Specificity of the Pyruvate Dehydrogenase Kinase for Pyruvate Dehydrogenase Component Bound to the Surface of the Kidney Pyruvate Dehydrogenase Complex and Evidence for Intracore Migration of Pyruvate Dehydrogenase Component†

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ABSTRACT: Using the bovine kidney pyruvate dehydrogenase complex we have investigated the mechanism whereby about three pyruvate dehydrogenase (active form) kinase molecules, tightly bound to the dihydrolipoyl transacetylase core, can rapidly phosphorylate and inactivate about 20 pyruvate dehydrogenase (active form) (PDH_a) tetramers which are also bound to the 60-subunit core. Evidence is presented that PDH_a kinase activity is not serviced by a process of dissociation and reassociation of PDH_a. Rapid inactivation of a full complement of PDH_a occurs at a rate exceeding the rate of dissociation of PDH_a, indicating that a PDH_a must move to the fixed kinase subunits without dissociating from the dihydrolipoyl transacetylase core. Consistent with that concept, at low concentrations of complex where a significant portion of PDH_a is free, bound PDH_a was inactivated at a rate equivalent to that at higher concentrations of complex, and free PDH_a was phosphorylated more slowly at a rate closely approximated by the rate of association of free PDH_a with the transacetylase core. Thus, with a low number of PDH_a molecules bound, PDH_a either is preferentially positioned for phosphorylation and inactivation by PDH_a kinase or can rapidly become so positioned without dissociating from the transacetylase core.

In addition to having three catalytic components, a distinctive feature of the pyruvate dehydrogenase complex from mammalian tissues (as well as other eucaryotic cells) is the presence of two regulatory enzymes, a kinase and a phosphatase. These serve to regulate the pyruvate dehydrogenase component by interconversion between an active (nonphosphorylated) form, PDH_a,[1] and an inactive (phosphorylated) form, PDH_b. The kinase is tightly bound to the dihydrolipoyltransacetylase core of the complex (Linn et al., 1972); the phosphatase associates with the core in a reversible reaction which requires Ca^{2+} (Pettit et al., 1972). A limited number of PDH_a kinase molecules, about three per core (Pratt & Roche, 1979), phosphorylate and inactivate a full complement of PDH_a molecules (20 $\alpha_2\beta_2$ tetrameric units per core in the kidney complex).[2] Similarly, a low number of PDH_b phosphatase molecules (one per core; M. L. Pratt and T. E. Roche, unpublished results) can rapidly and completely reactivate a full complement of PDH_b molecules. Furthermore, when PDH_a or PDH_b is present at levels in excess of binding sites on the transacetylase, inactivation or reactivation continues at a high rate and may involve exchange of product and substrate forms of PDH at specific sites on the surface of the complex (Pratt et al., 1979). Kinase molecules do not dissociate from the core under a variety of chaotropic conditions that completely remove the other components of the complex. Ca^{2+}-facilitated binding of PDH_b phosphatase to the core occurs even when all other components of the complex are present and bound. Thus it seems likely that these converter enzymes are located at specific and limited sites on the transacetylase core.

Clearly, phosphorylation or dephosphorylation requires the close approach of the appropriate substrate form of PDH to the appropriate converter enzyme; it is likely, for instance, that the PDH component must move to sustain PDH_a kinase activity. The first requisite for such a mechanism, mobility of PDH, has been established (Cate & Roche, 1979; Cate et al., 1980). Furthermore, Pratt et al. (1979) have described results consistent with the hypothesis that kinase activity is sustained by movement of PDH_a into and movement of PDH_b out of sites adjacent to a fixed PDH_a kinase. Here we present evidence that dissociation of PDH_a from distant sites and reassociation at sites near the kinase is too slow to account for the observed rates of phosphorylation. PDH_a kinase is supported by a more rapid process involving intracore movement of PDH_a.

Experimental Procedures

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

A highly purified preparation of bovine kidney pyruvate dehydrogenase complex (14.5 μmol of NADH min^{-1} mg^{-1}) 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.

