Heterogeneity of Binding Sites for the Pyruvate Dehydrogenase Component on the Dihydrolipoyl Transacetylase Core of Bovine Kidney Pyruvate Dehydrogenase Complex†

Douglas R. Brandt, Thomas E. Roche,* and Mary L. Pratt‡

ABSTRACT: We have characterized the dissociation equilibrium constant (K_d) and the rate constants of association and dissociation for the binding of the pyruvate dehydrogenase component (PDH) to the dihydrolipoyl transacetylase component of kidney pyruvate dehydrogenase complex. We have found about 7 high-affinity sites ($K_d = 1.5 \times 10^{-11}$ M) and about 13 low-affinity sites ($K_d = 2.5 \times 10^{-8}$ M) with negative cooperativity for binding of PDH at the weaker sites. The high-affinity sites show a much higher rate constant for association of PDH with the core than do the low-affinity sites. Catalytic turnover strengthens binding of PDH at high-affinity sites when the complex is preincubated in the presence of thiamin pyrophosphate (TPP). In the absence of TPP, appreciably tighter binding occurs at the low-affinity sites ($K_d = 5.9 \times 10^{-10}$ M). TPP weakens PDH binding at these sites with a half-maximal effect at about 5 μM TPP. In addition to TPP, increased ionic strength or 1–3 mM Mg^{2+} (with an enhanced effect of Mg^{2+} at higher ionic strengths) weakens PDH binding at low-affinity sites with a corresponding increase in the rate constant for dissociation. Further studies will be required to determine whether site heterogeneity contributes to dynamic processes in the function and regulation of the pyruvate dehydrogenase complex.

Pyruvate dehydrogenase complexes from mammalian tissues comprise three catalytic and two regulatory components. The core component, dihydrolipoyl transacetylase, is a large aggregate which is composed of 60 subunits that form a pentagonal dodecahedron (Barrera et al., 1972; Reed, 1974; Kresze & Ronft, 1980; Bleile et al., 1981).[1] The remaining two catalytic components and the two regulatory components bind to the transacetylase core.

The pyruvate dehydrogenase component is regulated by interconversion between an active, nonphosphorylated form (PDH_a)[2] and an inactive, phosphorylated form (PDH_b). Previous studies from this laboratory have demonstrated that PDH_a is mobile (Cate & Roche, 1979) and can exchange between transacetylase cores in a process limited by the rate of dissociation (Cate et al., 1980). When the transacetylase core is the limiting component, very high ratios of PDH_b to PDH_a are required to decrease the overall reaction catalyzed by the complex (Pratt et al., 1979). Since PDH_a catalyzes the rate-limiting step, reductive acetylation (Cate et al., 1980), the above observation of Pratt et al. (1979) indicates that PDH_a binds in preference to PDH_b at sites on the transacetylase core where the pyruvate dehydrogenase component can engage in catalysis. Pratt et al. (1979) also presented evidence that the mobility of PDH_a and the dissociation of PDH_b facilitate the phosphorylation reaction which is catalyzed by a specific kinase. Approximately three kinase molecules are very tightly associated with a transacetylase core (Pratt & Roche, 1979) and are probably immobile.

We now report further studies on the association of PDH_a with the transacetylase core. We have determined the dissociation constant (K_d) and the rate constants for dissociation (k_d) and association (k_a) under a variety of conditions. We have found that there are high- and low-affinity sites for the association and that binding at these sites is differentially affected by catalytic turnover, thiamin pyrophosphate, Mg^{2+}, and ionic strength.

Experimental Procedures

Materials. 3-(*N*-Morpholino)propanesulfonic acid (Mops), NAD (grade III), thiamin pyrophosphate (TPP), dithiothreitol, leupeptin, and *N*-ethylmaleimide were purchased from Sigma. CoA, ATP, and NADH were obtained from P-L Biochemicals, Inc. Pig heart dihydrolipoyl dehydrogenase was purchased from Boehringer-Mannheim.

A highly purified preparation of bovine kidney pyruvate dehydrogenase complex [12.9 μmol of NADH/(min mg)] was prepared as previously described (Roche & Cate, 1977). Resolved components were prepared by the method of Linn et al. (1972) except that the pyruvate dehydrogenase component was not crystallized.

Molecular Weights of Components and Estimation of the Composition of the Kidney Pyruvate Dehydrogenase Complex. The following molecular weights were used to calculate molar concentrations of components: 1.54×10^5 for the pyruvate dehydrogenase component, 3.1×10^6 for the dihydrolipoyl transacetylase core, and 1.10×10^5 for dihydrolipoyl dehydrogenase (Barrera et al., 1972).

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‡ Present address: Department of Pharmacology, University of Texas Medical School, Houston, TX 77025.

[1] Other models for the subunit structure of the dihydrolipoyl transacetylase have been proposed (Hamada et al., 1975; Sugden & Randle, 1978; Machicao & Wieland, 1980), but the 60-subunit model, consistent with electron micrographs (Reed & Oliver, 1968), has been clearly established by recent studies (Kresze & Ronft, 1980; Bleile et al., 1981). The recent work explained the anomalous migration of transacetylase subunits in sodium dodecyl sulfate–polyacrylamide gel electrophoresis which was the cornerstone for alternative models.

[2] Abbreviations: PDH, pyruvate dehydrogenase component; PDH_a, active (nonphosphorylated) form of the pyruvate dehydrogenase component; PDH_b, inactive (phosphorylated) form of the pyruvate dehydrogenase component; NEM, *N*-ethylmaleimide; TPP, thiamin pyrophosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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The ratio of PDH_a to transacetylase in purified samples of the complex was determined from densitometric scans of $NaDodSO_4$ gel electrophoretograms. The relation between sample weight and staining intensity was first determined for each component separately. Aliquots containing 0.5–12 μg of resolved components were subjected to electrophoresis; the gels were stained with Coomassie Brilliant Blue, destained uniformly by flow-through interconnected gel chambers, and scanned on a Gilford spectrophotometer. The areas under the bands were integrated and plotted against the weight of the samples. The calibrated plots gave different slopes for different components but were linear in each case. Samples of complex (2.0–20.0 μg) were subjected to electrophoresis, and the gels were stained, destained, and scanned. The areas under the bands were converted to molar ratios by using the molecular weights determined by Barrera et al. (1972). We determined that there were 20 PDH_a tetramers per transacetylase core. With an additional contribution of about 2.0×10^5 per complex due to PDH_a kinase, a molecular weight of 7.0×10^6 was estimated for the active complex, and this was used for calculating concentrations of complex. There should not be more than a 5% error in n values estimated by the above procedure assuming molecular weights determined by Barrera et al. (1972) are accurate.

