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LOCALIZATION OF PROTEIN SEGMENTS AFFECTING CONFORMATION OF DEOXYADENOSINE KINASE FROM LACTOBACILLUS ACIDOPHILUS R-26

DISSERTATION
Presented in Partial Fulfillment of the Requirement for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Shenyuan Guo, B.S.

The Ohio State University
1998

Dissertation Committee:
Professor David H. Ives, Adviser
Professor Smita S. Patel
Professor Richard P. Swenson
Professor George A. Marzluf

Approved by
Adviser
Biochemistry Department
ABSTRACT

Deoxyadenosine kinase (dAK)/deoxyguanosine kinase (dGK) and dAK/deoxycytidine kinase (dCK) are heterodimeric kinase pairs initiating the obligatory salvage pathway of DNA precursor synthesis for *Lactobacillus acidophilus* R-26. Each kinase subunit has its distinct deoxynucleoside specificity. The $V_{\text{max}}$ of dAK is heterotropically activated by the primary deoxynucleoside substrate of its heterologous kinase partner. The principal objective of this study is to understand the structural bases for this V-type heterotropic activation and for the conformational differences between dAK and dGK (or dCK).

dAK is inactive without associating with either dGK or dCK subunits, which are almost fully active without dAK, revealing dAK’s unique conformation. dAK subunits heterodimerize with dCK with a dissociation constant of 40 nM. The heterotropic activation of dAK occurs in existing heterodimers, and is not due to additional dAK heterodimerization. The adverse effects of substrates or end-products on the 3-Å cross-linking of
subunits suggest that the active site of dAK is near the heterodimer interface, although those ligands could also have exerted their effects allosterically.

The Ras G-2 (switch 1)-like sequence is important, in cis, during dAK's heterotropic activation, and is partially responsible for the conformational differences between dAK and dGK. A P155A mutation of the Ras-like sequence in the dAK subunit of dAK/dGK heterodimer cis-activated dAK two fold, with a corresponding reduction of the heterotropic activation by dGuo. dAK with the P155A mutation would still be inactive without associating with dGK. A 50-amino acid segment of dGK, including the Ras-like sequence, was “transplanted” into dAK without substantially altering dAK’s $V_{\text{max}}$ and $K_m$ values, but the heterotropic activation of dAK was completely abolished. Moreover, this chimeric dAK is more like a dGK in that its $K_s$ for the leading substrate was increased, suggesting the formation of an intermediate enzyme species along the kinetic pathway.

We hypothesize that the dependence of the dAK activity on dGK is analogous to the GTPase activation of Ras by RasGAP.
Dedicated to my parents
ACKNOWLEDGMENTS

I would like to gratefully express my appreciation to my adviser Professor David H. Ives, for his guidance, support, patience, understanding, encouragement, and assistance throughout these years! My research work would be impossible without his constant cultivation.

Many thanks also go to Dr. Seiichiro Ikeda, for his helpful discussions, and for his sharing tea with me during lunch time.

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I will also remember the stimulating time I had with Drs. Grace Ma, Young-Soo Hong, and Ning Ma in our lab.

I thank Mr. Jeff Kobelt for his kindness and friendship.

Finally, I must express my indebtedness to my parents for their constant love and willing sacrifice, things I can never repay in full!
VITA

June 12, 1970
Born - Shanghai, China

March 1993
Bachelor of Science, magna cum laude, with distinction in Biochemistry, The Ohio State University, Columbus, Ohio

March 1993 - present
Graduate Teaching/Research Associate Department of Biochemistry The Ohio State University, Columbus, Ohio

PUBLICATION


FIELDS OF STUDY

Major Field: Biochemistry

Studies in Protein Engineering, Enzyme Kinetics, and Molecular Biology with Professors David H. Ives, Smita S. Patel, Richard P. Swenson and George A. Marzluf
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<tr>
<td>Ade</td>
<td>adenine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATCase</td>
<td>aspartate transcarbamoylase</td>
</tr>
<tr>
<td>CHES</td>
<td>2-[N-Cyclohexylamino]ethanesulfonic acid</td>
</tr>
<tr>
<td>Chi</td>
<td>chimera</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>Cyt</td>
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<tr>
<td>dAdo</td>
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<td>dCyd kinase</td>
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</tr>
<tr>
<td>dCyd</td>
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<td>DTSSP</td>
<td>3,3'-dithiobis[sulfosuccinimidyl-propionate]</td>
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<td>guanine</td>
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<td>SDS</td>
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<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
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The page also includes the following abbreviations:

- PR: PR 5-phosphoribosyl-α-pyrophosphate
- RF: reverse phase
- SDS: sodium dodecyl sulfate
- TES: N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid
- Thy: thymine
- TK: dThd kinase
- Tris: tris-(hydroxymethyl)-aminomethane
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<td>uridine 5'-triphosphate</td>
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CHAPTER 1

INTRODUCTION

1.1 Perspective and Related Work.

1.1.1 General observations on allosteric regulation.

**K-type versus V-type.** Allosteric proteins are essential for a cell to regulate its biological functions, including the carbohydrate metabolism, nucleotide metabolism, and amino acid metabolism, etc. By subjecting the rate-determining step(s) of a metabolic pathway to allosteric control, a cell can respond to its changing environment promptly. An allosteric effect may be executed through subunit-subunit interactions, when the binding or turnover of one ligand (substrate) at one site is affected by the binding of another ligand (effector) at a different site of the protein. The effect is homotropic if the two ligands are the same; otherwise, the effect is heterotropic. Allosteric regulation can affect either a protein's affinity for its...
The concerted model versus the sequential model. An integrated model of allostery was first introduced by Monod et al. (1,2) in the Monod-Wyman-Changeux (MWC) concerted model designed to explain the cooperative binding of oxygen by hemoglobin. The model assumes allostERIC proteins to be oligomers and to exist in either the T (taut) state or the R (relaxed) state, with the T state having a lower affinity for ligands. The Koshland-Nemethy-Filmer (KNF) sequential model (3), proposed later, assumes that the conformational change induced by ligand-binding is transmitted to the neighboring vacant subunit across the subunit interface through subunit-subunit interactions, resulting in the better alignment of functional amino acid residues.

The conformational change versus oligomer dissociation / formation. Subunit-subunit interactions either can occur within the existing oligomers or may be realized upon oligomer association / dissociation (4). The transition of bacterial phosphofructokinase from the R- to the T- state involves the conformational replacement of an active site arginine side chain by glutamic acid, so that the binding of the substrate fructose 6-
phosphate is impeded due to electric repulsion (5). However, the activity of mammalian phosphofructokinases is regulated by association-dissociation rather than by conformational changes alone (6).

"Intrinsic" versus "induced" (latent) cooperativity/allostery.

Oligomeric proteins are potentially capable of subunit interactions, but not all of them are cooperative in ligand binding or catalysis. *E. coli* aspartate transcarbamoylase (ATCase) has been studied as a prototype of an allosteric enzyme (7). It contains two catalytic trimers (c₃) and three regulatory dimers (r₂). This enzyme shows the positive, *homotropic*, and cooperative binding of both substrates, aspartate and carbamoyl phosphate. Moreover, ATCase is *heterotropically* inhibited by OTP but activated by ATP; both ATP and OTP bind to the same site of the regulatory subunits (8-15). Catalytic trimers without regulatory subunits are non-cooperative (7), but site-directed mutagenesis of active-site residues have induced homotropic cooperativity within the catalytic trimer alone, without dependence on regulatory subunits (16).

Moreover, even though the *E. coli* native ornithine carbamoyltransferase is nonallosteric *in vivo*, a site-directed mutation of the
active site also induced positive substrate cooperativity. Therefore, allostery of some oligomeric proteins may be a latent property, to be induced only when needed to balance physiological functions of the oligomers (16).

**Zooming-in.** A cell requires four deoxynucleoside triphosphates (dNTPs) as precursors for DNA synthesis, and therefore a balanced supply of the four dNTPs is essential for proper DNA replication and repair. Perturbations of dNTP pools may lead to increased genetic point mutations and even cell death, as reviewed by Reichard (17). In most cells, a balanced dNTP pool is achieved through two coordinated processes: the de novo pathway and the substrate cycle salvage pathway employing enzymes such as deoxynucleoside kinases and 5'-nucleotidases (17).

1.1.2 The de novo pathway.

*E. coli* purine nucleotide biosynthesis. The de novo biosyntheses of purine and pyrimidine nucleotides are tightly controlled by mechanisms of both feedback inhibition and feed-forward activation. For example, during *E. coli* purine nucleotide biosynthesis (18,19), ADP and GDP are
mixed inhibitors for phosphoribosylpyrophosphate (PRPP) synthase. In addition, PRPP amidotransferase is allosterically and feed-forwardly activated by PRPP, but binds ATP, ADP, and AMP at one inhibitory site and GTP, GDP, and GMP at another. Moreover, GTP and ATP energize the synthesis of AMP and GMP, respectively, from IMP (Figure 1.1). Consequently, this control network achieves a regulated overall production of purine nucleotides, with relative amounts of ATP and GTP finely coordinated.

**E. coli pyrimidine nucleotide biosynthesis.** For *E. coli* pyrimidine nucleotide biosynthesis, aspartate transcarbamoylase (ATCase) is heterotropically inhibited by CTP (the end-product of pyrimidine nucleotide synthesis pathway), and is heterotropically activated by ATP (one of the two end-products of purine nucleotide biosynthesis pathway) (20). Therefore, the feedback inhibition of ATCase by CTP regulates the overall CTP and UTP biosynthesis, and the ATP activation of ATCase acts to couple the rates of synthesis of purine and pyrimidine nucleotides (Figure 1.2).
Aspartate + GTP

GDP + Pi

Adenylosuccinate

IMP

AMP

XMP

Glutamine + ATP + H₂O

Glutamate + AMP + PPi

GMP

Figure 1.1: Balanced production of GMP and AMP from IMP in *E. coli*. 

6
Figure 1.2: Regulation of the pyrimidine nucleotide biosynthesis pathway in *E. coli*. 
From ribonucleotides to deoxyribonucleotides. The synthesis of four dNTPs from respective NDPs is accomplished sequentially by the ribonucleoside diphosphate reductase and nucleoside diphosphate kinases. Allosteric control effects ensure the essential responsiveness of both *E. coli* and mammalian ribonucleotide reductase toward the levels of various nucleotides (21) (Figure 1.3). The purine ribonucleotide ATP stimulates the reduction of pyrimidine ribonucleotides CDP and UDP. Thymidylate synthase methylates dUMP to dTMP. dTTP then inhibits further CDP and UDP reduction, but stimulates the GDP reduction. The subsequent dGTP then shuts down the reduction of CDP, UDP, and GDP, but stimulates the production of the other purine deoxynucleotide dADP and hence dATP. As dATP accumulates, it inhibits all NDP reduction unless displaced by the enough high concentration of ATP. The intracellular balance between the two pyrimidine deoxynucleotides is maintained by Cyd/dCyd deaminase (22-24) or deoxycytidylate deaminase (25-28), allosterically activated by dCTP but inhibited by dTTP.
Figure 1.3: Regulation of ribonucleotide reductase in *E. coli*. 
1.1.3 The salvage pathway.

As reviewed by Reichard (17), the intracellular dNTP pools can also be fine-tuned by combined actions of a) nucleoside permease (29) catalyzing in- and out-flux of nucleosides, b) 5'-nucleotidases (30-32) catabolizing ribonucleotides and deoxynucleotides, c) deaminases for base interconversions (33), d) nucleoside phosphorylases or nucleoside transglycosylases (now nucleoside 2-deoxyribosyl transferase) generating nucleosides from bases (33), and e) deoxynucleoside kinases phosphorylating various deoxynucleosides (17).

Unlike de novo routes of nucleotide biosynthesis with features widely-conserved among different organisms, the salvage routes are far more diverse in nature and distribution. But the regulation features of deoxynucleoside kinases such as thymidine kinase (34-38) and deoxycytidine kinase (39) testify to the importance of salvage pathway, as pointed out by Kornberg (33).
1.1.3.1 Thymidine kinase.

E. coli thymidine kinase. E. coli thymidine kinase (TK), the only deoxynucleoside kinase in this organism, is specific for dThd and dUrd but not dCyd, and is subject to a) the potent end-product inhibition by dTTP, and b) the allosteric activation by dCDP as well as other deoxynucleoside di- and tri-phosphates (including dATP, dGTP, and dCTP) which accumulate when the dTTP supply is lacking (34,35). dCDP activates the enzyme by both decreasing its $K_m$ for dThd and increasing the $V_{max}$, suggesting that the activator binding site is separate from the substrate binding sites. High concentrations of ATP also decrease the $K_m$ (dThd) of E. coli TK, presumably by binding to the dCDP site. The dTTP inhibition is competitive with dThd, but noncompetitive with ATP. In the presence of dCDP, the dTTP exerts a greater inhibitory action, suggesting separate sites for dTTP and dCDP. E. coli TK undergoes "dimerization" of the two dimers, forming a tetrameric complex of 90 KDa, upon the binding of either type of effector (37). This oligomerization is accompanied by a more specific conformational change at the oligomer level, which is correlated with the very effect (e.g., stimulatory or inhibitory) of each deoxynucleotide. According to the zone sedimentation experiments, the dTTP-induced
oligomer has a more compact structure than the dCDP-induced one.

**Vaccinia virus TK.** Vaccinia virus (VV) TK (a homotetrameric complex of 80 KDa (40)) shares substantial homology, and is grouped in the same structural subdivision, with homotetrameric pox / eukaryotic / bacterial TKs (41,42). It can only phosphorylate dThd and its closely related analogs (e.g., bromo-deoxyuridine). It uses ATP but not GTP, CTP, nor UTP as the phosphate donor in vitro (43). Black and Hruby (44) have identified the probable dTTP binding site in domain IV (113FQRKP). Upon the mutation of either Q114D or Q114H, negative feedback inhibition on VV TK by dTTP (noncompetitive with dThd (45)) is lost, while the activity is unaffected. Q114H and Q114D proteins can still be eluted from an ATP-agarose affinity column with dTTP (albeit more dTTP appears to be required), indicating that dTTP still can bind to the mutants. [note: If the end-product inhibition were due to dTTP sterically occupying the substrate binding sites, then dTTP would inhibit this mutant VV TK.]

**Human TK1.** Human cytosolic thymidine kinase (TK1) is also a homotetrameric complex, with a native molecular mass of 96 KDa (46). It phosphorylates dThd and dUrd, and most nucleoside triphosphates except
dTTP and CTP (47,48) can be phosphate donors. Besides being a phosphate donor, ATP also functions as a positive effector, inducing from the dimer a more stable tetramer with higher affinity for the substrate (e.g., dThd) in a time and enzyme concentration dependent manner (49). Human cytosolic TK1 is more strongly inhibited by dTTP than *E. coli* TK is (41).

**Human TK2.** Surprisingly, human TK (TK2) exists in a monomeric form even in the presence of 2 mM ATP or 2 mM dThd (48). It phosphorylates dThd with negative cooperativity, but dCyd and dUrd can be substrates with normal Michaelis-Menten kinetics. Both ATP and CTP can be phosphate donors. dTTP *competitively* feedback inhibits the enzyme, while dCTP shows a negative cooperative inhibition pattern (47,48,50).

**Herpes simplex virus TK.** Herpesviral TKs exist as homodimers, and like human TK2, it can also phosphorylate dCyd (51,52). dTTP inhibition of Herpesviral TK is not as sensitive as that of human TKs. The unique ability of herpesviral TKs to phosphorylate dCyd may reflect their proposed origin from a captured cellular dCK gene (53). [Note: Regardless of the validity of the proposal, herpesviral TK does share...
significant sequence homology with human dCK as well as dGK and TK2 (see later).] The three-dimensional structure of thymidine kinase from Herpes simplex virus type I (HSV-1) has been obtained by two independent groups (54,55). According to Wild et al. (54,56) (Figure 1.4), helices a4 (centered at residue 131) and a6 (centered at 190) are at the center of the large, nearly flat, and exceptionally nonpolar dimer interface. The interface also includes residues from loop connecting a2 (centered at 87)-a3 (centered at 103), part of the loop connecting a9 (centered at 292)-a10 (centered at 314), and the ends of helices a10 (centered at 314) and a12 (centered at 364). The glycine rich loop (P-loop) connecting β-strand b1 (centered at 52) and helix a1 (centered at 67) forms the giant anion hole accommodating the β-phosphoryl group of ATP. The ribose moiety of thymidine points toward this anion hole. While Asp 162 of the DRH motif co-ordinates the Mg^{2+}, Tyr 172 of CYP motif stacks against the thymine base. Arg 222 of the arginine rich motif (residues 216 to 222) is at the active site (55). Thymidine binds closer to the dimer interface than ATP does. Recently, novel drug-specific HSV-1 TKs have been created upon random mutagenesis of the two regions right before the DRH and CYP motifs, respectively (57).
Figure 1.4: The secondary structure assignment of HSV-1 TK.

E, β-strand; S, turn without hydrogen bond; H, α-helix, T, turn with hydrogen bond  [Adapted from Figure 2 of (56)].
1.1.3.2 Deoxynucleoside kinases other than thymidine kinase.

**Human deoxycytidine kinase.** Homodimeric human "cytosolic" deoxycytidine kinase (dCK) has an apparent molecular weight of 60 KDa (39,58-60). Recently, dCK has been demonstrated to be actually located in nucleus (61). Human dCK binds dCyd with higher affinity, but phosphorylates it with lower $V_{max}$ than for dAdo and dGuo. Consequently, dCyd effectively inhibits phosphorylation of other substrates. All three nucleoside substrates show characteristic biphasic kinetics. It suggests that phosphorylation at two sites negatively cooperate with each other (62), or that two sites have different substrate affinities (59,63). Kim and Ives (64) found ATP to be the first substrate to bind and ADP the first product to leave, while the data from Datta et al. (65) suggested a random Bi-Bi mechanism. dCTP potently inhibits dCK activity (64-68), being competitive with ATP and noncompetitive with dCyd, supporting Kim and Ives' proposal. Moreover, when UTP instead of ATP is the phosphate donor, human dCK exhibits lower $K_m$ and $V_{max}$ values for dCyd, and dCTP's inhibition now becomes less potent and *uncompetitive* with dCyd (66,68). It is unclear if both phosphate donors contribute to regulatory mechanisms *in vivo* (39).
**Human deoxyguanosine kinase.** Human mitochondrial deoxyguanosine kinase (dGK) is a homodimeric enzyme with an apparent molecular weight of 58 KDa (69). The subunit molecular weight, according to the cDNA sequence, should be 28 KDa after expected protease cleavage of the mitochondrial translocation signal peptide (70). Human dGK phosphorylates dGuo, dAdo, and dIno (69-71). At physiological pH, dTTP seems to be a better phosphate donor than ATP (69). dGTP and dGDP both potently inhibit human dGK (69).

