Multienzyme Complexes

Lester J. Reed

Clayton Foundation Biochemical Institute and Department of Chemistry, University of Texas at Austin, Austin, Texas 78712

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Enzymes are biological catalysts that, like all catalysts, speed the approach to chemical equilibrium without shifting the equilibrium position itself. All enzymes now known are globular proteins. They range in organization and complexity from enzymes consisting of a single folded polypeptide chain, to oligomeric or multichain enzymes, to enzymes containing both catalytic and regulatory subunits, to multienzyme complexes, and to arrays of membrane-bound enzymes such as the electron-transport system of mitochondria.

Multienzyme complexes are aggregates of enzymes that catalyze two or more steps in a metabolic sequence. They have been observed to participate in reactions involving a number of very different substrates, and they have been found in a variety of biological systems. The multienzyme complexes that have been most thoroughly described are composed of two to six functionally related enzymes and have molecular weights ranging from a few hundred thousand to several million.

The components of several of these multienzyme complexes have been resolved and, in a few cases, it has been possible to assemble particles resembling the native complexes from their separate parts. The particles contain definite amounts of each enzyme, and the component proteins are apparently organized in a specific and regular way. The architecture, assembly, and function of multienzyme complexes are interesting in themselves. Moreover, it is reasonable to suppose that holding enzymes with related activities together serves similar ends in relatively simple particles and in the more elaborate arrays of membrane-bound enzyme systems. Attention to multienzyme complexes may be repaid by insight into the general advantages a cell obtains from the specific ordering of its enzymes.

The organization of functionally related enzymes into a complex might be expected to affect the efficiency, or the regulation, or both features of the enzyme system.[1] A complex might be more efficient in catalyzing a multistep metabolic sequence than would a collection of independent enzymes. In other words, complex formation provides a means of concentrating catalysts rather than having them randomly distributed in the cell. An intermediate produced by one enzyme would probably encounter the next enzyme in a sequence more rapidly if the two enzymes were physically associated with each other than if they were structurally independent. A complex would be particularly efficient if the metabolic intermediates were strongly bound to it and could not readily escape before the series of reactions was complete.

In some cases, the activity of one component of a multienzyme complex is modified by its association with the others. For example, the activities of the *Escherichia coli* tryptophan synthetase components are altered by complex formation; the reactions involving substrates of the tryptophan pathway are catalyzed more efficiently, while the serine deaminase activity of the B protein is suppressed.[2] The substrate specificity of anthranilate synthetase is broadened by complex formation to include glutamine as an ammonia donor.[3] The efficiency of the two regulatory enzymes, pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase, is increased by attachment to dihydrolipoyl transacetylase.[4,5]

This situation could arise if a catalytic conformation were imposed on one enzyme by its association with another, or if residues of more than one polypeptide chain participated in the active site that catalyzes a particular reaction. There may be complexes in which the activity of one component depends absolutely on the presence of another; in such cases, it would be impossible to resolve the complex into independently active components.

If the formation of a complex were necessary either to produce a species with any activity at all or to elevate the efficiency of a pathway to a physiologically useful level, then the ligand-controlled association of the members of a complex could provide a mechanism for the control of the pathway.[5]

Complex formation offers a way of segregating enzymes that would otherwise compete for the same metabolite, and such metabolic channeling has been invoked frequently in speculations on the role of multienzyme complexes in the cellular economy. The argument is that an intermediate produced by an enzyme that is a member of a complex would be acted upon preferentially by another enzyme in the aggregate and would not be freely available to en-

Lester J. Reed was born in New Orleans, La., in 1925. He received a B.S. in Chemistry from Tulane University and a Ph.D. in Organic Chemistry from the University of Illinois. After 2 years as a research associate at Cornell University Medical College in the laboratory of V. du Vigneaud, he joined the faculty of the University of Texas in 1948. He is now Professor of Chemistry and Director of the Clayton Foundation Biochemical Institute.

