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ENHANCED DISSOCIATION OF PYRUVATE DEHYDROGENASE FROM THE PYRUVATE DEHYDROGENASE COMPLEX FOLLOWING PHOSPHORYLATION AND REGULATORY IMPLICATIONS

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Summary. We have shown that the active form of the pyruvate dehydrogenase (PDH_a) component exhibits at least a 9-fold greater affinity for sites on the dihydrolipoyl transacetylase core of the pyruvate dehydrogenase complex than does the inactive (phosphorylated) form of pyruvate dehydrogenase (PDH_b). Consistent with a higher rate of dissociation for PDH_b than for PDH_a, free PDH_a rapidly replaces PDH_b whereas, even at high levels, free PDH_b only slowly replaces PDH_a. Dissociation of newly formed PDH_b, during phosphorylation by the immobile PDH_a kinase, leads to an increased association of free PDH_a as observed by protection against inactivation of the complex, even though PDH_a kinase activity is increased.

The pyruvate dehydrogenase component of the pyruvate dehydrogenase complex is regulated by interconversion between an active, nonphosphorylated form (PDH_a) and an inactive, phosphorylated form (PDH_b) (1). Recent studies from this laboratory have demonstrated that PDH_a can exchange between sites on the dihydrolipoyl transacetylase core (2) and that this exchange, which is limited by the rate of dissociation of PDH_a, is rapid enough to be an important process in the regulation of PDH (3). PDH movement may be particularly important in the phosphorylation reaction catalyzed by PDH_a kinase, a converter enzyme tightly associated with the dihydrolipoyl transacetylase core. In the present communication we show that PDH_b has a much lower affinity for the transacetylase core than PDH_a and describe results consistent with the rapid replacement of PDH_b by PDH_a during catalysis by PDH_a kinase. The significance of the decreased affinity of PDH_b in the reactions catalyzed by both PDH_a kinase and PDH_b phosphatase is discussed.

MATERIALS AND METHODS

Highly purified preparations of bovine kidney and heart pyruvate dehydrogenase complex (13.8 and 18.3 μmol NADH/min/mg, respectively) were obtained by procedures previously described (4,5). Catalytic components of bovine kidney

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a, active nonphosphorylated form of the pyruvate dehydrogenase component; PDH_b, inactive, phosphorylated form; MOPS, 3-(N-morpholino)propanesulfonate.

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complex were obtained by resolution according to the procedures of Linn et al. (5). Pig heart dihydrolipoyl dehydrogenase was obtained from Boehringer-Mannheim. [γ^{32}P]ATP was obtained from New England Nuclear. To prepare PDH_b, resolved PDH_a was incubated with 65 µg (gradient experiments) or 16 µg (Fig. 1) transacetylase-kinase subcomplex/mg PDH_a for 16 hr at 20° in 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM $MgCl_2$, 35 mM phosphate buffer, pH 7.2, and 0.2 mM [γ^{32}P]ATP (~200,000 cpm/nmol). Using the same level of ATP, phosphorylated pyruvate dehydrogenase complex was prepared by incubating the complex for 20 min at 30° in Buffer A: 40 mM MOPS (adjusted to pH 7.2 with KOH) containing 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM EGTA. Following these incubations, excess ATP was removed by addition of hexokinase and glucose to final concentrations of 50 units/ml and 10 mM, respectively. ADP, glucose, glucose-6-phosphate and phosphate were removed by chromatography of 0.3 ml samples on a 0.9 x 12 cm G-50 Sephadex column equilibrated with Buffer A. The three 0.3-ml fractions containing the highest level of ^{32}P-labeled protein were pooled.

In gradient experiments, samples were equilibrated at 20° for 20 min, and applied to a linear 10 to 30% sucrose gradient (4.95 ml) in Buffer A. Centrifugation was conducted at 20° for 85 min at 40,000 rpm in a SW 50.1 rotor. Following centrifugation, the contents of each tube were fractionated by drop count into 0.30 to 0.36 ml fractions. As appropriate, aliquots of each gradient fraction were assayed for NAD-reduction activity and/or ^{32}P-radioactivity. The capacity of PDH_b samples to be reactivated was tested by treatment with PDH_b phosphatase for 20 min at 30° by the procedures of Pettit et al. (6). The activity of PDH_a regenerated was invariably somewhat higher in a reconstitution assay (7) than that of the PDH_a used to prepare PDH_b.