1.1.3.3 Categorizing deoxynucleoside kinases.

**Sequence conservation among kinases.** There are at least two main families of deoxynucleoside kinases: a) the poxviral / eukaryotic / bacterial TK family (e.g., *E. coli* TK, vaccinia virus TK and human TK1), and b) herpes TK and human dCK family (e.g., *Lactobacillus* deoxynucleoside kinases, HSV-1 TK, and human dCK, dGK, and TK2). Only the ATP binding glycine rich (P-loop) domain is conserved in *both* families (41,72).

Within the second family of deoxynucleoside kinases, there are three
distinctively conserved motifs (70,72): ATP binding site, DRH or ERS motif, and arginine rich motif. In addition, a leucine rich motif is identified in human dCK, dGK, and TK2. This leucine zipper type of motif has been postulated to participate in the protein-protein interaction, although no supporting experimental data have been provided so far.

On the other hand, Traut (73) has proposed a consensus motif for the nucleotide-binding “regulatory site” based on an analysis of three-dimensional structures of E. coli aspartate carbamoyltransferase regulatory subunit, E. coli phosphofructokinase, and yeast phosphoglycerate kinase. For the first two proteins, the regulatory site is completely separate from the catalytic site, while the regulatory site of phosphoglycerate kinase overlaps with the phosphate acceptor site. The proposed “regulatory motif” is also present in E. coli and human thymidine kinases, human deoxycytidine kinase, and mouse uridine kinase (Figure 1.5), thus suggesting an additional common structural feature shared by both families of deoxynucleoside kinases.
a)  VEAI KR GT VID<sup>19</sup>  

b)  

c)  VFIR VDFNV PLD<sup>29</sup>  

d)  FGAI LNLVP LAE<sup>144</sup>  

e)  EOGI P L E YLE<sup>206</sup>  

a), *E. coli* aspartate carbamoyltransferase regulatory subunit.  b), *E. coli* phosphofructose kinase.  c), Yeast phosphoglycerate kinase.  d), Human TK1.  e), Human dCK. Residues in bold are those shown to interact with the nucleotide in deciphered structures. Identical residues or conservative replacements in other sequences are underlined.

Figure 1.5: Sequences corresponding to the proposed nucleotide-binding “regulatory” site (73).
Interestingly, the probable dTTP binding site in domain IV
($^{113}$FQRKP) identified by Black and Hruby (44) in VV TK is at the immediate
N-terminus of the segment 1 of the above mentioned putative motif. The
functions of this motif in those proteins remain to be confirmed upon
deciphering their own three-dimensional structures, but it is worth
mentioning that the putative nucleotide-binding “regulatory site” in human
deoxyctydine kinase follows immediately the arginine-rich motif postulated
to participate in the phosphate transfer (74).

The importance of deoxynucleoside kinase studies. We envision
(D.H. Ives, personal communication,(70)) that comparative studies of
related enzymes (e.g., HSV1-TK, human dCK and dGK, and heterodimeric
dAK/dGK from Lactobacillus acidophilus R-26 (74)) will eventually lead to
engineered nucleoside kinases valuable as prodrug activators in anti-
cancer gene therapy.
1.2. Previous Work/Knowledge on Deoxynucleoside Kinases from *Lactobacillus acidophilus* R-26.

1.2.1 *Lactobacillus acidophilus* R-26 and its deoxynucleotide metabolism.

*L. acidophilus* R-26, obtained from the American Type Culture Collection (ATCC 11506), is a gram positive thermophilic bacterium with the optimal growth temperature between 35-45°C. Lactic acid is the major fermentative end product of its glucose metabolism (75). Lacking a functional ribonucleotide reductase to couple NTP and dNTP synthases, this bacterium has an absolute requirement for a single deoxynucleoside of any kind, as well as for a purine and a pyrimidine. Nucleoside 2-deoxyribosyl transferase (trans-N-glycosidase) provides deoxyribose for all four deoxynucleosides from a single source of deoxynucleoside (76-79), by transferring the deoxyribosyl unit from base to base. While *E. coli* uses Cyd/dCyd deaminase (22-24) to enrich resources for the thymidylate pathway, *L. acidophilus* R-26 possesses a deoxycytidylylate deaminase (80). The latter enzyme is subject to strong inhibition by dTTP, and the inhibition is allosterically overcome by dCTP (28). Thymidylate synthase, catalyzing
the conversion of dUMP to dTMP, is subject to only weak product inhibition by dTMP, and neither the activity nor the synthesis of the enzyme is regulated in prokaryotes including various *Lactobacillus* species (81).

All four deoxynucleoside kinases are present in *L. acidophilus* R-26 (82). The principal pathways (83) of *L. acidophilus* R-26 deoxynucleotide metabolism are summarized in Figure 1.6.

The absence of a functional ribonucleotide reductase uncouples the NTP and dNTP syntheses in *L. acidophilus* R-26. The regulatory mechanisms balancing all four dNTPs associated with the reductase are then missing. *L. acidophilus* R-26 deoxynucleotide metabolism possibly has some new features that compensate for this defect. Deoxynucleoside kinases, a group of enzymes catalyzing the first step of phosphorylation of deoxynucleosides, are of particular interest in this regard.
Major enzymes involved are: a) nucleoside 2-deoxyribosyl transferase, b) deoxynucleoside kinases, c) deoxycytidylate deaminase, d) thymidylate synthetase, and e) thymidine kinase.

Figure 1.6: Principal pathways of deoxynucleoside metabolism in *L. acidophilus* R-26 (83).
1.2.2 Deoxynucleoside kinases: deoxyadenosine kinase, deoxyguanosine kinase, and deoxycytidine kinase from *L. acidophilus* R-26.

Durham and Ives (82) separated *L. acidophilus* R-26 thymidine kinase from the other three deoxynucleoside phosphorylation activities through calcium phosphate-gel batch precipitation and Sephadex G-150 chromatography. The thymidine kinase is effectively inhibited by dTTP, but the inhibition is reversed by dCTP. In a parallel fashion, deoxycytidine, deoxyguanosine, and deoxyadenosine phosphorylating activities are effectively inhibited by the homologous deoxynucleoside triphosphate; the inhibition is reversed by UTP or dUTP.

During purification of the latter three phosphorylating activities using calcium phosphate-gel, dAdo phosphorylating activity was preferentially inactivated. This is the first evidence for a dAdo binding site separate from the sites for dGuo or dCyd.

During Sephadex G-150 chromatography, dAdo phosphorylating activity emerged slightly earlier than that for dGuo and dCyd. Based on
more recent subunit composition data (see later), we think that Durham was dealing with dGK and dCK subunits partially disassociated from dAK. This possibility also explains the modest 40-60% activation of dAK by 2 mM of dGuo or dCyd (instead of the 4-fold activation of dAK consistently observed in later studies).

Reznik and Ives (84,85) also observed an apparent dissociation of the enzyme from a molecular weight species of 50 KDa to 35 KDa in sucrose gradient sedimentation, preparative electrophoresis, and Biogel P-100 chromatography. This conversion from a high to a low molecular weight form was unidirectional, and was prevented by ATP, dATP, dUTP, and dCTP at a concentration of 2mM or less. The low molecular weight form contains a lower specific activity of deoxyadenosine kinase compared to the deoxycytidine kinase activity. [note: Based on my studies on the subunit interactions between dAK and dGK (or dCK) (to be discussed later), subunit dissociation and subsequent dAK denaturation may account for the observed irreversible conversion.]

Deibel and Ives (86,87), employing Blue-Sepharose affinity chromatography and differential elution by MgATP, resolved the *L. acidophilus* R-26 deoxynucleoside kinases into two fractions: one enriched
in dAK and dCK activities, and the other in dAK and dGK activities. For the first fraction, there were two measurable $K_m$'s for dAdo (5 $\mu$M and $>$1000 $\mu$M), two $K_m$'s for dGuo (4 $\mu$M and 80 $\mu$M) but only one $K_m$ for dCyd (5 $\mu$M).

For the second fraction, there were two $K_m$'s for dAdo (5 $\mu$M and $>$500 $\mu$M), two $K_m$'s for dCyd (5 $\mu$M and 490 $\mu$M), and one $K_m$ for dGuo (5 $\mu$M). Thus, the separation between two activity fractions is not complete. The origins for these $K_m$ values became clear only after researchers in this laboratory separately expressed and kinetically dissected the individual subunits (shown later in Table 1.3).

While the dAK activity is always associated with and inseparable from dCK throughout the purification procedure, these activities reside at two separate catalytic sites, as indicated by the following observations: a) the different responses of dAK and dCK towards thermal inactivation and photo oxidation by rose bengal, b) preferential loss of the dAK activity, and c) activation of the dAK activity up to 500% by dCyd. [Note: The dAdo homotropic activation of dAK in the dCK rich fraction accompanied by a dramatically increased $K_m$ (dAdo) is now understood as dAdo binding to the dCyd site on dCK (discussed later).]
However, the very limited quantities available of pure protein prevented the SDS-PAGE analysis from verifying / rejecting the speculation that dAK and dCK constitute a multifunctional single polypeptide.

The molecular weights estimated by gel filtration are 50 ± 4 KDa for the enzymes of either fraction. A lower molecular weight form was also demonstrated, with a rapid loss of the dAK activity [Note: Again, this suggests dissociation of dAK from dGK or dCK, to be discussed later].

The kinetic mechanism of dCK is random Bi Bi. Like dAK and dGK, dCK is subject to feedback inhibition by its specific distal product, dCTP. However, dCTP can also serve as a moderately effective phosphate donor to dCyd, in the absence of ATP. In the presence of ATP, dCTP is linearly competitive with variable dCyd, but gives complex and nonlinear kinetics with variable ATP.

At pH 7.8, dCK exhibits its highest $V_{\text{max}}$ and lowest $K_m$ (dCyd). On the other hand, dAK has a pH optimum near 9.5, but a second pH optimum is apparent at pH 7.8 in the presence of heterotropic activator (dCyd). (Figure 1.7)
The dAdo concentrations are: \(\triangle\-\triangle\) for 48.2 \(\mu\text{M}\), and \(\cdots\cdots\) for 96.5 \(\mu\text{M}\). In the presence of heterotropic activator dCyd (381 \(\mu\text{M}\)), dAdo concentrations are: \(\triangle\-\triangle\) for 48.2 \(\mu\text{M}\), and \(\cdots\cdots\) for 96.5 \(\mu\text{M}\). Specific activity is measured by units/mg, as described in “Experimental Procedures.” [Figure from Deibel (84).]

Figure 1.7: Effects of pH variation on the dAK specific activity of dAK/dCK.
Chakravarty et al. (88) achieved the base-line separation of dAK/dCK from dAK/dGK by eluting Blue Sepharose with a bisubstrate mixture: 0.5 mM dCyd plus 1 mM ATP for dAK/dCK, and 1 mM dGuo and 5 mM ATP for dAK/dGK. Like dAK/dCK, the active sites for dAK and dGK are separate from each other, based on the following observations: a) preferential loss of the dAK activity during purification, b) each of the two deoxynucleosides stimulates the turnover of the other (but to different extents), c) turnovers at the two sites are additive, and d) selective chemical inactivation of dGK by 5'-[p-(fluorosulfonyl)-benzoyl]adenosine.

The kinetic mechanism of dGK is random Bi Bi, with dAK following an ordered Bi Bi path (ATP binding first and ADP leaving first). Moreover, dAK and dGK possess different kinetic parameters (89) (Table 1.1).

Based on a single band corresponding to a molecular weight species of 56 KDa upon silver staining of the SDS-PAGE, dAK/dGK was suggested to be a monomeric kinase with two separate active sites. However, this conclusion was decisively refuted by the work on affinity purified enzymes carried out later by Ikeda et al. (90) (discussed later).
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* Plots converge on abscissa.

* Plots converge in upper left hand quadrant.

Table 1.1: Comparisons of dAK and dGK kinetic characteristics (89).
Ikeda and Ives (91) synthesized four dNp₄A bisubstrate-type inhibitors of the respective four deoxynucleoside kinases from *L. acidophilus* R-26, and used each of them to construct affinity media for the purification of four kinases. TK was the only kinase quantitatively and specifically retained by dTp₄A column alone. With the other three dNp₄A columns used separately, two nearly equal amount of the dAK activity were found to be associated with dGK and dCK, respectively.

In the absence of other heterotropic activators, dCp₄A or dGp₄A activated the dAK activity up to 2-fold. This observation is consistent with the previous idea that dAK/dGK and dAK/dCK each have two distinct active sites for two different kinase activities.

Ikeda *et al.* (92) further carried out comparative kinetic inhibition studies with natural dNTP end products and dNp₄A bisubstrate analogs. Dissociation constants for the enzyme-substrate complexes were determined for each of these different inhibitors (Table 1.2).
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</tr>
<tr>
<td></td>
<td>MgATP</td>
<td>C</td>
<td>2.0</td>
<td></td>
<td>MgATP</td>
<td>C</td>
<td>0.9</td>
</tr>
</tbody>
</table>

- For dAK of dAK/dCK.
- For dAK of dAK/dGK.
- “C” for “competitive”; “NC” for “noncompetitive.”

Table 1.2: Comparisons of inhibition constants of deoxynucleoside kinases by dNp$_4$A and dNTP (92).
For both types of inhibitors, the deoxynucleoside moiety provides the basis for the inhibition specificity, and the phosphate groups (a total of four negative charges at pH 8.0) likely interact with the ATP site and reinforce the affinity of the molecule as an inhibitor. The adenosine portion of the dNp₄A type inhibitor does not further reinforce the binding affinity, and instead may even interfere with the proper alignment of phosphate groups with the enzyme's ATP site. This makes dNTP a more potent inhibitor ($K_i=0.4-3 \mu M$) than the corresponding dNp₄A ($K_i=1.4-9.2 \mu M$). As revealed by the inhibition pattern, dGK and dCK each follow random Bi Bi kinetic mechanisms, while dAK follows an ordered Bi Bi pathway.

Ikeda et al. (90) therefore constructed a new affinity medium with dCTP linked to Sepharose through an additional phosphate group between the terminal phosphate of dCTP and a hexyl group. The affinity purified protein was analyzed on SDS-PAGE, yielding a single band corresponding to a molecular weight species of only 26 KDa. Thus, the native protein is composed of two subunits of similar size. Since the N-terminal amino acid sequences of the two subunits differ slightly, dAK and dCK may be different subunits. This notion was then elegantly confirmed by functional assignments of subunits by means of limited proteolysis controlled by specific end-product inhibitors (93).
Two pairs of kinases (dAK/dGK and dAK/dCK) were each separated, on SDS-PAGE at pH 6.6, into a fast-moving component (F) and a slow-moving component (S). N-terminal amino acid sequences of all four components were obtained (Figure 1.8). The specific end-product inhibitor dNTP made the corresponding subunit resistant to trypsin digestion, but left the heterologous subunit susceptible to trypsin. The complete digestion of the subunit was accompanied by its complete inactivation. The phosphorylation function was thus assigned to each subunit of the two pairs of kinases. While the two slow-moving components are both dAK, the two fast-moving components are dGK and dCK, respectively (Figure 1.8).

An intrinsic conformational difference between dAK and dGK was revealed by the following observations (94): a) chaotropic salts such as guanidine-HCl and NaI in low concentrations each increase the $V_{\text{max}}$ of dAK, as dGuo does, while slightly inactivating dGK; b) the rate of trypsin proteolytic inactivation of dAK is significantly slower than that of dGK, but is accelerated by dGTP; c) specific labeling of dGK's dGuo site by [8-$^{14}$C]-8-azido-Ade permanently activates dAK two fold, while inactivating dGK.
Figure 2: Comparison of N-terminal amino acid sequences of the subunits of kinase complex I (dCyd kinase/dA do kinase(I)) and II (dGuo kinase/dA do kinase(lt)) from L. acidophilus R-26, and subunit assignment. "*" in a sequence represents a residue position where it was not possible to identify any amino acid with certainty. Matching amino acids are shown on the lines between subunit sequences.

[Figure 2 from Ikeda et al., (93)]

Figure 1.8: Comparison of N-terminal amino acid sequences of subunits from native dAK/dGK and dAK/dCK.
A cloning probe (74) was constructed based on the N-terminal 28 residues of the mixed dAK and dCK subunits (90), but upon application of this probe, tandem genes for the dAK and dGK subunits instead were cloned from *L. acidophilus* R-26 (Figure 1.9).

The tandem *dak-dgk* genes are preceded by a common endogenous promoter, and a transcription terminator follows the translational termination codon for *dgk*. Both *dak* and *dgk* genes contain an independent Shine-Dalgarno ribosome-binding sequence for subunit translation. Thus, the two subunits are transcribed as a polycistronic messenger RNA and then translated separately. [Note: According to later repeated efforts of DNA sequencing, it is found that a sequencing error was made just upstream of the Shine-Dalgarno sequence for the dAK translation in the published DNA sequence data. Fortunately, this error has not affected any studies so far. A correction of the relevant sequence appears in Appendix A.]

