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Subunit Binding in the Pyruvate Dehydrogenase Complex from Bovine Kidney and Heart†

Tyzz-Liang Wu‡ and Lester J. Reed*

ABSTRACT: Binding of pyruvate dehydrogenase (E_1) and dihydrolipoamide dehydrogenase (E_3) to the isolated dihydrolipoamide acetyltransferase (E_2) core of the pyruvate dehydrogenase complex from bovine heart and kidney was investigated with equilibrium, competitive binding, and kinetic methods. E_2, which consists of 60 subunits arranged with icosahedral 532 symmetry, apparently possesses six equivalent, noninteracting binding sites for E_3 dimers. It is proposed that each E_3 dimer extends across 2 of the 12 faces of the E_2 pentagonal dodecahedron. The equilibrium constant (K_d) for dissociation of E_3 from E_2 is about 3 nM, and the dissociation rate constant is about 0.057 min^{-1}. For E_1, K_d is about 13 nM, and the dissociation rate constant is about 0.043 min^{-1}. Extensive phosphorylation of E_1 (about three phosphoryl groups per E_1 tetramer) increases K_d to about 40 nM.

The mammalian pyruvate dehydrogenase complex is composed of multiple copies of three major components: pyruvate dehydrogenase (E_1),[1] dihydrolipoamide acetyltransferase (E_2), and dihydrolipoamide dehydrogenase (E_3). The E_2 component forms a structural core, composed of 60 subunits arranged with icosahedral 532 symmetry in a pentagonal dodecahedron like particle, to which E_1 and E_3 are bound by noncovalent bonds [for a review, see Reed & Pettit (1981)]. In solution, uncomplexed E_1 is a tetramer ($\alpha_2\beta_2$) and E_3 is a homodimer. The bovine kidney pyruvate dehydrogenase complex contains about 20 E_1 tetramers and about 6 E_3 dimers, whereas the heart complex contains about 30 E_1 tetramers and about 6 E_3 dimers. The kidney complex can bind about 10 additional E_1 tetramers, but neither complex can bind additional E_3 dimers (Barrera et al., 1972; L. Hamilton, P. Munk, and L. J. Reed, unpublished data). It is difficult to reconcile the presence of only six E_3 dimers (or 12 E_3 polypeptide chains) with the apparent icosahedral 532 symmetry of the E_2 core.

It is interesting to note in this connection that in the native pyruvate dehydrogenase complex from *Escherichia coli*, 24 E_1 chains and 12 E_3 chains are attached to the E_2 core, which is composed of 24 subunits arranged with 432 symmetry in a cubelike particle (Eley et al., 1972). E_3 is present in the complex as dimers (Reed et al., 1975; Coggings et al., 1976). One E_3 dimer is thought to be located on each of the six faces of the E_2 core (Oliver & Reed, 1982). Binding studies (Reed et al., 1975) indicated that the *E. coli* E_2 core can bind up to 24 E_1 dimers in the absence of E_3 and up to 24 E_3 dimers in the absence of E_1. When both E_1 and E_3 are present, the binding stoichiometry approaches 12 E_1 dimers and 6 E_3 dimers. This stoichiometry is the same as that of the native complex and produces maximum activity. Thus, although there appears to be a binding site (domain) on each of the 24 E_2 subunits for an E_1 dimer and a separate binding site on each E_2 subunit for an E_3 dimer, steric hindrance between the relatively bulky E_1 and E_3 dimers apparently prevents the E_2 core from binding 24 molecules of each ligand.

To gain further insight into the molecular basis of the unequal ratio of E_3 and E_2 subunits in the mammalian pyruvate dehydrogenase complex, we studied binding of E_3 and E_1

† From the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712. *Received July 26, 1983.* Abstracted from the Ph.D. dissertation of T.-L. Wu, The University of Texas at Austin (Wu, 1982). Suppported in part by Grant GM06590 from the National Institutes of Health, U.S. Public Health Service.

‡ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations: E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; M_r, molecular weight; EDTA, ethylenediaminetetraacetate.

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to the E_2 core by using equilibrium, competitive binding, and kinetic methods. The results obtained are reported here.

