Pyruvate Dehydrogenase Kinase Isoform 2 Activity Stimulated by Speeding Up the Rate of Dissociation of ADP†

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ABSTRACT: Pyruvate dehydrogenase kinase 2 (PDK2) activity is stimulated by NADH and NADH plus acetyl-CoA via the reduction and reductive acetylation of the lipoyl groups of the dihydrolipoyl acetyltransferase (E2) component. Elevated K^+ and Cl^- were needed for significant stimulation. Stimulation substantially increased both k_{cat} and the K_m for ATP; the fractional stimulation increased with the level of ATP. With an E2 structure lacking the pyruvate dehydrogenase (E1) binding domain, stimulation of PDK2 was retained, the K_m for E1 decreased, and the equilibrium dissociation constant for ATP increased but remained much lower than the K_m for ATP. Stimulation of PDK2 activity greatly reduced the fraction of bound ADP. These results fit an ordered reaction mechanism with ATP binding before E1 and stimulation increasing the rate of dissociation of ADP. Conversion of all of the lipoyl groups in the E2 60mer to the oxidized form ($E2_{ox}$) greatly reduced k_{cat} and the K_m of PDK2 for ATP. Retention over an extended period of time of a low portion of reduced lipoyl groups maintains E2 in a state that supported much higher PDK2 activity than short-term (5 min) reduction of a large portion of lipoyl groups of $E2_{ox}$, but reduction of $E2_{ox}$ produced a larger fold stimulation. Reduction and to a greater extent reductive acetylation increased PDK2 binding to E2; conversion to $E2_{ox}$ did not significantly hinder binding. We suggest that passing even limited reducing equivalents among lipoyl groups maintains E2 lipoyl domains in a conformation that aids kinase function.

Tailored control of the activity of the mitochondrial pyruvate dehydrogenase complex (PDC)[1] determines whether glucose and glucose-linked substrates (glycogen, lactate, citric acid cycle intermediates, and amino acids forming pyruvate or citric acid cycle intermediates) are converted to acetyl-CoA in mammalian tissues (*1*−*5*). The fraction of active (nonphosphorylated) PDC is controlled in a highly specific and responsive manner to meet the specific needs of diverse tissues in different nutritional states and during exercise. Upregulation of the PDC reaction supports the oxidative use of carbohydrate to meet energy demands of neural tissues, exercising muscles, and other tissues such as heart when carbohydrate is abundant. Conversion of excess carbohydrate to fatty acids also requires enhanced PDC activity in liver, adipose, and mammary tissues. Many neural tissues and red blood cells are critically dependent on glucose as a fuel. In mammals, formation of acetyl-CoA by the PDC reaction constitutes an irretrievable loss of carbohydrate. Therefore, downregulation of PDC activity constitutes a crucial means of conserving body carbohydrate reserves. Four pyruvate dehydrogenase kinase (PDK1, PDK2, PDK3, PDK4) and two pyruvate dehydrogenase phosphatase (PDP1, PDP2) isoforms function in adjusting the phosphorylation state of PDC (*3*, *5*−*9*). The differential expression of these isoforms in different tissues (*3*−*5*, *9*−*11*) in combination with their very different effector sensitivities (*3*, *11*−*13*) supports the required adaptive, tissue-specific control.

The intramitochondrial NADH/NAD^+ and acetyl-CoA/CoA ratios increase due to the PDC reaction and the degradation of fatty acids and ketone bodies (*1*−*4*). Hormones that increase fatty acid oxidation elevate these product to substrate ratios within mitochondria and speed up PDK-catalyzed inactivation of PDC. This means of rapidly downregulating PDC constitutes the primary means for lowering consumption of glucose-linked substrates. It is augmented under conditions of starvation by overexpression of PDK4 in many tissues (*3*−*5*, *10*, *11*), leading to greatly reduced PDC activity.

PDK2 is the most abundant and most universally distributed of the four PDK isoforms (*5*, *10*, *11*). PDK2 binds to the inner lipoyl domain (L2 domain) of the E2 subunit (*12*, *14*, *15*). Lipoyl domain binding enhances kinase activity by providing a kinase with greater access to the large complement of E1 bound on the E2 60mer (*3*, *12*, *16*−*21*, *38*). PDK2 is also the most responsive to stimulation by NADH and acetyl-CoA (*11*−*13*). Stimulation of PDK activity occurs

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[1] Abbreviations: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; E2, dihydrolipoyl acetyltransferase component; L1 domain, NH_2-lipoyl domain of E2; L2 domain, interior lipoyl domain of E2; $E2_{ox}$, E2 with all lipoyl groups in the oxidized form; ΔBE2, E2 construct lacking the E1 binding domain; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; AUC, analytical ultracentrifugation; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate.

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by an indirect mechanism. The intramitochondrial NADH/NAD^+ and acetyl-CoA/CoA ratios are translated by the reversible E3 and E2 reactions to thereby determine the levels of oxidized, reduced, and acetylated lipoyl groups (*3*, *12*, *19*−*25*). Using bovine kidney kinases, significant stimulation is retained with a peptide substrate or using free E1 and free lipoyl domains as long as E3 is available to catalyze lipoyl reduction using NADH as a substrate and the lipoyl domain-free inner core of E2 ($E2_l$) is available to catalyze the acetylation of reduced lipoyl groups using acetyl-CoA as a substrate (*24*). Acetylation of the L2 domain of E2 stimulates PDK2 activity by up to 3-fold (*12*). Reductive acetylation of the free L2 monomer gives only a small stimulation of PDK2 activity, but a substantial stimulation is achieved upon acetylating this domain in a glutathione *S*-transferase−L2 dimer (*12*).

In the companion paper (*38*), we present evidence that ADP dissociation is a slow step in PDK2 catalysis and that pyruvate inhibits kinase-catalyzed PDC inactivation by binding to PDK2·ADP. Here, we provide evidence, using kinetic and binding studies, that reductive acetylation stimulates PDK2 activity by speeding up ADP dissociation. We also find that full oxidation of lipoyl groups has an unexpectedly large effect in reducing kinase activity. The kinetic patterns in this and the companion paper (*38*) are fit well by an ordered reaction. Insights were gained into how reductive acetylation alters PDK2 function from kinetic studies using a modified E2 construct (ΔBE2) that cannot bind E1. Use of this construct allowed the level of this protein substrate to be varied while substantial stimulation of kinase activity could still be observed.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human PDK2 (*12*) and E3 (*26*) were prepared as described previously. The same procedure (*14*) was used to prepare E2, E2·E3BP, an E2 construct with the E1-binding domain removed (ΔBE2), and an E2·E3BP construct in which the lipoylated lysines in all three lipoyl domains (L1 and L2 domains of E2 and L3 domain of E3BP) were substituted with alanine (AAA-E2·E3BP). To prepare E2 with only oxidized lipoyl groups ($E2_{ox}$), 2−4 mg of E2 was treated with 2 mM NAD^+ and 20 μg of E3 for 5 min at 22 °C, and then gel filtration was conducted using a 15 × 0.4 cm Sephacryl S400 column. $E2_{ox}$ from the lead side of the peak was free of E3; E2 does not bind E3 in the absence of the E3BP component (*27*, *28*). The plasmid constructs used for expressing the various E2 constructs and E1 with a removable His tag will be described elsewhere.[2] E1 was prepared free of TPP as previously described (*12*). [γ-^{32}P]-ATP and [α-^{32}P]ATP were purchased from New England Nuclear.