Regions conserved between dAK and dGK as well as among other groups of proteins include (Figure 1.10): a) a glycine-rich region (P-loop) characteristic of ATP-binding sites (90), b) the DRS motif, putatively homologous with part of the nucleoside binding site for viral TK (95), c) an
Fig. 3. Homology of derived amino acid sequences of dAdo kinase (dAK) and dGuo kinase (dGK). 61% identity in the 215-amino acid overlap (DNASer AANIV). A colon (:) denotes amino acids that are positively related using the probability of acceptable mutation matrix, a blank denotes negatively related amino acids, and a period (.) indicates a neutral relationship. Consensus regions, sites i, ii, iii, and iv, described in the text, are indicated in bold.

[Figure 3 from Ma et al., (74)]

Figure 1.9: Homology of derived amino acid sequences of cloned dAK and dGK.
Figure 1.10: Multiple sequence alignment of cloned dAK and dGK with mammalian dCK.

[Figure 4 from Ma et al., (74)]
arginine-rich region widely conserved among nearly all sequenced
deoxynucleoside kinases, as well as among adenylate kinases (95,96), and
d) a Ras G-2 like region conserved in Ras-like proteins (97,98). Moreover,
dAK and particularly dGK, share moderate homology with mammalian
deoxycytidine kinases (Figure 1.10), especially at the first three motifs.

A site-directed mutation, R79K (99), in the DRS motif of dGK
increased dGK's $K_m$ (dGuo) nearly 10 fold, but stimulated dAK's $V_{max}$ nearly
29-fold, with only a moderate increase of dAK's $K_m$. The magnitude of
dAK's $V_{max}$ and $K_m$ changes is reminiscent of the heterotropic activation of
dAK upon dGuo or dGTP binding to dGK. The residue Arg-79 of dGK
might reside at the contact area of dAK and dGK, and participate in dGuo
binding, thus affecting dAK's catalytic turnover rate either directly or
indirectly. [note: However, some mistakes were made in interpreting the
dGK subunit's $K_m$ values for its two substrates. The data were subjected to
recalculation, and the results appear in Appendix B.]

In order to obtain a dCK-specific probe to clone the $dck$ gene,
internal dCK peptides were derived from tryptic digestion and subjected to
peptide sequencing (100). Upon sequencing 63% of the residues spanning
the whole native dCK subunit, the dCK peptide was found to differ from
dGK only at the very N-terminus (dCK is M-IVL, while dGK is -TVIVL), as
previously revealed (93). Furthermore, mass spectrometry measurements
revealed native dGK and dCK subunits to be identical in mass adjusted for
the first three residues. Accordingly, the \textit{dgk} gene was engineered into a
synthetic \textit{dck} gene from which dCK can be expressed in \textit{E. coli}. This dCK
specifically phosphorylates dCyd, just like dGK being specific for dGuo
(Table 1.3).

Moreover, dCyd or dCTP binding to this engineered dCK activates
the associated dAK heterotropically as in native dAK/dCK, and dCTP (but
not dGTP) exhibits potent end-product inhibition of this dCK activity. One
of the biggest challenges remaining is to identify the \textit{in vivo} mechanism by
which two polypeptides of distinctive substrate specificities are derived

In summary, while \textit{Lactobacillus} thymidine kinase is readily
separable from the other three kinase activities (82), dAdo kinase (dAK)
exists as a heterodimer with either a dGuo kinase (dGK) or a dCyd kinase
(dCK) subunit (86,88,93,101). The dAK activity in either heterodimer is
Table 1.3: Comparisons of some kinetic properties between homodimeric dGK and dCK (100).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (app) (μM)</th>
<th>$V_{max}/K_m$</th>
<th>Substrate</th>
<th>$K_m$ (app) (μM)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGuo</td>
<td>5.8</td>
<td>220</td>
<td>dGuo</td>
<td>110</td>
<td>2.6</td>
</tr>
<tr>
<td>dAdo</td>
<td>240</td>
<td>0.8</td>
<td>dAdo</td>
<td>1800</td>
<td>2.3</td>
</tr>
<tr>
<td>dCyd</td>
<td>1800</td>
<td>0.7</td>
<td>dCyd</td>
<td>2.8</td>
<td>230</td>
</tr>
</tbody>
</table>

* [MgATP] = 10 mM.
only 1/7 to 1/10 of the activity of dGK or dCK, but the activity of dAK is heterotropically activated 3 to 5 fold by dGuo or dCyd, respectively. In contrast, the activity of dGK or dCK is affected only slightly by dAdo. Therefore, the combined output of these two heterotropically-regulated dimers provides the nearly equal quantities of dAMP, dCMP, and dGMP needed for DNA precursor synthesis (88).

The amino acid sequences of dGK and dCK are identical except at their extreme N-termini, which determine their deoxynucleoside specificity. These polypeptides are now believed to be alternative processing products of the dgk gene (100). From here on, I will consider the conformations of dGK and dCK to be essentially identical within their respective heterodimers, in the context of heterotropic activation. The dGK and dCK are kinetically and structurally parallel, and in a "relaxed" conformation (S. Ikeda, personal communication) (88,94,101), whereas dAK is in a "constrained" conformational state that "relaxes" upon heterotropic activation. The "constrained" state of dAK was inferred from experiments such as chemical modification (86), limited proteolysis, activation by chaotrophic salts, and affinity labeling (87). Obviously, the largely unidirectional heterotropic activation of dAK by dGuo or dCyd reflects the unique conformational states of dAK and its heavy dependence on dGK for...
optimal catalytic turnover.

### 1.3. Experimental Rationale, Targets, and Principal Results.

Subunit-subunit interaction is the basis for a wide spectrum of regulatory mechanisms controlling and coordinating chemical events *in vivo* (102,103). The stereochemical nature of these allosteric interactions has been elucidated in great detail in several protein systems over the past 60 years (7).

*Lactobacillus acidophilus* R-26 is an organism lacking the essential ribonucleotide reductase for the deoxynucleotide *de novo* pathway, and it instead possesses all four deoxynucleoside kinases to generate building blocks of DNA through salvage pathways (82). A set of unique kinase regulatory mechanisms to maintain a balanced dNTP pool can be expected. And it is certainly of interest to uncover those responsible structural elements, particularly those involved in heterotropic activation.

Moreover, because *Lactobacillus* kinases share considerable sequence homology with human dCK, dGK and TK2, the findings in this
study will contribute to the understanding of structure-function relationship in this latter group of clinically important kinases. Furthermore, when the kinase is to be engineered for future use in the gene therapy against cancer, the goal should be turning it into a "super" kinase. This "suicide" kinase should not only possess unique specificity for the prodrug, but also phosphorylate the prodrug efficiently so as to minimize any side effects. As the initial step towards this long-term goal, studies on heterotropic activation may reveal critical structural requisites for efficient catalysis. Any trans-requirement should be not only satisfied but also surpassed by cis-modification and optimization.

Heterotropic activation of both subunits in the heterodimer is achieved through subunit-subunit interactions involving the subunit interface. Short-range (3 Å) chemical cross-linking studies were carried out to explore the interface of the dAK/dGK heterodimer and to detect conformational changes occurring at the interface during the heterotropic activation.

To dissect the nearly "one-way" interdependence leading to optimal conformations for both subunits of a heterodimer, unmodified dAK and dGK (or dCK) were expressed separately. Thus, the impact of in vitro
heterodimer reconstitution on each subunit's catalytic competence can be studied. Moreover, the prevalence of ligand-induced dimerization (or dimer dissociation) in other enzyme systems (4) also warrants such a study.

Both dAK and dGK contain a segment (site iv) homologous in varying degrees to the G-2 loop (Switch I) of p21ras protein (74), which, in Ras, is responsible for GTP binding and interaction with the GTPase Activating Protein (GAP) (97). The proline residue within this loop is of particular interest, because it is believed to stiffen the loop (104). Since dAK is in a "constrained" state, a site-directed proline/alanine substitution was made within this loop in an attempt to "relax" its conformation.

The tandem dak-dgk structural genes of Lactobacillus acidophilus R-26 have already been cloned; the derived amino acid sequences of dAK and dGK are 61% identical. The remaining sequence divergences must therefore account for any differences in conformation and substrate specificity of the two polypeptides. Thus, the cloned tandem dak-dgk genes present a unique opportunity for investigating the structural basis of dAK's and dGK's contrasting a) substrate specificities, b) kinetic mechanisms, and c) conformational properties. Because dak and dgk are more than 60% identical in their DNA sequences, constructing chimeric genes by
splicing *dak* and *dgk* at certain points might yield "hybrid" proteins, which, if active, may possess blended and even novel properties. In particular, such "transplants" can help identify structural elements responsible for the different catalytic efficiencies between dAK and dGK (or dCK).

Figure 1.11 illustrates the overall relation between objectives and approaches of this study, and the principal observations and conclusions are summarized as follows:

a) dAK’s conformation differs from that of dGK in that dAK expressed independently of dGK (or dCK) was virtually inactive.

b) dAK heterodimerized, with high affinity, with dGK (or dCK) (the apparent $K_d$ being 40 nM). This restored dAK’s activity and enabled the V-type heterotropic activation of dAK by dGuo (or dCyd), mediated through its subunit-subunit interaction with dGK (or dCK).

c) In comparison, the heterodimerization of self-active dGK (or dCK) with dAK only moderately stimulated the catalytic turnover rate of dGK (or dCK).

d) Heterotropic activation was not due to additional heterodimer formation induced by the ligand.
Figure 1.11: The overall relation between objectives and approaches.
e) A conformational change of the Ras switch l-like site iv motif on dAK was involved in modulating dAK's catalytic turnover rate in cis during heterotrophic activation. According to this model of Ras-RasGAP interaction, the active sites of dAK and dGK may be in close proximity to the subunit interface of the heterodimeric dAK/dGK.

f) The peptide segment between amino acid residues 120 and 170 was the cis structural element responsible for the heterotrophic activation of dAK; this segment includes such motifs as the arginine-rich site iii (identical for both dAK and dGK) and a Ras switch l-like site iv (slightly different between dAK and dGK).

g) The heterotrophic activation of dAK was accompanied by an increase in the dissociation constant for the dAK•MgATP, with accumulation of the intermediate enzyme species along the kinetics pathway.

h) Ma et al. (100) found that the presence or absence of Thr2 and Val3 is the sole determinant in differentiating the substrate specificity of dGK and dCK, and I found that these two residues did not affect the specificity of dAK.

i) The peptide segment between amino acid residues 120 and 170 was involved in modulating the turnover. The structural basis for the substrate specificity of dAK or dGK must lie between residues 19 and 119.
CHAPTER 2
EXPERIMENTAL PROCEDURES

2.1 Culturing \textit{E. coli} cells for plasmid miniprep or cloned kinase expression.

\textit{E. coli} XL1-blue (from Stratagene) or MV 1190 (from BioRad) cells transformed with pUC19 (Life Technologies) or pBlueScript (Stratagene) containing the desired kinase gene were grown in LB broth with 100 \( \mu \text{g/ml} \) ampicillin overnight at 37°C. For the plasmid miniprep purpose, the overnight culture was used directly. When kinase enzymes were to be purified, the overnight culture was used to inoculate the main culture (in LB, 100 \( \mu \text{g/ml} \) ampicillin) to an O.D. (at 600 nm) of 0.1. The cells in the main culture were grown at 37°C until the O.D. reached 1.0 and then harvested in a Sorvall RC-5B centrifuge (Du Pont Instruments) at 4,000 g for 30 minutes at 4°C. Collected cells were extracted for kinases immediately or stored in the \(-80°C\) freezer.
2.2 Extraction of cloned kinases.

Every 5 g (wet weight) harvested cells were resuspended in 50 ml extraction buffer (0.1 M Tris-HCl, pH 8.0 at 4°C, 3 mM EDTA). Sonication was then carried out on an ice-water mixture using the fine-tuned high intensity ultrasonic liquid processor (Sonics and Materials, Inc.) at 65% amplitude and a 2-second pulse for 5 minutes. Cell debris were removed upon centrifugation at 3,000 g for 30 minutes at 4°C. Ammonium sulfate crystals were slowly added to the supernatant to reach a 70% saturation over a period of 20 minutes with continuous stirring, and stirring for an additional 15 minutes. Proteins were then precipitated upon centrifugation at 12,000 g for 30 minutes at 4°C. The pellet was then dissolved in 15 mM potassium phosphate at pH 8.0, containing 50% glycerol. This ammonium sulfate fraction was stored at -20°C.

2.3 Protein Concentration Determination.

Protein concentrations were determined by the Bradford method (105). Bovine serum albumin was used to obtain the standard curve which was linear up to an A_{595} of 0.4. Concentrated protein samples were diluted
with distilled water, and an aliquot of 0.8 ml was mixed with 0.2 ml Bio-Rad dye concentrate. The absorbance at 595 nm was then measured upon 5-minute incubation after thorough mixing. Protein samples of unknown concentration were appropriately diluted so that an absorbance around 0.2 was routinely obtained.

2.4 Enzyme assays.

One unit of kinase activity is defined as 1 nmol of deoxynucleoside 5'-monophosphate formed per minute. The kinase reaction was carried out in a total volume of 40 μl containing the diluted kinase protein, 20 μM deoxynucleoside (Sigma), and 10 mM ATP (Sigma)-Mg²⁺ in 0.1 mM Tris-HCl, 5% glycerol, pH 8.0 at 20°C. [³H]dAdo, [³H]dGuo, or [³H]dCyd (Moravek Biochemicals) was included up to 0.5 μCi per assay to allow the product detection using the anion-exchange-disk method (106). The kinases were usually diluted so that a substrate turnover of less than 20% was achieved upon stopping the kinase reaction with 0.1 M formic acid. Specific activity is expressed as units per mg of protein. Substrate concentrations were varied for steady-state kinetics analysis as indicated in each case, but each reaction mixture contained a constant amount of
radioactive deoxynucleoside. Apparent $K_m$ values were measured with the other substrate at its saturation. True $K_m$ and $K_s$ values were obtained from the secondary plots of the slopes or Y-axis intercepts of the primary double reciprocal plots, as described (107).

2.5 Filter binding assay to determine the ligand binding affinity.

The overall procedure has been described by Patel and Hingorani (108). Nitrocellulose membrane circles (from Schleicher & Schuell) were wetted with 0.5 N NaOH, and were then rinsed with water and stored in membrane wash buffer (0.1 M Tris-HCl, 10% glycerol, pH 8.0 at 23°C) until use. Right before applying the assay mixture to the membrane on the filtration apparatus, the membrane was washed with 0.5 ml wash buffer. Each assay mixture contained 0.3 $\mu$M dCK, along with increasing amounts of radiolabeled dOTP (0, 0.5, 1.0, 3.0, 10.0 $\mu$M) in a total volume of 20 $\mu$l. After a 5-minute incubation at room temperature (23°C), 10 $\mu$l of the assay mixture was applied onto the membrane and filtered through the assembly (whereas the remaining mixture of each assay was saved for later determination of the percentage of ligand retained by dCK). The membrane was immediately washed again with 0.5 ml wash buffer. In order to determine the nonspecific ligand binding to the membrane, control
assays were carried out in which protein dilution buffer (15 mM potassium phosphate, 5% glycerol, pH 8.0) replaced dCK. The membrane circles were dissolved in ScintiVerse liquid scintillation counting cocktail (Fisher Scientific) overnight. Radioactivity retained by the membrane was measured by the scintillation counter (Beckman). The dissolved nitrocellulose did not quench the counting appreciably.

2.6 SDS-PAGE with Laemmli buffer system.

For most purposes such as visualizing protein purity or inspecting the effects of chemical cross-linking, SDS-PAGE was carried out essentially according to Laemmli (109). Briefly the system consists of the following routines: a) Separating gel: 12% acrylamide in 0.375 M Tris-HCl, pH 8.8 at 23°C; b) Stacking gel: 4% acrylamide, 0.125 M Tris-HCl, pH 6.8 at 23°C. In preparing both the separating and the stacking gels, polymerization catalysts ammonium persulfate and TEMED were double the recommended amount, and the degassing step was routinely omitted; c) Sample buffer (4X): 62.5 mM Tris-HCl, pH 6.8 at 23°C, 2% (w/v) SDS, 8 mM DTT, and 10% glycerol. Protein samples were diluted appropriately so that the sample buffer became 1X. The samples were then denatured by heating at 100°C for 5 minutes. Upon cooling, 1 μl saturated...
bromophenol blue solution was added to each 25μl protein sample; d) 5X running buffer was prepared by combining 4.5 g Tris base, 21.6 g glycine, and 1.5 g SDS, and diluting to 500 ml with dH₂O. This running buffer was diluted to 1X before use; e) Upon finishing the electrophoresis, the gel was stained for 30 minutes in the staining solution consisting of 0.1% (w/v) Coomassie Blue R-250, 40% (v/v) methanol, and 10% (v/v) acetic acid; f) The stained gel was then destained overnight in the destaining solution [10% (v/v) methanol and 7.5% (v/v) acetic acid]; g) The destained gel could then be dried.

2.7 Drying SDS-PAGE gels.

The destained SDS-PAGE gel was soaked in gel drying solution (45% (v/v) methanol and 5% (v/v) glycerol) with agitation for 15 minutes. Cellophane membrane sheets (Novel Experimental Technology) were wetted in the drying solution. The gel was then placed between two membrane sheets in a drying frame (with air bubbles squeezed out) and left at room temperature to dry overnight.
2.8 SDS-PAGE with MZE 3328.IV buffer system.

For the purpose of separating the two subunits of heterodimeric dAK/dGK, an alternative version of SDS-PAGE was performed according to the procedures described by Moos (110-114). The system consists of the following unique components: a) Separating gel: 12% acrylamide, 0.12 M BisTris-HCl, pH 6.61, prepared a day before use; when overnight polymerization was complete, in order to exhaust the remaining free radicals, pre-electrophoresis was performed in separating gel buffer with the addition of 0.1 mM sodium thioglycolic acid and 0.1% (w/v) SDS at 8 mA for 30 minutes; b) Stacking gel: 4% acrylamide, 0.125 M Tris-HCl, pH 6.80; c) Upper tank buffer: 0.113 M BisTris-HCl, 44 mM TES, pH 7.25; d) Lower tank buffer: 63 mM BisTris-HCl, pH 5.90; e) Electrophoresis was performed at a constant current in the range of 8 to 12 mA.

