# *Escherichia coli* Pyruvate Dehydrogenase Complex: Particle Masses of the Complex and Component Enzymes Measured by Scanning Transmission Electron Microscopy†

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ABSTRACT: Particle masses of the *Escherichia coli* pyruvate dehydrogenase (PDH) complex and its component enzymes have been measured by scanning transmission electron microscopy (STEM). The particle mass of PDH complex measured by STEM is $5.28 \times 10^6$ with a standard deviation of $0.40 \times 10^6$. The masses of the component enzymes together with their standard deviations are $(2.06 \pm 0.26) \times 10^5$ for the dimeric pyruvate dehydrogenase ($E_1$), $(1.15 \pm 0.17) \times 10^5$ for dimeric dihydrolipoyl dehydrogenase ($E_3$), and $(2.20 \pm 0.17) \times 10^6$ for dihydrolipoyl transacetylase ($E_2$), the 24-subunit core enzyme. The latter value corresponds to a subunit molecular weight of $(9.17 \pm 0.71) \times 10^4$ for $E_2$. The subunit molecular weight measured by polyacrylamide gel electrophoresis in sodium dodecyl sulfate is $8.6 \times 10^4$. STEM measurements on PDH complex incubated with excess $E_3$ or $E_1$ failed to detect any additional binding of $E_3$ but showed that the complex would bind additional $E_1$ under forcing conditions (high concentrations with glutaraldehyde). The additional $E_1$ subunits were bound too weakly to represent binding sites in an isolated or isolable complex. The mass measurements by STEM are consistent with the subunit composition 24:24:12 when interpreted in the light of the flavin content of the complex and assuming 24 subunits in the core enzyme ($E_2$).

The pyruvate dehydrogenase complex of *Escherichia coli* is a multienzyme complex that catalyzes the decarboxylation and dehydrogenation of pyruvate concomitant with the reduction of $NAD^+$ and acetylation of CoA[1] to *S*-acetyl-CoA.

The enzyme complex is composed of multiple copies of three enzymes, $E_1$, $E_2$, and $E_3$ (Reed & Cox, 1970), which act in concert according to eq 1–5 to catalyze the overall reaction, eq 6.

$$H^+ + CH_3COCOO^- + E_1\cdot TPP \rightarrow CO_2 + E_1\cdot CH_3C(OH){=}TPP \quad (1)$$

$$E_1\cdot CH_3C(OH){=}TPP + \text{lipoyl-}E_2 \rightarrow E_1\cdot TPP + S\text{-(acetyldihydrolipoyl)-}E_2 \quad (2)$$

$$S\text{-(acetyldihydrolipoyl)-}E_2 + CoA \rightleftharpoons \text{acetyl-CoA} + \text{dihydrolipoyl-}E_2 \quad (3)$$

$$\text{dihydrolipoyl-}E_2 + E_3\cdot FAD \rightleftharpoons \text{lipoyl-}E_2 + \text{dihydro-}E_3\cdot FAD \quad (4)$$

$$\text{dihydro-}E_3\cdot FAD + NAD^+ \rightleftharpoons E_3\cdot FAD + NADH + H^+ \quad (5)$$

$$CH_3COCOO^- + CoA + NAD^+ \rightarrow CO_2 + \text{acetyl-CoA} + NADH \quad (6)$$

Twenty-four identical $E_2$ subunits arranged in cubic symmetry constitute the structural core of the complex to which multiple copies of $E_1$ and $E_3$ are noncovalently bound (Eley et al., 1972; Reed, 1974). The number of bound $E_1$ and $E_3$ subunits and the molecular weight of the complex itself have not been generally agreed upon. Molecular weight values of 4.6–4.8 million for the complex have been measured by both sedimentation velocity ultracentrifugation and low-angle laser scattering techniques (Eley et al., 1972; Koike et al., 1960; Angelides et al., 1979). However, Danson et al. (1979) reported a $M_r$ of 6.1 million from the diffusion coefficient measured by laser light intensity fluctuation spectroscopy. A limited heterogeneity in the particle size of the complex has also been suggested from the active enzyme centrifugation method (Schmitt & Cohen, 1980). The molecular values of the component enzymes $E_1$ and $E_3$ have been reported in several studies to be 90 000–100 000 for $E_1$ and 54 000–60 000 for $E_3$ (Eley et al., 1972; Vogel, 1977; Koike et al., 1963; Angelides et al., 1979). The molecular weight of $E_1$ was also deduced from the gene sequence of *E. coli* K12 as 99 474, which agrees with the above value (Stephens et al., 1983a). The same group computed the molecular weight of $E_2$ as 65 959 from the gene sequencing experiment (Stephens et al., 1983b). Molecular weight values of $E_2$ ranging between 65 000 and 89 000 have been reported from other laboratories employing other techniques of physical measurement (see Discussion and Table V).

On the basis of molecular weight measurements as well as $E_1$, $E_2$, $E_3$, and cofactor contents of the PDH complex, the stoichiometry of the complex as purified was postulated to be 24 $E_1$·TPP:24 $E_2$:12 $E_3$·FAD (Moe & Hammes, 1974; Shepherd & Hammes, 1977; Collins et al., 1977; Eley et al., 1972).

