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Steginsky, Corazon Anonuevo

ESCHERICHIA COLI ALPHA-KETOGLUTARATE DEHYDROGENASE COMPLEX
STUDY OF MECHANISM AND SPECIFICITY

The Ohio State University
Ph.D. 1983

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ESCHERICHIA COLI \( \alpha \)-KETOGLUTARATE DEHYDROGENASE COMPLEX
STUDY OF MECHANISM AND SPECIFICITY

DISSertation

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

By
Corazon Anonuevo Steginsky, B.S.Chem.

* * * *

The Ohio State University
1983

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Advisor
Department of Chemistry
to my parents
ACKNOWLEDGEMENTS

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FIELD OF STUDY

Major Field:  Biochemistry

Research:  Mechanism and Specificity of α-Ketoglutarate Dehydrogenase Complex. Dr. P.A. Frey, Adviser
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CHAPTER I

INTRODUCTION

α-Ketoglutarate dehydrogenase complexes (α-KGDC) have been isolated from E.coli (Koike et al, 1960), Acinetobacter sp. (Parker and Weitzman, 1972), cauliflower florets (Poulsen and Wedding, 1970), Blowfly muscle (Hansford, 1972), Saccharomyces cerevisiae (Hirabayashi and Harada, 1971) and various mammalian tissues (Sanadi et al, 1952; Massey, 1960; Hirashima et al, 1967; Ishikawa et al, 1966; Sanadi and Littlefield, 1951; Stanley and Perham, 1980; Furuta et al, 1971). They are multienzyme complexes, i.e. aggregates of different functionally related enzymes bound together by non-covalent forces into highly organized structures.

α-KGDC was partially purified from E.coli as two enzyme fractions A' and B using a procedure which involves manganous chloride precipitation of nucleic acids followed by ammonium sulfate fractionation (Korkes et al, 1951). Fraction B was shown to contain an NAD-linked dihydrolipoic dehydrogenase (Hager and Gunsalus, 1953). Later, Reed and co-workers (Koike et al, 1960) isolated α-KGDC from E.coli extracts using a series of purification steps which included protamine sulfate fractionation, ultracentrifugation, and isoelectric precipitation. The isolated complex had a molecular weight of 2.4 million, exhibited partial dependence (50%) on added TPP in the NAD
reduction assay and contained 11 moles of bound (\(^{35}\text{S}\)) lipoic acid and 10 moles of FAD per mole of the complex.

Structural information, including subunit and cofactor stoichiometry, of an enzyme complex is vital for eventually achieving an understanding of its mechanism of action. Investigations of this sort have been performed in various laboratories.

*E. coli* \(\alpha\)-KGDC has been separated into three enzymes: \(\alpha\)-ketoglutarate dehydrogenase (\(E_1\)), a TPP requiring enzyme with a molecular weight of 94,000; dihydrolipoyl transsuccinylase (\(E_2\)) which contains covalently bound lipoic acid and has a molecular weight of 40,000; and dihydrolipoyl dehydrogenase (\(E_3\)), a flavoprotein with a molecular weight of 56,000 (Pettit et al., 1973). The complex consists of about 12 \(\alpha\)-ketoglutarate dehydrogenase chains (i.e. six dimers), 24-transsuccinylase chains and about 12 dihydrolipoyl dehydrogenase chains (i.e. six dimers). The mammalian \(\alpha\)-KGDC resembles the *E. coli* complex in appearance and subunit composition (Tanaka et al., 1972). Reconstitution studies of the \(\alpha\)-KGDC from *E. coli* indicated that the \(\alpha\)-ketoglutarate dehydrogenase and the flavoprotein do not combine with each other but that each of these components combines with the transsuccinylase (Mukherjee et al., 1965). When the native complex, \(\alpha\)-ketoglutarate dehydrogenase-transsuccinylase subcomplex or transsuccinylase core was incubated with dihydrolipoyl dehydrogenase a maximum of 36 flavoprotein chains (18 dimers) were bound to the transsuccinylase. However, maximum activity was observed when only 12 flavoprotein chains (6 dimers) were attached to \(E_2\). It was also observed that the order of binding of the flavoprotein and \(\alpha\)-
ketoglutarate dehydrogenase to the transsuccinylase did not affect the subunit composition of the reconstituted complexes (i.e. 10E₁; 24E₂; 36E₃). The transsuccinylase core did not have the ability to bind more than 12 α-ketoglutarate dehydrogenase chains (6 dimers) (Pettit et al, 1973).

The complex isolated by Reed's group in 1973 contained 12 lipoyl moieties measured by the number of (¹⁴C) succinyl groups bound to the enzyme when the complex reacted with (5-¹⁴C) α-ketoglutarate in the presence of TPP and Mg²⁺. Flavin analysis which involved measuring the absorbance of neutralized trichloroacetic acid extracts at 450 nm before and after reduction with dithionite indicated 10 moles of flavin per mole of the complex.

Reed later reported that there are 24 instead of 12 protein bound lipoyl moieties. Briefly, their experiment consisted of succinylating α-KGDC with (5-¹⁴C) α-ketoglutarate in the presence or absence of N-ethylmaleimide (NEM). Of these 24 (¹⁴C) succinyl groups incorporated, 99% were associated with E₂ as determined by SDS gel electrophoresis and 94% were CoA labile (in the absence of NEM). NEM was proposed to stabilize the enzyme bound succinyl groups. The low degree of succinylation obtained earlier was attributed to the lability of enzyme bound succinyl groups. In addition, when (¹⁴C) NEM was used to label the enzyme 22 moles of the radioactive NEM were incorporated in an α-ketoglutarate dependent fashion. NEM is known to alkylate the sulfhydryl groups of lipoic acids (Collins and Reed, 1977).

Angelides and Hammes demonstrated that there are 12.6 decarboxylase sites per enzyme complex of molecular weight 2.5 x 10⁶ as determined by
titration and inactivation of the α-ketoglutarate component with thiamin thiazolone pyrophosphate. Modification of lipoic acids in the transsuccinylase component with N \(^{(3)H}\)-ethylmaleimide revealed 22.8 lipoic acids per enzyme complex. Virtually full activity was observed with only 8 FAD per enzyme complex out of a total of 12 (Angelides and Hammes, 1979). These observations were in agreement with Reed's findings.

Reed and co-workers determined the number of protein bound lipoyl moieties in α-KGDC by a more direct method. This consisted of addition of a known amount of \([8,8-^2H_2]\) lipoic acid to a protein sample, acid hydrolysis of the protein to release bound lipoic acid, extraction of the lipoic acid, and subsequent conversion to methyl-6,8- bis (benzylthio)octanoate for GC-MS analysis. One lipoyl moiety per transsuccinylase polypeptide chain was found (White et al, 1980).

All these findings indicate a subunit stoichiometry of 12E\(_1\), 24E\(_2\), 12E\(_3\) and cofactor stoichiometry of 12TPP, 24 lipoic acids, 12 FAD.

The transsuccinylase component of α-KGDC serves both structural and catalytic functions. The transsuccinylase core has been crystallized. Electron micrographs and their optical diffraction patterns in combination with X-ray diffraction data suggested an octahedral (432) symmetry with trimers of E\(_2\) situated near the vertices of a truncated cube (De Rosier et al, 1971, De Rosier and Oliver, 1971). Dimers of E\(_1\) bind to the E\(_2\) core at 6 locations possibly along the edges of the cube on the 2-fold axes. Flavoprotein dimers bind to six sites of the cube perhaps on the 4-fold axes (Metzler, 1977).

The present knowledge of the mechanism of decarboxylation and
oxidation of α-ketoglutarate has emerged from a variety of experiments involving both the native complex as well as the resolved enzyme system. The study of partial reactions, their cofactor dependence and response to inhibitors as well as synthesis of model compounds served as the basis for the proposal by Gunsalus and independently by Reed that the conversion of α-ketoglutarate, CoA and NAD⁺ to CO₂, succinyl CoA and NADH occur via the following sequence of reactions:

\[
\begin{align*}
\alpha\text{-ketoglutarate} + E_1[TPP] & \rightarrow CO_2 + E_1[\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}] \\
E_1[\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}] & \rightarrow E_1[TPP] + E_2[\text{Succinyl-Lip-SH}] \\
E_2[\text{Succinyl-Lip-SH}] + CoA & \rightarrow E_2[\text{Lip-} (SH)_2] + \text{Succinyl CoA} \\
E_2[\text{Lip}(SH)_2] + E_3[\text{FAD,ox}] & \rightarrow E_2[\text{Lip} S_2] + E_3[\text{FAD,red}] \\
E_3[\text{FAD,red}] + NAD^+ & \rightarrow E_3[\text{FAD,ox}] + NADH + H^+ 
\end{align*}
\]

The second step in this scheme seems to be the rate limiting step in the overall reaction of the complex (Mukherjee et al, 1965).

Decarboxylation Reaction (1). The decarboxylation reaction involves cleavage of α-ketoglutarate to CO₂ and TPP bound "active aldehyde". It is known that the H-atom on position 2 of thiazolium salts is labile and exchanges with D₂O non-enzymically very quickly in a hydroxide catalyzed process (Breslow, 1957; Breslow, 1958). It is accepted that TPP ionizes at position 2 and the resulting zwitterion reacts with the carbonyl group of the substrate, forming an intermediate which subsequently undergoes decarboxylation to form the "active
aldehyde" (Scheme 1). The occurrence of this step is inferred from the following experimental observations: (1) TPP dependent, CoA independent exchange between $^{14}$CO$_2$ and the carboxyl group of α-ketoglutarate is catalyzed by porcine heart α-ketoglutarate oxidase. (2) In the absence of an oxidant, α-ketoglutarate is decarboxylated by porcine heart α-KGDC to form succinic semialdehyde, which was assayed as the 2,4-dinitro-

Scheme 1

phenylhydrazone (Stumpf et al., 1947; Sanadi et al., 1952). (3) Oxidation of α-ketoglutarate with ferricyanide as electron acceptor requires TPP but not bound lipoic acid, CoA or NAD$^+$. Ferricyanide apparently oxidizes the "active aldehyde" to the acid (Massey, 1960; Koike and Reed, 1960). (4) Resolution of E.coli α-KGDC yielded α-ketoglutarate dehydrogenase (E$_1$) which catalyzes the following reaction (Mukherjee et al., 1965):
\[ \alpha\text{-ketoglutarate} + 2 \text{Fe(CN)}_6^{3-} + \text{H}_2\text{O} \rightarrow \text{succinate} + \text{CO}_2 + 2 \text{Fe(CN)}_6^{4-} + 2 \text{H}^+ \]  

(5) In the oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase complex (PDC), a multienzyme system which is very similar to \(\alpha\text{-KGDC}\); \(\alpha\)-hydroxyethyl-thiamin pyrophosphate and \(\alpha\)-lactylthiamin pyrophosphate have been isolated and characterized (Krampitz et al, 1958; Krampitz et al, 1961; Holzer et al, 1960; Holzer and Beaucamp, 1961). (6) Carbon dioxide is one of the products of the reaction.

Reduction and Acylation of the lipoyl moieties (2). The next step in the proposed scheme is the reductive succinylation of the lipoyl groups in \(E_2\). Highly purified \(\alpha\text{-KGDC}\) contains lipoic acids bound in amide linkage to the \(\alpha\)-amino group of a lysine residue in the \(E_2\) active site (Nawa et al, 1960). A succinyl enzyme intermediate is formed by the reaction of \(\alpha\text{-ketoglutarate}\) with its dehydrogenase complex in the absence of \(\text{NAD}^+\) and \(\text{CoA}\) (Sanadi et al, 1959, Sanadi, 1963). There are 2 mechanisms which are generally proposed to account for reductive acylation in \(\alpha\text{-ketoacid dehydrogenase complexes}\). The first mechanism involves reduction of the disulfide group of lipoamide by \([\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}]\) producing an acyl-TPP intermediate which then transfers the acyl group to dihydrolipoamide (Scheme 2). This mechanism was proposed on the basis of the known ability of \(\text{Fe(CN)}_6^{3-}\) to oxidize an \(E_1\)-active semialdehyde complex. When this reaction was carried out in \(\text{HPO}_4^{2-}\), a hydroxamic acid precursor which was thought to be succinyl phosphate, was produced (Sanadi, 1963).

Two facts raise questions about Scheme 2. Both inhibition by arsenite, which forms a complex with dihydrolipoyl groups, and reduction
Scheme 2

Scheme 3
of FAD in dihydrolipoyl dehydrogenase (E₃) require the presence of CoA as well as α-ketoglutarate. Since succinyl dihydrolipoyl groups produced by reaction of α-ketoglutarate in the absence of CoA cannot react with arsenite or reduce FAD, it appears that the presence of CoA promotes both processes by permitting deacylation of succinyl dihydrolipoyl groups, generating dihydrolipoyl groups according to Equation 3. The dihydrolipoyl groups would react with arsenite or reduce FAD. Since Scheme 2 generates dihydrolipoyl groups without the participation of CoA, these facts can be compatible with Scheme 2 only if acylation of reduced lipoyl groups occurs at a much faster rate than the reaction with arsenite or reduction of FAD.

The second mechanism involves nucleophilic attack of [γ-carboxy-α-hydroxypropyl]TPP carbanion on the disulfide group of oxidized lipoamide, generating a hemithioacetal. Although there is no experimental evidence supporting this mechanism (Scheme 3), it was proposed based on the reactivity of disulfide bonds towards nucleophiles (March, 1977) and on the observation that hemithioacetal groupings are formed in certain enzyme catalyzed oxidation reactions, i.e. with glyoxylase and glyceraldehyde-3-phosphate dehydrogenase (Walsh, 1979). Subsequent expulsion of the TPP carbanion constitute the actual oxidation step generating the succinyl thioester linkage with lipoamide.

Acyl transfer reaction (3). This step involves nucleophilic displacement of dihydrolipoyl enzyme by CoA. Mono-S-succinyl-dihydrolipoic acid is produced enzymatically by coupling succinic thiokinase and E.coli fraction A'(E₁-E₂ subcomplex), a reversal of
reaction (3). Succinic thiokinase generates succinyl CoA:

\[
\text{succinate} + \text{ATP} + \text{CoASH} \longrightarrow \text{succinyl CoA} + \text{ADP} + \text{HPO}_4^-
\]
and transfer of succinyl group to dihydrolipoic acid is catalyzed by fraction A' :

\[
\text{succinyl CoA} + \text{lip}(\text{SH})_2 \longrightarrow \text{succinyl-S-lip-SH} + \text{CoASH}
\]
The thioester has been characterized as 6-S-succinyl-dihydrolipoic acid (Reed, 1966).

**Electron transfer reactions (4,5).** The fourth and fifth steps in the proposed reaction scheme involves the transfer of two electrons from each lipoyl group to NAD via dihydrolipoyl dehydrogenase, \(E_3\cdot\text{FAD}\). As observed spectrophotometrically, the flavin of the pig heart \(\alpha\)-KGDC is reduced in the presence of \(\alpha\)-ketoglutarate and CoA and reoxidized by added NAD\(^+\). The reduction is inhibited by arsenite (Massey, 1960).

Dihydrolipoyl dehydrogenase has been isolated in purified form from \(\alpha\)-KGDC of various sources including *E. coli* (Koike et al, 1960) and was shown to catalyze the following reaction (Equation 7).

\[
\text{Lip(SH)}_2 + \text{NAD}^+ \longrightarrow \text{Lip S}_2 + \text{NADH} + \text{H}^+
\]  
In addition to FAD, dihydrolipoyl dehydrogenase also contains a disulfide that interacts with the flavin (Koike et al, 1960b). This disulfide has been implicated in the catalytic action of the enzyme. Matthews et al (Matthews et al, 1977) proposed the participation of a base in the active site of porcine heart lipoamide dehydrogenase. The base was proposed to catalyze electron transfer according to the following mechanism (Scheme 4).
This mechanism predicts pH independent rates of reduction of the enzyme by dihydrolipoamide at pH values above the pK of the base on the oxidized enzyme. The rate of enzyme reduction by dihydrolipoamide, i.e. \( E + \text{Lip(SH)}_2 \rightarrow E\text{H}_2 + \text{LipS}_2 \), was found to be constant from pH 5.5 to pH 8.1 (with the higher pH representing the upper limit of the pH range where the oxidized enzyme is stable) indicating that the pK\(_a\) of BH\(^+\) is less than 5.5. This group also proposed that the base should be in its protonated form for enzymatic reduction of lipoamide, i.e. \( \text{EH}_2 + \text{Lip S}_2 \rightarrow E + \text{Lip(SH)}_2 \), and presented evidence that the pKa for the thiolate/BH\(^+\) ion pair is 7.9 (Scheme 4e).

While a stable flavin charge transfer complex was observed when
dihydrolipoyl dehydrogenase from pig heart, beef liver, dog fish liver, spinach leaves and yeast was reduced with excess NADH or dihydrolipoamide, as evidenced by increased absorbance in the 500-600 nm region with a peak at 530 nm, that isolated from E.coli showed no such increases (Koike et al, 1960 b). However, Williams later discovered that small quantities of charge transfer complex are indeed transiently formed when lipoyl dehydrogenase isolated from E.coli (B strain or the acetate requiring mutant, M 191-6) is reduced by NADH or dihydrolipoamide. The rapid formation of the charge transfer complex is followed by slow, further reduction to the fully reduced form. These observations suggest that the charge transfer complex formed in E.coli lipoyl dehydrogenase is less stable than those from other sources. This may explain the higher sensitivity of the E.coli enzyme to inhibition by excess NADH (Wiliams, 1965).

According to the accepted mechanism of α-KGDC, it is necessary for the enzyme bound lipoyl moiety to interact with γ-carboxy-α-hydroxypropyl-TPP of the dehydrogenase, with the as yet unknown site for succinyl transfer to CoA, and with the prosthetic disulfide group of the dihydrolipoic dehydrogenase. These interactions occur within a complex in which there is restricted movement of the individual enzymes and no dissociation of intermediates. The attachment of lipoic acid via an amide linkage to the ε-amino group of a lysine residue at the transsuccinylase active site provides a flexible arm of approximately 14A for the reactive dithiolane ring (Reed, 1966).

A mechanism has been postulated in which a single lipoic acid rotates among the three catalytic sites, this requires the sites to be
no farther apart than 28 Å (Reed, 1974).

Fluorescence measurements by Angelides and Hammes (Angelides and Hammes, 1979) indicated that the distance between the decarboxylase site and lipoic acid is 32 Å, between lipoic acids it ranges from 24 to 41 Å, between lipoic acid and FAD it is 22 Å and between the decarboxylase site and FAD it is 33 Å. The fluorescence emission spectrum of the complex with the lipoic acids modified with N-(3-pyrene) maleimide shows no evidence of excimer formation and the fluorescence polarization is very low suggesting little interaction between the labeled lipoic acids. For a single lipoic acid to cover the measured distances between catalytic sites, it is necessary either for the lipoyl arm to be stretched as far as possible with the experimental uncertainties accounting for the distances being larger than 28 Å or a conformational change and/or intervening group transfer occur in the succinylation of lipoic acids.