2.9 Electroblotting of Protein for N-terminal protein sequencing.

Kinase subunits separated by SDS-PAGE with the MZE 3328.IV buffer system were electrophoretically blotted onto the Immobilon-P membrane (from Millipore) at room temperature (23°C), following the procedures as generally described by Kyhse-Andersen (115). Filter papers
(Whatman #1) soaked with anode buffer 1 (0.3 M Tris, 20% (v/v) methanol, pH 10.4) were stacked on the bottom electrode, followed by a stack of filter papers soaked in anode buffer 2 (0.025 M Tris, 20% (v/v) methanol, pH 10.4). The immobilon membrane wetted in 20% (v/v) methanol was placed upon the stack, followed by the unstained acrylamide gel. Filter papers soaked with cathode buffer (40 mM 6-amino-n-caproic acid, 25 mM Tris, 20% (v/v) methanol, 0.005% SDS, pH 9.4) were then stacked onto the gel, followed by the cathode. During the whole process, care was taken not to trap air bubbles between the stacks. Electroblotting was carried out at a starting current of 0.8 mA/cm² for two hours. The completion of the blotting process was signified by the disappearance of prestained standard protein markers from the gel. The concentration of SDS used in the system should be optimized in order for the membrane to retain the maximum amount of protein. The membrane was then stained in 0.1% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol / 10% (v/v) acetic acid for 15 minutes, and destained in 40% methanol / 10% acetic acid for 15 minutes. The membrane was then dipped quickly in 90% methanol / 7% acetic acid, and allowed to air dry at room temperature.
2.10 N-terminal amino acid sequence analysis.

Polypeptides blotted onto the membrane were excised and submitted to the Ohio State University Biopolymer Facility for N-terminal amino acid sequence analysis. The analysis was based on gas-phase Edman degradation chemistry.

2.11 Synthesis and purification of oligonucleotides.

Oligonucleotides (trityl-off) were synthesized by the Ohio State University Biotechnology Center Nucleic Acid Facility. Deprotected oligonucleotides were supplied in NH₄OH solutions. n-Butanol (1 ml, ACS reagent grade) was mixed thoroughly with 0.1 ml oligo solution in NH₄OH by vortexing for 15 seconds. Upon centrifuging at 12,000 rpm (in Beckman Microfuge E) for 1 minute, the n-butanol phase was discarded. The oligonucleotide pellet was washed with 1 ml of ice-cold 80% ethanol. The pellet was dried at room temperature for 15 minutes and dissolved in 50 μl dH₂O, and then stored at -20°C. If it was necessary to remove salts from the preparation, the above purified oligo in dH₂O was applied to a Bio-Rad Bio-Spin 6 chromatography column equilibrated with dH₂O.
2.12 Plasmid DNA miniprep.

For the purpose of restriction mapping analysis, plasmid DNA was purified according to the procedures described (116), with slight modifications. Overnight culture, 1.5 ml, was centrifuged at 12,000 g for 30 seconds. The medium was removed and the pellet suspended in the remaining medium. Freshly prepared Solution II (0.2 ml of 0.2 N NaOH / 1% (w/v) SDS) was then added to the cell suspension, and mixed by inverting 5 times. The mixture was left on ice for 5 minutes. Ice-cold Solution III (0.15 ml of 60 ml 5 M potassium acetate / 11.5 ml glacial acetic acid / 28.5 ml autoclaved dH₂O) was added and mixed gently, and mixture was left on ice for 3 to 5 minutes. Upon collecting the supernatant, RNase A (DNase free) was added to reach a final concentration of 1 μg/ml. After a 30-minute incubation at 37°C, the mixture was subjected to phenol / chloroform (1:1) extraction once, and chloroform extraction once. Double-stranded DNA was precipitated with two volumes of ethanol for two minutes at room temperature. The DNA was collected by centrifugation at 12,000 g for 5 minutes and washed with ice-cold 80% ethanol. The plasmid pellet was then dissolved in 30 μl dH₂O and stored at -20°C.

Alternatively, for purposes such as preparing PCR templates and
automatic DNA sequencing, plasmid DNA was purified using a QIAprep
spin miniprep kit, following the procedures recommended by the
manufacturer.

2.13 Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis was routinely carried out in 0.8% (w/v)
agarose gel in 0.5X TBE (116). TBE (5X) was prepared by mixing 27 g
Tris-Base, 13.75 g boric acid, and 1.86 g EDTA, diluting to 500 ml with
dH$_2$O. Sample buffer (6X) included 0.25% (w/v) bromophenol blue and
40% (w/v) sucrose. The DNA sample was mixed with sample buffer so that
the sample buffer final concentration was 1X. Electrophoresis was carried
out at a constant voltage of 100 V. The gel was stained in dH$_2$O containing
0.5 $\mu$g/ml ethidium bromide, and destained with dH$_2$O.

2.14 Purification of DNA fragments from agarose gel electrophoresis.

In order to purify DNA fragments from the agarose gel, 1% (w/v) low
melting point agarose was used instead of regular 0.8% (w/v) agarose.
Upon staining and destaining, the gel containing the desired DNA fragment
was excised and melted at 65°C. The DNA fragment was retained by and then eluted from QIAGEN tip-5 anion exchange column, following procedures described by the manufacturer. Alternatively, regular agarose gel was used, and DNA fragments were extracted using Qiaquick gel extraction kit.

2.15 DNA ligation.

Vector DNA, 0.5-1.0 μg, and as much insert DNA as possible were mixed, incubated at 45°C for 5 minutes and placed immediately on ice for 5 minutes. Ligase reaction buffer (Life Technologies) (containing in final concentration: 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, and 5% (w/v) polyethylene glycol-8000) and one unit of T4 DNA ligase (Life Technologies) were then added. dH₂O was used to adjust the total volume to 20 μl. The ligation reaction was carried out at room temperature overnight.

2.16 Competent E. coli cell preparation and DNA transformation.

The procedures are described in the manual accompanying the Muta-Gene phagemid in vitro mutagenesis kit (version II) (Bio-Rad). The
cells were treated with 100 mM MgCl₂ and then 100 mM CaCl₂. Competent cells were resuspended in 85 mM CaCl₂ and 15% glycerol, and stored at -70°C. Defrosted competent cells, 0.3 ml, were mixed with a maximum of 15 μl of ligation product. In case of the plasmid transformation, a 5 μl- aliquot of DNA solution from the miniprep was usually sufficient. Upon gentle mixing, transformation was allow to proceed for 45 minutes on ice. The cells were then heat-shocked at 42°C for 3 minutes, and then cooled on ice for 5 minutes. The cells were cultured in LB at 37°C for 1 hour before being plated onto LB/agar plates containing 50 to 100 μg/ml ampicillin.

2.17 Manual DNA sequencing using the dideoxy method.

DNA sequencing was carried out using the dideoxy method by Sanger et al. (117), employing the Sequenase Version II kit (USB), following the procedures recommended by the manufacturer. The desired primer (1 pmol) was annealed to the template (5 μg) upon heating at 75°C for 3 minutes and cooling to room temperature over a period of 45 minutes. dATP[α-³⁵S] was used for detection, and ddNTP was used to terminate the DNA synthesis. Sequencing polyacrylamide gel (8%) was prepared from Long Ranger gel solution (AT Biochem). The gel was dried using a gel dryer (Bio-Rad, Model 583) and autoradiographed on X-ray film (Kodak X-
2.18 Automated DNA sequencing.

Automated DNA sequencing was carried out by the Ohio State University Campus Chemical Instrument Center Biopolymer Facility or by the Biotechnology Center Nucleic Acid Facility, using Applied Biosystems Model 373A sequencer, employing dye terminators. The templates were purified using a QIAprep spin column kit, and sequencing primers were desalted using Bio-Spin 6 columns (Bio-Rad). The computer-identified sequences were proof-read manually to ensure correct identifications.

2.19 Estimation of the approximate dissociation constant \((K_d)\) of dAK/dCK.

Independently-expressed inactive dAK was partially purified as an ammonium sulfate fraction, while dCK was further purified by gel filtration chromatography. The latter involved applying a dCK ammonium sulfate fraction (50 mg total protein in 8 ml) to a Sephacryl S-200 HR (Pharmacia) gel permeation column (2.5 cm × 148 cm, 726 ml volume) equilibrated with
elution buffer containing 15 mM potassium phosphate (pH 8.0) and 5% glycerol. About 75% of the dCK activity was recovered with the dCK protein peak (about 80% pure, as estimated from SDS-PAGE), with a protein concentration of about 0.08 mg/ml, as determined by the Bradford method (105) using dye reagent from Bio-Rad. The molar concentration of dCK was then calculated, based on its subunit molecular weight. Various amounts of dCK were mixed with fixed amounts of dAK, incubated on ice for 10 minutes, and then diluted and incubated at 20°C for further 5 minutes. Aliquots (10 μl each) were then taken from each diluted mixture for assay (at 20°C) of the dAK activity (total assay volume of 40 μl) in the presence of 400 μM dCyd, which both activated the dAK subunit and blocked the detection of secondary dAK activity of the heterodimeric dCK protein. The progress of the dAK and dCK heterodimerization was monitored by this measurement of the dAK activity, since the total catalytic turnover of dAdo within the 30 minute assay period should be linearly correlated with the amount of heterodimeric dAK/dCK in equilibrium. The data were then fitted to Equation 2.1, with the assumption that dCK used during titration existed as monomers before associating with dAK.

\[
AC = \frac{(K_a + C_T + A_T)/2 - [(K_a+C_T +A_T)^2/4 - (A_T \times C_T)]^6}{(Equation 2.1)}
\]
where $K_d$ is the dissociation constant of heterodimeric dAK/dCK, $C_T$ is the total dCK concentration added each time, $A_T$ is the total dAK concentration, and $AC$ is the concentration of heterodimeric dAK/dCK in equilibrium.

Alternatively, the data were fitted into Equation 2.2, assuming that dCK may also exist as homodimers before the heterodimer reconstitution:

$$AC = A_T \times [(K_d^2 + 8 \times K_{d2} \times C_T)^{1/2} - K_{d2}] / [4 \times K_{d1} + (K_d^2 + 8 \times K_{d2} \times C_T)^{1/2} - K_{d2}] \quad \text{(Equation 2.2)}$$

Correspondingly, $K_{d1}$ is the dissociation constant of dAK/dCK, while $K_{d2}$ is the dissociation constant for dCK/dCK. Derivation of Equation 2.2 is shown in Appendix C.

2.20 Determination of the heterotropic dAK activation by dGuo or dCyd during the titration of dAK with dGK or dCK.

All three polypeptides were partially purified as ammonium sulfate fractions. Various amounts of dGK or dCK were incubated on ice for 10 minutes with fixed amounts of dAK, diluted and assayed for the dAK
activity in the presence or absence of dGuo or dCyd (400 μM), respectively. Both dGK and dCK active sites have a secondary dAK activity (but with a much larger $K_m$ for dAdo than for the homologous deoxynucleoside), regardless of whether dGK or dCK is expressed independently of dAK (100), or is in the form of heterodimer (results based on the secondary slopes of plots of $1/v$ versus $1/[dAdo]$; S. Ikeda, personal communication). Therefore, these secondary dAK activities should be blocked by saturating dGK and dCK with their more tightly-binding primary deoxynucleoside substrates. When the dAK activity was to be assayed without dGuo or dCyd, a control was included in which the secondary dAK activity of free dGK or dCK was assayed. Hence, the dAK activity due solely to dAK in the heterodimer was calculated by subtracting the secondary dAK activity of either the dGK or dCK protein from the total dAK activities of the heterodimer.

2.21 Mono-Q anion exchange chromatography.

The anion exchange chromatography was carried out using the Waters 625 LC System equipped with a Pharmacia Mono-Q column. The column was equilibrated with Buffer A containing 15 mM potassium
phosphate, pH 8.0, and 20% glycerol. Upon sample application, the column was washed with Buffer A, and the kinase activities were eluted at 0.15 mM NaCl developed by a linear NaCl gradient.

2.22 HPLC-Reverse Phase (RF) chromatography.

HPLC-RF chromatography was carried out on the Waters 625 LC System fitted with a Vydac 214TP54 (C-4) column (Vydac, Hesperia, CA). The dAK and dGK subunits were separated by a linear acetonitrile gradient developed by Buffer A [0.06% (v/v) trifluoroacetic acid in water], and Buffer B [80% (v/v) acetonitrile, 0.054% (v/v) trifluoroacetic acid, in water].

2.23 Preparation of Blue-Sepharose.

Cibacron blue 3G-A substituted agarose was prepared according to the method of Böhme et al. (118), with slight modifications.

2.24 Purification of dAK/dGK for chemical cross-linking studies.

Heterodimeric dAK/dGK was expressed and extracted as described.
earlier. An ammonium sulfate fraction containing 169 mg of total protein was loaded onto the gel permeation column (as described above), and 15-ml fractions were collected. Five fractions containing at least 80% of the dAK/dGK activities were identified.

Those active fractions were then pooled and further purified on a Blue-Sepharose column (2.5 cm × 2.5 cm), as described previously (86), with modifications. Aliquots of dAK/dGK active fractions (in 15 mM potassium phosphate, 5% glycerol, pH 8.0) were adjusted to pH 6.6 by mixing 10 volumes of enzyme fraction with 0.7 volumes of pH-adjusting buffer (150 mM monopotassium phosphate/5% glycerol, pH 2.6). The pH-adjusted fractions were further diluted 2 fold with wash buffer 1 (15 mM potassium phosphate, 5% glycerol, pH 6.6) before application to the Blue-Sepharose column at a flow rate of 2 ml/min. At a flow rate of 2.5 ml/min, the column was washed with 90 ml wash buffer 1, then with 250 ml wash buffer 2 (15 mM potassium phosphate, 15 mM AMP, 5% glycerol, pH 8.0), and 150 ml wash buffer 3 (15 mM potassium phosphate, 5% glycerol, pH 8.0). The dAK/dGK protein was finally eluted with 100 ml elution buffer (15 mM potassium phosphate, 10 mM ATP, 5% glycerol, pH 8.0), and the first eight 10-ml fractions were pooled and concentrated to ~1 ml with CentriPlus ultrafiltration units from Amicon, resulting in dAK/dGK protein
(80% pure, as estimated from SDS-PAGE) at a concentration of ~1 mg/ml.

2.25 Chemical cross-linking.

The cross-linker stock solution, 1,5-difluoro-2,4-dinitrobenzene (DFDNB, from Pierce) in acetone, was freshly prepared before each use. Each reaction, in a total volume of 100 μl (pH 8.0), contained 15 mM potassium phosphate (or, 45 mM potassium phosphate, to maintain pH when dGTP or dATP was introduced), 10% glycerol, 0.1 mg/ml dAK/dGK, and 0.05 mM DFDNB (added last). Whenever reactions were carried out in parallel, the reaction mixtures contained identical concentrations of potassium phosphate. The reactions were allowed to proceed for 1 hour at room temperature (22.5°C), and were quenched with 50 mM (final concentration) Tris-HCl (pH 8.0). When necessary, the reaction mixtures were passed through Bio-Spin 6 columns (Bio-Rad) to remove possible remaining DFDNB and other salts. Finally, 35 μl of the reaction mixture was analyzed by SDS-PAGE (12% gel), using the Laemmli buffer system (109). The gels were stained with Brilliant Blue R (Sigma) and dried, and
2.26 Construction of Chimera I.

Chimera I was formerly called the independent chimeric dAK. As shown in Figure 2.1, the phagemid pBlueScript (+) KS (Stratagene) construct (74) containing the cloned tandem \textit{dak-dgk} genes was cleaved at two \textit{Sty I} restriction sites with endonuclease from BRL, and the residual phagemid was re-ligated to yield the \textit{Chi-I} construct (Figure 2.2 A), following isolation and purification on Qiagen columns. \textit{Chi-I} encodes a polypeptide of dAK spliced to dGK at residue 170.
Figure 2.1: Construction of *Chimera 1*.
Numbers indicate the position of amino acid residues.

Shadowed boxes represent gene fragments of \textit{dgk} origin.

Figure 2.2: Schematic illustrations of chimeric gene constructs and the unmodified \textit{dak-dgk} gene.
2.27 Construction of Chimera I'.

The plasmid pBlueScript (+) KS containing the dak gene was linearized with a single cut by Sty I (Life Technologies). On the other hand, the dak-dgk tandem gene construct cloned in pBlueScript(+) KS was also treated by Sty I, and the resulting smaller Sty I fragment was isolated from the agarose gel using the Qiagen gel extraction kit and ligated to the above linearized dak construct to yield Chi-I'. This Chi-I' construct (Figure 2.2 B), like dak/dgk (Figure 2.2 F), is also tandem in nature. It contains an unmodified dak gene followed by a chimeric dgk gene encoding a dGK spliced to dAK at residue 170.

2.28 Construction of Chimera II.

The Chi-II gene (Figure 2.2 C) encodes a chimeric polypeptide Chi-II where the N-terminal half of dAK is spliced to the C-terminal half of dGK at residue 120. It was constructed using the “recombinant PCR” techniques as generally described (119) (Figure 2.3).
c, Primer 1. d, Primer 2. a, Primer 3. b, Primer 4.

Figure 2.3: "Recombinant" PCR.
pfu DNA polymerase was purchased from Stratagene and employed following the procedures recommended by the manufacturer. In PCR-1, the independently cloned *dak* (120) served as the template, and Primer 1 (5'-CCC CCC CGG GGT ACC AGC ATC TAT CTT ACA-3') and Primer 2 (5'-ATC AGG ATT CTT CTT TGG ATT ACC AGG TGC-3') functioned as the upstream and downstream primer, respectively. In PCR-2, the independently cloned *dgk* (100) served as the template, and Primer 3 (5'-GCA CCT GGT AAT CCA AAG AAG AAT CCT GAT-3') and Primer 4 (5'-CCC CCG GGG TAC CCC AAA ATC AGT TAA CAG-3') served as the upstream and downstream template. PCR products from the above two reactions were purified using Qiaquick PCR purification kit, and were joined and amplified in a third PCR reaction, using Primer 1 and Primer 4 as the upstream and downstream primer respectively. The PCR product from this reaction was purified and digested with Kpn I, and finally cloned into pBlueScript(+) KS as confirmed by restriction mapping with Kpn I and Xba I. The *Chi-II* gene was then sequenced to its entirety by the Ohio State University Biopolymer Facility.
2.29 Construction of Chimera III.