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[1] Abbreviations: PDH complex, pyruvate dehydrogenase complex; $E_1$, pyruvate dehydrogenase; $E_2$, dihydrolipoyl transacetylase; $E_3$, dihydrolipoyl dehydrogenase; STEM, scanning transmission electron microscopy; TPP, thiamin pyrophosphate; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; $M_r$, molecular weight; SD, standard deviation; CoA, coenzyme A.

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However, a stoichiometry of 48 $E_1$:24 $E_2$:24 $E_3$ as the fully constituted complex was also proposed by Bates et al. (1977), who reported a considerable degree of variability in the $E_1$ content of the complex from preparation to preparation. This stoichiometry would correspond to a $M_r$ of 8 million for the fully assembled complex.

In this paper we report on measurements of the particle mass of the PDH complex and the molecular weights of the component enzymes by scanning transmission electron microscopy. These values and the functional lipoic acid and FAD contents of this complex are compatible with chain ratios of 24:24:12 for $E_1$:$E_2$:$E_3$ in the purified complex.

## Experimental Procedures

*Materials.* [1,5-$^{14}$C]Glutaraldehyde with a specific activity of $1.59 \times 10^5$ cpm/$\mu$mol was synthesized from [$^{14}$C]KCN purchased from Amersham according to the procedure of Arciprete et al. (1972). Other chemicals were of analytical grade or equivalent and used without further purifications.

*Purification and Resolution of PDH Complex.* The PDH complex was purified and resolved by following the published procedures (Reed & Willms, 1966; Speckhard & Frey, 1975; Bleile et al., 1979) in the continuous presence of 5 mM PMSF and 1 mM EDTA to minimize proteolysis. Typically, 175 mg of the complex was purified from 1300 g of *E. coli* Crookes strain. The specific activity was 48 units/mg of protein under the assay conditions described by Maldonado et al. (1972). One unit of activity was defined as the production of 1 $\mu$mol of NADH/min under the assay conditions.

*SDS–Polyacrylamide Gel Electrophoresis.* The purity of the PDH complex and the resolved components was verified by electrophoresis in gels polymerized with 7.5% acrylamide in the presence of 0.1% SDS. The protein was denatured by incubation for 5 min at 100 °C in the presence of 1% SDS and 0.1 M mercaptoethanol. The gel electrophoresis followed the procedure of Weber & Osborn (1969). Only the well-resolved component enzymes were used for the molecular weight measurements by STEM. For measurement of the molecular weight by SDS gel electrophoresis, gels were prepared from 6% acrylamide and run with the high molecular weight standards obtained from Bio-Rad Laboratories. The molecular weight values of the standards ranged from 45 000 to 116 000.

All protein concentrations were measured by the method of Lowry et al. (1951) in standard solutions of PDH complex whose concentrations were correlated by $A_{280}$ and dry weight measurements. When applied to cross-linked PDH complex, the Lowry procedure was correlated with the dried weight of cross-linked complex.

*Preparation of Protein Samples for STEM.* Protein specimens were prepared for analysis in the STEM by cross-linking at a concentration of 1.5 mg mL$^{-1}$ with 0.25 wt % glutaraldehyde for 15 min at 25 °C in 2 mM potassium phosphate buffer (pH 7.0). Sodium borohydride, 2 mol/mol of glutaraldehyde, dissolved in 0.3 M potassium phosphate buffer at pH 8.0 was added. The final concentration of the potassium phosphate buffer was 0.1 M. After 20 min at 4 °C, 8 molar equiv of sodium pyruvate was added to quench the excess sodium borohydride. The reaction mixture was left for 60 min at 4 °C. The cross-linked protein was isolated by ultracentrifugation for 3.5 h at 100000$g$ when the protein sample was mainly the PDH complex. For samples of $E_1$ or $E_3$ proteins, a Sephadex G-25 column (0.7 cm × 17 cm) was used to isolate the protein. For samples of $E_2$ core, dialysis overnight at 4 °C against 0.02 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 5 mM PMSF was employed. When excess $E_1$ or $E_3$ was added to the PDH complex prior to cross-linking, 48 equiv of $E_1$ or $E_3$ was added, calculated assuming the molecular weight of the PDH complex was 5 million; and they were incubated for 60 min at 4 °C before being cross-linked with glutaraldehyde. The effectiveness of cross-linking was confirmed by 7.5% polyacrylamide gel electrophoresis as described above. When completely cross-linked, no protein bands penetrated the gel.

*Preparations of Specimens for STEM Analysis.* Specimens of cross-linked proteins were prepared by absorption onto carbon films supported by titanium grids. Freshly prepared carbon films were coated onto deionized water and picked up on holey carbon films supported on titanium grids. Samples were prepared by first placing a 2.5-$\mu$L drop of tobacco mosaic virus (TMV) solution (30 $\mu$g/mL) on the grid for 30 s. All but 0.5 $\mu$L of TMV solution was removed by touching the edge of the grid with filter paper, and a 2.5-$\mu$L drop of sample buffer was added as a wash. After a second such wash, 2.5 $\mu$L of sample was injected into the buffer already on the grid and allowed to diffuse to the grid surface for 1 min. After this time, the grid was washed 4 times with deionized and distilled water or a solution of 0.02 M $NH_4^+HCO_3^-$ (~10 s/wash). The wash buffer was wicked to less than 0.1-mm thickness, and the specimen was frozen by plunging it into liquid nitrogen slush (to avoid boiling at the surface of the grid). Six such specimens were placed in an ion-pumped vacuum system, pumped to $<10^{-8}$ Torr, and warmed at 1 °C/min until the chamber pressure rose above $10^{-8}$ Torr. The temperature was then held essentially constant at approximately −100 °C for 4–8 h until all ice sublimed and the pressure fell below $10^{-8}$ Torr, whereupon 1 °C/min warming resumed to 20 °C. The freeze-dried samples were transferred under vacuum to the STEM and observed at −160 °C with a cold stage. STEM images were recorded as described in Mosesson et al. (1981).