Kinase Activity Assays. PDK activity was measured in duplicate or triplicate as the initial rate of incorporation of [^{32}P]phosphate into E1 using 0.1 mM [γ-^{32}P]ATP (150−500 cpm/pmol) at 30 °C. PDK2 assays conducted in the presence of E2 routinely used 0.04−0.05 μg of PDK2, 10 μg of E2, 10−14 μg of E1, and 1−2 μg of E3 in 25 μL final volume. Assays performed in the absence of E2 or presence of ΔBE2 or AAA-E2·E3BP were conducted with 0.2−0.4 μg of PDK2 with other proteins added at the indicated levels. Incorporation of [^{32}P]phosphate into E1 was terminated after a 60 s reaction time (see footnote 3 in ref *38*). Assays that paralleled binding studies used more PDK2, E2, and E1 (see below), lower reaction temperatures, and a shorter reaction time (12 s) to prevent depletion of E1 with effector stimulatory activity. Most assays were conducted with buffer A (pH 7.4) containing 113 mM Hepes−Tris, pH 7.4, 60 mM KCl, 30 mM K-Hepes, 2 mM $MgCl_2$, 0.2 mM EDTA, and 13.5 ± 2.7 mM K^+ and 7.7 ± 1.5 mM phosphate from additions of E2 (in 50 mM potassium phosphate, pH 7.2) and E1 (in 50 mM potassium phosphate, pH 7.5). The compositions of assay buffers evaluating salt requirements for observing stimulation of PDK2 activity by NADH and acetyl-CoA were as shown in Figure 1. To minimize reduction of lipoyl groups, no dithiothreitol was included in the assay buffer although a low level was introduced since E1 was prepared in the presence of 1 mM dithiothreitol; this E1/buffer contributed 35−40% of the volume when concentrated protein components are preincubated but only 10−14% of the final reaction mixture volume. In most assays assessing product stimulation, NADH and NAD^+ were introduced at final levels of 0.6 and 0.2 mM, respectively, 60 s prior to ATP. When included, acetyl-CoA was added at a final level of 50 μM 20 s prior to ATP. All assays contained E3 as indicated above. When the NADH/NAD^+ ratio or the acetyl-CoA/CoA ratio was varied, constant total pools of 1.0 or 0.4 mM, respectively, were maintained. All results were supported by more than one experiment. These often used different enzyme preparations and may involve some variation in conditions. Other conditions were as indicated in figure and table legends. Average rates from assays, performed at least in duplicate, are reported. Data analyses were performed as described in the companion paper (*38*). Repetitions or closely related experiments gave results that agreed within experimental error with the results reported.

Binding of Adenine Nucleotides. Binding of [α-^{32}P]ATP under nonturnover conditions and parallel tests of [γ-^{32}P]-ATP or α-^{32}P-labeled ATP under turnover conditions were conducted using the cold-trap method (*29*) by the dilution procedure modified as described in the companion paper (*38*) but with E3 additionally included (18 μg of E2, 2 μg of PDK2, and 6 μg of E3 in nonturnover experiments or 39 μg of E1, 39 μg of E2, 6 μg of E3, and 2 μg of PDK2 in turnover experiments). Effectors such as NADH plus NAD^+ and acetyl-CoA were added as described under the kinetic assays above. For turnover conditions, reactions were allowed to proceed following addition of ATP for only 12 s at 22 °C before a 45 μL sample was diluted into 0.9 mL of 20 mM potassium phosphate buffer, pH 7.0, containing 5% glycerol that was maintained at −3 °C. Subsequent steps and analyses were as described in the companion paper (*38*).

Sedimentation Velocity Studies. Sedimentation velocity experiments were conducted as previously described (*14*, *28*) using an Optima XL-I ultracentrifuge using the An-60 Ti rotor at 20 °C. Sedimentation was monitored with interference optics, and the sample and reference solutions were precisely matched in concentrations including those of absorbing materials (NADH, NAD^+, ADP, etc.) and positions of minisci using double sector cells with a capillary connection. Transfer of a small amount of solvent from the

[2] Y. Hiromasa, H. Bao, X. Yan, X. Gong, A. Yakhnin, J. Dong, S. A. Kasten, L. Hu, T. Peng, J. C. Baker, M. Sadler, and T. E. Roche, manuscript in preparation.

reference side that is loaded in slight excess occurred during a short period of centrifugation at 10000 rpm (*14*). The run was stopped and the cell gently rotated to attain even mixing of solvent and sample throughout the sample side, and then the run was restarted and completed with final loading of ~400 μL per cell. The buffer used in all studies was 50 mM potassium phosphate, pH 7.5, containing 0.5 mM EDTA. Sedimentation at 35000 rpm was conducted with continuous collection of interference scans at a 1 min interval. Sedimentation data were analyzed using DCDT+ software version 1.16 provided by J. S. Philo (www.jphilo.mailway-.com) (*30*). The sedimentation coefficients were calculated by using the $g(s^*)$ fitting function in DCDT+ software (*30*, *31*). Buffer density and viscosity were calculated by using Sednterp version 1.08 (www.jphilo.mailway.com). The level of PDK2 bound to E2 was estimated from the increase in the rapidly sedimenting complex due to bound component (fringe ratio method, typical experimental error ± 15% with 95% confidence interval) (*14*, *28*). The $g(s^*)$ analysis in DCDT+ software gave the $g(s^*)$ distribution and the extrapolation to the initial fringe. The initial fringe of the complex fraction was calculated above the 20 S region of the $g(s^*)$ distribution. The level of bound PDK2 was also estimated by the change in S as previously standardized (*14*); estimates of PDK2 bound from measured ΔS changes vary in repetitions by no more than ±6%, but the calibration PDK2 bound per ΔS change depends on other techniques, and this introduces an additional error of about ±8% so the estimated overall error is about ±13%. Given the inherent experimental errors, there was good agreement between the ΔS and fringe ratio methods in estimating bound PDK2 (Figure 5 legend).

E3 Recycling Assay and Reductive Acetylation To Measure Reduced Lipoyl Groups. The E3 recycling reaction was used to measure functional lipoyl groups and, as modified below, to estimate the proportion of reduced/oxidized lipoyl groups. Using NADH as a substrate, E3-catalyzed reduction of E2 lipoyl groups is coupled to their reoxidation by reacting with 0.15 mM 5,5′-dithiobis(2-nitrobenzoate) (DTNB) (*32*, *33*). In this case, the chemical reoxidation of reduced lipoyl groups (i.e., E2 as a lipoyl domain source) rather than E3 was made rate limiting so that the reaction was proportional to the level of reacting lipoyl groups. In a 200 μL reaction volume, this involved using 2.5 μg of E3 and ~5 μg of the E2 source. This was used to evaluate retention of reactive lipoyl groups in $E2_{ox}$ and to evaluate the level of lipoyl groups reduced by different dithiothreitol treatments using the following subtractive procedure. Dithiothreitol was used to reduce lipoyl groups of E2 under the indicated conditions (time, temperature, and level) followed by reacting all reduced lipoyl groups with 20 mM *N*-ethylmaleimide (200 mM concentrate contained 10% ethanol) for 1 min. E2 was then separated from unreacted *N*-ethylmaleimide by passage through a 10 cm Sephacryl S-300 column at room temperature. Unmodified (oxidized) lipoyl groups that did not react with *N*-ethylmaleimide were then measured by the above E3 recycling reaction. All of the above steps were performed in 50 mM potassium phosphate (pH 7.5) containing 0.5 mM EDTA.

The level of reduced lipoyl groups in the standard E2 preparation was evaluated by measuring the level of reductive acetylation of E2 using 100 μM [1-^{14}C]acetyl-CoA and 30 s reaction time at 30 °C. The level of acetyl groups covalently attached to the protein was measured as previously described (*23*, *24*). The same assay procedure was used to assess E2 acetylation under selected ratios in which the acetyl-CoA to CoA ratio was varied at a fixed NADH to NAD^+ ratio (E1 and E3 but not PDK2 included with E2).

