Activated Function of the Pyruvate Dehydrogenase Phosphatase through Ca\(^{2+}\)-facilitated Binding to the Inner Lipoyl Domain of the Dihydrolipoyl Acetyltransferase*

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Micromolar Ca\(^{2+}\) facilitates \(-10\)-fold enhancement of pyruvate dehydrogenase phosphatase (PDP) activity by aiding the association of PDP with the dihydrolipoyl acetyltransferase (E2) component. Connected by linker regions, E2 consists of two lipoyl domains, the NH\(_2\)-lipoyl domain (L1) and the interior lipoyl domain (L2), and a pyruvate dehydrogenase component binding domain surrounding a 60-mer inner core. Using recombinant constructs of L1 or L2, E2-enhanced PDP activity was markedly decreased by L2 but not by L1, effectively competing with intact E2 in Ca\(^{2+}\)-dependent binding of PDP (half-maximal reduction at 2.0 \(\mu\)M L2 versus 6.7 \(\mu\)M E2 subunit). Using L2 fused to glutathione S-transferase resulted in direct Ca\(^{2+}\)-dependent binding of PDP to L2 (K\(_m\) \(-1.7 \mu\)M L2). Affinity-bound glutathione S-transferase-L2 was used to purify PDP to homogeneity by selective binding and elution by Ca\(^{2+}\)-chelation.

The large activity enhancement of PDP by E2 was eliminated by enzymatic removal of lipotes from E2 and restored by their enzymatic reintroduction. The critical role of the L2 lipoyte is not in binding of PDP to E2, since PDP was still bound by delipoylated L2, and delipoylated L2 inhibited E2-enhanced PDP activity, although lipoylated L2 was more effective in each of these tests. Thus, pyruvate dehydrogenase complex activity is increased by enhanced availability of PDP to its E2-bound, phosphorylated pyruvate dehydrogenase substrate as a consequence of the Ca\(^{2+}\)-facilitated interchange of PDP among the mobile L2 domains and an essential (undetermined) step engaging the L2 lipoyte.

Mammalian pyruvate dehydrogenase complex (PDC)\(^1\) is organized around a 60-subunit dihydrolipoyl acetyltransferase (E2) structure, which consists of four independently folded domains connected to each other by mobile linker regions 20–30 amino acids in length (1–3). Association of 20 trimers of the COOH-terminal domain of E2 produces a central cavity in the shape of a dodecahedron. Exterior to this inner core assembly, after the first linker region, each E2 has a 5-kDa domain that binds the pyruvate dehydrogenase (E1) component (4, 5); 20–30 E1 tetramers \((\alpha_β\) \(β_2\)) bind per E2\(_\alphaβ\). Then, set off by two more linker regions are two \(-10\)-kDa lipoyl domains, an interior one (L2), and an NH\(_2\)-terminal one (L1). An E3-binding protein (E3BP) is similar to E2 in consisting of three linker connected domains (6) in which the distinct inner domain of E3BP binds the inner domain of E2 (7, 8) apparently inside the dodecahedron cavity\(^2\) and connects by a linker region to an exterior E3 binding domain (10), followed by a linker-connected lipoyl domain.

PDC is regulated by interconversion of E1 between a nonphosphorylated, active form and a phosphorylated, inactive form (E1b). Pyruvate dehydrogenase phosphatase (PDP) catalyzes the Mg\(^{2+}\)-requiring, Ca\(^{2+}\)-stimulated dephosphorylation and activation of E1 (11–14). Removal of phosphates from the \(α\) subunit of E1 can occur with resolved E1b but is enhanced manyfold when E1b and PDP associate with the E2 core. PDP binds E2 via an interaction that requires Ca\(^{2+}\) (15) to be provided at a micromolar level (16). The magnitude of this enhancement (typically \(7–16\)-fold) depends on the level of E1b used, since kinetically it results primarily from a large decrease in the K\(_m\) of PDP for E1b that results from concentrating PDP along with E1b at the surface of the E2 core (15). Ca\(^{2+}\) also causes about a 2-fold decrease in the K\(_m\) of PDP for Mg\(^{2+}\) (17).