Preparation of N-Ethylmaleimide-Inactivated Pyruvate Dehydrogenase Complex. Samples of the pyruvate dehydrogenase complex were inactivated to greater than 98% by minor modification of the procedure of Cate et al. (1980). The procedure results in selective inactivation of the transacetylase component by reaction of reduced lipoyl moieties with *N*-ethylmaleimide (NEM). The complex (1.0 mg/mL) was treated with 0.10 mM NEM for 10 min at 30 °C in a buffer containing 40 mM Mops-K (pH 7.2), 0.50 mM EDTA, and 0.50 mM NADH. Dithiothreitol was added to a final concentration of 0.50 mM to quench the reaction. Unless otherwise indicated, the product was diluted to 0.50 mg/mL with 40 mM Mops-K (pH 7.2).

Titration of Inactivated Pyruvate Dehydrogenase Complex with Resolved Dihydrolipoyl Transacetylase. To assess the effect of NEM treatment upon the binding of pyruvate dehydrogenase by dihydrolipoyl transacetylase, inactivated pyruvate dehydrogenase complex (as prepared above, 1.0 mg/mL) was titrated with a stock solution containing dihydrolipoyl transacetylase (2.0 mg/mL) and dihydrolipoyl dehydrogenase (0.40 mg/mL) in 40 mM Mops-K (pH 7.2) and 0.10 mM EDTA. Treated complex (10.0 μg) was incubated for 5 min at 30 °C with various levels of dihydrolipoyl transacetylase core ranging from 4.0 to 60.0 μg. Then, half of the incubation volume was assayed for production of NADH as described by Linn et al. (1972). Activity due to a small amount of the pyruvate dehydrogeanse component in the resolved dihydrolipoyl transacetylase and the residual activity of the NEM-treated complex were subtracted from the observed rate. These background rates never exceeded 6% of the observed activity.

Determination of the Rate Constant for Dissociation-Limited Intercore Movement of the Pyruvate Dehydrogenase Component. The rate constant for dissociation of PDH from complex containing NEM-inactivated cores was determined in the presence of excess dihydrolipoyl dehydrogenase and dihydrolipoyl transacetylase by measuring the increase in NAD^+-reduction activity as PDH_a moved from inactive to active cores (Cate et al., 1980). To conserve resolved transacetylase in the present studies, a lower ratio of active to inactive transacetylase (i.e., 4.5:1 rather than 10:1 or 20:1) was used; this lower ratio gave similar results in determinations of k_d.[3] Dissociation was invariably a first-order process. Unless otherwise indicated, assays were initiated by the addition of 5.0 μg of inactivated complex to a cuvette containing 10.0 μg of dihydrolipoyl transacetylase, 2.0 μg of dihydrolipoyl dehydrogenase, 0.50 mM $MgCl_2$, 43.3 mM Mops, 39.0 mM K^+, 0.24 mM CoA, 5.3 mM cysteine hydrochloride, 2.0 mM pyruvate, 2.0 mM NAD, and 0.20 mM TPP in a final volume of 1.0 mL. The ionic strength was 0.055 M, and the final pH was 7.3. Other conditions were varied as described in the legends.

Determinations of the Fractional Binding of PDH_a for a Wide Range of Concentrations of Complex and of Rate Constants for Association and Dissociation. We assumed that the rate of the overall reaction catalyzed by the associated system in the presence of excess levels of the third catalytic component, the dihydrolipoyl dehydrogenase, was a linear function of the amount of PDH_a bound to the dihydrolipoyl transacetylase core. To test this assumption, pyruvate dehydrogenase complex at 1.0 mg/mL (where >99% PDH_a is bound) was incubated at 30 °C with varying amounts of transacetylase. Equilibrium distributions develop as previously demonstrated in a mass transport experiment (Cate & Roche, 1979). With 3–20 PDH_a bound per transacetylase core, activity was proportional to the bound PDH_a.

A Farrand ratio 2-spectrofluorometer was used to measure rates of NADH production from 0.015 to 3.0 μM min^{-1} and a Gilford 252 spectrophotometer for rates in the overlapping range from 0.30 to 150 nmol/(mL min). Both instruments were used in conjunction with Omniscribe recorders. Unless otherwise indicated, experiments were conducted at 30 °C in buffer A which contained 40 mM potassium phosphate, 0.20 mg/mL BSA, 2.0 mM EDTA, 2.0 mM dithiothreitol, 7.5 μg/mL dihydrolipoyl dehydrogenase, 0.50 μg/mL leupeptin, 2.5 mM $MgCl_2$, 0.80 mM NAD^+, 0.080 mM TPP, 0.25 mM CoA, and 5.3 mM cysteine hydrochloride. Buffer A had an ionic strength of 0.115 M and a final pH of 7.1.

The following procedures (designated P-1–3 to simplify Results) allowed measurement of the fractional binding of PDH_a to the transacetylase core for a wide range of concentrations of complex under a variety of conditions.

In procedure P-1, for concentrations of complex below 0.25 μg/mL (0.036 mM), the dissociation of PDH_a to equilibrium was monitored by measuring NADH production continuously as catalytic activity decreased after sudden dilution of a stock solution of the complex into buffer A containing 1.0 mM pyruvate. Even at the highest level of complex tested, less than 6.0% of the CoA and 2.0% of the NAD were converted to products by the time the dissociation of the complex reached equilibrium (20–25 min). Thus, neither substrate depletion nor product inhibition significantly altered the observed rate profile. The stock solution of complex at 10.0 μg/mL was maintained at 4 °C in buffer A minus substrates, cofactors, cysteine hydrochloride and dihydrolipoyl dehydrogenase. The level of dissoication of PDH_a in this stock solution was evaluated as the ratio between its initial catalytic rate and the initial catalytic rate of an equivalent amount of enzyme diluted from 1.0 mg/mL where the PDH_a is entirely bound. Typically,

[3] Our analysis of the rate constant of dissociation (k_d) of PDH_a from NEM-inactivated cores assumes that it is equivalent to the k_d value for dissociation from active cores. Assuming $d(PDH_a)/dt = 0$ yields $k_d t = (L)_T/[(L)_T + (L)_T^*] \ln [(PL)_{eq}/[(PL)_{eq} - (PL)_t]]$ where $(L)_T$ and $(L)_T^*$ are the total concentrations of PDH_a binding sites on active and inactive cores, respectively. $(PL)_{eq}$ and $(PL)_t$ equal the concentration of PDH_a binding to active cores at equilibrium and at any time t and are proportional to the observed catalytic rates at equilibrium and at any time t, respectively.

in the stock solution 90% PDH_a is bound. From the complete dissociation profiles, rate constants of association and dissociation were calculated as described in the Appendix.