*Particle Mass Measurements by Use of STEM.* Particle masses of the PDH complex and its component enzymes were measured at the STEM facility at Brookhaven National Laboratory. Data analysis was by use of the interactive computer-graphics display system described by Hainfeld et al. (1982). Included with each specimen were particles of tobacco mosaic virus, which served as internal molecular weight standards. The mass per unit length of this virus is accurately known; and it is easily distinguished from PDH complex and other proteins by its morphology.

## Results

*Cross-Linking of PDH Complex.* Preliminary particle mass measurements on the PDH complex by STEM clearly revealed the necessity for cross-linking the complex before proceeding with specimen preparation and STEM analysis. Images obtained on un-cross-linked specimens were highly irregular and appeared against a dense background of dissociated subunits. This finding was hardly surprising considering the large number of subunits in this complex; and it necessitated chemical cross-linking of the complex prior to preparing specimens for particle mass measurements. Chemical cross-linking, apart from physically stabilizing the complex, complicates both the preparation of specimens and the analysis of data. Cross-linking artifacts include the potential transfer of subunits from one particle complex to another, leading to microheterogeneity, as well as the enhancement of particle masses due to the masses of the cross-linking entities. Indeed, we observed a high degree of microheterogeneity in particle masses when we carried out cross-linking with 1% glutaraldehyde for 60 min, a fairly standard procedure used in the

Table I: Amount of [1,5-$^{14}$C]Glutaraldehyde Bound to PDH Complex

| | cpm/$\mu$g of protein | glutaraldehyde/protein (wt %) |
|---|---|---|
| PDH[a] | 42.73 | 2.7 |
| PDH + $E_1$[b] | 42.41 | 2.7 |

[a] PDH complex at 1.5 mg/mL was cross-linked with [1,5-$^{14}$C]-glutaraldehyde as described under Experimental Procedures. [b] The procedure was as above except that an additional 48 equiv of $E_1$ was added to the PDH complex prior to reaction with glutaraldehyde.

preparation of negatively stained specimens for conventional transmission electron microscopy.

We chose glutaraldehyde as a cross-linking agent for these studies because it could be prepared in $^{14}$C-labeled form from $^{14}CN^-$, so that the amount incorporated into our specimens could be accurately measured and the particle masses corrected for the cross-linker content. The two main artifacts that had to be controlled with glutaraldehyde were the problem of microheterogeneity resulting from the use of high concentrations and long reaction times and the problem of the chemical reversibility of this cross-linker when minimally cross-linked specimens were subjected to dilution or mounted specimens to washing procedures. We devised a cross-linking procedure that minimizes or eliminates these problems.

To stabilize the glutaraldehyde cross-links we used $NaBH_4$ to reduce the aldimine groups formed reversibly between glutaraldehyde and the lysine $\epsilon$-$NH_2$ groups of the proteins. We then established minimal cross-linking conditions by varying the glutaraldehyde concentration and exposure time prior to addition of $NaBH_4$. We assessed cross-linking effectiveness by polyacrylamide gel electrophoresis in SDS, the criterion for effectiveness being that an adequately cross-linked complex would not release any subunits upon denaturation in SDS, so no protein bands would penetrate the gel. We found by this procedure that minimal cross-linking conditions for this complex at 1.5 mg mL$^{-1}$ are 0.25% glutaraldehyde at pH 7 for 15 min at 25 °C followed by reduction with $NaBH_4$; and we used this procedure in preparing proteins for analysis in STEM.

Table I shows the amount of [1,5-$^{14}$C]glutaraldehyde bound to the PDH complex under our cross-linking conditions. The data show that about 2.7% of the total mass in these samples is contributed by [1,5-$^{14}$C]glutaraldehyde. Inasmuch as up to 10% of the total mass could be [1,5-$^{14}$C]glutaraldehyde if all available lysines were reductively alkylated, 2.7% seems to be a reasonable value for a minimally effective degree of cross-linking.

Our estimate of a maximum of 10% is based on the lysine content of the complex (Eley et al., 1972) and the assumption that maximum reductive alkylation would correspond to two molecules of glutaraldehyde per lysine, as well as the assumption of no irreversible incorporation of polymeric glutaraldehyde into the protein. Our finding of 2.7% corresponds to the cross-linking of about half the lysines, assuming one glutaraldehyde to two lysine residues in a cross-link. With half of the lysines cross-linked by five carbon spacers, one can expect a few interchain cross-links in each subunit, given that the lysines are in general surface residues. For example, of the 50 lysine residues in $E_1$, approximately 25 are involved in cross-links; and if only two to five are interchain, the cross-linking should be effective. Since $E_1$, $E_2$, and $E_3$ have similar lysine contents (Eley et al., 1972; Bleile et al., 1979; Williams et al., 1967), a similar incorporation of cross-links occurs in the isolated proteins, and we have applied this correction to all of the STEM measurements reported in this paper.