Measurement of PDH_a Intercore Movement. NEM-inactivated PDC was prepared, and data were analyzed as previously described (Brandt et al., 1983). Five micrograms of inactive commplex was added to 1.0-mL final volume in a

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[1] 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; PDC, pyruvate dehydrogenase complex; NEM, *N*-ethylmaleimide; TPP, thiamin pyrophosphate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

[2] Phosphorylation of one α-subunit per $\alpha_2\beta_2$ unit of PDH_a appears to lead to inactivation of an entire PDH tetramer (Yeaman et al., 1978).

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cuvette at 15 °C containing 10.0 μg of dihydrolipoyl transacetylase, 2.0 mM $MgCl_2$, 0.24 mM CoA, 5.3 mM cysteine hydrochlorides 2.0 mM pyruvate, 2.0 mM NAD, 0.2 mM thiamin pyrophosphate (TPP), and 0.5 mM EDTA in a 50 mM potassium phosphate buffer (pH 7.2). The influence of solvent ionic strength and/or viscosity on movement was studied by adding KCl and/or glycerol (final concentrations 100 mM and 10% v/v, respectively).

Kinase Activity Compared to Intercore Movement. For studies on changes in PDH_a activity done in parallel with investigations on PDH_a intercore movement, complex (1.0 mg/mL) was incubated for 1 min at 15 °C in a buffer containing 0.50 mM EDTA, 1.50 mM $MgCl_2$, and 50 mM potassium phosphate (pH 7.2). Glycerol and/or KCl was used to manipulate ionic strength and viscosity as described above. Phosphorylation was initiated by the addition of ATP to a final concentration of 0.10 mM, and aliquots of the reaction mixture were assayed for NADH production after 15 and 60 s by the method of Hucho et al. (1972).

Effect of PDH_b Binding to Transacetylase in Dilute Solutions in the Presence and Absence of TPP. These and subsequent studies were conducted in buffer B consisting of 50 mM Mops-K (pH 7.2), 50 mM KCl, 2.0 mM EDTA, 2.5 mM $MgCl_2$, 0.20 mg/ml BSA, 2.0 mM dithiothreitol, and 0.50 μg/mL leupeptin (ionic strength = 0.11 M).

To test the effect of PDH_b on PDH_a binding, phosphorylated (and inactivated) complex was prepared by treating complex (1.0 mg/mL) in buffer B with ATP (0.10 mM) for 3 min at 30 °C. Active complex (also denoted mock-treated complex) was treated in the same manner except that ATP was omitted. Hexokinase and glucose (final concentrations 25 units/mL and 10 mM, respectively) were added to both incubation mixtures, and the samples were put on ice after a 2-min additional incubation. Active complex and a sample containing equal amounts of active and inactive complex (denoted "mixed complex") were incubated for 10 min at 30 °C and then put on ice. After treatment, the activity of mock-treated complex was essentially unchanged, and the activity of the mixed complex was 51.1 ± 0.9% of the mock-treated complex, showing that ATP was completely hydrolyzed and the inactivation reaction stopped by the hexokinase/glucose treatment. Moreover, the presence of PDH_b does not reduce the activity of the PDH_a present in active complex when assayed at 1.0 mg/mL.

The effect of PDH_b on PDH_a binding to catalytic sites was further assessed in dilute solutions by incubating both the mock-treated and mixed complex at 2.0 μg/mL in the presence or absence of TPP (0.20 mM) for 20–35 min at 30 °C in buffer B. Activity measurements were taken in triplicate both prior to and subsequent to incubation. The fraction PDH_a bound was taken to be equal to the ratio of the equilibrium and initial rates as described in the preceding paper (Brandt et al., 1983).

Determination of the Fractional Binding of PDH_a. PDH_a binding was measured as outlined previously (Brandt et al., 1983). Samples were incubated in buffer B (μ = 0.11 M) for 10–15 min at 30 °C in the presence or absence of TPP. Low enzyme concentrations (≤1 μg/mL complex) were assayed in buffer B (final volume 1.0 mL) by the addition of concentrated substrates and dihydrolipoyl dehydrogenase. Higher enzyme concentrations (≥10 μg/mL) were assayed by dilution of complex in buffer B into buffer B plus substrates.

