Effect of Substitutions in the Thiamin Diphosphate-Magnesium Fold on the Activation of the Pyruvate Dehydrogenase Complex from Escherichia coli by Cofactors and Substrate*

(Received for publication, January 29, 1996, and in revised form, September 16, 1996)

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The homotropic regulation of the Escherichia coli pyruvate dehydrogenase multienzyme complex (PDHc) by its coenzyme thiamin diphosphate and its substrate pyruvate was re-examined with complexes containing three and one lipoyl domains per E2 chain, and several variants of the latter, containing substitutions in the putative thiamin diphosphate fold of E1 (G231A, G231S, C259S, C259N, and N258Q). It was found that all of the E1 variants had significantly reduced specific activities, as reported elsewhere (Russell, G. C., Machado, R. S., and Guest, J. R. (1992) Biochem. J. 287, 611–619). In addition, extensive kinetic studies were performed in an attempt to determine the effects of the amino acid substitutions on the Hill coefficients with respect to thiamin diphosphate and pyruvate. All but one of the variants were incapable of being saturated with thiamin diphosphate, even at concentrations > 5 mM. Most importantly, the striking activation lag phase lasting for many seconds in the parental complexes containing three and one lipoyl domains per E2 chain was totally eliminated in the variants. Furthermore, activation by the coenzyme was localized to the E1 subunit, because resolved E1 exhibits virtually the same behavior during the activation lag phase as does the complex. In the parental complexes two distinct lag phases could be resolved, the duration of both decreases with increasing ThDP concentration. A mechanism that is consistent with all of the kinetic data on the parental complexes involves rapid equilibration of the first ThDP with the E1 dimer, followed by a slow conformational equilibration, that in turn is followed by slow addition of the second ThDP to form the fully activated dimer. When the diphosphate site is badly impaired, the binding affinity is very much reduced, this perhaps eliminates the slow step leading to the activated dimer form of the E1.

The pyruvate dehydrogenase complex (PDHc) is a multienzyme complex that is found in both prokaryotic and eukaryotic organisms and is of great importance in carbohydrate metabolism. The enzyme complex catalyzes the oxidative decarboxylation of pyruvate in the following overall reaction according to Koihe et al. (2).

\[
\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{Acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \tag{1}
\]

In Escherichia coli three different enzyme components are involved in the above reaction: pyruvate dehydrogenase, utilizing thiamin diphosphate (ThDP) as a cofactor (EC 1.2.4.1; E1); dihydrolipoamide transacetylase, which contains covalently-bound lipoyl groups (EC 2.3.1.12; E2); and lipoamide dehydrogenase, containing tightly bound FAD (EC 1.8.1.4; E3). The multienzyme complex performs the following series of reactions according to Gunsalus (3), Massey (4), and Reed (5).

\[
\begin{align*}
\text{Pyruvate} + \text{E1-ThDP-Mg}^2+ &\rightarrow \text{E1-hydroxyethylidene-ThDP-Mg}^2+ + \text{CO}_2 \\
&\rightarrow \text{E1-hydroxyethylidene-ThDP-Mg}^2+ + \text{E2-lipoamide} \\
&\rightarrow \text{E1-ThDP-Mg}^2+ + \text{E2-acetyldihydrolipoamide} \\
&\rightarrow \text{E2-acetyldihydrolipoamide + CoA} \\
&\rightarrow \text{E2-dihydrolipoamide + acetyl-CoA} \\
\end{align*}
\]

\[
\begin{align*}
\text{E2-dihydrolipoamide} + \text{E3-FAD} &\rightarrow \text{E2-lipoamide + E3-FADH}_2 \\
\text{E3-FADH}_2 + \text{NAD}^+ &\rightarrow \text{E3-FAD + NADH} \tag{2–6}
\end{align*}
\]

The complex consists of multiple copies of each component, an ideal polypeptide stoichiometry being: 24 E1, molecular weight 99,474 (6); 24 E2, molecular weight 65,959 (7); and 12 E3, molecular weight 50,554 (8); for a total calculated molecular weight of 4.57 × 10^6 daltons.