RESULTS AND DISCUSSION

The effects of addition of PDH_a to samples of phosphorylated pyruvate dehydrogenase complex and addition of PDH_b to nonphosphorylated complex were investigated under conditions of enzymatic turnover (i.e., in NAD-reduction assays). Bovine heart complex was primarily used in these studies because it has a full complement of PDH (30 $\alpha_2\beta_2$ units per complex [5]) although similar results were obtained with the kidney complex. An immediate and appreciable increase in the activity of highly phosphorylated pyruvate dehydrogenase complex (>2.5 ^{32}P/PDH) was observed upon addition of resolved PDH_a, even at low ratios of PDH_a to the PDH_b of the complex. The extent of reaction was similar when protein components were preincubated prior to assay. On the other hand, PDH_b caused only a small and slowly developing decrease in the activity of nonphosphorylated heart complex at large ratios (10:1) of PDH_b to the PDH_a of the complex. These observations indicate that PDH_a has a higher affinity for the dihydrolipoyl transacetylase core and that PDH_b dissociates more rapidly than PDH_a.

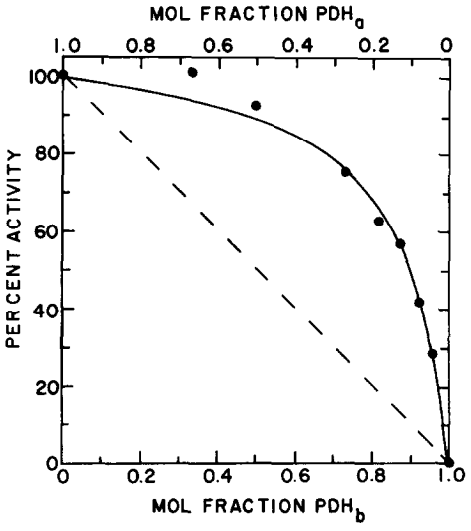


Fig. 1. Plot of the relative NAD-reduction activity at different mole fractions of PDH_a and PDH_b. At the mole fractions of PDH_a and PDH_b indicated, 75 µg of PDH was incubated with 1.2 µg of dihydrolipoyl transacetylase in 62.5 µl of buffer A for 12 min at 30°. In a 1.0 ml assay containing 10 µg of dihydrolipoyl dehydrogenase, 50 µl of each incubation mixture was assayed for NAD-reduction activity. The activity of the sample, in which PDH_a had a mole fraction of 1.0, was 23.5 nmol NADH/min and was assigned a value of 100%. The dashed and solid lines, respectively, represent theoretical curves for relative affinities of PDH_a:PDH_b of 1:1 and 9:1.

To determine the relative affinities of PDH_a and PDH_b, a constant amount of PDH, at various ratios of PDH_b to PDH_a, was incubated with a fixed level of dihydrolipoyl transacetylase. The amount of dihydrolipoyl transacetylase was sufficiently small so that quantities of PDH_a or PDH_b were saturating at all ratios used. Each mixture was assayed for NAD-reduction activity in the presence of a saturating level of dihydrolipoyl dehydrogenase and the resultant activities were plotted as a function of the mole fraction of PDH_a and PDH_b (Fig. 1). PDH_a exhibits at least a 9-fold greater affinity than PDH_b for sites on the dihydrolipoyl transacetylase core. This is a minimum difference since dephosphorylation of PDH_b by treatment with PDH_b phosphatase produced PDH_a with 30% greater activity than the PDH_a from which it was prepared.

If the higher affinity of PDH_a than PDH_b for the core of the complex does not require the presence of substrates or enzymatic turnover, similar results would be expected from experiments in which the complex is separated from free PDH by sedimentation through a sucrose gradient. Fig. 2 shows results