Kinase Activity Associated with Complex Equilibrated at Various Concentrations in the Presence or Absence of TPP. PDH_a kinase activity was correlated with the fraction of PDH_a bound at equilibrium for concentrations of complex from 0.10 μg/mL to 1.0 mg/mL. Complex was incubated 15 min at 30 °C in buffer B and the fraction of bound PDH_a determined as described above and in the preceding paper (Brandt et al., 1983). PDH_a kinase activity was determined by initiation of the reaction with ATP (final concentration 0.10 mM). For complex at concentrations of 10.0 μg/mL and above, aliquots were assayed for NADH production by the method of Hucho et al. (1972) at the times indicated in the figure legends. For complex at concentrations less than 10.0 μg/mL, residual activity of the complex was measured following the addition of hexokinase (25 units), glucose (10.0 μmol), dihydrolipoyl dehydrogenase (6.5 μg), and concentrated substrates to a final volume of 1.00 mL. With these levels of hexokinase and glucose, ATP was completely hydrolyzed, in less than 2 s as determined with luciferase. The substrates were added as a 200-fold concentrate of freshly mixed CoA, NAD^+, and TPP. Pyruvate was added separately as a 100-fold concentrate. The final concentrations of these substrates and cofactors were as indicated above. Activity increases with time as free PDH_a associates with complex; kinase activity was based on the maximum rate achieved. For these dilute samples, the maximum rate does not reflect total PDH_a but the amount of PDH_a tetramer bound. The amount of bound PDH_a is, however, proportional to the total present; control experiments established that the fraction of PDH_a bound does not change as the amount of PDH_b increases. Thus, kinase activity could be determined directly from the equilibrium catalytic rate before and after inactivation of PDH_a by the kinase. In addition, inactivation was monitored in some studies by assays in which excess transacetylase core (8.7 μg/mL) was added prior to initiation of catalytic turnover. Under those conditions, 99% of the PDH_a was bound.

Evaluation of the Rate Constants of Association of Free PDH_a with Transacetylase following Phosphorylation of Bound PDH_a and Determination of Whether That Association Limits PDH_a Kinase Activity. Since with dilute samples of complex, kinase preferentially inactivates bound PDH_a (as detailed under Results), NADH production increases with time in subsequent assays of overall activity as a new equilibrium of bound and free PDH_a is achieved in the presence of the PDH_b formed. The time course can be evaluated to determine the rate constants for association (k_a) and dissociation (k_d). Equations for these rate constants were derived by assuming that dissociation or movement of PDH_b does not limit this process. Decreases in k_a values from those found at these concentrations of complex in the absence of PDH_b are interpreted as resulting from PDH_b interference. Such interference by a full complement of PDH_b is not detected at high concentrations of complex and free PDH_a in the absence of TPP (Pratt et al., 1979). The time course for the association of PDH_a to the dihydrolipoyl transacetylase cores can be fitted to eq 1 (PL) is the concentration of the catalytic unit produced

$$k_{at} = \frac{(PL)_{eq}}{(P)_T(L)_T - (PL)_{eq}^2} \times \ln \frac{(PL)_{eq}[(L)_T(P)_T - (PL)_{eq}(PL)_t]}{(L)_T(P)_T[(PL)_{eq} - (PL)_t]} \quad (1)$$

by tetramer binding to core, and (P) and (L) are the levels of PDH_a and transacetylase binding sites, respectively. The subscripts eq and t refer respectively to the concentration of the indicated species at equilibrium and at any time t. The subscript T refers to the total concentration of the indicated species which, in the case of PDH_a, must be corrected for the PDH_b present.

The rates of association, determined from these analyses, were compared with the rate at which dilute complex phosphorylated PDH_a after initially bound complex was depleted and only free PDH was available for phosphorylation. Since, as detailed under Results, initially bound PDH_a is preferentially phosphorylated and inactivated, the rate of phosphorylation subsequent to the inactivation of that PDH_a would be limited by the rate of association of PDH_a if the rate of inactivation exceeds the rate of association. The inactivation time course subsequent to conversion of the initially bound PDH_a to PDH_b [designated $(P)_B$ below] can be fit by the expression where $(P)_T$ equals the total PDH, $(L)_T$ equals the

$$\% \text{ inactivation} = \frac{(P)_B + (P)_F[1 - \exp[-k_a t(L)_T]}{(P)_T} \times 100 \quad (2)$$

number of preferred (high-affinity) sites available on the transacetylase, and $(P)_F$ equals the number of initially free PDH_a. The fraction of free PDH_a that has undergone inactivation at any time t is described by the expression $(P)_F \exp[-k_a t(L)_T]$ where k_a is the calculated value which best fits the observed data.