In procedure P-2a, complex at concentrations between 0.0098 and 20.0 μg/mL (0.0014–2.9 nM) was equilibrated in buffer A in the absence of catalytic turnover; equilibrium between bound and free PDH_a was generally obtained within 10–20 min.[4] Pyruvate was added as a 100× concentrate to initiate turnover. Any readjustment in the dissociation equilibrium was followed by monitoring NADH production. For the highest concentration of complex, a sample was equilibrated in buffer A, then a sample diluted into a cuvette containing buffer A plus pyruvate, and the initial velocity measured (procedure P-3).

In many studies (P-2b), the complex was preincubated in the absence of TPP, in which case separate concentrates of pyruvate (as above) and TPP (as a 200× concentrate) were added simultaneously to initiate turnover. The endogeneous activity of complex in the absence of added TPP is indicated in the appropriate figure legends as the TPP dependence (i.e., the difference in catalytic activity in the presence and absence of TPP divided by the activity in the presence of TPP). The basis of this approach is the very tight binding of active aldehyde intermediate, α-hydroxyethylthiamin pyrophosphate, at the step preceding the rate-limiting step of reductive acetylation (Cate et al., 1980). The TPP was captured on the complex by catalytic turnover in assay mixture lacking TPP was based on rates of NADH production being constant for at least 40 s and equivalent specific activities determined following dilution of the complex to different levels (5–20 μg/mL).

In procedure P-2c, buffer A′ (in which 40 mM Mops-K replaced potassium phosphate to lower the ionic strength to 0.054 M) was used to investigate the influence of ionic strength, substrates, and cofactors on PDH_a binding. Activity was initiated by addition of pyruvate and, as required, other concentrated substrates or cofactors.

Results

Effects of Ionic Strength and $MgCl_2$ on Dissociation of PDH_a. The rate constant of dissociation (k_d) of the pyruvate dehydrogenase component from NEM-inactivated dihydrolipoyl transacetylase core can be determined in the presence of excess levels of active core by measuring the increase with time of the NAD-reduction activity, as PDH_a moves from inactive to active cores (Cate et al., 1980). This approach allows measurement of the rate-limiting dissociation step in intercore movement of the pyruvate dehydrogenase component at fairly high levels of the complex (2–10 μg/mL).

Nearly equivalent binding to NEM-inactivated and untreated cores was observed when the redistribution reached equilibrium. For instance, at the ratio used in the studies described below, 4.5 active cores per inactive core, 81% of the maximal activity was achieved at equilibrium which is very close to 81.8% calculated assuming PDH has equal affinity for active and inactive cores. This result could mean that the rate constants for association and dissociation did not change after NEM treatment or that both rate constants happened to be affected in the same proportion. In either case we shall assume that conditions altering the rate constant for dissociation (k_d) from NEM-inactivated cores would elicit similar changes in dissociation from active cores.

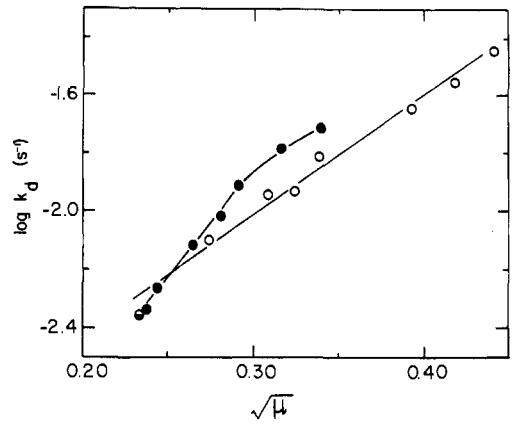

FIGURE 1: Effect of ionic strength (μ) on the rate constant for dissociation (k_d) of PDH_a. The ionic strength was varied with either $MgCl_2$ (●) or KCl (○). Methods of preparation of NEM-inactivated complex and assay conditions for following PDH_a dissociation were as described under Experimental Procedures.

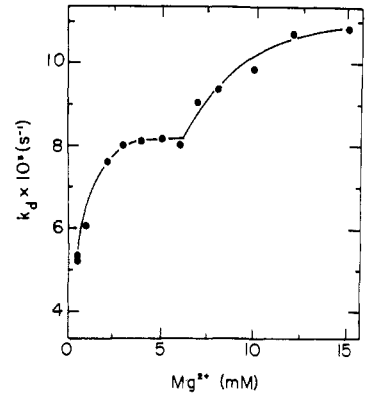

FIGURE 2: Effect of Mg^{2+} concentration on the rate constant for dissociation (k_d) of PDH_a at constant ionic strength. The ionic strength was maintained at 0.099 M by altering the amount of KCl. Preparation of inactive complex and other assay conditions were as described under Experimental Procedures.

We found that log k_d increased linearly with the square root of the ionic strength (Figure 1). This is the observed relationship for changes in electrostatic interactions of ionic species in dilute solution (cf. Weston & Schwarz, 1972) and clearly indicates that ionic interactions are important in the association of PDH_a with the transacetylase core. The linear plot has a slope of 4.1 with a correlation coefficient of 0.991. The magnitude of the slope suggests that multiple ionic interactions are involved between subunits. The observation is interesting because interconversion of this component by phosphorylation and dephosphorylation might lead to changes in electrostatic interactions.

Low levels of $MgCl_2$ gave a significant increase in slope over that achieved with KCl (Figure 1). The slope for the data points corresponding to $MgCl_2$ levels between 0.50 and 10.0 mM ($\mu^{1/2}$ = 0.234–0.291) was 7.8. In further studies, the concentration of Mg^{2+} was varied between 0.50 and 15.0 mM while maintaining a constant ionic strength by compensating reduction in KCl. As shown in Figure 2, an unusual multiphasic response with two plateau regions was observed at a total ionic strength of 0.10 M. Further studies over a range of ionic strengths gave qualitatively similar responses; however, the increase in k_d for a change in $MgCl_2$ from 0.50 to 5.0 mM was appreciably greater at higher ionic strength while the

[4] Longer incubations in buffer A or buffer A′ did not appear to change the equilibrium activities, which indicates that equilibrium was achieved. Prolonged incubation was generally not done to avoid enzyme inactivation. Any error introduced by not reaching equilibrium would result in reporting too large a fraction of PDH_a bound and, therefore, tighter than correct binding.