Table II: Particle Mass of PDH Complex

| sample no. | mean $M_r$ ± SD[a] |
|---|---|
| 1[b] | (5.00 ± 0.36) × $10^6$ |
| 2[b] | (5.71 ± 0.30) × $10^6$ |
| 3[c] | (5.20 ± 0.41) × $10^6$ |
| 4[c] | (5.20 ± 0.52) × $10^6$ |

[a] Mean molecular weight of 120–180 particles. Histograms are shown in the supplementary material (see paragraph at end of paper regarding supplementary material). [b] Samples of two isolates purified in the absence of PMSF and EDTA. [c] Samples of two isolates purified in the presence of PMSF and EDTA.

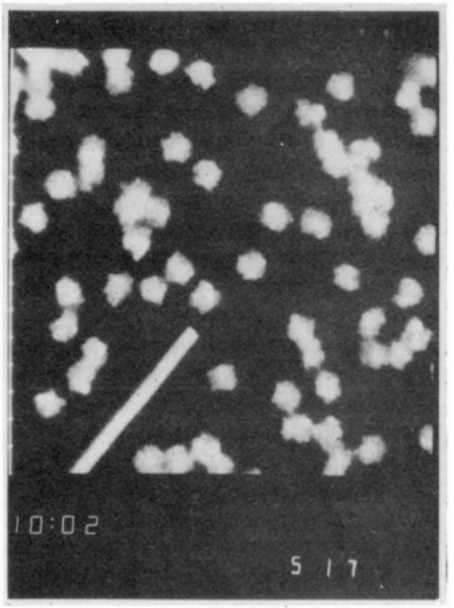


FIGURE 1: STEM micrograph of the *E. coli* PDH complex. The rod structure is tobacco mosaic virus added to the specimen as an internal mass standard. The observed area corresponds to 5120 Å × 5120 Å.

*Particle Weight of PDH Complex.* Particle weights measured by STEM on four different preparations of the PDH complex are set forth in Table II. Samples 1 and 2 were purified without added protease inhibitors, while samples 3 and 4 were purified in the presence of 1 mM EDTA and 5 mM PMSF. The overall particle weights do not vary with the presence or absence of protease inhibitors during purification of the complex; however, as shown later, the molecular weight of dihydrolipoyl transacetylase is very much dependent upon the presence of protease inhibitors. The mean molecular weight value (±SD) for these four preparations of the complex is (5.28 ± 0.40) × $10^6$.

A typical STEM micrograph of the PDH complex is shown in Figure 1. In the particle mass analysis the masses of individual particles are measured by a procedure that involves the subtraction of the background signal. Note the percentage of individual particles in the field and the low and constant background. The SD in particle mass measurements arises from any microheterogeneity that may exist among the particles and the presence of extraneous scattering entities in the background, such as adventitious contaminants.

*Molecular Weight of Component Enzymes.* The molecular weight values measured by STEM of $E_1$, $E_2$, and $E_3$ resolved and purified from the PDH complex are presented in Table III. The molecular weight values of $E_1$ and $E_3$, isolated as dimers, are in agreement with the published values measured by other methods (Eley et al., 1972; Vogel, 1977; Koike et al., 1963; Angelides et al., 1979). The larger of the two values for $E_2$ was obtained on a sample that had been resolved from

Table III: Molecular Weight of Component Enzymes

| enzyme | mean $M_r \pm$ SD[a] |
|---|---|
| $E_1$ (dimer) | $(2.06 \pm 0.26) \times 10^5$ |
| $E_3$ (dimer) | $(1.15 \pm 0.17) \times 10^5$ |
| $E_2$ (24 subunits)[b] | $(2.20 \pm 0.17) \times 10^6$ |
| $E_2$ (24 subunits)[c] | $(1.60 \pm 0.21) \times 10^6$ |

[a] Mean molecular weight of 69–335 molecules or particles. Histograms are shown in the supplementary material. [b] Purified in the presence of PMSF and EDTA. [c] No protease inhibitors present during purification.

the PDH complex in the presence of 1 mM EDTA and 5 mM PMSF. The smaller value was obtained on $E_2$ that had been resolved in the absence of added protease inhibitors. Other samples resolved without protease inhibitors had molecular weight values as small as $1 \times 10^6$. $E_2$ is known to be labile to proteolysis (Bleile et al., 1979; Hale & Perham, 1979) and may be especially labile during the resolution process, which involves the use of urea.

Since $E_2$ is known to consist of 24 identical subunits, the subunit molecular weight would be $(9.17 \pm 0.70) \times 10^4$ on the basis of the larger STEM measurement or $(6.67 \pm 0.87) \times 10^4$ on the basis of the smaller measurement in Table III. Values comparable to both of these have been measured by other techniques. The position of $E_2$ in our SDS–polyacrylamide gels is consistent with a molecular weight between 80 000 and 90 000 when compared with the positions of $E_1$ and $E_3$.

