Lipoyl Domain-based Mechanism for the Integrated Feedback Control of the Pyruvate Dehydrogenase Complex by Enhancement of Pyruvate Dehydrogenase Kinase Activity*

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To conserve carbohydrate reserves, the reaction of the pyruvate dehydrogenase complex (PDC) must be down-regulated when the citric acid cycle is provided sufficient acetyl-CoA. PDC activity is reduced primarily through increased phosphorylation of its pyruvate dehydrogenase (E1) component due to E1 kinase activity being markedly enhanced by elevated intramitochondrial NADH: NAD⁺ and acetyl-CoA:CoA ratios. A mechanism is evaluated in which enhanced kinase activity is facilitated by the build-up of the reduced and acetylated forms of the lipoyl moieties of the dihydrolipoyl acetyltransferase (E2) component through using NADH and acetyl-CoA in the reverse of the downstream reactions of the complex. Using a peptide substrate, kinase activity was stimulated by these products, ruling out the possibility kinase activity is increased due to changes in the inactivation state of its substrate, E1 (thiamin pyrophosphate). Each E2 subunit contains two lipoyl domains, an NH₂-terminal (L1) and the inward lipoyl domain (L2), which were individually produced in fully lipoylated forms by recombinant techniques. Although reduction and acetylation of the L1 domain or free lipoamide increased kinase activity, those modifications of the lipoate of the kinase-binding L2 domain gave much greater enhancements of kinase activity. The large stimulation of the kinase generated by acetyl-CoA only occurred upon addition of the transacetylsyl-catalyzing (lipoyl domain-free) inner core portion of E2 plus a reduced lipoate source, affirming that acetylation of this prosthetic group is an essential mechanistic step for acetyl-CoA enhancing kinase activity. Similarly, the lesser stimulation of kinase activity by just NADH required a lipoate source, supporting the need for lipoate reduction by E3 catalysis.

Complete enzymatic delipoylation of PDC, the E2-kinase subcomplex, or recombinant L2 abolished the stimulatory effects of NADH and acetyl-CoA. Retention of a small portion of PDC lipoylates lowered kinase activity but allowed stimulation of this residual kinase activity by these products. Reintroduction of lipoyl moieties, using lipoyl protein ligase, restored the capacity of the E2 core to support high kinase activity along with stimulation of that activity up to 3-fold by NADH and acetyl-CoA. As suggested by those results, the enhancement of kinase activity is very responsive to reductive acetylation with a half-maximal stimulation achieved with ~20% of free L2 acetylated and, from an analysis of previous results, with acetylation of only 3–6 of the 60 L2 domains in intact PDC. Based on these findings, we suggest that kinase stimulation results from modification of the lipoate of an L2 domain that becomes specifically engaged in binding the kinase. In conclusion, kinase activity is attenuated through a substantial range in response to modest changes in the proportion of oxidized, reduced, and acetylated lipoyl moieties of the L2 domain of E2 produced by fluctuations in the NADH: NAD⁺ and acetyl-CoA:CoA ratios as translated by the rapid and reversible E3 and E2 reactions.

In mammalian cells, the pyruvate dehydrogenase complex (PDC) controls the oxidative utilization of glucose (1). Flux through this reaction results in a net depletion of body carbohydrate reserves. The activity of PDC must be reduced when fatty acids or ketone bodies are being preferentially used to provide 2-carbon units for oxidative energy production by citric acid cycle/oxidative phosphorylation systems, a routine situation in many organs. Furthermore, under conditions of starvation or diabetes, the activity of PDC is reduced to a minimal level to conserve carbohydrates essential for the brain and other specialized tissues/organs. To achieve its critical role in cellular fuel conservation, the PDC reaction is controlled primarily by a highly regulated phosphorylation/dephosphorylation cycle which is carried out by dedicated kinase and phosphatase components. Phosphorylation of the pyruvate dehydrogenase (E1) component inactivates the complex and dephosphorylation reactivates the complex.

Shortly after PDC was shown to be regulated by this interconversion (2), the capacity of fatty acids and ketone bodies to promote inactivation of PDC was demonstrated in intact tissues (3, 4) and in studies with intact mitochondria (5, 6). Studies with purified complex (7–9) and isolated mitochondria (10–12) found that increases of the PDC reaction products

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The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; E1a, active, nonphosphorylated form of E1; E2, dihydrolipoyl acetyltransferase component; E2L1 or L1, NH₂-terminal lipoyl domain of E2; E2L2 or L2, internal lipoyl domain of E2; E2N, E1 binding domain; E2D, diglomer forming, transacetylase catalyzing COOH-terminal domain of E2; PDK1 or just K1, major (in bovine kidney) catalytic subunit of pyruvate dehydrogenase kinase, formerly designated by K; PDK2 or K2, minor catalytic subunit of pyruvate dehydrogenase kinase, formerly designated by K; E3, dihydrolipoyl dehydrogenase; E3BP or X, E3-binding protein or protein X; TTP, thiamin pyrophosphate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid.
Mechanism of Kinase Stimulation

FIG. 1. Domain structure of the dihydrolipoyl acetyltransferase (E2) component and interactions with the kinase and E1 components. E2 has four globular domains consisting of an NH2-terminal lipoyl domain, L1, an inner lipoyl domain, L2, an E1 binding domain, and a core-forming, transacytase-catalyzing inner domain at the COOH-terminal end (18-20). The kinase binds to the L2 domain by a domain-specific and lipoyl prosthetic group-requiring interaction (26). E1, an \( \alpha_1 \beta_2 \) tetramer, binds to the B domain of E2 via its \( \beta \)-subunit (18).

promote an increase in the proportion of PDC in the phosphorylated (inactive) state. With purified complex, the activity of the kinase is greatly enhanced upon elevation of the NADH: NAD⁺ ratio and the acetyl-CoA:CoA ratio along with a reciprocal reduction in phosphatase activity as the NADH:NAD⁺ ratio is elevated (7).