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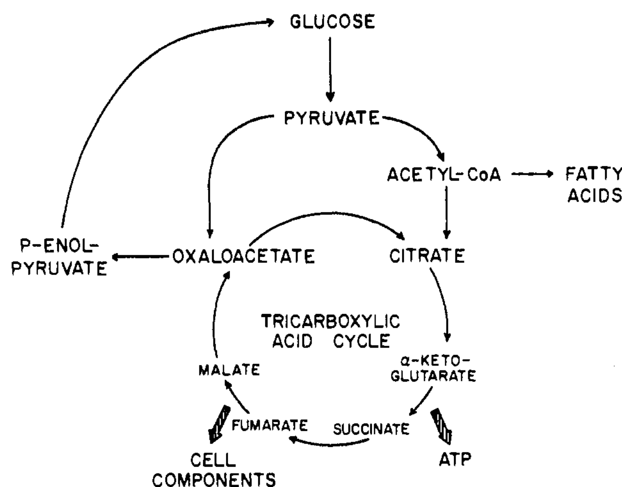
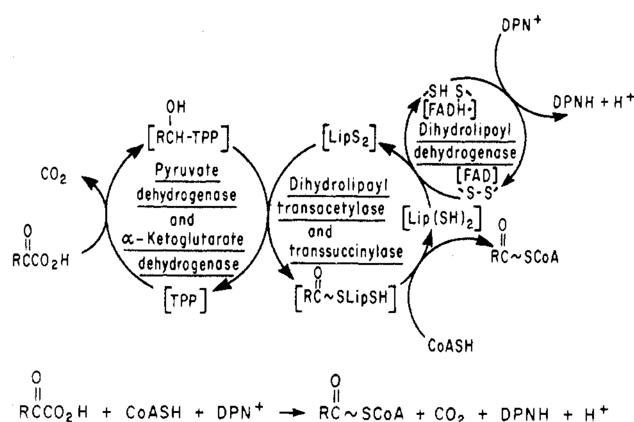


Figure 1. Diagram illustrating central positions of pyruvate and α-ketoglutarate in cellular metabolism. The abbreviations used are: CoA, coenzyme A; ATP, adenosine 5′-triphosphate.



$$RCCO_2H + CoASH + DPN^+ \rightarrow RC{\sim}SCoA + CO_2 + DPNH + H^+$$

Figure 2. Reaction sequence in pyruvate and α-ketoglutarate oxidation. The abbreviations used are: TPP, thiamine pyrophosphate; $LipS_2$ and $Lip(SH)_2$, lipoyl moiety and its reduced form; CoASH, coenzyme A; FAD, flavine adenine dinucleotide; DPN^+ and DPNH, diphosphopyridine nucleotide and its reduced form.

zymes that are not present in the complex. The complexes involved in aromatic amino acid synthesis in fungi provide a well-documented example of this kind.[6] If the intermediates formed along a metabolic sequence are strongly bound to the complex catalyzing the entire pathway, then the intermediates can be directed unambiguously in only one direction. This feature is exhibited by the α-keto acid dehydrogenase complexes[1b] and by the yeast fatty acid synthetase.[7]

What follows is a description of a few multienzyme complexes that have been investigated in this laboratory. The characteristics of these complexes may serve to illuminate analogous features of other organized enzyme systems.

Structure and Function of α-Keto Acid Dehydrogenase Complexes

Enzyme systems that catalyze a lipoic acid mediated oxidative decarboxylation of pyruvic and α-ketoglutaric acids have been isolated from microbial and eukaryotic cells as functional units with molecular weights in the millions. Two classes of complexes have been obtained, one specific for pyruvate, the other for α-ketoglutarate. Much of the interest in these multienzyme complexes comes from their key positions in metabolism (Figure 1). Pyruvate is an intermediate in the biosynthesis of fats (lipogenesis), carbohydrates (gluconeogenesis), and certain amino acids, and its complete oxidation *via* the tricarboxylic acid cycle is a major source of energy. Tricarboxylic acid cycle intermediates, including α-ketoglutarate, are also converted to precursors of protein, porphyrins, and nucleic acids.[8]

The α-keto acid dehydrogenase complexes possess distinctive structures[9] and catalyze a coordinated sequence of reactions (Figure 2).[10] Several of them have been separated into their component enzymes and have been reassembled from the isolated enzymes. Each of these complexes contains a core, consisting of dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase, to which pyruvate dehydrogenase or α-ketoglutarate dehydrogenase and dihydrolipoyl dehydrogenase (a flavoprotein) are joined by noncovalent bonds.[9] Thus, the core enzyme plays both a catalytic and a structural role. The designs of the dihydrolipoyl transacetylases and transsuccinylases appear to be based on cubic point group symmetry;[11] these core enzymes consist of either 24 or 60 subunits.