Experimental Procedures

Materials. The pyruvate dehydrogenase complex was isolated from bovine heart and kidney mitochondria and was separated into its component enzymes as described previously (Linn et al., 1972; Pettit & Reed, 1982; Pettit et al., 1983). The E_2 core preparations used in these studies contained a small amount of tightly bound kinase, approximately two molecules per molecule (60 subunits) of E_2. [^{3}H]- and [^{14}C]formaldehyde (40 and 42 mCi/mmol, respectively) were purchased from New England Nuclear, [γ-^{32}P]ATP (20 Ci/mmol) was from Amersham/Searle, hexokinase was from Boehringer Mannheim, sodium cyanoborohydride was from Aldrich, and Sepharose 6B and Sephadex G-25 were from Pharmacia. All other reagents and materials were of the purest grade available commercially.

Radiolabeling of Proteins. Reductive methylation of E_2 and E_3 in the presence of [^{3}H]- or [^{14}C]formaldehyde and sodium cyanoborohydride was carried out by a modification of the procedure described by Dottavio-Martin & Ravel (1978). To a solution of 4 mg of protein in 1.0 mL of buffer A (20 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mM $MgCl_2$) was added 1.5 μmol of radioactive formaldehyde (60 000 cpm/nmol) followed by 0.4 mg of sodium cyanoborohydride. The mixture was incubated at 25 °C for 40 min and then passed through a column (1 × 15 cm) of Sephadex G-25 equilibrated with buffer A. The protein was dialyzed overnight against the same buffer. The enzymatic activities of radiolabeled E_2 and E_3 were about 95% of those of untreated samples, as determined by reconstituting the pyruvate dehydrogenase complex (Bleile et al., 1981) and measuring overall activity of the reconstituted complex (Pettit & Reed, 1982). As many as 10 radiolabeled methyl groups were incorporated per E_2 subunit of M_r 52 000 and 40–50 methyl groups per E_3 subunit of M_r 55 000.

Several attempts to label E_1 by reductive methylation resulted in inactivation of the enzyme. Therefore, E_1 was phosphorylated to different degrees in the presence of [γ-^{32}P]ATP and a small amount of kidney E_2–kinase subcomplex (1% by weight of E_1) (Yeaman et al., 1978). Heavily phosphorylated E_1 was prepared by adding 0.2 mL of 5 mM [γ-^{32}P]ATP (20 000–100 000 cpm/nmol) and 60 μg of E_2–kinase subcomplex to 6 mg of E_1 in 0.8 mL of buffer A containing 0.5 mM dithiothreitol. The reaction was allowed to proceed at 25 °C for 2 h and then terminated by adding 0.35 mL of 1 M glucose and 30 μg of hexokinase to scavenge the ATP. Excess reagents were removed by gel filtration over Sephadex G-25. Lightly phosphorylated E_1 was prepared similarly, except that a lower concentration of ATP (0.3 mM in the final solution) was used and the incubation period was reduced to 30 min.

Protein was determined by the method of Lowry et al. (1951) with crystalline serum albumin as the standard. The values obtained were multipled by 0.99 for E_1, 1.09 for E_2, and 0.79 for E_3. These correction factors were determined by amino acid analysis and by refractometry in the analytical ultracentrifuge (Babul & Stellwagen, 1969).

Equilibrium Binding by Gel Filtration. The equilibrium gel filtration procedure of Hummel & Dreyer (1962) was used. A Sepharose 6B column (0.9 × 30 cm) was equilibrated at 25 °C with different concentrations of radiolabeled E_3 or E_1. A suitable amount of the E_2 core was carefully applied to the column, which was then eluted with the same E_3 or E_1 solution used to equilibrate the column. Fractions of seven drops (0.46 mL) were collected directly in scintillation vials, 10 mL of Amersham/Searle ACS scintillation fluid was added, and radioactivity was determined with a Beckman LS-230 scintillation counter.