Metabolic inhibitors of PDHc include reduced nicotinamide adenine dinucleotide (NADH), acetyl-CoA, and guanosine triphosphate (GTP) (9, 10, 11, 12). Mammalian PDHc is regulated in a more complex fashion by phosphorylation and dephosphorylation (5) of the α subunit of the tetrameric E1 component (α2β2). PDHc is also inhibited by various substrate inhibitors, including 2,3,5,6-tetrachloro-1-p-benzoquinone (TCBQ), 3-aminopyridine (3-AP), and 3-hydroxy-2-naphthoic aldehyde (HNA).
analouges including bromopyruvate (13), fluoropyruvate (14, 15), phosphate analogues of pyruvate (16, 17), mono- and bifunctional arenoxides (18, 19, 20), branched chain keto acids (21), and tetrahydrothiamin pyrophosphone (22).

Although no x-ray structure has been published for an E1 component, amino acid sequence alignments have indicated that they contain a β-turn-α-turn-β structural motif that is responsible for binding the thiamin diphosphate and MgII cofactors (23). This prediction was borne out by the corresponding three-dimensional structures of pyruvate decarboxylase (PDC) from yeast (see Dyda et al. (24) for PDC from Saccharomyces uvarum and Arjuman et al. (25) for PDC from Saccharomyces cerevisiae), transketolase from baker’s yeast (26) and pyruvate oxidase from Lactobacillus plantarum (27). Site-directed mutagenesis studies on PDC from Zymomonas mobilis (28, 29) confirmed the importance of this ThDP fold in maintaining enzyme activity. In particular, substitutions other than Asp to Gly at position 440 (the MgII binding site) led to inactive protein, as did the N467G substitution in the same region. These amino acids are part of the conserved DGDC N,N motif on all ThDP enzymes studied to date. The sequence alignment of nearly 20 ThDP-dependent enzymes also succeeded in identifying the likely region for ThDP binding in E1 of PDHc isolated from E. coli (23). Russell et al. (1) confirmed the importance of such a ThDP fold in PDHc, by showing that the E1 and PDHc activities of site-directed variants in this putative fold were affected. In this report, we extend the studies of Russell et al. to demonstrate that the corresponding substitutions not only diminish the enzyme activity, but also affect the activation of the enzyme by ThDP-MgII.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strain JRG1342 carrying plasmids encoding 3-lip PDHc (pGS523) (wild-type), 1-lip PDHc (pGS501), variant PDH complexes with mutations in E1 (G231A (pGS455), N258Q (pGS597), C259N (pGS596)), and strain JRG2800 carrying a plasmid with mutation in E1, G231S (pGS457), and strain JRG2600 carrying a plasmid with mutation in E1, G231S (pGS543), were from the laboratories of J. R. G.

Purification of the 1-Lip and 3-Lip PDH Complexes— Cultures were grown in the LB medium containing glucose (0.2%) and ampicillin (50 μg/ml) at 37°C. Expression of the PDH complexes was induced by the addition of isopropyl-1-thio-D-galactoside to 1 mM. The conditions for overproduction of the 3-lip PDH complex (wild-type) and several 1-lip additions of isopropyl-1-thio-D-galactoside to 1 mM were used for the near-UV (250–350 nm) region. The wave-

Circular Dichroism—(CD) studies were performed on a Jobin Yvon Dichrograph Mark VI spectrophotometer at 25°C. A 1.0 cm path length cell was used for the near-UV (230–350 nm) region. The wave-

Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (35).

RESULTS

ThDP Binding by 3-Lip and 1-Lip PDH Complexes—In Table 1 are presented kinetic parameters for the 3-lip and 1-lip PDH complexes, calculated from fitting the steady-state rates for different concentrations of ThDP using the Hill equation. A more detailed analysis using Edie-Scatchard and Lineweaver-