E. coli **Pyruvate Dehydrogenase Complex.** The *E. coli* (Crookes strain) pyruvate dehydrogenase complex has been separated into three enzymes,[12,13] pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase, and the complex has been reassembled from the isolated enzymes.[13,14] The subunit composition of the complex is summarized in Table I. The complex consists of about 24 pyruvate dehydrogenase chains (*i.e.*, 12 dimers), 24 transacetylase chains, and about 12 flavoprotein chains (*i.e.*, 6 dimers).[15] Each transacetylase chain apparently contains one molecule of covalently bound lipoic acid. Electron micrographs of the *E. coli* pyruvate dehydrogenase complex show a polyhedral structure with a diameter of about 300 Å (Figure 3A). The transacetylase has the appearance of a cube (Figure 3B), and its design appears to be based on octahedral (432) symmetry (Figure 3C). Presumably, the pyruvate dehydrogenase dimers are

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(15) M. H. Eley, G. Namihira, L. Hamilton, P. Munk, and L. J. Reed, *Arch. Biochem. Biophys.*, 152, 655 (1972). The *E. coli* K12 dihydrolipoyl transacetylase is reported to consist of 16 apparently identical polypeptide chains of molecular weight about 80,000 [O. Vogel, B. Hoehn, and U. Henning, *Proc. Nat. Acad. Sci. U. S.*, 69, 1615 (1972)]. However, it seems that 16 subunits are incompatible with the cube-like appearance of the transacetylase in the electron microscope and with the fact that the *E. coli* dihydrolipoyl transsuccinylase, which closely resembles the *E. coli* dihydrolipoyl transacetylase in appearance (*cf.* Figure 3B,F), has been shown by X-ray diffraction, electron microscopic, and physical and chemical studies to consist of 24 subunits.[11,20]

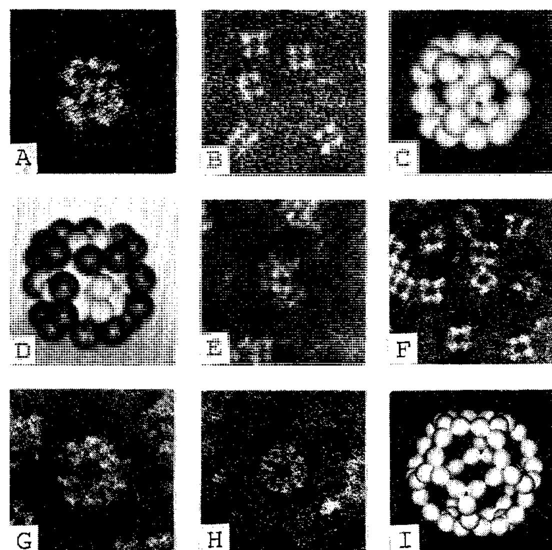

Figure 3. Electron micrograph images and interpretative models. (A) *E. coli* pyruvate dehydrogenase complex; (B) *E. coli* dihydrolipoyl transacetylase, the core enzyme of the pyruvate dehydrogenase complex; (C) model of the *E. coli* transacetylase and transsuccinylase having 24 spherical subunits placed on the vertices of a truncated cube; (D) model of the *E. coli* pyruvate dehydrogenase complex. The 12 pyruvate dehydrogenase dimers are placed on the twofold positions (*i.e.*, on the edges) of the transacetylase cube, and the 6 dihydrolipoyl dehydrogenase dimers are placed on the fourfold positions (*i.e.*, in the faces); (E) *E. coli* α-ketoglutarate dehydrogenase complex; (F) *E. coli* dihydrolipoyl transsuccinylase, the core enzyme of the α-ketoglutarate dehydrogenase complex; (G) bovine kidney pyruvate dehydrogenase complex; (H) bovine kidney dihydrolipoyl transacetylase; (I) model of the mammalian transacetylase having 60 spherical subunits placed on the vertices of a pentagonal dodecahedron. The electron micrographs were taken by Robert M. Oliver. The samples were negatively stained with sodium methyl phosphotungstate. Magnification is 300,000×.

located at the 12 twofold positions (*i.e.*, on the edges) of the transacetylase cube, and the dihydrolipoyl dehydrogenase dimers are located at the 6 fourfold positions (*i.e.*, in the faces) (Figure 3D).

The structural genes for the pyruvate dehydrogenase and the transacetylase, and apparently for the favoprotein as well, are closely linked on the *E. coli* chromosome, and biosynthesis of the complex begins with the biosynthesis of the pyruvate dehydrogenase component.[16,17]

It is evident from Figure 2 that the lipoyl moiety undergoes a cycle of transformations, *i.e.*, reductive acetylation, acetyl transfer, and electron transfer. These transformations involve the interaction of the lipoly moiety, which is covalently bound to the transacetylase, with α-hydroxyethylthiamine pyrophosphate, which is bound to pyruvate dehydrogenase, and with FAD, which is bound to dihydrolipoyl dehydrogenase. The lipoyl moiety must also interact with CoA at a site on the transacetylase. Highly favorable positioning of the three enzymes and, by inference, of their coenzymes or prosthetic groups must be assumed in order to account for the occurrence of the overall reaction.