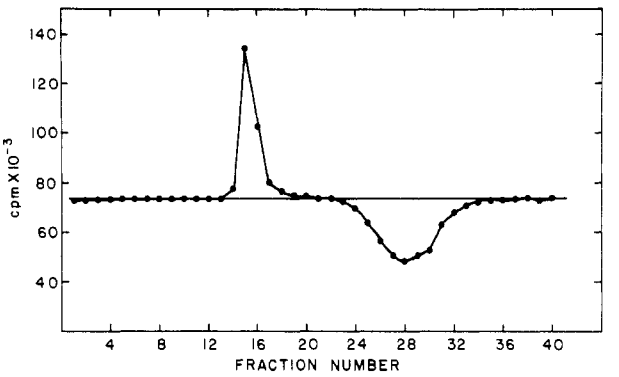

FIGURE 1: Measurement of binding of E_3 to the E_2 core by the Hummel and Dreyer technique. A Sepharose 6B column (0.9 × 30 cm) was preequilibrated and eluted at 25 °C with 103 nM ^{14}C-labeled E_3 in buffer A (20 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mM $MgCl_2$). A solution of 129 pmol of the E_2 core in 50 μL of the same buffer was applied to the column. Fractions (0.46 mL) of the eluate were collected directly in scintillation vials and counted.

Measurement of Kinetic Dissociation Rates. The procedure was a modification of that described by Henderson & Wang (1972). A Sepharose 6B column (0.9 × 15 cm or 0.9 × 30 cm) was equilibrated at 25 °C with 300 nM unlabeled E_3 in buffer A containing 0.5 mM dithiothreitol. A mixture of ^{14}C-labeled E_3 and ^{3}H-labeled E_2 (molar ratio 20:1) was incubated at 25 °C for 10 min and then applied to the column. Elution with the same unlabeled E_3 solution was resumed immediately. Fractions of three drops (0.2 mL) from the 15-cm column or seven drops (0.46 mL) from the 30-cm column were collected in scintillation vials and counted. The elution times of the peak fractions were also recorded. The experiment was repeated at different flow rates by adjusting the hydrostatic pressure. Dissociation rates for E_1 from E_1E_2 subcomplex were determined by the same procedure with ^{32}P-labeled E_1 and ^{3}H-labeled E_2 (molar ratio 100:1).

Results

Equilibrium Binding. Binding of E_3 to the isolated E_2 core as measured by equilibrium gel exclusion chromatography is illustrated in Figure 1. Establishment of a well-defined plateau between the peak and the trough in this elution profile indicates that a true binding equilibrium between the two proteins was established. The area of the trough was used to calculate the amount of E_3 bound. Correction was made for dilution by the unlabeled sample (Fairclough & Fruton, 1966). A Scatchard plot (Scatchard, 1949) shows (Figure 2) that, in the absence of E_1, the E_2 core, which consists of 60 apparently identical subunits, has six apparently identical and noninteracting binding sites for E_3 dimers, with a dissociation constant (K_d) of about 3 nM. When a saturating concentration of E_1 (160 nM) was present in both the preequilibrating solution and the E_2 sample, neither the binding constant nor the binding stoichiometry for E_3 was affected (Figure 2).

Binding sites on the E_2 core for E_1 tetramers also appeared to be identical and noninteracting (Figure 3), with K_d values of about 13 nM for lightly phosphorylated E_1 (average of 1.1–1.4 phosphoryl groups per molecule) and about 40 nM for heavily phosphorylated E_1 (average of 2.5–2.9 phosphoryl groups per molecule). The binding stoichiometry for E_1 proved difficult to determine. With different preparations of the

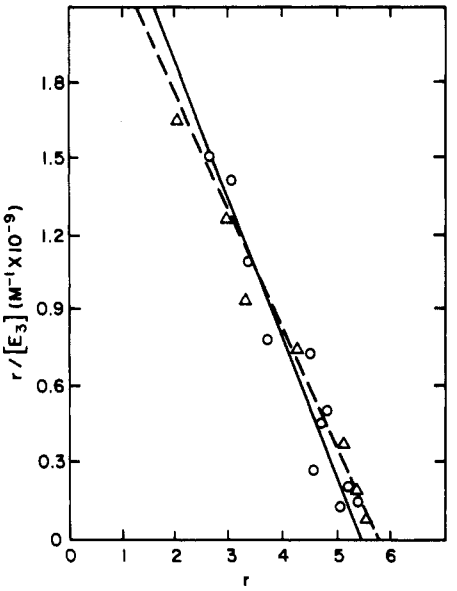

FIGURE 2: Plots of $r/[E_3]$ vs. r, where r is the number of moles of E_3 bound per mole of E_2 and $[E_3]$ is the concentration of free E_3. The data were obtained in the absence (○) and presence (△) of E_1 by the Hummel and Dreyer technique. The amounts of the E_2 core used varied from 6.5 to 129 pmol, and the concentrations of ^{14}C-labeled E_3 ranged from 1.2 to 110 nM. Where indicated (△) 160 nM E_1 was present in the preequilibrating solution and was also incubated with E_2 for 10 min before the sample was applied to the column.