PDP is composed of two subunits (16, 18). Its catalytic subunit (\(M_\text{r}\) 52,600) is in the phosphatase 2C class but shares only about 20% sequence identity with rat cytosolic \(α\) and \(β\) isoforms of phosphatase 2C (19). The other subunit (\(M_\text{r}\) 96,000) serves a regulatory role by affecting the concentration of Mg\(^{2+}\) required for PDP activity (20). This subunit is a flavoprotein (FAD), and its sequence is distantly related to the mitochondrial flavoprotein dimethylglycine dehydrogenase (14), which functions in choline degradation. Spermine and, to a lesser degree, other polyamines reduce the K\(_m\) of PDP for Mg\(^{2+}\) (21), probably by reversing the effect of the regulatory PDP subunit (20). The effect of spermine appears to be a direct effect on the phosphatase (22). The concentration dependence of PDP for Mg\(^{2+}\) is also reduced in permeabilized mitochondria prepared from insulin-treated adipose tissue (23).

A major interest of our laboratory is how the E2 core binds and aids the function of the kinase and phosphatase components. We have recently established that the kinase preferentially binds to the L2 domain of E2 and that L2 must retain its lipoyl cofactor for that association to occur (24). We have also found that the reduction and acetylation of the L2 lipoyte

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**The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase complex component; E1b, phosphorylated, inactivated E1; E2, dihydrolipoyl acetyltransferase component; PDCb, phosphorylated, inactivated PDC; L1 domain, NH\(_2\)-lipoyl domain of E2; L2 domain, interior lipoyl domain of E2; H1, hinge or linker region connecting L1 and L2 in E2; PDP, pyruvate dehydrogenase phosphatase; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein; GST, glutathione S-transferase; GSH, glutathione; PAGE, polyacrylamide gel electrophoresis; MOPS-K, 3-(N-morpholino)propanesulfonic acid-K\(^-\).
markedly enhances kinase activity (25). Previously, we have used protease Arg C to progressively remove the outer domain regions of E3BP (then termed protein X) and then those of the E2 core and observed a loss of the capacity for enhanced PDP function correlated with removal of the outer domains of E2 (22). In cleaving E2, protease Arg C removed the lipoyl domain region of E2 and in a separate cleavage removed the E1 binding domain. Here we have found that E1b binding to E2 makes it a better substrate for PDP, established that Ca$^{2+}$-dependent binding of PDP to E2 occurs at the L2 domain of E2 and characterized the affinity of this interaction, uncovered a critical role for the L2 lipoate for E2-enhanced PDP activity, and used the specific PDP-L2 interaction to purify PDP.

**EXPERIMENTAL PROCEDURES**

**Materials**—The bovine kidney PDC and, from the complex, the resolved E1 and E2-E3BP kinase subcomplex were prepared by standard procedures (26, 27). The E2 lipoyl domain region was selectively removed from E2-E3BP kinase by treatment with Clostridium histolyticum collagenase, and a kinase (K) and E3BP (E3BP-K) complex were prepared by standard procedures (28). The indicated levels of GST-H1 were used as described previously (29). Bovine kidney PDP was purified through the DEAE chromatography step according to the procedure of Pratt et al. (18) to yield a stable preparations in which PDP constituted 20–50% of the protein. The preparation used in most studies had an initial specific activity of 350 units/mg (units defined below); PDP activity, following free estimation of the same level of kinase, was very stable. Recombinant constructs of the lipoyl domains of human PDC E2 were prepared both fused to glutathione S-transferase (GST) and as free structures as described previously (28). The E2 amino acid sequences of the constructs used were L1(1–98), L2(120–233) L1 H1 L2(1–233) and H1 L2(98–233), with H1 designating the first hinge (or linker region) connecting L1 and L2. Enterococcus faecalis lipoproteinase was prepared by minor modification of the procedures of Suzuki and Reed (29), and lipoyl protein ligase, made as described by Green et al. (30), was kindly provided by John Guest (University of Sheffield, Sheffield, Sheffield, United Kingdom).