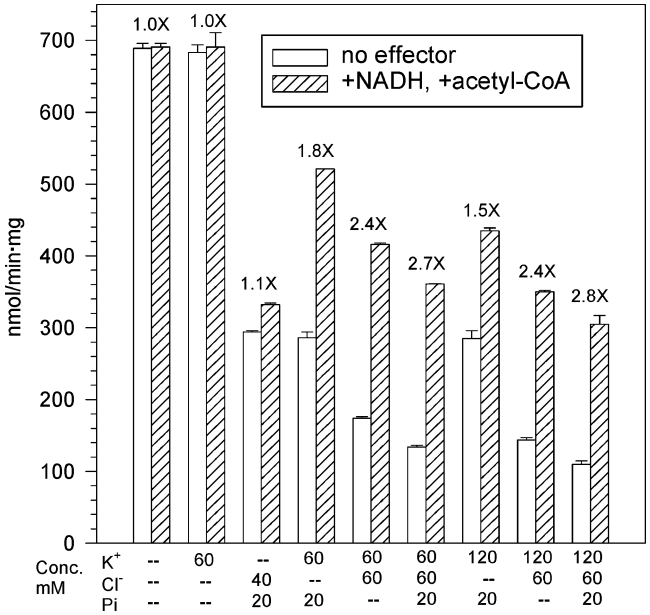

FIGURE 1: Variation in the effects of NADH plus acetyl-CoA on PDK2 activity with variation in buffer conditions. Tris and Hepes were used as counterions and in combination to provide ionic strength as needed. The change in PDK2 activity was evaluated with and without addition of 0.6 mM NADH plus 0.2 mM NAD^+ at 60 s and 50 μM acetyl-CoA at 20 s prior to initiation of PDK2 activity by addition of [γ-^{32}P]ATP to a final concentration of 0.1 mM. Reaction mixtures also contained 14 μg of E1, 10.5 μg of E2, 2 μg of E3, and 0.05 μg of PDK2. The reactions at 30 °C activity were terminated after 60 s. Other conditions were as described under Experimental Procedures.

RESULTS AND DISCUSSION

Ion Requirement and Changes in Kinetic Parameters with Acetyl-CoA and NADH Stimulation. Our goal is to understand how changes in the reduction and oxidation state of the lipoyl groups of E2 alter PDK2 activity. Figure 1 shows the changes in PDK2 stimulation assessed with a variety of buffer conditions using 0.1 mM [γ-^{32}P]ATP. The levels of various ions added are indicated at the bottom of Figure 1; no added salt corresponds to buffer B (companion paper) and includes approximately 7.5 mM phosphate and 13 mM K^+ from the additions of E1 and E2. Neither use of buffer B nor additional inclusion of 60 mM K^+ ion elicited significant stimulation of PDK2 activity. The inclusion of 60 mM Cl^- or 20 mM phosphate (μ = 30 mM for Cl^- and 35 mM for phosphate) reduced PDK2 activity and, when 60 mM K^+ was also included, gave 2.4- and 1.8-fold stimulations. However, even the combination of phosphate and Cl^- did not support significant stimulation of PDK2 activity in the absence of elevated K^+. Including both anions with 60 mM K^+ gave a 2.7-fold stimulation but substantially decreased the control activity due to the inhibitory effects of both anions. Therefore, both the inhibitory effects of an anion and inclusion of K^+ are required for NADH/acetyl-CoA stimulation of PDK2 activity. The level of Cl^- needed for significant stimulation

Table 1: Changes in Kinetic Parameters of PDK2 in Phosphorylating E2-Bound E1 with Different Treatments of E2 That Change the Status of Reduction of the Lipoyl Groups of E2[a]

E2 treatment	V_{max} (nmol·min^{-1}·mg^{-1})	K_m(ATP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m[c] (s^{-1} M^{-1})
expt 1[b]				
none	595 ± 40	39.5 ± 3	0.91	2.3×10^4
expt 2 (Figure 2)				
none	595 ± 25	38.5 ± 2.5	0.91	2.4×10^4
NADH/NAD^+	840 ± 25	47.0 ± 3	1.3	2.7×10^4
NADH/NAD^+ + acetyl-CoA	2060 ± 90	116 ± 8	3.15	2.7×10^4
expt 3 (Figure 3)				
$E2_{ox}$	75 ± 5	7.65 ± 0.9	0.115	1.5×10^4
$E2_{ox}$ + 2 mM dithiothreitol (2 min)	110 ± 15	11.3 ± 2.2	0.17	1.5×10^4
$E2_{ox}$ + NADH/NAD^+ (1 min)	140 ± 8	10.9 ± 1	0.21	2.0×10^4

[a] All assays were conducted in buffer A under the conditions described in the figure legends. [b] Buffer A results in experiment 3 were from Table 1 of the companion paper (*38*). [c] The experimental errors in k_{cat}/K_m values are less than ±0.35.

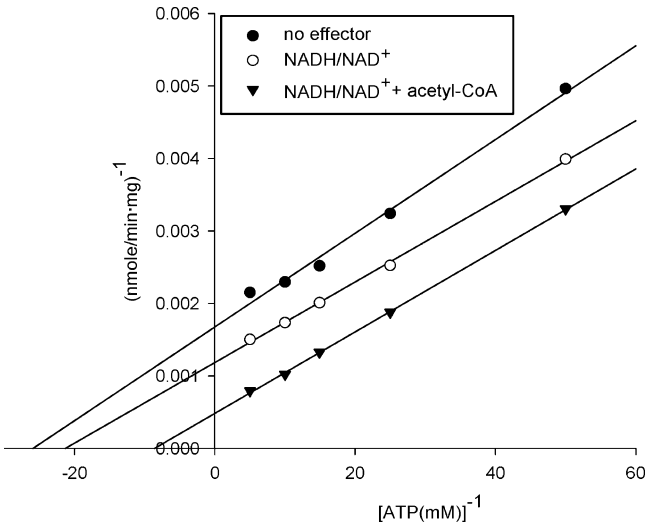

FIGURE 2: Double reciprocal plots showing the variation in PDK2 activity with the ATP level and also with additional treatments to reduce and reductively acetylate E2's lipoyl groups. PDK2 assay mixtures contained 14 μg of E1, 10 μg of E2, 1 μg of E3, and 0.05 μg of PDK2. Duplicate assays for each condition were performed with reaction mixtures containing no effectors, 0.6 mM NADH plus 0.2 mM NAD^+ for 60 s, or this addition and 50 μM acetyl-CoA for 20 s prior to adding the indicated levels of [γ-^{32}P]ATP. Other conditions were as described under Experimental Procedures.

is physiological, but the introduction of 20 mM phosphate added to 7.5 mM phosphate (a physiological level) is beyond that found in mitochondria; additional phosphate is not included in the studies below, which were conducted with buffer A.