Collins and Reed (Collins and Reed, 1977) proposed that the lipoyl
groups in α-KGDC act as a network functioning as an acyl group and electron pair relay system through thiol-disulfide and acyl transfer reactions among all of the lipoic moieties. Their experiment consisted of blocking about 9 out of 12 $E_1$ with thiamin thiazolone pyrophosphate and reacting the complex with (5-¹⁴C) $\alpha$-ketoglutarate in the presence of TPP. Rapid incorporation of about 20 succinyl groups per molecule of the complex was observed. In cross-linking experiments Collins and Reed observed monomeric as well as dimeric transsuccinylase species when α-KGDC was incubated with NADH in the presence of N,N'-p-phenylenedimaleimide. No larger oligomeric species were observed due to the presence of only one lipoic moiety per chain; and no transsuccinylase dimer was detected in the absence of NADH.

A diagram of Reed's proposal is shown below.

It will be interesting to do a kinetic experiment under conditions where some $E_1$ are blocked as described by Reed (Collins and Reed, 1977) to see whether the rate of acyl and electron transfer among lipoic acids is
catalytically competent.

Perham and Roberts (Perham and Roberts, 1981) inferred the presence of polypeptide chains with high intramolecular mobility from proton nmr data. Tryptic treatment of the complex removes the lipoic acid containing regions of E₂ leaving most of the mobile region and a highly assembled complex with the apparent molecular weight of E₂ falling from 50,000 to 36,000. The presence of a mobile region to which the lipoic acids are attached would make it easier for the lipoic acids to span the different active sites in the complex in the swinging arm model of lipoyl group interaction with E₁ and E₃. The proposal of a network of interacting lipoyl moieties by Reed (Collins and Reed, 1977), if catalytically significant, might, therefore, be facilitated by protein structural mobility as well as mobility in the lipoyl structure.

The findings of Reed and co-workers (Stepp et al., 1981) were in agreement with those of Perham and Roberts' (Perham and Roberts, 1981). E₂ consists of two functionally distinguishable domains, a compact one responsible for the integrity of the quaternary structure of the complex and containing the E₁ and E₃ binding sites, and a mobile lipoyl domain which contains the enzyme bound lipoyl moiety. The mobile and compact domains are attached through a trypsin sensitive region so that limited tryptic digestion separates them. The domain has a molecular weight of 28,000 and the lipoyl domain a molecular weight of about 11,000. Reed's data show that release of the lipoyl domains is faster than loss of enzymatic activity, leading him to propose a model in which each E₁ and E₃ is serviced by at least two lipoyl domains and interaction of the lipoyl groups with the E₁ and E₃ active sites
involves movement of lipoyl domains as well as rotation of lipoyl residues:

They further proposed that the transsuccinylase region is in the subunit binding domain.

Experimental evidences suggest that only half of the lipoyl groups in pyruvate dehydrogenase complex are catalytically significant. Frey and co-workers' measurement of NADH yield of the reduced, acetylated complex yielded 5.2 nmoles of NADH per mg of the complex indicating that only 24 of the 48 lipoyl moieties transfer electron equivalents to $E_3$ (Frey et al, 1978). Akiyama and Hammes using rapid mixing quench techniques showed that although 48 molecules of ($^{14}\text{C}$) acetyl groups are incorporated when PDC is incubated with (3-$^{14}\text{C}$) pyruvate in the presence of TPP and Mg$^{2+}$, there are 2 distinct rates of incorporation with about half of the acetylation occurring through each process and with only the fast reaction having catalytic significance (Akiyama and Hammes, 1980). Since α-KGDC is very similar to PDC, a model which involves interaction of only 12 out of the 24 lipoic acids with $E_1$ and $E_3$ is
possible.

The porcine heart $\alpha$-KGDC was found to be inhibited by the products succinyl CoA and NADH (Smith et al., 1974). The inhibition by succinyl CoA was independent of the NADH:NAD ratio and competitive with CoASH. At saturating levels of $\alpha$-ketoglutarate and NAD$^+$, the $K_m$ for CoASH was 2.7 $\mu$M and the $K_i$ for succinyl CoA was 6.9 $\mu$M. Inhibition by NADH was non-competitive with respect to NAD$^+$, CoA or $\alpha$-ketoglutarate except at high NAD$^+$ concentrations, where reciprocal plots were nonlinear and the inhibition pattern for NADH vs. NAD$^+$ changed from noncompetitive to competitive (Lawlis and Roche, 1981b). At high $\alpha$-ketoglutarate and CoA concentrations, the $K_m$ for NAD was 21 $\mu$M and the $K_i$ for NADH was 4.5 $\mu$M (Lawlis and Roche, 1981a). The bovine kidney $\alpha$-KGDC was resolved and the effect of NADH on the rate of decarboxylation of $\alpha$-ketoglutarate was investigated. NADH inhibited the partial reaction (Lawlis and Roche, 1981b) indicating that this effector at least in part, alters the activity of the complex by binding at an allosteric site on the $\alpha$-ketoglutarate dehydrogenase component.

A different form of inhibition by NADH was observed in the case of the _E. coli_ lipoamide dehydrogenase. This inhibition was partially reversed by NAD$^+$, consistent with reduction by NADH of the active 2-electron reduced enzyme intermediate to the inactive 4-electron reduced form. The reversal by NAD$^+$ was due to reoxidation of the 4-electron reduced form back to the active 2-electron reduced enzyme. Reversal of inactivation by NAD$^+$ was only partial since a) NAD$^+$ bound to the oxidized enzyme giving rise to a dead end complex and b) the presence of both NADH and NAD led to accumulation of the binary enzyme pyridine
nucleotide complexes, i.e. $\text{EH}_2\cdot\text{NADH} \rightarrow \rightarrow \text{EH}_4\cdot\text{NAD}$ which are intermediates in the two-electron to four electron reduction of the enzyme and thus are not on the catalytic pathway. The inhibition by NADH becomes significant as the ratio of $\text{NAD}^+/\text{NAD}^+ + \text{NADH}$ falls below 0.85. This NADH inhibition of $\alpha$-KGDC may serve as a control for the flux of metabolites through the TCA cycle (Wilkinson and Williams, 1981).

Regulation of $\alpha$-KGDC by ATP/AMP was also observed. The activity of the complex from cauliflower florets (Wedding and Black, 1971) and Acinetobacter sp. (Parker and Weitzmann, 1972) is markedly stimulated by AMP. Hansford observed that $\alpha$-KGDC from blowfly flight muscle mitochondria is inhibited by ATP and this inhibition is reversed by ADP or AMP (Hansford, 1972). ATP increases the $K_m$ for $\alpha$-ketoglutarate without altering maximal velocity of porcine $\alpha$-KGDC (McCormack and Denton, 1979) and bovine $\alpha$-KGDC (Lawlis and Roche, 1981a) whereas ADP decreased this parameter.

$\text{Ca}^{+2}$ decreases the $K_m$ for $\alpha$-ketoglutarate of porcine heart $\alpha$-KGDC (McCormack and Denton, 1979) and bovine kidney $\alpha$-KGDC (Lawlis and Roche, 1981) with no effect on maximal velocity. This effect of $\text{Ca}^{+2}$ was postulated to occur via a $\text{Ca}^{+2}$ binding protein (similar to Calmodulin). The rate of decarboxylation reaction by bovine kidney $\alpha$-KGDC was stimulated by $\text{Ca}^{+2}$, thus it is possible that the regulation of the complex is achieved by binding at an allosteric site on the $\alpha$-ketoglutarate dehydrogenase component of the complex.

This dissertation is divided into three parts. Chapter III describes experiments investigating active site coupling in $\alpha$-
ketoglutarate dehydrogenase complex. Chapter IV describes studies on the mechanism of succinyl and electron transfer from α-ketoglutarate dehydrogenase ($E_1\cdot$TPP) to dihydrolipoyl transsuccinylase ($E_2\cdot$Lip $S_2$). Chapter V addresses the question of whether pyruvate can act as a substrate for α-ketoglutarate dehydrogenase complex.
CHAPTER II
MATERIALS AND METHODS

Materials

Substrates, Coenzymes, Coenzyme Analog. Potassium \( \alpha \)-ketoglutarate (\( \alpha \)-KG), sodium pyruvate, cysteine, S-succinyl CoA, NAD\(^+\), NADH, TPP and CoA were purchased from Sigma. Thiamin Thiazolone Pyrophosphate was a gift of Dr. Ronald Kluger from the Department of Chemistry, University of Toronto.

Chemicals. Sodium glyoxylate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), Bicine, Trizma, Bisacrylamide, Sodium Dodecyl Sulfate (SDS), Phenylmethyl sulfonyl fluoride (PMSF), and tetrasodium ethylenediamine tetra-acetic acid (EDTA) were obtained from Sigma. Sodium Arsenite and semicarbazide hydrochloride were obtained from MCB. Acrylamide was purchased from Eastman Kodak, phenol, was from Fisher Scientific Co., N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate from Canalco, Triethylamine from Aldrich, Insta Gel from Packard, and Aquasol from New England Nuclear.

Resins for Column Chromatography. DEAE Sephadex was purchased from Sigma. Dowex Ag 50 WX-8 was obtained from Bio Rad.

Radiochemicals. Sodium (5-\(^{14}\)C)\( \alpha \)-ketoglutarate and sodium (2-\(^{14}\)C) pyruvate were purchased from Amersham Corporation. The specific...
radioactivities of (5-\(^{14}\)C)\(\alpha\)-ketoglutarate and (2-\(^{14}\)C) pyruvate were determined as follows: concentrations of stock solutions were measured spectrophotometrically using NAD\(^+\) reduction assay (Section A of Methods) in the presence of \(\alpha\)-KGDC and PDC respectively. Samples of known \(^{14}\)C content were diluted with a known amount of the corresponding cold \(\alpha\)-keto acid and converted to the semicarbazone (Shriner et al, 1956). The derivative was crystallized to constant specific radioactivity. The measured specific radioactivity was corrected for dilution by added carrier to obtain the specific radioactivity of \(\alpha\)-ketoacid in the stock solution.

Enzymes. \(\alpha\)-Ketoglutarate Dehydrogenase Complex was purified from E.Coli Crooke's strain (ATCC 8739) grown according to the method of Korkes (Korkes, 1955). The cells were harvested at 4\(^\circ\)C with a Sorvall continuous flow centrifuge. The purification method used was Speckhard and Frey's modification (Speckhard & Frey, 1975) of Reed's procedure (Reed & Mukherjee, 1969). Two minor changes in the purification procedure were introduced, namely: 1) addition of 1% RNA, pH 6.2 (0.007x Volume of the crude extract) to the buffer used to resuspend the protamine sulfate pellet increased the yield and (2) isoelectric precipitation in 0.2 pH increments beginning at pH 5.9 gave a better separation of \(\alpha\)-KGDC and PDC. Purified enzyme was dissolved in 20 mM potassium phosphate buffer, pH7 containing 1 mM PMSF and 1 mM EDTA, frozen in liquid N\(_2\) and stored at -70\(^\circ\)C. PMSF and EDTA had no effect on enzyme activity as determined by NAD\(^+\) reduction assay. The purified preparations had a specific activity of 20 to 25 \(\mu\)moles of NADH formed per minute per mg of protein at 30\(^\circ\)C.
Succinic thiokinase was obtained from Sigma.

METHODS

A. Substrate Assays. The concentrations of the following were determined spectrophotometrically in 0.1 M potassium phosphate buffer, pH 7 using the given extinction coefficients at the appropriate wavelength:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Extinction Coefficient (mM^{-1}cm^{-1})</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA</td>
<td>16.84</td>
<td>257</td>
</tr>
<tr>
<td>(Dawson et al, 1969)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPP</td>
<td>8.55</td>
<td>267</td>
</tr>
<tr>
<td>(Butler et al, 1977)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD</td>
<td>17.8</td>
<td>259</td>
</tr>
<tr>
<td>(Dawson et al, 1969)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>6.2</td>
<td>340</td>
</tr>
<tr>
<td>(Horecker and Kornberg, 1969)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTPP</td>
<td>10.6</td>
<td>234</td>
</tr>
<tr>
<td>(Butler et al, 1977)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α-ketoglutarate and pyruvate concentrations were determined using the NAD\(^+\) reduction assay (Reed & Mukherjee, 1969). Succinyl CoA concentration was obtained by subtracting the concentration of free sulfhydryl in solution measured by the DTNB assay in 0.1 M sodium bicinate buffer at pH 8.0 using the extinction coefficient of 13.6 mM\(^{-1}\)cm\(^{-1}\) at 412 nm (Ellman, 1959), from the measured total adenosine using the extinction coefficient of 15.4 mM\(^{-1}\)cm\(^{-1}\) at 260 nm (pH 7) (Cha

B. Enzyme Assay. Overall activity of α-KGDC was assayed spectrophotometrically by measuring the rate of NADH appearance as described by Reed (Reed & Mukherjee, 1969).

C. S-(4-\textsuperscript{14}C) Succinyl CoA Synthesis. S-(4-\textsuperscript{14}C) Succinyl CoA was synthesized from (5-\textsuperscript{14}C)α-ketoglutarate and CoA using α-KGDC in the presence of TPP, Mg\textsuperscript{2+} and NAD\textsuperscript{+}. To a test tube containing 4.0 mL of 0.12 M potassium phosphate buffer at pH 8.0 were added 36.0 mg CoA (40.0 μmole), 294.0 mg NAD\textsuperscript{+} (443.1 μmole), 0.24 mg TPP (0.5 μmole) and 1.0 mg MgSO\textsubscript{4} (8.3 μmole). The pH of the reaction mixture was adjusted to 8.0. A 0.1 mL aliquot of a 13.7 mg/mL α-KGDC solution (about 1.4 mg) was added and the reaction was initiated by addition of 49.0 μmole of (5-\textsuperscript{14}C) α-ketoglutarate. The reaction proceeded at 25°C; 1 μL aliquots were withdrawn at various times and tested for free sulfhydryl concentration using the DTNB assay. When the reaction was about 90% complete, the pH of the solution was adjusted to 5.0 by addition of 1.0 N acetic acid. The mixture was centrifuged for 5 minutes at high speed using a clinical centrifuge. The supernatant fluid was subjected to DEAE Sephadex A25 column chromatography as described and illustrated in Figure 1.

The fractions containing S-(4-\textsuperscript{14}C) succinyl CoA, as determined by A\textsubscript{260} and radioactivity profile were pooled and passed through Dowex Ag50WX8, H\textsuperscript{+} form (100-200 mesh) at 4°C and vacuum distilled to a syrup which was then lyophilized to obtain solid S-(4-\textsuperscript{14}C) succinyl CoA.

Specific radioactivity was determined by counting a known quantity of S-(4-\textsuperscript{14}C) succinyl CoA as described in Section E. Succinyl CoA
Figure 1.
S-(4-\textsuperscript{14}C) Succinyl CoA Purification.

After centrifugation of the reaction mixture for S(4-\textsuperscript{14}C)succinyl CoA synthesis, the supernatant fluid was applied to a 1.5 cm x 21.0 cm DEAE Sephadex A25 column, which was eluted with 0.5 M sodium formate buffer, pH 3.0 at a flow rate of 1.2 mL/min. Column chromatography was done at 4°C. 24.0 mL fractions were collected. Absorbance at 260nm was determined and radioactivity was analyzed as described in Section E.

- - 260 nm Absorbance

O - O cpm/mL
concentration was determined as described in Section A, as well as by measuring the amount of free sulfhydryl using DTNB assay before and after arsenolysis by succinic thiokinase (Cha & Parks, 1964). Arsenolysis was carried out for 20 minutes at 25°C in 0.1 M tris-acetate buffer, pH 7.4 containing 20 mM MgSO₄, 0.19 M sodium arsenate and 0.01 mg succinic thiokinase (about 0.1 unit). Both methods of succinyl CoA concentration determination gave the same result. The specific radioactivity of S-(4-¹⁴C) succinyl CoA was the same as that of the starting material: (5-¹⁴C) α-ketoglutarate. No α-ketoglutarate could be detected by the NAD reduction assay under conditions in which 0.1% contamination could be detected.

If the specific radioactivity of S-(4-¹⁴C) succinyl CoA was not satisfactory after column chromatography, the sample was further purified by paper chromatography using Whatman 3MM filter paper with methanol: 88% formic acid: H₂O (16:3:1) solution as the solvent. S-(4-¹⁴C) succinyl CoA was eluted from the paper with 0.5 M formic acid. The eluted sample was lyophilized to obtain the powder.

D. Isolation of Radiochemically labeled α-KGDC. To determine the amount of protein bound radioactive groups, phenol extraction or filter paper-TCA precipitation method was used. Gel filtration was attempted but was not successful due to the lability of enzyme bound succinyl groups.

In the phenol extraction method, 0.2 to 0.4 mL reaction mixtures were quenched with 0.6 to 0.8 mL phenol saturated water making the volume of the aqueous layer 1.0 mL. One mL of water-saturated phenol was added. The protein was extracted into the phenol layer by vigorous
mixing of the two liquid phases using a vortex mixer. Aqueous and phenol layers were separated by low speed centrifugation using a clinical centrifuge. The phenol layer was washed with equal volumes of phenol saturated water until the wash was free of radioactivity.

In the filter paper-TCA precipitation method, 50 µL reaction mixture was applied on a dry 1.5 cm x 1.5 cm piece of Whatman 3 MM filter paper that had previously been soaked with 10% TCA and dried. The paper containing the sample was dried, rinsed with 10% TCA until the wash was free of radioactivity, rinsed with two 10 mL portions of ethanol, dried and counted as described in Section E.

E. Measurement of Radioactivity. Radioactivity in water-saturated phenol and on filter paper was counted in 8.0 mL Insta Gel using a Packard TriCarb 3320 or Tricarb C2425 liquid scintillation spectrometer. The same results were obtained whether or not the protein sample on filter paper was digested with 1.0 mL of 0.2 N sodium hydroxide at 50°C for 1 hour and subsequently neutralized with 2N hydrochloric acid prior to counting. One (1.0) mL aqueous samples (i.e. α-ketoglutarate, pyruvate or succinyl CoA solution used as standard) were counted in 8.0 mL Insta Gel.

Column eluates as well as aqueous washings of phenol and filter paper were analyzed by adding 1.0 mL sample to 15.0 mL of Aquasol or 15.0 mL of scintillation fluid (0.7% PPO, 0.03% POPOP, 10% naphthalene in 1,4-dioxane) and counted in a Packard Tricarb 460C or Beckman LS-100C liquid scintillation spectrometer.

Semicarbazones of α-ketoglutarate and pyruvate were dissolved in 3.0 mL of hot water, cooled to room temperature and counted in 8.0 mL of
Insta Gel.