**PDP Assays—Phosphatase activity was determined by measuring the increase in PDC activity or the release of $\gamma$-P from PDCb or resolved E1b. To prepare phosphorylated PDC, 5–10 mg of complex was incubated at 30°C with 0.5 mM ATP in 50 mM MOPS-K (pH 7.5), 0.5 mM EDTA, 2 mM dithiothreitol, and 1.2 mM MgCl$_2$ for 5 min, followed by at least 30 min at 4°C. $\gamma$-P-E1 (6–8 mg) was prepared in the same buffer by incubation of 50 mg of E2 kinase/mg of E1 for 30 min at 30°C, followed by overnight on ice. $\gamma$-PATP was initially at least 400 (as determined by gel filtration of PDC or resolved E1, at least 50% of $\gamma$P phosphates were incorporated per E1 tetramer. ATP was then hydrolyzed by treatment with hexokinase and glucose, followed by further dialysis with EDTA reduced to 0.1 mM. To remove E2 kinase from E1b, this structure (saturated with bound E1b) was pelleted by centrifugation in a Ti-50.2 rotor at 35,000 rpm for 90 min and excess E1b recovered in the supernatant. The protein concentrations of phosphorylated substrates were measured by the BCA technique as described previously (28).

The rate of increase in PDC activity by PDP (1 unit = an increase of 1 mmol NADH/min) was estimated by minor modifications of the procedure of Pratt et al. (18). In a final volume of 25 l of buffer A (40 mM MOPS-K, final pH 7.4, 0.1 mM EDTA, 0.4 mM dithiobitol, 1.2 mM Ca$^{2+}$, 1.0 mM Mg$^{2+}$, 1.0 mM EGTA, and 0.2 ml/ml Pluronic F-68) and 0.04 1 m and of buffer containing 40 mM MOPS-K, pH 7.5, 1.2 mM Ca$^{2+}$, 1.0 mM EDTA, 0.4 mM dithiobitol, and 2 ml/mg Pluronic F-68. Studies were conducted in the absence and presence of Mg$^{2+}$ (2.0 mM Mg$^{2+}$ plus 0.2 mM EDTA when added). After 120 s, 50–51 ml samples held at held at room temperature (24°C) or on ice were transferred to calibrated 0.5-ml Eppendorf tubes containing 25 ml of the same buffer in the same basic temperature. These were mixed by vortex for 10 s and during the next 10 s transferred to an Eppendorf 5415 C centrifuge, and then the gel beads with the bound GST construct were pelleted at 14,000 rpm for 10 s. PDP activity was determined for duplicate 5-ml samples of each supernatant. Non-specific binding was evaluated in the same conditions using the same level of GST as GST-H1-L2. We found a wide variation in the capacity of different GSH-Sepharose lots to rapidly and nearly completely bind GST or the GST construct (residual GST activity in the supernatant measured as described previously; Ref. 28). The preparation used (Pharmacia Biotech Inc., lot 238620) reproducibly bound >92% of the highest level of the GST construct used under the above conditions, whereas most lots bound only 65–90% of the GST activity. Variation was primarily in the rate of binding rather than the extent of binding; the basis of the difference is not understood. Minor corrections of data were made assuming the same proportion of PDP was bound to the small portion of GST-H1-L2 (determined as GST activity that remained in the supernatant) and did not pellet with the GSH-Sepharose.

**Purification of PDP—** GST-L2-GSH-Sepharose (3 mg) was bound to 1.5 ml of GSH-Sepharose and then equilibrated in 40 mM MOPS-K, 1.2 mM Ca$^{2+}$, 1.0 mM EGTA, 2.0 mM MgCl$_2$, 0.2 mM EDTA, and 10 mg/ml Pluronic F68. To 1 ml of this lipoyl domain-bearing gel, 80–160 units of PDP, typically 0.5–1 mg protein, prepared through the DEAE concentrator column step previously described, were incubated at 4°C in 2 ml of the above buffer for 30 min with mild shaking. Free PDP activity was <2% of the original level. This gel was then layered over 0.5 ml of GST-H1-L2-GSH Sepharose washed with 10 ml of the above buffer and then eluted at 25 ml/min with the above buffer modified to contain no Ca$^{2+}$, 1.5 mM EGTA, and 10% glycerol (v/v). PDP activity was determined for each fraction by spectrophotometric assay, and individual fractions were frozen and stored at –20°C.