| Complex | Substitution | Specific activity | Relative activity | Km | n
|---------|--------------|------------------|------------------|----|----|
| 3-Lip PDHc | 8.43 | 100 | 0.0026 | 0.92 |
| E1 (PDHc) | 0.0043 | 0.17 | 1.3 |
| E1 (resolved) | 0.0106 | 0.034 | 1.0 |
| 1-Lip PDHc | 6.86 | 100 | 0.0028 | 0.99 |
| E1 (PDHc) | 0.0017 | 0.19 | 1.2 |
| 1-Lip PDHc | 0.067 | 0.97 | Unsat.
| G231S | 0.0119 | 0.03 | 1 |
| G231A | 0.0057 | 0.89 | 1.26 |
| N258Q | 1.18 | 17.0 | Unsat.
| C259N | 1.85 | 27.0 | Unsat.
| C259S | 3.94 | 58.0 | 0.037 |

a Activity of 3-lip, 1-lip, and variant PDH complexes was determined using the NADH assay in the presence of 0.2 mM ThDP. E1 activity in the 3-lip, 1-lip PDH complexes or of resolved E1 was assayed according to E1-specific assay.

b Hill coefficient.

c Apparent Km.

d Unsat., not saturated at 5–10 mM ThDP.

The overall enzymatic reaction for pyruvate oxidase from E. coli (30) was initiated either by adding enzyme to a mixture of all other components, or by adding CoA. Steady-state velocities were taken from the linear portions of the progress curves after completion of the lag phases (if present). One unit of activity is defined as the amount of NADH produced (umol/min/mg of protein).

The E1 activities in the complexes or of the resolved E1 component were measured according to McNally, Motter, and Jordan (31). The reaction mixtures contained: 0.05 M Tris, pH 7.7, 5.0 mM pyruvate, 12 mM magnesium chloride, 0.2 mM ThDP, and 1.25 mM each of 4,4′-dithiopyridine and 4-mercaptopyridine. Reactions were initiated by the addition of enzyme and monitored at 390 nm at 27°C using the Cobas Bio centrifugal analyzer. Specific activities were calculated using εmax = 72,200 M–1 cm–1 for the reaction product, N-acetyl-4-thiopyridine (32). The activity of resolved E1 was also measured after reconstitution with excess E2–E3 subcomplex using the NADH assay for PDHc activity.

The lag phases ( τ) were determined graphically as described by Horn and Biswasanker (33). ThDP concentrations were determined using ε723 = 7,500 M–1 cm–1 (34), and protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin as standard.

The Michaelis-Menten constant Kon, the apparent Km, and Hill coefficient (nH) were obtained by fitting the steady-state rates at different concentrations of ThDP or pyruvate with the Hill equation and the logarithmic form of the Hill equation using the Delta Graph (Prol) and CA-Cricket Graph computer programs. The steady-state rates were also analyzed using the Edie-Scatchard and Lineweaver-Burk plots. The kinetic parameters represent the mean of at least three independent experimental data sets.

Gel Filtration High Performance Liquid Chromatography— Gel filtration high performance liquid chromatography was performed on a TSK G3000-SW column (7.5 mm × 60 cm) equilibrated with 50 mM potassium phosphate, pH 7.5 containing 0.2 M Na2SO4 and 0.1% NaN3. The column was calibrated using molecular weight standards (Pharmacia): ovalbumin (43,000), bovine serum albumin (67,000), aldolase (158,000), and ferritin (440,000).

Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (35).

Circular Dichroism—(CD) studies were performed on a Jobin Yvon Dichrograph Mark VI spectrophotometer at 25°C. A 1.0 cm path length cell was used for the near-UV (230–350 nm) region. The wave-

In Table 1 are presented kinetic parameters for the 3-lip and 1-lip PDH complexes, calculated from fitting the steady-state rates for different concentrations of ThDP using the Hill equation. A more detailed analysis using Edie-Scatchard and Lineweaver-

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Burk plots revealed deviations from hyperbolic behavior at concentrations of ThDP, 1 mM. ThDP has been reported to exhibit different allosteric regulatory properties at different pyruvate concentrations (36). Thus at 0.2 mM pyruvate, no cooperativity was observed over the entire ThDP concentration range tested (data not shown), whereas at 0.08 mM pyruvate, the 3-lip PDHc exhibited positive cooperativity ($n_H = 1.46$) for binding ThDP at ThDP concentrations $< 1 \mu$M, and no cooperativity ($n_H = 1.0$) at ThDP concentrations $> 1 \mu$M, in good agreement with Bisswanger (36). However, with 2 mM pyruvate, the binding of ThDP by 3-lip PDHc showed no cooperativity at ThDP concentrations $< 1 \mu$M, and weak negative cooperatively ($n_H = 0.79$) at ThDP concentrations $> 1 \mu$M (Fig. 1). The increasing cooperativity of 3-lip PDHc observed with decreasing concentrations of both ThDP and pyruvate clearly confirm that 3-lip PDHc is regulated by both the cofactor and the substrate. The regulatory properties of 1-lip PDHc were similar to those of the 3-lip PDHc (data not shown).