Table I: Influence of Ionic Strength on the Mg^{2+}-Facilitated Increase in the Rate Constant of Dissociation (k_d) for Dissociation of PDH_a from NEM-Inactivated Dihydrolipoyl Transacetylase[a]

ionic strength (M)	k_d(5.0 mM Mg^{2+})/ k_d(0.5 mM Mg^{2+})	k_d(10.0 mM Mg^{2+})/ k_d(5.0 mM Mg^{2+})
0.084	1.17	1.19
0.099	1.55	1.20
0.145	1.80	1.17

[a] Values for k_d were determined at the Mg^{2+} concentrations indicated, and the ionic strength was adjusted as necessary with KCl to levels indicated.

increase in k_d for levels of Mg^{2+} above 5 mM appeared to be independent of ionic strength (Table I). This effect of Mg^{2+} on k_d may be significant since it is enhanced by increasing the ionic strength into a near-physiological range (μ = 0.145 M, Table I) and since it is greatest in the range 0.5–3 mM Mg^{2+} which is within the physiological range for intramitochondrial free Mg^{2+}.

The above results indicate that at nearly physiological ionic strengths (0.14 M) and typical intramitochondrial levels of Mg^{2+} (2.0 mM), the k_d for dissociation of PDH_a would be about 0.025 s^{-1}. These studies were conducted with 2.94 nM active cores and with 0.7 nM complex containing inactivated cores. Control studies have shown that the k_d values do not change significantly in studies using an 8-fold higher level of active transacetylase, showing that the rate of association does not contribute significantly to the observed rates of reactivation. However, a nearly linear dependence of k_d on the level of inactive core was observed and could not be fitted by equations derived under a variety of assumptions. Extrapolation to infinite dilution yielded a k_d value very close to values obtained with low concentrations of complex by the method described below which utilized only active core. We developed these procedures to clarify further the nature of PDH_a binding and to evaluate the dissociation equilibrium constant(s) for PDH_a binding. Values for k_d (as well as the rate constant for association, d_a) were also determined but only at much lower concentrations of complex than were used in the above approach.

Determination of Binding Constants for the Association of PDH_a with Dihydrolipoyl Transacetylase Cores. We investigated dissociation of PDH_a from purified kidney complex for a very wide range of concentrations of complex (from 0.0014 to 32 nM) by altering our procedure (P-1 to P-3) for different concentrations of complex. The different procedures were applied to overlapping concentration ranges to allow comparison of data obtained in various ways. A particularly important observation for very dilute pyruvate dehydrogenase complex was that activity loss with time could, indeed, be attributed to dissociation of PDH_a since addition of an excess level of resolved transacetylase resulted in complete recovery of activity.

In Figure 3, a Scatchard plot is shown which describes PDH_a binding to transacetylase cores for samples of complex equilibrated in buffer A. Two classes of PDH binding sites are observed with intrinsic dissociation constants, K_d, of 1.5 $\times 10^{-11}$ M and 2.5 $\times 10^{-8}$ M. There appear to be about 7 high-affinity sites and about 13 lower affinity sites. The dashed line is a calculated line for a smooth transition based on the above dissociation constants. The K_d for the tight binding sites (i.e., K_1) was calculated by a least-squares fit to data in that region, and the K_d for the weaker binding sites (i.e., K_2) was estimated by successive approximation of the transition and lower region. K_2 equals half the lower slope in agreement with

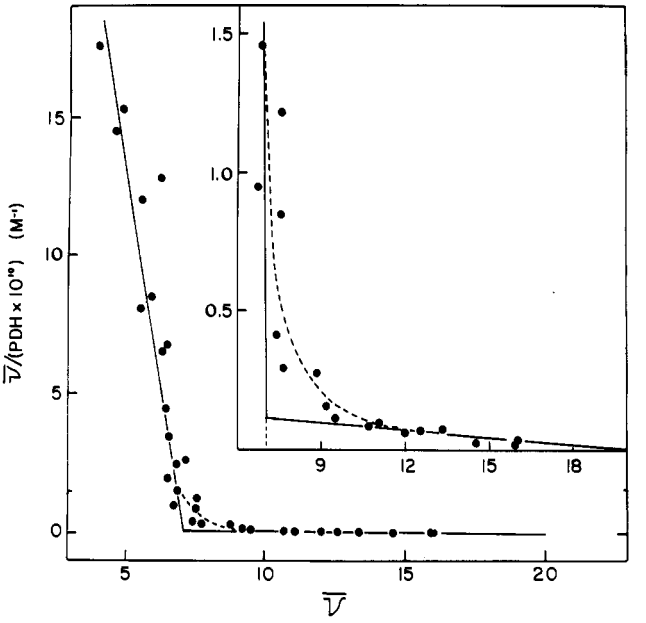

FIGURE 3: Scatchard plot for the binding of PDH_a to transacetylase core in the presence of TPP. PDH_a binding data were collected over a wide range of concentrations of complex (1.4 pM to 32.1 nM) by using procedures P-1, P-2a, and P-3 described under Experimental Procedures. Note location on the ordinate of zero and the additional mark denoting the upper range of the insert. The insert is an expansion of the data for the transition region and for the entire region for low-affinity sites. The fraction bound (ν) is given in terms of moles bound per mole of core, and PDH is used to symbolize the concentration of free PDH_a.

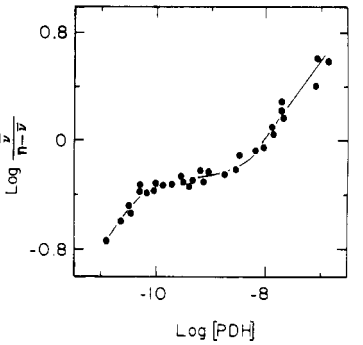

FIGURE 4: Hill plot for the binding of PDH to transacetylase core in the presence of TPP. The value used for n, the total number of binding sites, was based on figure 3. On the abscissa, the molar concentration of free PDH is given. Other conditions were as described in Figure 3.

the equations of Klotz & Hunston (1971). These K_d values are site-specific dissociation constants which were calculated assuming two classes of noninteracting sites. If interactions between classes of sites (i.e., negative cooperativity) contribute to the observed data, the constants determined would not constitute site-specific dissociation constants (Ferguson-Miller & Koppenol, 1981).