Quench corrections on phenol and filter paper samples in Insta Gel were made by adding 0.10 mL of (14C)-counting standard [50 mM(5-14C) α-ketoglutarate or 50 mM(2-14C) pyruvate] to each of the previously counted samples. The quench percentage was calculated as follows:

\[
\% \text{ quench} = \frac{cpm_{\text{std}} + cpm_{\text{sample}} - cpm_{\text{std}} + cpm_{\text{sample}}}{cpm_{\text{std}} + cpm_{\text{sample}}} \times 100
\]

The quench corrected sample cpm is then given by the folowing equation:

\[
\text{corrected cpm} = \text{observed cpm} + \text{observed cpm} \left( \frac{\% \text{ quench}}{100} \right)
\]

F. Sodium DodecylSulfate (SDS) - Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (Weber & Osborn, 1969). A 10% gel solution was prepared by dissolving 1.0 g of acrylamide, 27 mg of bisacrylamide and 10 mg of ammonium persulfate in 10 mL of 10 mM sodium phosphate buffer at pH 7 containing 6M urea and 1% SDS. Gel formation was initiated by addition of 5 mL of N,N,N',N'-tetramethylethylenediamine. The resulting solution was quickly pipetted into 0.6 cm x 11 cm silanized gel tubes; and the tops were layered with water saturated n-butanol. Gels were left to stand overnight. The n-
butanol layers were rinsed off with water before the gels were used.

Samples were prepared by mixing 25 to 100 µg of protein with 10 mM sodium phosphate buffer, pH 7 containing 6 M urea and 1% SDS such that the total volumes were 100 µL. The samples were heated for 10 minutes at 90°C, and bromphenol blue added to a final concentration of 1%. A drop of glycerol was added; and the samples were applied to gels that had been pre-electrophoresed for 30 minutes.

Electrophoresis was carried out at 4 mA/gel with 10 mM sodium phosphate buffer at pH 7 containing 0.1% SDS. Electrophoresis was stopped as soon as the bromphenol blue dye reached the bottom of the gels. Gels were removed from the tubes and stained for 2 hours in 0.2% Coomasie brilliant blue in methanol-acetic acid-water (50.0:7.5:42.5 V/V) solution. Gels were destained in methanol-acetic acid-water (5.0:7.5:87.5 V/V) solution.

G. Protein Measurement. Protein dry weight determination of the purified α-KGDC was done as follows: 16 mg of α-KGDC in 1.5 mL of 20 mM potassium phosphate buffer, pH 7 was dialyzed exhaustively against 2 mM potassium phosphate buffer at pH 7. The protein concentration of a portion of the dialyzed sample was determined using the Lowry procedure (Milleu, 1959). 1.0 mL of the protein sample was dried in a vacuum desiccator along with 1.0 mL of the dialysis buffer. The two vessels were dried to constant weight and the weights of buffer salts and protein determined. Protein concentration using dry weight was found to be 95.6% that obtained using the Lowry procedure with bovine serum albumin as the standard protein.

H. FAD reduction. Anaerobic reduction of the flavoprotein was
carried out using the procedure of Speckhard and Frey (Frey et al., 1978). The spectra were measured using anaerobic cuvettes equipped with side pouches for addition of the reducing agent (Fig. 2). The main chamber contained α-KGDC, TPP, MgSO₄ and CoA. The reducing agent (α-KG or pyruvate) was placed in the side pouch. The system was flushed repeatedly with Argon, the visible spectrum of the oxidized enzyme was obtained, the cuvette was tipped to add the reductant to the main chamber, the reaction mixture was mixed thoroughly and the spectrum of the reduced enzyme complex was measured using a Norelco-Unicam SP800 double beam recording spectrophotometer.
Figure 2

Cuvette for Anaerobic Experiments.

The cuvette used in anaerobic reduction experiments was a pyrex one centimeter path length cuvette with a ground glass tapered joint. The top assembly was made in the glass shop of the Ohio State University, Department of Chemistry. The top is covered with a serum stopper. Two metal needles were inserted into the stopper to flush the system with Argon. These metal needles were removed at the end of the purging process.
FIGURE 2

- SERUM STOPPER

1 cm PATH LENGTH
PYREX CUVETTE WITH GROUND GLASS JOINT

SIDE POUCH

8.2 cm

1.2 cm
CHAPTER III
Active Site Coupling in α-KGDC

In the case of a multienzyme complex such as α-KGDC, the achievement of a clear understanding of the chemical communications among the components requires knowledge of the numbers of different protein chains and associated coenzymes, as well as of whether all the coenzymes are catalytically functional. The latter is especially important when coenzymes and enzymic components are present in unequal numbers.

This chapter describes experiments in which the catalytic pathway has been exploited to measure the number of active site cofactors, i.e., lipoyl groups for E$_2$ and FAD for E$_3$, that interact with the other enzyme components of the complex. The results provide information about the stoichiometry of active sites within the complex.

1. Anaerobic Reduction of FAD by Substrates

Dihydrolipoyl dehydrogenases isolated from α-ketoglutarate dehydrogenase complexes of various sources have been extensively studied.

It is well known that the flavin noncovalently associated with dihydrolipoyl dehydrogenase is FAD. This is inferred from the UV-Vis absorption spectrum and paper chromatographic analysis of neutralized perchloric acid extracts (Koike et al., 1960a) and also from the reactivation of the apoprotein induced by added FAD but not FMN (Koike
et al, 1960b). The absorption spectrum of FAD, dihydrolipoyl dehydrogenase, and α-ketoglutarate dehydrogenase complex have been obtained. Table 1 lists the wavelengths corresponding to the maxima, minima and shoulders of these spectra in the 320 nm to 700 nm region. When the flavin is bound to the enzyme, the maximum at 450 nm is red-shifted, the shoulders on both sides of the 450 nm peak are increased considerably, and the minimum at 405 nm, as well as the peak at 375 nm are blue-shifted.

The flavin contents of dihydrolipoyl dehydrogenase and α-KGDC have been determined by measuring the absorbancy of neutralized trichloroacetic acid extracts at 450 nm. It has also been estimated in intact complexes by measuring A$_{455}$ before and after reduction with dithionite. A change in the extinction coefficient of 10.4 mM$^{-1}$cm$^{-1}$ upon reduction was used for both the free and bound flavin (Beinert and Page, 1957). Quantitation of the FAD content of the complex was also carried out by flavin fluorescence (Angelides and Hammes, 1978, Angelides and Hammes, 1979) measurement.

In this section, anaerobic reduction of α-KGDC bound flavin was accomplished using α-ketoglutarate as the reducing system. The degree of substrate-induced bleaching at 455 nm was used to calculate the functional FAD.

The visible spectrum of the complex in the presence of TPP, Mg$^{2+}$ and CoA is the same as that of the complex alone (Koike et al, 1960a).

It is apparent upon comparison of Figures 3 and 4 that the visible spectrum of an oxidized enzyme preparation purified in the absence of PMSF and EDTA differs significantly from that of one purified in the
Table 1. Spectral features of FAD in the 320 nm to 700 nm region.

<table>
<thead>
<tr>
<th>Form of FAD</th>
<th>Wavelength (nm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>maxima</td>
<td>minima</td>
<td>shoulders</td>
</tr>
<tr>
<td>afree</td>
<td>375,450</td>
<td>405</td>
<td>both sides of 450</td>
</tr>
<tr>
<td>b$_{E3}$ associated</td>
<td>359,456</td>
<td>395</td>
<td>435,480</td>
</tr>
<tr>
<td>c$_{a-KGDC}$ associated</td>
<td>350,455</td>
<td>395</td>
<td>425,480</td>
</tr>
</tbody>
</table>

a (Williams, 1965)

b$_{E3}$ = Dihydrolipoyl Dehydrogenase (Koike et al, 1960b)

c (Koike et al, 1960 a)
presence of these protease inhibitors. While the band positions are essentially the same, the spectrum of the enzyme purified in the presence of protease inhibitors is "cleaner" with deeper minima and more prominent maxima.

Another significant difference was the CoA dependence for reduction of FAD by α-ketoglutarate. The reduction illustrated in Figure 3 with enzyme purified in the absence of PMSF and EDTA occurred in the absence of added CoA. Although CoA was present in the experiment of Figure 3, it proved not to be required for the reduction. As shown by Figure 4, however, enzyme purified in the presence of the protease inhibitors exhibited an absolute dependence upon added CoA for the FAD to be reduced by α-ketoglutarate.

Using the decrease in absorbance at 455 nm, the difference in the extinction coefficient of the oxidized and reduced flavin and the protein concentration, the amount of the flavin reduced by α-ketoglutarate in the presence of CoA was calculated to be 3.7 and 4.5 nmoles per mg of the complex in two determinations, the first using enzyme purified in the absence of PMSF and EDTA and the second using enzyme purified in the presence of the protease inhibitors.

The bleached spectra in Figures 3 and 4 are those of the 4-electron reduced form of the flavoprotein which is known to be catalytically non-functional (Williams, 1965). It is produced by a secondary reduction of catalytically functional 2-electron reduced form. Although it does not appear on the catalytic pathway, the 4-electron reduced form contains FADH$_2$ and is bleached, which facilitates the measurement of FAD. Since it is derived from the catalytically functional 2-electron reduced form.
Figure 3
Anaerobic Reduction of FAD in α-KGDC with Added CoA

Using the special glass cuvette depicted in Figure 2, anaerobic reduction of α-KGDC was carried out as follows: a 0.9 mL solution consisting of 4.5 mg α-KGDC, 0.05 μmole of TPP, 0.05 μmole of MgSO₄ and 0.10 μmole of CoA in 20 mM potassium phosphate buffer at pH 7.0 was placed in the cuvette chamber. Potassium α-ketoglutarate, 0.24 μmole in 0.1 mL of 20 mM potassium phosphate buffer at pH7 was placed in the side pouch. The cuvette was purged of oxygen by passing argon through the solutions for 30 minutes. The spectrum (350-700 nm) of the enzyme in the main chamber was measured; α-ketoglutarate in the side pouch was mixed thoroughly with the solution in the main chamber and the spectrum was again rescanned.

A reference was used for anaerobic reduction experiments. The reference cuvette contained the same solution as the main chamber of the sample cuvette except that α-KGDC was omitted. α-Ketoglutarate (0.1 mL of a 2.4 mM solution) was added to the reference cuvette at the same time that α-ketoglutarate in the side pouch of the sample cuvette was mixed with the solution in the main chamber.

The results are reported as $E_{1\%}$, i.e. absorbance of 10 mg α-KGDC per mL of solution.

○ — ○ oxidized enzyme
○ — ● reduced enzyme
Figure 4

Coenzyme A Dependence in the Reduction of FAD in α-KGDC

The cuvette contained 2.8 mg α-KGDC (purified in the presence of 1 mM PMSF and 1 mM EDTA), 0.05 μmole TPP, 0.05 μmole MgSO₄ and 0.10 μmole CoA in 0.90 mL of 0.02 M potassium phosphate buffer at pH 7. Potassium α-ketoglutarate, 0.24 μmole in 0.1 mL of 0.02 M potassium phosphate buffer at pH 7 was placed in the side pouch. The cuvette was purged of oxygen by passing argon through for 30 minutes. The absorption spectrum (350-700 nm) of the enzyme in the main chamber was measured (•-•). α-ketoglutarate in the side pouch was mixed thoroughly with the solution in the main chamber and the spectrum was rescanned (X-X).

The reference solution was prepared as described in Figure 3.

The foregoing experiment was carried out in the absence of CoA. The spectrum (350-700 nm) of the enzyme after α-ketoglutarate was mixed with the solution in the main chamber is shown (O-O).

The results are reported as $E_{1\%}$, i.e. absorbance of 10 mg α-KGDC per mL of solution.
Figure 4

Absorbance vs Wavelength (nm)

Absorbance

Wavelength (nm)
of E₃-FAD, its appearance in experiments involving α-ketoglutarate as the reducing agent reflects the presence of "functional" FAD.

Comparison of the amount of functional flavin measured here (3.7 to 4.5 nmoles FAD/mg complex) with Reed's (4.0 nmoles/mg) and Hammes (4.8 nmoles/mg) measurements of total α-KGDC flavin (Koike et al., 1960a, Angelides and Hammes, 1979, Pettit et al., 1973) indicates that all the flavoprotein in the complex can be reduced by dihydrolipoyl groups in E₂. Thus, all the dihydrolipoyl dehydrogenases in the complex are coupled to the transsuccinylase component.

Since the FAD in α-KGDC purified in the absence of PMSF and EDTA is reducible by α-ketoglutarate in the absence of added CoA, it is most likely that such preparations of the complex contain endogenous CoA. It is not known how the presence of PMSF and EDTA during purification leads to the removal of CoA from the complex, but it is possible that CoA might react with PMSF to displace fluoride, forming phenylmethylsulfonyl-CoA. The latter would be inactive as a substrate.

2. Succinylation of α-KGDC by (5-¹⁴C) α-ketoglutarate.

An intermediate in the reaction catalyzed by the α-KGDC is a succinyl-dihydrolipoyl transsuccinylase, in which the succinyl groups are bonded to the dihydrolipoyl moieties associated with this component of the complex. The lipoyl coenzyme is covalently bonded to this enzyme through the ε-amino group of a lysine residue. The succinyl-intermediate arises by means of reductive succinylation of the lipoyl coenzyme by γ-carboxy-α-hydroxypropyl-TPP following the decarboxylation step. In the absence of CoA and NAD⁺ this intermediate should
accumulate until all of the lipoyl groups have been reduced and succinylated.

When (5-\(14^C\)) \(\alpha\)-ketoglutarate is the substrate under reductive succinylation conditions, i.e. in the absence of CoA and NAD\(^+\), the (4 - \(14^C\)) succinyl groups serve as a means of measuring the lipoic acid content of the enzyme. In such experiments L.J. Reed and his coworkers have obtained two different results. When the complex was incubated with (5-\(14^C\)) \(\alpha\)-ketoglutarate for 2 to 5 min at 25°C under aerobic conditions, 4.8 nmoles of succinyl groups per mg of the complex were covalently bound (Pettit et al., 1973). However, when the incubation conditions were changed to 1 min. at 4°C under \(N_2\) with or without added NEM, 9.8 nmoles of succinyl groups per mg of the complex were bound. The low result was attributed to instability of enzyme bound succinyl groups at 25°C. NEM was thought to stabilize the succinyl groups.

In the light of these observations, optimum conditions for \(\alpha\)-KGDC succinylation by (5-\(14^C\)) \(\alpha\)-KGDC were investigated to determine the maximum number of (\(14^C\)) succinyl groups that can be incorporated into the complex.

Figure 5 shows the dependence of the extent of succinylation of the complex on the concentration of (5-\(14^C\)) \(\alpha\)-ketoglutarate. Maximum incorporation is observed when the concentration of \(\alpha\)-ketoglutarate (0.292 mM) is at least 10-fold in excess of the enzymic lipoyl groups.

There was no difference in the extent of succinyl groups incorporated when the reaction was carried out at 4°C or at 25°C nor under aerobic or anaerobic conditions. The effect of the nature of buffer system as well as the pH on the degree of succinylation of \(\alpha\)-KGDC
Figure 5
Dependence of $^{14}\text{C}$ Succinyl Group Incorporation on $\alpha$-Ketoglutarate Concentration.

$\alpha$-KGDC (2.8 mg/mL) was placed at 25°C for 30 sec with 0.074 mM to 0.73 mM (5-$^{14}$C) $\alpha$-ketoglutarate in the presence of 1 mM TPP and 1 mM MgSO$_4$ in 0.2 mL of 10 mM potassium phosphate buffer at pH 7. The reaction was quenched by adding 0.8 mL of phenol saturated water. Labeled enzyme complex was isolated by phenol extraction and counted as described in the methods section.
FIGURE 5

(5-$^{14}$C) α-KETOGLUTARATE CONCENTRATION (mM)

nmol (14C)SUCCINYL / mg α-KGDC
by (5-\textsuperscript{14}C) \(\alpha\)-ketoglutarate are shown in Table 2. At pH 7, 9.1 nmoles of \((4-\textsuperscript{14}C)\) succinyl groups were incorporated per mg of the complex using 5 mM potassium phosphate buffer. When the concentration of this buffer was increased to 10 mM, 8.6 nmoles of \((4-\textsuperscript{14}C)\) succinyl groups were isolated per mg of the complex. It is apparent from these results that the potassium phosphate concentration in the range of 5 to 10 mM does not significantly affect the extent of succinylation of the complex. At pH 8, 8.2 and 8.1 nmoles \((4-\textsuperscript{14}C)\) succinyl groups per mg of the complex were obtained when the concentration of sodium bicinicate buffer was 5 mM and 10 mM, respectively. The concentration of sodium bicinicate has no effect on the amount of succinyl groups incorporated into the complex by \((5-\textsuperscript{14}C)\) \(\alpha\)-ketoglutarate. There is no substantial difference in the degree of succinylation at pH 7 as compared with that at pH 8.

According to the accepted mechanism, succinyl groups attached to the lipoyl groups in \(E_2\) should be CoA labile. As shown in Table 2, 83 to 94% of the enzyme bound succinyl groups are CoA labile (7.1 to 7.8 nmoles/mg complex) at the indicated CoA concentration, which is 29-fold in excess of \(\alpha\)-ketoglutarate. The extent of desuccinylation with CoA appears to be the same at pH 7 and 8 as well as at 5 mM and 10 mM concentrations of the buffer systems used.

The maximum level of succinylation in these experiments was somewhat less than the maximum reported by Reed and coworkers. In view of the known lability of succinyl thioesters, it was decided to examine the effect of repeated and prolonged phenol extraction on the level of \textsuperscript{14}C incorporation. To do this the concentration of \((\textsuperscript{14}C)\) label in the phenol layer was followed as a function of the number of times the
Table 2. Effect of pH and buffer system on the degree of succinylation of \( \alpha \)-KGDC with (5-\( ^{14} \)C) \( \alpha \)-ketoglutarate.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>nmoles (4-( ^{14} )C) succinyl group/mg complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-CoA(^a)</td>
</tr>
<tr>
<td>5 mM potassium phosphate, pH7</td>
<td>9.1</td>
</tr>
<tr>
<td>10 mM potassium phosphate, pH7</td>
<td>8.6</td>
</tr>
<tr>
<td>5 mM sodium bicinate, pH8</td>
<td>8.2</td>
</tr>
<tr>
<td>10 mM sodium bicinate, pH8</td>
<td>8.1</td>
</tr>
</tbody>
</table>

\(^a\)Succinylation of \( \alpha \)-KGDC with (5-\( ^{14} \)C) \( \alpha \)-ketoglutarate. \( \alpha \)-KGDC (2.0 mg/mL) was reacted at 25°C for 30 sec with 0.21 mM (5-\( ^{14} \)C) \( \alpha \)-ketoglutarate in the presence of 0.25 mM TPP and 0.50 mM MgSO\(_4\) in 0.2 mL of the indicated buffer. The reaction mixture was quenched by adding 0.8 mL of phenol-saturated water. (4-\( ^{14} \)C) Succinylated complex was isolated by phenol extraction and counted.