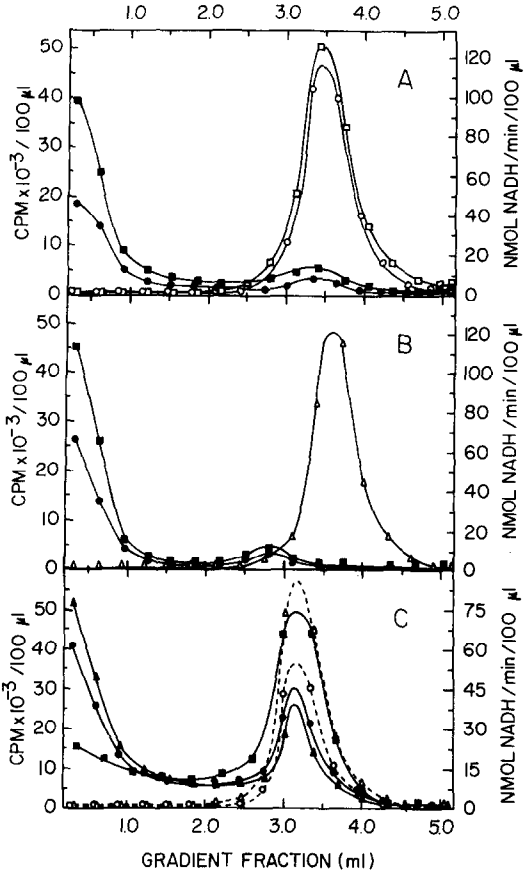


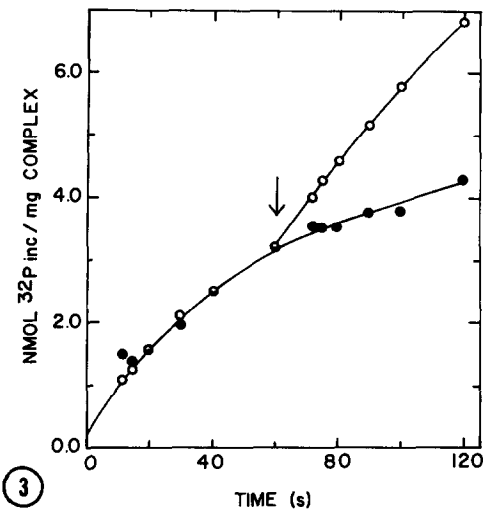
Fig. 2. Linear sucrose gradient analysis of PDH_b and PDH_a equilibrated, respectively, with the active and inactive forms of the heart pyruvate dehydrogenase complex. Samples (0.19 to 0.3 ml) were equilibrated in Buffer A and aliquots (0.14 to 0.2 ml) were analyzed by linear sucrose gradients as described in Materials and Methods. PDH_b and phosphorylated pyruvate dehydrogenase complex, respectively, contained 2.1 and 2.7 phosphoryl groups per PDH and retained 3% and 1.5 % of their original activities. NAD-reduction activity is designated with open symbols (and dashed lines, Panel C) and ^{32}P-radioactivity is designated with closed symbols. Panel A shows gradient analyses of 99 µg (o,●) or 198 µg (□,■) PDH_b with 100 µg of heart pyruvate dehydrogenase complex. The corresponding control samples for each of these levels of PDH_b (● or ■) and for the complex (Δ) are shown in Panel B. Panel C shows gradient analyses on 77 µg of phosphorylated heart complex (■) and this level of complex in the presence of 32 µg (o,●) and 64 µg (Δ,▲) of PDH_a. The PDH_a used in these experiments had a specific activity (in a reconstitution assay) of 45% that of PDH_a in the kidney pyruvate dehydrogenase complex from which it was resolved. Effective concentrations of resolved PDH are based on its relative activity.

from gradient experiments on mixtures of PDH_b and nonphosphorylated complex (Panel A) and mixtures of PDH_a and phosphorylated complex (Panel C). Comparison of the mobilities of phosphorylated (Panel C, ■) and nonphosphorylated (Panel B, Δ) samples of heart complex shows that enhanced dissociation of PDH_b results

in slower sedimentation of phosphorylated complex. Addition of PDH_b to non-phosphorylated complex at effective (c.f., Legend, Fig. 2) ratios of 0.9 and 1.8 PDH_b:PDH_a did not displace PDH_a from the heart complex (Panel A). The small peaks with intermediate mobilities in the control samples of PDH_b (Panel B, ●,■) were due to association of PDH_b with the transacetylase-kinase subcomplex used to prepare PDH_b. For the mixtures of nonphosphorylated complex and PDH_b, dissociation of PDH_a from nonphosphorylated complex and reassociation with the transacetylase-kinase subcomplex slightly decreased the mobility of native complex and increased the mobility of added subcomplex (Panel A). NAD-reduction assays indicate no loss in PDH_a from higher molecular weight fractions. The PDH_b associated with higher molecular weight fractions did not exceed the available sites (i.e., those not occupied by PDH_a) on transacetylase cores.

Addition of PDH_a to phosphorylated complex (Panel C), at effective ratios of 0.4 and 0.8 PDH_a:PDH_b, increased the NAD-reduction activity to 29% and 54% of the activity of nonphosphorylated complex. The activity of the corresponding gradient fractions summed to 29% and 48% respectively. Thus PDH_a was effective in displacing PDH_b and the position of the equilibrium was maintained during sedimentation through the gradient. The degree of displacement of PDH_b is somewhat less than would be predicted from the reciprocal experiment described above. Although the basis for this is not clear, it is probably related to the fact that resolved PDH_a has a lower specific activity than the PDH_a of the isolated complex. Clearly PDH_a is much more effective in displacing PDH_b from native complex than PDH_b is in displacing PDH_a, and this difference does not require the presence of substrates or catalytic turnover by the complex.