The plasmid pBlueScript(+) KS containing Chi-II was linearized with a single cut by Sty I and ligated with the smaller Sty I fragment cleaved from the unmodified dak/dgk tandem genes (120). Therefore, Chi-III (Figure 2.2 D) is also a tandem gene construct, containing a chimeric gene encoding a "dAK" subunit with residues 120-170 replaced by the counterparts from dGK, followed by an unmodified dgk gene. The ligation product was confirmed by restriction mapping with Sty I and Xba I, and by DNA sequencing (by Biopolymer Facility).

2.30 Construction of Chimera IV.

The gene for Chi-IV (Figure 2.2 E) was constructed in the same way as Chi-II, except that Primer 2 (5'-GGC TGA CTT AAT ACT AGC TAA ACG GTG GTT-3') and Primer 3 (5'-AAC CAC CGT TTA GCT AGT ATT AAG TCA GCC-3') were specifically designed for this particular case. Upon subcloning into the pBlueScript vector, the Chi-II gene was sequenced to its entirety (by Biopolymer Facility).
2.3.1 Site-directed P155A and S156T substitutions of dAK in the dAK/dGK heterodimer.

The mutagenesis was carried out using the Muta-Gene phagemid in vitro mutagenesis kit (version 2) from Bio-Rad. In order to specifically mutagenize the *dak* gene without altering the homologous sequence in *dgk*, *Chi-I* (Figure 2.2 A) served as the template. The mutagenesis of two residues likely to be functional was carried out using the primers 5'-CTT TTA AAC TTG CAT CGG TTG-3' and 5'-GTA ATC TTT TAA AGT TGG ATC GGT TG-3' to produce mutant strains expressing dAK(P155A) and dAK(S156T), respectively. Mutation was confirmed by the dideoxy manual DNA sequencing. Upon obtaining the desired mutants, a mutant *dak-dgk* construct was assembled by resplicing the *Sty I* fragment from normal *dak-dgk* into the *Sty I* site of the mutant *Chi-I* construct (i.e., the tandem gene sequence is restored, except for the mutation within dak, reversing the steps shown in Figure 2.1). DNA sequencing and assays for the dGK activity were carried out to identify colonies with the *Sty I* insert.
CHAPTER 3

SUBUNIT-SUBUNIT INTERACTION

3.1 Independently expressed dAK was inactive until associated with dGK.

With the primer 5'-CCA TAT TGG ACC GCA GTT TTG CT_C GTT AAC TAG TTT AAĄ TTC CCT-3', the dgk structural gene was completely looped out from the original tandem dak-dgk construct (120). DNA sequencing results provided by Ning Ma (unpublished data) confirmed that the dgk gene was deleted, leaving only the dak structural gene immediately followed by the transcription terminator of the original tandem genes (Figure 3.1).
The arrow indicates the connection between Nucleotide 654 and Nucleotide 1346. Nucleotides are numbered according to Figure 1 of (74).

Figure 3.1: Partial DNA sequence of the independent dak gene

(unpublished data provided by Ning Ma).
Consequently, the *dak* gene in this construct should be transcribed and translated just as it is in the tandem *dak-dgk* genes, but without the *dgk* gene product. The dAK protein, thus expressed, was enzymatically inactive until the heterodimeric enzyme form was reconstituted *in vitro* with independently expressed dGK or dCK polypeptides (Table 3.1). The dAK activities were assayed for the dAK, dGK or dCK subunit, and for *in vitro*-reconstituted dAK/dGK or dAK/dCK heterodimer. Independently-expressed dAK was enzymatically inactive until the heterodimer formation. The secondary dAK activity of dGK or dCK obtained in the absence of dGuo or dCyd was subtracted from total dAK activities of dAK/dGK or dAK/dCK, yielding the dAK activity solely from the dAK subunit of the heterodimer. In the presence of dGuo or dCyd, detection of the secondary dAK activity of either heterodimeric dGK or dCK, respectively, was completely blocked, so that the dAK activity measured was that from the activated dAK subunit of the heterodimer.
<table>
<thead>
<tr>
<th>Polypeptide Composition</th>
<th>dAK Activity&lt;sup&gt;a&lt;/sup&gt; without dGuo</th>
<th>dAK Activity&lt;sup&gt;a&lt;/sup&gt; with dGuo</th>
<th>Heterotropic Activation by dGuo</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAK</td>
<td>0.48</td>
<td>0.48</td>
<td>(38-0.48) / (18-6.4) = 3.2</td>
</tr>
<tr>
<td>dGK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>dAK+dGK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polypeptide Composition</th>
<th>dAK Activity&lt;sup&gt;a&lt;/sup&gt; without dCyd</th>
<th>dAK Activity&lt;sup&gt;a&lt;/sup&gt; with dCyd</th>
<th>Heterotropic Activation by dCyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAK</td>
<td>0.48</td>
<td>0.48</td>
<td>(155-0.75) / (69-36) = 4.7</td>
</tr>
<tr>
<td>dCK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>dAK+dCK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69</td>
<td>155</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> (units x 10<sup>4</sup>) The dGuo or dCyd concentration was 400 μM.

<sup>b</sup> The total amount of dGK or dCK protein was in stoichiometric excess of dAK.

Table 3.1: The activity of dAK subunit was dependent on the dGK or dCK subunit.
Like the wild type enzymes (87,88), the dAK activity of reconstituted dAK/dGK or dAK/dCK heterodimer was further activated 3-5 fold heterotropically by dGuo or dCyd, respectively (Table 3.1). Although both dGK and dCK, expressed separately from dAK, have a secondary dAK activity, the secondary $K_m$ (dAdo) is 1-2 orders of magnitude higher than the $K_m$ value for the respective primary deoxynucleoside substrate, dGuo or dCyd (Table 1.3) (100). In the heterodimeric form, dGK or dCK's secondary $K_m$(dAdo) is also estimated to be at least 1,000 $\mu$M (S. Ikeda, personal communication), while the $K_m$ for the primary deoxynucleoside is less than 10 $\mu$M (100). Therefore, detection of the secondary dAK activity of either heterodimeric dGK or dCK is easily blocked by saturating (e.g., 400 $\mu$M) dGuo or dCyd, respectively. In the absence of the heterotropic activator, the secondary dAK activity of free dGK or dCK was determined and subtracted from total dAK activities of the heterodimer obtained from reconstitution. This approximates the dAK activity of the dAK subunit in the heterodimer before heterotropenic activation.

Furthermore, the secondary dAK activity of the free dGK or dCK protein amounts to 2 to 7% of the primary dGK or dCK activity when assayed with only 20 $\mu$M dAdo. And dGK or dCK in the heterodimer contributes about 30% of assayed dAK activities from the heterodimer.
Therefore, when the 3 to 5 fold heterotropic activation is observed in wild type enzymes (where dGK or dCK is nearly in equal stoichiometry amount of dAK), this trivial secondary dAK activity of dGK or dCK can be ignored even if it is not blocked. The fact that the calculated heterotropic activation factor (Table 3.1) falls within the range observed in the wild type heterodimers further validates the calculation shown.

The observation that dAK by itself had no discernable dAK activity parallels the situation with Ras of G proteins. In the interaction between Ras proteins and GAP, GAP directly activates GTPase activity of Ras ~100,000 fold (98,121,122) by supplying one positive charge and better positioning other important residues, both essential for transition state stabilization (123). Furthermore, in heterotrimeric G proteins, when both the Gα GTP-binding core domain ("R-2") and a GAP-like Gα insert domain ("G-2") are separately expressed as recombinant proteins, "G-2" stimulates the GTPase activity of "R-2" under conditions where neither alone hydrolyzes GTP (98). In another GTPase family including proteins involved in protein translocation across membranes, a G-2 loop-containing protein called FtsY again has no measurable GTPase activity alone unless combined with Ffh-4.5S ribonucleoprotein (124).
3.2 Independently expressed dGK or dCK was only partially dependent on dAK for activity.

The independently expressed dGK (data not shown) or dCK (Figures 3.2, A and B) was almost fully active, being activated only an additional 50-100% upon heterodimer reconstitution \textit{in vitro}. Only the $V_{\text{max}}$ was increased with no significant changes in the apparent $K_m$ for either the deoxynucleoside substrate or ATP-Mg$^{2+}$. This relatively modest dependence of the dGK or dCK activity upon the dAK subunit parallels the small heterotropic activation ($\sim$10\%) of heterodimeric dGK (Figures 3.2, C and D) or dCK by dAdo (data not shown). The heterotropic activation is also a $V_{\text{max}}$ effect with no changes in the apparent $K_m$ of either the deoxynucleoside substrate (87,88) or ATP-Mg$^{2+}$, within experimental errors (Figures 3.2, C and D).
Figure 3.2: The activity of dGK or dCK was only partially dependent on the dAK protein.
The dGK's V-type nature of its heterotropic activation by dAdo can be predicted theoretically, assuming $K_m$ values approximating the $K_a$ values (Figure 3.3). The square stands for the dAK subunit, and the circle for dGK. "A" denotes bound dAdo, and "G" bound dGuo. $K_1$ represents dGK's affinity for dGuo when dAdo does not bind to dAK. $K_2$ stands for dAK's affinity for dAdo when dGK is bound with dGuo. $K_3$ represents dAK's affinity for dAdo when dGK is free. $K_4$ stands for the affinity of dGK for dGuo when dAK is bound with dAdo. The equation $K_1 \times K_2 = K_3 \times K_4$ applies. Since dGuo binding to dGK does not change dAK's affinity for dAdo, $K_2$ equals $K_3$. Therefore, $K_1$ must be equal to $K_4$. It means that dAdo binding to dAK should not affect dGuo binding to dGK in the heterodimeric dAK/dGK.
Figure 3.3: Linked function analysis.
3.3 Heterotropic activation was not caused by induced heterodimer formation.

The finding that dAK expressed without dGK was enzymatically inactive until the heterodimer formation raises one question: does the deoxynucleoside substrate strengthen the inter-subunit affinity of the heterodimer, driving the equilibrium towards heterodimer formation, and thus activating dAK and dGK? The independent cloning of \textit{dak}, \textit{dgk}, and \textit{dck} provided the means to answer this question. During the \textit{in vitro} heterodimer reconstitution, the amount of dAK protein was fixed while dGK or dCK amount was varied incrementally so that dAK was titrated by the formation of heterodimers. However, the heterotropic activation of dAK by dGuo or dCyd appeared as usual even when all the dAK subunits existed in the heterodimeric form with either dGK or dCK, respectively (Figure 3.4). Such an observation indicates that heterotropic activation is not due to the formation of additional heterodimeric dAK, because otherwise the heterotropic activation of dAK would be diminished by excess dGK (or dCK). Therefore, heterotropic activation must be due to a conformational fine-tuning of structural element(s) affecting catalytic turnover mediated through the interface of any \textit{existing} heterodimeric molecules.
Figure 3.4: Heterotropic activation was not caused by the ligand-induced heterodimer formation.
The interaction between dAK and dGK (or dCK) occurs in two stages. The first stage starts immediately after protein synthesis, as two subunits associate into a heterodimer, reciprocally changing each other’s catalytic efficiency, but to different extents, as determined by their contrasting conformations (94). The subunit-subunit interaction is used to achieve heterotropic activation in the second stage. Binding of a homologous deoxynucleoside substrate causes a conformational change transmitted to the neighboring subunit, thereby fine-tuning the subunit interaction of any existing heterodimer, producing an additional increase in $V_{\text{max}}$ heterotropically.

3.4 Estimation of the heterodimer equilibrium dissociation constant.

Experiments to estimate the dissociation constant of dAK/dCK were based on the following observations: a) dAK was inactive by itself (Table 3.1), b) detection of the secondary dAK activity of heterodimeric dCK should be completely blocked by saturating dCyd, and c) the affinity between dAK and dGK (or dCK) was unaltered by dGuo (or dCyd) (Figure 3.4). Furthermore, since dAK was active only in the heterodimeric form, the equilibrium concentration of dAK/dCK should be directly proportional to
the amount of dAdo converted to dAMP during the 30 minute assay period. Therefore, the heterodimer formation was monitored directly by measuring the dAK activity of the "revived" and heterotropically activated dAK subunit. The data obtained were then fitted into Equation 2.1, yielding a $K_d$ of $4 \times 10^{-8}$ M. An identical $K_d$ value (Figure 3.5) was also obtained through Equation 2.2 which assumes that the dCK proteins used for the titration were in the homodimeric form before associating with dAK.

Since the heterodimer formation was monitored by measuring the dAK activity from the dAK subunit in the heterodimer, the $K_d$ value should be an apparent $K_d$ measured under the assay conditions: 100 mM Tris-HCl, 5% glycerol at 20°C. This estimated $K_d$ should be reasonably close to the true value in vivo, partially because nearly 100% of the dCK molecules used for the titration were active (S. Ikeda, unpublished results which revealed that all of the enzyme protein was retained on, and then biospecifically eluted from, a dCTP-Sepharose column). In the chemical cross-linking reactions (described in a later section), where both dAK and dGK were present at concentrations of about $2 \times 10^{-6}$ M, nearly 100% of dAK and dGK should be in the heterodimeric form according to this $K_d$. 
Purified dCK protein was used to titrate dAK, in the presence of saturating dCyd (400 μM) to block detection of the secondary dAK activity from heterodimeric dCK. Hence, the measured dAK activity was solely from heterotropically activated dAK of the formed heterodimer and served to monitor the progress of the titration. The data were fitted by SigmaPlot™ with Equation 2.2.

Figure 3.5: Estimation of the dissociation constant ($K_d$) for the heterodimer.
dCK subunit can also take the place of dGK in dAK/dGK heterodimers. Such displacement was detected by dCyd’s (instead of dGuo’s) ability to heterotropically activate dAK’s activity (Table 3.2). dGK in dAK/dGK has weak affinity for dCyd, therefore, 0.4 mM dCyd activated dAK of dAK/dGK only 20-45%. On the other hand, dCyd activated dAK of dAK/dCK 5 to 6-fold.

3.5 Thermostability of dAK and dGK.

In the absence of any ligands, the thermostability of dAK and dGK was different from each other. With ATP removed from dAK/dGK samples with Bio-Spin 6, the kinases were incubated either at room temperature (23°C) or at 37°C, at different pH conditions (6.6, 7.5, and 9.6). Aliquots were taken after different incubation intervals, diluted 120-fold, and assayed for dAK and dGK activities in Tris-HCl buffer (pH 8.0) at room temperature. Figure 3.6 shows that incubation temperature had a much more profound effect on the enzyme stability than the chosen pH had.
Protein Component  | dAK Activity (CPM) | dAK Activity (CPM) in the Presence of dCyd (400 μM) | Heterotropic Activation by dCyd (400 μM) \\
---|---|---|---
 dAK/dGK | 1675 | 2382 | 1.4 \\
 dCK | 20379 | 269 | - \\
 dAK/dGK + dCK | 25875 | 9860 | 5.9 \\

Table 3.2: dCK subunit can take the place of dGK in dAK/dGK heterodimers.
Figure 3.6: Differential thermostability of dAK and dGK.
At room temperature, both dAK and dGK were stable. But at 37°C, dAK lost more than 80% of its activity within the first 5 minutes, but dGK lost less than 40% of its activity within the same period, and showed no further dramatic activity loss during the following 2 or more hours. This two-phased loss of dGK activity, as well as contrasting patterns of dAK and dGK inactivation, suggests that a heterodimer dissociation process might be involved in the thermo denaturation. Moreover, it seems likely that unfolding of dAK was either the cause or the consequence of the heterodimer dissociation. The dissociated dAK could not be “rescued" by the addition of excess dGK in the assay condition (Figure 3.7), indicating thermal denaturation.

The above observations may shed light on the observed lability of dAK during conventional purification processes (82,86,89). This lability is somewhat puzzling when one recognizes that dGK is actually more labile than dAK when under attack by the chemical cross-linking reagent DFDNB (to be shown later), by the chemical modification reagent Rose Bengal (86), or by tryptic digestion (94). Such tendency for dAK to be differentially inactivated during purification might be explained if, during conventional chromatographic procedures (usually in the absence of any biological ligands), dAK subunits tend to dissociate from dGK and be denatured.
Figure 3.7: dAK inactivation at 37°C involved thermal denaturation.
3.6 Chemical cross-linking studies.

To identify interfacial domains, 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Figure 3.8), with an arm-length of only 3 Å, was used to cross-link dAK and dGK subunits in the normal heterodimer. Under the experimental conditions, the activities of dAK and dGK should be stable for two hours, but upon the addition of DFDNB (0.05 mM), dGK lost its activity more quickly than dAK (Figure 3.9). Within one hour, more than 80% of the dGK activity was lost, while only 50% was lost from dAK.

This difference between dAK and dGK parallels their relative rates of inactivation by Rose Bengal-induced photo oxidation (86), or trypsin proteolysis, and also reflects their opposite response to dilute chaotropic salts (94). DFDNB is a reagent with a preference for -NH₂ and phenolic groups, but is also capable of reacting with sulfhydryls (present in neither dAK nor dGK) and imidazole groups (125). The differential inactivation of dAK and dGK could be due to their contrasting conformation, or different reactivity of the amino acid side chain at a particular position, or both.

A successful cross-linking event requires the same cross-linker to react with both subunits. But some of the cross-linkers may have reacted
Figure 3.8: The structure of DFDNB.
The dAK and dGK activities of heterodimeric dAK/dGK (0.1 mg/ml) were monitored over time, under the cross-linking conditions (15 mM potassium phosphate, 10% glycerol, pH 8.0), either with DFDNB or without it (as a control).