Most assays conducted in this paper do not include more than 0.1 mM dithiothreitol in the final assay. E3-catalyzed reduction of the lipoyl groups of E2 using 0.6 mM NADH and 0.2 mM NAD^+ increased both the V_{max} and the K_m of PDK2 for ATP (Figure 2, experiment 2 in Table 1). The additional step of E2-catalyzed acetylation of dihydrolipoyl groups caused a marked increase in PDK2 activity due to V_{max} being increased by 3.45-fold to 2060 ± 90 nmol·min^{-1}·mg^{-1}. Reductive acetylation also significantly elevated the K_m for ATP by 3-fold so that, despite the large increase in V_{max}, there was a very small apparent increase (15%) in k_{cat}/K_m (Table 1, experiment 2; the experimental errors overlap). In the absence of stimulatory effectors, the absolute values of k_{cat} and K_m are close to those shown in experiment 1. As described and analyzed in detail under the overall analysis, fractional stimulation due to reductive acetylation steadily increased with the ATP concentration. As indicated in that analysis, the combined increase in k_{cat} and K_m fit stimulation resulting from an increase in the rate of dissociation of ADP. That is also supported by binding studies below.

Sustained Effects of Oxidation of Lipoyl Groups. To better understand the effects of changes in the oxidation state of lipoyl groups on PDK2 activity, we wanted to fully convert lipoyl groups to the oxidized form. Pettit et al. (*34*) reported that PDKs were thiol-sensitive enzymes based on treating E2-bound PDK with low levels of 5,5′-dithiobis(2-nitrobenzoate) (DTNB). DTNB is particularly effective in converting reduced lipoyl groups to the oxidized form; this occurs in a two-step reaction. The initial disulfide exchange step links a 5-thio-2-nitrobenzoate (TNB) by a disulfide; this TNB is very rapidly released by formation of the internal disulfide (oxidized) form of the lipoyl group. E2 was treated with a low level (20−50 μM) DTNB, and then free DTNB is fully removed by gel filtration. In comparison to untreated E2, PDK2 activity supported by the DTNB-treated E2 was reduced by >4-fold. However, this was not the only cause of loss of kinase activity. Pretreating PDK2 with this level of DTNB followed by gel filtration also decreased PDK2 activity by 3-fold. These two states of inhibition differed in that the effect of DTNB treatment of PDK2 was mostly reversed by a 2 min treatment with 2 mM dithiothreitol. In contrast, there was a slow and less complete regain of effective E2 activation of PDK2 activity when DTNB-treated E2 was treated with 2−4 mM dithiothreitol or E3/NADH (data not shown).

E2 with fully oxidized lipoyl groups was prepared without modifying other thiols by E3-catalyzed oxidation of lipoyl groups using 2 mM NAD^+, followed by gel filtration to remove NAD^+ (see Experimental Procedures). Using the same PDK2 as in the Figure 2 experiment, this NAD^+/E3-treated E2 ($E2_{ox}$) supported a much lower V_{max} and K_m for ATP (Figure 3) (experiment 3 in Table 1). This experiment employed our standard approach of incubating PDK2 with E2 and E1 for at least 1 h at 4 °C. Exposure to 2 mM dithiothreitol for 2 min or E3-catalyzed reduction for 1 min increased PDK2 activity (Figure 3, experiment 3 in Table 1), but the rates were well below those supported by untreated E2 with no reductant added (control Figure 2, experiment 2 in Table 1). The low V_{max} associated with $E2_{ox}$ is coupled again to a decrease in the K_m of PDK2 for ATP (Figure 3, experiment 3 in Table 1). The V_{max} supported by $E2_{ox}$ is below that observed in the absence of an E2 source in the

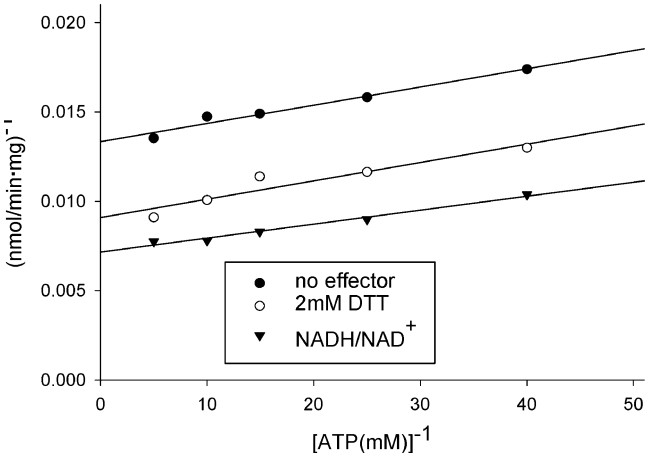

FIGURE 3: Double reciprocal plots showing the variation in PDK2 activity with the ATP level using an E3/NAD$^+$-pretreated E2 ($E2_{ox}$) with no treatment and additional treatments with dithiothreitol and NADH/E3. Assay mixtures contained the same level of components as in the legend to Figure 2 except that $E2_{ox}$ replaced E2. When indicated, components were treated with 2 mM dithiothreitol for 120 s or 0.6 mM NADH plus 0.2 mM NAD$^+$ for 60 s prior to initiating kinase activity.

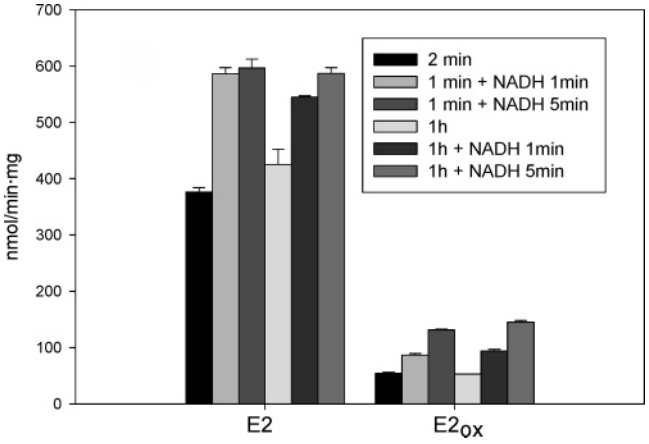

FIGURE 4: Effects of treatment of E2 and $E2_{ox}$ with NADH for 1 or 5 min to control activities when PDK2 is or is not preincubated with the E2 source for 60 min. Other conditions were as described in the legend to Figure 2.

companion paper (*38*). However, using the same level of E1 (3.6 μM) and 0.1 mM ATP, the rate of phosphorylation of free E1 present was ~3.5-fold slower than the rate in this study using $E2_{ox}$−E1. Therefore, $E2_{ox}$ is still aiding access of PDK2 to E1.

We compared the initial rates of PDK2 reaction supported by E2 and $E2_{ox}$ when PDK2 either was added to incubation mixtures equilibrated at 30 °C or underwent the standard 60 min preincubation with either E2 along with E1 and E3 at 4 °C. With either background control activity with PDK2 supported by $E2_{ox}$ remained low, and treatment with 0.6 mM NADH plus 0.2 mM NAD$^+$ for 1 or 5 min did not increase the activity of PDK2 incubated with $E2_{ox}$ to a level as high as that supported by untreated E2 in the absence of effectors (Figure 4). The longer treatment with NADH did lead to a larger fractional activation with the $E2_{ox}$. With the standard E2, there was not a significant increase in PDK2 activity due to the longer treatment with NADH. Korotchkina et al. (*13*) reported a very low PDK2 activity that, after 5 min treatment with NADH, underwent a similar fractional stimulation to that observed with $E2_{ox}$. It seems likely that these investigators used E2 preparations in which the lipoyl groups were almost entirely in the oxidized form. In a cyclic E3 assay in which lipoyl groups were the limiting component (see Experimental Procedures), $E2_{ox}$ supported as high a rate as untreated E2 (data not shown). Therefore, the slow development of a stimulatory effect by NADH/E3 treatment was not due to slower E3-catalyzed reduction of lipoyl groups or less lipoyl groups being available for the E3 reaction.