\(^b\)Desuccinylation of (\( ^{14} \)C) succinyl-\( \alpha \)-KGDC with CoA. The procedure is as described for Part (a) except 6.1 mM CoA was added at the end of the 30 sec incubation of \( \alpha \)-KGDC with (5-\( ^{14} \)C) \( \alpha \)-ketoglutarate. One minute after CoA addition, the reaction mixture was quenched by adding 0.8 mL of phenol-saturated water. The amount of protein-bound (4-\( ^{14} \)C) succinyl group was assayed using the phenol extraction technique.
phenol layer was washed with water. A large decrease in radioactivity was observed initially, followed by a slower, continued decrease (Fig. 6). The continued gradual loss of radioactivity in the phenol layer with continued washing could be an indication of slow hydrolysis of succinyl groups from the enzyme. This would complicate the analysis by yielding low values of enzyme bound succinyl groups. An approximate correction could be made by extrapolating data obtained after many washes, for example 13 to 15 in Fig. 6, to zero washes. Such an extrapolation in Fig. 6 would give a value of about 9.6 nmoles of succinyl groups per mg of protein. This is quite a long and questionable extrapolation.

The results of using the filter paper - TCA precipitation method to isolate protein bound radioactivity are shown in Table 3. The values obtained were higher using this procedure. At a CoA concentration of 3.0 mM (33 fold in excess of \(\alpha\)-ketoglutarate), an average of 8.5 nmoles of \(^{14}\text{C}\) succinyl groups/mg complex were CoA labile. An average of 11.0 nmoles \(^{14}\text{C}\) succinyl groups/mg complex are removed by 17.6 mM (196 fold in excess of \(\alpha\)-ketoglutarate) coenzyme A. The amount of label incorporated in the absence of CoA (12.6 to 13.3 nmole/mg complex) is larger than the number of reported lipoyl groups (10 nmole/mg complex). These larger values may be due to acylation of nucleophile in the enzyme other than the lipoyl groups, as well as a reflection of uncertainties in experimental measurements.

The filter paper - TCA precipitation method was used for the remainder of the labeling experiments for the following reasons: (1) the radioactivity associated with the protein sample remained constant
Figure 6

Stability of (14C) Succinyl-α-KGDC During Phenol Extraction

α-KGDC (2.0 mg/mL) was permitted to react at 25°C for 30 sec with 0.21 mM (5-14C) α-ketoglutarate in the presence of 0.25 mM TPP and 0.50 mM MgSO4 in 0.4 mL of 5 mM potassium phosphate buffer at pH 7. The reaction mixture was quenched by addition of 1.6 mL of phenol-saturated water. The protein was extracted into 2.0 mL of water-saturated phenol and the phenol layer was washed many times with equal volumes of phenol saturated water. Aliquots of the phenol layer (0.2 mL) were withdrawn and assayed for (14C) content after the numbers of washes indicated in the figure. Results are plotted as nmoles (14C)/mg of the complex vs. number of washes.
Figure 6

mmoles (14C) mg α-KGDC in the PHENOL LAYER

NUMBER OF WASHES

FIGURE 6
Table 3. Measurement of $\alpha$-KGDC bound (4-$^{14}$C) succinyl groups by filter paper-TCA precipitation.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>nmoles (4-$^{14}$C) succinyl/mg complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{\text{a}}$CoA $^{\text{b}}$</td>
</tr>
<tr>
<td>Set 1</td>
<td></td>
</tr>
<tr>
<td>5 mM potassium phosphate, pH 7</td>
<td>12.6</td>
</tr>
<tr>
<td>10 mM potassium phosphate, pH 7</td>
<td>13.3</td>
</tr>
<tr>
<td>Set 2</td>
<td></td>
</tr>
<tr>
<td>5 mM potassium phosphate, pH 7</td>
<td>12.6</td>
</tr>
<tr>
<td>10 mM potassium phosphate, pH 7</td>
<td>13.3</td>
</tr>
</tbody>
</table>

$^{\text{a}}$Succinylation of $\alpha$-KGDC with (5-$^{14}$C) $\alpha$-ketoglutarate. $\alpha$-KGDC (0.8 mg/mL) was combined at 25°C with 0.09 mM (5-$^{14}$C) $\alpha$-ketoglutarate in the presence of 0.25 mM TPP and 0.50 mM MgSO$_4$ in 85 μL of the indicated buffer. After 30 sec, 50 μL of the reaction mixture was applied to a dry piece of 1.5 x 1.5 cm Whatman 3 MM filter paper previously soaked in 10% TCA and dried in the air. The non-protein bound radioactivity was washed from the paper and the labeled protein sample was counted as described in the methods section.

$^{\text{b}}$Desuccinylation of (4-$^{14}$C) succinyl-$\alpha$-KGDC with CoA. The procedure was as described for Part (a), except that 3.0 mM CoA (Set 1) or 17.6 mM CoA (Set 2) was mixed with the reaction mixture at the end of the 30 sec incubation of $\alpha$-KGDC with (5-$^{14}$C) $\alpha$-ketoglutarate. One minute later 50 μL of the reaction mixture was assayed for protein bound (4-$^{14}$C) succinyl groups.
with 5-8 washes of 5 ml portions of 10% TCA, (2) Succinyl CoA is quite soluble in phenol and (3) this method could be carried out more quickly than the phenol extraction technique.

The data presented here show that maximum succinylation of α-KGDC by (5-\(^{14}\)C) α-ketoglutarate can be carried out at 25°C under aerobic conditions when the α-ketoglutarate concentration is at least 10 fold in excess of the lipoyl groups present in the reaction mixture. The average number of succinyl groups incorporated is 10.5 nmoles (8-13 nmoles) per mg of the complex at least 8.7 of which (7.1-11.6 nmoles) are CoA labile.

It is pertinent to consider whether all of the succinyl groups incorporated are CoA-labile but that this lability was not demonstrated in the present experiments. Note that in Table 3 the CoA-labile counts were dependent upon [CoA] and corresponded to around 11 nmoles per mg of protein at the highest CoA level. This may be regarded as a maximum estimate of the CoA-labile succinyl-dihydrolipoyl groups, since the CoA level was more than 2 x 10^3 times the concentration of protein-bound succinyl groups and there was adequate time for the reaction to go to completion.

A reasonable assessment of all of the experiments is that the lipoic acid content of the complex is between 9.5 and 11 nmoles per mg of protein. The lower value is derived from the rather long extrapolation of data in Figure 6 and the larger is from Table 3.

3. Succinylation of α-KGDC by (4-\(^{14}\)C)Succinyl CoA

Lipoyl groups in the complex become succinylated by reversal of Steps 3-5 of the overall catalytic pathway. The relevant equations (3-
5) are given below for clarity.

\[
E_3[\text{FAD,ox}] + \text{NADH} + H^+ \rightarrow E_3[\text{FAD,red}] + \text{NAD}^+ \quad (5)
\]

\[
E_3[\text{FAD,red}] + E_2[\text{Lip S}_2] \rightarrow E_3[\text{FAD,ox}] + E_2[\text{Lip(SH)}_2] \quad (4)
\]

\[
E_2[\text{Lip(SH)}_2] + (4-^{14}\text{C})\text{succinyl CoA} \rightarrow E_2[(4-^{14}\text{C})\text{succinyl-Lip-SH}] \quad (3)
\]

The substrates formally necessary for these reactions are NADH and succinyl CoA. However, in the actual experiments NAD$^+$ was also added to maintain the ratio of NAD$^+$ to NADH at 3.6. This prevented inactivation of E$_3$ FAD that would result from formation of the 4-electron reduced state (Wilkinson and Williams, 1981). NADH alone would have produced the inactive form. TPP and MgSO$_4$ were also present in the reaction mixture to prevent the possibility of acyl transfer from lipoyl groups to nucleophile(s) in the E$_1$ active site. The reaction was carried out at 4°C instead of at 25°C to decrease the spontaneous rate of succinyl CoA hydrolysis, which would reduce the effective concentration in the reaction mixture.

The results are given in Table 4. In the absence of NADH a maximum of 0.1 to 0.9 nmoles of (4-^{14}\text{C}) succinyl groups were incorporated into the protein, while 11.0 nmoles/mg of the complex were covalently bound when NADH was present. Therefore, a maximum number of 10 nmoles (4-^{14}\text{C}) succinyl groups are incorporated in an NADH dependent manner per mg of the complex.

If the sites succinylated are the lipoyl groups in E$_2$, which seems to be the case as indicated by the requirement for NADH, then all lipoyl
Table 4. Succinylation of α-KGDC by (4-14C) succinyl CoA.

<table>
<thead>
<tr>
<th>(4-14C)succinyl CoA Concentration (mM)</th>
<th>nmoles (14C) succinyl/mg complex</th>
<th>-NADH (^a)</th>
<th>+NADH (^b)</th>
<th>NADH dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>.025</td>
<td>0.1</td>
<td>5.4</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>.035</td>
<td>0.3</td>
<td>6.3</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>.067</td>
<td>0.3</td>
<td>7.2</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>.080</td>
<td>0.4</td>
<td>8.2</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>.111</td>
<td>0.3</td>
<td>8.0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>.250</td>
<td>0.9</td>
<td>11.0</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>.500</td>
<td>0.1</td>
<td>10.1</td>
<td>10.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)(4-14C) Succinyl CoA was combined with a solution containing α-KGDC (0.8 mg/ml), TPP (2.9 mM), MgSO₄ (4.7 mM) and NAD (8.8 mM) in 10 mM potassium phosphate buffer, pH 7 at 4°C for 30 sec. 50 μL of the 85 μL reaction mixture was assayed for protein bound (4-14C) succinyl using the filter paper-TCA precipitation method.

\(^b\)(4-14C) Succinyl CoA was combined with a solution containing α-KGDC (0.8 mg/ml), TPP (2.9 mM), MgSO₄ (4.7 mM) and NAD (8.8 mM) in 10 mM potassium phosphate buffer, pH 7 at 4°C. The reaction was initiated by NADH (2.4 mM) addition making the total volume of the reaction mixture 85 μL. 30 sec after NADH was added, 50 μL of the reaction mixture was analyzed for protein bound (4-14C) succinyl using the filter paper-TCA precipitation method.
groups in $E_2$ are reduced by the action of $E_3\cdot FAD$, allowing for succinyl transfer to these groups from succinyl CoA.

The maximum number of sites labeled by (5-$^{14}$C) $\alpha$-ketoglutarate, 9.5 to 11 nmoles per mg of complex, and (4-$^{14}$C)succinyl-CoA, 10 nmoles per mg, are essentially the same. It is most likely that these are the lipoyl groups and that the complex contains about 10 nmoles of lipoic acid per mg of protein. All of these lipoyl groups are readily reduced by NADH, showing that all are coupled to dihydrolipoyl dehydrogenase.

4. NADH yield of reduced, succinyalted $\alpha$-KGDC.

Another method by which the coupling between $E_2$ and $E_3$ can be investigated is to measure the amount of NADH formed under the conditions of a single turnover. It is not possible to isolate the active, fully reduced, succinylated complex because of the intrinsic tendency of succinyl thioesters to lose succinyl groups, leading to re-oxidation of non-acylated dihydrolipoyl groups. However, a fully reduced, succinylated complex might be generated in situ using the required excess of $\alpha$-ketoglutarate, followed by removal of unreacted $\alpha$-ketoglutarate by means of the carboligase reaction catalyzed by $E_1$. This uses glyoxylate as the "active aldehyde" acceptor (Schlossberg et al, 1970):

$$E_1[\text{OOCCCH}_2\text{CH}_2\text{CH-TPP}] + \text{HCCOO}^- \rightarrow \text{OOCC(CH}_2)_2\text{CCHCOO}^-$$

2-hydroxy-3-ketoadipate

$$\text{OOCC(CH}_2)_2\text{CCHCOO}^- + H^+ \rightarrow \text{OOCC(CH}_2)_2\text{CCH}_2\text{OH} + \text{CO}_2$$

5-hydroxy-4-ketovalerate
If all the E\textsubscript{2} lipoyl groups are coupled to E\textsubscript{3} FAD, addition of CoA and NAD\textsuperscript{+} to the succinylated complex could lead to formation of NADH in the same amount as the number of E\textsubscript{2} lipoyl groups succinylated by \(\alpha\)-ketoglutarate.

In a typical experiment two spectrophotometric cuvettes were prepared, a sample cell and a reference cell each containing buffer (potassium phosphate at pH 7), \(\alpha\)-KGDC, TPP, MgSO\textsubscript{4} and NAD\textsuperscript{+}. \(\alpha\)-ketoglutarate followed by glyoxylate 30 sec later were added to each cuvette and the cuvettes were incubated at 27°C for specific lengths of time. After recording the absorbance at 340 nm, CoA was mixed into the sample cuvette and an equal volume of buffer into the reference cuvette and the absorbance at 340 nm again recorded. The difference in absorbance measurements was corrected for dilution effects and end absorbance due to CoA. The extinction coefficient of NADH at 340 nm (6.2 m\textsuperscript{M}\textsuperscript{-1}cm\textsuperscript{-1}) and the protein concentration were used to calculate the amount of NADH formed per mg of \(\alpha\)-KGDC.

The results of such experiments carried out in 0.12 M potassium phosphate buffer are shown as a plot of nmoles NADH produced per mg of the complex versus the time at which CoA was added (Fig. 7). At 1 minute, a large amount of NADH was formed indicating that some unreacted \(\alpha\)-ketoglutarate was still present in the system. After 2 minutes the data plot as a straight line with a small negative slope. Extrapolation
Figure 7
NADH Yield of Reduced Succinylated α-KGDC.

The sample and reference cuvettes contained α-KGDC (0.42 mg), TPP (0.5 μmole), MgSO₄ (0.5 μmole) and NAD⁺ (1.5 μmole) in 0.12 M potassium phosphate buffer at pH 7. α-Ketoglutarate (0.065 μmole) followed by glyoxylate (0.10 μmole) 30 sec later were added to each cuvette. After incubation at 27°C for the lengths of time indicated the A₃₄₀ was recorded. A 50 μL aliquot of 8 mM CoA was added to the sample cuvette and an equal volume of buffer to the reference cuvette, making the total volume of each solution 1.0 mL. The absorbance at 340 nm was again read. The difference in A₃₄₀ corrected for dilution effects and end absorbance due to CoA, corresponded to the amount of NADH produced. (x) aerobic reaction.

Anaerobic sample and reference cells were prepared using the special cuvette shown in Figure 2. The main chamber contained α-KGDC (0.42 mg), TPP (0.5 μmole) MgSO₄ (0.5 μmole) and NAD⁺ (1.5 μmole) in 0.75 mL of 0.12 M potassium phosphate buffer, pH 7. α-Ketoglutarate (0.065 μmole) in 0.10 mL of the same buffer was placed in the side pouch. The cells were flushed with argon for 30 min at 4°C. Buffer, glyoxylate (1 mM) and CoA (8 mM) were also purged of oxygen with argon. The temperature of each cell was then allowed to reach 27°C using a water bath. α-Ketoglutarate was mixed with the solution in the chamber. 0.1 mL glyoxylate was added to each cuvette. The absorbance at 340 nm was measured, 0.05 mL of 8 mM CoA was added to the sample cuvette and an equal volume of buffer was added to the reference cuvette making the total volume of each solution 1.0 mL (Airtight Hamilton syringes were used for adding glyxolate, CoA and buffer to the cuvettes). The absorbance at 340 nm was again determined. NADH yield of reduced, succinylated complex was then calculated. ( ) anaerobic reaction.
of this line to zero time gives an intercept of 4.3 nmoles of NADH/mg complex, accounting for only half the number of electron equivalents from the dihydrolipoyl groups. To ensure that this did not result from oxidation of NADH or dihydrolipoyl groups by O2, the experiment was also carried out under anaerobic conditions. The results were the same as those obtained from experiments carried out in air. The low yield of NADH might be due to enzyme inactivation by the side products formed in the reaction mixture. By varying the α-ketoglutarate concentration, this possibility could be examined as in Figure 8, since this would cause the concentration of the side product to vary. The data were then plotted as NADH yield versus concentration of α-ketoglutarate.

The plot in Figure 8 can be explained as follows: at low α-ketoglutarate concentration, there is not enough α-ketoglutarate to fully succinylate the enzyme. Thus the increase in NADH production is a manifestation of an increase in the extent to which the complex is succinylated. The plateau region, 4.5 nmoles NADH/mg complex, corresponds to α-ketoglutarate concentration levels where the enzyme is fully succinylated and any excess α-ketoglutarate is completely removed from the system by the side reaction. The concentration of side products varies but does not affect the amount of NADH formed. The fast rate of increase in NADH yield at high α-ketoglutarate concentration is a reflection of unreacted α-ketoglutarate in the reaction mixture at the end of the incubation time.

These findings are similar to those in Figure 7 and so do not support the hypothesis that the low NADH yields results from the effects of side products. The results obtained in these experiments can be
Figure 8

NADH Yield of Reduced Succinylated α-KGDC .

Effect of α-Ketoglutarate Concentration.

The reaction mixture and procedure are similar to that of Figure 7 except for (1) varying α-ketoglutarate concentration and (2) fixed incubation time: 3 minutes at 27°C. All reactions were carried out under aerobic conditions.
**Figure 8**

![Graph showing the relationship between α-ketoglutarate concentration (mM) and nmoles NADH/mg α-KGDC.](image-url)
interpreted to mean that only half the dihydrolipoyl groups are oxidized by $E_3\cdot$FAD. However, this interpretation is inconsistent with the results of NADH-dependent labeling by (4-$^{14}$C) succinyl-CoA, which showed that all $E_2$ lipooyl groups can be reduced by NADH via $E_3\cdot$FAD. Some other explanation must be sought for the anomalous results.

Since all the lipooyl groups in $E_2$ are initially reduced and succinylated by $\alpha$-ketoglutarate (as indicated by data in Section 2), loss of half of the electron equivalents is indicated. There are two ways by which electron equivalents can be lost: (1) since NAD$^+$ is originally present in both cuvettes and $\alpha$-ketoglutarate and glyoxylate are added to both sample and reference cuvettes, any electron transfer to NAD$^+$ due to hydrolysis of lipooyl bound succinyl groups before CoA addition will not be detected; (2) if the reaction between $E_1$ and $E_2$ is reversible, the presence of glyoxylate as an "active semialdehyde" acceptor can favor the reverse reaction, i.e. transfer of electrons and succinyl groups from $E_2$ to $E_1$, forming $E_1[\gamma$-carboxy-\(\alpha\)-hydroxypropyl-TPP]. This could in turn form a condensation product with glyoxylate as shown by Reactions 8-10. The following section deals with these possibilities.

5. **Possible cause of the apparent loss of reducing equivalents.**

In the foregoing section, the NADH yield of the reduced, succinylated complex was less than the total number of lipooyl groups in $E_2$. It can not be said that only half of the lipooyl moieties are coupled to $E_3\cdot$FAD, since succinyl CoA labeling experiments indicate coupling of all lipooyl residues in $E_2$ with $E_3\cdot$FAD. One possible
explanation for this low NADH yield is loss of enzyme bound succinyl groups and electron equivalents in a hydrolytic reaction:

\[
\begin{align*}
H_2O & \quad E_2[\text{succinyl-Lip-SH}] \longrightarrow E_2[\text{Lip(SH)}_2] + \text{succinate} \\
& \quad E_3\text{FAD} \\
E_2[\text{Lip(SH)}_2] + \text{NAD}^+ & \longrightarrow E_2[\text{LipS}_2] + \text{NADH} + \text{H}^+ 
\end{align*}
\] (11)

(12)

Since both the sample and reference cuvette contained TPP, MgSO_4, NAD^+, \(\alpha\)-ketoglutarate and glyoxylate before CoA addition, transfer of electron equivalents to NAD^+ via \(E_3\text{FAD}\) after hydrolysis would not be observed. Thus, when CoA was added to the sample cuvette, only the remaining succinyl groups and electron equivalents would lead to NADH formation in the sample cuvette.