**Lipoamidase Binding Site**—The removal and reintroduction of lipoyl groups of phosphorylated PDC were executed by minor modifications of developed procedures (24, 25). $^{32}$P-PDCb was treated with lipoamidase (300 μg/mg) in 50 mM sodium phosphate (pH 7.5) at 30°C. Samples of PDCb were removed at the indicated time points and were estimated for changes in phosphatase activity (25) and lipoyl content (5 μg). Lipoyl content was estimated by determining the change in E2 activity using a cyclic assay (26) carried out in microplate wells containing 50 mM sodium phosphate (pH 7.5), 1 mM EDTA, 0.3 mM NADH, 0.1 mM NAD$^+$, and 0.2 mM 5,5’-dithiois(2-nitrobenzoate) in a final volume of 200 μl. The change in absorbance at 405 nm (ε = 27.2 A$^2$ mmol$^{-1}$ cm$^{-1}$) was measured with time using a UV Max microplate reader. The very low residual E3 activity observed following lipoamidase treatment was comparable to the E3 activity obtained by addition of a level of lipote equivalent to those used in the lipoyl domains in PDCb. (Preliminary experiments using PDC not labeled with $^{32}$P confirmed that loss of E3 activity correlated with loss of acetylation sites.) After 150 min, 0.4 mM phenylmethylsulfonyl fluoride was added, the incubation continued for 12 min to inactive lipoylase, followed by addition of dithiothreitol to 0.5 mM to quench.
the reactivity of residual phenylmethylsulfonyl fluoride, and dialyzed into 40 mM MOPS-K (pH 7.5) and 0.2 mM EDTA. SDS-PAGE analysis (33) showed no change in the pattern of the delipoylated and relipoylated PDC preparations, except that delipoylated E2 had a mobility slightly slower than the original or relipoylated E2. The level of lipoylation was evaluated by comparing acetylation of lipoyl moieties of 8.7-μg samples of the PDC preparations using [1-32P]ATP (preparation used to make PDCb to maintain the radiospecific activity due to any further phosphorylation), 0.1 mM lipolate, and 1.5 mM MgCl₂ (25). EDTA was added to 1.5 mM to terminate the reaction along with leupeptin and aprotinin to 1 μg/ml, followed by dialysis for 48 h against 40 mM MOPS-K (pH 7.5) and 0.2 mM EDTA. SDS-PAGE analysis (33) showed that no change in the pattern of the delipoylated and relipoylated PDC preparations, except that delipoylated E2 had a mobility slightly slower than the original or relipoylated E2. The level of lipoylation was evaluated by comparing acetylation of lipoyl moieties of 8.7-μg samples of the PDC preparations using [1-32P]acetate and CoA in the presence of NADH along with removal of CuA formed by the α-ketoglutarate dehydrogenase complex reaction as described previously (25, 28). PDC samples were stored at −80 °C for 8 weeks prior to measurements of acetylation, so 32P counts were low; corrections for 32P were made from the reactivity of residual phenylmethylsulfonyl fluoride, and dialyzed into 40 mM MOPS-K (pH 7.5) and 0.2 mM EDTA. SDS-PAGE analysis (33) showed no change in the pattern of the delipoylated and relipoylated PDC preparations, except that delipoylated E2 had a mobility slightly slower than the original or relipoylated E2. The level of lipoylation was evaluated by comparing acetylation of lipoyl moieties of 8.7-μg samples of the PDC preparations using [1-32P]acetate and CoA in the presence of NADH along with removal of CuA formed by the α-ketoglutarate dehydrogenase complex reaction as described previously (25, 28). PDC samples were stored at −80 °C for 8 weeks prior to measurements of acetylation, so 32P counts were low; corrections for 32P were made from the reactivity of residual phenylmethylsulfonyl fluoride, and dialyzed into 40 mM MOPS-K (pH 7.5) and 0.2 mM EDTA. SDS-PAGE analysis (33) showed no change in the pattern of the delipoylated and relipoylated PDC preparations, except that delipoylated E2 had a mobility slightly slower than the original or relipoylated E2. The level of lipoylation was evaluated by comparing acetylation of lipoyl moieties of 8.7-μg samples of the PDC preparations using [1-32P]acetate and CoA in the presence of NADH along with removal of CuA formed by the α-ketoglutarate dehydrogenase complex reaction as described previously (25, 28). PDC samples were stored at −80 °C for 8 weeks prior to measurements of acetylation, so 32P counts were low; corrections for 32P were made from the reactivity of residual phenylmethylsulfonyl fluoride, and dialyzed into 40 mM MOPS-K (pH 7.5) and 0.2 mM EDTA. SDS-PAGE analysis (33) showed no change in the pattern of the delipoylated and relipoylated PDC preparations, except that delipoylated E2 had a mobility slightly slower than the original or relipoylated E2. The level of lipoylation was evaluated by comparing acetylation of lipoyl moieties of 8.7-μg samples of the PDC preparations using [1-32P]acetate and CoA in the presence of NADH along with removal of CuA formed by the α-ketoglutarate dehydrogenase complex reaction as described previously (25, 28). PDC samples were stored at −80 °C for 8 weeks prior to measurements of acetylation, so 32P counts were low; corrections for 32P were made from