A possible molecular basis of these interactions was suggested by our finding[18] that the lipoyl moiety

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Table I

Subunit Composition of Pyruvate and α-Ketoglutarate Dehydrogenase Complexes[a]

Enzyme	Mol wt	Subunits No.	Subunits Mol wt	Subunits per molecule of complex
E. coli PDC	4,600,000			
Pyruvate dehydrogenase	192,000	2	96,000	24
Transacetylase	1,700,000	24	65,000–70,000	24
Flavoprotein	112,000	2	56,000	12
E. coli KGDC	2,500,000			
α-KG dehydrogenase	190,000	2	95,000	12
Transsuccinylase	1,000,000	24	42,000	24
Flavoprotein	112,000	2	56,000	12
Bovine kidney PDC	7,000,000[b]			
Pyruvate dehydrogenase	154,000	2	41,000	40
		2	36,000	40
Transacetylase	3,100,000	60	52,000	60
Flavoprotein	110,000	2	55,000	10
Kinase	?	?	~50,000	~5
Phosphatase	100,000	1	100,000	~5

[a] The abbreviations used are: PDC, pyruvate dehydrogenase complex; KG, ketoglutarate; KGDC, α-ketoglutarate dehydrogenase complex. [b] Does not include the phosphatase, which dissociates from the complex during its purification.

is bound in amide linkage to the ϵ-amino group of a lysyl residue in the transacetylase (and in the transsuccinylase) (Figure 4). This attachment provides a flexible arm of about 14 Å for the reactive dithiolane ring, conceivably permitting the lipoly moiety to rotate among the catalytic centers of the three different enzymes that comprise the complex (Figure 5). The results of a recent kinetic analysis of the mammalian pyruvate dehydrogenase complex are consistent with this proposed mechanism.[19]

E. coli **α-Ketoglutarate Dehydrogenase Complex.** The *E. coli* α-ketoglutarate dehydrogenase complex (Figure 3E) has been separated into three enzymes, α-ketoglutarate dehydrogenase, dihydrolipoly transsuccinylase, and dihydrolipoyl dehydrogenase.[20] The dihydrolipoyl dehydrogenase component of the α-ketoglutarate dehydrogenase complex and the flavoprotein isolated from the *E. coli* pyruvate dehydrogenase complex are very similar, if not identical.[21] The subunit composition of the *E. coli* α-ketoglutarate dehydrogenase complex is summarized in Table I. It consists of about 12 α-ketoglutarate dehydrogenase chains (*i.e.*, six dimers), 24 transsuccinylase chains, and about 12 flavoprotein chains (*i.e.*, six dimers).[20,22]

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(22) The mammalian α-ketoglutarate dehydrogenase complex [N. Tanaka, K. Koike, M. Hamada, K.-I. Otsuka, T. Suematsu, and M. Koike, *J. Biol. Chem.*, 247, 4043 (1972)] resembles the *E. coli* complex in appearance and subunit composition.

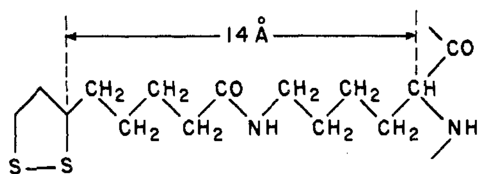

Figure 4. Functional form of lipoic acid in the *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes. The carboxyl group of lipoic acid is bound in amide linkage to the ϵ-amino group of a lysyl residue, providing a flexible arm about 14 Å for the reactive dithiolane ring.

The appearance of the transsuccinylase in the electron microscope (Figure 3F) is strikingly similar to that of the *E. coli* transacetylase (Figure 3B). The *E. coli* transsuccinylase has been crystallized. Electron micrographs and their optical diffraction patterns in combination with X-ray diffraction data were used to analyze the packing of the transsuccinylase cores in the crystal.[11] The cores were found to be packed edge to edge in a face-centered-cubic lattice, *F*432. In this arrangement the octahedral symmetry of the transsuccinylase core is directly displayed in the crystal. A three-dimensional map at 38-Å resolution has been produced from electron micrographs, and an 18-Å resolution map has been constructed from the X-ray data. The distribution of matter in the electron density maps together with the information about the subunit composition establish that the transsuccinylase consists of 24 globular units arranged near the vertices of a truncated cube (Figure 3C).