Accordingly, the E1a kinase has a crucial role wherein it throttles down PDC activity in response to increases in the mitochondrial acetylation and reduction potentials. Not only are NADH and acetyl-CoA produced during mitochondrial oxidation of all fuels, they are direct products of the PDC reaction. This laboratory presented evidence that this control of kinase activity initially involves these product to substrate ratios being translated by competitive utilization in the downstream reactions of the complex which, in turn, adjusts the fraction of the complex's lipoyl prosthetic groups in the oxidized versus reduced versus acetylated forms (13-17). Specifically, our mechanism proposes that kinase down-regulates PDC activity due to NADH reacting in the reverse of the dihydrolipoyl dehydrogenase (E3) reaction and acetyl-CoA reacting in the reverse of the dihydrolipoyl acetyltransferase (E2) reaction. Typically a 60-80% enhancement in kinase activity occurs upon lipoate reduction and up to a 3-fold enhancement following lipoate acetylation. The potential for understanding the molecular basis of this control has greatly improved with new insights into the structure of E2 subunits and the unusual nature of the association of the kinase with E2.

The E2 subunits of mammalian PDC have four domains connected by relatively large (2-3 kDa) and highly mobile linker regions Fig. 1 (18-20). Sixty COOH-terminal inner domains (E2) associate to form a dodecahedral inner core which catalyzes the transacytase reaction. Each inner domain is connected to 3 globular domains by linker or hinge regions. The globular domains consist of two ~10-kDa lipoyl domains (E2,1 and E2,2) or L1 and L2) and an E1-binding domain (E2,1) located between the inner core and the lipoyl domain region.

The subunits of E1a kinases of PDC and the branched-chain \( \alpha \)-keto acid dehydrogenase complex are related to procaryotic histidine kinases but not to the extramitochondrial serine and tyrosine kinases of eukaryotes (21-23). PDC kinase binds to the lipoyl domain region of E2 through an association that requires the lipoyl prosthetic group (24-26). Using lipoylated and delipoylated forms of recombinant L1 and L2 of human PDC-E2 (27), the kinase was shown to bind preferentially to the lipoylated L2 (Fig. 1) (26). The E1a kinase was also shown to interchange rapidly between L2 structures (26). To account for tight binding and rapid interchange, a dynamic "hand over hand" mechanism is proposed in which a dimeric kinase alternates between being bound to one and two L2 domains. This interchange and catalytic function of the kinase exert their combined effects in the limited space at the surface of the complex where the a kinase molecule and many E1 components are tightly bound to the mobile outer domains of E2. The capacity of continuously bound kinase to phosphorylate bound E1 components more rapidly than free kinase can phosphorylate free E1 is termed E2-activated kinase function.

The aim of this work is to determine the compulsory components, domains, and catalytic processes involved in kinase stimulation by NADH and acetyl-CoA, and to evaluate the relative capacities of the recombinant L1 and L2 in mediating the stimulation of kinase in the presence or absence of E2-activated kinase function. We have found that stimulation occurs with a peptide substrate of the kinase; that a lipoyl source must be available for catalytic reduction by E3 or acetylation by E2; that the L2 lipoyl domain is much more effective in mediating kinase stimulation than the L1 domain; and that the kinase is remarkably sensitive to the level of acetylation of L2. The "Discussion" integrates these observations and draws new mechanistic conclusions based on these and previous results.

EXPERIMENTAL PROCEDURES

Materials—Standard procedures were used to prepare: bovine kidney pyruvate dehydrogenase complex (28), the E2-X-K(2) subcomplex (27), the B subunit, the lipoyl domain region (E2, fragment) of bovine E2 (30). The inner core of bovine E2 was prepared using trypsin treatment followed by pelleting through a sucrose layer in the presence of 5 \( \mu \)g/ml soybean trypsin inhibitor (31). The individual lipoyl domains, L1(1-98) and L2(120-233) of human E2 were expressed in Escherichia coli as fully lipoylated structures fused to glutathione S-transferase and purified to homogeneity as fusion protein free domains as described by Liu et al. (27). Lipoprotein ligase of E. coli was overproduced in E. coli strain TM202 which contains the expression plasmid pTM70, and purified as described (33). The E3 component from porcine heart was from Sigma. Kinase pentacaptopoepptide substrate, YHHGSHMSDPGVSGYRT with the sequential sites of phosphorylation designated by S1 and S2, was prepared in the Biotechnology core facility at Kansas State University and shown to give a single symmetric peak in reverse phase HPLC, to have the correct sequence by automated Edman degradation, and correct mass by FAB mass spectrometry. \( [\gamma-^32P]ATP, [1-^{14}C]acetyl-CoA, and [2-^{14}C]pyruvate were obtained from DuPont NEN.\)