The question of whether lipoyl groups are fully succinylated by \(\alpha\)-ketoglutarate in the presence of glyoxylate also arises. Incomplete succinylation could cause the low NADH yield under the conditions described in Section 4.

To investigate whether NADH formation occurs when \(\alpha\)-ketoglutarate is incubated with the enzyme in the presence of TPP, MgSO_4 and NAD^+, two cuvettes were prepared each containing TPP, MgSO_4, NAD^+ and \(\alpha\)-KGUC in 0.12 M potassium phosphate buffer at pH 7. The baseline absorption spectrum of the reaction mixture in the region 310 to 390 nm was recorded, \(\alpha\)-ketoglutarate was mixed into the sample cuvette and an equal volume of buffer into the reference cuvette. The spectrum was recorded every minute for 4 mins. To correct for the change in spectrum due to \(\alpha\)-ketoglutarate absorption in this region, the procedure described above was carried out in the absence of \(\alpha\)-KGDC in the cuvettes. The change in absorption spectrum due to the reaction between the complex and \(\alpha\)-
ketoglutarate was calculated as follows:

\[ A_{\Delta \text{corr}} = (A_t - A_b) - (A_t' - A_b') \]

where: \( A_{\Delta \text{corr}} \) = change in absorbance at a given wavelength due to the reaction between \( \alpha \)-KGDC and \( \alpha \)-ketoglutarate.

\( A_{b'} \) = baseline absorbance of a solution containing TPP, MgSO\(_4\), NAD\(^+\) and \( \alpha \)-KGDC.

\( A_b \) = baseline absorbance of a solution containing TPP, MgSO\(_4\) and NAD\(^+\).

\( A_{t'} \) = absorbance after \( \alpha \)-ketoglutarate addition to a solution containing TPP, MgSO\(_4\), NAD\(^+\) and \( \alpha \)-KGDC at a given time.

\( A_t \) = absorbance after \( \alpha \)-ketoglutarate addition to a solution containing TPP, MgSO\(_4\) and NAD\(^+\) at a given time.

Although there are changes in the spectrum with time when \( \alpha \)-ketoglutarate is incubated with \( \alpha \)-KGDC in the presence of TPP, MgSO\(_4\) and NAD\(^+\) (Figure 9) the change does not correspond to the NADH absorption spectrum in this region.

After the initial increase in absorbance at 340 nm, a steady decrease is observed (Table 5). According to the measured NADH yield of the reduced, succinyllated \( \alpha \)-KGDC (Figure 7), only 10.5 moles and 9.5 moles of NADH per mole of the enzyme were produced at the end of the 2-minute and 3-minute reaction times, respectively. Assuming that the enzyme is fully succinyllated initially (i.e. 24 molecules of succinyl groups per particle of the complex) as indicated by labeling experiments (Section 2), the results depicted in Figure 7 indicate a loss of 13.5 moles of succinyl groups and the corresponding electron equivalents at the end of 2 minutes and an additional loss of 1.0 mole of succinyl
Figure 9

Change in Absorbance Spectrum when α-Ketoglutarate is incubated with α-KGDC in the Presence of TPP, MgSO₄ and NAD⁺

The sample and reference cuvette each contained 0.73 μmole TPP, 0.73 μmole MgSO₄ and 2.18 μmole NAD⁺ in the absence or presence of 0.6 mg α-KGDC in 0.12 M potassium phosphate buffer at pH 7 at 25°C. The baseline spectrum in the region 310-390 nm was obtained. 0.1 μmole of α-ketoglutarate was added to the sample cuvette and an equal volume of buffer to the reference cuvette making the total volume of each solution 1.0 mL. The spectrum was measured every minute for 4 minutes.

The absorbance values obtained after addition of α-ketoglutarate were corrected for baseline absorbance. The corrected absorbance measured in the absence of enzyme was subtracted from that obtained in the presence of enzyme to determine the effect of incubation of α-ketoglutarate with α-KGDC in the presence of TPP, MgSO₄ and NAD⁺ on the spectrum of the reaction mixture. These differences in corrected absorbances (ΔAcorr) are plotted versus the wavelength.

0—0 0.5 min

0—0 1.5 min

0—0 2.5 min

0—0 3.5 min

0—0 4.5 min
Figure 9

A_\text{corr} vs. Wavelength (nm)

-0.002
-0.001
0.000
0.001
0.002
0.003
0.004

340
380
Table 5. Change in $A_{340}$ when $\alpha$-ketoglutarate is incubated with $\alpha$-KGDC in the Presence of TPP, MgSO$_4$ and NAD$^+$.  

<table>
<thead>
<tr>
<th>time(min)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.0018</td>
<td>0.0037</td>
<td>0.0013</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0014</td>
<td>0.0023</td>
<td>0.0003</td>
</tr>
<tr>
<td>3.0</td>
<td>0.0007</td>
<td>0.0009</td>
<td>-0.0031</td>
</tr>
</tbody>
</table>

A. The materials and procedure are similar to those described in Fig. 9. The $A_{\Delta \text{corr}}$ at 340 nm corresponding to the specified reaction times are given.

B. The materials and procedure are similar to those described in Fig. 9, except 1.22 mg of $\alpha$-KGDC and 0.2 µmole of $\alpha$-ketoglutarate were used. The $A_{\Delta \text{corr}}$ at 340 nm corresponding to the specified reaction times are given.

C. The materials and procedure are similar to those described in Fig. 9 except 1.22 mg of $\alpha$-KGDC and 0.2 µmole of $\alpha$-ketoglutarate were used and the reaction was carried out under anaerobic conditions using the special cuvettes depicted on Fig. 2. The sample and reference cuvette each contained TPP, MgSO$_4$, NAD$^+$ and $\alpha$-KGDC in 0.12 M potassium phosphate buffer at pH 7 in the main chamber. $\alpha$-Ketoglutarate was placed in the side pouch of the sample cuvette and an equal volume of buffer in the side pouch of the reference cuvette. The cells were flushed with argon for 30 minutes, the baseline absorbance at 340 nm was obtained, the solution in the side pouch was mixed with that in the main chamber and the absorbance at 340 nm was measured every minute. The $A_{\Delta \text{corr}}$ at 340 nm corresponding to the specified reaction times are given.
group and the corresponding electron equivalents at the end of 3 minutes (i.e. a total loss of 14.5 moles of succinyl groups at the end of 3 minutes). If the loss of reducing equivalents was due to hydrolysis, an increase in $A_{340}^{nm}$ of 0.0203 unit for an enzyme concentration of 0.61 mg/mL or 0.0406 unit for an enzyme concentration of 1.22 mg/mL should be obtained at the end of 2 minutes with an additional increase of 0.0015 unit for an enzyme concentration of 0.61 mg/mL or 0.0030 unit for an enzyme concentration of 1.22 mg/mL at the end of 3 minutes under the reaction conditions described in Table 5. However, an increase of 0.0014 unit for an enzyme concentration of 0.61 mg/mL and 0.0023 unit for an enzyme concentration of 1.22 mg/mL were obtained at the end of 2 minutes (Table 5). While experiments described in Figure 7 predict an increase in $A_{340}$ with time if the loss of reducing equivalents was due to hydrolysis, Table 5 shows a decrease in $A_{340}$ with time. The low increase in $A_{340}$ observed at the end of 2 minutes as well as the decrease in $A_{340}$ with time can not be explained by NADH oxidation since similar results are obtained under anaerobic conditions. Therefore, hydrolysis of lipoyl bound succinyl groups cannot account for the apparent loss of reducing equivalents.

The change in $A_{340}$ (Table 5), as well as the absorbance spectrum in the 310nm-390 nm region (Figure 7) could be due mostly to the formation of products from the carboligase side reaction catalyzed by $E_1$.

Table 6 contains the data on succinylation of $\alpha$-KGDC with (5-$^{14}$C)$\alpha$-ketoglutarate followed by incubation with glyoxylate for periods indicated on the table. Within 30 seconds of incubation of $\alpha$-KGDC with
Table 6. Effect of Glyoxylate on the succinylation of α-KGDC by (5-14C) α-ketoglutarate.

<table>
<thead>
<tr>
<th>time of incubation</th>
<th>nmoles (4-14C) succinyl/mg enzyme</th>
<th>-CoAa</th>
<th>+CoAb</th>
<th>CoA labile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>12.5</td>
<td>1.4</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>6.5</td>
<td>1.3</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>5.5</td>
<td>1.0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>4.4</td>
<td>0.0</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>5.0</td>
<td>0.3</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>5.2</td>
<td>0.2</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

aEffect of glyoxylate on succinylation of α-KGDC by (5-14C) α-ketoglutarate. α-KGDC (0.07 mg) reacted at 25°C for 30 sec. with 0.01 μmole (5-14C) α-ketoglutarate in the presence of 0.1 μmole TPP, 0.1 μmole MgSO4, 0.3 μmole NAD+ in 0.12 M potassium phosphate buffer, pH 7. 5 μL of 4 mM glyoxylate was added making the total volume 100 μL and the reaction mixture incubated for 1 to 10 min. 50 μL of the reaction mixture was assayed by the filter paper-TCA precipitation method for protein bound radioactivity.

bEffect of glyoxylate on desuccinylation of (4-14C) succinyl-α-KGDC with CoA. The materials and procedure are the same as in (a), except 10 μL of 8 mM CoA were added after incubation with glyoxylate. 1 min after addition of CoA, 50 μL of the reaction mixture were assayed by the filter paper-TCA precipitation method for protein-bound radioactivity.
(5-14C)α-ketoglutarate in the presence of appropriate cofactors, complete succinylation of the complex occurred (Table 6, time = 0 min). Only 6.5 out of 12.5 nmoles (14C) succinyl per mg of the complex remained when (4-14C) succinyl-α-KGDC was treated with glyoxylate for 1.0 minute. There was a gradual decrease in the amount of radioactivity associated with α-KGDC between 1.0 min to 5.0 min of incubation of the radiolabeled complex with glyoxylate. After 5.0 min the (4-14C) succinyl groups associated with the enzyme leveled off to 4.9 nmoles per mg of the complex. 0.8 mM CoA is sufficient to remove 80-100% of the enzyme bound succinyl groups.

To determine whether the decrease in the amount of (14C) succinyl groups associated with α-KGDC is a result of the interaction of (14C) succinyl-α-KGDC with glyoxylate, the complex was incubated with (5-14C) α-ketoglutarate for the lengths of time indicated on Table 7 in the absence of glyoxylate. A maximum of 19 nmoles (4-14C) succinyl are incorporated per mg of the complex, however, only 8-10 nmoles are CoA labile. The non-CoA labile groups might be bound to some other nucleophiles in the enzyme.

The data in Table 7 show that glyoxylate causes the loss of enzyme bound succinyl groups. This can explain the low NADH yield in the experiments of Section 4. A possible explanation for this observation is that glyoxylate causes the reversal of reaction between E1 and E2 due to the formation of side product(s). The relevant reactions are described by equations 13 and 14.
Table 7. Effect of prolonged incubation of α-KGDC with (5-14C) α-ketoglutarate on the amount of (4-14C)-succinyl groups covalently associated with the enzyme.

<table>
<thead>
<tr>
<th>time of incubation with α-ketoglutarate (min)</th>
<th>nmoles (14C) succinyl/mg enzyme</th>
<th>-CoA^a</th>
<th>+CoA^b</th>
<th>CoA labile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td>12.4</td>
<td>4.3</td>
<td>8.1</td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>17.0</td>
<td>7.1</td>
<td>9.9</td>
</tr>
<tr>
<td>3.5</td>
<td></td>
<td>19.1</td>
<td>10.3</td>
<td>8.8</td>
</tr>
<tr>
<td>5.5</td>
<td></td>
<td>18.3</td>
<td>9.3</td>
<td>9.0</td>
</tr>
</tbody>
</table>

^aSuccinylation of α-KGDC with (5-14C) α-ketoglutarate. The materials and procedure are the same as those in Table 6a except (5-14C) α-ketoglutarate reacted with the enzyme for periods of time indicated on this table instead of 30 sec and glyoxylate was not present in the reaction mixture.

^bDesuccinylation of (4-14C) succinyl α-KGDC. The materials and procedure are similar to those in Table 6b except (5-14C) α-ketoglutarate reacted with α-KGDC for various lengths of time (indicated on the table) instead of 30 sec, and glyoxylate was not present in the reaction mixture.
\[ E_2[\text{succinyl-Lip-SH}] + E_1[\text{TPP}] \rightarrow E_2[\text{Lip } S_2] + E_1[\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}] \] (13)

\[ E_1[\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}] + \text{glyoxylate} \rightarrow \text{condensation product(s)} \] (14)

This process explains the loss of both succinyl groups and electron equivalents from the complex.
Discussion

The flavin coenzyme of the complex has been reduced with dithionite either in the isolated form (FAD) in neutralized acid extracts of $\alpha$-KGDC (Pettit et al, 1973) or in association with $\alpha$-KGDC (Koike et al, 1960a) to determine the flavin content of the complex. It has also been reduced while bound to purified dihydrolipoamide dehydrogenase with dihydrolipoamide, NADH or dithionite as reducing agents (Koike et al, 1960b, Williams, 1965) to study the intermediates involved in the catalytic pathway.

For Section 1 of this chapter, reduction of the $\alpha$-KGDC bound flavin using $\alpha$-ketoglutarate and CoA as the electron donating system was carried out to determine the number of flavins that are reducible by the lipoyl groups in $E_2$. This was found to be 3.7-4.5 nmoles/mg complex which is equal to the reported flavin content of $\alpha$-KGDC, 4.0-4.8 nmoles/mg complex with 1 FAD/E$_3$ active site (Koike et al, 1960a, Angelides and Hammes, 1979, Pettit et al, 1973). It is therefore concluded that all the dihydrolipoyl dehydrogenase in the complex are coupled to the dihydrolipoyl transsuccinylase core.

The purification procedure used yielded enzyme samples with associated Coenzyme A as inferred from reduction of the flavin in the absence of added CoA. This CoA contamination was removed during purification in the presence of PMSF and EDTA.

An average of 10.5 nmoles (4-$^{14}$C) succinyl groups were covalently incorporated per mg of the complex by reaction with $\alpha$-ketoglutarate in the absence of CoA, which is in agreement with Reed's observation (Collins and Reed, 1977). (4-$^{14}$C) succinyl CoA introduced 10 nmoles of
(4-\textsuperscript{14}C) succinyl groups per mg of the complex in an NADH dependent manner. These data indicate that all the transsuccinylase subunits interact with the \( \alpha \)-ketoglutarate dehydrogenase component as well as with the dihydrolipoyl dehydrogenase component.

To verify the coupling between dihydrolipoyl transsuccinylase and dihydrolipoyl dehydrogenase the NADH yield of the reduced, succinylated complex was measured. This unexpectedly turned out to correspond to only about half the reducing equivalents thought to be present. The low yield was ultimately accounted for by the loss of succinyl groups and electron equivalents through the reversal of the reaction between \( \alpha \)-ketoglutarate and dihydrolipoyl transsuccinylase under conditions where there is no \( \alpha \)-ketoglutarate to resuccinylate the lipoyl residues but where there is glyoxylate to form condensation product(s) with the TPP bound "active aldehyde" in \( E_1 \). This reversal of reaction between \( E_1 \) and \( E_2 \) has been observed by Gunsalus (Gunsalus, 1954) for \textit{E. coli} fraction A (partially purified pyruvate dehydrogenase-transacetylase subcomplex). Incubation of acetaldehyde (as acceptor for the aldehyde formed), acetyl phosphate, dihydrolipoate, TPP, MgCl\(_2\), CoA and phosphotransacetylase with \textit{E. coli} fraction A led to oxidation of dihydrolipoate and formation of acetoin.

The fact that approximately half the succinyl groups and reducing equivalents are lost during the first 1 to 2 minutes and the other half are retained for the next 8 minutes, raises questions about the relative rates of succinyl and electron transfer from \( E_1 \) to \( E_2 \). Are there one or two classes of lipoyl groups as determined by their rate of interaction?
with $E_1$? If there are two classes, do they both show catalytic competence?

It may not be necessary to propose two classes of lipoyl groups. The observation described in the preceding paragraph may be explained by a lower $K_m$ of $E_1$ for oxidized lipoic acid. Thus, after the first 12 lipoyl moieties transfer succinyl and electrons back to $E_1$•TPP, the oxidized lipoic acid can act as a dead end inhibitor for the reverse reaction.

The most important findings in this chapter are (1) all the flavoproteins in the complex are coupled to $E_2$ and (2) all the transsuccinylase components are coupled to $E_3$. 
CHAPTER IV
Hydrolysis of Succinyl CoA

It is evident from the available experimental data on the mechanism of α-KGDC that the transfer of succinyl groups and electrons from α-ketoglutarate dehydrogenase (E₁) to dihydrolipoyl transsuccinylase (E₂) is still poorly understood. This chapter addresses the question of whether the transfer of succinyl groups and electron pairs from E₁ to E₂ occur via a stepwise or concerted mechanism.

Enhanced CoA production from succinyl CoA in the presence of α-KGDC, NADH and TPP was observed in this laboratory. This could be due to two major processes:

1) nucleophilic displacement of CoA by protein sulfhydryl groups:
\[ E{-}\text{SH} + \text{succinyl CoA} \rightarrow E{-}\text{S-succinyl} + \text{CoASH} \] (15)

2) reversal of the sequence of steps involved in the oxidative decarboxylation of α-ketoglutarate, as defined by equations 16-20:
\[ E₃[\text{FAD,ox}] + \text{NADH} + H^+ \rightarrow E₃[\text{FAD,red}] + \text{NAD}^+ \] (16)
\[ E₃[\text{FAD,red}] + E₂[\text{Lip S₂}] \rightarrow E₃[\text{FAD,ox}] + E₂[\text{Lip(SH)₂}] \] (17)
\[ E₂[\text{Lip(SH)₂}] + \text{succinyl CoA} \rightarrow E₂[\text{succinyl-S-Lip-SH}] + \text{CoASH} \] (18)
\[ E₂[\text{succinyl-S-Lip-SH}] + E₁[\text{TPP}] \rightarrow E₂[\text{Lip(SH)₂}] + E₁[\text{succinyl-TPP}] \] (19)
\[ \text{E}_2 \text{lip(SH)2} \rightarrow \text{E}_1 \gamma \text{c} \]  
\[ \text{E}_1 [\text{succinyl-TPP}] \rightarrow \text{E}_1 [\text{TPP}] + \text{succinate} \]  
\[ \text{H}_2 \text{O} \rightarrow \text{E}_1 [\text{TPP}] + \text{succinate} \]  
\[ \text{E}_2 [\text{succinyl-S-Lip-SH}] + \text{E}_1 [\text{TPP}] \rightarrow \text{E}_2 [\text{Lip S2}] + \]  
\[ \text{E}_1 [\gamma \text{-carboxy-\alpha-hydroxypropyl-TPP}] \]  

CoA formation via Equation 15 does not require added cofactors, however, a higher concentration of CoA may be found in the presence of NADH due to the reduction of the disulfides in E₃ and the lipoyl groups in E₂ increasing the enzyme sulfhydryl group which can displace CoA from succinyl CoA.

