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RECOLLECTIONS

From lipoic acid to multi-enzyme complexes

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I shall retrace the high points of a trail of research that I have had the pleasure of establishing in association with many collaborators. This trail has led from the isolation and identification of a microbial growth factor to the structure, function, and regulation of α-keto acid dehydrogenase complexes. The high points of this trail in the 1950s were the isolation, characterization, and synthesis of lipoic acid and identification of its functional form. In the late 1950s and into the 1960s the trail led to the isolation, resolution, and reconstitution of the *Escherichia coli* pyruvate and α-ketoglutarate dehydrogenase complexes, characterization of their component enzymes, and elucidation of their macromolecular organization. In the late 1960s part of our research effort was directed toward isolating and characterizing the bovine pyruvate dehydrogenase (PDH) complex. In the early stage of this investigation we found that the complex is regulated by phosphorylation and dephosphorylation. Resolution of the mammalian PDH complex and characterization of its component enzymes, including the kinase and the phosphatase, continued in the 1970s and early 1980s. We also obtained evidence that the dihydrolipoamide acetyltransferase components of the *E. coli* and bovine PDH complexes possess a multi-domain structure. In the 1980s we isolated and characterized the bovine branched-chain α-keto acid dehydrogenase complex and the phosphatase that regulates its activity. In the late 1980s and early 1990s we cloned and disrupted the genes encoding the components of the *Saccharomyces cerevisiae* PDH complex and used protein engineering techniques to study structure–function relationships. In the mid-1990s we cloned, sequenced, and expressed cDNAs encoding the two subunits comprising bovine PDH phosphatase and gained a deeper understanding of their nature and regulation (Fig. 1).

This trail of discovery started in the spring of 1949, about six months after I joined the faculty of the Department of Chemistry at The University of Texas. At that time I started working on the isolation of a factor that replaced acetate in the growth medium for certain lactic acid bacteria. Research on the acetate-replacing factor was initiated by Esmond Snell at the University of Wisconsin, continued with a graduate student, Beverly Guirard, after Esmond moved to The University of Texas, and then pursued by Milton Getzendaner, a graduate student under Roger Williams' supervision. I inherited this project in the spring of 1949. We established that this factor is widely distributed in animal, plant, and microbial cells and that animal liver is a rich source. The factor is tightly bound to liver protein and is released only after hydrolysis in acid or base. At that time pharmaceutical companies were processing large amounts of pork and beef liver to obtain extracts suitable for treatment of pernicious anemia. Fresh liver was extracted with warm water, and the residual liver proteins and fatty material were dried and sold as an animal feed supplement. Arrangements were made with Eli Lilly and Company to obtain liver residue, and we developed procedures for extracting and purifying the acetate-replacing factor.

In the late 1940s and early 1950s several other groups were trying to isolate factors that were similar to, if not identical with, the acetate-replacing factor. These factors included the "pyruvate oxidation factor" of O'Kane and Gunsalus that was necessary for oxidation of pyruvate to acetate and carbon dioxide by *Streptococcus faecalis*; "protogen," an unidentified growth factor for a

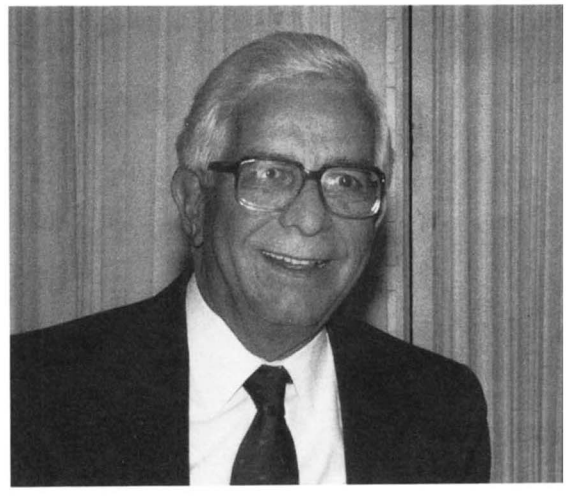

Fig. 1. Lester Reed.