Although the transsuccinylase consists of 24 very similar polypeptide chains, it can accept only 12 lipoyl moieties and only 12 α-ketoglutarate dehydrogenase chains.[20] The transsuccinylase can bind about 36 flavoprotein chains (*i.e.*, 18 dimers). However, nearly full activity is recovered when only about 12 chains (*i.e.*, six dimers) are bound. Clearly, binding of flavoprotein to the 24 potential sites on the transsuccinylase is not random.

A possible explanation of these findings is that a systematic perturbation of the structure of the transsuccinylase has reduced its symmetry from 432 to 23, so that instead of one set of 24 equivalent sites for each ligand the transsuccinylase has two sets of 12 equivalent sites. The perturbation need not be a large one, and the binding energies of the nonequivalent sites could be different. Preliminary image analysis of transsuccinylase–flavoprotein subcomplexes suggests that the flavoprotein dimers are located at the fourfold positions (*i.e.*, in the faces) of the transsuccinylase cube.[23] Further understanding of these distinctive features of the transsuccinylase and their functional significance must await a more detailed analysis of the structure of the α-ketoglutarate dehydrogenase complex.

Mammalian Pyruvate Dehydrogenase Complex. In mammalian cells the pyruvate dehydrogenase complex is found in mitochondria, apparently in the matrix space.[24] The pyruvate dehydrogenase complexes isolated from porcine heart[25] and from bovine

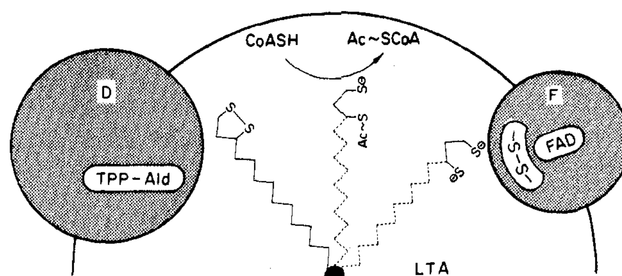

Figure 5. A schematic representation of the possible rotation of a lipoyllysyl moiety between α-hydroxyethylthiamine pyrophosphate (TPP-Ald) bound to pyruvate dehydrogenase (D), the site for acetyl transfer to CoA, and the reactive disulfide of the flavoprotein (F). The lipoyllysyl moiety is an integral part of dihydrolipoyl transacetylase (LTA).

kidney and heart[26] contain a core, consisting of dihydrolipoyl transacetylase, to which pyruvate dehydrogenase, dihydrolipoyl dehydrogenase, and two regulatory enzymes—a kinase and a phosphatase—are joined. The approximate subunit composition of the bovine kidney pyruvate dehydrogenase complex is summarized in Table I. The pyruvate dehydrogenase component of the complex, in contrast to the *E. coli* pyruvate and α-ketoglutarate dehydrogenases, possesses the subunit composition $\alpha_2\beta_2$.[27] The whole complex contains 60 transacetylase chains, about 40 of each of the two types of pyruvate dehydrogenase chains (*i.e.*, about 20 tetramers), about ten flavoprotein chains (*i.e.*, about five dimers), and about five kinase molecules.[27] Each transacetylase chain apparently contains one molecule of covalently bound lipoic acid. The molecular weight and subunit composition of the kinase are not yet known. However, a kinase polypeptide chain of molecular weight about 50,000 has been detected by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. Because the phosphatase tends to dissociate during purification of the complex, its exact stoichiometry has not been established.

The appearance of the bovine kidney pyruvate dehydrogenase complex in the electron microscope is shown in Figure 3G. The transacetylase component of the complex has the appearance of a pentagonal dodecahedron (Figure 3H),[25,28] and its design appears to be based on icosahedral (532) symmetry (Figure 3I). The tentative conclusion from electron microscopic studies is that the flavoprotein dimers are located at the fivefold positions (*i.e.*, in the faces) of the transacetylase pentagonal dodecahedron and that the pyruvate dehydrogenase tetramers are located at the twofold positions (*i.e.*, on the edges). The locations of the kinase and the phosphatase on the transacetylase are not yet known. It is obvious that the number of molecules of pyruvate dehydrogenase, flavoprotein, kinase, and phosphatase found in the bovine kidney pyruvate dehydrogenase complex (Table I) is less than the number of potential binding sites for these ligands on the transacetylase. The molecular basis of this phenomenon and its functional significance are under investigation.