Kinase Activity—E1a kinase activity was measured in duplicate or triplicate as the initial rate of \([^{32}P]phosphate incorporation from [\gamma-^{32}P]ATP, [1-^{14}C]acetyl-CoA, and [2-^{14}C]pyruvate into E1a tetramers in PDC or as the resolved E2-free component. Most assays were conducted in a final volume of 50 \( \mu l \) of buffer A; 50 \( \mu M \) MOPS-K, 20 \( \mu M \) potassium phosphate, 60 \( \mu M \) KCl, 2 \( \mu M \) MgCl₂, 0.4 \( \mu M \) EDTA, and 0.4 \( \mu M \) dihydroxyacetone phosphate (16, 17). Unless otherwise indicated, the levels for the following reagents were NADH and NAD⁺ at 3:1 ratio with final concentrations of 0.6 \( \mu M \) NADH and 0.2 \( \mu M \) NAD⁺ and acetyl-CoA or pyruvate at 50 \( \mu M \), L1 or L2 at 16 \( \mu M \), dihydrolipoamide at 20 to 200 \( \mu M \), and E2 at 2 \( \mu M \). Dihydrolipoamide was dissolved in \( \mathrm{H}_2\mathrm{O} \) containing 0.2 \( \mu M \) EDTA by warming the solution to 60 °C; it was critical that the stock solutions (0.2-2.0 \( \mu M \)) be maintained at room temperature (i.e. not placed on ice) to retain this ligand in solution. The latter was monitored with the E3 cycling reaction below. ATP was added last except in one experiment in which a concentrated fraction of delipoylated E2-X-K(2) subcomplex

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2 The E1 fraction was free of E2 based on SDS-PAGE separation, silver staining, and Western blot analysis using monoclonal antibodies that were specific for the L2 domain of E2. Silver staining of a 2-\( \mu g \) sample of E1 demonstrated a band that migrated at the position of the K1 subunit of the kinase. However, because of comigration with the \( \alpha \) subunit of E1 with the K2 subunit of the kinase, we were unable to determine whether this kinase subunit was also present in this prep.
was treated with ATP and lipoyl protein ligase and the subsequent kinase assays were performed without removal of residual ATP. In the latter series of assays, including those with parallel samples of untreated and delipoylated subcomplex, E1-kinase was added last to initiate kinase activity. For effective reductive acetylation of E2 lipoic acid domains by E1, E1 was preincubated with 50 μM TPP and diluted 10 or 20-fold into kinase mixtures lacking additional TPP. Kinase (and acetylation) assays conducted with CoA removal were performed using the conditions described under acetylation studies. All control assays were conducted in triplicate or quadruplicate and other assays at least in duplicate. In these and other assays the absolute deviations are shown. Reactions were terminated by spotting samples to dry trichloroacetic acid-containing Whatmann No. 3MM with

Reactions were started by adding 0.5 mM -ketoglutarate dehydrogenase complex in reaction mixtures containing 50 mM sodium phosphate buffer, pH 7.5, 1 mM -ketoglutarate and 50 μM lipoic acid, 0.5 mM ATP and 2 mM MgCl2. The acetylation and E3 assays (described above) were used to show that lipoyl moieties had been incorporated. Usually, excess ATP was removed by dialysis versus 20 mM potassium phosphate buffer, pH 7.0.

PDC Activity Studies—The effects of delipoylation or relipoylation of PDC and E2×K1K2 subcomplex samples were deduced from their PDC activities (33). E2×K1K2 subcomplex, 4 μM subcomplex was combined with 8 μM of E1 and 4 μM of E3 in 10 μl and incubated for 60 s at 30° C, and a 6 μl sample was added to a standard PDC assay. Since removal of lipoic groups has a disproportionately low effect on PDC activity at early stages, delipoylation is analyzed more accurately by determining changes in acetylation capacity (above). The fractional binding of TPP to E1 can be evaluated in PDC activity assays because bound TPP is continuously converted to the more tightly bound hydroxyethylidenetriamino pyrophosphate intermediate, the substrate of the rate-limiting reductive acetylation step (35, 36). The levels of TPP bound to E1 were limited by comparing the rates of PDC reaction obtained by diluting enzyme 200-fold as the last addition into assay mixtures lacking or containing TPP. Reconstituted PDC activity was measured with resolved E1 and excess E2×K1K2 subcomplex (10 μg) and E3 (4 μg) and limiting E1 (4 μg). The fractional E1-TPP is defined as the ratio of the highest activity observed in the absence of TPP to that observed in the presence of TPP.

The effects of changes in NADH:NAD+ and acetyl-CoA:CoA ratios on TPP binding were investigated. In preliminary experiments, acetyl-CoA was used in the same high lipoic acid-containing Whatmann No. 3MM with Reactions were terminated by spotting samples to dry trichloroacetic acid-containing Whatmann No. 3MM with

Mechanism of Kinase Stimulation

The requirements for NADH and acetyl-CoA stimulation of E1 kinase activity were evaluated via phosphorylating various substrates using the E2×K1K2 subcomplex as a source of kinase activity. As in assays below, NAD+ and NADH were used at a 1:3 ratio to prevent inhibition of E3 by its conversion to the 4 -reduced state. At this ratio, kinase activity is near maximally stimulated by NADH and maximally stimulated by the combination of NADH and acetyl-CoA. We used an E1 preparation that gave <1% activity in a PDC reaction mixture lacking TPP. As shown in Table 1, kinase activity was stimulated 50% by NADH and nearly 2-fold by the combination of NADH and acetyl-CoA in the phosphorylation of E1 whether or not E1 contained TPP cofactor. As expected (37), kinase activity was reduced by TPP. However, the stimulation has been due to reversing the inhibitory effect of TPP or activation via the formation of a reaction intermediate on E1 (e.g. hydroxyethylidenetriamino pyrophosphate formed from acetyl-lipoate by E2). Such PDC digestion produces a complex mixture of lipoic acid domains, the catalytic inner core of E2 and the trypsin-resistant

3 The E3 component uses the lipoic domain-associated lipoate much more effectively than the free lipoate in the cyclic E3 assay; accordingly there is little interference by lipoates released by lipoamidase from lipoamidase (J. Baker, S. Ravindran, and T. E. Roche, manuscript in preparation).