Results

We estimate that the catalytic rate constant for PDH_a kinase activity is of the order of 10^{-1} s^{-1} (for instance, we calculate a value of 0.27 s^{-1} from typical data at 1.0 mg/mL complex assuming three PDH_a kinase per complex) while k_d for PDH dissociation is of the order of 10^{-3}–10^{-2} s^{-1} (Cate et al., 1980; Brandt et al., 1983). These data suggest that PDH_a dissociation cannot contribute to PDH_a kinase activity. However, a cautious interpretation is required because the rate constant for PDH_a kinase was determined at high concentrations of complex, and the rate constants of dissociation were determined at low concentrations of complex (<0.20 μg/mL for dissociation profiles and maximally 10.0 μg/mL for dissociation from NEM-inactivated cores). We have attempted, in the present paper, to determine whether conditions increasing k_d also increase PDH_a kinase activity. We also investigated PDH_a kinase function at low concentrations of complex where kinase activity can be related directly to PDH_a association, and the relative utilization of bound and free PDH_a can be determined.

Correlation between Kinase Activity and Intercore Movement of PDH_a. In the preceding paper (Brandt et al., 1983) we observed that the rate constant for PDH_a dissociation increased with increasing ionic strength. Further studies were conducted to determine whether PDH_a kinase activity was increased under conditions which enhance the rate of PDH_a dissociation. Studies were conducted in 50 mM potassium phosphate (pH 7.2) which contains a sufficient concentration of K^+ to saturate specific effects of K^+ ion on PDH_a kinase activity (Roche & Reed, 1974; Pettit et al., 1975; Cate & Roche, 1978). Addition of further salt reduced PDH_a kinase activity, with 60 mM KCl causing only a 13% reduction and more chaotropic salts causing larger decreases at the same ionic strength. That level of KCl caused a 32% increase in the rate constant for dissociation of PDH_a. When similar studies were conducted in the presence of 20% glycerol which reduced both PDH_a kinase activity (19%) and the k_d for PDH_a dissociation (24%), 60 mM KCl reduced PDH_a kinase activity (23%) while appreciably increasing the k_d for PDH_a (122%). Thus, dissociation of PDH_a tetramers occurs at or below the rate of PDH_a kinase activity (Cate et al., 1980), and salt-enhanced PDH_a dissociation was associated with decreased PDH_a kinase activity. PDH_a dissociation does not appear to be a rate-limiting step in PDH_a kinase activity.

Table I: Influence of PDH_b on the Functional Binding of PDH_a at Equilibrium (γ_{eq})[a]

PDH_b (%)	TPP (μM)	γ_{eq} experimental	γ_{eq} simulated
0	0	0.82 ± 0.01	0.79[b]
50	0	0.92 ± 0.04	0.95[b]
0	200	0.39 ± 0.02	0.42[c]
50	200	0.42 ± 0.01	0.73[c]

[a] The concentration of complex was 2.0 μg/mL and data were measured in triplicate. Simulated data were calculated by using the binding constants given in the preceding paper (Brandt et al., 1983) based on the assumptions described in the text. Details of the experiment are described under Experimental Procedures. [b] $K_d = 1 \times 10^{-12}$ M, $n = 7$; $K_d = 5.9 \times 10^{-10}$ M, $n = 13$. [c] $K_d = 1.5 \times 10^{-11}$ M, $n = 7$; $K_d = 2.5 \times 10^{-8}$ M, $n = 13$.

Capacity of PDH_b To Displace PDH_a from Sites on the Dihydrolipoyl Transacetylase Where PDH_a Participates in the Overall Reaction. Pratt et al. (1979) presented evidence that PDH_a displaces PDH_b from sites on the transacetylase core when PDH_a and PDH_b are present in large excesses over transacetylase core; the observed effect was based on initial velocities measured after a 12-min preincubation of resolved components in a buffer lacking TPP. Additional studies (Table I) were required to evaluate the effects of PDH_b on PDH_a binding in the presence and absence of TPP. In contrast to the above studies, experiments described in the present paper and in the preceding paper (Brandt et al., 1983) were performed with the kidney complex where the PDH to core ratio was constant and the amount of PDH did not exceed the number of binding sites on the transacetylase core. In addition, some studies were carried out in the presence of TPP which loosened binding of PDH_a to a weaker class of binding sites on the transacetylase by about 40-fold (Brand et al., 1983).