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An interesting difference between the bovine kidney and heart dihydrolipoyl transacetylases has been observed.[29] Although the two transacetylases, and the two pyruvate dehydrogenases as well, are very similar and are functionally interchangeable, the kidney transacetylase apparently can bind only about 20 pyruvate dehydrogenase tetramers (either kidney or heart pyruvate dehydrogenase), whereas the heart transacetylase can bind about 30 pyruvate dehydrogenase tetramers. This difference is reflected in the molecular weights of the two pyruvate dehydrogenase complexes. The bovine heart pyruvate dehydrogenase complex has a molecular weight of about 9,000,000, whereas the kidney complex has a molecular weight of about 7,000,000. The molecular basis of this difference and its functional significance remain to be elucidated.

We have isolated and purified pyruvate dehydrogenase complexes from several other microbial and eukaryotic cells to compare their morphology, subunit composition, and regulatory properties.[30] Electron microscope and sedimentation velocity studies indicate that the pyruvate dehydrogenase complexes from pigeon breast muscle, *Neurospora crassa,*[31] *Saccharomyces cerevisiae,* and *Saccharomyces lactis* are similar in size and appearance (*i.e.,* icosahedral design) to the mammalian pyruvate dehydrogenase complex. However, icosahedral morphology is apparently not unique to the pyruvate dehydrogenase complexes from eukaryotic cells, since the pyruvate dehydrogenase complex from the bacterium, *Streptococcus faecalis,* resembles the mammalian complex rather than the *E. coli* complex in size and appearance.

Regulation of α-Keto Acid Dehydrogenase Complexes

In view of their central positions in metabolism (Figure 1), the pyruvate and α-ketoglutarate dehydrogenase complexes are likely candidates for metabolic regulation. Mechanisms for control over the activity of these complexes have indeed been found.

Product Inhibition. The activity of the pyruvate dehydrogenase complexes is inhibited by the products of pyruvate oxidation, acetyl-CoA and DPNH, and these inhibitions are reversed competitively by CoA and DPN^+, respectively.[19,32] In an analogous manner, the α-ketoglutarate dehydrogenase complexes are inhibited by succinyl-CoA and DPNH, and these inhibitions are competitive with respect to CoA and DPN.[33] These observations have led to suggestions that the activity of these multienzyme complexes may be regulated *in vivo,* at least in part, by the [acetyl-CoA]/[CoA] or [succinyl-CoA]/[CoA] ratios, and by the oxidation level of the DPN–DPNH pool.

The sites of acyl-CoA and DPNH inhibition are the transacetylase and flavoprotein components of the complexes, respectively. However, in certain organisms acetyl-CoA and DPNH apparently also act as feedback inhibitors of the α-keto acid dehydrogenase component of the complexes. Thus, acetyl-CoA inhibition of the *E. coli* pyruvate dehydrogenase complex is competitive with respect to pyruvate,[34] and DPNH inhibition of the *Acinetobacter* sp. α-ketoglutarate dehydrogenase complex is competitive with respect to α-ketoglutarate.[35]

Feedback Regulation by Nucleotides. Since oxidation of pyruvate and α-ketoglutarate *via* the tricarboxylic acid cycle (Figure 1) leads to the generation of ATP, it should not be surprising that the activity of pyruvate and α-ketoglutarate dehydrogenase complexes is subject to regulation by the phosphorylation state (*i.e.,* the "energy charge"[36]) of the nucleotide pool. Thus, the activity of the pyruvate dehydrogenase component of the *E. coli* complex is stimulated by nucleoside monophosphates, and it is inhibited by guanosine triphosphate.[34] This latter inhibition is noncompetitive with respect to pyruvate, and it is reversed by guanosine diphosphate. The activity of the *E. coli* pyruvate dehydrogenase complex decreases with increasing energy charge of the adenylate pool, and this effect is maximal in the presence of acetyl-CoA.[37]

The activity of the α-ketoglutarate dehydrogenase complex derived from cauliflower florets[38] and from *Acinetobacter* sp.[35] is markedly stimulated by adenosine 5′-phosphate (AMP). AMP apparently promotes tighter binding of α-ketoglutarate dehydrogenase with its substrate, α-ketoglutarate, and with its coenzyme, magnesium thiamine pyrophosphate. Since the concentrations of AMP and ATP are inversely related in living cells, stimulation by AMP is thought to correspond in metabolic terms to negative feedback by ATP. Indeed, the α-ketoglutarate dehydrogenase complex derived from blowfly flight muscle mitochondria is inhibited by ATP, and this inhibition is reversed by adenosine 5′-diphosphate (ADP) or AMP.[39]

The rationale of this feedback control mechanism is that high levels of ADP and AMP in a cell would signify a low level of ATP (*i.e.,* little energy available). In such circumstances it would be advantageous to catalyze the oxidative processes of the tricarboxylic acid cycle at a high rate. On the other hand, when the levels of ADP and AMP are low, the α-ketoglutarate dehydrogenase complex will function less rapidly, apparently because it will bind its substrates less tightly. In these circumstances, α-ketoglutarate will be diverted into synthetic processes, *i.e.,* formation of glutamate, glutamine, and nitrogen compounds.