We also measured the subunit molecular weight of $E_2$ by SDS–polyacrylamide gel electrophoresis in gels prepared from 6% acrylamide and using markers ranging from 45 000–116 000 in molecular weight. An excellent straight line was obtained in the plot of log $M_r$ vs. migration distance (correlation factor −0.986) with $E_1$ and $E_3$ as internal markers from the PDH complex well correlated with the line. The migration distances of $E_2$ corresponded to a $M_r$ of 86 000 in two preparations of the PDH complex that had been purified in the presence of 1 mM EDTA and 5 mM PMSF. This value is the same, within error, as the subunit molecular weight computed from the larger STEM measurement.

*Binding of Additional Subunits by PDH Complex.* Inasmuch as the PDH complex is reported to vary in subunit composition, especially in its $E_1$ content, from preparation to preparation (Bates et al., 1977), we have carried out experiments designed to determine how the enzymatic activity and particle mass of the complex are altered by added $E_1$ and $E_3$ subunits. In the first experiments, we incubated the complex with added $E_1$ or $E_3$ subunits, in amounts corresponding to 48 additional subunits per particle of complex and then carried out the cross-linking with glutaraldehyde by the usual procedure. Any additional subunits bound by the complex should have been trapped by the cross-linking procedure, and their presence would be revealed by particle mass measurements.

The STEM data given in Table IV show no evidence of the capacity of the complex to bind additional $E_3$ subunits, since the particle masses are indistinguishable from those in Table II for the PDH complex. It is quite clear, however, that the complex binds additional $E_1$ subunits, since the mass analysis of relevant samples reproducibly reveals two classes of particles, one corresponding to the native complex and a larger species with a particle mass of $6.3 \times 10^6$. The histogram for one of these experiments is illustrated in Figure 2 and clearly shows two classes of particles.

It is doubtful whether the larger species in Table IV and Figure 2 represent functionally meaningful particles, since the additional 10–12 $E_1$ subunits are weakly bound. This is shown

Table IV: Particle Weights of PDH Complex Cross-Linked in the Presence of Added Component Enzymes

| sample | mean $M_r \pm$ SD[a] |
|---|---|
| PDH complex (a) + $E_3$ | $(5.30 \pm 0.37) \times 10^6$ |
| PDH complex (b) + $E_3$ | $(5.40 \pm 0.34) \times 10^6$ |
| PDH complex (a) + $E_1$[b] | $(5.3 \pm 0.27) \times 10^6$ |
| | $(6.2 \pm 0.23) \times 10^6$ |
| PDH complex (b) + $E_1$[b] | $(5.4 \pm 0.23) \times 10^6$ |
| | $(6.3 \pm 0.24) \times 10^6$ |

[a] Particle mass measurements were made on 85–178 particles. The histograms are shown in the supplementary material and Figure 2. Two different samples of PDH complex (a) and (b), both purified in the presence of 1 mM EDTA and 5 mM PMSF, were used in these experiments. [b] Two distinct particle masses were detected that differed in molecular weight by about $10^6$. See Figure 2 for a typical histogram.

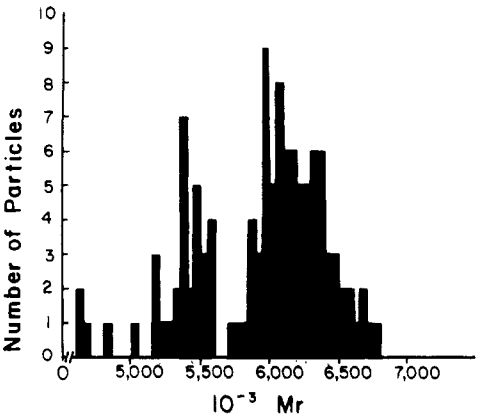


FIGURE 2: Particle mass histogram for PDH complex cross-linked with added $E_1$. Cross-linking and particle mass measurements are described under Experimental Procedures.

by the particle mass data themselves, since about 30% of the particles have the mass characteristic of the complex in Table II. Under the incubation conditions, 1–2 mg of protein $mL^{-1}$ for 60 min at pH 7.0 and 4 °C, all sites binding $E_1$ subunits with the reported dissociation constant $K_d$ of $10^{-11}$ M (Hale et al., 1979) would have been filled, and the subunits should have been trapped by the cross-linking procedure. Second, any functionally meaningful subunits bound with a $K_d$ of $10^{-11}$ M should remain bound when the complex is reisolated by ultracentrifugation prior to cross-linking; however, when this was done, the particle masses were in the low range, somewhat lower than those in Table II.

Another means by which functionally bound subunits may be detected is by activity measurements. Since the rate-limiting process of the overall reaction, eq 6, is the $E_1$ function in equations 1–2, binding of additional $E_1$ subunits at vacant $E_1$ sites should enhance the activity of the complex. As shown in Figure 3, a modest enhancement of activity (10%) can be detected when large amounts of $E_1$ are added to the complex. Shown as the inset to Figure 3 is a double-reciprocal plot of the enhanced rate, $\Delta v$, vs. $[E_1]$. The $K_m$ value extracted from this plot is 2.7 $\mu$M, which, if the enhanced activity is proportional to additional bound $E_1$ and the binding sites are equivalent, should be $K_d$ for the additional $E_1$ sites.