The studies, shown in Table I, were conducted with fully active complex or with complex in which half of the PDH was in the inactive form, PDH_b. Samples of complex were equilibrated at a total concentration of 2.0 μg/mL (2.86×10^{-10} M) where a portion of PDH_a is free. The fractional binding of PDH_a was determined as described under Experimental Procedures and in the preceding paper (Brandt et al., 1983). The experimental results were compared with values calculated assuming that PDH_b did not compete significantly with PDH_a at sites where PDH_a binds to participate in catalysis. If this assumption is correct, one would expect that conversion of part of the initial complement of PDH_a to PDH_b would increase the fractional binding of the remaining PDH_a.

The results are shown in Table I. In the absence of TPP, measured and calculated values agreed well, indicating that PDH_b did not inhibit PDH_a binding to sites on the transacetylase core where PDH_a participates in the overall reaction (Table I). PDH_b did appear to compete with PDH_a in the presence of TPP. Calculations using the constants determined in the previous paper (Brand et al., 1983) closely approximated the observed binding of PDH_a when PDH_b was absent but did not when half of the PDH was present as PDH_b. This indicated either that PDH_b competes directly with PDH_a for sites on the transacetylase core where PDH_a is engaged in catalysis or that PDH_b tetramers bound at neighboring sites sterically hinder PDH_a binding.

The data in the presence of TPP are consistent with nearly equivalent binding of PDH_a and PDH_b. This assumption also fits data observed over a range of PDH_a:PDH_b ratios (data not shown). For the experimental conditions where the ratio of total PDH to sites on the transacetylase and the sum con-

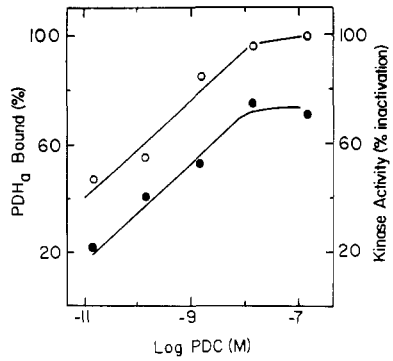

FIGURE 1: Correlation of PDH_a kinase activity with percent PDH_a bound to the transacetylase core in the absence of TPP. The percent PDH_a bound (O) and kinase activity at 15 s (●) were determined at the indicated concentrations of complex (PDC) by the methods described under Experimental Procedures.

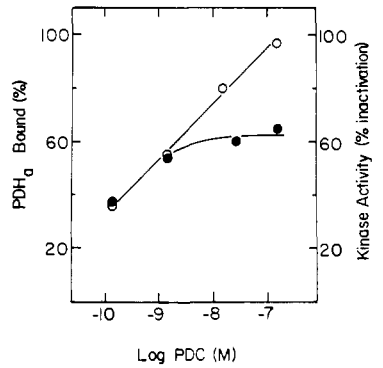

FIGURE 2: Correlation of PDH_a kinase activity with percent PDH_a bound to the transacetylase core in the presence of TPP. The percent PDH_a bound (O) and kinase activity at 30 s (●) were determined as described under Experimental Procedures. The TPP concentration was 0.20 mM, and the concentrations of complex (PDC) were as indicated.

centration of PDH_a and PDH_b are constant, equal competition of PDH_a and PDH_b results in a constant fractional binding of PDH_a (PDH_a bound/PDH_a total) as the PDH_a to PDH_b ratio is varied. Thus, in studies of PDH_a kinase activity at low concentrations of complex, residual equilibrium activities measured in the presence of TPP should approximate the percent inactivation of PDH_a since the fraction of PDH_a bound would be a constant fraction of the total PDH_a remaining.

Correlation between Kinase Activity and Percent PDH_a Bound. Above, we have shown a lack of correlation between changes in kinase activity and the rate of intercore movement. An interesting possibility is that kinase activity is maintained by a rapid movement of bound PDH_a along the surface of the cores. This hypothesis predicts that all bound PDH_a would be rapidly available to the kinase even when only a few PDH_a tetramers are distributed over the core. The remaining studies evaluate that possibility.