Regulation by Covalent Modification. Another regulatory mechanism, involving phosphorylation and dephosphorylation of the mammalian pyruvate dehydrogenase complex (Figure 6) was first demonstrated in this laboratory.[40] Phosphorylation and

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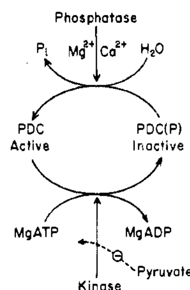

Figure 6. Interconversion of active and inactive (phosphorylated) forms of the mammalian pyruvate dehydrogenase complex (PDC). The dashed line indicates inhibition of the kinase by pyruvate.

concomitant inactivation of the complex are catalyzed by a $MgATP^{2-}$-requiring kinase, and dephosphorylation and concomitant reactivation are catalyzed by a Mg^{2+}-requiring phosphatase. The kinase is tightly bound to the transacetylase, whereas the phosphatase is not.[26,41a] In fact, the association of the phosphatase and the transacetylase is dependent on the presence of Ca^{2+} ions.[5] This control mechanism has been demonstrated with preparations of the pyruvate dehydrogenase complex from kidney, heart, liver, brain, and adipose tissue.[42] We have found no evidence that the *E. coli* or *S. faecalis* pyruvate dehydrogenase complexes or the mammalian or *E. coli* α-ketoglutarate dehydrogenase complexes are regulated by phosphorylation and dephosphorylation. However, the pyruvate dehydrogenase complexes derived from mitochondria of the fungus, *Neurospora crassa,*[30,43] and the yeast, *Saccharomyces lactis,*[30] are subject to regulation by this control mechanism.

The site of this covalent regulation is the pyruvate dehydrogenase component of the complex. Phosphorylation occurs on seryl residues in the α chain (mol wt 41,000) of bovine kidney and heart pyruvate dehydrogenase.[27] It appears that phosphorylation of only one α chain in the bovine kidney pyruvate dehydrogenase tetramer ($\alpha_2\beta_2$) results in inactivation of the tetramer.[44] Evidence has been obtained which suggests that the α chain catalyzes the decarboxylation of pyruvate to produce α-hydroxyethylthiamine pyrophosphate and that the β chain catalyzes the reductive acetylation of the lipoly moiety of the transacetylase with α-hydroxyethylthiamine pyrophosphate[45] (*cf.* Figure 2). The amino acid sequence around the phosphoserine residues in bovine kidney pyruvate dehydrogenase has been determined.[46]

Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg

Phosphorylation of the first seryl residue in this sequence results in inactivation of pyruvate dehydrogenase. The third seryl residue undergoes phosphorylation only after the first seryl residue is phosphorylated.[44,46] The physiological significance, if any, of this latter phosphorylation site is under investigation.

Some of the kinetic and regulatory properties of the purified pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase from bovine kidney and heart have been determined.[4] ADP inhibits the kinase competitively with respect to ATP. Pyruvate is the substrate for pyruvate dehydrogenase, and it also inhibits the kinase. Pyruvate is noncompetitive with ATP. The concentration of Mg^{2+} (about 2 m*M*) required for half-maximal activity of the phosphatase is approximately 100 times the concentration of $MgATP^{2+}$ (about 0.02 m*M*) required for half-maximal activity of the kinase.

Recent studies indicate that Ca^{2+} is required in addition to Mg^{2+} for pyruvate dehydrogenase phosphatase activity.[5,41b,47] In the presence of Ca^{2+} the phosphatase binds to the transacetylase, thereby facilitating the Mg^{2+}-dependent dephosphorylation of the phosphorylated pyruvate dehydrogenase.[5] Half-maximal activity of the phosphatase is observed at a free Ca^{2+} concentration of about 1 μM. It is possible that Ca^{2+} serves as a bridging ligand between the phosphatase and the transacetylase. Alternatively, Ca^{2+} may bind to either the phosphatase or the transacetylase, producing a conformational change that facilitates binding of the phosphatase. The binding of both the phosphatase and the kinase to the transacetylase apparently increases the affinity of these two regulatory enzymes for their respective protein substrates.[4,5]

Although a variety of physiologically significant compounds have been tested, including adenosine cyclic 3′,5′-monophosphate (cyclic AMP), we have been unable to detect regulation of the bovine kidney and heart kinase and phosphatase by any substances other than those mentioned above. The results obtained with the purified pyruvate dehydrogenase system suggest that the activity of the kinase may be regulated *in vivo* by the intramitochondrial concentration of pyruvate and the ATP/ADP ratio, and that the activity of the phosphatase may be regulated by the intramitochondrial concentration of uncomplexed Mg^{2+} and Ca^{2+}.[42] The concentrations of uncomplexed Mg^{2+} and Ca^{2+} in the mitochondrial matrix may be determined, at least in part, by the ATP/ADP ratio, since ADP forms a much weaker complex with these divalent cations than does ATP.