Lester J. Reed was born in New Orleans, Louisiana, in 1925 and received a B.S. in chemistry from Tulane University in 1943. He was awarded a Ph.D. in organic chemistry from the University of Illinois in 1946 under R.C. Fuson. He was a research associate in biochemistry under Vincent du Vigeaud at Cornell University Medical College from 1946 to 1948. He joined the Department of Chemistry at The University of Texas in 1948 as an Assistant Professor. He is currently Ashbel Smith Professor of Chemistry and Biochemistry. He was Director of the Clayton Foundation Biochemical Institute from 1963 to 1996. Dr. Reed is a member of the National Academy of Sciences and the American Academy of Arts and Sciences. He received the Eli Lilly & Co. Award in Biological Chemistry of the American Chemical Society in 1958, an honorary doctorate from Tulane University in 1977, and the Merck Award of the American Society for Biochemistry and Molecular Biology in 1994.

protozoan, *Tetrahymena geleii*, that was being purified by Stokstad, Jukes, and associates at Lederle Laboratories; and the "B. R. factor" of Kline and Barker required for growth of *Butyribacterium rettgeri* with lactate as the fermentable carbon source.

In the fall of 1950, a collaboration with Gunsalus and the Lilly Research Laboratories was undertaken to isolate the acetate-replacing/pyruvate oxidation factor. The Lilly group adapted and scaled up isolation procedures developed by us, and concentrates of the growth factor that were 0.1 to 1% pure were sent to us for further processing. One of the most exciting times in my life occurred on or about March 15, 1951, when I obtained the first pale-yellow crystals of the factor. The amount was minute, only about 3 mg. It was partially characterized and given the trivial name α-lipoic acid (Reed et al., 1951). The isolation procedure involved a 300,000-fold purification. A total of approximately 30 mg of crystalline lipoic acid was eventually isolated. We estimated that approximately 10 tons of liver residue were processed to obtain this small amount of the pure substance. NMR and mass spectrometers were not available in those days, but it was possible to establish that lipoic acid is either 6,8-, 5,8-, or 4,8-dithiooctanoic acid. That the correct structure is 6,8-dithiooctanoic acid (1,2-dithiolane-3-valeric acid) was established by synthesis of DL-lipoic acid, first achieved by the Lederle group (Reed, 1957). I was intrigued by this simple, yet unique substance and wanted to know more about its biological function, i.e., with what and how did it function in living cells. We, therefore, set about establishing this part of the trail, which turned out to be even more exciting than the isolation and characterization of lipoic acid. Elucidation of the mechanism of oxidative decarboxylation of α-keto acids is a fascinating chapter in modern biochemistry. I shall review briefly the major developments in this story.

The equation shown below represents the coenzyme A and NAD^+-linked oxidative decarboxylation of α-keto acids. In addition to CoA and NAD^+, thiamin diphosphate, a divalent metal ion, protein-bound lipoic acid, and FAD are required.

$$RCOCO_2^- + CoASH + NAD^+ \rightarrow RCOSCoA + CO_2 + NADH.$$

With a few notable exceptions, prior to 1950 pyruvate and α-ketoglutarate oxidation had been studied with particulate preparations from animal tissues and micro-organisms that were unsuitable for detailed analysis. However, these studies, notably those of Peters and his associates at Oxford, including Ochoa, had shown that thiamin diphosphate is required by enzymes that catalyze a decarboxylation of α-keto acids. Other important developments in the late 1940s and early 1950s were Lipmann's discovery of coenzyme A, Stadtman's discovery of phosphotransacetylase and elucidation of the reaction catalyzed by this enzyme, and Lynen's demonstration of the thioester linkage in acetyl-CoA.

Solubilization of bacterial and animal α-keto acid oxidation systems in the early 1950s in the laboratories of Ochoa and Green was a significant advance. Korkes, Gunsalus, and Ochoa demonstrated that dismutation of pyruvate by enzyme preparations from *E. coli* and *S. faecalis* required a divalent metal ion, thiamin diphosphate, CoA, and NAD^+. They succeeded in separating the pyruvate oxidation system of *E. coli* into two components, designated Fraction A and Fraction B. Jaganathan and Schweet isolated a pyruvate oxidation system from pigeon breast muscle in a highly purified state, with an apparent molecular weight of about 4 million. These preparations were shown subsequently to reduce NAD^+ and to acetylate CoA. I remember Dick Schweet telling me about the skepticism expressed by some well-known enzymologists concerning the nature and purity of his "pyruvic oxidase" preparations. One prominent enzymologist suggested that Schweet had isolated a membrane fragment, and that if he continued with the purification he would eventually obtain a soluble enzyme with a respectable molecular weight. Seymour Kaufman showed that dismutation of α-ketoglutarate by soluble preparations from pig heart required NAD^+ and CoA and that one of the products was succinyl CoA. Sanadi and Littlefield isolated the α-ketoglutarate oxidation system from pig heart as a highly purified preparation with an apparent molecular weight of 2 million and showed that NAD^+ and CoA were the natural electron and acyl acceptors.