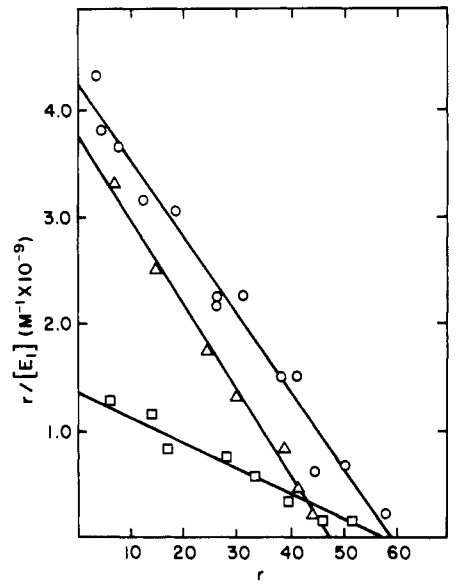

FIGURE 3: Plots of $r/[E_1]$ vs. r, where r is the number of moles of E_1 bound per mole of E_2 and $[E_1]$ is the concentration of free E_1. Binding of E_1 was measured by the Hummel and Dreyer technique. The amounts of the E_2 core used varied from 1.3 to 387 pmol, and the concentrations of ligands were (○) 1.1–240 nM lightly phosphorylated E_1 in the absence of E_3, (□) 5–300 nM heavily phosphorylated E_1 in the absence of E_3, and (△) 2–200 nM lightly phosphorylated E_1 in the presence of 70 nM E_3.

isolated E_2 core, whether from heart or kidney, the values ranged from 35 to 55 molecules of E_1 bound per molecule of the E_2 core. Somewhat fewer E_1 molecules were bound in the presence than in the absence of E_3 (Figure 3).

Competitive Binding of Labeled and Unlabeled Enzymes. To confirm that chemical modification of E_1 and E_3 by attachment of the radioactive probes did not significantly affect protein–protein interactions, competitive binding experiments were performed. A mixture of radiolabeled and unlabeled E_3 or E_1 was used to preequilibrate and to elute the Sepharose 6B column. The presence of unlabeled E_3 caused proportional

Table I: Competition between Radiolabeled and Unlabeled E_3 Binding to E_2[a]

labeled E_3		unlabeled and labeled E_3			
[B] (nM)	r_B[b]	[A] (nM)	[B] (nM)	r_B[b]	K_A (nM)[c]
9.92	4.13 ± 0.08	5.85	4.85	1.92 ± 0.04	3.83
3.71	2.69 ± 0.11	1.95	2.06	1.40 ± 0.06	4.13

[a] In the experiment performed with labeled E_3 only, a column packed with Sepharose 6B was preequilibrated and eluted with ^{14}C-labeled E_3 of the specified concentration. A sample of E_2 (8–13 pmol) was applied to the column, and fractions (0.46 mL) were collected and counted. An r_B value was calculated from the radioactivity in the trough. In the experiment performed with labeled and unlabeled E_3, a mixture of ^{14}C-labeled E_3 and unlabeled E_3 was used to preequilibrate and to elute the column. Symbols: A and B, unlabeled and labeled E_3; r_B, number of labeled E_3 dimers bound per molecule of the E_2 core; K_A, dissociation constant for unlabeled E_3. [b] Average of two experiments. [c] Calculated from eq 1 and 3 with $n = 6.07$ and $K_B = 4.66$ nM. These values were obtained from the experiment performed with labeled E_3 only.