The results obtained with the purified pyruvate dehydrogenase system have been confirmed and extended with rat fat-cell[48] and liver[49] mitochondria. Conditions that increase the intramitochondrial pyruvate concentration or decrease the ATP/ADP ratio result in activation of pyruvate dehydrogenase. These observations are consistent with inhibition of pyruvate dehydrogenase kinase by pyruvate and

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ADP, respectively, or possibly with activation of pyruvate dehydrogenase phosphatase by Mg^{2+} and Ca^{2+}.

Regulation of the interconversion of the phosphorylated and nonphosphorylated forms of pyruvate dehydrogenase in rat adipose tissue by insulin[48,50] and in rat heart, liver, and kidney by long-chain fatty acids[51] has been reported. Insulin apparently increases the proportion of the nonphosphorylated form of pyruvate dehydrogenase, and this effect is antagonized by adrenaline and by adrenocorticotropin. Metabolic states (diabetes, starvation) associated with increased concentrations of plasma free fatty acids result in an increase in the proportion of the phosphorylated form of pyruvate dehydrogenase. It would appear that these hormonal and metabolic effects on the interconversion of the phosphorylated and nonphosphorylated forms of pyruvate dehydrogenase are indirect. It seems possible that these effects may be mediated through changes in the intramitochondrial concentrations of pyruvate, ADP, ATP, Mg^{2+}, and Ca^{2+}.

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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Naturally Occurring Substances. Alkaloids

Ernest Wenkert,* Jasjit S. Bindra, Ching-Jer Chang, David W. Cochran,[1a] and Fred M. Schell[1b]

Department of Chemistry, Indiana University, Bloomington, Indiana 47401

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Research on the chemistry of organic natural products has undergone extraordinary acceleration since the advent of nuclear magnetic resonance spectroscopy in the 1950s. For the most part, this forceful tool of structure analysis has been focused on the hydrogen nucleus (pmr spectroscopy) and hence has been limited by a narrow, *ca.* 10 ppm range of spectral detail. However, recent advances in the pmr field, *e.g.*, the use of paramagnetic shift agents,[2] as well as the expansive, pioneering efforts of Lauterbur, Stothers, Grant, Roberts, and others[3] in the ^{13}C natural abundance magnetic resonance area (cmr spectroscopy), have broadened greatly the scope and utility of nmr spectroscopy for the determination of structures of natural substances.

Historically, natural product chemists have adjusted rapidly to new analytical techniques as soon as instruments have been mass produced. Since this period of development of cmr spectroscopy has arrived, broad acceptance of this new, powerful tool of analysis awaits only the accumulation of chemical shift data on compounds representative of all types of natural products. A huge data bank of the chemical shifts of alkaloids,[4] amino acids and peptides,[5] antibiotics,[6] cannabinoids,[7] carbohydrate derivatives,[8] prostaglandins,[9] steroids,[10] and terpenes[11]

Ernest Wenkert is a graduate of the University of Washington (B.S., M.S.) and Harvard University (Ph.D.). For 10 years he was at Iowa State University, and since 1961 he has been at Indiana University where he is presently Herman T. Briscoe Professor of Chemistry. Besides the ^{13}C nmr studies reported in this Account, Professor Wenkert is involved in the structure determination and synthesis of natural products and the study of the mechanisms of synthetically interesting organic reactions.

Jasjit S. Bindra, a graduate of Agra University in India, was a postdoctoral fellow in the Wenkert group at Indiana University during 1969–1971 and is now a research chemist at Chas. Pfizer and Co., Inc.

Ching-Jer Chang, David W. Cochran, and Fred M. Schell were graduate students with Professor Wenkert and received their Ph.D. degrees in 1973, 1971, and 1972, respectively. Dr. Chang is now Assistant Professor in the Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University; Dr. Cochran, Postdoctoral Fellow in the Department of Radiological Sciences, School of Hygiene, Johns Hopkins University; and Dr. Schell, Assistant Professor in the Department of Chemistry, University of Tennessee.

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