To better understand our results, the level of reduced lipoyl groups in E2 and dithiothreitol-treated E2 was evaluated. On the basis of acetylation by [1-^{14}C]acetyl-CoA (*23*, *24*), about six to nine lipoyl groups in untreated E2 are in the reduced form. To evaluate the level of reduced lipoyl groups, dithiothreitol-treated E2 was reacted with excess *N*-ethylmaleimide, and then this thiol-specific alkylating agent was removed as described under Experimental Procedures. The level of nonalkylated (i.e., oxidized) lipoyl groups was then measured by using the lipoyl groups as the limiting component in the E3 cycling reaction. A 2 min treatment at 30 °C with 2 mM dithiothreitol reduced the level of available lipoyl groups by 30−35%. Using 4 mM dithiothreitol, nonalkylated lipoyl groups represented 28%, 15%, and 7.2% after 20 min, 60 min, and 14 h treatments at 4 °C. Assuming oxidized dithiothreitol does not exceed 0.1 mM, the 14 h value fits the expected equilibrium constant $K_{eq} = (DTT_{red})$-$(lipoyl_{ox})/(DTT_{ox})(lipoyl_{red}) \geq 3$ (i.e., dihydrolipoyl is a slightly stronger reductant than dithiothreitol).

In combination, our results indicate that storage of E2 with a small portion of lipoyl groups in the reduced form has a large effect on the capacity of E2 to enhance PDK2 function. Apparently some process that is only slowly reversed occurs when the full complement of lipoyl groups is oxidized. As indicated in the companion paper, the preparations of E2 that supported particularly high PDK2 activity (experiments 1 and 2 in Table 1 of the companion paper) were exposed to 10 mM rather than our now standard use of 1 mM mercaptoethanol in the gel filtration step that preceded pelleting E2 and then dissolving in a buffer free of thiols. The 2 min exposure at 30 °C to 2 mM dithiothreitol [used in the companion paper (*38*)] leads to reduction of 30−35% of lipoyl groups, independent of those preparation conditions. However, different E2 preparations did not provide similar support of PDK2 activity. We suggest that the intramolecular transfer of reducing equivalents among the lipoyl groups of an E2 60mer by disulfide exchange (*35*, *36*) is important for maintaining the capacity to enhance kinase activity. The L2 domain preferentially binds PDK2 (*14*, *15*) and supports enhanced PDK2 function (*12*). Although the lipoyl group availability for reaction with E3 does not change, it seems likely that over time the conformation of the L2 domain is altered when its lipoyl group stays in the more hydrophobic, oxidized state.

PDK2 Binding to E2 with Fully Oxidized, Reduced, and Reductively Acetylated Lipoyl Groups. Previously, we found that reduction of E2's lipoyl groups enhanced binding of PDK2 to E2 (*14*). Since the maximum velocities supported by $E2_{ox}$ were greatly reduced, we evaluated whether there was a change in PDK2 binding to $E2_{ox}$ by sedimentation velocity studies (Figure 5). The E2 used in these studies had a slightly higher *S* value than those used in previous studies (*14*, *28*) due to incomplete removal of poly(ethylenimine) (MW 60000); however, this had no effect on PDK2 binding

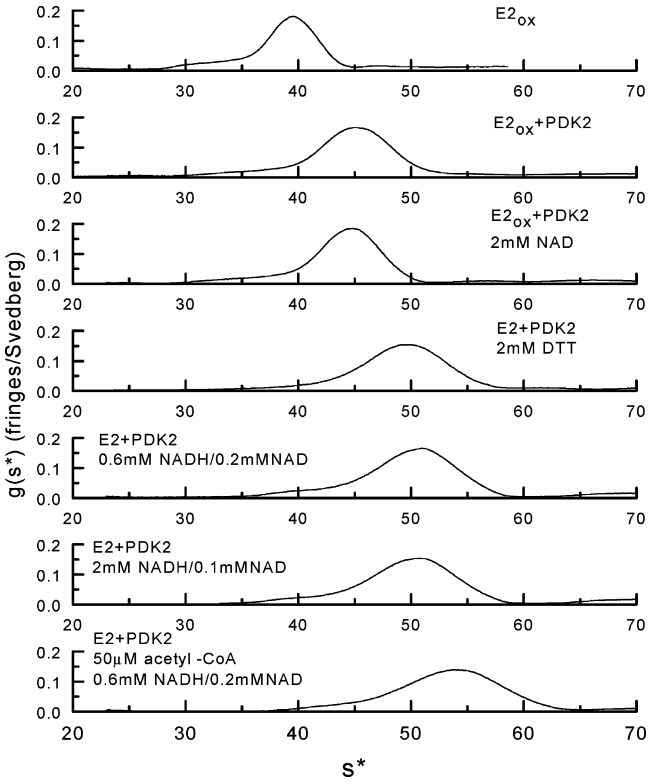

FIGURE 5: Sedimentation profiles for E2 plus PDK2. The $g(s^*)$ profiles are shown that were derived from analysis of sedimentation velocity studies performed with 0.121 μM $E2_{ox}$ and 4.07 μM PDK2 in 50 mM potassium phosphate, pH 7.5, and 0.5 mM EDTA. The bottom five sedimentation runs had 0.1 μg of E3; $E2_{ox}$ was used throughout but E2 is not labeled as $E2_{ox}$ when the subsequent treatments reduced lipoyl groups. Sedimentation was conducted at 35000 rpm at 20 °C. Other conditions and procedures for data analysis were as described under Experimental Procedures. From the panels from top (starting at the second panel) to bottom, the estimates of bound PDK2 per E2 60mer using the ΔS procedure were 6.2, 6.0, 11.3, 12.7, 12.7, and 16.2 (estimated value error ± 13%); using the fringe ratio procedure these estimates were 8.7, 7.8, 10.7, 14.4, 12.5, and 15.6 (estimated value error ± 15%).

as compared to earlier studies (*14*). In all cases, 30 PDK2 dimers were added per E2 60mer which was present at 0.12 μM. The extent of binding of PDK2 to $E2_{ox}$ (see legend to Figure 5) was not significantly decreased from that observed in earlier studies with untreated E2 (*14*). In agreement with those studies with E2, reduction of lipoyl groups of $E2_{ox}$ increased PDK2 binding from about ~7 dimers per E2 60mer to about 12 PDK2 bound per E2 60mer. There was little difference in binding capacity when the NADH/NAD^+ ratio was increased from 3 to 20 or when 2 mM dithiothreitol was used as a reductant (Figure 5).

Previously, we had not evaluated the effect of reductive acetylation (*14*) because of a concern that disulfides might form between acetyllipoyl groups. At least with 0.5 mM EDTA in the buffer there was not a significant level of intermolecular disulfides formed following reductive acetylation. There was an increase to ~16 PDK2 dimers bound per reductively acetylated E2 60mer (Figure 5, bottom panel). This supports the acetyl group directly participating in interactions that enhance PDK2 binding to a lipoyl domain.

Using the K_d from binding of a large complement of PDK2 to E2·E1 in AUC studies (*14*), we estimate that only 55% of PDK2 would be bound under our standard assay conditions (94% with E2·E1 with reduced lipoyl groups). Nevertheless, PDK2 activity decreased much more than 50% with $E2_{ox}$, and reduction of lipoyl groups failed to restore high activity while restoring binding of PDK2 to the level observed with untreated E2 (*14*). Therefore, we conclude that decreased binding of PDK2 to $E2_{ox}$ is not the primary cause of reduced kinase activity.