Figure 3.9: Time-course of dAK and dGK inactivation by DFDNB.
with only one subunit, and thus prevented such a site from being linked to the other subunit via another linker molecule. Unfortunately, an alternative reagent, DTSSP (3,3'-Dithiobis[sulfosuccinimidyl-propionate], spacer arm 12Å), gave even lower cross-linking efficiency (data not shown).

Cross-linking by DFDNB was inhibited by substrates such as ATP and dAdo, and by both end-product inhibitors, dATP and dGTP (Figures 3.10, 3.11, and 3.12) (Table 3.4). Since both subunits have an ATP site, it is not clear if the effect of ATP on crosslinking is a function of both active sites or if it is mediated through a single subunit. Figure 3.13 shows that ATP protected both dAK and dGK against inactivation by DFDNB.
From left to right, Lane 1: Low-range molecular weight standards (Bio-Rad); Lane 2: dAK/dGK + DFDNB; Lane 3: dAK/dGK + DFDNB + ATP; Lane 4: dAK/dGK + DFDNB + AMP; Lane 5: dAK/dGK; Lane 6: Low-range molecular weight standards; Lane 7: Prestained low-range molecular weight standards (Bio-Rad). Ligand concentrations are indicated in Table 3.3. Top arrow indicates the cross-linked dAK/dGK. The bottom arrow indicates the monomeric dAK/dGK.

Figure 3.10: ATP reduced the cross-linking efficiency.
From left to right, Lane 1: Low-range molecular weight standards; Lane 2: dAK/dGK + DFDNB + ATP; Lane 3: dAK/dGK + DFDNB + AMP; Lane 4: dAK/dGK + DFDNB + dATP; Lane 5: dAK/dGK + DFDNB + dGTP; Lane 6: dAK/dGK + DFDNB; Lane 7: dAK/dGK + DFDNB + dGuo; Lane 8: dAK/dGK; Lane 9: Molecular weight standards; Lane 10: Prestained molecular weight standards. Ligand concentrations are indicated in Table 3.3. Top arrow indicates the cross-linked dAK/dGK. The bottom arrow indicates the monomeric dAK/dGK.

Figure 3.11: dATP and dGTP reduced the cross-linking efficiency.
From left to right, Lane 1: Molecular weight standards; Lane 2: dAK/dGK; Lane 3: dAK/dGK + DFDNB; Lane 4: dAK/dGK + DFDNB + ATP; Lane 5: dAK/dGK + DFDNB + dAdo; Lane 6: dAK/dGK + DFDNB + dGuo; Lane 7: Molecular weight standards; Lane 8: Prestained molecular weight standards. Ligand concentrations are indicated in Table 3.3. Top arrow indicates the cross-linked dAK/dGK. The bottom arrow indicates the monomeric dAK/dGK.

Figure 3.12: dAdo, but not dGuo, reduced the cross-linking efficiency.
Table 3.3: The effect of substrate or end-product inhibitor on the cross-linking efficiency, as quantitated by SigmaGel software.

<table>
<thead>
<tr>
<th>substrate or end-product inhibitor</th>
<th>cross-linking efficiency (%) by DFDNB (0.05 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>20</td>
</tr>
<tr>
<td>dGTP (1.6 mM)</td>
<td>3</td>
</tr>
<tr>
<td>dATP (1.6 mM)</td>
<td>6</td>
</tr>
<tr>
<td>ATP (10 mM)</td>
<td>6</td>
</tr>
<tr>
<td>dGuo (1.0 mM)</td>
<td>21</td>
</tr>
<tr>
<td>dAdo (1.0 mM)</td>
<td>7</td>
</tr>
<tr>
<td>AMP * (30 mM)</td>
<td>25</td>
</tr>
</tbody>
</table>

* As a control.
The cross-linking conditions were the same as indicated in Figure 3.9, except that 10 mM ATP was added in order to protect dAK/dGK against inactivation as indicated. The enzyme was subjected to 100-fold dilution immediately before the activity assay.

Figure 3.13: ATP protected both dAK and dGK against DFDNB inactivation.
Chemical cross-linking with a 3 Å linker offered an opportunity to study the interface of the heterodimeric dAK/dGK. By analogy with Ras-GAP interactions, the dGK subunit contacts the dAK subunit at dAK's active site affecting the transfer of the phosphoryl group. Hence, ATP as well as dATP should shield the triphosphate binding site and possibly prevent the chemical cross-linking. And this was found to be the case, along with the protection by dAdo. However, such observations do not provide the direct proof. It can still be argued that cross-linking events can be hindered allosterically through conformational changes induced by the ligand binding. Such arguments and the analogy are not mutually exclusive. Thus, the subunit cross-linking studies at least provided a definite picture, but at a low-resolution, of a conformational change occurring across the subunit-interface.
CHAPTER 4
IDENTIFYING THE STRUCTURAL ELEMENT PARTICIPATING IN THE HETEROTROPIC ACTIVATION

4.1 The effect of site-directed substitution, P155A or S156T, on dAK of heterodimeric dAK/dGK.

4.1.1 Introduction.

Analysis of the amino acid sequences derived from the tandem $dak$-$dgk$ genes reveals that dAK and dGK both contain a segment (residues 153-161 at site iv) homologous, in varying degrees, to the G-2 (switch 1) sequence of Ras proteins of the GTPase superfamily (Figure 4.1) (74). This type of sequence is so far unknown in any other deoxynucleoside kinase.

Within each GTPase family, the amino acid sequences corresponding to the G-2 region are highly conserved, but sequences vary
dAK or dGK's Ras G-2-like site iv is only a few residues downstream from the arginine-rich region, site iii. The P-loops of both dAK or dGK (site I) and of Ras (G-1 Sequence) are very near the N-terminus of each polypeptide.

Figure 4.1: Comparison of conserved sequences at sites I and iv of dAK and dGK with the G-1 and G-2 consensus sequences of Ras-like proteins.
between families within the superfamiiy. However, an invariant element of the G-2 sequence in all the GTPase families is the threonine residue interacting with the metal ion and the γ-phosphate (97,126). Noticeably, dAK and dGK have a serine and threonine residue respectively at position 156.

Interestingly, this Ras switch 1-like sequence of dAK is only 6 residues away from the arginine-rich site iii (residues 140-146). This arginine-rich region is also highly conserved in nearly all the other deoxynucleoside kinases sequenced so far. Arg 222 within this region of HSV-1 TK has been shown to contact the phosphates of ATP (56), and the corresponding arginine residue in site iii of dAK and dGK likely has a similar function (74). In an apparently equivalent manner, the transition state is stabilized by an arginine residue within the G-2 sequence among the α-subunits [e.g., G\textsubscript{ia1}] of G proteins (97,126-128). However, this catalytically important Arg is missing from the corresponding G-2 (switch 1) sequence of Ras (97,126,129), and provided to Ras in trans by its GAP (123). The association with GAP as well as the binding of GTP-Mg\textsuperscript{2+} is a function of this G-2 (switch 1) sequence of Ras proteins (97,130). The switch 1-loop of Ras changes its conformation upon the interaction with GAP. The proline residue within this sequence is structurally important
because it divides the conformational change of the loop into two parts, with the Cα of the proline residue remaining in the same position during the Ras-RasGAP interaction (Figure 4.2).

4.1.2 Effects of the substitutions.

Upon confirming the desired mutagenesis by DNA sequencing (Figures 4.3 and 4.4), the unaltered dAK/dGK strain, the dAK(P155A)/dGK strain, and the dAK(S156T)/dGK strain were cultured and harvested in parallel, the kinases were partially purified through ammonium sulfate precipitation, and dAK and dGK activities analyzed.

The dGK activities from all three strains were the same (~30 units/mg), whereas the dAK activity of dAK(P155A)/dGK was elevated 2-3 fold compared either with the wild type or with dAK(S156T)/dGK. Table 4.1 shows the effects of mutagenesis on the heterotropic activation. For dAK(P155A)/dGK, the dAK activity was permanently cis-activated 2 fold, with the heterotropic activation effect of dGuo accordingly reduced 2 fold.
Figure 4.2: The Cα of the proline residue remains in the same position during the Ras-RasGAP interaction.
TTTGAACAAATTATCAACCGATGCAAGTTTAAAA

The arrow indicates the mutagenic base at position 463. Nucleotides are numbered according to Figure 1 of (74).

Figure 4.3: DNA sequencing of dAK(P155A).
The arrow indicates the mutagenic base at position 467. Nucleotides are numbered according to Figure 1 of (74).

Figure 4.4: DNA sequencing of dAK(S156T).
### Table 4.1

<table>
<thead>
<tr>
<th>dAK Source</th>
<th>Relative dAK Activity</th>
<th>Relative dAK Activity</th>
<th>Heterotropic Activation Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without dGuo</td>
<td>With dGuo*</td>
<td></td>
</tr>
<tr>
<td>dAK/dGK</td>
<td>1.0^b</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>dAK(P155A)/dGK</td>
<td>2.0</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>dAK(S156T)/dGK</td>
<td>1.0^b</td>
<td>4.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

^a dGuo concentration was 400 µM.

^b dAK specific activity was 3 units/mg.

Table 4.1: The dAK activity of dAK(P155A)/dGK was permanently activated two fold, with a corresponding decrease in the heterotropic activation factor.
Both the wild type and the mutant dAK activities had the same maximum turnover potential when heterotropically activated, and there was only a 10% difference in the $K_m$ (dAdo) between wild type and dAK(P155A) (Table 4.2).

Since the Ras switch-1 loop changes its conformation during the Ras-RasGAP interaction, alteration of its conformation in dAK should affect subunit interaction and substrate turnover, if dAK-dGK interaction parallels that of Ras-RasGAP. Presumably, a proline/alanine substitution on dAK increased loop flexibility and altered dAK's interaction with dGK in a way partially mimicking the impact on dAK when dGuo binds to dGK. But the lack of an effect of the S156T mutation on dAK indicates that this particular amino acid replacement was not sufficient to convert dAK's conformational state to dGK's.

The effect of such a mutation on dAK is not surprising. Point mutations of subunit interface regions in other allosteric proteins, such as dimeric glutathione reductase from *E. coli* (131) and tetrameric pyruvate kinase of yeast (132), also alter protein conformation, intersubunit communication, and hence allosteric behavior and catalysis.
Table 4.2: $K_{m, app}(\text{dAdo})$ and $K_{m, app}(\text{ATP})$ of dAK in unmodified recombinant dAK/dGK and dAK(P155A)/dGK.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$K_{m, app}(\text{dAdo})$ ($\mu\text{M}$) $^a$</th>
<th>$K_{m, app}(\text{ATP})$ ($\mu\text{M}$) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAK/dGK</td>
<td>6.0±0.5</td>
<td>209±16</td>
</tr>
<tr>
<td>dAK(P155A)/dGK</td>
<td>6.7±0.4</td>
<td>160±18</td>
</tr>
</tbody>
</table>

$^a$ The average of three independent measurements.
4.2 The Effects of Mg$^{2+}$ and Mn$^{2+}$ on the kinase activities and heterotropic activation.

In Ras proteins, the switch-1 loop is involved in the metal ion and phosphate group binding, as well as in the Ras-RasGAP interaction. Table 4.3 compares the different requirement of dAK and dGK on metal ions. dGK had a stricter requirement for Mg$^{2+}$ in that the dGK activity was inactivated more than 60% in the presence of Mn$^{2+}$. But dAK exhibited a better tolerance for a different metal ion Mn$^{2+}$, retaining 80% of its activity when Mg$^{2+}$ was replaced. In addition, dAK's heterotropic response was correspondingly reduced. Such a comparison again reveals the conformational difference between dAK and dGK, with dAK's active site being less optimal and hence less demanding.
Table 4.3: The Effects of Mg$^{2+}$ and Mn$^{2+}$ on the kinase activities and heterotrophic activation.
4.3 Identifying the structural element involved in the intersubunit communication and heterotropic activation through chimera constructions.

4.3.1 Introduction.

Conformational changes occur at the intersubunit contact during the heterotropic activation, as revealed by limited proteolysis (94) and by the adverse effects of dAdo, dATP, dGTP, and ATP on the cross-linking efficiency (120). Both dAK and dGK contain, to different extents, a Ras G-2 loop (switch I)-like sequence (residues 153-161) immediately following an arginine-rich site (residues 140-146) identical between dAK and dGK. A mutation of the structurally important proline 155 within the Ras-like sequence of dAK permanently cis-activated dAK half-way towards its maximum activity potential, with a corresponding reduction in the magnitude of the heterotropic trans-activation by dGuo (120).

Studies of the Ras-RasGAP interactions have shown the switch I region to be at the protein-protein interface, and a part of the Ras GTPase active site (123). By analogy, we hypothesize that the Ras-like sequence
is involved in dAK/dGK intersubunit communication, specifically in heterotrophic activation.

Human dCK (60), dGK (70,71) and TK2 (133) have all been cloned, and they share sequence homology with the deoxynucleoside kinases from \textit{L. acidophilus} R-26 (133). Their subunits are of similar size, and they share sequence homology along the entire span of the polypeptide, and specifically at the P-loop, the D(E)RS motif, and the arginine-rich site (74). Unlike the \textit{Lactobacillus} kinases, however, those human kinases do not possess the Ras switch I-like sequence. Instead, just after the arginine-rich site they have a leucine-rich sequence which is thought to be involved in subunit-subunit interaction (72), but no experimental data have so far been presented illustrating the functions of this leucine rich sequence in the human enzymes.

To investigate the dAK polypeptide segment involved in the \textit{Lactobacillus} dAK/dGK subunit interface communication and its effects on the dAK catalysis \textit{in cis}, a peptide segment from residues 120 to 170 on dAK was replaced by its counterpart from dGK. Upon such a "transplant," the dAK activity was elevated about 40%, with its heterotropic response toward dGuo abolished. Moreover, the chimeric "dAK" started to behave
like dGK with a decreased affinity for MgATP. The data presented below demonstrate that at least one of the peptide segment involved in the intersubunit communication has been located.

4.3.2 The carboxyl-terminus of dGK downstream of residue 170 was not responsible for dGK's substrate specificity nor catalytic turnover rate.

Chimera-1 (Chi-I), encoded by Chi-I (Figure 2.2 A), contains the N-terminus of dAK and the C-terminus of dGK, joined at the residue 170. This chimera behaved like the independently-expressed unmodified dAK. Like dAK, Chi-I was inactive when expressed alone, but regained its dAK activity upon heterodimerizing with dGK (or dCK), and could be further activated heterotropically by the deoxynucleoside of its heterologous partner (Table 4.4).

Upon the P155A substitution, the heterotropic activation of Chi-I (P155A) was reduced by half (Table 4.4). Expressed alone, however, Chi-I (P155A) was still inactive, just like Chi-I and the unmodified dAK. Therefore, although the P155A substitution in dAK/dGK elevated the dAK
<table>
<thead>
<tr>
<th>Protein Composition</th>
<th>Heterotropic Activation Fold</th>
<th>Heterotropic Activation Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>When Heterodimerized with dGK</td>
<td>When Heterodimerized with dCK</td>
</tr>
<tr>
<td>dAK</td>
<td>3.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Chi-I</td>
<td>2.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Chi-I (P155A)</td>
<td>1.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 4.4: Comparisons among dAK, Chi-I and Chi-I (P155A).
basal activity (i.e., the activity before heterotrophic activation) two fold, the
dAK with the P155A substitution would also be inactive if expressed
without dGK.

A reciprocal chimera, heterodimeric Chi-I' encoded by Chi-l' (Figure
2.2 B), was constructed. In this case, unmodified dAK heterodimerizes with
a chimeric subunit, that contains the N-terminus of dGK spliced to the C-
terminus of dAK at residue 170. This chimera behaved essentially like
unmodified dAK/dGK, in terms of the ratio of the dGK and dAK basal
activities and the ability of dGuo to activate dAK (Table 4.5).

Since dAK is inactive by itself, and because dGK is only partially
active when expressed alone, the nearly unaltered ratio of dGK and dAK
basal activities suggests that the affinity between the two heterologous
subunits within Chi-l' could be about the same as the affinity between the
unmodified dAK and dGK subunits.

Restriction mapping analysis of the Chi-l' construct is shown in
Figure 4.5.
Table 4.5: Chi-1’ behaved essentially the same as dAK/dGK.
From left to right, Lane 1: λ/Hind III markers; Lane 2: Chi-I' + Sty I; Lane 3: dak-dgk + Sty I; Lane 4: Chi-I' + Nis I; Lane 5: dak-dgk + Nis I; Lane 6, λ/Hind III markers.

Figure 4.5: Restriction mapping analysis of pBlueScript KS (+) containing Chi-I'.

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4.3.3 The structural elements determining the substrate specificity and dAK's self-activity must reside N-terminal to residue 120.

The chemical cross-linking studies showed that conformational changes occur at the subunit interface during heterotropic activation, or that the subunit interface contributes directly to the active site of dAK (120), or both. A conformational change involving the Ras switch I-like sequence on the dAK subunit has also been implicated in the heterotropic activation of dAK. To further probe the peptide segments affecting the inter-subunit communication between dAK and dGK (or dCK), Chimera-I (Chi-I) encoded by Chi-I (Figure 2.2 C) was constructed. Figure 4.6 shows the restriction mapping analysis of the Chi-I construct, and Figure 4.7 shows the representative DNA sequence.
From left to right, Lane 1: \(\lambda/Hind\) III markers; Lane 2: \(Chi-II + Xba\) I; Lane 3: \(dak + Xba\) I; Lane 4: \(dak + Xba\) I; Lane 5: \(dak-dgk + Xba\) I; Lane 6: \(dgk + Xba\) I; Lane 7: pBlueScript vector only; Lane 8: \(\lambda/Hind\) III markers.

Figure 4.6: Restriction mapping analysis of pBlueScript KS (+) containing \(Chi-II\).
GCACCTGGTAATCCAAAGAAGAATCCTGAT
↑
The arrow indicates the connection between Nucleotide 360 and Nucleotide 1030. Nucleotides are numbered according to Figure 1 of (74).