decreases in the amounts of labeled E_3 bound to the E_2 core (Table I), resulting in lower binding stoichiometry for the labeled E_3 but little change in K_d. From each competition experiment, a K_d value for unlabeled E_3 can be calculated according to principles described by Englund et al. (1969), provided that labeled and unlabeled E_3 compete for the same binding sites and that the dissociation constant for labeled E_3 is known. Competitive binding is governed by the equations

$$K_A = [A](n - r_A - r_B)/r_A \quad (1)$$

$$K_B = [B](n - r_A - r_B)/r_B \quad (2)$$

where A is unlabeled E_3, B is labeled E_3, n is the number of identical, independent E_3 binding sites on the E_2 core, r is the number of ligand molecules bound per E_2 molecule, and K is the dissociation constant. Equation 2 can be rearranged to

$$r_A = n - r_B - (r_B K_B/[B]) \quad (3)$$

K_A and r_A are the only unknown quantities and can be calculated from eq 1 and 3. The results presented in Table I show that the dissociation constants for reductively methylated and native E_3 are very similar.

Results of similar experiments performed with both lightly and heavily phosphorylated E_1 are shown in Table II. Agreement is very good between the K_d values for unphosphorylated E_1 obtained from the two sets of experiments. It is evident that all forms of E_1 bind to the E_2 core at the same set of sites and that lightly phosphorylated and unphosphorylated E_1 have virtually identical binding affinities for the E_2 core. Heavily phosphorylated E_1, however, appears to bind to the E_2 core with an affinity about one-third that of the other two forms.

Determination of Dissociation Rate Constants. A reconstituted E_2E_3 subcomplex, prepared from ^3H-labeled E_2 and ^{14}C-labeled E_3, was applied to a Sepharose 6B column preequilibrated and eluted with unlabeled E_3. An elution profile showing dissociation of labeled E_3 from the E_2E_3 subcomplex is presented in Figure 4. A high molar ratio of E_3 to E_2 (about 20:1) was used in preparing the E_2E_3 subcomplex to ensure saturation of E_2 with E_3. As the large E_2E_3 subcomplex enters the gel matrix, it separates from the excess unbound, labeled E_3. Thereafter, any labeled E_3 that dissociates from the subcomplex would be extensively diluted with the high concentration of preexisting, unlabeled E_3. We estimated that

Table II: Competition between Phosphorylated and Unphosphorylated E_1[a]

	phosphorylated E_1		unphosphorylated and phosphorylated E_1			
expt	[B] (nM)	r_B	[A] (nM)	[B] (nM)	r_B	K_A (nM)[b]
I	6.21	11.8	3.06	3.47	1.38	15.4
	15.4	20.1	8.19	7.72	9.63	13.5
	72.1	34.6	40.2	36.3	17.5	18.7
						av: 15.9
II	8.25	6.93	3.92	4.75	3.32	12.6
	26.7	17.0	14.6	13.7	6.74	20.3
	102.9	32.4	61.2	58.6	11.7	22.5
						av: 18.5

[a] The experiments were performed as described in the legend of Table I. When only ^{32}P-labeled E_1 was used, the Sepharose 6B column was preequilibrated and eluted with labeled E_1 of the specified concentration. A sample of the E_2 core (3.2–258 pmol) was applied to the column, and fractions (0.46 mL) were collected and counted. In experiments I and II, the phosphorylated E_1 had on the average 1.26 and 3.07 phosphoryl groups per tetramer, respectively. In the experiments performed with labeled and unlabeled E_1, a mixture of the two forms was used to preequilibrate and to elute the column. Symbols: A and B, unphosphorylated and phosphorylated E_1; r_B, number of phosphorylated E_1 tetramers bound per molecule of the E_2 core; K_A, dissociation constant for unphosphorylated E_1. [b] Calculated from eq 1 and 3 with n = 42.1 and K_B = 16.2 nM for experiment I and n = 47.7 and K_B = 48.4 nM for experiment II. These values were obtained from the experiments performed with phosphorylated E_1 only.