CoA formation via Equations 16 to 20 should show dependence on active lipoyl residues in E₂ as well as on added NADH and TPP-Mg²⁺. Two hypothetical mechanisms by which succinyl groups and electrons from S-succinyl-dihydrolipoamide in E₂ could be transferred to E₁-TPP are discussed. The first possibility, as shown by Equations 19, 19a and 19b involves succinyl group transfer followed by electron transfer to TPP forming an E₁[succinyl-TPP] intermediate which is quite susceptible to nucleophilic attack. At room temperature, methanolysis of 3,4-dimethyl-2-(acetyl)-thiazolium nitrate is 56% complete within 10 minutes (Breslow and McNelis, 1960). The 3,4-dimethyl-2-(acetyl)-thiazolium iodide was rapidly deacylated in aqueous solutions at pH 7-8 to give acetic acid. The half-life in 0.02 M imidazole buffers, pH 7-8, was less than 30 seconds (Daigo and Reed, 1962). When α-hydroxyethyl TPP is oxidized by acetoacetate synthetase in the presence of ferricyanide at pH 6.2, the acetyl adduct of TPP is hydrolyzed instantaneously (Krampitz, 1970). The second possibility, as shown by Equation 20 involves simultaneous
transfer of succinyl groups and electrons to $E_1[TPP]$ forming $E_1[\gamma\text{-carboxy-}\alpha\text{-hydroxypropyl-TPP}]$ which is considerably more stable than $E_1[\text{succinyl-TPP}]$. Less than 1% of $\alpha\text{-hydroxyethyl TPP}$ was decomposed to acetaldehyde and TPP at pH 6.5 for 4 hours at 30°C (Krampitz, 1970).

Equations 19 and 19b will show turnover in $\alpha\text{-KGDC}$ catalyzed succinyl CoA hydrolysis within minutes since $E_1[\text{succinyl-TPP}]$ will be hydrolyzed as soon as it is formed, while Reactions 15 and 20 will not result in turnover.

The cofactor dependence of $\alpha\text{-KGDC}$ catalyzed succinyl CoA hydrolysis was investigated. In a typical experiment, a complete reaction mixture consisted of $\alpha\text{-KGDC}$, TPP, MgSO$_4$, NAD$^+$ and NADH in 50 mM potassium phosphate buffer at pH 7. Hydrolysis was initiated by the addition of succinyl CoA to this mixture. Six such reaction mixtures were prepared and the reaction time for each one was varied from 1 to 15 minutes allowing for the determination of free sulfhydryl group concentration in the reaction mixture at 6 different time points during the course of the reaction. Each reaction mixture was assayed for free sulfhydryl content as described in Figure 10.

The foregoing experiment with a complete reaction mixture was also carried out in the absence of NADH, TPP, MgSO$_4$ or NAD$^+$ from the reaction mixture. The rate of succinyl CoA hydrolysis in the absence of $\alpha\text{-KGDC}$ but in the presence of the cofactors was also determined and this nonenzymatic rate subtracted from all of the others. The reaction progress curves shown in Figure 11 were obtained. These curves showed that the enzymatic hydrolysis of succinyl-CoA is absolutely dependent upon the presence of NADH and substantially dependent upon added TPP.
Figure 10

Determination of the Sulfhydryl Group Concentration Using the DTNB Assay.

10 µL of succinyl CoA (10.0 mM) solution in 50 mM potassium phosphate buffer at pH 7 were mixed with 90 µL of 50 mM potassium phosphate buffer at pH 7 containing α-KGDC (0.078 mg), TPP (.29 µmole), MgSO₄ (.47 µmole) NAD⁺ (.88 µmole) and NADH (.24 µmole). The reaction mixture was allowed to stand at 25°C for a minute. At the end of the incubation time, 50 µL of the reaction mixture was assayed for the concentration of free sulfhydryl groups as follows: 1 ml of 1 mM DTNB in 0.1 M sodium bicinate buffer at pH 8.0 was placed in a cuvette and the baseline absorbance at 412 nm was obtained. 50 µL of the reaction mixture was added to this cuvette and the absorbance at 412 nm was monitored for 2 min. The change in absorbance at the instant of addition was then extrapolated from the plot of A₄12 vs. time as shown on this graph.
Figure 10

TIME (minutes)

412 nm ABSORBANCE

0.05

0.10
Effect of TPP, Mg$^{+2}$, NAD$^+$ and NADH on α-KGDC Catalyzed Succinyl CoA Hydrolysis

Six solutions each containing α-KGDC (0.078 mg), TPP (0.29 μmole) MgSO$_4$ (0.47 μmole) NAD$^+$ (0.88 μmole) and NADH (0.24 μmole) in 90 μL of 50 mM potassium phosphate buffer at pH 7 were prepared. 10 μL of succinyl CoA (10 mM) were added to each solution to initiate the enzyme catalyzed hydrolysis of succinyl CoA. The reaction was carried out at 25°C for various lengths of time (i.e. Solution 1 for 1.0 min, solution 2 for 3.0 min, etc.) as shown on the graph. At the end of the reaction time 50 μL of the reaction mixture was assayed for the concentration of free sulfhydryl group as described in Figure 10.

The foregoing experiment was carried out in the absence of TPP, MgSO$_4$, NAD$^+$, NADH or α-KGDC.

The change in absorbance at 412 nm (Δ$A_{412}$) at the instant of addition of the reaction mixture to DTNB was obtained as described in Figure 10. The Δ$A_{412}$ in the presence of the enzyme was corrected for the Δ$A_{412}$ in the absence of the enzyme. The corrected Δ$A_{412}$ is plotted vs. the reaction time.

- O-O α-KGDC plus all cofactors
- O-O α-KGDC plus all cofactors except TPP
- O-O α-KGDC plus all cofactors except NAD$^+$
- O-O α-KGDC plus all cofactors except Mg$^{+2}$
- •-• α-KGDC plus all cofactors except NADH
Fig. 11
The reaction observed in the absence of added TPP might be attributed to the endogenous TPP known to be present.

The initial rates calculated from the initial slopes of the curves in Figure 11 are summarized on Table 8. The data represent the average of 2 to 3 determinations. Ninety-two percent of the rate of α-KGDC catalyzed succinyl CoA hydrolysis is NADH dependent, 34% is dependent on added TPP, 62% on added MgSO₄ and 48% on added NAD⁺. According to Equations 16 to 20 the α-KGDC catalyzed succinyl CoA hydrolysis should be fully dependent on NADH, TPP and MgSO₄. The extent of the effect of added NADH on the rate of hydrolysis is in agreement with Equations 16 to 20. The isolated complex contains about 50% of its total TPP as indicated by the NAD reduction assay, which gave 55% of the complex activity in the absence of added TPP. This explains why the rate of hydrolysis without added TPP is 66% of the rate in the complete system. The dependence of the rate of succinyl CoA hydrolysis on added MgSO₄ is higher than the observed rate dependence of α-ketoglutarate oxidation as measured by the NAD⁺ reduction assay (33%). It is possible that succinyl-CoA or contaminating succinate may be complexing Mg²⁺ and limiting its availability for TPP-binding. The lower rate of hydrolysis in the absence of added NAD⁺ cannot easily be explained, but might have to do with the fact that NAD⁺ prevents the production of the inactive, 4-electron reduced state of the enzyme.

The rate of α-KGDC catalyzed succinyl CoA hydrolysis is dependent on the ratio of NAD⁺ to NADH and not on the absolute concentration of
Table 8. Effect of Cofactors on α-KGDC catalyzed succinyl CoA hydrolysis.

<table>
<thead>
<tr>
<th>Composition of the Reaction Mixture</th>
<th>Rate of hydrolysis (mM succinyl CoA/min per mg complex)</th>
<th>Rate of hydrolysis in complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>7.2 x 10^-3</td>
<td>1.00</td>
</tr>
<tr>
<td>-NADH</td>
<td>0.6 x 10^-3</td>
<td>.08</td>
</tr>
<tr>
<td>-TPP</td>
<td>4.7 x 10^-3</td>
<td>.66</td>
</tr>
<tr>
<td>-Mg+2</td>
<td>2.7 x 10^-3</td>
<td>.38</td>
</tr>
<tr>
<td>-NAD</td>
<td>3.7 x 10^-3</td>
<td>.52</td>
</tr>
</tbody>
</table>

A complete reaction mixture consisted of α-KGDC (.078 mg), TPP (0.29 μmole), MgSO₄ (0.47 μmole), NAD (0.88 μmole), NADH (0.24 μmole) and succinyl CoA (0.10 μmole) in 100 μL of 50 mM potassium phosphate buffer at pH 7 at 25°C. -NADH, -TPP, -MgSO₄ or -NAD indicate the absence from the reaction mixture of NADH, TPP, MgSO₄ or NAD, respectively.

The initial slopes of the $\Delta A_{412}$ vs. time plots shown in Figure 11 was obtained. Using the protein concentration, the extinction coefficient of the thiol anion (13.6 mM⁻¹cm⁻¹) and the corrected initial slope, the initial rate of hydrolysis of succinyl CoA under the reaction conditions described for each plot was calculated.

The rate of enzyme catalyzed hydrolysis (mM/min per mg complex) as well as the ratio of the rate of hydrolysis in the absence of a cofactor (NADH, TPP, MgSO₄ or NAD⁺) to that of the complete reaction mixture were tabulated. The values shown on the table represent the average of two to three experimental runs.
each nucleotide. As shown in Figure 12, the rate of hydrolysis at 4.4 mM NAD\(^{+}\) and 1.2 mM NADH is the same as the rate at 8.8 mM NAD\(^{+}\) and 2.4 mM NADH.

The requirement for TPP in the α-KGDC catalyzed hydrolysis of succinyl CoA is verified by the inhibition of the reaction by TTPP, Fig. 13 and Table 9. Preincubation of the enzyme with 0.49 mM TTPP in the presence of MgSO\(_4\) inhibited the rate of hydrolysis of succinyl CoA by 53% (Table 9) and the rate of α-KGDC oxidation by 50%. Doubling the concentration of TTPP in the preincubation mixture gave the same results. The activity of the complex towards succinyl CoA or α-ketoglutarate was not completely abolished by TTPP, presumably due to the presence of tightly bound TPP isolated with the complex and not displaced by TTPP.

The sensitivity of α-KGDC to arsenite has provided a means for elucidating the role of lipoic acid in succinyl CoA hydrolysis. The sulphydryl groups of dihydrolipoamide residues in the transsuccinylase react with arsenite to form a covalent adduct (Sanadi et al, 1959). Preincubation of α-KGDC with arsenite in the presence of NADH and NAD\(^{+}\) resulted to 77% reduction of enzyme activity toward succinyl CoA (Fig. 14, Table 10) and 75% reduction of the activity of the complex towards α-ketoglutarate. The residual activity may be due to the dissociation of the arsenite dithiol complex.

A second method was used to investigate the requirement for lipoic acids in the α-KGDC catalyzed succinyl CoA hydrolysis. The lipoic acids were covalently modified by NEM as described in Fig. 15. Inactivation by NEM completely abolished the ability of the complex to catalyze the
Figure 12

Effect of a Lower Concentration of NAD$^+$ and NADH on the Rate of α-KGDC Catalyzed Succinyl CoA Hydrolysis

The change in A$_{412}$, corrected for non-enzymatic hydrolysis as described in Figure 11, is plotted vs. the reaction time.

〇 — 〇 The same procedure as described for Figure 11 (〇 — 〇)

● — ● Similar procedure as in Figure 11 (〇 — 〇) except 0.44 µmole of NAD$^+$ and 0.12 µmole of NADH were used.
FIGURE 12
Figure 13.

Effect of TTPP on α-KGDC Catalyzed Succinyl CoA Hydrolysis

α-KGDC (3.12 mg/mL) was incubated with TTPP [0.49 mM (●●), or 0.98 mM (X-X)] and MgSO₄ (18.8 mM) in 50 mM potassium phosphate buffer at pH 7.0 (Solution A; total volume = 0.25 mL) at 4°C for 30 min. At the end of the incubation period, Solution A was transferred to a 25°C water bath and was allowed to equilibrate to this temperature for 5 min. 25 μL of Solution A were then added to 65 μL of 50 mM potassium phosphate buffer at pH 7 containing TPP (0.45 mM), NAD⁺ (13.5 mM) and NADH (3.7 mM). The reaction was started by addition of 10 μL of 10.0 mM succinyl CoA. The reaction was carried out at 25°C for various lengths of time. 50 μL of the reaction mixture were then added to 1mL of 1 mM DTNB in 0.1 M sodium bicinate buffer at pH 8.0. The change in absorbance at 412 nm at the instant of addition of the reaction mixture to DTNB was obtained and corrected for non-enzymatic hydrolysis as described in Figure 11. The progress curves are shown in this figure.

O—O ; As a control, α-KGDC (3.12 mg/mL) was incubated with MgSO₄ (18.8 mM) in 50 mM potassium phosphate buffer at pH 7 in the absence of TTPP. The procedure described in the preceding paragraph was followed.
<table>
<thead>
<tr>
<th>Concentration of TTPP in the preincubation mixture (mM succinyl CoA)</th>
<th>Rate of hydrolysis preincubated with TTPP (mM succinyl CoA/min per mg complex)</th>
<th>Rate of hydrolysis by α-KGDC preincubated without TTPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49 mM</td>
<td>$2.8 \times 10^{-3}$</td>
<td>0.47</td>
</tr>
<tr>
<td>0.98 mM</td>
<td>$2.8 \times 10^{-3}$</td>
<td>0.47</td>
</tr>
</tbody>
</table>

The experiments are described in Figure 13. The initial rate of succinyl CoA hydrolysis was obtained from the initial slope of each $\Delta A_{412}$ vs time plot in Fig 13, the protein concentration and the extinction coefficient of the thiol anion.

The rate of enzyme catalyzed hydrolysis (mM/min per mg complex) as well as the ratio of the rate of hydrolysis by α-KGDC preincubated with TTPP to that by α-KGDC preincubated without TTPP are tabulated. The values represent the average of 2 experimental runs.
Figure 14

Arsenite Inhibition of the $\alpha$-KGDC Catalyzed Succinyl CoA Hydrolysis

$\bullet$-$\bullet$; $\alpha$-KGDC (1.2 mg/mL) was incubated with NAD$^+$ (13.5 mM), NADH (3.7 mM) and sodium arsenite (1.5 mM) in 50 mM potassium phosphate buffer at pH 7.0 (Solution A, total volume = 0.65 mL) at 4°C for 30 minutes. During the course of the incubation, 65 $\mu$L portions of solution A were transferred to 6 small test tubes. At the end of 30 minutes these small tubes were transferred to a 25°C water bath and allowed to equilibrate for 5 min. Aliquots (25 $\mu$L) of 50 mM potassium phosphate buffer at pH 7 containing 11.8 mM TPP and 18.8 mM MgSO$_4$ were then added to each of the 65 $\mu$L portions of solution A. Succinyl CoA hydrolysis was started by addition of 10 $\mu$L of 10 mM succinyl CoA solution. Incubation was at 25°C for various times. Aliquots (50 $\mu$L) of each of the reaction mixtures were then assayed for free sulfhydryl as described in Figure 10. The change in $A_{412}$ was corrected for non-enzymatic hydrolysis as described in Figure 11 and is plotted vs. the reaction time.

$\circ$-$\circ$; the foregoing experiment was carried out in the absence of arsenite.
FIGURE 14
Table 10. Arsenite inhibition of the α-KGDC catalyzed succinyl CoA hydrolysis.

<table>
<thead>
<tr>
<th>Concentration of Arsenite in the preincubation mixture</th>
<th>Rate of hydrolysis by α-KGDC modified with arsenite/(mM succinyl CoA/min per mg complex)</th>
<th>Rate of hydrolysis by α-KGDC unmodified with arsenite</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$6.0 \times 10^{-3}$</td>
<td>1.00</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>$1.4 \times 10^{-3}$</td>
<td>.23</td>
</tr>
</tbody>
</table>

The experiments are described in Figure 14. The initial rate of succinyl CoA hydrolysis was obtained from the initial slope of each $A_{412}$ vs. time plot in Figure 14, the protein concentration and the extinction coefficient of the thiol anion.

The rate of enzyme catalyzed hydrolysis (mM/min per mg of the complex), as well as the ratio of the rate of hydrolysis catalyzed by α-KGDC modified with arsenite to that catalyzed by the unmodified complex are tabulated. The values represent the average of two experimental runs.
The lipoyl groups of α-KGDC (6 mg) were covalently modified with NEM (3 μmole) in the presence of NAD⁺ (1.2 μmole), TPP (3.0 μmole) and MgSO₄ (3.0 μmole) using NADH (0.3 μmole) as the reducing agent. The reaction was carried out in 1.0 mL of 50 mM potassium phosphate buffer at pH 7.0 at 27°C. Aliquots of the reaction mixture were withdrawn at various times and tested for enzyme activity using the NAD⁺ reduction assay. o—o

In a second reaction vessel, as a control experiment, α-KGDC (6mg) was incubated with NADH (0.3 μmole), NAD⁺ (1.2 μmole), TPP (3.0 μmole) and MgSO₄ (3.0 μmole) in 1.0 mL of 50 mM potassium phosphate buffer at pH 7 at 27°C. Aliquots of this solution were tested for enzyme activity again using the NAD⁺ reduction assay. ●—●

The rate of inactivation of the complex by NEM is plotted as residual α-KGDC activity versus time.

Figure 15
Modification of α-KGDC by NEM
Figure 15

% Residual Activity

Time (minutes)
hydrolysis of succinyl CoA (Fig. 16, Table 11).

\((1-^{14}\text{C})\text{Succinate}\) was identified as the hydrolysis product resulting from the decomposition of \((^{14}\text{C})\text{succinyl-CoA}\) in the presence of \(\alpha\)-KGDC, TPP, MgSO\(_4\), NAD\(^+\) and NADH. Two radioactive peaks were observed in a column chromatogram (Figure 17). The first radioactive peak and the \((1-^{14}\text{C})\text{succinate}\) sample obtained from the reaction of \((5-^{14}\text{C})\alpha-\text{ketoglutarate}\) with hydrogen peroxide exhibited the same elution volume in the chromatographic system described in Figure 17. Of the total radioactivity in the \((4-^{14}\text{C})\text{succinyl CoA}\) sample 74.4% was found in the first peak. The second radioactive peak (fractions 62-75) contained unreacted \((4-^{14}\text{C})\text{succinyl CoA}\). In the absence of \(\alpha\)-KGDC, a control reaction, 50.9% of total radioactivity was associated with the first peak (Figure 18). When the \((4-^{14}\text{C})\text{succinyl CoA}\) sample used for experiments described in Figures 17 and 18 was chromatographed in the system described in Figure 17, 48.5% of the total radioactivity was found to be associated with the first peak. Applying this correction on the data shown in Figures 17 and 18, 26% and 2.4% of the total radioactivity was associated with peak I with \(\alpha\)-KGDC in the reaction mixture and without \(\alpha\)-KGDC in the reaction mixture, respectively.