The approximately 10-fold higher affinity of PDH_a than PDH_b for the transacetylase core (Fig. 1) may contribute to the effective functioning of PDH_a kinase which is tightly bound to the transacetylase core. Following phosphorylation, dissociation of PDH_b may make sites where phosphorylation occurs more accessible to PDH_a. Results consistent with this concept are shown in Fig. 3. Addition of PDH_a to a sample of complex, in which significant phosphorylation



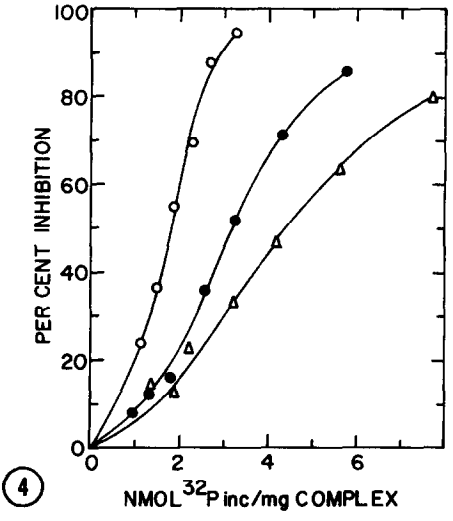


Fig. 3. PDH_a enhancement of PDH_a kinase activity following formation of appreciable PDH_b. PDH_a kinase activity was measured using 0.1 mM [γ ^{32}P]ATP in the presence of 0.2 mM ADP by procedures previously described (8) with the following modification. Two series of assays were conducted with PDH_a (o), at 1.3 mg/mg of kidney complex,or buffer (•) added at 60 s to each assay exceeding 60 s.

Fig. 4. Increased phosphorylation required to inactivate kidney pyruvate dehydrogenase complex in the presence of added PDH_a. Over a time range of 15 to 90 s, PDH_a kinase activity was concurrently measured as both incorporation of ^{32}P into protein and loss of NAD-reduction activity. Other conditions were as in Fig. 3 except that resolved PDH_a was added prior to initiation of the reaction at 0 (o), 0.88 (•), and 2.2 (Δ) mg PDH_a/mg pryuvate dehydrogenase complex. The relative initial rates for these mixtures were, respectively, 4.64, 5.36, and 7.52 nmol ^{32}P incorporated/min/mg of complex.

had occurred, immediately increased the rate of phosphorylation to 93% that of the initial rate (4.2 nmol/min/mg complex). The presence of additional PDH_a at the beginning of the phosphorylation reaction caused only a small increase in the initial rate of phosphorylation. Thus, under conditions where PDH_a kinase activity is limited by the availability of protein substrate, the sites associated with PDH_a kinase activity, presumably on the transacetylase core, are available for binding of PDH_a.

During the phosphorylation reaction, exchange of PDH_a for PDH_b would be expected to protect against inactivation of the complex. As shown in Fig. 4, this was observed when resolved PDH_a was added prior to initiation of phosphorylation at a level 2 or 5 times that associated with the isolated kidney complex. The rate of phosphorylation was increased but the rate of inactiva-

tion was decreased. This is clearly consistent with a fairly rapid replacement of the PDH_b formed by PDH_a. Since NAD-reduction assays were done concurrently with phosphorylation assays, it is not surprising that protection was somewhat less than predicted by the difference in affinities shown in Fig. 1. Newly formed PDH_b may have to undergo a conformational change before it becomes less tightly associated and the activity measured may not reflect an equilibrium distribution of bound PDH_a and PDH_b.

Thus, our results indicate that PDH_b has a lower affinity for the dihydrolipoyl transacetylase core of the complex due to a higher rate of dissociation for PDH_b than PDH_a. Previously, Cate *et al.* (3) have shown that the rate of dissociation of PDH_a is fast enough to make an important contribution to the functioning of PDH_a kinase. The results described above (Fig. 3 and 4) are consistent with dissociation of PDH_b facilitating PDH_a kinase activity. It is also likely that PDH_b phosphatase activity is enhanced by dissociation of PDH_b. Recent studies (Pratt and Roche, unpublished) have demonstrated that a large excess of PDH_a does not inhibit PDH_b phosphatase activity. This implies that either phosphatase utilizes free PDH_b or phosphatase activity requires movement of PDH_b to a specific site created by association of PDH_b phosphatase with the transacetylase core, a Ca^{2+} requiring step (6). This latter possibility is in accord with data suggesting that PDH_b phosphatase activity is enhanced by the binding of PDH_b to the transacetylase core (9).

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