Figure 4.7: Representative DNA sequencing data of Chi-II.
Chi-II is a hybrid construct with the N-terminal half of dAK joined at residue 120 with the C-terminal half of dGK. Compared with Chi-I (spliced at residue 170), 50 more dAK's amino acid residues have been replaced by the counterparts from dGK. This advance towards the N-terminus now includes conserved motifs such as the Ras switch I-like sequence (residues 153 to 161, different between dAK and dGK) and the arginine-rich site (residues 140 to 146, identical between dAK and dGK).

Interestingly, Chi-II was still inactive when expressed alone, and exhibited the dAK activity only when reconstituted into a heterodimer with either dCK or dGK. Therefore, the structural elements responsible for dAK's substrate specificity and for dGK's being self active (i.e., being active when expressed without dAK) must reside further upstream of residue 120. Unlike dAK or Chi-I, whose reconstituted dAK activity could be completely activated heterotropically, Chi-II's reconstituted dAK activity did not undergo heterotropic activation (Table 4.6).
Protein Construct | Relative dAK Activity Upon Heterodimer Reconstitution | Activation Fold by dCyd (400 μM)
---|---|---
dAK | 1.0$^b$ | 3.8$^b$
Chi-II | 1.4±0.4$^c$ | 1.2±0.3$^c$

$^a$ The heterodimer reconstitution was carried out with excess amount of dCK.

$^b$ The data were obtained from duplicates of one colony.

$^c$ The data were obtained from duplicates of three independent colonies.

Table 4.6: Chi-II no longer responded to the heterotropic "activator" after heterodimer reconstitution.
Chi-II’s inactivity impeded further kinetic studies, but helped in estimating the affinity between Chi-II and dCK. Earlier, the apparent dissociation constant ($K_a$) of the unmodified dAK/dCK was determined to be 40 nM, and the presence of dCyd did not affect the inter-subunit affinity (120). In the present study, in the presence of dCyd, the unmodified dAK and Chi-II were nearly equivalent in their affinity for dCK, the $K_a$ for Chi-II/dCK being about 100 nM (Figure 4.8).

4.3.4 The heterotropic activation of "dAK" in Chimera III was abolished.

In order to simplify the kinetic studies on Chi-II, Chi-III was constructed. Figure 4.9 shows the restriction analysis of Chi-III. The Chi-III (Figure 2.2 D) gene is a tandem construct, like dak-dgk (Figure 2.2 F), being transcribed into a poly-cistronic messenger RNA followed by then separate but presumably simultaneous translation of the two subunits. The subunits heterodimerize in vivo, either during or after translation. The peptide segment from residues 120 to 170 of the "dAK" subunit of Chi-III is substituted by the counterpart from dGK, while the dGK subunit of Chi-III is unmodified.
Figure 4.8: A comparison of dAK and Chi-II with regard to their affinities for dCK.
From left to right, Lane 1: \(\lambda/Hind\) III markers; Lane 2: Chi-III + Xba I; Lane 3: Chi-II + Xba I; Lane 4: dak-dgk + Xba I; Lane 5: \(\lambda/Hind\) III markers.

Figure 4.9: Restriction mapping analysis of pBlueScript containing the Chi-III gene.
The unmodified dAK/dGK and Chi-III were expressed in _E. coli_ and ammonium sulfate fractions prepared. While the dAK activity of dAK/dGK was heterotropically activated 4-fold by dGuo (at 400 \( \mu M \)) as usual, the dAK activity of Chi-III in the presence of dGuo was reduced by about 30% (Figure 4.10).

There are several possible reasons for this heterotropic deactivation:

a) dGuo binding to dGK may induce a conformational change, which in turn transmits to "dAK" of Chi-III across the interface, allosterically reducing the dAK activity; b) the "dAK" subunit of Chi-III may have a broad deoxynucleoside substrate specificity, allowing dGuo to compete with dAdo for the active site on "dAK", thus inhibiting the dAK activity isosterically; c) dGuo binding to dGK may cause partial dissociation of the otherwise associated "dAK" and dGK, reducing the dAK activity since "dAK" is inactive without associating with dGK or dCK.

The third possibility is discounted based on the lack of dCyd heterotropic activation observed for Chi-II when reconstituted into heterodimers with excess amount of dCK (Table 4.6). As shown above, in the presence of dCyd, Chi-II and dAK were equivalent in terms of their
Half of the reduction in the dAK activity of Chi-III occurred at a dGuo concentration corresponding to the $K_m$ (dGuo) value of dGK.

Figure 4.10: Differential responses of dAK activities from dAK/dGK and Chi-III towards increasing concentrations of dGuo.
affinity for dCK; with a molar excess of dCK and in the presence of dCyd, all the Chi-II subunits should be in the heterodimeric form with dCK. Therefore, the absence of the heterotropic activation of the reconstituted dAK activity of Chi-II (see Table 4.6) cannot be due to the heterodimer dissociation.

The second possibility was also excluded, according to the above mentioned observation (Figure 4.10). The dAK activity of Chi-III was deactivated half-way by dGuo at a concentration corresponding to the $K_m$ (dGuo) value (5-10 $\mu$M) of dGK. The "dAK" subunit in Chi-III could not have broadened its specificity to accommodate dGuo to any significant extent, since the dAK activity of Chi-III (assayed with 20 $\mu$M dAdo) would otherwise have been completely blocked by over 400 $\mu$M dGuo. Therefore, the apparent inhibition of the dAK activity must be due to dGuo binding to the dGK subunit.

The dGK subunit has a secondary dAK activity (100). Based on the secondary $K_m$ (dAdo) and $V_{max}$ of dAMP formation by the dGK subunit, the heterodimeric dGK can contribute about 30% of the total measured dAK activity from dAK/dGK. Therefore, the 30% inhibition of the dAK activity of Chi-III by dGuo is mostly due to the blocking of the secondary dAK activity.
of the dGK subunit. Consequently, the binding of dGuo to dGK has little, if any, effects on the "dAK" subunit of Chi-III. The heterotropic response of the "dAK" subunit has therefore largely been abolished by replacing the dAK polypeptide segment from residues 120 to 170 with the counterpart from dGK.

4.3.5 The "dAK" subunit of Chi-III had a relatively elevated dAK activity compared with the dAK of dAK/dGK.

By comparing the dAK activities from ammonium sulfate fractions of Chi-III and unmodified dAK/dGK, the dAK activity from Chi-III was found to be elevated by about 40% (Table 4.7).

Like the heterotropically activated dAK activity, this increased activity of "dAK" was also due to a $V_{\text{max}}$ effect since the $K_m$ values for the two substrates were not significantly changed (see below). With the abolishment of the heterotropic response for "dAK", this chimeric "dAK" would be expected to possess the maximum catalytic potential of an unmodified and heterotropically activated dAK. The 40% increase is less than the normal 300% activation, and one might suppose that this is due to
Table 4.7: The dAK activity of Chi-III was elevated while being subjected to apparent deactivation by heterotropic "activator".
weakened affinity between "dAK" and dGK. But this is unlikely, since Chi-II was essentially equivalent to dAK in its affinity for dGK (Figure 4.8). "dAK" of Chi-III was most likely equivalent to unmodified dAK in this regard as well.

The 40% increase in $V_{\text{max}}$ and the lack of significant changes of $K_m$ values together strongly suggest that the "dAK" polypeptide has not been detrimentally misfolded. In fact, the "dAK" subunit, with the residues 120-170 "transplanted" from dGK, has already started to behave like a dGK subunit in certain aspects.

4.3.6 Both the "dAK" subunit of Chi-III and the heterotropically activated dAK of dAK/dGK have weakened binding affinity for the substrate ATP-Mg$^{2+}$, like the dGK subunit.

The inhibition patterns of the bisubstrate analogue dATP (competitive with ATP and noncompetitive with dAdo, Figure 4.11) reveal that, for the "dAK" subunit in Chi-III, ATP-Mg$^{2+}$ was still the first substrate to bind. In this respect, the "dAK" subunit remains essentially a dAK subunit.
For the dAK activity of Chi-III, dATP was noncompetitive with dAdo (A), but competitive with ATP (B).

**Figure 4.11**: Inhibition patterns of the dAK activity of Chi-III by dATP.
However, unlike the double reciprocal plot of the unmodified dAK before heterotrophic activation (where the plot lines converged on the X-axis and $K_m = K_s$), the lines for the "dAK" converged on a point above the X-axis, due to a larger dissociation constant ($K_s$) for the Enzyme-MgATP complex (Figure 4.12 A, B, Table 4.8).

In other words, while dAK had a $K_m$ (ATP) value equal to $K_s$ (ATP), the "dAK" subunit of Chi-III had a largely unchanged $K_m$ (ATP) value but an increased $K_s$ (ATP). Most interestingly, heterotropically activated dAK also had an increased $K_s$ (ATP) value while its $K_m$ (ATP) remained unaltered (Figure 4.12 C, Table 4.8). Furthermore, dGK of dAK/dGK also had a $K_s$(ATP) larger than its $K_m$(ATP) (Figure 4.12 D, Table 4.8). Therefore, both the unmodified but heterotropically activated dAK and the "dAK" of Chi-III have started to behave like dGK in their weakened affinity for the substrate ATP-Mg$^{2+}$. 
Figure 4.12: Representative double reciprocal plots for kinase activities of different subunit constructs.
Subunit Type & $K_m$ (MgATP) ($\mu$M)$^a$ & $K_s$ (MgATP) ($\mu$M)$^a$ & $K/K_m$ \\
--- & --- & --- & --- \\
dAK of dAK/dGK & 170 (103$^b$) & 186 (120$^b$) & 1.1 (1.2$^b$) \\
"dAK" of Chi-III & 366 (68$^b$) & 1786 (2982$^b$) & 4.9 (43.9$^b$) \\
dAK of dAK/dGK +dGuo & 207 & 520 & 2.5 \\
dGK of dAK/dGK & 315 & 1945 & 6.2 \\

$^a$ Average values of at least two independent experiments on ammonium sulfate fractions of kinases, unless otherwise indicated.

$^b$ Values from a single experiment on gel filtration purified kinases

Table 4.8. Comparisons of $K_m$ (MgATP) and $K_s$ (MgATP) values of different subunits.
4.3.7 pH Dependence of the kinase activities.

Figure 1.7 shows the effect of heterotropic activation on the pH dependence of the dAK activity. The presence of dCyd induces the second maximal dAK activity around pH 7.8. Such a pattern was reproduced in a different buffer system using TES and CHES (Figure 4.13, A and B). Moreover, the dAK activity of Chi-III also showed a second pH optimum around pH 8, in the absence of any heterotropic ligand (Figure 4.13, C and D). Such an observation provides an additional dimension in comparing the heterotropically activated dAK with the chimeric "dAK" of Chi-III. Elucidating the structural and kinetic basis of such a pH-dependence pattern will help better understand the kinase reaction mechanism.

Heterodimerization of dCK with dAK also induced a second maximal dCK activity around pH 8 (Figure 4.14, A and B). Is it possible that both heterotropic activation and heterodimerization induce similar structural and kinetic changes?
A, dAK of dAK/dCK; B, Heterotropically activated dAK of dAK/dCK; C, dAK of dAK/dGK; D, "dAK" of Chi-III.

Figure 4.13: pH dependence of the dAK activity.
A, dCK of dCK/dCK; B, dCK of dAK/dCK.

Figure 4.14: pH dependence of the dCK activity.
4.3.8 G proteins, HSV-1 thymidine kinase, adenylate kinase, and *Lactobacillus* deoxynucleoside kinases----a case of a Rosetta Stone.

The three dimensional structures of G proteins (134,135), adenylate kinases and HSV-1 thymidine kinase (54-56) have all been well revealed and correlated to their catalytic functions. However, a better understanding of the structure-function relationship of the *Lactobacillus* deoxynucleoside kinases still awaits a high resolution X-ray solution. Meanwhile, sequence conservations shared among those proteins provide some clues as to which portions of the *Lactobacillus* kinases are of functional importance. Part of the inferred information is consistent with the results previously reported of the *Lactobacillus* kinases, while other inferences suggest intriguing future experimental exploration.

Each subunit of the heterodimeric *Lactobacillus* deoxynucleoside kinases has an arginine rich motif followed immediately by a Ras switch I-like sequence. This arginine rich motif is also conserved in HSV-1 TK (Figure 4.15) (along with its family members human dCK, dGK and TK2) and has been shown to interact with the ATP molecule (56). In G\(\alpha_{11}\), one of the \(\alpha\) subunits of the heterotrimeric G proteins, transition state stabilization
<table>
<thead>
<tr>
<th>Residues</th>
<th>dAK (74)</th>
<th>dGK (74)</th>
<th>Ras (104,123)</th>
<th>G_{i1} (127)</th>
<th>HSV-1 TK (56)</th>
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<tr>
<td>8 GPIGAGKS</td>
<td>78 DRS</td>
<td>140 RIQKRGR</td>
<td>153 TDPSLKD</td>
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<td>dGK (74)</td>
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<td>G_{i1} (127)</td>
<td></td>
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<tr>
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<td>HSV-1 TK (56)</td>
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<tr>
<td>56 GPHGMGKT</td>
<td>162 DRH</td>
<td>216 RLAKRQR</td>
<td>HSV-1 TK (56)</td>
<td></td>
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</tr>
</tbody>
</table>
is carried out by arginine 178 within G\(\alpha_1\)'s own switch I (G-2) sequence (127) (Figure 4.15). Ras protein does not possess this counterpart arginine residue in its own switch I (Figure 4.15), but arginine 789 inserted by GAP almost identically mimics the position of arginine 178 of G\(\alpha_1\) (123). Like G\(\alpha\) subunits, *Lactobacillus* kinases possess *in cis* arginine residues presumably also involved in transition state stabilization. This arginine-rich motif is followed immediately by a Ras switch I-like sequence (Figure 4.15). dAK exhibited a weakened affinity for MgATP upon heterotropic activation, and Chi-III's abolishment of heterotropic activation was also accompanied by a decreased affinity between "dAK" and MgATP (Figure 4.12) (Table 4.8). Both examples suggest that heterotropic activation may involve a conformational change that shifts the binding preference away from the ground state, with the formation of intermediate enzyme species along the kinetic pathway.

In G proteins, a highly conserved aspartate residue from G-3 participates in coordinating Mg\(^{2+}\) through a water molecule (134) (Figure 4.15). A similar function is carried out in HSV-1 TK by aspartate 162 of Motif-3 (\(^{161}\)FDRHP) (56) (Figure 4.15). This aspartate 162 (55) is at the same position as aspartate 93 of adenylate kinase (136) and aspartate 80 (from G-3) of *E. coli* elongation factor Tu (one of the G proteins) (137). At
first glance, *Lactobacillus* deoxynucleoside kinases do not appear to have G protein's G-3-like sequences, yet together with HSV-1 TK (Figure 4.15) and human dCK, dGK and TK2, they share the D(E)RS(H) motif (70-72,133). The *Lactobacillus* deoxynucleoside kinase activity was virtually eliminated when the aspartic residue in the $^{78}$DRS motif was mutated to alanine, glutamate, or asparagine residue (99). In HSV-1 TK, 10 residues downstream of aspartate 162, tyrosine 172 stacks against the thymine base of the substrate, and this tyrosine residue can be functionally replaced by phenylalanine (138). In *Lactobacillus* deoxynucleoside kinases, as well as in the human kinases, 10 residues downstream of aspartate (glutamate) residue of the D(E)RS motif also lies the residue phenylalanine 88. The functional importance of this phenylalanine residue remains to be assessed by mutagenic or X-ray studies.

According to the data available from mutagenesis (99,120) and from the chimera constructions presented above, as well as the presence of conserved motifs, heterodimeric *Lactobacillus* deoxynucleoside kinases possess at least three out of the five nucleotide binding motifs -- or their functional equivalents -- commonly identified in G-proteins. Listed in order from N- to C-terminus, those three motifs in *Lactobacillus* kinases are the
P-loop, the G-3 equivalent DRS motif, and the Ras G-2 (switch I)-like sequence. During the Ras-GAP interaction and its induced GTPase action, the conformational changes in switch I and switch II (which is just downstream of G-3) are coupled, since both regions are involved in binding ATP and Mg\(^{2+}\) (135) (Figure 4.16). A 50-amino acid segment of *Lactobacillus* dAK including the Ras switch I-like sequence is involved in the intersubunit communication, affecting dAK’s activity in cis. An additional chimera (Chi-IV, encoded by *Chi-IV*, Figure 2.2 E) was constructed such that dAK was spliced to dGK at residue 65 just N-terminal to the \(^{78}\)DRS motif. The DNA sequence analysis is shown in Figure 4.17.

Unfortunately, this new chimera was inactive even when reconstituted into heterodimers with dGK or dCK. Splicing at this position must have created a distortion in the dAK tertiary structure rendering it inactive.

A question still remains as to which segment on dGK is the *in trans* structural element for dAK, transmitting the conformational change to dAK upon heterotropic activation. Intriguingly, an R79K substitution on dGK has a profound stimulatory effect on dAK’s catalytic turnover rate (99). Is dGK’s \(^{78}\)DRS motif, upon modification, undergoing a conformational change
GDP and AlF$_3$ mimic the transition state of GTP hydrolysis at the active site of the Ras-RasGAP complex. [Figure adapted from (123).]
AACCACCATTAGCTAGTATTAAGTCAGCC

The arrow indicates the connection between Nucleotide 192 and Nucleotide 862. Nucleotides are numbered according to Figure 1 of (74).