Kinase activity[3] was determined over a wide range of concentrations of complex, giving a range of fractional binding of PDH_a (Brandt et al., 1983). For assays in which TPP was absent, the kinase reaction was allowed to proceed 15 s wheras a 30-s period was used in the presence of TPP, an inhibitor of the kinase (Roche & reed, 1972). There was a strong correlation between the initial rate of PDH_a inactivation and the percent PDH_a bound both in the absence of TPP (Figure 1) and in the presence of TPP (Figure 2). Furthermore, in the absence of TPP, the reaction rate saturated only when a full complement of PDH was bound (Figure 1). This suggests that virtually all the bound PDH_a is rapidly available to the kinase. In contrast, the maximum rate of PDH_a kinase was lower and was reached at a lower fractional binding (~60%) in the presence of TPP. In a study of kinase activity conducted in the absence of TPP, excess transacetylase core was added after termination of kinase activity, so that essentially all PDH_a was bound in subsequent assays of the overall reaction. The same pattern for changes in kinase activity with fractional binding of PDH_a was observed.

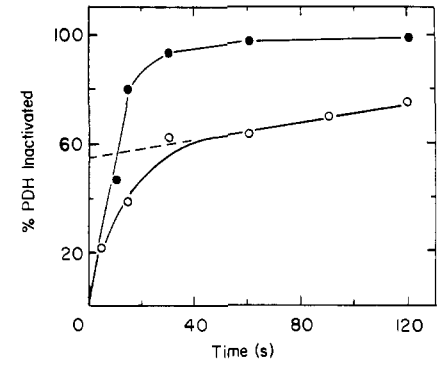

FIGURE 3: Time course of inactivation of PDH_a by PDH_a kinase in the absence of TPP at a high and low concentration of complex. The time course of kinase activity was determined for concentrations of complex of 1.0 mg/mL (0.143 nM) (●) and 1.0 μg/mL (0.143 pM) (O). All other conditions are described under Experimental Procedures.

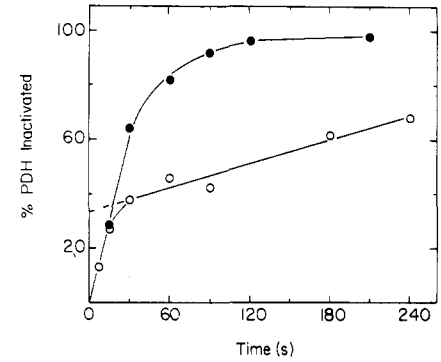

FIGURE 4: Time course of inactivation of PDH_a by PDH_a kinase in the presence of TPP at a high and low concentration of complex. The time course of kinase activity was determined for concentration of complex of 1.0 mg/mL (0.143 nM) (●) and 1.0 μg/mL (0.143 pM) (O) in the presence of 0.20 mM TPP. All other conditions are described under Experimental Procedures.

The experiments shown in Figures 3 and 4 suggest that initially bound PDH_a is inactivated much more rapidly than unbound component. In these experiments, the loss of PDH_a activity over an extended period was compared at a high (1.0 mg/mL; 1.43×10^{-7} M) and at a low (1.0 μg/mL; 1.43×10^{-10} M) concentration of complex. The comparison was done in the absence (Figure 3) and in the presence (Figure 4) of TPP. At the higher concentration, nearly all of the PDH_a was bound. At the lower concentration of complex, 35% of the PDH was bound when TPP was present and 60% when it was not. In the absence of TPP, the initial rates for inactivation of PDH_a were identical for complex incubated at 1.0 μg/mL and 1.0 mg/mL. At 1.0 mg/mL, more than 95% of the PDH_a was inactivated rapidly. At the lower concentration, the initial rapid burst of inactivation was followed by continued inactivation at a greatly diminished rate (Figure 3). The slow rate of inactivation of PDH_a after 40 s for 1.0 μg/mL complex

[3] Loss of overall activity with the time has been used as a standard velocity assay of PDH_a kinase, and we also use that terminology. More precisely percent inactivation with time has units of s^{-1} and constitutes a measure of a rate constant rather than a rate.

extrapolates back to 55% on the ordinate, which closely approximates the percent PDH_a initially bound.