The next important development was the isolation and characterization of lipoic acid described above. The presence of a disulfide linkage in lipoic acid recalled the interesting results of Peters and co-workers, who had observed a rather specific inhibition of the pigeon brain pyruvate oxidation system by trivalent arsenicals, particularly Lewisite, and a reversal of this toxic action by the dithiol 2,3-dimercaptopropanol (British anti-Lewisite, BAL), but not by monothiols. They postulated the existence of a dithiol structure as part of the pyruvate oxidation system. These results were duplicated by Gunsalus and associates with *S. faecalis* cells, and interpreted as indicating the involvement of dihydrolipoic acid in pyruvate oxidation. Gunsalus proposed that lipoic acid underwent a cycle of reactions in α-keto oxidation comprising reductive acylation, acyl transfer, and electron transfer. Lipoic acid was visualized as functioning after diphosphothiamin and before CoA and NAD^+. Gunsalus, Hager, and associates obtained evidence for this proposal using lipoic acid and derivatives thereof in substrate amounts. They demonstrated that *E. coli* Fraction A contained a lipoyl transacetylase and that Fraction B contained a lipoyl dehydrogenase. In the late 1950s, Vince Massey showed that the lipoyl dehydrogenase component of the pig heart α-ketoglutarate dehydrogenase complex is identical with Straub diaphorase, a flavoprotein described in 1939. Mechanistic studies by Massey and later by Charles Williams elucidated the catalytic mechanism. Model experiments conducted by Ronald Breslow with thiamin and analogs thereof led him to propose a mechanism of thiamin diphosphate action. 2-(1-Hydroxyethyl)thiamin diphosphate was proposed to be "active acetaldehyde." This hypothesis was confirmed and extended by enzymic studies carried out by Lester Krampitz and by Helmut Holzer and their associates.

In my laboratory, we developed mild procedures for purification of the pyruvate and α-ketoglutarate oxidation systems from *E. coli*. By the late 1950s, Masahiko Koike succeeded in isolating these enzyme systems as highly purified functional units with molecular weights in the millions (Koike et al., 1960). It was very exciting to see in the analytical ultracentrifuge of my friend and collaborator at NIH, Bill Carroll, a major symmetrical peak for each of the two highly purified preparations, and that the boundary of the yellow color of the flavoprotein was associated with the main peak. The molecular weights of these multi-enzyme units were determined to be 4.8 and 2.4 million, respectively. By careful, and persistent work over a period of several years, we dissected the pyruvate and α-ketoglutarate dehydrogenase complexes into their component enzymes and reassembled the large functional units from the isolated enzymes (Koike et al., 1963). We demonstrated that each of these functional units is composed of multiple copies of three enzymes, a pyruvate or α-ketoglutarate decarboxylase-dehydrogenase (E_1), a dihydrolipoyl acetyltransferase or succinyltransfer-

ase (E_2), and the flavoprotein, dihydrolipoyl dehydrogenase (E_3). These three enzymes, acting in sequence, catalyze the reactions shown in Figure 2. E_1 catalyzes both the decarboxylation of the α-keto acid (reaction 1) and the subsequent reductive acylation of the lipoyl moiety that is covalently bound to E_2 (reaction 2). E_2 catalyzes the acyl transfer to CoA (reaction 3), and E_3 catalyzes the re-oxidation of the dihydrolipoyl moiety with NAD^+ as the ultimate electron acceptor (reactions 4 and 5).

Hayao Nawa showed in the late 1950s that the lipoyl moiety in the *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes is attached in amide linkage to the ϵ-amino group of a lysine residue (Nawa et al., 1960). An enzyme that hydrolyzes the lipoyllysyl linkage, lipoamidase, as well as an ATP-dependent enzyme that reincorporates the lipoyl moiety, i.e., a lipoate-protein ligase, were detected in *S. faecalis* extracts and partially purified. We proposed that this linkage provides a flexible arm, about 14 Å in length, for the reactive 1,2-dithiolane ring, permitting the lipoyl moiety to rotate among the catalytic sites of the three component enzymes of each complex. This is the so-called "swinging-arm" active-site coupling mechanism. Some 15 years later, Richard Perham and Cees Veeger and their associates attached a spin label to the protein-bound lipoyl moieties and showed by ESR spectroscopy that the lipoyl moieties exhibited considerable rotational mobility.