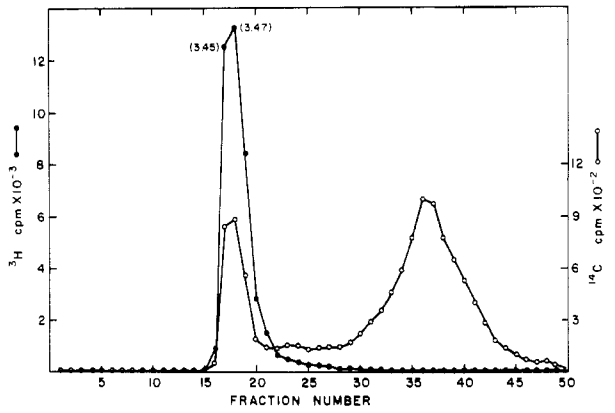

FIGURE 4: Dissociation of E_3 from E_2E_3 subcomplex. A mixture of 13.5 pmol of ^3H-labeled E_2 and 273 pmol of ^{14}C-labeled E_3 in 60 μL of buffer A containing 0.5 mM dithiothreitol was incubated at 25 °C for 10 min. The mixture was applied to a Sepharose 6B column (0.9 × 30 cm or 0.9 × 15 cm) that had been preequilibrated at 25 °C with a 300 nM solution of unlabeled E_3 in the same buffer. Fractions of 0.46 mL for the larger column and 0.20 mL for the smaller column were collected and counted. The calculated E_3 to E_2 molar ratio in the peak fraction (r) is given in parentheses.

after the subcomplex moved more than 5% of the length of the column, concentrations of labeled E_3 in the proximity of the subcomplex were less than 1% of the concentration of unlabeled E_3. Only at early stages of the elution, when the subcomplex had not been completely separated from unbound labeled E_3, were these ideal conditions not met. However, at that stage the subcomplex presumably was still saturated with E_3, and few vacant sites were available for reassociation with labeled E_3. Therefore, it is reasonable to neglect the reassociation of labeled E_3 with the E_2 core and to describe the process as a first-order, irreversible reaction:

$$E_2(E_3^*)_r \rightarrow E_2(E_3^*)_{r-1} + E_3^*$$

The number of labeled E_3 molecules (E_3^*) bound per E_2 molecule, r, in the peak fraction was calculated from radioactivity in the fraction and the known specific radioactivities of E_2 and E_3. By running the same Sepharose 6B column at different rates, it is possible to measure the rate of dissociation of labeled E_3 from the E_2 core (Henderson & Wang, 1972). The intercept at the ordinate in a plot of ln r vs. elution time of the subcomplex (Figure 5A) gives the binding stoichiometry of the original $E_2E_3^*$ subcomplex, and the negative slope is the dissociation rate constant (k_d). The k_d value for E_3^* is about 0.057 min^{-1}, corresponding to a half-life of about 12 min.

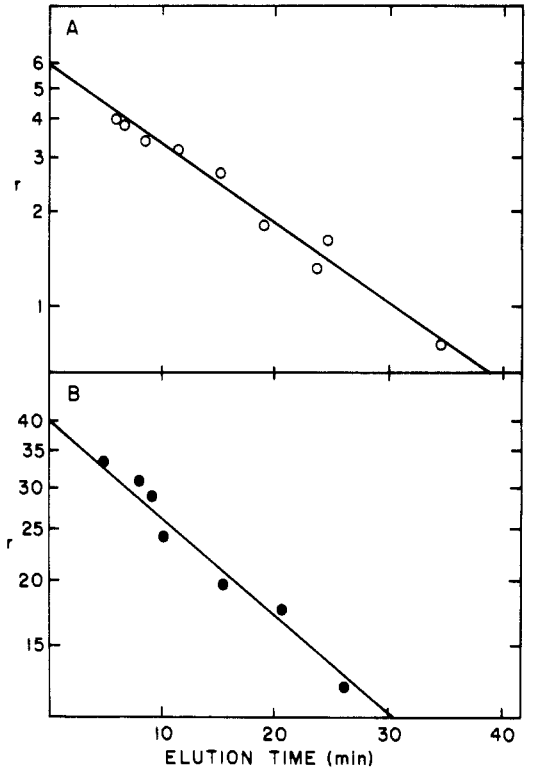

FIGURE 5: First-order plots of r vs. elution time, where r is the number of moles of E_3 (A) or E_1 (B) bound per mole of E_2. The r values were calculated from the radioactivity in the peak fraction, as illustrated in Figure 4.

The binding stoichiometry, six, is in excellent agreement with results of the equilibrium binding studies.