TPP dramatically loosens PDH_a binding at the weaker class of binding sites on the transacetylase core (Brandt et al., 1983), and it also alters the initial rate for kinase activity at high enzyme concentrations (Roche & Reed, 1972). It was, therefore, of interest to determine the effect of TPP on the time course of PDH_a inactivation for complex at 1.0 mg/mL and 1.0 μg/mL. As shown in Figure 4, the initial rates of PDH_a inactivation were slower than in Figure 3 due to the effect of TPP, but similar initial rates were observed at the high and low concentrations of complex. Following the initial rate, the ensuing rate at 1.0 μg/mL extrapolates to 33% on the ordinate, which again closely approximates the percent PDH_a bound at that concentration.

In either the presence or absence of TPP, complex at 1.0 μg/mL exhibited no enzymatic activity upon initiation of the overall reaction after the kinase had inactivated greater than 60% of the total PDH_a. Subsequent to initiation of catalysis, the rate of NADH production increased from zero to a maximal value. This observation indicates that the rapid phase of the kinase reaction inactivated all the initially bound PDH_a and that recoery of catalytic activity reflected the reequilibration of free PDH_a with the complex.

These observations clearly indicate that the kinase preferentially inactivated PDH_a bound to the core component and suggest that subsequent kinase activity was limited by the rate of association of PDH_a. In all of the above studies, kinase activity, at low concentrations of complex, was based on the maximmal rates achieved in the presence of TPP, and therefore, these rates should be directly proportional to the fraction of active PDH_a (see earlier section). Analysis of the rate of association of free PDH_a and correlation of this rate to the rate of inactivation of initially free PDH_a are given below.

The results in the presence of TPP differ in a significant respect from those obtained without TPP. At 1.0 mg/mL the rate of inactivation of PDH_a diminished after about 60% PDH was inactivated (Figure 4). This result suggests that accumulation of PDH_b makes PDH_a less available to the kinase. If PDH_a with TPP bound were simply a poorer but available substrate, there would not be a falloff in rate until the amount of PDH_a was greatly reduced. The observation in Figure 2 that PDH_a kinase activity, in the presence of TPP, saturates at 60% PDH_a bound may also result in part from PDH_b interference. Such interference was indicated by the data in Table I and from analysis of the rates of binding of free PDH_a given below.

Studies on the Rate of Association of PDH_a Subsequent to Kinase Activity. As indicated above, following initiation of catalysis, the rate of NADH production increases as free PDH_a binds until a new equilibrium is attained. When this profile of NADH production following inactivation by PDH_a kinase was analyzed, the rate of association of PDH_a was determined under conditions of catalytic turnover. Equation 1 for the calculation of the rate constants of association (k_a) is given under Experimental Procedures. That equation assumes PDH_a binds at high-affinity sites without PDH_b interference. The value for k_a of $(6 \pm 1) \times 10^6$ M^{-1} s^{-1} was obtained from analysis of time courses where 60–90% of the total PDH_a was inactivated. Since the k_a for high-affinity sites determined in the preceding paper (Brandt et al., 1983) was 1.2×10^8 M^{-1} s^{-1}, the above value indicates some interference of PDH_b in the binding of PDH_a.

An association rate constant was calculated which gave the best fit for the slow rate of inactivation of free PDH_a (at 1.0 μg/mL complex) subsequent to the inactivation of bound PDH_a in the presence of TPP (Figure 4). By use of eq 2 given under Experimental Procedures and with the assumption that binding only occurred at high-affinity sites, a k_a of 3×10^6 M^{-1} s^{-1} was calculated. Thus, there is good agreement of this value and the k_a values calculated above. Becuase of the limiting assumptions, little significance can be placed on the absolute values, but the agreement suggests PDH_b hinders both activity regain and kinase inactivation of free PDH_a by slowing the rate of association. The agreement of the two rate constants also suggests that there must be an efficient mechanism for PDH_a becoming available to the kinase even when only a single PDH_a tetramer is bound in the presence of PDH_b. It should be noted that Pratt et al. (197) showed that, during a time course of inactivation of complex at 1.0 mg/mL, maximal kinase activity resumes when a large excess of PDH_a is added after most of the PDH_a initially present has been converted to PDH_b. Thus PDH_b formed does not prevent efficient utilization of PDH_a where a large excess of PDH_a is present.