These were exciting times for us in the late 1950s and early 1960s. We visualized the *E. coli* PDH complex as an organized mosaic of enzymes. To obtain evidence for this hypothesis, we turned to electron microscopy. I contacted Humberto Fernandez-Moran, who was then at the Massachusetts General Hospital, and arranged to bring a sample of the PDH complex to his laboratory. This was in January of 1962, and was indeed a memorable occasion. When our sample was negatively stained with phosphotungstate to provide contrast and then examined in the electron microscope, we saw a beautifully organized structure. The particles seen in the electron microscope had a diameter of about 300 Å, and there was a definite indication of subunits arranged in a regular manner (Fernandez-Moran et al., 1964). Within about two years, we set up an electron microscopy laboratory in the Biochemical Institute at The University of Texas. Electron microscopy studies were carried out by my associate Robert Oliver, X-ray crystallographic studies by collaborators David DeRosier and Marvin Hackert, and sedimentation equilibrium molecular weight determinations by Petr Munk. The results demonstrated that both the acetyltransferase and the succinyltransferase (E_2) consist of 24 apparently identical polypeptide chains arranged as eight trimers in a cube-like particle exhibiting octahedral (432) symmetry. Multiple copies of E_1 and E_3 are attached to E_2 by noncovalent bonds. In the PDH complex, 12 E_1 dimers and 6 E_3 dimers are apparently arranged, respectively, on the 12 edges and in the six faces of E_2 (Reed, 1974).

In the late 1960s part of our research effort was directed toward isolation and characterization of the mammalian pyruvate and α-ketoglutarate dehydrogenase complexes, which are localized to mitochondria, within the inner membrane–matrix compartment. Procedures were developed for preparation of mitochondria on a large scale from bovine kidney and heart (with the advice and assistance of my friend and colleague, Dan Ziegler), and relatively mild procedures were developed to isolate the pyruvate and α-ketoglutarate dehydrogenase complexes from the mitochondrial extracts. In the course of attempts to stabilize these complexes in crude extracts of bovine kidney mitochondria, Tracy Linn observed that the PDH complex, but not the α-ketoglutarate dehydrogenase complex, underwent a time-dependent inactivation in the presence of ATP. A systematic investigation revealed that the bovine kidney and heart PDH complexes are regulated by a phosphorylation-dephosphorylation cycle (Linn et al., 1969). Phosphorylation and concomitant inactivation of the complex is catalyzed by an ATP-dependent kinase, which is tightly bound to the complex, and dephosphorylation and concomitant reactivation is catalyzed by a Mg^{2+}-dependent phosphatase, which is loosely attached to the complex. It seemed curious at the time (1968) that inactivation of the PDH complex by phosphorylation had not been detected earlier. The explanation may lie in a remark by Henry Lardy after receiving a preprint of our paper on the phosphorylation and inactivation of the PDH complex. (This finding) "explains why we have never been able to get pyruvate to be oxidized in submitochondrial particles, because we invariably add ATP to keep things in the 'optimum' state." This control mechanism was subsequently confirmed in the laboratories of Otto Wieland, Philip Randle, S.E. Severin, and other investigators with preparations of the PDH complex from other mammalian tissues and from pigeon breast muscle, plant tissue, and *Neurospora crassa*.