**RESULTS**

**Effect on PDP Activity of Removal of the E2 Lipoyl Domain Region but not Its E1 Binding Domain**—E2 enhances the rate of dephosphorylation of 32P-P-E1b (μM) by −10-fold, but reduction of free Ca²⁺ to a subnanomolar level by chelation with EGTA eliminates nearly all of this activation. Rahmatullah et al. (22) found that loss of E2-activated PDP activity correlated with removal of the outer domains of E2 and not with removal of the outer domains of E3BP. However, although the approach used (protease Arg C) first removed the outer domain of E3BP, it cleaved linker regions on both sides of the E1 binding domain of E2, so E1 binding was also being progressively lost. Treatment with collagenase selectively removes the bilipoyl domain region of E2, and the resulting E2ΔB-E3BP subcomplex binds E1. E2ΔB-E3BP (5 μg) gave a 43% stimulation of PDP activity at 10 mM Mg²⁺ (from 30 ± 1.5 to 43 ± 1.1 nmol of [32P]PO₄ released/min/mg of PDP) and nearly a 2-fold stimulation at 0.5 mM Mg²⁺ (from 5.35 ± 0.7 to 11.9± 2 nmol of [32P]PO₄ released/min/mg). Accordingly, binding of E1b to E2 improves the presentation of E1b as a substrate to PDP. Mechanisms involving restriction of the movement and orientations of bound E1b (entropic effect) or a conformational change in bound E1b could contribute to this enhanced dephosphorylation.

**Inhibition of PDP by Lipoyl Domains**—Since removal of the outer lipoyl domain region of E2 prevents most of the E2-activated PDP function, it seemed likely that the Ca²⁺-dependent interaction of PDP with E2 was at a lipoyl domain. Fig. 1 shows the effects of 5 and 15 μM L1, L2, H1-L2, and L1-H1-L2 on the rate of PDP dephosphorylation of 32P-P-PDC in the presence of a saturating level of Ca²⁺. L2-containing structures (L2, H1-L2, and L1-H1-L2) reduced E2-activated phosphatase activity, with >67% reduction due to 5 μM L2 or H1-L2 and >60% due to the bilipoyl domain L1-H1-L2 structure, and 15 μM L2 or H1-L2 reduced the rate of dephosphorylation to ~16%, with a slightly higher activity (~19%) with this level of L1-H1-L2. In marked contrast, L1 had little if any effect on E2-enhanced phosphatase activity at either concentration. Assuming inhibition resulted solely from free L2 competing in binding PDP with the L2 domains of the assembled PDCb E2 (subunit concentration, ~6.4 μM), the finding that 5 μM free L2 gives more than 50% inhibition would indicate that free L2 competes very effectively. Fig. 1b also shows the effect of L2 on PDP activity when Ca²⁺ was chelated by EGTA. Removal of free Ca²⁺ greatly diminished PDP activity, and little, if any, inhibition was detected with 15 μM L2, supporting the prospect that L2 inhibition results from selective binding of PDP to L2 in a Ca²⁺-requiring process.