Using PDHc from *E. coli* with low concentrations of ThDP in the incubation medium and saturating concentrations of pyruvate, Bisswanger (36) showed that the progress curve for the overall reaction (i.e. formation of NADH) exhibits an activation lag phase of several minutes before the steady-state rate is reached. The duration of the lag phase was found to decrease with increasing concentration of ThDP.

In our studies the progress curves for the overall reaction using 3-lip and 1-lip PDHc complexes exhibited lag phases in the presence of less than 25 $\mu$M ThDP when the reaction was initiated by the addition of enzyme (see Fig. 2A for typical results). With increasing ThDP concentration, the lag phase shortened (Fig. 2B). At low ThDP concentrations, the lag phase could be several minutes even at saturating pyruvate concentrations. On the other hand, at high ThDP concentrations the lag phase was not apparent on the seconds scale, even at low pyruvate concentrations. Therefore, the ThDP concentration is the major affecter for the lag phase.

That true steady state was attained subsequent to the lag phase was also demonstrated as follows (data not shown). A reaction, resulting in a progress curve such as shown in Fig. 2A, was initiated by the addition of 1 $\mu$M ThDP. Once the presumed steady state was reached, saturating ThDP was

**FIG. 1.** Hill plots of the ThDP dependence of the steady-state rates of 3-lip PDHc (wild type) at 27 °C. Reaction rates were assayed in 0.25 ml Tris-HCl, pH 8.0, containing 2.0 mM Mg(II), 2.5 mM NAD$^+$, 2.6 mM DTT, 0.13 mM CoA and pyruvate at 2.0 (○) mM or 0.08 mM (+). The reaction was initiated by the addition of 2.5 $\mu$g of enzyme.

**FIG. 2.** A, typical progress curve for NADH production in the overall PDHc reaction with 3-lip PDHc at 0.5 $\mu$M ThDP concentration. Reaction conditions were the same as in Fig. 1. Concentration of pyruvate was 2.0 mM. B, ThDP dependence of the lag phase for 3-lip PDHc. Conditions were the same as in Fig. 1, concentration of pyruvate was 2.0 mM. The reaction was initiated by the addition of 1 $\mu$g of enzyme. C, ThDP dependence of the lag phase for resolved E1. 6.6 $\mu$g of E1 was used for measuring the reaction rates at 380 nm in 0.25 ml of reaction medium containing 2.0 mM pyruvate, 4.0 mM Mg(II), 1.0 mM 4,4-dithiodipyridine, 1.0 mM 4-mercaptopyridine, and 16 $\mu$g of bovine serum albumin.
added to the reaction (0.2 mM final concentration), and the steady-state rate was measured again. This rate was identical within experimental error to the steady-state rate observed on initial addition of 0.2 mM ThDP. The attainment of steady-state rates subsequent to the lag phase was demonstrated at 0.08 and 2.0 mM pyruvate concentrations. These experiments also excluded the intervention of protein inhibition during the measurements.

Activation of E1 in the PDH Complex by ThDP—The activation of the E1 component of the 3-lip and 1-lip PDH complexes by ThDP was investigated using the E1-specific assay in order to eliminate the possibility that E2-E3 catalyzed reactions contribute to the lag phase. For ThDP, the experimental results yielded an apparent $K_m = 0.19$ mM and $n_H = 1.2$ for the 1-lip PDHc, and an apparent $K_m = 0.17$ mM and $n_H = 1.3$ for the 3-lip PDHc (Table I). The apparent $K_m$ values obtained for ThDP by assaying the E1-specific model reaction are very high for both complexes compared to those obtained by assaying the overall reaction. The kinetic curves for ThDP exhibit weak positive cooperativity although the activation lag phase was still observed with the E1-specific assay.