Effects of Reductive Acetylation Using ΔBE2. Using an E2 lacking the E1 binding domain (ΔBE2), we evaluated the change in kinetic properties of PDK2 using free E1 as a substrate with and without reductive acetylation. At either extreme in the substrate levels (20 μM ATP plus 3.14 μM E1 or 200 μM ATP plus 5.23 μM E1), the inclusion of 30 μg of ΔBE2 caused only a 22 ± 2% increase in PDK2 activity (Table 2).[3] Reductive acetylation gave significant stimulation even when only 10 μg of ΔBE2 was used. Larger stimulations of PDK2 were facilitated by acetylated ΔBE2 at 200 μM ATP than at 20 μM ATP (Table 2). In Figure 6, the effect of reductive acetylation of ΔBE2 on the kinetic parameters of PDK2 was evaluated at three levels of E1 with a range of ATP concentrations. The lines were generated by fitting the data to the ordered mechanism (*38*); constants derived from this fit and from replots (e.g., intercepts versus $[E1]^{-1}$) agreed closely. These gave $V_{max} = 750 \pm 80$ nmol·min^{-1}·mg^{-1} and a K_m for E1 of 51 ± 12 μM. The position of the crossover point being below the *x* axis confirms that $K_d < K_m$ for ATP. The intersect for the crossover point on the *x* axis (equals $-1/K_d$) gives a K_d of 6.2 ± 0.5 μM for ATP. From both the total fit and the *y* axis intersect value for the crossover point [$y_c = 1/V_{max}(1 - K_{mA}/K_{dA}) = -0.0215$ (nmol·min^{-1}·mg^{-1})$^{-1}$], a K_m for ATP is estimated to be 105 ± 15 μM.

In the absence of reductive acetylation of ΔBE2, PDK2 activity was evaluated with the same concentrations of ATP as in Figure 6 and 4.5, 7.5, and 10.5 μM E1 (data not shown). As in Table 2, the specific activities were lower at comparable substrate concentrations than with reductively acetylated ΔBE2. ΔBE2-supported PDK2 activity definitely had a lower K_d for ATP of 3.4 ± 1 μM, based on the crossover point (at -0.295 μM^{-1} on the *x* axis); the crossover point on the *y* axis was -0.030 (nmol·min^{-1}·mg^{-1})$^{-1}$. The K_m for E1 was >80 μM, which is higher than the K_m of PDK2 for E1 supported by acetylated ΔBE2 and significantly higher than the K_m for E1 (26 μM) of free PDK2 (*38*) (see overall analysis). Due to the high K_m for E1, only lower limits could be specified for the other kinetic parameters from this study conducted with 30 μg of ΔBE2 (V_{max} > 500 nmol·min^{-1}·mg^{-1}; the K_m for ATP > 40 μM). The probable near K_m range of ΔBE2-bound PDK2 for E1 is 15−30 mg/mL (100−200 μM E1); 1.60 mg/mL = 10.5 μM was the highest level used. With ΔBE2, the elevated K_m of PDK2 for E1 apparently contributes to kinase specific activities being lower than with acetylated ΔBE2 (Table 2). In contrast to these results with ΔBE2, a change in the dependence of E2-bound PDK2 for E2-bound E1 is not detected due to reductive acetylation since E1 is already provided very efficiently to PDK2.

[3] Assuming that binding of PDK2 to ΔBE2 is equivalent to binding to E2, our AUC studies (*14*) predict that, even with 30 μg of ΔBE2 in 25 μL (0.385 μM), only 65% of PDK2 (added at 0.39 μM PDK2 per ΔBE2) will be bound. However, further increasing the level of ΔBE2 did not significantly increase PDK2 activity. On the basis of the increased affinity demonstrated in Figure 5, the fraction of bound PDK2 is predicted to be >95% following reductive acetylation of ΔBE2.

Table 2: Effect of Addition of ΔBE2 on PDK2 Activity with and without Reductive Acetylation[a]

E1 (μM)	NADH + acetyl-CoA effectors[b]	PDK2 activity (nmol·min^{-1}·mg^{-1})					
		no ΔBE2		10 μg of ΔBE2 (0.126 μM)[c]		30 μg of ΔBE2 (0.38 μM)[c]	
		20 μM ATP	200 μM ATP	20 μM ATP	200 μM ATP	20 μM ATP	200 μM ATP
3.14	none	16.8	19.8	18.6	23.4	20.6	24.6
3.14	included			25.8 (1.4)	38 (1.6)	29.6 (1.4)	41 (1.7)
5.23	none	26.5	29.7			32	37
5.23	included					42 (1.3)	66 (1.8)

[a] Experimental conditions not indicated in the table were as described under Experimental Procedures. The deviations from the average values shown were within ±5%. [b] NADH, NAD$^+$, and acetyl-CoA were added at 0.6, 0.2, and 0.05 mM, respectively. [c] Fold stimulated.

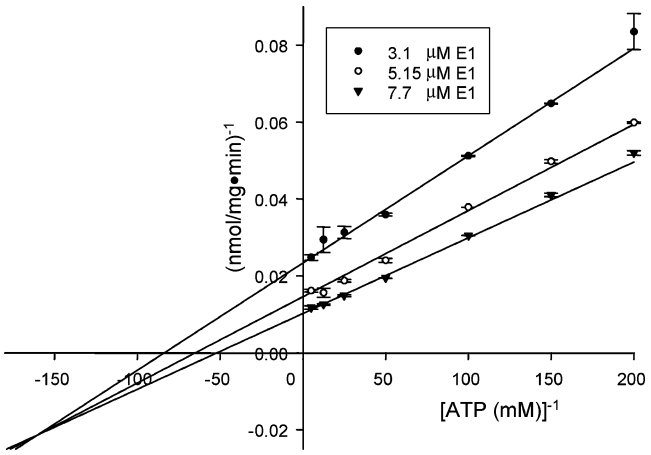

FIGURE 6: Double reciprocal plots showing the variation in PDK2 activity with the ATP following reductive acetylation of ΔBE2. Assays were conducted with 30 μg of ΔBE2, 4 μg of E3, and 0.4 μg of PDK2 with 3.12, 5.2, or 7.79 μM E1. A mixture of 0.6 mM NADH plus 0.2 mM NAD$^+$ was added 60 s and 50 μM acetyl-CoA 20 s before adding [γ-^{32}P]ATP at the indicated concentration.

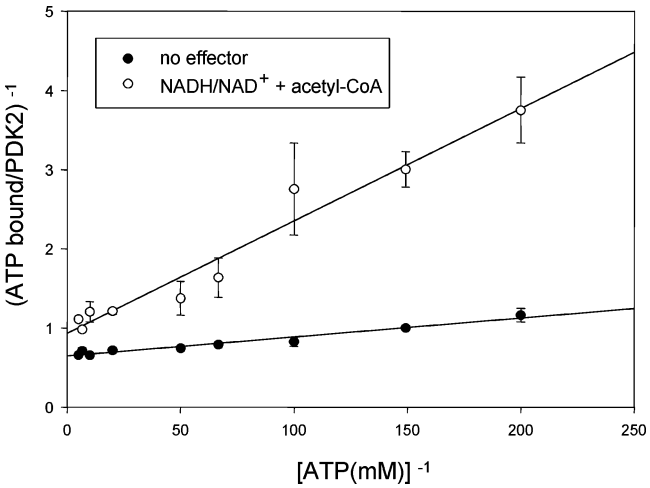

FIGURE 7: Klotz plot of binding of [α-^{32}P]ATP by PDK2 with and without reductive acetylation of E2. Binding of ATP was evaluated with 2 μg of PDK2, 2 μg of E3, and 18 μg of E2. For reductive acetylation of E2, 0.6 mM NADH plus 0.2 mM NAD$^+$ was added 60 s and 50 μM acetyl-CoA 20 s before the indicated level of [α-^{32}P]ATP. Binding was evaluated as described under Experimental Procedures.