**Concentration Dependence for L2 Inhibition**—Fig. 2 shows the concentration dependence for L2 inhibition with 21 μg of 32P-P-PDC (~10 μg of E2, 6.7 μM E2 subunit). Fig. 2, inset, presents the same data in a Dixon plot. Half-maximal inhibition occurred at ~20 μM L2, which is below the E2 subunit concentration. The solid lines are theoretical fits derived as-
assuming that L2 inhibition resulted from competitive binding of L2 and intact E2 subunits. The excellent fit supports the operation of a simple competition between E2 and L2 and yields a computed optimum ratio for the equilibrium dissociation constants for the formation of L2-PDP and E2-PDP complexes of 0.335 (the ratio is calculated as described in the legend to Fig. 2). The results strongly suggest that free L2 competes very effectively with the intact E2 subunits of PDC for the binding of the PDP, thereby eliminating rapid dephosphorylation of E1b bound to the E2 in PDC.

Inhibition by GST-H1zL2—The preparation of lipoyl domains fused to GST affords a simple method for evaluating direct binding of PDP to L2. The use of GST-H1zL2 was selected because the L2 domain is separated from GST by an intervening mobile linker region (H1). Fig. 1a shows that inhibition of E2-activated PDP activity by 5 and 15 μM GST-H1zL2 (monomer concentration) was reduced (55 and 77% inhibition, respectively), significantly less than by L2 alone. Not only might GST (and L1 in L1-H1-L2) physically interfere with PDP-L2 encounters, but, because the fusion protein is a dimeric structure (GST-H1zL2), it would lower the effective concentration and slow the rate of diffusion. Nevertheless, GST-H1-L2 was clearly still very effective and was used in tests of direct binding of PDP to L2.

FIG. 2. Change in E2-activated PDP activity with increasing L2. PDP assays were conducted with 21 μg of 32P-PDC in the presence of 10 mM Mg2⁺ with the indicated levels of L2 (construct amino acid sequence 120–233). Other conditions were as described in “Experimental Procedures.” Inset, data replotted in a Dixon plot. The lines shown in both figures are theoretical fits for the ratio of the equilibrium binding constants of PDP with L2 over that of PDP with E2 subunits: 

\[
K_d^{L2}/K_d^{E2} = \frac{([L2]_t) ([E2]_t)}{([PDP]_t)} - \frac{([L2-PDP]) ([E2])}{([PDP]) + ([L2-PDP]) ([L2-PDP])}
\]

This equation assumes that free PDP (not bound to L2 or E2) is not significant (at a fixed E1, the level of E2 present in PDC used gives near maximal stimulation of the kinase). The observed activity is calculated as: 

\[
\frac{([PDP]_t) ([L2-PDP])}{([L2]_t) ([PDP]_t)} \frac{V_{max}}{V_{min}}
\]

where \(V_{max}\) is the activity in the absence of L2, and \(V_{min}\) is the extrapolation of the best fit line to infinite L2. L2-PDP is derived from the best \(r\) value fit of the data obtained by iteratively solving for L2-PDP at all L2 levels from the quadratic form of the first equation; \(r = 0.335\) gave the best fit.

FIG. 3. Binding of PDP to GST-H1zL2. GST-H1-L2 or GST (2–6 μM subunit concentration) was incubated with 0.26 units of PDP followed by separation of the fusion protein-PDP complex and determination of unbound PDP activity using the conditions and approach described under “Experimental Procedures.” On average, 28% of PDP nonspecifically bound to GSH-Sepharose in the presence of GST. Absolute deviations are shown, and the optimal line and its deviation were determined from a linear least squares analysis.

FIG. 4. Purification of PDP. a, profile for elution of PDP activity from a 1.5-ml GSH-Sepharose-GST-H1-L2 column (total units in 55-μl fractions) for fractionation of 0.5 mg (85 units) of a DEAE fraction of PDP as described under “Experimental Procedures.” b, SDS-PAGE pattern (33) for the 2.8-μg DEAE fraction and 0.36-μg purified PDP (PDPc, catalytic subunit; PDPr, regulatory subunit) developed by silver staining (34).
Ca$^{2+}$-dependent Binding of PDP to L2—Using a buffer containing 1.2 mM Ca$^{2+}$ plus 1.0 mM EGTA, PDP was selectively bound by GST-H1-L2 anchored on GSH-Sepharose but not by GST so anchored. PDP was then selectively eluted by washing with 1.0 mM EGTA. Thus, direct Ca$^{2+}$-dependent binding was demonstrated. Furthermore, we found tight binding not only required Ca$^{2+}$ but was markedly enhanced by the presence of 2.0 mM Mg$^{2+}$ (e.g. the fractional binding of PDP by 5 μM GST-H1-L2 was reduced from 72 to 41% when Mg$^{2+}$ was not included).