Analysis of these data, in a Hill plot, is shown in Figure 4. By curve fitting of these data using the K_d values and a number of sites determined in Figure 3 and assuming no site–site interactions, we closely fit the data for the tight binding sites; however, data for the weak binding site (Hill coefficient = 0.58) deviated significantly from the simulated curve, indicating negative cooperatively in the binding of PDH_a at the weaker sites. This suggests that, when the PDH_a binding sites on transacetylase core are heavily occupied, protein–protein interactions between PDH_a tetramers are increased which reduce their affinity for the core.

Table II: Influence of Catalytic Turnover on PDH_a Binding at High-Affinity Sites on the Transacetylase Core in the Presence of TPP and Determination of Rate Constants for Binding at High-Affinity Sites[a]

concn of complex ($M \times 10^{-12}$)	K_1 (nonturnover) ($M \times 10^{-11}$)	K_1 (turnover) ($M \times 10^{-11}$)	k_a ($M^{-1}\ s^{-1} \times 10^8$)	k_d ($s^{-1} \times 10^{-3}$)
3.57	7.7	0.73	1.0	0.7
7.14	5.3	0.82	1.3	1.1
14.29	11.7	0.94	1.2	1.1
av value	8 ± 2	0.83 ± 0.06	1.2 ± 0.1	1.0 ± 0.1

[a] Complex was incubated at the indicated concentrations for 15 min in the absence of turnover (procedure P-2a). Binding constants in the absence of catalysis were evaluated from initial slopes subsequent to the addition of pyruvate, and binding constants in the presence of catalysis were determined from the final slopes after equilibrium rate was achieved. Rate constants were determined as outlined in the Appendix. Other conditions were as listed under Experimental Procedures.

Effects of Catalytic Turnover on PDH_a Binding. At very low concentrations of complex, we have observed that catalytic turnover enhances PDH_a binding at high-affinity sites. Thus, when complex was incubated for 15 min, which allowed PDH association to achieve equilibrium in the absence of turnover, and turnover was initiated, the activity increased with time. As shown in Table II, values for K_1 were 10 times smaller in the presence of catalytic turnover. Values for K_1, in the absence of turnover, were based on the initial rates observed upon addition of pyruvate to samples that had acieved equilibrium in the absence of turnover (procedure P-2a) and were calculated with an n for high affinity sites of 7 which was determined by Scatchard analysis. Values for K_1, in the presence of turnover, were determined from the final catalytic rate after the association reaction had reached a new equilibrium, generally in about 3–5 min. This final rate was very close to that observed from dissociation profiles at the same concentration. (K_1 values were slightly smaller than that calculated from Figure 3.) Thus, essentially the same final association equilibrium was achieved when approached from either direction (i.e., by following the activity decrease in the presence of turnover to a constant rate after dilution of a sample into an assay mix or by following the activity increase to a constant rate upon addition of pyruvate to a dilute sample allowed to equilibrate in the absence of turnover). This clearly indicates that the final catalytic rates observed do indeed reflect attainment of equilibrium.

The enhanced binding of PDH_a with turnover appears to be associated only with high-affinity sites since it was not observed at concentrations of complex greater than 0.3 μg/mL where the tight sites, but not the weak ones, are already saturated in the absence of turnover. The possibility that an effect was not observed at higher protein concentrations due to a rapid association was eliminated by equilibrating parallel samples in this concentration range in buffer A and then adding pyruvate to one and diluting an equivalent sample to a much lower concentration in buffer A plus pyruvate. Observed initial rates were proportional to the dilution, and thus there did not appear to be a portion of free PDH_a that rapidly associates at the higher concentration upon initiation of turnover since such an association should have been detected in the very dilute solution.

Rate Constants of Association and Dissociation. Because of the low concentrations at which complex was equilibrated, the increase in activity after turnover began resulted from PDH_a binding exclusively at high-affinity sites. Thus, the time course could be analyzed to determine the rate constants of association and dissociation for binding of PDH_a at high-affinity sites (Table II). Essentially identical values were calculated for a 4-fold concentration range of complex which indicates that these values are good estimates of the rate constants for binding at high-affinity sites. The high value for the rate constant of association indicates that binding at high-affinity sites occurs at a nearly diffusion-limited rate.

Table III: Determination of Rate Constants for PDH_a Binding at Low-Affinity Sites[a]

concn of complex ($M \times 10^{-12}$)	k_a ($M^{-1}\ s^{-1} \times 10^5$)	k_a ($s^{-1} \times 10^{-3}$)
7.14	2.6	6.4
14.29	1.5	3.6
21.43	1.4	3.4
28.57	2.2	5.6
35.71	2.9	7.3
av value	2.1 ± 0.6	5.3 ± 0.8

[a] Catalytic turnover was initiated by the dilution of the stock solution of complex (1.45 nM) into buffer A plus pyruvate (1.0 mM) to the final concentrations listed below (procedure P-1). NADH production was followed spectrofluorometrically during the period required for PDH_a dissociation to come to equilibrium. The data were analyzed as outlined in the Appendix. The equilibrium concentration of PDH_a bound at low-affinity sites was calculated from the K_2 values determined in Figure 3.

From time courses for dissociation of PDH_a (procedure P-1) for complex diluted from 1.43 nM (10.0 μg/mL) to a range of 7.14–35.7 pM, net release of PDH_a occurs almost exclusively from low-affinity sites, and analysis of the time course yields k_a and k_d values for low-affinity sites. Data in Table III show the variation in rate constants determined at several concentrations of the complex. Average values of $2.1 \times 10^5\ M^{-1}\ s^{-1}$ for k_a and $5.3 \times 10^{-3}\ s^{-1}$ for k_d were obtained. Compariing these values to those for high-affinity sites indicates that, under conditions of catalytic turnover, the rate constant for dissociation is only about 5 times larger for dissociation from low-affinity sites while the rate constant of association is 10^3 times smaller. Thus, the transition to lower affinity sites would appear to result primarily from increased occupancy causing more interference during the process of association.

Effect of Substrates, Thiamin Pyrophosphate, and Ionic Strength on PDH_a Binding. The influence of individual substrates or cofactors on PDH_a binding was investigated by procedure P-2c. Even in the absence of other substrates, effects of pyruvate could not be tested in the absence of catalytic turnover because dead-end inhibition develops.[5] In studies on low-affinity sites (i.e., at 1.43 nM complex) NAD^+ and CoA did not significantly alter the equilibrium fraction of PDH_a bound (i.e., 0.85–0.90); however, TPP reduced the fraction bound to about 0.56, and the same reduction of binding was observed in the presence of absence of NAD^+ and CoA. When the ionic strength was increased from 0.054 to 0.154 M, the fraction bound in the absence of TPP was reduced to about 0.71, but the fractional level of PDH bound in the presence of TPP did not change.