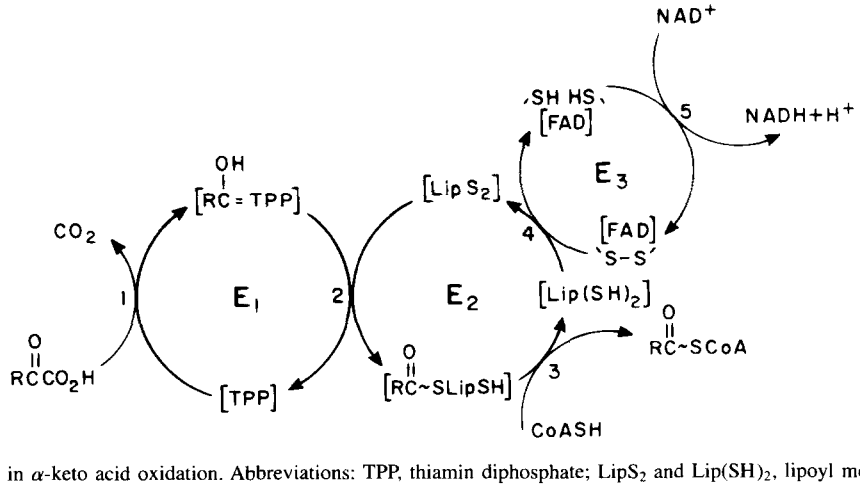

Fig. 2. Reaction sequence in α-keto acid oxidation. Abbreviations: TPP, thiamin diphosphate; $LipS_2$ and $Lip(SH)_2$, lipoyl moiety and its reduced form.

Over a period of several years our group separated the bovine kidney and heart PDH complexes into their component enzymes, including the kinase and the phosphatase, and characterized the individual enzymes (Linn et al., 1972). The bovine heart PDH complex has a molecular weight of about 9.5 million. Its subunit composition is now known to be 60 E_2 subunits, 30 E_1 tetramers ($\alpha_2\beta_2$), and 12 E_3 dimers, which are positioned on the E_2 core by 12 E_3-binding protein (protein X) monomers. The $E_1\alpha$ subunit undergoes phosphorylation and dephosphorylation. The appearance of E_2 in the electron microscope is that of a pentagonal dodecahedron, and its design is based on icosahedral (532) symmetry. We proposed that the E_1 tetramers are located on the 30 edges and the E_3 dimers in the 12 faces of the pentagonal dodecahedron.

A novel architectural feature of dihydrolipoamide acyltransferases was revealed initially in our laboratory by limited proteolysis of the *E. coli* acetyltransferase and by electron microscopy. Dennis Bleile found in the late 1970s that trypsin cleaved the acetyltransferase, which contained radioactive lipoyl moieties, into two large fragments (Bleile et al., 1979). One fragment, designated the lipoyl domain, contained the covalently bound lipoyl moieties and exhibited an extended structure. The other tryptic fragment exhibited a compact structure and contained the active site, the intersubunit binding sites, and the binding sites for E_1 and E_3. The assemblage of compact catalytic domains constitutes the inner core of E_2, conferring the cube-like appearance in the electron microscope. The two domains are connected by a trypsin-sensitive hinge region. We suggested that movement of lipoyl domains and not simply rotation of lipoyllysyl moieties provides the means to span the physical gaps between catalytic sites on the complex. These early findings on the domain structure of dihydrolipoamide acyltransferases were confirmed and extended by studies involving molecular genetics, limited proteolysis, and proton NMR spectroscopy in the laboratories of John Guest and Richard Perham. Briefly, the amino-terminal segment possesses one, two, or three lipoyl domains, followed by a domain that is involved in binding E_3 and/or E_1, and then by a catalytic domain that contains the active site as well as additional subunit binding sites. The domains are connected by flexible segments or hinge regions that are rich in alanine, proline, and charged amino acid residues. Recently, Wim Hol and associates determined the crystal structure at 2.6 Å resolution of the cube-like inner core of the dihydrolipoamide acetyltransferase from the *Azotobacter vinelandii* PDH complex. Richard Perham and associates used multi-dimensional NMR to determine the three-dimensional solution structures of the lipoyl domain and the E_1/E_3-binding domain of the acetyltransferase from *Bacillus stearothermophilus*. Hol and associates also determined the crystal structure of this E_3-binding domain complexed with an E_3 dimer. These structures provide a deeper understanding of how the lipoyl domain can move between the active sites of E_2 and E_3 in the PDH complex.

In the late 1970s Flora Pettit and Steve Yeaman purified to apparent homogeneity and characterized the bovine branched-chain α-keto acid dehydrogenase complex. In the 1980s Zahi Damuni isolated and characterized the phosphatase that participates in the regulation of this complex, and he also isolated and characterized a potent heat-stable inhibitor of the phosphatase.