Figure 4.17: Representative DNA sequence data for Chi-IV.
propagated to dAK, or is dGK's 79DRS directly participating in dAK's catalysis *in trans*? X-ray data are indispensable to answer this question.
CHAPTER 5

THE N-TERMINAL SEQUENCE OF dAK EXPRESSED IN *E. coli*

5.1 The N-terminal amino acid sequences of dAK and dGK expressed in *E. coli*.

dAK/dGK purified through ammonium sulfate precipitation, gel filtration chromatography, and Mono-Q anion-exchange chromatography were separated into subunits on SDS-PAGE employing the MZE 3328.IV buffer system. The two polypeptides were then electroblotted onto Immobilon-P. Upon staining, the amount of dAK appeared to be only 1/10 of dGK (data not shown). This difference was not caused by the differential retention of polypeptides by the membrane, because an SDS-PAGE gel of the same preparation also revealed the protein amount difference (data not shown). The preferential loss of dAK subunits most likely have occurred during anion-exchange chromatography. The membrane sections containing the desired polypeptides were excised and
submitted to the Ohio State University Biochemical Instrument Center for
the N-terminal amino acid sequence analysis. The concurrent quantitation
of each amino acid residue confirmed the 10-fold difference in the peptide
amount. The analyzed N-terminal amino acid sequences of dAK and dGK
are shown in the second column of Table 5.1.

In comparison with the amino acid sequences deduced from the dak
and dgk genes, the first methionine residue of both polypeptides was
cleaved from the kinase polypeptides expressed in E. coli. This
observation is consistent with the finding that Thr in the second position
tends to stimulate the removal of the initial Met (139). When Ma et al.
(100) deleted the second and third codons of the dgk gene to create a
synthetic dck gene, the expressed dCK polypeptide retained its initial Met;
Ile at the second position apparently instructed the retention of the initial
Met (139).
<table>
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<th>Subunit</th>
<th>Sequenced N-terminus</th>
<th>Calculated MH*</th>
<th>MALDI MH*</th>
<th>Δ</th>
<th>Nanospray MH* ± σ</th>
<th>Δ</th>
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<tr>
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<td>MIVLSG...</td>
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<td>24,499</td>
<td>0</td>
<td>24,501 ± 1.7</td>
<td>+2</td>
</tr>
<tr>
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<td>+5</td>
<td>24,500 ± 2.3</td>
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<td>dGK of Cloned dAK/dGK</td>
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<td>26,194</td>
<td>+9</td>
<td>26,187 ± 2.8</td>
<td>+2</td>
</tr>
</tbody>
</table>

Table 5.1: Comparisons between wild-type and cloned dAK subunits.
5.2 MALDI and nanoelectrospray mass spectrometry of dAK and dGK expressed in *E. coli*.

Heterodimeric dAK/dGK was purified to at least 85% purity through ammonium sulfate precipitation, gel filtration chromatography, and Blue-Sepharose pseudo-affinity chromatography. The dAK and dGK subunits were then separated on HPLC-RF chromatography (Figure 5.1). The subunits were finally subjected to molecular mass measurements by MALDI and nanoelectrospray mass spectrometry, and the results are summarized in Table 5.1.

N-terminal processings of dAK are different in *E. coli* and in *L. acidophilus* R-26. For the native dAK expressed in *L. acidophilus* R-26, the second and third amino acid residues are apparently deleted and the initial Met is retained or regained. Therefore, the recombinant dAK and native dAK differ at their extreme N-termini. However, both dAK polypeptides exhibit the same substrate specificity and $K_m$ values, the same basal dGK and dAK activity ratio, and the same degree of heterotropic activation by dGuo. The second and third amino acid residues are therefore not responsible for the dAK specificity and heterotropic activation by dGuo.
Figure 5.1: Separation of dAK and dGK subunits on HPLC-RF chromatography.
(This is surprising because the same residues determine the substrate specificity of dGK and dCK.) But still one question remains: is the presence of these two residues the cause of dAK being inactive when expressed alone? It is necessary to delete the second and third codons of \textit{dak} to unambiguously answer this question. However this has not been done in this study, because it has long been observed that native dAK polypeptides were labile during conventional purifications. The native dAK will very likely be inactive without dGK, just like the cloned dAK expressed in \textit{E. coli}. 
CHAPTER 6

CONCLUSIONS, DISCUSSION, AND HYPOTHESES

6.1 Recent revelations on Ras-RasGAP interactions.

The mechanism of GTPases activation by their respective GAPs has recently been elucidated (123,140,141). Crystallographic studies on such complexes as Ras-RasGAP, G\textsubscript{a}-RGS4, and RhoA-RhoGAP (GAPs in bold) convincingly reveal convergent forces shaping three structurally divergent proteins into GTPase Activating Proteins (GAPs) such that, in complex with their respective G-proteins, they carry out GTP hydrolysis by nearly identical mechanisms! Based on the studies of those three complexes, two aspects of the efficient GTP hydrolysis mechanism have been understood: a) An arginine finger pointed from those "morphologically different hands" (142) occupies an almost identical position in each to stabilize the transition state of GTP hydrolysis; b)
Stabilization of additional active site residues from switch I and switch II (such as the threonine and glutamine residue, respectively) is achieved upon the G-protein and GAP association, so that the attacking nucleophilic water molecule and the β-, γ-phosphates are more optimally aligned for the reaction. The convergence is not surprising given the common GTP-binding fold of those G-proteins.

6.2 Comparisons between *Lactobacillus* kinases and GTPases.

Deoxynucleoside kinases of *L. acidophilus* R-26, catalyzing the transfer of γ-phosphoryl group to the 3'-hydroxyl group of the deoxynucleoside substrate, should share considerable similarities with G-proteins in their active sites. The kinases and GTPases share sequence motifs such as the glycine-rich P-loop (G-1 in G proteins) and Ras switch 1-like sequence (G-2 in G proteins). Although those *L. acidophilus* R-26 kinases do not possess the exact G-3 motif, they have a putative \(^{78}\text{DRS}\) motif, the aspartate residue of which most likely participates in the Mg\(^{2+}\) coordination in the same manner as the aspartate residue of G-3 in G-proteins. The possible importance of this \(^{78}\text{DRS}\) motif is further supported by conservation of the phenylalanine residue at position 88. Pivotal for
understanding the catalytic mechanisms of this group of kinases, the $V_{\text{max}}$ heterotrophic activation of dAK must employ some features already revealed in interactions between G-proteins and their respective GTPase activity regulators. Because of the sequence conservations, particularly the Ras switch 1-like motif, any similarity shared by G-proteins and *L. acidophilus* R-26 deoxynucleoside kinases should be considered as an example of divergence.

6.3 Experimental evidence for the function of the Ras-like sequence in *Lactobacillus* kinases.

The Ras switch 1-like sequence plays a role in the dAK heterotrophic activation. Switch 1 of Ras proteins changes its conformation to optimize the positions of γ-phosphate and Mg$^{2+}$ ion. If this sequence also functions in dAK, altering the conformation of this sequence should affect the kinase activity. The P155A mutation cis-elevated dAK $V_{\text{max}}$ two-fold, with a corresponding reduction of the heterotrophic activation effect by dGuo. Because the maximum dAK activity potential of dAK(P155A)/dGK was not changed, the substitution has not caused a global misfolding. Since this mutation had an effect on the heterotrophic activation, the conformation
change of this motif must be involved in the heterotopic activation.

But a question still remains: why didn't the P155A mutation fully cis-activate the dAK $V_{\text{max}}$, with a concomitant complete abolishment of the heterotropic activation? A generic answer is that P155A substitution alone has not achieved, to the fullest extent, the conformational change attainable by heterotropic activation.

6.4 A preliminary steady-state analysis of the heterotropic activation of dAK.

The Ras-RasGAP interaction stabilizes the transition state of GTP hydrolysis. But the steady state rate of GTP hydrolysis remains unaltered, and is determined by the product GDP release. Therefore, one possible answer to the above mentioned question is that heterotropic activation is a combined effect of transition state stabilization and product release acceleration. And the P155A substitution might have achieved mainly only one aspect, possibly the transition state stabilization of the phosphoryl group transfer. Presteady state kinetics studies are necessary to identify a) the rate-limiting step(s) of the kinase reaction and b) the exact step(s)
accelerated during heterotropic activation, neither of which could be explored satisfactorily by the steady state kinetic studies carried out thus far.

Using the King-Altman procedure, Plapp has derived the rate equation for the complete Ordered Bi Bi mechanism (143), which is suitable for the dAK catalysis represented by the following scheme:

\[ \begin{align*}
E &\rightleftharpoons E\cdot\text{MgATP} \\
&\rightleftharpoons E\cdot\text{MgATP}\cdot\text{dAdo} \\
&\rightleftharpoons E\cdot\text{MgADP}\cdot\text{dAMP} \\
&\rightleftharpoons E\cdot\text{dAMP} \\
&\rightleftharpoons E
\end{align*} \]

(Scheme 6.1)

Accordingly, \( K_s(\text{MgATP}) = \frac{k_2}{k_1}, \)

\[ K_m(\text{MgATP}) = \frac{k_5k_7k_9}{(k_1(k_5k_7+k_5k_9+k_7k_10))}, \]

and \( k_{\text{cat}} = \frac{k_5k_7k_9}{(k_5k_7+k_5k_9+k_7k_9+k_7k_10)}. \) During heterotropic activation, \( k_{\text{cat}} \) is increased 4 fold while the \( K_m(\text{MgATP}) \) value is essentially unchanged, therefore, \( k_1 \) must experience a 4 fold increase as well. Since \( K_s(\text{MgATP}) \) is increased 2.5 fold upon heterotropic activation, \( k_2 \) must be increased 10 fold.

On the other hand, if the product release step(s) is/are the slowest and thus rate-limiting, the dAK reaction can then be simplified by the following scheme:
\[ K_s(\text{MgATP}) [\text{dAdo}] K' k_4 \]

\[ E \rightleftharpoons E \cdot \text{MgATP} \rightleftharpoons E \cdot \text{MgATP} \cdot \text{dAdo} \rightleftharpoons E \cdot \text{MgADP} \cdot \text{dAMP} \rightarrow E \]

(Scheme 6.2)

where \([E \cdot \text{MgATP} \cdot \text{dAdo}] = [\text{dAdo}] K [E \cdot \text{MgATP}]\), and \([E \cdot \text{MgADP} \cdot \text{dAMP}] = K' [E \cdot \text{MgATP} \cdot \text{dAdo}]\). According to Fersht (144), \(K_s(\text{MgATP}) = K_s(\text{MgATP}) / (1 + [\text{dAdo}] K + [\text{dAdo}] K')\), and \(k_{\text{cat}} = k_4 [\text{dAdo}] K K' / (1 + [\text{dAdo}] K + [\text{dAdo}] K K')\).

Upon heterotropic activation, \((1 + [\text{dAdo}] K + [\text{dAdo}] K K')\) is increased 2.5 fold. Since \(k_{\text{cat}}\) is increased 4 fold, then \(k_4 [\text{dAdo}] K K'\) should experience a 10 fold increase. This analysis reveals that even if the product release is the rate-limiting step being accelerated, it might not be the only step to be affected during the heterotropic activation.

For the "dAK" subunit in Chi-III, based on the relevant values from Table 4.7 and Table 4.8, \(k_1\) is reduced by 30% compared to the counterpart value of dAK/dGK. Therefore, the \(k_2\) value of "dAK" in Chi-III must be increased 7 fold compared with the \(k_2\) value of heterodimeric dAK/dGK.

According to Scheme 6.2, for "dAK" of Chi-III, \((1 + [\text{dAdo}] K + [\text{dAdo}] K K')\) is increased 5 fold. Hence, for the "dAK" of Chi-III, \((k_4 [\text{dAdo}] K K')\) as a whole is increased 7 fold. Just like the heterotropic activation of dAK, the step(s) before the product release involved in the catalysis of the "dAK" of Chi-III
might also be accelerated.

6.5 The significance of studies on *Lactobacillus* kinases.

**On the studies of GTPases.** Having the Ras switch 1 (G-2)-like sequence right after the arginine-rich motif which most likely acts as the built-in “arginine finger” *in cis*, the deoxyadenosine kinase is more like α subunits of trimeric G proteins which also possess a built-in arginine finger. While X-ray crystallographic studies have just been initiated for the *Lactobacillus* deoxynucleoside kinases, ample structural data have already been collected for G-proteins, providing a point of reference for the kinase studies. But such benefits will occur not just in a one-way fashion. Further structure-function studies on those kinases will surely broaden and deepen our understanding of the GTP-binding fold, a common theme shared by varying G-proteins. In fact, it is those many studies on different G proteins that eventually revealed the strikingly similar catalytic mechanisms of these GTPases. Without variations, there will surely be no theme.

**On the rational drug design against cancer.** The significance of studies on *Lactobacillus* kinases goes well beyond the realm of G-proteins.
Deoxynucleoside kinases share sequence homology with human dCK, dGK, and TK2, suggesting that they might follow a common folding pattern, but with variations. *Lactobacillus* deoxynucleoside kinases can serve as the frame-work to engineer novel kinases in gene therapy against cancer (D.H. Ives, personal communication). Any structural basis of the substrate specificity difference between *Lactobacillus* and human deoxynucleoside kinases can be exploited in rational drug design and gene therapy, in order to maximize drug potency and minimize any side effect.

Meanwhile, the catalytic efficiency is also of importance for the engineered kinases. *Lactobacillus* dAK and dGK (or dCK) differ in their $V_{max}$, and dAK's $V_{max}$ can be heterotropically activated to its maximal potential. Elucidating the structural basis for the $V_{max}$ difference between dAK and dGK (or dCK) and for the heterotropic activation of dAK is the very first step towards maximizing the catalytic turnover rate. Observations made during this study on the Ras switch I-like sequence is of importance in this regard. The conformation of this motif is involved in enhancing the catalytic efficiency of dAK. Moreover, "transplanting" a segment including the Ras switch I-like sequence from dGK into dAK achieved some aspects of heterotropic activation and converted dAK to dGK to certain degree.
Another observation, which can be exploited to maximize the catalytic efficiency, is the apparent pH-dependence of the kinase activities. The kinase activity has thus far been routinely assayed at pH 8.0; however, *Lactobacillus* kinases exhibited a higher activity around pH 9.5 (a condition not realistic physiologically) regardless of the homo-dimeric, hetero-dimeric, and heterotropically-activated forms (Figures 4.13 and 4.14). It is of interest to identify the mechanism(s) of such pH dependence, and accordingly to optimize the kinase efficiency with the aids of X-ray studies. One of the fundamental hypotheses behind above proposed endeavors is that any trans-requirements for efficient catalysis can be fulfilled and even surpassed by cis-structural modification.

On the studies of human deoxynucleoside kinases. Last but not least, human dCK possesses, instead of the Ras switch I-like sequence, the putative “regulatory motifs” proposed by Traut (73). The validity of the proposal depends on the actual three dimensional structure of human dCK, yet to be deciphered. The apparently common motif may not serve the exact same function in all the different proteins harboring it, including *E. coli* aspartate carbamoyltransferase regulatory subunits, *E. coli* phosphofructokinase, and yeast phosphoglycerate kinase. It is however interesting to
note that, in *Trypanosoma brucei* phosphoglycerate kinase (145), the equivalent arginine 39 within the so called "regulatory motif" has been proposed to *directly* stabilize the transferred phosphoryl group. Because this "regulatory motif" is situated right after the arginine-rich motif in human dCK, in a position very closely approximating that of the Ras-like sequence in *Lactobacillus* dAK, can this "regulatory motif" also *directly* participate in the phosphoryl group transfer by human dCK?
BIBLIOGRAPHY


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37. Iwatsuki, N. and Okazaki, R. (1967) *J. Mol. Biol.* 29, 139-154


176


Corrected DNA Sequence of the Cloned Tandem *dak-dgk*

Gene Reported by Ma, *et al.* (74)

`aaactatatatTgtagaaagaacgtg`

S/D sequence for dAK translation is underlined, and “t” missing in the published sequence is in bold capital.
APPENDIX B

Corrected $K_{m,\text{app}}$ and $K_s$ Values for Different dGK Subunits

Reported by Hong, et al., (99)

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<th>UMCE</th>
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<tr>
<td>$K_{m,\text{app}}(\text{dAdo})$ ($\mu$M)</td>
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<td>/</td>
<td>/</td>
</tr>
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<td>100</td>
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<td>$K_s(\text{ATP})$ ($\mu$M)</td>
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<td>dGK</td>
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<td>8,000</td>
<td>13,300</td>
<td>15,000</td>
</tr>
</tbody>
</table>
APPENDIX C

Derivation of Equation 2.2

\[ A + G = AG, \quad K_{d1} = [A][G]/[AG] \ldots \ldots 1) \]

\[ G + G = GG, \quad K_{d2} = [G][G]/[GG], \text{ or } [G] = ([GG]K_{d2})^{\frac{1}{2}} \ldots \ldots 2) \]

\[ [A]_T = [A] + [AG] \ldots \ldots 3) \]

\[ [G]_T = [G] + 2[GG] + [AG] \ldots \ldots 4) \]

\[ K_{d1}/K_{d2} = [A][GG]/([G][AG]) = [GG]([A]_T - [AG])/([AG]([GG]K_{d2})^{\frac{1}{2}}) \]

\[ \therefore [GG]^{1/2} = K_{d1}[AG]/(K_{d2}^{1/2}([A]_T - [AG]) \ldots \ldots 5) \]

Combine 2), 4), and 5),

\[ [G]_T = ([GG]K_{d2})^{\frac{1}{2}} + 2[GG] + [AG] = K_{d1}[AG]/([A]_T - [AG]) + (2/K_{d2})(K_{d1}[AG]/([A]_T - [AG]))^2 + [AG] \]

\[ \therefore K_{d1}[AG]/([A]_T - [AG]) = ((K_{d2}^2 + 8K_{d2}[G]_T)^{\frac{1}{2}} - K_{d2})/4 \]

If \([G]_T\) is much larger than \([AG]\), then:

\[ K_{d1}[AG]/([A]_T - [AG]) = ((K_{d2}^2 + 8K_{d2}[G]_T)^{\frac{1}{2}} - K_{d2})/4 \]

\[ \therefore [AG] = ((K_{d2}^2 + 8K_{d2}[G]_T)^{\frac{1}{2}} - K_{d2})[A]_T/((K_{d2}^2 + 8K_{d2}[G]_T)^{\frac{1}{2}} - K_{d2} + 4K_{d1}) \]

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