The dependence of the reduction in binding of PDH at low-affinity sites on TPP concentration is shown in Figure 5. Half-maximal reduction in PDH_a binding occurred at about 5 μM TPP which corresponds well to the dissociation constant

[5] Incubation with pyruvate leads to acetylation of complex (and in the presence of excess TPP accumulation of reaction products such as acetoin and acetolactate). Following acetylation, the activity of the complex decreases with time, apparently due to a slowly developing dead-end inhibition. We have been unable to find conditions to prevent this inactivation.

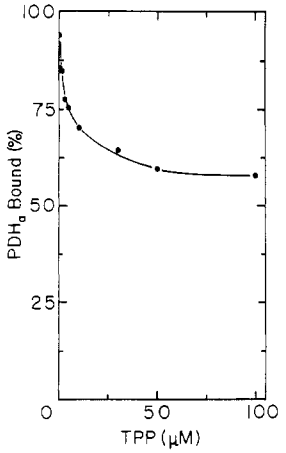

FIGURE 5: Concentration dependence for the effect of TPP on PDH_a binding to transacetylase core. Complex was incubated at 2.9 nM (20 μg/mL) with varying levels of TPP and buffer A lacking CoA and NAD. After incubation, 160-μL aliquots were assayed for NADH production, and the percent PDH_a bound (94% in the absence of TPP) was determined as described under Experimental Procedures (procedure P-2c). The TPP dependence of the enzyme used was 85%.

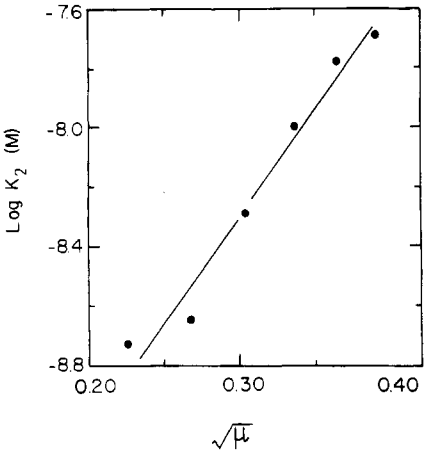

FIGURE 6: Effect of ionic strength (μ) on the dissociation constant (K_d) for binding PDH_a to the transacetylase core. Complex was incubated at a final concentration of 1.4 nM (10 μg/mL) in buffer A′ lacking CoA, NAD, and TPP. KCl was used to increase the ionic strength. Catalytic turnover was initiated by the addition of a freshly mixed 200× concentrate of CoA, NAD^+, and TPP and a 100× pyruvate concentrate (procedure P-2c). The K_d values were determined as described under Experimental Procedures. The TPP dependence of the enzyme used was 85%.

for TPP binding by PDH in the bovine kidney complex (Roche & Reed, 1972).[6]

The effect of ionic strength on PDH_a binding to low-affinity sites was investigated in the absence of TPP. Complex containing a low level of endogenous TPP (85% dependence) was incubated at a low (7.1 pM) and at a high (1.4 nM) concentration without added TPP. At the lower concentration of complex (i.e., for PDH_a binding to higher affinity sites), there was little, if any, effect of ionic strength on PDH binding. However, at the high concentration of complex (reflecting

[6] The data in Figures 3 and 4 reflect equilibrium under conditions of catalytic turnover in the presence of saturating TPP. Bound TPP would be present almost entirely as α-hydroxyethylthiamin pyrophosphate since this intermediate accumulates prior to the rate-limiting step of reductive acetylation (Cate et al., 1980). The concentration dependence for TPP determined in Figure 5 involves equilibration of samples in the absence of catalytic turnover, procedure P-2c. Equilibrium in the absence of turnover was also required for data presented in Figure 7 where procedure P-2b was used.

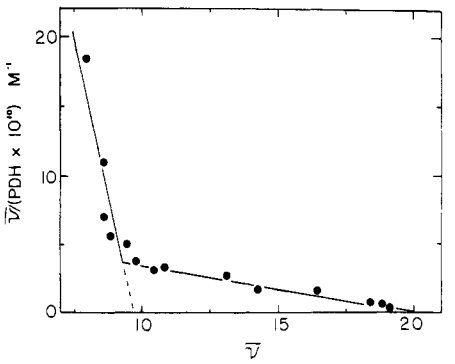

FIGURE 7: Scatchard plot for the binding of PDH_a to transacetylase core in the absence of TPP. PDH_a binding was determined for complex concentrations of 3.6 pM to 14.3 nM by using the approaches described under Experimental Procedures (procedure P-2b). The fraction PDH_a bound (ν) is given in moles bound per mole of core, and PDH is used to symbolize the concentration of free PDH_a. The TPP dependence of the enzyme used was 93%.

low-affinity sites), log K_2 increased in a linear manner with $\mu^{1/2}$ (Figure 6) in studies in the absence of substrates and cofactors. The observed dependence is similar to the increase in the rate constant for dissociation, k_d, shown in Figure 1 and probably reflects an enhanced rate of dissociation due to decreased electrostatic interactions between PDH_a and the transacetylase core with increasing ionic strength. The presence of TPP at 80 μM eliminated the effect of ionic strength on K_d values for low-affinity sites but did not eliminate the effect on the rate constant of dissociation of PDH_a from NEM-inactivated cores. This suggests that the complete effect of TPP may require unmodified lipoyl domains. In the absence of added TPP, tighter binding was observed than for studies reported in Figures 3 and 4 and is characterized below.

PDH_a Binding in the Absence of TPP and Catalytic Turnover. The binding curves shown in Figures 3 and 4 describe PDH_a binding in the presence of TPP and catalytic turnover. Since TPP reduced PDH_a binding to the transacetylase core at least at low-affinity sites and since catalytic turnover enhanced PDH_a binding at high-affinity sites, further studies were conducted to ascertain whether there were distinct high- and low-affinity sites in the absence of TPP and catalytic turnover (procedure P-2b).