A second estimate for $K_d$ can be obtained from the data in Figure 2. From the ratio of the two species and assuming they were effectively trapped by the cross-linker, it is possible to calculate the $K_d$ as $4.5 \times 10^{-5}$ M.

Both of the above values for $K_d$ are unrealistically large for a multienzyme complex that could be isolated and characterized as an intact entity. The additional binding of $E_1$ must involve secondary sites on the PDH complex that are not

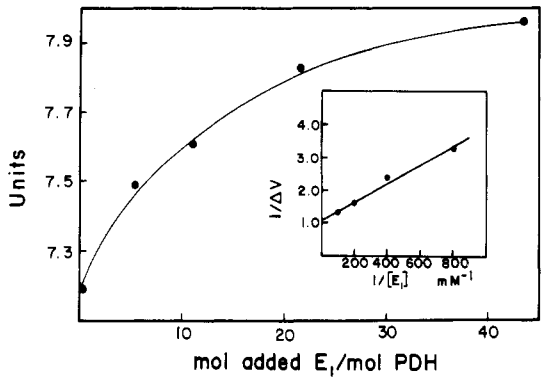


FIGURE 3: Activation of PDH complex by added $E_1$. PDH complex at 1.2 mg $mL^{-1}$ was incubated with various amounts of $E_1$ for 10 min at 25 °C in 50 mM $Na^+$-bicine buffer (pH 8.0). After 10 min, the sample was assayed for overall PDH complex activity as described under Experimental Procedures. The amount of $E_1$ added was 1.24, 2.46, 4.88, and 9.69 $\mu$M final concentration. A $M_r$ of 5.3 million was used for the PDH complex in calculating the moles of $E_1$/moles of PDH ratio. The inset is the double-reciprocal of rate enhancement vs. $[E_1]$.

normally involved in the action of this enzyme. The fact that these $E_1$ interactions slightly enhance overall activity does not necessarily mean that the additional $E_1$ is binding at sites equivalent or even comparable to those occupied by $E_1$ in the purified complex. The enzyme would certainly catalyze reaction 1 regardless of the location of the binding sites on the PDH complex. Enhancement of overall activity suggests, but does not prove, that added $E_1$ can couple with $E_2$ to catalyze reaction 2. This too would not necessarily involve binding of $E_1$ at normal binding sites, since the structural mobilities of the lipoyl groups and the lipoyl binding domains in $E_2$ could ensure effective coupling even when $E_1$ is weakly bound at secondary sites (Collins & Reed, 1977; Bates et al., 1977; Danson et al., 1978).

## Discussion

The particle mass of the *E. coli* PDH complex measured in STEM is reproducibly $(5.28 \pm 0.40) \times 10^6$, and those of the component enzymes are $(1.03 \pm 0.13) \times 10^5$ for $E_1$, $(5.75 \pm 0.09) \times 10^4$ for $E_3$, and either $(9.17 \pm 0.70) \times 10^4$ (STEM) or $8.9 \times 10^4$ (SDS–PAGE) for $E_2$. These values are compatible with the chain ratios 24:24:12 proposed by Eley et al. (1972) and Angelides et al. (1979). The calculated particle mass of the complex on the basis of these ratios and the molecular weight of the components would be $5.36 \times 10^6$ or $5.23 \times 10^6$, depending upon which $E_2$ molecular weight is used. These values are in excellent agreement with the measured particle mass.

Despite the agreement, these measurements do not themselves establish the subunit composition or even that the complex has a definite composition. This is because of the number of subunits in the complex and the fact that the SD in the particles mass is about $\pm 0.4 \times 10^6$. A large number of compositional models would fit the range $(5.28 \pm 0.40) \times 10^6$ for the particle mass, even with the constraint of 24 $E_2$ subunits. An additional constraint is the FAD content, which is 2.4 nmol $mg^{-1}$ and reflects the $E_3$ content (Speckhard & Frey, 1975). This corresponds to 12 mol of FAD per particle of mass $5.3 \times 10^6$. Taking error into account, the average particle should therefore contain $12 \pm 1$ subunits of $E_3$. This severely limits the $E_1$ content to about $24 \pm 2$ per particle. It is reasonable to conclude, therefore, that the average composition is 24:24:12 for $E_1$:$E_2$:$E_3$. Available data cannot exclude a modest degree of microheterogeneity; however, nothing in the data suggests the existence of heterogeneity.

In view of the claim that the fully consisted complex contains 48 $E_1$ and 24 $E_3$ subunits and has a $M_r$ of $8 \times 10^6$ (Bates et al., 1977), we have considered the possibility that the complex we purify is deficient in $E_1$ and $E_3$ subunits. To investigate this we combined purified $E_1$ or $E_3$ subunits with the PDH complex at pH 7 and subjected the mixtures to chemical cross-linking with glutaraldehyde followed by $NaBH_4$. Particle mass measurements revealed no additional $E_3$ binding and only weak $E_1$ binding to secondary sites. These were forcing conditions involving high protein concentrations, prolonged incubation, and irreversible chemical cross-linking. If particles with 48 $E_1$ subunits or 24 $E_3$ subunits can form spontaneously, we should have detected them. All of our results imply that the PDH complex we isolate is fully constituted.