Fraction 16 from Figure 17 was rechromatographed through a second DEAE Sephadex A25 column developed as described in Figure 19. Again, the product of the enzyme catalyzed succinyl CoA hydrolysis had the same elution volume as the authentic \((1-^{14}\text{C})\) succinate sample (Figure 19) in the second chromatographic system.

The reciprocal plot of the rate of \(\alpha\)-KGDC catalyzed succinyl CoA hydrolysis as a function of succinyl CoA concentration is shown in
Figure 16

NEM Inhibition of the α-KGDC Catalyzed Succinyl CoA Hydrolysis

When the activity of the complex which was treated with NEM was less than 1% that of the control, 6 μmoles of β-mercaptoethanol were added to each of the reaction vessels described in Figure 15. Each reaction solution was then centrifuged at 36,000 rpm for 3 hours using a type 40 rotor in a refrigerated Spinco ultracentrifuge. The protein pellets were redissolved in 20 mM potassium phosphate buffer at pH 7 and dialyzed exhaustively at 4°C against 20 mM potassium phosphate buffer at pH 7. The protein concentrations of the two solutions were determined by the Lowry procedure using BSA as the standard protein.

○○○, similar procedure as in Figure 11 (○○○) except the enzyme sample (0.095 mg) from the control experiment described in Figure 15 was used.

○○○, similar procedure as in Figure 11 (○○○) except the NEM modified enzyme (0.082 mg) described in Figure 15 was used.

The change in A412, corrected for non-enzymatic hydrolysis as described in Figure 11, is plotted vs. the reaction time.
TIME (minutes)

FIGURE 16
Table 11. NEM inhibition of the α-KGDC catalyzed succinyl CoA hydrolysis.

<table>
<thead>
<tr>
<th>Concentration of NEM in the preincubation mixture (mM)</th>
<th>Rate of hydrolysis (mM succinyl CoA/min per mg complex)</th>
<th>Rate of hydrolysis by NEM modified α-KGDC</th>
<th>Rate of hydrolysis by unmodified α-KGDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3 x 10^{-3}</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>3.0 mM</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The experiments are described in Figure 16. The initial rate of succinyl CoA hydrolysis was obtained from the initial slope of each $A_{412}$ vs. time plot in Figure 16, the protein concentration and the extinction coefficient of the thiol anion.

The rate of enzyme catalyzed hydrolysis (mM/min per mg of the complex), as well as the ratio of the rate of hydrolysis catalyzed by NEM modified α-KGDC to the rate of hydrolysis catalyzed by unmodified α-KGDC are tabulated.
A 1.5 mL solution containing 50 mM potassium phosphate buffer at pH 7 containing 2.0 μmole of TPP, 5.0 μmole of MgSO₄, 8.0 μmole of NAD⁺, 2.0 μmole of NADH and 7.7 mg of α-KGDC was incubated with 0.5 μmole of (4-¹⁴C) succinyl CoA at 25°C. After 10.0 minutes, the pH of the reaction mixture was adjusted to 5.0 with 1 M formic acid and centrifuged for 5.0 min at medium speed using a table top clinical centrifuge. The pH of the resulting supernatant liquid was adjusted to 3.0 with 1 M formic acid. The supernatant fluid was then applied to a 0.7 cm x 13.0 cm DEAE Sephadex A25 column which was packed and pre-equilibrated with water. The column was washed with 10.0 mL of water, 10.0 mL of 0.1 M sodium formate buffer at pH 3.0, 10.0 mL of 0.6 M sodium formate buffer at pH 3.0 and finally 50.0 mL of 1.0 M sodium formate buffer at pH 3.0. The column was developed at 25°C with a flow rate of 1.0 mL per 4 min. One mL fractions were collected.

A 0.1 mL aliquot of each fraction was added to 5.0 mL of Insta Gel containing 3.0 mL of water and counted using a Tricarb C2425 liquid scintillation spectrometer. The cpm/mL values are plotted versus the fraction number (●-●).

The absorbance at 260 nm of each fraction was obtained and plotted versus the fraction number (○-○).
Figure 17

(cpm/mL) x 10^-3

Fraction Number

260 nm Absorbance
Figure 18

Non-Enzymatic Hydrolysis of \((4-^{14}\text{C})\) Succinyl CoA.

Elution Profile of the Reaction Mixture.

The materials and procedure are similar to those described in Figure 17 except \(\alpha\)-KGDC was not included in the reaction mixture.

The cpm/mL (●●) and \(A_{260}\) nm (○○) are plotted versus the fraction number.
Figure 19
Elution Profile of (1-\textsuperscript{14}C) Succinate and the Product of α-KGDC Catalyzed Succinyl CoA Hydrolysis

Two DEAE Sephadex A25 columns were packed and pre-equilibrated with water. The first column was loaded with the (1-\textsuperscript{14}C) succinate sample at pH 7.6 obtained from the reaction of (5-\textsuperscript{14}C)α-ketoglutarate with hydrogen peroxide and purified using the chromatographic system described in Figure 17. The other column was loaded with fraction 16 at pH 7.6 obtained from the experiment described in Figure 17 and which contained the product of the α-KGDC catalyzed (4-\textsuperscript{14}C) succinyl CoA hydrolysis. The two columns were developed with 10.0 mL of water, 15.0 mL of 0.1 M triethylammonium bicarbonate (TEAB) at pH 7.6, 15.0 mL of 0.3 M TEAB at pH 7.6 and 15.0 mL of 0.6 M TEAB at pH 7.6 at 25°C with a flow rate of 1 mL per 4.0 min. One mL fractions were collected.

The cpm/mL vs. fraction number plots of the two columns are superimposed in this figure.

\begin{itemize}
  \item [●●] (1-\textsuperscript{14}C) succinate
  \item [○○] product of α-KGDC catalyzed (4-\textsuperscript{14}C) succinyl CoA Hydrolysis
\end{itemize}
Fig. 20. The $K_m$ and $V_{max}$ as determined from the slope and intercept of the reciprocal plot using the least squares method are $9.3 \times 10^{-5}$ M and $2.0 \times 10^{-2}$ μmole/min per mg of the complex.
The materials and procedure are similar to those described in Figure 11 (- - ) except the concentration of succinyl CoA was varied. For succinyl CoA concentrations between 0.025 mM to 0.070 mM, the volume of the reaction mixture was increased to 150 μL (from 100 μL) while maintaining the concentrations of the cofactors and the enzyme and 100 μL of the reaction mixture were assayed. The initial rate of the α-KGDC catalyzed succinyl CoA hydrolysis was obtained as described on Table 8 for each succinyl CoA concentration. The data is plotted as 1/rate, (μmole succinyl CoA/min per mg complex)$^{-1}$ vs. 1/succinyl CoA concentration, (mM)$^{-1}$. 

Figure 20

Rate of Succinyl CoA Hydrolysis as a Function of Succinyl CoA Concentration
Figure 20

1 / rate of hydrolysis (mole succinyl CoA/min per mg)⁻¹

1 / succinyl CoA concentration (mM)⁻¹
Discussion

The experimental results show that the α-KGDC catalyzed succinyl CoA hydrolysis is fully dependent on active lipoyl residues and NADH, and partially dependent on TPP (34%-53%, average value = 44%), MgSO₄ (62%) and NAD⁺ (48%). These observations indicate that the enzyme catalyzed succinyl CoA hydrolysis occur via the reversal of the sequence of steps involved in the oxidative decarboxylation of α-ketoglutarate (Equations 16-20).

The two mechanisms of electron and succinyl group transfer between $E₁\cdot$TPP and $E₂\cdot$Lip are depicted as pathways a and b below:

Scheme 5
Pathway (a) involves a concerted transfer of electrons and succinyl group generating a hemithioacetal while pathway (b) involves transfer of succinyl group followed by electrons forming a succinyl-TPP intermediate. Both pathways when reversed produce \(\gamma\)-carboxy-\(\alpha\)-hydroxypropyl]-TPP. This could conceivably decompose to succinic semialdehyde, though it almost certainly does not, by analogy with Krampitz's findings that less than 1\% of \(\alpha\)-hydroxyethyl-TPP was decomposed to acetaldehyde and TPP at pH 6.5 for 4 hours at 30°C (Krampitz, 1970).

In the presence of excess succinyl CoA, Scheme 6 shows that the succinyl group is trapped as succinyl-TPP, according to pathway b, when the dihydrolipoyl group is resuccinylated by succinyl CoA.

![Scheme 6](image_url)
Succinyl-TPP has then no alternative but to undergo hydrolysis generating succinate and TPP. In two chromatographic systems (DEAE Sephadex A25 developed with a stepwise gradient of sodium formate buffer at pH 3.0 and DEAE Sephadex A25 developed with a stepwise gradient of TEAB at pH 7.6), the product of α-KGDC catalyzed succinyl CoA hydrolysis has the same elution volume as the authentic (1-14C) succinate sample. Although succinic semialdehyde is expected to co-elute with succinic acid at pH 3.0, the two should be separated when rechromatographed at pH 7.6. These observations suggest that the product of the enzymatic reaction is succinate instead of succinic semialdehyde and support pathway b (b1 and b2) as the reductive succinylation mechanism.

The series of reactions: succinylation of reduced lipooyl residues by succinyl CoA, succinyl transfer from lipooyl groups to TPP, hydrolysis of succinyl-TPP, can be repeated many times, leading to a continuous turnover in succinyl CoA hydrolysis catalyzed by the enzyme. Assuming a MW of 2.5 x 10^6 and 12 E1•TPP sites per particle of the complex, the calculated V_max indicate that the turnover number for the hydrolysis reaction is 4 moles succinyl CoA per minute per mole of active site.
CHAPTER V
Pyruvate as a substrate for α-KGDC

α-KGDC preparations were reported to be slightly contaminated by pyruvate dehydrogenase complex (PDC) due to observed small activity of the enzyme preparations towards pyruvate. This α-keto acid was oxidized at a rate approximately 2% of that of α-ketoglutarate. To determine the extent of PDC contamination, anaerobic reduction of the flavin in PDC using pyruvate and CoA as the reducing system appeared to offer a reasonable chance of success. The PDC content of a given α-KGDC preparation could then be calculated from the difference in the extinction coefficient of the oxidized and the reduced flavin as well as the molecular weight and subunit composition of PDC.

The result of such an experiment is given in Figure 21; comparison with Figure 3 of Chapter III shows that pyruvate and α-ketoglutarate reduced the flavin in the enzyme preparation to the same extent; 3.7 nmols of FAD per mg of the protein sample were reduced by pyruvate in the presence of TPP, MgSO₄ and CoA. It appears that pyruvate can be oxidized by α-KGDC. This chapter deals with the question of whether pyruvate oxidation by this enzyme follows the same sequence of steps as the oxidation of α-ketoglutarate.
Using the special glass cuvette shown in Figure 2, anaerobic reduction of α-KGDC was carried out as follows: 4.5 mg α-KGDC, 0.05 μmole of TPP, 0.05 μmole of MgSO₄, and 0.10 μmole of CoA were placed in the cuvette chamber in 0.90 mL of 0.02 M potassium phosphate buffer at pH 7. Sodium pyruvate (0.1 mL of 2.4 mM) in 0.02 M potassium phosphate buffer at pH 7 was placed in the side pouch. The cuvette was freed of O₂ by purging with argon for 30 minutes. The spectrum (350-700 nm) of the enzyme in the main chamber was measured, pyruvate in the side pouch was mixed thoroughly with the solution in the main chamber and the spectrum was rescanned.

A reference was used for this anaerobic reduction experiment. The reference cuvette contained the same solution as the main chamber of the sample cuvette except for α-KGDC. 0.10 mL of 2.4 mM pyruvate solution was added to the reference cuvette when pyruvate in the side pouch of the sample cuvette was mixed with the solutions in the main chamber.

The results are reported as E₁₀, i.e. absorbance of 10 mg α-KGDC per 1 mL of solution.

- Oxidized enzyme
- Reduced enzyme
1. Acetylation of α-KGDC by (2-14C) pyruvate.

The dependence of the amount of (1-14C) acetyl group incorporated into the complex on the concentration of (2-14C) pyruvate was investigated. The complex was permitted to react with the indicated concentration of (2-14C) pyruvate in the presence of TPP and MgSO₄ at 25°C for 30 seconds. Assuming that there are 24 lipooyl residues per particle of the complex that can covalently bind acetyl groups and that all the (1-14C) acetyl groups isolated with the complex are associated with the lipooyl moieties in E₂, the data show that when pyruvate is present in an amount (18.19 mM) which is more than 2,000 times the concentration of lipoic acid, only 83% of the lipooyl moieties are acetylated at the end of 30 seconds (Fig. 22). This led to time dependence study of acetyl group incorporation by (2-14C) pyruvate.

At a concentration of 1.54 mM (2-14C) pyruvate, complete acetylation is observed after 3 min (Fig. 23). When 3.0 mM CoA is combined with the acetylated complex, generated as in the first 3 min of Figure 23 for 1.0 min. at 25°C, only 0.67 nmole out of 9.96 nmoles (1-14C) acetyl groups remained bound per mg of the complex. Since 9.29 nmole (1-14C) acetyl groups were CoA labile, we can conclude that 9.29 nmoles of lipooyl residues per mg of the complex are acetylated.

2. (1-14C) Acetyl CoA formation from (2-14C) pyruvate.

It can be inferred from the flavin reduction experiment that electrons from pyruvate can be shuttled to the dihydrolipoyl dehydrogenase. Acetylation of α-KGDC by (2-14C) pyruvate indicate that pyruvate is decarboxylated by the complex and the acetyl groups and electrons from the α-ketoglutarate dehydrogenase subunit are transferred
Acetylation of $\alpha$-KGDC as a Function of (2-^{14}C) Pyruvate Concentration

$\alpha$-KGDC (0.72 mg/mL) was incubated in 95 $\mu$L of 10 mM potassium phosphate buffer at pH 7.0 with 0.22 mM TPP, 0.45 mM MgSO$_4$ and the indicated concentration of (2-^{14}C) pyruvate for 30 sec at 25°C. The amount of protein bound (1-^{14}C) acetyl groups was determined by the filter paper-TCA precipitation method.
Figure 23
Acetylation of α-KGDC by (2-^{14}C) Pyruvate
as a Function of Reaction Time

α-KGDC (0.72 mg/mL) was incubated in 95 μL of 10 mM potassium phosphate buffer at pH 7.0 with 0.22 mM TPP, 0.45 mM MgSO₄ and 1.54 mM (2-^{14}C) pyruvate for 0.5 to 10.0 min (as indicated in this figure) at 25°C. The amount of (1-^{14}C) acetyl residue associated with the complex was determined by the filter paper-TCA precipitation method.
Figure 23

nmole $^{14}$C ACETYL / mg $\alpha$-KGDC

TIME (minutes)

8II
to the lipoyl moieties in the transsuccinylase. The lability to CoA of the acetyl groups incorporated into the complex is an indirect proof that the lipoyl bound acetyl groups are transferred to CoA to form acetyl CoA. An experiment which directly showed the formation of acetyl CoA from the action of α-KGDC on (2-14C) pyruvate was performed. α-KGDC was permitted to react with (2-14C) pyruvate in the presence of TPP, MgSO4, CoA and NAD+ in 50 mM potassium phosphate buffer at pH 7.0. One mL aliquots of the reaction mixture were taken at times indicated in Figures 24 to 28, quenched and subjected to ion exchange chromatography, according to the method developed by Fluornoy (Fluornoy, in progress), as described in Figure 24. This chromatographic procedure separates the three radioactive components of the reaction mixture namely (1-14C) acetate (peak I), (2-14C) pyruvate (peak II), and (1-14C) acetyl CoA which co-elutes with CoA (peak III). The NAD+ and TPP peaks can be observed when the absorbance at 260 nm is plotted versus the fraction number. These peaks were identified by D. Fluornoy (Fluornoy, in progress). His method involved determination of the individual elution profiles of known samples of NAD+, TPP, CoA, acetyl CoA, (1-14C) acetate and (2-14C) pyruvate using the chromatographic procedure described in Figure 24.

The amount of (1-14C) acetyl CoA in the reaction mixture was expressed as the percentage of total radioactivity associated with the CoA-acetyl CoA peak. This is plotted versus the reaction time (Figure 30). These values were obtained by taking the difference between the amount of radioactivity that was associated with the CoA-acetyl CoA peak when α-KGDC was present in the reaction mixture and when the complex was
not added to the reaction mixture. 2.2% of total radioactivity was present in the CoA-acetyl CoA peak when α-KGDC was not included in the reaction mixture (Figure 29). An increase in (1-^{14}C) acetyl CoA with increase in reaction time is observed (Figure 30).

In a similar experiment, the rate of NADH formation in a reaction mixture described in Figure 24, was monitored by following the change in absorbance of the solution at 340 nm (Figure 31). Both graphs (^{14}C) acetyl CoA formation, Figure 30 and NADH formation, Figure 31, show that the increase in radioactivity associated with the CoA-acetyl-CoA peak is accompanied by an increase in the absorbance at 340 nm; indicating that NADH is generated at the same time that acetyl-CoA is formed in the reaction mixture. No loss in the activity of the complex toward α-ketoglutarate was observed at the end of 45 minutes.

0.5% to 0.6% more radioactivity than what was originally present in the (2^{14}C) pyruvate sample was found associated with the acetate peak at the end of 30 min and 45 min respectively. Whether this is a result of an enzymatic or a non-enzymatic hydrolysis of acetyl CoA needs to be investigated further.

3. Determination of K_m and V_max of α-KGDC for pyruvate.

The rate of pyruvate oxidation by the α-ketoglutarate dehydrogenase complex was measured by the NAD reduction assay. Rate measurements were carried out at saturation levels of cofactors, using two different constant levels of α-KGDC concentrations (6.87 x 10^{-2}mg/mL and 1.37 x 10^{-1}mg/mL) at varying pyruvate concentrations (Fig. 32). The average K_m and V_max obtained from the Lineweaver and Burk plots of the data using
Figure 24

α-KGDC Catalyzed (1-14C) Acetyl CoA Formation from (2-14C) Pyruvate

A 4.68 mL solution of 50 mM potassium phosphate buffer at pH 7 containing 0.26 μmole of TPP, 0.50 μmole of MgSO₄, 6.05 μmole of CoA, 5.02 μmole of NAD⁺ and 1.37 mg of α-KGDC was mixed with 0.52 mL of 2.44 mM (2-14C) pyruvate solution in 50 mM potassium phosphate buffer at pH 7.0 to initiate the reaction at 30°C. After 5 min, a 1.0 mL aliquot was taken and the pH adjusted to 5.0 with 1 M formic acid and the resulting mixture centrifuged for 5 min at medium speed using a tabletop clinical centrifuge. The pH of the supernatant fluid was adjusted to 3.6 and the solution was applied to a 0.7 cm x 13 cm DEAE Sephadex A25 column which was packed and pre-equilibrated with water. The column was washed with 11.0 mL of H₂O followed by 17.0 mL of 0.1 M triethyl ammonium formate (TEAF) buffer at pH 3.6 and finally with 17.0 mL of 0.6 M TEAF buffer at pH 3.6. The column was developed at 25°C with a flow rate of 1 mL per 4 minutes. One mL fractions were collected.