Lightly phosphorylated E_1 (average of 1.2 phosphoryl groups per molecule) dissociated from the $E_1^*E_2$ subcomplex with a k_d of 0.043 min^{-1}, corresponding to a half-life of 16 min (Figure 5B). The binding stoichiometry for E_1^* varied with different preparations of the E_2 core, but in each case, the values agreed with those obtained with the Hummel and Dreyer method.

Titration of E_1E_2 Subcomplex with E_3. E_1E_2 subcomplex, prepared by mixing E_1 and E_2 in a molar ratio of 82:1, was exposed to increasing amounts of E_3, and the mixtures were analyzed for overall reconstituted pyruvate dehydrogenase complex activity (Pettit & Reed, 1982). The plot of activity vs. molar ratio of E_3 to E_2 was curvilinear (Figure 6). Maximum activity was observed at a molar rato of about 6:1.

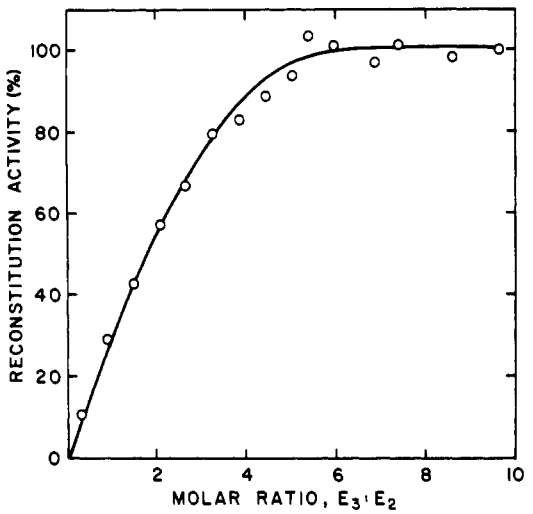

FIGURE 6: Titration of E_1E_2 subcomplex with E_3. Mixtures containing 214 pmol of E_1, 2.6 pmol of E_2, and increasing amounts of E_3 in 50 μL of 20 mM potassium phosphate buffer, pH 7.0, were incubated at 30 °C for 10 min. Triplicate 10-μL aliquots were assayed for reconstituted pyruvate dehydrogenase complex activity.

Discussion

The data reported in this paper indicate that the acetyltransferase (E_2) core of both the bovine kidney and heart pyruvate dehydrogenase complexes possesses six apparently identical and noninteracting binding sites for dihydrolipoamide dehydrogenase (E_3) dimers with a K_d of about 3 nM. These values were obtained by equilibrium gel exclusion chromatography with E_3 labeled with [^{14}C]methyl groups. The presence of a saturating concentration of pyruvate dehydrogenase (E_1) affected neither the K_d value nor the binding stoichiometry of E_3. Results of competitive binding with unlabeled E_3 demonstrated that the values of the binding parameters obtained with radiolabeled E_3 are very similar to those of the native protein. The dissociation rate measurement (Henderson & Wang, 1972) provides an independent method of determining the binding stoichiometry. Because the gel filtration column is preequilibrated with a high concentration of unlabeled E_3, the E_2E_3 subcomplex is saturated with E_3 at all times. By monitoring the dissociation of labeled E_3 at different time intervals, it is possible to obtain the number of labeled E_3 molecules originally bound to the E_2 core.

The possibility that the flavoprotein E_3 is bound to the E_2 core as 12 monomers rather than 6 dimers seems unlikely in view of the fact that rather severe conditions are required to dissociate this flavoprotein into monomers and the monomer is inactive with lipoate (van Muiswinkel-Voetberg et al., 1973). Also, at a concentration as low as 3 nM, E_3 did not dissociate into monomers as analyzed by gel filtration on Sephadex G-200 (data not shown). Finally, a species corresponding to E_3 dimer was detected by two-dimensional polyacrylamide gel electrophoresis analysis of bovine heart pyruvate dehydrogenase complex that had been cross-linked with either dithiobis(succinimidyl propionate) or dithiobis(phenyl azide) (Wu, 1982). These observations suggest that E_3 is present in the mammalian pyruvate dehydrogenase complex as dimers, as demonstrated previously with the *E. coli* pyruvate dehydrogenase complex (Reed et al., 1975; Coggins et al., 1976). Binding of 6 rather than 60 E_3 molecules can be reconciled with the apparent icosahedral symmetry of the mammalian E_2 core if we assume that each E_3 dimer extends across 2 of the 12 faces of the E_2 pentagonal dodecahedron, thereby sterically hindering binding of additional E_3 dimers in those two faces (Reed et al., 1980).