4 The binding to E1 of TPP (37) or of thiaminmthiazolone pyrophosphate, an analog of the transition state intermediate, hydroxyethylidenetiamino pyrophosphate (14), inhibits phosphorylation of E1 by the kinase. To evaluate the effect of the hydroxyethylidenetriamino pyrophosphate intermediate on kinase activity, Cary (39) tested the effect of addition of TPP to PDC in the presence of low pyruvate plus a level of acetyl-CoA and NADH that gave a high phosphorylation of lipoic acid moieties (and high stimulation of the kinase). Addition of 50 μM TPP reduced kinase activity by 60% due to formation of hydroxyethylidenetriamino pyrophosphate which, due to the short reaction time (40 s) and low pyruvate used (50 μM), was minimally converted to acetic acid and acetolactate. This inhibition by this tightly bound intermediate is similar to that by a saturating level of TPP binding to E1 (37) and indicates stimulation of kinase activity does not result from releasing the kinase of inhibition by E1-bound TPP.
E3, all of which are required for kinase stimulation (see below).

Because some stimulation by acetyl-CoA is detected in the absence of NADH, the level of incorporation of covalently attached acetyl groups from [1-14C]acetyl-CoA was determined in the presence or absence of unlabeled ATP under the conditions of kinase assays (Table I). Without NADH, exposure to submillimolar levels of dithiothreitol allows acetylation of a small portion of the lipoyl groups by acetyl-CoA. The low stimulation of kinase that occurs in conjunction with the low level of acetylation in the absence of NADH agrees with previous studies (15–17) (cf. “Discussion”). Thus, our results eliminate the possibility that kinase stimulation is due to changes in the form of TPP interacting with the E1 substrate but they are consistent with the participation of lipoyl prosthetic groups.

Pyruvate Stimulation—Stimulation by pyruvate was first reported by Cooper et al. (41). Support for this stimulation occurring via reductive-acetylation of lipoyl moieties stems from the findings that adding low levels of pyruvate to TPP-containing PDC generates an increased kinase activity equivalent to that with NADH plus acetyl-CoA and that pyruvate is occurring via reductive-acetylation of lipoyl moieties stems from the findings that adding low levels of pyruvate to TPP.

| Additions | Kinase activity (fold change) | Acetylation |
|-----------|------------------------------|-------------|
|           | -TPP                         | +TPP        |
| None      | 2.08 ± 0.25 (1)              | 1.61 ± 0.14 (1) |
| Pyruvate (50 mM) | 2.04 ± 0.06 (0.98) | 1.71 ± 0.3 (1.06) |
| E2 fragment (1 mM) | 1.89 ± 0.19 (0.91) | 1.38 ± 0.08 (0.86) |
| Pyruvate (50 mM), E2 fragment (1 mM) | 2.01 ± 0.09 (0.97) | 2.81 ± 0.03 (1.75) |

Rationale for TPP and lipoyl domain sources for the stimulation of E1 kinase activity

Requirements for TPP and lipoyl domain sources for the stimulation of E1a kinase by pyruvate

Assays were conducted in buffer A using 25 μg of E1a kinase (kinase-specific activity was 0.29 nmol · min⁻¹ · mg⁻¹) 0.29 nmol in 20 mM potassium phosphate buffer. When TPP was included, E1a kinase concentration was incubated with 50 μM TPP for several minutes and then diluted 20-fold into kinase assay mixtures to give a final TPP concentration of 2.5 μM. The E2L fragment containing both lipoyl domains of E2 was prepared as indicated under “Experimental Procedures.” Other conditions were as indicated under “Experimental Procedures.”

Mechanism of Kinase Stimulation

Stimulation of kinase activity of the E2·X·K1K2 subcomplex using E1 free of TPP, E1-TPP, or peptide substrate

Assay mixtures (50 μl final) contained 15 μg of E2·X·K1K2 subcomplex, 2 μg of E3, and as substrate 25 μg of E1 (3.2 μM) free of TPP, 25 μg of E1 incubated with 50 μM TPP and diluted 1 to 10 into kinase assays (E1-TPP), or 25 μg of pentadecapeptide substrate (0.3 mM). The mixture of NADH and NAD⁺ was added 60 s and acetyl-CoA 20 s before [γ-32P]ATP (420 cpm/pmol), and reactions were terminated after 40 s with E1 as a substrate and after 120 s with peptide substrate. Other steps were performed as described under “Experimental Procedures.” Activity values with peptide are rounded to nearest 0.05 nmol/min/mg except the control average value (no addition) which had a low deviation in triplicate assays.

| Additions               | Kinase activity (fold change) | Acetylation |
|------------------------|------------------------------|-------------|
|                        | nmol/ min mg subcomplex      | nmol/ min mg pentadecapeptide | nmol (1-14C) acetyl incorporated mg subcomplex |
| None                   | 8.5 ± 0.0 (1.0)              | 6.6 ± 0.2 (1.0) | 0.193 ± 0.003 (1.0) |
| NADH (0.2 mM), NAD⁺ (0.1 mM) | 12.8 ± 0.5 (1.5) | 10.3 ± 0.5 (1.55) | 0.43 ± 0.04 (2.2) |
| Acetyl-CoA (0.1 mM)     | 11.0 ± 0.2 (1.3) | 8.3 ± 0.3 (1.25) | 0.515 ± 0.04 (2.7) |
| NADH (0.2 mM), NAD⁺ (0.1 mM), acetyl-CoA (0.1 mM) | 14.8 ± 0.3 (1.75) | 14.6 ± 0.1 (2.22) | 1.05 ± 0.02 (5.4) |

E3, which all of which are required for kinase stimulation (see below).