The molecular weight values for $E_1$ and $E_3$ are in agreement with those measured by other techniques, but our values for the PDH complex and the 24 subunit $E_2$ core are higher than those obtained by others using the sedimentation velocity or light scattering techniques. We believe that our measurements are accurate and reflect the true masses of the particles we isolate. The discrepancies can be rationalized in several ways, none of which alters our conclusion that the complex we isolate is intact and contains approximately 24 subunits of $E_1$, 24 of $E_2$, and 12 of $E_3$.

Our measurements for the complex and the core could be high because the cubic symmetry of the core creates the possibility of a vacant space at the center, which could accumulate matter of some sort during the assembly process. This matter would be detected in the STEM measurements and give falsely high particle masses for the complex and core protein without affecting the mass measurements carried out on purified $E_1$ and $E_3$. One might expect this also to be detected in the sedimentation velocity measurements, which give a $M_r$ of 4.6 million for the complex (Eley et al., 1972). However, it would not necessarily appear in sedimentation velocity measurements, since the sedimentation coefficient of this complex is reported to be essentially unaffected by the removal of the large tryptic fragment A from the 24 subunit $E_2$ core (Bleile et al., 1979). This degradation results in a mass loss of between 0.77 and 1.15 million with no effect on the sedimentation coefficient, and so raises additional questions about the reliability of the sedimentation coefficient as a measure of molecular weight in this case. If extraneous matter is included within the core, then our calculated molecular weight for $E_2$ subunits (91 000 ± 7000) is high by an unknown amount. Our measurement of 86 000 for $E_2$ subunits by SDS–PAGE is well within the standard deviation of the STEM value.

Bleile et al. (1979) have suggested that the SDS–PAGE technique gives anomalously high values for $E_2$. They measured the mass of tryptic fragment A from $E_2$ as 46 000–48 000 by SDS–PAGE and 31 600 by sedimentation equilibrium analysis and concluded that the lower value is correct. Fragment D, the other major tryptic product, also give a higher mass by SDS–PAGE (36 000–39 000) than by sedimentation equilibrium (28 700–30 500). The lower $M_r$ values were consistent with molecular masses of the $E_2$ core (1.55 millon) and $E_2$ subunits (60 000) measured by sedimentation equilibrium (Bleile et al., 1979). Therefore, their mass measurements by equilibrium sedimentation were internally consistent but at variance with their SDS–PAGE data.

Falsely high values of molecular weight for $E_2$ could also arise from incomplete resolution of $E_1$ or $E_3$ from the core. We believe that this cannot account for our results, however, since our SDS gels of $E_2$ stained with Coomassie brilliant blue

Table V: Molecular Weight Reported for $E_2$

| $M_r$ | method of measurement | ref |
|---|---|---|
| 60 500 | sedimentation equilibrium | Bleile et al., 1979 |
| 65 959 | gene sequencing | Stephens et al., 1983b |
| 70 000 | sedimentation equilibrium | Eley et al., 1972 |
| 76 000 | SDS–PAGE | Angelides et al., 1979 |
| 80 000 | SDS–PAGE | Vogel et al., 1971 |
| 83 000 | SDS–PAGE | Vogel et al., 1977 |
| 86 000 | SDS–PAGE | Vogel et al., 1977 |
| 86 000 | SDS–PAGE | this study |
| 89 000 | SDS–PAGE | Gebhardt et al., 1978 |
| 91 000 | STEM | this study |

dye do not reveal the presence of significant amounts of other components. This is a sensitive test because $E_2$ stains more lightly than either of the other components, and using the staining coefficients of Angelides et al. (1979), we estimate that our preparations of $E_2$ core contain less than one subunit of $E_1$. No flavin from $E_3$ can be detected. Furthermore, in other experiments using $E_2$ core protein reconstituted with an average of one $E_1$ or $E_3$ dimer per core, the expected heterogeneity among the particles can easily be detected in the STEM measurements, which show particle masses ranging upward from 2 million for the core and corresponding to species with one, two or three additional dimers of $E_1$ or $E_3$. The $E_2$ core itself is quite homogeneous.

The gene sequence specifying the primary structure of $E_2$ translates into a protein with a molecular mass of 66 000 (Stephens et al., 1983b) in essential agreement with the sedimentation equilibrium value of Bleile et al. (1979) and significantly smaller than that we find by STEM and SDS–PAGE. Our results can be correlated with this size $E_2$ subunit only by assuming SDS gel electrophoresis gives a value too high by 20 000 and that each intact core contains extraneous matter corresponding to a mass of 500 000. The STEM and gene sequence values could both be correct if the gene that was isolated for sequencing from a transducing phage is a truncated version of the gene specifying this enzyme in *E. coli* Crookes strain. This protein is a product of gene duplication that contains at least three potential lipoyl binding domains, so a truncated protein would retain activity.

The molecular weight of $E_2$ remains controversial. Recent values listed in Table V range from 60 000 to 90 000. It is worth noting that the $E_2$ core is labile to proteolysis, especially during resolution from $E_3$, which involves the use of 4 or 5 M urea. In our hands, the core resolved in the absence of proteolytic inhibitors has a mass of 1.6 million (Table III), corresponding to a subunit mass of 67 000.