As expected if the rate of ADP dissociation increases, reductive acetylation of ΔBE2 probably increased the K_m for ATP (~105 μM); however, with the comparison being only a lower limit (>40 μM at saturating E1), that is not established. Another indication that dissociation of adenine nucleotides was probably increased by reductive acetylation is the increase in the K_d for ATP from 3.4 to 6.2 μM. This trend is confirmed by binding studies below; indeed, the estimated increase due to reductive acetylation of E2 is larger.

Effects of Product Stimulation on the Binding of ATP. In the absence of E1 (i.e., turnover), treatment of E2·PDK2 with NADH/E3 plus acetyl-CoA caused a definite decrease in the affinity of [α-^{32}P]ATP for PDK2; the K_d for ATP increased from 4 ± 1 to 15 ± 3 μM (Figure 7). There also was a decrease in the number of binding sites from $n = 1.55 \pm 0.1$ to 1.07 ± 0.1. Therefore, acetylation of E2 reduced the affinity of PDK2 for ATP by about 3-fold, but it was still relatively tight. The reduction in *n* suggests that an allosteric interaction in the PDK2 dimer hinders ATP binding at the second site in association with stimulation of PDK2 activity by reductive acetylation.

Under conditions of catalytic turnover, stimulation by NADH reduced and stimulation by NADH plus acetyl-CoA appeared to eliminate binding by 0.1 mM [α-^{32}P]ATP (Figure 8). The large reduction in bound adenine nucleotides with NADH and acetyl-CoA was not observed when the nonlipoylated (alanine substituted at the lipoylated lysine) E2·E3BP was used, so NADH and acetyl-CoA do not influence binding of ATP to PDK2. With 0.2 mM ATP, binding was

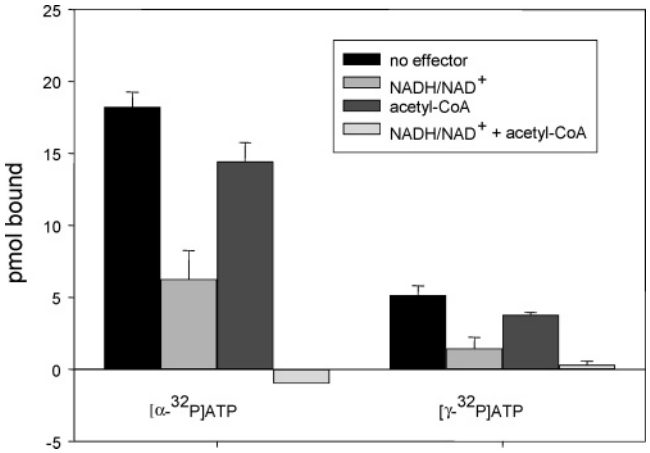

FIGURE 8: Effects of reduction and acetylation of E2 on the binding of adenine nucleotides during catalytic turnover. The amounts of [γ-^{32}P]ATP and of α-^{32}P-labeled ATP plus ADP retained by PDK2 were determined with 2 μg of PDK2, 39 μg of E1, 39 μg of E2, and 6 μg of E3 after 12 s of PDK2 catalysis at 22 °C. Binding was determined as described under Experimental Procedures (*38*).

detected under conditions of product stimulation, and there was little difference between the level of binding with [α-^{32}P]ATP (0.33 ± 0.07/PDK2) and [γ-^{32}P]ATP (0.29 ± 0.03/PDK2), indicating that ATP rather than ADP was primarily bound. As described under the overall analysis, these results are consistent with the rate of dissociation of ADP being elevated and suggest that the association of ATP

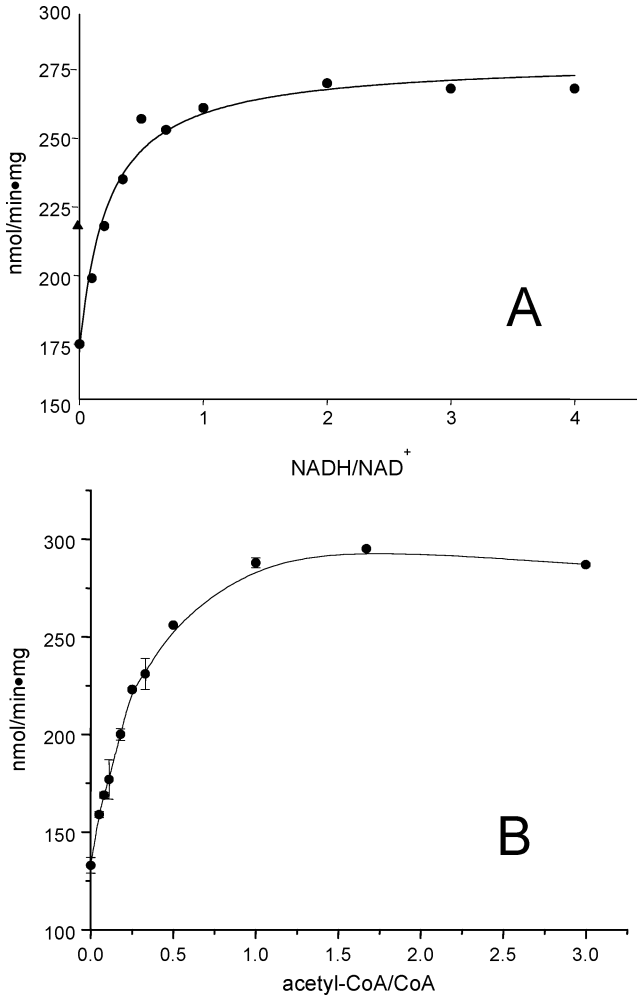

FIGURE 9: Variation in PDK2 activity with a change in the ratios of NADH to NAD^+ and acetyl-CoA to CoA. PDK2 assays were conducted with 14 μg of E1, 10 μg of E2, 1 μg of E3, and 0.05 μg of PDK2. In varying the NADH to NAD^+ ratio a constant pool of 1 mM pyridine nucleotide was used, and in varying the acetyl-CoA to CoA ratio a constant pool of 0.5 mM acetyl-CoA plus CoA was used along with an NADH to NAD^+ ratio of 0.35 NADH to NAD^+ ratio. The mixtures of NADH plus NAD^+ were added 60 s and the mixtures of acetyl-CoA plus CoA 30 s prior to 0.1 mM [γ-^{32}P]ATP.

is a rate-limiting step for stimulated PDK activity at concentrations of ATP below 0.1 mM. That analysis also considers possible deficiencies of the binding assay, particularly under conditions of turnover, following reductive acetylation.

Variation in PDK2 Activity with Product to Substrate Ratios. The effects of variation in the NADH/NAD^+ and acetyl-CoA/CoA ratios on PDK2 activity were evaluated using constant pools of total NADH plus NAD^+ of 1 mM and acetyl-CoA plus CoA of 0.5 mM. Figure 9A shows the change in PDK2 activity as the NADH/NAD^+ ratio is increased. Half-maximal stimulation was supported by the 0.24 NADH/NAD^+ ratio. This is a higher ratio than required for half-maximal stimulation of bovine kidney or porcine liver PDKs (*37*). Figure 9B shows the change in activity with a change in the acetyl-CoA/CoA ratio in the presence of the 0.35 NADH/NAD^+ ratio. Half-maximal stimulation was observed at the 0.23 acetyl-CoA/CoA ratio. This is a somewhat higher ratio than was required with the PDKs associated with bovine kidney PDC (*37*). The condition giving the half-maximal increase in activity led to about one-sixth of the lipoyl groups being acetylated, based on incorporation of acetyl groups using [1-^{14}C]acetyl-CoA (data not shown). Near-maximal stimulation is achieved with about one-third of the lipoyl groups undergoing acetylation.