Activation of Resolved E1 by ThDP—No cooperativity was observed with ThDP and resolved E1 ($n_H = 1.0, K_m = 0.034$ mM; see Table I) according to the E1-specific assay. The regulatory properties could not be investigated at low coenzyme concentration due to the low activities observed with the E1-specific assay at ThDP concentrations $< 0.005$ mM. Nevertheless, the absence of cooperativity at ThDP concentration $> 0.005$ mM differs from both the weak negative cooperativity ($n_H = 0.79$) observed with the overall PDHc assay at $> 1$ mM ThDP, and from the weak positive cooperativity ($n_H = 1.2, 1.3$) observed with the E1-specific assay for 1-lip PDHc and 3-lip PDHc. However, there was still a detectable lag phase at low ThDP concentrations, and as observed with the 3-lip PDHc (Fig. 2B), the size of the lag phase declines to a diminishing extent with increasing ThDP until saturated (Fig. 2C). These results provide excellent support for the view that the ThDP-dependent lag phase reflects changes taking place solely in the E1 component. It was also shown that the resolved E1 component and the variant E1-G231A are dimers irrespective of ThDP concentration. The apoE1 applied to the TSK G3000-SW column had the same retention time (34.75 min) as the E1 component and the variant E1-G231A are dimers irrespective of ThDP binding does not reflect interactions between the E1 and the E2-E3 subcomplex, or dimerization of two E1 subunits. The influence of association-dissociation of the E1 subunit with the E2-E3 subcomplex on the lag phase was not investigated in our laboratory. However, Biaswanger (36) reported that a lag phase due to the reassociation of E1 with the E2-E3 subcomplex is independent of the concentration of ThDP. It also appears unlikely that 1-lip, 3-lip, or variant PDH complexes contain a weakly bound E1 component after ultracentrifugation at 100,000 $	imes$ g for 3–4 h. Russell et al. (1) reported that the polypeptide chain stoichiometry of E1p/E2p/E3 in the sedimented 1-lip PDHc was approximately 0.3:1:0.1:3.4, showing a superimposition of fast protein liquid chromatography.

Kinetic Analysis of the ThDP-dependent Lag Phase—For 1-lip and 3-lip PDH complexes a plot of $1/\tau$ versus [ThDP] (where $\tau$ is the duration of the lag phase) is linear at ThDP concentrations $> 1$ mM, but deviates from linearity at concentrations $< 1$ mM (Fig. 3). The data suggest that interaction of ThDP with the apoenzyme in the ThDP concentration range investigated certainly involves more than a single step to reach a catalytically active species. Two lag phases were observed, both ThDP concentration-dependent. At small ThDP concentrations ($< 1$ mM), the duration of the lag phase was longer than that at ThDP concentrations $> 1$ mM. Based on these data, a minimal mechanism can be proposed for ThDP binding at low ThDP concentrations (Fig. 3, inset): rapid equilibrium binding followed by a slow isomerization. In all of these discussions, a dimeric E1 is assumed.

\[
(\text{E1})_2 + (\text{ThDP} \cdot \text{Mg(II)}) \rightarrow_k K_1 (\text{E1})_2 \cdot (\text{ThDP} \cdot \text{Mg(II)})
\]

\[
\text{REACTION 7}
\]

Assuming that the first equilibration step is fast compared to the subsequent isomerization step, $k_1$, $k_2$, and $k_{-2}$ can be obtained from the lag phase using Equations 1 and 2 (39) and are presented in Table II.

\[
1/\tau = (k_2 K_1 [\text{ThDP}]) (1 + K_1 [\text{ThDP}]) + k_{-2}
\]  

\[
1/[1/(\tau) - k_{-2}] = 1/k_2 + 1/[k_1 K_1 [\text{ThDP}]]
\]

The curve generated with the constants in Table II is superimposed on the experimental data in Fig. 3 (inset) showing a good fit.