Fig. 3 shows the change in PDP binding with the level of GST-H1-L2 in the presence of 0.2 mM free Ca$^{2+}$ and 1.8 mM free Mg$^{2+}$. In this experiment, the fusion protein and PDP were incubated together, followed by rapid mixing with GSH-Sepharose and pelleting of the Sepharose beads. An apparent binding constant of about 1.75 ± 0.4 μM L2 is obtained from Fig. 3. Thus we have obtained direct support for PDP binding to L2; however, this binding is weaker than expected from analysis of activity studies or Fig. 2 (see “Discussion”). Our conditions of rapidly executing the steps of binding of the fusion protein to beads and removing the GST structures while minimizing the volume of the beads prevented use of higher concentrations of GST-H1-L2 at room temperature. In one study on ice, GST-H1-L2 was rapidly and nearly completely bound by the affinity gel at a somewhat higher fusion protein level. At the reduced temperature, the binding was somewhat tighter over the same concentration range as Fig. 3, (K_d, ~0.8 μM), and a weaker class of binding sites was detected at higher levels of the fusion protein (K_d, ~3 μM). The many data points in Fig. 2 are closely fit by a single binding constant and not fit well by two classes of sites; therefore, we surmise that a technical deficiency in the binding tests is occurring at the higher levels of fusion protein. Consistent with somewhat tighter binding at reduced temperatures, slower dissociation of PDP from GST-H1-L2 at 6 °C than room temperature was found in developing the conditions for PDP purification.

Purification of PDP—Tests revealed that PDP could be maintained fully bound to the L2-bearing column after washing with 5 column volumes in the presence of Ca$^{2+}$ and Mg$^{2+}$ at 6 °C, and that a transition to buffer containing EGTA and no added Ca$^{2+}$, followed by slow elution, gave good recovery of PDP activity in a fairly sharp peak (Fig. 4a). This process was used in the purification of the PDP. Fig. 4b shows the pattern of SDS-PAGE-separated PDP eluted from GSH-Sepharose-GST-H1-L2. Only PDP subunits were observed, indicating that essentially homogeneous PDP (specific activity, 660 units/mg)$^3$ was obtained using this affinity purification step.

Effect of Delipoylation on PDP Activity and Binding to L2—Fig. 5a shows that delipoylation of $^{32}$P-PDC on PDP activity leads to a marked reduction in PDP activity. Fig. 5b shows the associated loss in the reduction of lipoates in PDCb by E3 catalysis as the PDCb is delipoylated. The loss of E2-activated PDP activity was more pronounced as the last portion of lipoates was removed. Reintroduction of lipoates through lipoyl protein ligase catalysis to a level that gave two-thirds as high an acetylation capacity as the control PDCb (Fig. 6b) restored high PDP activity (Fig. 6a). The only change in SDS-PAGE patterns was a slight decrease in E2 mobility following delipoylation that was reversed on relipoylation.$^4$ This suggests that the lipoyl group of the L2 domain plays an essential role in PDP function.

To assess whether delipoylation led to a loss of PDP binding to the L2 domain following delipoylation, the effects of delipoylated L2 versus lipoylated L2 in inhibiting dephosphorylation of PDCb were compared. Fig. 7a shows that 13 μM delipoylated L2 was somewhat less effective than 13 μM L2 but still significantly inhibited PDP dephosphorylation of E1b in the intact complex. Fig. 7b compares the binding by 4 μM lipoylated and delipoylated GST-H1-L2. Delipoylation reduced but did not prevent binding of the phosphatase. The marked reduction in the capacity of delipoylated E2 to activate PDP activity cannot be explained by reduced PDP binding. E1b binding was fully retained in delipoylated PDCb (data not shown). Thus, lipoyl groups of E2 must have a specialized role in facilitating high PDP activity (see “Discussion”).

**DISCUSSION**

Hormones that signal cellular events that place increased energy demands often initiate signal transduction pathways that increase cellular Ca$^{2+}$ derived either from intracellular stores or from transient extracellular import. The rise in cytoplasmic Ca$^{2+}$ to a near micromolar level is coupled by specialized import-export transporters that generate a similar in-

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$^3$ We have observed specific activities as high as 1500 units/mg in freshly prepared PDP, but this specific activity is not maintained under storage conditions that we have used to date.