Because some stimulation by acetyl-CoA is detected in the absence of NADH, the level of incorporation of covalently attached acetyl groups from [1-14C]acetyl-CoA was determined in the presence or absence of unlabeled ATP under the conditions of kinase assays (Table I). Without NADH, exposure to submillimolar levels of dithiothreitol allows acetylation of a small portion of the lipoyl groups by acetyl-CoA. The low stimulation of kinase that occurs in conjunction with the low level of acetylation in the absence of NADH agrees with previous studies (15–17) (cf. “Discussion”). Thus, our results eliminate the possibility that kinase stimulation is due to changes in the form of TPP interacting with the E1 substrate but they are consistent with the participation of lipoyl prosthetic groups.

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could still be stimulated nearly 2-fold by NADH plus acetyl-CoA but the activity remained well below the fully lipoylated PDC control (Fig. 2). The delipoylated PDC1 or PDC2 showed no stimulation of kinase activity relative to the control subcomplex. Other conditions were as described under "Experimental Procedures." The control sample of subcomplex was given parallel incubations. Kinase assays included 25 μg of E1; pyridine nucleotides were added 60 s and acetyl-CoA 20 s prior to [γ-32P]ATP, and the reactions were terminated after 60 s.

with a low level of acetylation of lipoytes reduced during storage of the complex in 0.5 mM dithiothreitol. SDS-PAGE analysis of the lipoamidase treated PDC1 or PDC2 showed no changes in the protein pattern indicating that there was no proteolysis during the lipoamidase treatments.

Lipoamidase and Lipoyl Protein Ligase Treatment of E2X-K1K2—The requirement for lipote could be confirmed by reattaching the cofactor but E1 must be absent to avoid its phosphorylation in the ATP-dependent lipoyl-protein ligation reaction. E2X-K1K2 was incubated with or without lipoamidase for 210 min and then lipoamidase-inactivated with PMSF as described under "Experimental Procedures" and the legend to Fig. 3. In Fig. 4, capacity of the L1 and L2 domains of E2 to support effector stimulation of kinase activity. Kinase assays were conducted with 25 μg of E1 containing low kinase, and, as indicated, human recombinant L1 or L2 domains were included at 16 μM. Effectors were added at the levels and times given in Fig. 3. The solid bar shows kinase activity in the absence of L1 or L2. Other conditions were as described under "Experimental Procedures."
Mechanism of Kinase Stimulation

In kinase and acetylation assays, the acetylation of 16 μM L2 catalyzed by the E2, oligomer was carried out with removal of CoA formed by the α-ketoglutarate dehydrogenase complex reaction under the conditions described under "Experimental Procedures." Acetyl-CoA was included at levels from 1.5 to 30 μM and was completely consumed in acetylation of L2 at all but the two highest concentrations (based on the measured extents of acetylation). NADH and NAD+ were present at 0.25 mM. Other conditions were as described under "Experimental Procedures."

![Graph](image)

**Fig. 5.** Relationship between the degree of acetylation of the L2 domain and stimulation of kinase activity. In kinase and acetylation assays, the acetylation of 16 μM L2 catalyzed by the E2, oligomer was carried out with removal of CoA formed by the α-ketoglutarate dehydrogenase complex reaction under the conditions described under "Experimental Procedures." Acetyl-CoA was included at levels from 1.5 to 30 μM and was completely consumed in acetylation of L2 at all but the two highest concentrations (based on the measured extents of acetylation). NADH and NAD+ were present at 0.25 mM. Other conditions were as described under "Experimental Procedures."

subcomplex. Delipoylation of the subcomplex reduced kinase activity and abolished the rate enhancing effects of NADH and acetyl-CoA (Fig. 3), but these were to a large extent restored following ligase treatment.

Lipoyl Domain Role and Requirement for Acetyltransferase Reaction—To investigate the relative effectiveness of E2's lipoyl domains, the capacity of recombinant human lipoyl domains (L1 and L2) in mediating kinase stimulation was evaluated with resolved E1 in the presence of E3 and in the presence or absence of lipoyl domain-free E2 (Fig. 4). Kinase activity was increased slightly by NADH with the L1 domain but to a much larger extent by the L2 domain. In the absence of E2, acetyl-CoA had no effect, alone, nor did it enhance the effect of NADH alone. In the presence of acetyl transferase catalyzing E2, kinase activity was stimulated by the combination of NADH and acetyl-CoA with the L1 domain, but not to the extent achieved with just NADH with the L2 domain (Fig. 4). An even larger (nearly 3-fold) enhancement of kinase activity was observed when a combination of effectors was added with L2 in the presence of E2. Since there seems to be no possible role of E2, other than catalyzing the transacylation reaction, the results provide definitive support for the view that the stimulating effects of acetyl-CoA are mediated by lipoyl acetylation. In the L2 + E2, series (Fig. 4), the enhancement by acetyl-CoA, alone, occurred with acetylation of L2 (below). The results establish a preferential role for L2 in mediating kinase stimulation in keeping with the preferential binding of the kinase to the L2 domain (26) (cf., "Discussion").

In parallel experiments, the levels of acetylation of lipoyl domains under the conditions used for studying kinase stimulation were evaluated. With the L2 domain at 20 s (time of ATP addition after acetyl-CoA addition) and at 80 s (the time the kinase reactions were terminated) 0.11 and 0.25 acetyl groups were incorporated per L2 in the presence of E2, but in the absence of NADH. Whereas in the presence of NADH/NAD+ (Fig. 4), the enhancement by acetyl-CoA, alone, occurred with acetylation of L2 (below). The results establish a preferential role for L2 in mediating kinase stimulation in keeping with the preferential binding of the kinase to the L2 domain (26) (cf., "Discussion").