Discussion

Our studies on the bovine kidney pyruvate dehydrogenase complex have investigated the mechanism whereby about 3 PDH_a kinase molecules, tightly bound to the dihydrolipoyl transacetylase core, can rapidly phosphorylate and inactivate about 20 PDH_a tetramers which are also bound to the 60-subunit core. Lack of correlation between changes in the rate of dissociation of PDH_a (measured as intercore movement) and the rate of PDH_a inactivation by the kinase indicated that a process of dissociation and reassociation of PDH_a does not serve to sustain kinase activity.

We have presented several observations supporting the concept that kinase activity is facilitated by movement of the PDH_a component to sites where an α-subunit of the PDH tetramer is readily phosphorylated by the kinase. Furthermore, our observations would appear to require a molecular mechanism in which PDH_a maintains contact with the transacetylase core as it moves to the kinase. The level of rapidly inactivated PDH_a decreased as the fractional level of bound PDH_a decreased. However, the initial rate of PDH_a inactivation was not reduced for dilute samples of complex in which a major fraction of PDH_a was free. That result indicates that all PDH_a kinase molecules are accessible to PDH_a when only a few PDH_a molecules are bound to the transacetylase. Since all molecules of PDH_a kinase are tightly bound to the core, then bound PDH_a molecules must either be positioned adjacent to the kinase or have a facile mechanism for moving to adjacent sites. If movement of PDH_a is required for the initial turnover, over data suggest that such movement is not rate limiting since reduced occupancy by PDH_a on the transacetylase core did not reduce the initial rate of PDH_a kinase activity. Further studies will be required to determine whether the presence of high- and low-affinity sites (Brandt et al., 1983) contributes to the two-dimensional translational diffusion of PDH_a on the surface of the transacetylase core.

Not only did inactivation of bound PDH_a occur in a highly preferential manner but also free PDH_a molecules were rapidly inactivated upon binding to the core. Three observations with complex at 1.0 μg/mL support that conclusion. First, following the period of rapid inactivation of bound PDH_a, no activity was initially detected when kinase activity was terminated and the overall reaction initiated; activity developed with time during a process of reequilibration as free PDH_a molecules bound to the transacetylase core. Second, a rate constant of association determined from the recovery of catalytic activity

agrees with a rate constant derived by fitting the time course for inactivation of initially free PDH_a. Thus it would appear that, under those conditions, the rate of inactivation of free PDH_a is limited by the rate of association. Finally, extrapolation of the slow inactivation of free PDH_a to zero time showed that the rapidly inactivated fraction was the same as the initial fraction of bound PDH_a, as would be expected if bound PDH_a was phosphorylated preferentially.

Bleile et al. (1981) have established that both of the domains of the transacetylase core, the inner-structural domain and the large flexible extension to which lipoyl groups are bound, are required for binding of the $\alpha_2\beta_2$ tetramer of PDH. One could envision association and dissociation of an $\alpha\beta$ region of a PDH tetramer with the flexible extensions as a mechanism contributing to intracore movement.

In previous studies, Pratt et al. (1979) observed that, when large excesses of PDH_a and PDH_b are available to transacetylase core, PDH_a is bound at least 10-fold better. Those results were based on competitive binding in the absence of TPP at concentrations of component that would result in saturation of all sites on the transacetylase core. In studies with dilute complex, we have found that, in the presence of TPP, PDH_b competes more effectively with PDH_a; PDH_b reduced both the equilibrium binding of PDH_a and the rate constant for association of PDH_a. It seems likely that the TPP-induced decrease in binding of PDH_a (Brandt et al., 1983) contributes to enhanced competition by PDH_b. Further work is needed to determine whether bound PDH_b decreases PDH_a binding, in the presence of TPP, by a competitive interaction at the same class of sites or by steric interaction between PDH_a and PDH_b bound at adjacent but different sites on the transacetylase core.

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Registry No. PDH, 9014-20-4; pyruvate decarboxylase, 9001-04-1; PDH kinase, 9074-01-5.

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