$^4$ Unusual mobility decreases with removal of lipoates from lipoyl domain structures are readily observed for native gels, but mobility decreases have also been observed by SDS-PAGE analyses in some cases (28). The latter shifts probably result from a different cause than those occurring in native gel patterns as previously discussed (28).
The Experimental Procedure was conducted as described under "Experimental Procedures."
not releasing PDP; Ref. 23). Furthermore, PDP may reversibly interact with sites other than PDC E2, particularly when the intramitochondrial free Ca\(^{2+}\) concentration is low.

For the L2-PDP interaction to yield a large enhancement in PDP activity, we have found that the lipoyl domains of the assembled E2 core must retain their lipoyl moieties. Loss of PDP binding by L2 was a possible explanation for the loss of E2-activated PDP function. However, significant (albeit reduced) Ca\(^{2+}\)-independent inhibition and binding of PDP were still observed with delipoylated L2 structures. Therefore, the complete removal of E2 enhancement of PDP activity after delipoylating the E2 core implies that processes other than simply binding and concentrating PDP and E1b are facilitated by the E2 core. Since L1 did not affect PDP activity, it seems unlikely that the L1 lipoyl group contributes to E2-activated PDP activity. Yang and Roche\(^7\) have recently found that a recombinant oligomer E2 structure lacking L1 can activate PDP activity, establishing that the L2 lipoate can serve. An interesting and reasonable possibility is that the L2 lipoate somehow augments encounters of PDP with E1b at the surface of E2. A potential role of the lipote would be to support efficient delivery of the phosphatase to E1b by the lipoyl cofactor of the PDP-carrying L2 interacting with E1b in a manner similar to the E1 lipoyl domain interaction occurring in the reductive acetylation reaction (step 2 catalyzed by E1 in the overall PDC reaction). This would require that PDP be tethered to L2 in a way that does not interfere with the highly specific L2-E1 interaction while simultaneously aiding the positioning of the active site of the P\(_c\) subunit of PDP to efficiently dephosphorylate E1b.

The small enhancement in PDP activity observed when E1b is bound by E2\(_{260}\), which lacks the L2 binding site, implies a consequential effect of this binding, since localizing E1 on this assembled structure may actually reduce the frequency of diffusion-based encounters relative to free E1b interacting with free PDP. Binding of E1b to E2 may induce a conformational change in E1b that exposes its sites of phosphorylation or may restrict E1b positions in a way that increases the probability of productive encounters with a freely diffusing PDP.

Schemes involving anchoring the entire E2 core were developed for purification of PDP (16, 18). However, these were expensive preparations and had a lower capacity for binding PDP (16, 18). However, these were still observed with delipoylated L2 structures. Therefore, the complete removal of E2 enhancement of PDP activity after delipoylating the E2 core implies that processes other than simply binding and concentrating PDP and E1b are facilitated by the E2 core. Since L1 did not affect PDP activity, it seems unlikely that the L1 lipoyl group contributes to E2-activated PDP activity. Yang and Roche\(^7\) have recently found that a recombinant oligomer E2 structure lacking L1 can activate PDP activity, establishing that the L2 lipoate can serve. An interesting and reasonable possibility is that the L2 lipoate somehow augments encounters of PDP with E1b at the surface of E2. A potential role of the lipote would be to support efficient delivery of the phosphatase to E1b by the lipoyl cofactor of the PDP-carrying L2 interacting with E1b in a manner similar to the E1 lipoyl domain interaction occurring in the reductive acetylation reaction (step 2 catalyzed by E1 in the overall PDC reaction). This would require that PDP be tethered to L2 in a way that does not interfere with the highly specific L2-E1 interaction while simultaneously aiding the positioning of the active site of the P\(_c\) subunit of PDP to efficiently dephosphorylate E1b.

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Schemes involving anchoring the entire E2 core were developed for purification of PDP (16, 18). However, these were expensive preparations and had a lower capacity for binding PDP (possibly because multiple cross-linking between the matrix and E2\(_{260}\) aggregates interfered with PDP interactions). The use of GST-H1\(_1\) affords high recovery of essentially ho-