Hormonal regulation of the mammalian PDH complex is particularly fascinating because it involves signal transduction not only across the cell membrane but also across the inner mitochondrial membrane to target the PDH phosphatase and, consequently, the PDH complex, located in the mitochondrial matrix. It is now known that the major regulators of the phosphatase activity are Ca^{2+} and Mg^{2+}, which involve the hormones epinephrine and insulin, respectively. In the early 1970s our group partially purified PDH phosphatase from bovine heart and kidney mitochondria and showed that it requires Mg^{2+} or Mn^{2+} for activity. Denton, Randle, and Martin subsequently reported that Ca^{2+} stimulated the activity of the phosphatase in the presence of Mg^{2+}. Flora Pettit and Tom Roche in our group showed that Ca^{2+} mediates translocation of the phosphatase to the E_2 component of the PDH complex, presumably in proximity to its substrate, phosphorylated E_1, thereby increasing the rate of dephosphorylation. This Ca^{2+}-mediated translocation apparently is the molecular basis of the epinephrine-induced activation of PDH phosphatase observed by Richard Hansford, Richard Denton, and other investigators.

In the early 1980s Martin Teague, Flora Pettit, and co-workers purified PDH phosphatase to near homogeneity and showed that it consists of a Mg^{2+}-dependent and Ca^{2+}-stimulated catalytic subunit (50 kDa; PDPc) and a flavoprotein of unknown function (100 kDa; later designated PDPr) (Teague et al., 1982). Zahi Damuni showed that polyamines, particularly spermine, increase the sensitivity of PDH phosphatase to Mg^{2+}. Denton and associates subsequently showed that insulin stimulates the activity of PDH phosphatase in adipose tissue by increasing the sensitivity of the phosphatase to Mg^{2+}. Spermine apparently mimics the insulin effect. The function of PDPr remained a mystery until Janet Lawson recently cloned and expressed cDNA encoding PDPc. By comparing the properties of recombinant PDPc and the native PDH phosphatase heterodimer (PDPc bound to PDPr), we obtained insight into the function of PDPr. Jiangong Yan found that PDPr decreases the sensitivity of PDPc to Mg^{2+} and that spermine increases the sensitivity of PDH phosphatase but not PDPc to Mg^{2+}, apparently by interacting with PDPr (Yan et al., 1996). We interpret these observations to indicate that PDPr blocks or distorts the Mg^{2+}-binding site of PDPc and that spermine produces a conformational change in PDPr (allosteric effect) that reverses its inhibitory effect. These observations raise the intriguing prospect that an insulin-induced allosteric effect on PDPr may underlie its stimulation of PDH phosphatase activity.

To gain further understanding of structure–function relationships in eukaryotic PDH complexes, we initiated in the late 1980s molecular genetic studies of the PDH complex in the yeast *Saccharomyces cerevisiae*. The genes encoding the five proteins comprising the complex ($E_1\alpha$, $E_1\beta$, E_2, E_3BP, and E_3) were cloned, sequenced, expressed, and disrupted. Studies on E_3-binding protein (E_3BP) confirmed and extended previous studies of Tom Roche and of Gordon Lindsay and their associates with the protein X component of the bovine PDH complex. E_3BP and E_2 apparently evolved from a common ancestor. E_3BP possesses an amino-terminal lipoyl domain, followed by an E_3-binding domain, and then by a carboxyl-terminal domain that is involved in anchoring E_3BP to the inner core of E_2. Binding studies in conjunction with cyroelectron microscopy and three-dimensional image reconstruction in collaboration with James Stoops and Timothy Baker and their associates revealed a unique structural organization of the *S. cerevisiae* PDH complex and, by analogy, of the mammalian complex (Stoops et al., 1977). E_2 consists of 20 cone-shaped trimers at the vertices of a pentagonal dodecahedron. There are 12 large openings that lead into a central cavity. It was generally believed that the other components of these complexes are bound on the outside of the E_2 scaffold. By contrast, our results show that E_3BP binds near the tips of the E_2 trimers within the central cavity and anchors an E_3 dimer inside each of the 12 pentagonal faces of E_2.

Our finding that the E_2 structure, with 532 molecular symmetry, can physically accommodate only one BP-E_3 complex in each of its 12 pentagonal-shaped faces provides a satisfactory explanation of the unique polypeptide chain ratio in the *S. cerevisiae* and mammalian PDH complexes (60 $E_1\alpha$:60 $E_1\beta$:60 E_2:12 BP:24 E_3).

I hope these recollections have given some appreciation of the thrill and excitement I have experienced in establishing this trail of research from lipoic acid to the structure, function, and regulation of the α-keto acid dehydrogenase complexes. I have been accompanied in the various stages of this journey by excellent associates, including undergraduate, graduate, and postdoctoral students, technicians, and members of the senior staff of the Biochemical Institute, and by collaborators at other universities and institutes.

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