As shown in Figure 7, two classes of sites were observed, and the apparent K_d for low-affinity sites was 5.9×10^{-10} M which is 42 times tighter than that observed in the presence of TPP.[6] In addition, negative cooperativity was not detected for these sites. Consistent with TPP weakening PDH_a binding, catalytic turnover decreased with time for higher concentrations of complex at which there are changes in occupancy of low-affinity sites. The final rate indicated the same fraction bound as reported in Figure 3.

Again, a transition to tighter binding was observed at low levels of binding. Attempts were made to fit the data to calculated Scatchard plots, assuming the presence of two classes of independent binding sites. The data were most closely approximated by seven high-affinity sites with $K_d = 1 \times 10^{-12}$ M. That is, the affinity of both classes of sites for PDH appears to be increased in the absence of TPP. However, even this best fit was unsatisfactory; in particular, the model predicted a more gradual transition between the two limbs of the Scatchard plot than was observed.

In contrast to samples equilibrated in buffer A with TPP, samples in buffer A lacking TPP did not exhibit an increase in activity with time when catalytic turnover was initiated by adding substrate and TPP. In fact a slight decrease in rate (completed in 5 min) ensued which probably reflected reduced

binding at low-affinity sites. Thus, to account for the activity increase noted earlier for complex equilibrated in the presence of TPP, turnover reverses (10-fold) the decrease in PDH_a binding at high-affinity sites caused by TPP. As noted above, TPP caused a much larger decrease in binding at low-affinity sites, and catalytic turnover did not tend to reverse this effect of TPP.

Discussion

The PDH_a component catalyzes the rate-limiting step in the overall reaction catalyzed by the pyruvate dehydrogenase complex (Cate et al., 1981), and we have found that, in the presence of excess dihydrolipoyl dehydrogenase, the fractional binding of PDH_a component to the dihydrolipoyl transacetylase core could be determined from the fractional catalytic activity at binding equilibrium for a given concentration of complex. In all our studies we have used intact complex rather than the resolved component as a source of PDH_a because we have been unable to obtain resolved PDH_a with a specific activity (as the limiting component in reconstitution assays) greater than 60% of that for PDH associated with the intact complex. In the presence of TPP, analysis of the fraction bound in a Scatchard plot indicated about 7 high-affinity and 13 low-affinity sites for binding of PDH_a to the dihydrolipoyl transacetylase core with apparent negative cooperativity for binding of PDH_a at low-affinity sites.

In addition to the large differences in affinity, we have detected other distinct features for the high- and low-affinity binding sites. In the presence of TPP, catalytic turnover strengthened binding at high-affinity sites but not at low-affinity sites. On the other hand, Mg^{2+} and increased ionic strength increased the rate of dissociation from low-affinity sites. In the absence of TPP, increased ionic strength decreased equilibrium binding at low-affinity sites but did not detectably alter PDH_a binding at high-affinity sites.

Initial velocities for samples equilibrated in the absence of TPP indicated that PDH_a binding was about 40 times tighter at low-affinity sites than it was in the presence of TPP, with negative cooperativity not detected. When TPP was absent, the experiments could not be fitted well by a simple model using two classes of sites, but the data appear to show a transition to a class of even tighter binding high-affinity sites than was observed in the presence of TPP.

Kresze & Ronft (1980) and Bleile et al. (1981) have established that the subunits of the dihydrolipoyl transacetylase in the bovine kidney and heart complex are composed of two domains, an inner-structural domain (M_r 26 000) and a large acidic extension with a bound lipoyl moiety (M_r 28 000). Furthermore, Bleile et al. (1981) presented evidence that both the inner and outer domains were required for binding of PDH_a. This contrasts with the *Escherichia coli* complex in which PDH binds tightly to an analogous inner-structural domain (Bleile et al., 1979). The PDH component in the *E. coli* complex apparently does not dissociate at a significant rate (Hale et al., 1979), in contrast to PDH in the kidney complex (Cate & Roche, 1979; Cate et al., 1980, and data herein).

On the basis of the amino acid composition (Barrera et al., 1972) and migration in isoelectric focusing gels (T. E. Roche, unpublished results), the α-subunit of PDH_a has a rather basic isoelectric point, $pI > 7.5$. As noted above, the lipoyl domain of the transacetylase is rather acidic, $pI = 4.6$ (Bleile et al., 1981), and association of these structural units by ionic interactions seems likely. The enhanced dissociation of PDH_a elicited by millimolar Mg^{2+} or increased ionic strength (with KCl) observed in the present work as well as the large changes in affinity of PDH_a upon phosphorylation (Pratt et al., 1979) may involve changes in the electrostatic interaction between these structural domains. In addition, since TPP appreciably reduces PDH_a affinity and essentially eliminates the effect of ionic strength on binding of PDH_a at low-affinity sites, it seems likely that TPP alters an ionic interaction in reducing PDH_a binding.

In addition to determination of equilibrium constants, various approaches were used to determine the rate constants for binding of PDH_a at high- and low-affinity sites on the transacetylase core. Since with samples equilibrated at <0.02 nM, initiation of turnover resulted in further binding at high-affinity sites, analyses of such time courses allowed us to segregate the rate constants for binding at high-affinity sites. A very high k_a value of 1.2×10^8 M^{-1} s^{-1} was determined. With only mild orientation requirements, this value would appear to be diffusion limited (Schmitz & Schurr, 1972). The 60-subunit transacetylase core approximates a fixed surface with several sites available for binding of the PDH_a tetramer. This very high rate of association suggests that, with about five PDH_a's bound, additional binding of PDH_a is not restricted to the remaining high-affinity sites. An interesting possibility is that, upon binding at a low-affinity site, PDH_a moves to a high-affinity site without dissociating from the surface of the dihydrolipoyl transacetylase. Such two-dimensional diffusion (i.e., intracore movement) appears to facilitate PDH_a kinase activity as shown in the following paper (Brandt & Roche, 1983).

We have also conducted studies on bovine heart complex which contains 30 PDH_a's per core in the purified complex. A larger number of sites for binding PDH_a was observed with a similar pattern for binding of PDH_a in the presence of TPP, i.e., a two-phase Scatchard plot (Brandt, 1981). Further studies are required to determine why the core of the heart complex, which seems very similar to the analogous structure from kidney, binds so much PDH_a.