Overall Analysis of Kinetic and Binding Studies. We have found that product stimulation increases V_{max} and the K_m for ATP. In the opposite direction, a pretreatment that converted all of the lipoyl groups in E2 to the oxidized form supports greatly reduced PDK2 activity and a greatly reduced K_m for ATP versus the standard E2 preparations. Therefore, all conditions found to increase k_{cat} also increase the K_m for ATP and vice versa. Again, that is most easily explained and fit by an ordered mechanism in which ATP binds first and the dissociation of ADP is rate limiting in the absence of stimulatory effectors. In the absence of E1, about a 3.75-fold increase in the binding constant for ATP was observed due to reductive acetylation of E2. On the basis of kinetic studies, $k_{cat}/K_m = k_1$ was not significantly changed due to reductive acetylation of E2. Therefore, these data indicate that the rate of ATP dissociation is increased nearly 4-fold. It seems likely there will be at least an equivalent increase in the rate of dissociation of ADP due to reductive acetylation.

Another trend is discerned in the data for Figure 2 from analyzing the changes in the ratios of stimulated to nonstimulated activity with an increasing ATP concentration. For a change from 20, 40, 67, 100, 200 μM to saturating ATP, respectively, stimulations of 1.5, 1.7, 1.9, 2.25, 2.7, and 3.4 were observed, respectively. While not a typical pattern for an activating condition, this is precisely what is predicted by the proposed mechanism. By fitting the data with reasonable changes in kinetic constants,[4] a 4.7-fold increase in just the rate of ADP dissociation gives a calculated stimulation of 1.5, 1.75, 2.0, 2.2, 2.6, and 3.5, respectively, at 20, 40, 67, 100, 200 μM and saturating ATP, respectively. This is a very good fit of the above data.

The finding that reductive acetylation of ΔBE2 increases PDK2 activity again confirms that E1 need not be bound to E2 for observing kinase stimulation (*12*, *24*). Since only limits for kinetic constants could not be determined in the study with ΔBE2, we can most meaningfully compare kinetic properties when E1 is varied with free PDK2 (*38*) and PDK2 bound to acetylated ΔBE2. Assuming the ordered reaction, the increase in k_{cat} from 0.26 to 1.15 s^{-1} requires minimally that the rate of dissociation of ADP exceed these values. The change in the K_m for ATP from about 5 μM for the free PDK2 to about 106 μM is partially explained by a decrease in the rate of association of ATP with acetylated ΔBE2 activated PDK2 (i.e., $k_{cat}/K_m = k_1$ decreases from 6.5×10^4 M^{-1} s^{-1} for free PDK2 to 1.11×10^4 M^{-1} s^{-1}). Using rate constants as defined in the reaction scheme in Figure 8 of

[4] The relative rates are directly proportional to the ratio of $1/((1/k_1'[ATP]) + (k_5 + k_7'/k_5k_7'))$ to $1/((1/k_1[ATP]) + (k_5 + k_7/k_5k_7))$ where the prime values are those after stimulation by NADH and acetyl-CoA [rate constants as in Figure 8 of the companion paper (*38*)]. To fit the greater level of ADP bound than ATP under condition turnover in the absence of effectors, k_5 must be at least $4 \times k_7$ but cannot exceed $10 \times k_7$. On the other hand, it is very difficult to fit the observed product stimulation (3.46 increase in k_{cat}) by a speed up in ADP dissociation if k_5/k_7 is not at least 10. Therefore, for the nonstimulated PDK2, we used a ratio of 10 as an approximation of the initial k_5/k_7 ratio and $k_7 = 1.0$ s^{-1} (k_7 must be >0.91 s^{-1} and <1.14 s^{-1}) (*38*).

the companion paper (*38*), a further increase of 4.5 in this K_m must still come from the rest of this parameter [$k_5k_7/(k_5 + k_7)$]; this and the related 4.5-fold increase in k_{cat} could again be explained by a speed up in the rate of dissociation of ADP (k_7 step). One result does not yield to this explanation. With ΔBE2 (not acetylated), the higher K_m of PDK2 for E1 cannot be due to a slower k_7 step.[5]

Under conditions of turnover with 0.1 mM ATP being used as substrate, the greatly reduced binding of adenine nucleotides following reductive acetylation of E2 (Figure 8) was in the direction predicted, but the change was beyond that expected. The falloff to maximally one ATP being bound per dimer following reductive acetylation (Figure 7) may limit the extent of binding to PDK2. An ordered mechanism supports (via the k_1 step) some PDK2·ATP accumulation with 0.1 mM ATP (k_1[ATP] = 2.7 s^{-1} exceeds the observed v = 1.5 s^{-1}). With 0.2 mM ATP, a low level of bound ATP was observed, but k_1[ATP] = 5.4 s^{-1} exceeds not only the observed velocity by 2.8-fold but also the k_{cat} of stimulated PDK2 by 1.7-fold. Even under these stimulatory conditions, the dissociation of ATP is estimated to proceed at a rate much slower than k_{cat} = 3.2 s^{-1} (i.e., $k_2 = k_1K_d$ = 0.4 s^{-1}). While less than the expected level of ATP was bound, there was a marked change from mainly ADP being bound to primarily ATP being bound with 0.2 mM ATP following reductive acetylation. We cannot eliminate the possibility that, under conditions of turnover and product stimulation, a conformational transition occurs in PDK2 that allows some adenine nucleotide to be released from the active site during the washing steps at −3 °C.

Just as two-way communication is indicated between the ATP/ADP binding site and the pyruvate binding site (*38*), our results indicate that reductive acetylation limits the time frame for adenine nucleotide being held at the active site, and in the other direction, AUC studies demonstrated that lipoyl domain binding is weakened by adenine nucleotides being bound at the active site (*14*). It seems likely that the latter specifically involves weakening of binding of the lipoyl prosthetic group rather than the lipoyl domain.

In summary our kinetic and adenine nucleotide binding data fit the dissociation of ADP changing from a limiting step to a faster process upon reductive acetylation of the lipoyl groups of E2. Besides increases in k_{cat} and the K_m for ATP that fit this interpretation, reductive acetylation led to enhanced binding of PDK2 by E2 and a somewhat reduced but still tight affinity of PDK2 for ATP. The kinetic study with acetylated ΔBE2 allowed an overview of kinetic parameters of stimulated PDK2, which confirmed that the K_d for ATP remains much lower than the K_m for ATP. When all of the lipoyl groups of E2 are converted to the oxidized form, this alters E2 so that it has a greatly reduced capacity to enhance PDK2 activity. This state is only slowly reversed upon reduction of the lipoyl groups. We suggest that this requires a change in the conformation of the inner lipoyl domain of E2 subunits that binds PDK2 most effectively (*14*).

[5] In the control study without reductive acetylation of ΔBE2, the K_m for E1 (minimal value 80 μM, probable value 100−200 μM) is higher than found for free PDK2 (*38*) (~26 μM) or PDK2 binding to acetylated ΔBE2 (~51 μM). For an ordered reaction $K_{mB} = k_7(k_4 + k_5)/k_3(k_5 + k_7)$. Therefore, for PDK2 bound to ΔBE2, the higher K_m for E1 requires that the rate of association of E1 (k_3 step) decreases or, if equilibrium binding of E1 is established ($k_4 \gg k_5$), that the rate of dissociation of E1 increases. Slow dissociation of ADP (k_7 step) must also be compensated by such a change to give the higher K_m of PDK2 for E1 with ΔBE2.

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