**Fig. 6.** Relative effects of dihydrolipoamide and acetylated-dihydrolipoamide to L1 and L2 on kinase activity and capacity of dihydrolipoamide to support lipoyl domain effects. Where indicated by key bar legends, dihydrolipoamide, L1, and L2 were added to assays at 20 μM. E1-kinase was added to reaction mixtures containing 1 μg of E3 and all reactants except dihydrolipoamide, acetyl-CoA, and ATP. Dihydrolipoamide was added 40 s and acetyl-CoA 20 s before ATP, and other additions were made as described in the legend to Fig. 4 and under "Experimental Procedures."

Degree of Acetylation of L2 Versus the Extent of Stimulation of the Kinase—The above results suggest a progression in kinase stimulation with L2 acetylation. This relationship was evaluated studying kinase activity and acetylation, in parallel, under conditions of controlled acetylation by the acetyl-CoA being completely consumed (Fig. 5). Low concentrations of acetyl-CoA were incubated with the L2 domain in the presence of E2, E3, and a 1:1 ratio of NADH:NAD+ along with the α-ketoglutarate dehydrogenase complex plus α-ketoglutarate to convert CoA into succinyl-CoA. The measured extents of acetylation confirmed that, at all but the highest level (24 μM), all the [1-14C]acetyl-CoA was consumed in acetylating the L2 domain (16 μM). The enhancement of kinase activity increased with the level of acetylation with a half-maximal stimulation observed when ~20% of the L2 were acetylated (Fig. 5). Though a low proportion of free L2, this is higher proportion of L2 acetylated than is required in the intact complex as explained under “Discussion.” The decline in stimulation at high levels of acetylation is also consistent with the latter studies with intact complex (15–17).

Effects of Dihydrolipoamide and Acetyl-dihydrolipoamide and Requirement for a Lipoyl Source for NADH Stimulation of Kinase Activity—Previously reported stimulation of PDC-kinase by dihydrolipoamide (13, 14) could involve reduction of lipoyl moieties in the complex through E3 catalysis or disulfide exchange. We were unable to do experiments in the absence of E3 because of trace E3 in our E1 and resolved kinase preparations. (E3 is very active enzyme and only one round of turnover is needed to reduce the lipoate of L1 or L2.) With no lipoyl domain source present, E1-kinase was increased by dihydrolipoamide with a maximal stimulation at 50 μM of 40–55%.

Previously Liu et al. (26) found L1 and L2 inhibited kinase activity at levels greater than 20 μM. For direct comparison, effects of lipoate sources (dihydrolipoamide, L1, and L2) at 20 μM, alone and in combination, were determined to evaluate the direct and supportive roles of dihydrolipoamide. Addition of 20 μM dihydrolipoamide to E1-kinase without a lipoyl domain source or with L1 or L2 gave small but reproducible enhancements (15–40% ±L1; 30–60% +L2) of kinase activity (Fig. 6, open bars). NADH did not stimulate in the absence of a lipoate source (first series, solid bar); indeed, the mixture of NADH +
acetyl groups transfer from L-isomer to incorrect D-isomer and lipoamide immediately preceded the kinase assay since stimulation was not detected with stored preparations in which lipoamide immediately preceded the kinase assay since stimulation was not detected with stored preparations. It was important that acetylation of dihydrolipoamide resulted from reduction of the lipoate group of L2. The higher stimulation by dihydrolipoamide in the presence of NADH alone. These results indicate that with NADH than with dihydrolipoamide and the combination of L2 did not yield the full capacity for stimulation (Fig. 7). The previous lack of full recovery in the ligase treatment of delipoylated E2-X.KK2 (Fig. 3) was probably due to loss or modification of some of the kinase during these treatments. The requirement for L2's lipoate while not exposing the kinase to lipoamidase and lipoate protein ligase, delipoylated and relipoylated preparations of L2 were made. Delipoylation of the L2 domain abolished its capacity to potentiate acetyl-CoA stimulation of the kinase and ligase treatment restored the full capacity for stimulation (Fig. 7). The previous lack of full recovery in the ligase treatment of delipoylated E2-X.KK2 (Fig. 3) was probably due to loss or modification of some of the kinase during these treatments. The requirement for L2's lipoyl group and for E2, catalysis seems to constitute incontrovertible evidence that kinase stimulation is mediated by acetylation most effectively via the L2 domain of E2.

Effect of Delipoylation and Relipoylation of L2 Domain—To test the importance of L2's lipoate while not exposing the kinase to lipoamidase and lipoate protein ligase, delipoylated and relipoylated preparations of L2 were made. Delipoylation of the L2 domain abolished its capacity to potentiate acetyl-CoA stimulation of the kinase and ligase treatment restored the full capacity for stimulation (Fig. 7). The previous lack of full recovery in the ligase treatment of delipoylated E2-X.KK2 (Fig. 3) was probably due to loss or modification of some of the kinase during these treatments. The requirement for L2's lipoyl group and for E2, catalysis seems to constitute incontrovertible evidence that kinase stimulation is mediated by acetylation most effectively via the L2 domain of E2.

Direct Product Inhibition of the PDC Reaction—The mechanism of product inhibition of PDC under low salt conditions (43) and the effects of higher ionic strengths on the PDC reaction have been characterized (44). However, at physiological K+ levels, the effects of varying NADH:NAD+ and acetyl-CoA:CoA ratios have been characterized for the kinase reaction (27) but not the PDC reaction. The results in Fig. 8 show that half-maximal inhibition of PDC is observed at an NADH:NAD+ ratio of 0.42 (●) in the absence of acetyl-CoA; and at an acetyl-CoA:CoA ratio of 11.2 (●) in the absence of NADH and at 3.55 in combination with a fixed NADH:NAD+ ratio of 0.1 (△). The strongest direct product inhibition of PDC was found with a half-maximal effect at a low NADH:NAD+ ratio of 0.125 when the acetyl-CoA:CoA ratio was held at 1 (Fig. 8, ○). However, near-maximal stimulation of the kinase to switch off bovine kidney PDC occurs with an NADH:NAD+ ratio of 0.1 at a 10-fold lower acetyl-CoA:CoA ratio. We conclude that much higher ratios of products to substrates are required for direct product inhibition of bovine kidney PDC than for speeding PDC inactivation by enhancing kinase activity (cf. "Discussion").