A particular advantage of the STEM measurements is that, since individual particle masses are evaluated, the histograms can reveal microheterogeneity. Perham and co-workers report marked variation with respect to subunit composition in the PDH complex from *E. coli* K12 (Bates et al., 1977; Hale & Perham, 1979; Packman et al., 1984). In the technique they use for assessing subunit ratios, lysine residues are exhaustively radioamidinated and the radiolabeled subunit bands counted after excision from SDS–polyacrylamide gels. This is a powerful technique when the lysine contents of the subunits are known, as they are for the PDH complex. However, the validity of this technique is undermined if subunits are proteolytically nicked. The reported ratios by this technique are 1.2–1.5 for $E_1$:$E_2$ and 0.6–0.8 for $E_3$:$E_2$ in several preparations of the complex. These ratios would correspond to masses ranging from 6.2 million to 6.6 million, assuming a mass of 2.2 million for $E_2$ and chain ratios of 1.2:1:0.8 to 1.5:1:0.6 for $E_1$:$E_2$:$E_3$. The range is 5.5 million to 5.9 million if the $E_2$ mass is 1.5 million. Both of these mass ranges are higher than what we observe, so we rule out the chain ratios of Perham and co-workers for the complex we isolate. It is unlikely that the K12 and Crookes strain complexes differ significantly since they have the same flavin contents and give identical band patterns on SDS–PAGE (Speckhard & Frey, 1975). And we observe no significant variation in mass from one preparation to another.

The maximum microheterogeneity permitted by our data can be estimated. If it is assumed that all of the SD is attributable to microheterogeneity and that heterogeneity results from competition between $E_1$ and $E_3$ dimers for binding sites about the 24-subunit $E_2$ core, the subunit compositions would range between 18:24:18 and 30:24:6 for $E_1$:$E_2$:$E_3$. The true composition must be much closer to 24:24:12, however, since the SD cannot be attributed solely to microheterogeneity. The SD is determined largely by variations in the background signals, which must be subtracted as an *average* background from the signals obtained from each particle. Variations in the background appear as SD in the particle masses. The contribution of errors in measurement to the SD is much smaller (1%–2%). It is significant that the percent SD in the particle mass measurements on the PDH complex are similar to those observed for the component enzymes, where there is no question of heterogeneity. Therefore, the SD must be attributed largely to variations in background and not to microheterogeneity. However, minor variations in composition among particles could be masked by the background.

A major advantage of using STEM for molecular weight measurement is that the actual distribution of the masses for many individual particles is obtained rather than a single average value. STEM has been applied to a wide variety of proteins and macromolecules for measurements of molecular weight with a good correlation to the known values (Wall & Hainfeld, 1984).

## Supplementary Material Available

Histograms showing particle mass measurement of the PDH complex and of the PDH complex cross-linked in the presence of $E_3$ and histograms showing molecular weight measurements of $E_1$, $E_2$, and $E_3$ (5 pages). Ordering information is given on any current masthead page.

**Registry No.** PDH complex, 9014-20-4; $E_1$, 9001-04-1; $E_2$, 9032-29-5; $E_3$, 9001-18-7.

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# Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Iso-Branched Fatty Acids. 1. Differential Scanning Calorimetric Studies†

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ABSTRACT: The thermotropic phase behavior of aqueous dispersions of phosphatidylcholines containing one of a series of methyl iso-branched fatty acyl chains was studied by differential scanning calorimetry. These compounds exhibit a complex phase behavior on heating which includes two endothermic events, a gel/gel transition, involving a molecular packing rearrangement between two gel-state forms, and a gel/liquid-crystalline phase transition, involving the melting of the hydrocarbon chains. The gel to liquid-crystalline transition is a relatively fast, highly cooperative process which exhibits a lower transition temperature and enthalpy than do the chain-melting transitions of saturated straight-chain phosphatidylcholines of similar acyl chain length. In addition, the gel to liquid-crystalline phase transition temperature is relatively insensitive to the composition of the aqueous phase. In contrast, the gel/gel transition is a slow process of lower cooperativity than the gel/liquid-crystalline phase transition and is sensitive to the composition of the bulk aqueous phase. The gel/gel transitions of the methyl iso-branched phosphatidylcholines have very different thermodynamic properties and depend in a different way on hydrocarbon chain length than do either the "subtransitions" or the "pretransitions" observed with linear saturated phosphatidylcholines. The gel/gel and gel/liquid-crystalline transitions are apparently concomitant for the shorter chain iso-branched phosphatidylcholines but diverge on the temperature scale with increasing chain length, with a pronounced odd/even alternation of the characteristic temperatures of the gel/gel transition. Our observations can be rationalized by assuming that the stable conformations characteristic of these lipids at temperatures below the gel/gel transition temperature and their conformation in the liquid-crystalline state are such that direct interconversions between the two states are improbable and that such interconversions must proceed via an intermediate state or states.

Fatty acids containing a single methyl branch near the methyl terminus of the hydrocarbon chain are abundant and widespread constituents of the membrane lipids of eubacteria. In addition to their occurrence in all species of the genus *Bacillus* which have been examined thus far, methyl iso- and anteiso-branched fatty acids are also found in eight other genera of Gram-positive and in four genera of Gram-negative eubacteria. In those bacterial species in which they occur, methyl iso- and anteiso-branched fatty acids are normally the predominant components of the membrane lipids, accounting for between 65% and 95% of the total esterified fatty acid; even-chain saturated fatty acids, especially palmitic acid,

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