Some final comments are useful regarding routine spectrophotometric assays of the kidney complex at the usual level of 1–5 μg of complex/mL. We have found that the slow falloff in rate normally observed is greatly reduced by addition of excess transacetylase core (D. R. Brandt and T. E. Roche, unpublished results). This result is consistent with the reduced affinity of PDH_a for transacetylase core during catalytic turnover in the presence of TPP. In this study, as with all other experiments reported in the present work, excess dihydrolipoyl dehydrogenase was present. The dissociation of that component from the dihydrolipoyl transacetylase could also be detected when excess levels of the flavoprotein were not added. Since the dihydrolipoyl dehydrogenase does not catalyze a rate-limiting step in the overall reaction, the falloff in rate due to its dissociation cannot be simply interpreted to define a dissociation constant for the dihydrolipoyl dehydrogenase.

Acknowledgments

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Appendix

If a change in PDH_a binding is restricted to one of the two classes of transacetylase binding sites, the approach to equilibrium of pyruvate dehydrogenase binding to the transacetylase core is defined by

$$\frac{d(PL)}{dt} = k_a(P)_t(L)_t - k_d(PL)_t \quad (1)$$

where the subscript t refers to the concentration at time t. (P) and (L) are the free concentrations of pyruvate dehydrogenase tetramer and the available class of core binding sites, respectively; (PL) is the concentration of active complex produced by tetramer binding of those sites. Since at equilibrium

$$k_a(P)_{eq}(L)_{eq} = k_d(PL)_{eq} \quad (2)$$

where the subscript eq refers to the concentration at equilibrium, eq 1 can be rearranged as follows after substituting in the appropriate conservation equations:

$$\frac{d(PL)}{dt} = \frac{k_a}{(PL)_{eq}}[(PL)_{eq} - (PL)_t][(P)_T(L)_T - (PL)_t(PL)_{eq}] \quad (3)$$

where the subscript T refers to the total concentration. (For low-affinity sites the total concentration of PDH_a must be corrected for PDH_a bound at high-affinity sites.) Integration of eq 3 from zero time to time t is of the form

$$\int_{(PL)_i}^{(PL)_t} \frac{d(PL)}{a + b(PL) + c(PL)^2} = \frac{k_a t}{(PL)_{eq}} = \Big|_{(PL)_i}^{(PL)_t} \frac{1}{q^{1/2}} \ln \frac{2c(PL) + b - q^{1/2}}{2c(PL) + b + q^{1/2}} \quad (4)$$

where $(PL)_i$ equals the initial concentration of tetramer bound to the available class of transacetylase sites, $a = (P)_T(L)_T \cdot (PL)_{eq}$, $b = -(PL)_{eq}^2 - (P)_T(L)_T$, $c = (PL)_{eq}$, and $q^{1/2} = (b^2 - 4ac)^{1/2} = (P)_T(L)_T - (PL)_{eq}^2$. Making the appropriate substitutions and inserting the limiting values of PL yields eq 5.

$$k_a t = \frac{(PL)_{eq}}{[(P)_T(L)_T - (PL)_{eq}^2]} \times \ln \frac{[(PL)_{eq}(PL)_t - (P)_T(L)_T][(PL)_i - (PL)_{eq}]}{[(PL)_{eq}(PL)_i - (P)_T(L)_T][(PL)_t - (PL)_{eq}]} \quad (5)$$

The equation for $k_d t$ is obtained by taking the product of $k_a t$ and K_d or may be derived from eq 1 after appropriate substitution for k_a from eq 2 and is equal to eq 6.

$$k_d t = \frac{[(P)_T - (PL)_{eq}][(L)_T - (PL)_{eq}]}{[(P)_T(L)_T - (PL)_{eq}^2]} \times \ln \frac{[(PL)_{eq}(PL)_t - (P)_T(L)_T][(PL)_i - (PL)_{eq}]}{[(PL)_{eq}(PL)_i - (P)_T(L)_T][(PL)_t - (PL)_{eq}]} \quad (6)$$

Rate constants were determined by least-squares fitting of the calculated value $k_a t$ (or $k_d t$) vs. time. $(PL)_t$ is directly proportional to $\Delta A_{340}/\Delta t$ for high-affinity transacetylase sites; for low-affinity sites, the rate due to PDH_a bound at high-affinity sites was subtracted from the total rate.

Registry No. PDH, 9014-20-4; TPP, 154-87-0; pyruvate decarboxylase, 9001-04-1; dihydrolipoyl transacetylase, 9032-29-5; Mg, 7439-95-4.

References

Barrera, C. R., Namihira, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 327–342.

Bleile, D. M., Munk, P., Oliver, R. M., & Reed, L. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4385–4389.

Bleile, D. M., Hackert, M. L., Pettit, F. H., & Reed, L. J. (1981) *J. Biol. Chem. 256*, 514–519.

Brandt, D. R. (1981) Ph.D. Dissertation, Kansas State University.

Brandt, D. R., & Roche, T. E. (1983) *Biochemistry* (following paper in this issue).

Cate, R. L., & Roche, T. E. (1979) *J. Biol. Chem. 254*, 1659–1665.

Cate, R. L., Roche, T. E., & Davis, L. C. (1980) *J. Biol. Chem. 255*, 7556–7562.

Ferguson-Miller, S., & Koppenol, W. H. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 4–7.

Hale, G., Bates, D. L., & Perham, R. N. (1979) *FEBS Lett. 104*, 343–346.

Hamada, M., Otsuka, K., Tanaka, N., Ogasahara, K., Koike, K., Hiraoka, T., & Koike, M. (1975) *J. Biochem. (Tokyo) 78*, 187–197.

Klotz, I. M., & Hunston, D. L. (1971) *Biochemistry 10*, 3065–3069.

Kresze, G.-B., & Ronft, H. (1980) *Eur. J. Biochem. 112*, 589–599.

Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 327–342.

Machicao, F., & Wieland, O. H. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1093–1106.

Pratt, M. L., & Roche, T. E. (1979) *J. Biol. Chem. 254*, 7191–7196.

Pratt, M. L., Roche, T. E., Dyer, D. W., & Cate, R. L. (1979) *Biochem. Biophys. Res. Commun. 91*, 289–296.

Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.

Reed, L. J., & Oliver, R. M. (1968) *Brookhaven Symp. Biol. No. 21*, 397–412.

Roche, T. E., & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun. 48*, 840–846.

Roche, T. E., & Cate, R. L. (1977) *Arch. Biochem. Biophys. 183*, 664–677.

Schmitz, K. S., & Schurr, J. M. (1972) *J. Phys. Chem. 76*, 534–545.

Sugden, P. H., & Randle, P. J. (1978) *Biochem. J. 173*, 659–668.

Weston, R. E., Jr., & Schwarz, H. A. (1972) in *Chemical Kinetics*, pp 165–171, Prentice-Hall, Englewood Cliffs, NJ.