**DISCUSSION**

Randle and co-workers (45, 46) pointed out the importance of feedback control of PDC in satisfying metabolic needs and presented evidence for direct product inhibition of the PDC reaction. After regulatory interconversion of PDC between ac-
Mechanism of Kinase Stimulation

Fig. 9. Model of proposed steps in kinase stimulation. The proportion of L2 domains of the E$_{260}$ core having oxidized, reduced, or acetylated lipoates responds to changes in the NADH:NAD$^+$ ratio and acetyl-CoA:CoA ratio via the rapid and reversible reactions catalyzed by E3 and E2, respectively. Our model proposes that a change from the kinase binding to one or two L2 containing only oxidized lipoate (nonstimulated K state) to interacting with an L2 containing a reduced or acetylated lipoate results in the modified lipoates allosterically inducing conformational changes that generate the progressively more active K$^+$ or K** states, respectively.

tive and inactive forms (2) became known, Randle’s laboratory contributed to evidence (cf. Introduction) that the products of PDC reaction influence the proportion of PDC in the active form, through diverse studies with purified complex isolated mitochondria, and intact tissues (1, 8, 9, 47). In their studies with purified porcine heart PDC, no stimulation by acetyl-CoA beyond that of NADH was detected, and they speculated that enhanced kinase activity might be due to the removal of an inhibition of kinase activity caused by the absence of an interaction between oxidized lipoate and the E1 substrate (8, 9). Although not the correct mechanism and detection of an effect of acetyl-CoA required use of higher K$^+$ concentration (see below), the suggestion of a role for changes in the intermediate status of lipoyl groups was insightful as was their linking of the stimulatory effect of pyruvate (41) to the effect of products (9). This laboratory presented the initial and much subsequent evidence that reduction and acetylation of lipoyl prosthetic groups of PDC constituted essential steps in the operation of a sensitive signal translation process whereby increases in the NADH:NAD$^+$ and acetyl-CoA:CoA ratios markedly enhanced kinase activity (13–17). However, elucidation of the minimal requirements for the operation of this control could be accomplished only following a greatly enhanced understanding of the organization of the complex, the preparation of individual lipoyl domains of PDC-E2, and the availability of lipomodase and lipoyl protein ligase, combined with the selective use of peptide substrate and free forms of lipoamide.

Using these tools, we have found that the marked enhancement of kinase activity by acetyl-CoA requires a lipoate source and its reduction, the catalytic domain of E2, and a peptide substrate. These requirements conclusively support a change in the kinase mediated by catalytically forming acetyl-dihydrolipoate. Accumulation of this intermediate explains the similarity strong stimulation of kinase activity by low pyruvate via E1(TPP) catalysis. The direct stimulation by dihydrolipoamide, its capacity to replace NADH in potentiating acetyl-CoA stimulation, and the complete lack of an effect of NADH on kinase activity in the absence of a lipoate source strongly favor NADH boosting kinase activity through its use in the E3 reaction. We have established that this intervention is abolished by complete removal of lipoates from intact E2 subunits or lipoyl domains. Of particular importance for understanding the operation and regulation of the kinase is our finding that the kinase-binding L2 domain is much more effective than E2’s L1 domain or lipoamide in mediating kinase stimulation. The 3-fold increase in kinase activity generated by reductive acetylation of recombinant L2 is comparable to the change in kinase activity produced by acetylation of lipoyl moieties in the intact complex. Reduced L1 was only slightly more effective than free dihydrolipoamide when compared at 20 µM, and 50 µM dihydrolipoamide gave at least an equivalent stimulation, suggesting the structure of L1 does not contribute significantly to these effects. At higher levels L1 inhibits kinase activity (26). Thus, the L2 domain must have structural features that facilitate its dedicated roles in binding the kinase, producing enhanced E1 phosphorylation within the confines of the complex, and further increasing kinase activity upon reduction and acetylation of its lipoyl group. This raises the question of the linkage between these L2-supported actions of the kinase.

Detaching lipoates of the E$_{260}$ core removes the capacity of the E2 core to give a severalfold increase in E1 phosphorylation (24). Via lipoyl-dependent binding to the L2 domain, rapid “hand over hand” interchange of a kinase dimer between lipoyl domains apparently eliminates constraints normally associated with binding that is as tight as exists between E2 and the kinase (25, 26). It is significant that a lipomodase treatment of PDC, which left only a few lipoates and caused close to full loss of this E2-enhanced kinase function, still allowed marked stimulation of the residual kinase activity upon acetylation of those few domains retaining lipoyl groups (PDC$_1$ series in Fig. 2). This result forcefully suggests that the kinase has moved to the limited number of lipoylated L2 domains and, furthermore, that their acetylation (during a short-lived dissociation and reassociation of the kinase) facilitated kinase stimulation. Based on this continued stimulation when very few E2 subunits retain lipoate, we hypothesize that maximal effector stimulation is mediated by an allosteric effect induced by a reductively acetylated L2 domain that becomes engaged in binding of the kinase (i.e. not by interaction of an acetylated prosthetic group on a neighboring lipoyl domain that is not engaged in binding the kinase). Such a highly specific interaction is further supported below.