0.2 mL of each fraction was added to 15.0 mL of Aquasol containing 0.8 mL of H₂O and counted using a Beckman LS 100C liquid scintillation spectrometer. The cpm/mL values are plotted versus the fraction number (●●●). The absorbance at 260 nm of each fraction was obtained using a Cary 118 C double beam spectrophotometer. The data are plotted as $A_{260}$ versus the fraction number ( ○○ ).
Figure 24

260 nmabsorbance

(Fraction Number

( cp /mL) x 10^-3

FRECTION NUMBER

FIGURE 24
Figure 25

α-KGDC Catalyzed (1-^{14}C) Acetyl CoA Formation
from (2-^{14}C) Pyruvate

The same materials and procedure as described in Figure 24 were used except 1.0 mL portion of the reaction mixture was taken at the end of 10 minutes.

- o-o cpm/mL vs. fraction number
- •• A_{260} vs. fraction number
Figure 25

260 nm ABSORBANCE

(cpm / mL) \times 10^{-3}

FRACTION NUMBER

FIGURE 25
α-KGDC Catalyzed (1-^{14}C) Acetyl CoA Formation from (2-^{14}C) Pyruvate

The same materials and procedures as described in Figure 24 were used except 1.0 mL of the reaction mixture was taken at the end of 15 minutes.

α-occpm/mL vs. fraction number

•• A\textsubscript{260} nm vs. fraction number
FIGURE 26

260 nm ABSORBANCE

100

80

60

40

20

0

(cpm / mL) x 10^{-3}

10 20 30 40

FRACTION NUMBER
Figure 27

α-KGDC Catalyzed (1-\(^{14}\)C) Acetyl CoA Formation from (2-\(^{14}\)C) Pyruvate

The same materials and procedure as described in Figure 24 were used except 1.0 mL of the reaction mixture was taken at the end of 30 minutes.

\(\text{\(\infty\)}\) cpm/mL vs fraction number

\(\bullet\bullet\) A\(_{260}\) vs. fraction number
FIGURE 27

Fraction Number vs. Absorbance at 260 nm
The same materials and procedures as described in Figure 24 were used except 1.0 mL of the reaction mixture was taken at the end of 45 minutes.

- o o cpm/mL vs. fraction number

- • • A[260] vs. fraction number
Figure 28

260 nm ABSORBANCE

(cpm / mL) x 10^-3

FRACTION NUMBER

10 20 30 40

100 80 60 40 20 0

-1.0

-2.0

-3.0
Figure 29

Elution Profile of the Reaction Mixture for the Acetylation of α-KGDC

0.935 mL of 50 mM potassium phosphate buffer at pH 7.0 containing 0.05 μmole of TPP, 0.10 μmole of MgSO₄, 1.21 μmole of CoA and 1.00 μmole of NAD⁺ was mixed with 0.105 mL of 2.44 mM (2-¹⁴C) pyruvate solution in 50 mM potassium phosphate buffer at pH 7.0. After adding 80 μL of 1 M formic acid, the mixture was subjected to DEAE Sephadex A25 column chromatography as described in Figure 24.

The radioactivity and absorbance at 260 nm of each fraction were determined as described in Figure 24.

○○ cpm/mL vs. fraction number

●● A₂₆₀ vs. fraction number
Figure 29

260 nm ABSORBANCE

(cpm/mL) x 10^{-3}

FRACTION NUMBER

FIGURE 29
The amount of (1-$^{14}$C) acetyl CoA in the reaction mixture is expressed as percentage of total radioactivity in the reaction mixture. The data were obtained from Figures 24 to 28 and plotted as % of total radioactivity associated with the CoA-acetyl CoA peak versus reaction time. The values shown in this figure have been corrected for the amount of radioactivity [2.2% of total radioactivity of the (2-$^{14}$C) pyruvate sample, Figure 29], which was associated with the CoA-acetyl CoA peak in the original sample of (2-$^{14}$C) pyruvate.
% TOTAL RADIOACTIVITY ASSOCIATED with CoA-ACETYL CoA PEAK
Figure 31

NADH Yield in the Oxidation of Pyruvate by α-KGDC

0.935 mL of 50 mM potassium phosphate buffer at pH 7.0 containing 0.05 μmole of TPP, 0.10 μmole of MgSO₄, 1.21 μmole of CoA and 1.00 μmole of NAD was mixed with 0.100 mL of 2.01 mM sodium pyruvate solution in 50 mM potassium phosphate buffer at pH 7.0. The reaction was carried out in a pyrex cuvette with 1.0 cm path length and the change in absorbance at 340 nm was monitored.
FIGURE 31

TIME (minutes)

340 nm ABSORBANCE
the least squares method to calculate the slope and the intercept are 
$6.91 \times 10^{-4} \text{M} \ [6.82 \times 10^{-4} \text{M at } (\alpha-\text{KGDC }) = 6.87 \times 10^{-2} \text{ mg/mL}];$
$7.00 \times 10^{-4} \text{M at } (\alpha-\text{KGDC }) = 1.37 \times 10^{-1}\text{mg/mL}]$ and $8.2 \times 10^{-2} \mu\text{mole/min/mg}$ 
$[8.2 \times 10^{-2} \mu\text{mole/min/mg at } (\alpha-\text{KGDC }) = 6.87 \times 10^{-2}\text{mg/mL} \text{ and at } (\alpha-\text{KGDC })$
$= 1.37 \times 10^{-1}\text{mg/mL}], \text{ respectively.}$

Since the $K_m$ and $V_{\text{max}}$ for $\alpha$-ketoglutarate of the E. coli $\alpha$-KGDC were not found in the literature, these were determined at two different constant $\alpha$-KGDC concentrations (Fig. 33). The values obtained are $7.2 \times 10^{-5} \text{M} \ [7.5 \times 10^{-5} \text{M at } (\alpha-\text{KGDC }) = 6.8 \times 10^{-4}\text{mg/mL}; 6.8 \times 10^{-5} \text{M at } (\alpha-$
$\text{KGDC }) = 1.36 \times 10^{-3}\text{mg/mL}]$ for the $K_m$ and $20.9 \mu\text{mole/min/mg} \ [18.9$
$\mu\text{mole/min/mg at } (\alpha-\text{KGDC }) = 6.8 \times 10^{-4}\text{mg/mL}; 22.8 \mu\text{mole/min/mg at } (\alpha-$
$\text{KGDC }) = 1.36 \times 10^{-3} \text{mg/mL}]$
Figure 32

$K_m$ and $V_{max}$ Determination Using Pyruvate as Substrate

Rate measurements were carried out at 30°C in 50 mM potassium phosphate buffer at pH 8.1 containing CoA (0.095 mM), cysteine (2.75 mM), NAD$^+$ (2.08 mM), TPP (0.19 mM) and MgSO$_4$ (1.0 mM) in the presence of two different constant amounts of $\alpha$-KGDC:

- $6.87 \times 10^{-2}$ mg/mL
- $1.37 \times 10^{-1}$ mg/mL

at varying pyruvate concentrations. The data were plotted according to Lineweaver and Burk (Lineweaver and Burk, 1934).
FIGURE 32

$\text{Yield NADH/min per mg } \alpha\text{-KGDC}$

$\text{PYRUVATE CONCENTRATION (mM)}^{-1}$

$\text{(\textmu mole NADH/min per mg } \alpha\text{-KGDC})^{-1}$
Figure 33

K_m and V_max Determination Using α-Ketoglutarate as Substrate

Rate measurements were carried out at 30°C in 50 mM potassium phosphate buffer at pH 8.1 containing CoA (0.095 mM), cysteine (2.75 mM), NAD^+ (2.08 mM), TPP (0.19 mM) and MgSO_4 (1.00 mM) in the presence of two different constant amounts of α-KGDC:

- ○ 6.8 x 10^{-4} mg/mL
- ● 1.36 x 10^{-3} mg/mL

at varying α-ketoglutarate concentrations. The data were plotted according to Lineweaver and Burk (Lineweaver and Burk, 1934).
Figure 33

(μmole NADH / min per mg α-KGDC)⁻¹

1/α-KETOGLUTARATE CONCENTRATION (mM)⁻¹
DISCUSSION

Our experimental results indicate that acyl and electron transfer pathways with pyruvate, as the substrate follow the normal catalytic scheme of the enzyme, i.e.:

\[ \text{E}_1[\text{TPP}] + \text{pyruvate} \rightarrow \text{E}_1[\alpha\text{-hydroxyethyl-TPP}] + \text{CO}_2 \]
\[ \text{E}_1[\alpha\text{-hydroxyethyl-TPP}] + \text{E}_2[\text{Lip S}_2] \rightarrow \text{E}_2[\text{acetyl-S Lip-SH}] + \text{E}_1[\text{TPP}] \]
\[ \text{E}_2[\text{acetyl-S-Lip-SH}] + \text{CoA} \rightarrow \text{E}_2[\text{Lip(SH)}_2] + \text{acytly CoA} \]
\[ \text{E}_2[\text{Lip(SH)}_2] + \text{E}_3[\text{FAD, ox}] \rightarrow \text{E}_2[\text{Lip S}_2] + \text{E}_3[\text{FAD, red}] \]
\[ \text{E}_3[\text{FAD, red}] + \text{NAD}^+ \rightarrow \text{E}_3[\text{FAD, ox}] + \text{NADH} + \text{H}^+ \]

Pyruvate is two carbons shorter than \( \alpha \)-ketoglutarate and lacks the second carboxyl group that \( \alpha \)-ketoglutarate has. This difference in the primary structures of these \( \alpha \)-ketoacids results in a 10 fold lower affinity of \( \alpha \)-KGDC for pyruvate compared to \( \alpha \)-ketoglutarate. It would be interesting to know the relative effects of the chain length and the presence of a second carboxylate group on the affinity of the complex for an \( \alpha \)-ketoacid. This type of information will provide a better understanding of the nature of the decarboxylating site in \( \text{E}_1 \)

The rate of oxidation of \( \alpha \)-ketoglutarate is about 250 times faster than the rate of oxidation of pyruvate. It is not possible to tell at this time whether the rate of decarboxylation, the rate of transfer of acyl groups and electrons from \( \text{E}_1[\text{TPP}] \) to \( \text{E}_2[\text{Lip S}_2] \), and the rate of acyl transfer to CoA are affected by the nature and length of the \( \alpha \)-ketoacid chain to the same extent or whether the rate of one step is decreased considerably more than the others. It is apparent, however,
that none of these steps has an absolute requirement for all the structural features of α-ketoglutarate.
CHAPTER VI
DISCUSSION

The total number of flavin associated with \( \alpha \)-KGDC has been measured by various laboratories and was found to be 4.0 to 4.8 nmoles per mg of the complex (Pettit et al, 1973, Angelides and Hammes, 1979). In this dissertation, reduction of flavin by \( \alpha \)-ketoglutarate provided a means to measure the number of catalytically functional flavin in the complex which was found to approach a limiting value of 4.5 nmoles per mg of the complex. This finding supports Hammes' interpretation that the full activity observed with less than the native flavin content is an indication that lipoic acid oxidation is not the rate limiting step in the overall catalytic reaction rather than an indication that not all of the complex associated flavin are catalytically functional.

The results of studies involving coupling of \( \alpha \)-lipoyl groups in dihydrolipoyl transsuccinylase with the other two enzyme components of the complex show that although there are similarities in the structure and mechanism of action of the \( \alpha \)-ketoglutarate and pyruvate dehydrogenase multienzyme complexes, there are also important differences between them. Bates et al reported inequivalent interactions of dihydrolipoyl transacetylase with a given pyruvate dehydrogenase dimer in pyruvate dehydrogenase complex (Bates et al, 1977). Frey et al observed that only half of \( \alpha \)-lipoyl moieties in PDC are coupled to the
dihydrolipoyl dehydrogenase of the complex (Frey et al., 1978). The findings in this work indicate that all the \( \alpha \)-lipoyl groups (10 nmol/mg complex) in the dihydrolipoyl transsuccinylase of \( \alpha \)-KGDC are coupled to the \( \alpha \)-ketoglutarate dehydrogenase as well as the dihydrolipoyl dehydrogenase components of the complex. However, measurement of the NADH yield of the reduced, succinylated complex as well as succinylation of the complex with (5-\( ^{14} \)C)\( \alpha \)-ketoglutarate in the presence of glyoxylate show that half of the succinyl groups and corresponding electron equivalents associated with the lipoyl groups in the transsuccinylase are lost during the first two minutes, possibly due to the reversal of the reaction between \( E_1 \) and \( E_2 \), while the other half are lost at a much slower rate. These two apparent rates in the reverse reaction between \( E_1 \) and \( E_2 \) may be a manifestation of a higher affinity of \( E_1 \) for the non-succinylated lipoyl moieties favoring the formation of \( E_1[TPP]-E_2[Lip \ S_2] \) complex over \( E_1[TPP]-E_2[\text{succinyl-Lip-SH}] \) complex thus inhibiting further loss of succinyl groups and electron equivalents, rather than an indication of two classes of lipoic acids with respect to their interaction with \( E_1 \), since Reed et al. (Collins and Reed, 1977) reported that all 24 succinyl acceptor sites in \( \alpha \)-KGDC are charged with \( \alpha \)-ketoglutarate in the presence of only a few functionally active \( \alpha \)-ketoglutarate dehydrogenase chains.

Although the role of TPP in the oxidative decarboxylation of \( \alpha \)-ketoglutarate is known, its exact mode of action is not. The initial step of the reaction of TPP with \( \alpha \)-ketoglutarate involves ionization of the thiamin to the carbanion which in turn adds to the carbonyl group of the substrate. This covalent intermediate formed with TPP makes
possible the development of a negative charge, by decarboxylation, on the carbon atom of the substrate which was formerly the carbonyl carbon, due to resonance stabilization of the negative charge involving the thiazolium ring:

\[
\begin{align*}
  &\text{CH}_2\text{CH}_2\text{COO}^- \\
  &\text{C-OH} \\
  &\text{S} \quad \text{N-} \quad \text{R}_1 \\
  &\text{R}_2 \quad \text{R}_3
\end{align*}
\]

This carbanion-enamine intermediate can react with an oxidizing agent to give acyl-thiamine which can then transfer the acyl group to an acceptor. Both oxidation and acyl transfer occur when this carbanion-enamine intermediate reacts with \( \alpha \)-lipoamide. It is not known, however, whether these two reactions occur in one step or two steps.

The data on succinyl-CoA hydrolysis presented in this work suggest the formation of a succinyl-TPP intermediate when \( E_2 \) [succinyl-Lip-SH] reacts with \( E_1[TPP] \). By microscopic reversibility of reaction mechanisms, this observation supports the two step mechanism of reductive succinylation of the \( \alpha \)-lipoyl groups. Walsh (Walsh, 1979) favored the one step reductive succinylation because of the known reactivity of disulfide bonds toward nucleophiles. He suggested that the carbanion formed in the decarboxylation of the substrate can attack the disulfide bond of the lipoyl residues forming a hemithioacetal
grouping which can subsequently expel the TPP carbanion generating the thioester linkage with lipoamide. However, in view of the relative stability of the two resonance forms (carbanion and enamine) of the $E_1$ TPP bound intermediate, it is quite likely that the enamine has a larger contribution to the hybrid resonance structure making the hybrid resonance structure less nucleophilic than the carbanion resonance form. Our finding that succinyl-TPP is formed in the reaction between $E_2$ [succinyl-Lip-SH] and $E_1$ [TPP] in the presence of excess succinyl CoA together with the reported ability of ferricyanide to oxidize the TPP bound carbanion-enamine intermediate to acyl-TPP constitute a strong evidence that the reductive succinylation of lipoamide occur in two steps.

The binding of a substrate molecule to an enzyme active site involves interaction of certain parts of the substrate molecule with residues in the enzyme active site. The positions and chemical nature of these active site residues determine the specificity of the enzyme. Compounds possessing a structure analogous to that of the substrate will be able to bind and possibly react with the enzyme active site.

Examination of the $\alpha$-ketoglutarate molecule reveals three types of functional groups; the carboxyl group on each end of the molecule, the carbonyl group, and the two methylene carbons at the $\beta$ and $\gamma$ positions. Ionic interactions and/or H-bonding could serve to anchor $\alpha$-ketoglutarate to the enzyme active site. A comparison of the structure of pyruvate with that of $\alpha$-ketoglutarate show that a second ionic interaction at the $\alpha$-ketoglutarate dehydrogenase active site is not possible for pyruvate which contains a methyl group instead of two.
methylene and carboxyl groups found in α-ketoglutarate. The shorter chain length and the absence of a second carboxyl group at the gamma position in pyruvate resulted to a ten-fold lower affinity of α-KGDC for pyruvate.

The experimental results in this dissertation show that in spite of the difference in the structure of pyruvate and the native substrate, the oxidative decarboxylation of pyruvate still follows the normal catalytic scheme of the enzyme although at a rate which is 250 times slower than that of the native substrate.

While α-KGDC oxidatively decarboxylates pyruvate, PDC does not act on α-ketoglutarate. This is not surprising since a small substrate in an active site which can accommodate a larger hydrocarbon chain should not cause unfavorable steric interactions with active site residues.

In summary, the most important findings in this work are:
1. All the FAD associated with the complex are catalytically functional.
2. All the α-lipoyl moieties in the transsuccinylase components are coupled to α-ketoglutarate dehydrogenase as well as to dihydrolipoyl dehydrogenase.
3. The reductive succinylation of α-lipoamide occur in two steps.
4. Pyruvate act as a substrate for α-KGDC. This information can be very useful for further mechanistic studies of α-KGDC. Pyruvate, being a slow substrate, may lead to formation of larger steady state concentrations of certain intermediates in the catalytic pathway, which would allow for easier detection of these intermediates.
This dissertation research provided a better understanding of the mechanism of \( \alpha \)-ketoglutarate dehydrogenase complex, an insight into the most important structural aspects for a molecule to act as a substrate of the complex and a possible tool for further studies on the mechanism of the complex.
REFERENCES

Fluornoy, D., Ph.D. Dissertation, University of Wisconsin, in progress.
Horecker, B.L. and Kornberg, A., J. Biol. Chem., 175, 385 (1948).


Pettit, F.H., Hamilton, L., Munk, P., Namihira, G., Eley, M.H., Willms,
Reed, L.J., in Comprehensive Biochemistry, 14, 99 (Florkin, M., and
Sanadi, D.R., in The Enzymes, 7, 307 (Boyer, P.D., Lardy, H. and
(1959).
(1959).
(1952).
(1961).