Consistent with the binding data are the results of reconstitution experiments, in which E_1E_2 subcomplex was titrated with E_3 and the activity of the reconstituted complex was measured. A convex titration profile was obtained, with maximum activity observed at an E_3 to E_2 molar ratio of about 6:1 (Figure 6). These results are very similar to those obtained when *E. coli* E_1E_2 subcomplex was titrated with *E. coli* E_3 (Reed et al., 1975). It appears that of the six E_3 dimers that bind to the E_1E_2 subcomplex, the initial E_3 dimers bound produce proportionally greater overall activity than do subsequent E_3 dimers. A possible explanation of these results is that there are multiple, interconnected pathways from E_1 subunits to the few E_3 subunits in the pyruvate dehydrogenase complex involving an extensive lipoyl–lipoyl interaction network (Hackert et al., 1983a,b).

The equilibrium constant for dissociation of lightly phosphorylated E_1 (average of 1.2 phosphoryl groups per tetramer) from a reconstituted E_1E_2 subcomplex is about 13 nM, and the dissociation rate constant is 0.043 min^{-1}. Heavily phosphorylated E_1 (average of 2.7 phosphoryl groups per tetramer) has a somewhat lower affinity for the E_2 core, with K_d = 40 nM. Competitive binding experiments showed that the binding affinity of unphosphorylated E_1 is about the same as that of lightly phosphorylated E_1 and that all three species of E_1 apparently bind to the same set of sites on the E_2 core. We have been unable, however, to determine unambiguously the stoichiometry of binding of E_1 to isolated E_2 by the gel filtration method. Depending on the particular preparation of the E_2 core used, whether from kidney or heart, the number of E_1 tetramers bound per molecule of the E_2 core varied from 35 to 55. The cause of this variation is not known. Binding of either 30 E_1 tetramers, one on each of the 30 edges of the E_2 pentagonal dodecahedron, or 60 E_1 tetramers, one on each E_2 subunit, would be consistent with the apparent 532 symmetry of the E_2 core. It should be noted that all of the E_1 preparations used in these experiments had high enzymatic activity and did not contain aggregates of E_1.

Roche and co-workers (Cate & Roche, 1979; Cate et al., 1980; Pratt et al., 1979) provided evidence for intermolecular movement of E_1 molecules between E_2 cores of both the bovine kidney and heart pyruvate dehydrogenase complexes and proposed that shuttling of E_1 molecules between a few tightly bound pyruvate dehydrogenase kinase molecules on the E_2 core may play an important role in regulation of E_1 activity. In support of this proposal, they reported (Pratt et al., 1979) that unphosphorylated E_1 exhibits at least a 9-fold greater affinity than heavily phosphorylated E_1 for their binding sites on the E_2 core and that the first-order dissociation rate constant for unphosphorylated E_1 is in the range 1.3–2.2 min^{-1}. In contrast, our data show the difference in binding affinities of unphosphorylated and heavily phosphorylated E_1 is only about 3-fold, and the dissociation rate constant determined for E_1, 0.043 min^{-1}, is markedly lower than the value reported by Cate et al. (1980). In a recent paper, Brandt et al. (1983) reported that bovine kidney E_2 contains two sets of binding sites for E_1, about 7 high-affinity sites with K_d = 15 pM and about 13 low-affinity sites with K_d = 25 nM in the presence of the coenzyme thiamin pyrophosphate and K_d = 0.59 nM in the absence of coenzyme. These results are at variance with our finding of only one set of binding sites for E_1 on the E_2 core, with K_d = 13 nM (in the absence of thiamin pyrophosphate). It should be noted that the results of Roche and co-workers are based on an indirect method of measuring E_1 binding to E_2, i.e., measurement of changes in overall enzymatic activity (NADH production with pyruvate as substrate) accompanying

dissociation or association of E_1 and E_2, in the presence of excess E_3.

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Registry No. E_2, 9032-29-5; E_3, 9001-18-7; pyruvate decarboxylase, 9001-04-1; pyruvate dehydrogenase, 9014-20-4.

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