Beside E1 catalyzed reductive-acetylation giving a nearly equal stimulation to acetyl-CoA (13–15), α-ketobutyrate (38) gives a lesser stimulation similar to that of propionyl-CoA at low levels of propionylation (16). In the absence of a lipoyl domain source, kinase phosphorylation of E1 is not stimulated at low pyruvate (Ref. 17, miniprint section); and here we show
that TPP and a lipoyl domain source are needed to achieve pyruvate stimulation. These findings raise the question as to whether the E1 reaction or the downstream (E2,E3) reactions determine the relative proportion of lipoates in the oxidized, reduced and acetylated forms in the complex. Since E1 catalyzes the rate-limiting step (35, 36), under most metabolic conditions, this distribution should be determined by the NADH:NAD⁺ ratio and the acetyl-CoA:CoA ratios and kinase activity should primarily respond to their fluctuation.

With purified porcine liver and bovine kidney PDC, half-maximal stimulations of kinase activities occur at ratios of NADH:NAD⁺ of 0.03 and 0.05, respectively, and, with 2 mM dithiothreitol as a reducing agent, at ratios of acetyl-CoA:CoA of 0.1 and 0.17, respectively (28). Even lower acetyl-CoA:CoA (<0.1) give half-maximal stimulation when a NADH:NAD⁺ ratio of 0.1 is provided to reduce lipoates (data not shown). At these low ratios, there is minimal product inhibition of the PDC reaction by NADH plus acetyl-CoA (cf. the 0.1 value on the Δ curve, Fig. 8). Significant direct product inhibition occurs with low NADH:NAD⁺ ratio when the acetyl-CoA:CoA ratio was held at a relatively high level of 1 (Fig. 8, ○). Acetyl-CoA:CoA ratios have been generated at and above this range with isolated mitochondria and are proposed to contribute to direct PDC inhibition (48); depletion of CoA may also inhibit PDC due to buildup of fatty acyl-CoAs (49). While these probably contribute to a fine tuning role on a small portion of active PDC, enhanced phosphorylation should effectively throttle down PDC activity before onset of these extremes.

A prior, the lesser enhancement of kinase activity by just the conversion of lipoates from the oxidized to the reduced form could involve either removal of an inhibitory interaction of oxidized lipoates, an enhancement of kinase activity by gain of a positive allosteric interaction of the reduced form, or a change in the thiol disulfide state of a kinase subunit. Studies with E2-bound kinase found that the kinase is sensitive to thiol reagents (13, 52), effects probably due to changes in the oxidation-reduction state of the lipoate of L2 domains. Although there is a small increase in alkylation of cysteines of the K1 subunit following treatment of E2-X:kinase-E3 with NADH, this was not rapidly reversed by excess NAD⁺ which opposes kinase stimulation. Thus, a mediator role for changes in the thiol-disulfide status of the kinase is not indicated. Furthermore, our finding that free dihydrolipoamide and to a greater extent acetylated dihydrolipoamide directly stimulated kinase activity in the absence of a lipoyl domain source, eliminating the prospect that stimulation results from removal of an inhibitory effect by the oxidized form of lipoate. As modeled in Fig. 9, we conclude that kinase activity is enhanced through direct positive allosteric interactions of the reduced or the acetylated form of an L2 lipoate and we further suggest that L2 specificity derives from the L2 domain engaged in binding the kinase.

Consistent with stimulation occurring at low ratios of acetyl-CoA to CoA, half-maximal and near-maximal stimulation of kinase associated with the intact bovine kidney PDC are achieved with only 7–10 and 22–26 acetyl groups incorporated, respectively, per PDC (15–17). With the recent knowledge that there are 3 lipoyl domains, two on E2 and one on the E3-binding protein giving a total of at least 126 lipoates per E2, it seems likely that no more than 12 and possibly as few as 7 acetyl groups are incorporated per PDC into the kinase binding L2 domain for near-maximal stimulation, and as few as 3 and no more than 6 are incorporated for half-maximal stimulation. Even at fairly low levels of acetylation, both E2 and E3BP are acetylated (42). Furthermore, analysis by autoradiography of the acetylation of elastase-generated polypeptides of L1 and L2, that were identified on blots by L1- and L2-specific monoclonal antibodies (27), demonstrated that both lipoyl domains of E2 are acetylated well with low levels of acetylation (S. Rahmatullah and T. E. Roche, unpublished results). There appeared to be a somewhat higher acetylation of L2 but the complex distribution of L1- and L2-derived polypeptides made that judgment uncertain.

In conclusion, our results strongly support E3-catalyzed reduction and E2-catalyzed acetylation of lipoyl moiety of the kinase binding inner (L2) domain of E2 mediating a marked enhancement in kinase activity. The effectiveness in intact PDC of acetylation a low proportion of L2 domains in enhancing the phosphorylation of many bound E1 by a single tightly bound kinase activity would be reinvestigated.

6 J. Baker and T. E. Roche, unpublished results.

7 Maeng et al. (54) have presented evidence that there are 12 protein X (E3BP) in yeast PDC using an area densiometric analysis approach. Using a less accurate densiometric approach, Jilka et al. (42) determined that there are only 6 E3BP in mammalian PDC but that should be reinvestigated.
bound kinase molecule is probably accomplished by inter-L2 domain movement of the kinase combined with a stronger interaction of the kinase with acetylated L2 domains. Several studies on kinase function and regulation are consistent with a change in a K⁺-requiring process ultimately occurring in kinase stimulation. A candidate mechanism involves counteracting K⁺-strengthened ADP binding to the kinase to speed up slow dissociation of this product from the active site of the kinase.

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