At ThDP concentrations $> 1$ mM, a plot of $1/\tau$ versus [ThDP] is linear (Fig. 3), suggesting that a single-step binding mechanism accounts satisfactorily for these data, leading to the expanded mechanism (Reaction 8). The rate constants $k_3$ and $k_{-3}$ could be obtained from Equation 3 and are also presented in Table II.

\[
(\text{E1})_2 + (\text{ThDP} \cdot \text{Mg(II)}) \rightarrow_k K_1 (\text{E1})_2 \cdot (\text{ThDP} \cdot \text{Mg(II)})
\]

\[
\text{REACTION 8}
\]

\[
1/\tau = k_3 [\text{ThDP}] + k_{-3}
\]
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| Enzyme                  | $K_d$ | $k_2$ | $k_{-3}$ | $k_3$ | $k_{-3}$ |
|-------------------------|-------|-------|----------|-------|----------|
|                         | $\mu M$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ |
| 3-Lip PDHc              | $1.17 \times 10^6$ | 0.0112 | 0.0038 | $(0.713 \pm 0.057) \times 10^3$ | $(0.758 \pm 0.064) \times 10^{-2}$ |
| 1-Lip PDHc              | $1.33 \times 10^6$ | 0.0076 | 0.0061 | $(0.703 \pm 0.106) \times 10^3$ | $(0.869 \pm 0.026) \times 10^{-2}$ |
| C259N variant 1-lip PDHc|       |       |         | $(0.279 \pm 0.0216) \times 10^3$ | $(1.44 \pm 0.327) \times 10^{-2}$ |

$^a$ Binding constant.
$^b$ Rate constants of ThDP binding.
$^c$ Rate constants of ThDP dissociation. Values of $k_3$ and $k_{-3}$ are average of three to four different preparations of PDHc and C259N variant 1-lip PDHc.

ThDP-dependent Lag Phase in the Presence of Pyruvate—In the experiments analyzed above, the lag phase for the 1-lip and 3-lip PDH complexes was determined by assaying the overall reaction in the presence of 2 mM pyruvate. According to some reports, pyruvate concentration may affect the binding affinity for ThDP (40), as well as the duration of the lag phase (36). In further experiments, 1-lip PDHc was preincubated for different times with ThDP (1 or 5 $\mu M$) in the absence or in the presence of 2 mM pyruvate, and the reaction was initiated by the addition of all other components required for the overall NADH assay, except ThDP. The duration of the lag phase was found to decrease with increasing preincubation time and independent of the presence of 2 mM pyruvate in the preincubation medium (Fig. 4A). To investigate the influence of pyruvate on the binding affinity of PDHc for ThDP, 1-lip PDHc was preincubated for different times as described above at a ThDP concentration near saturation (11 $\mu M$) in the absence and presence of 2 mM pyruvate and the initial rates were fitted to Equation 4 (40, 41).

\[
v_t = (v_v - v_i) \exp(-(k_{-3}[\text{ThDP}] + k_3)t) + v_i \quad \text{Eq. (4)}
\]

$v_v$ and $v_i$ are the initial rates at a preincubation time $t$, at time zero and at infinite time, respectively. The kinetic constants obtained from this fitting were: $k_3 = 0.749 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$; $k_{-3} = 0.921 \times 10^{-2} \text{ s}^{-1}$ in the absence, and $k_3 = 0.832 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$; $k_{-3} = 0.311 \times 10^{-2} \text{ s}^{-1}$ in the presence of 2 mM pyruvate.

The $K_d$ for ThDP calculated in the presence of 2 mM pyruvate is 3.73 $\mu M$, very similar to the $K_d$ for ThDP obtained for the overall reaction (2.8 $\mu M$). The $K_d$ for ThDP in the absence of pyruvate is 12.29 $\mu M$. These kinetic constants calculated from the initial rates are in good agreement with those from the pre-steady-state experiments shown in Table II. Hübner et al. recently reported (42) a $K_d = 17 \mu M$ for ThDP of in the presence of, and 1.4 $\mu M$ in the presence of, 2 mM pyruvate (from steady-state kinetic measurements). Considering the differences in enzyme source, protocols used, etc., these numbers are in satisfactory accord with those obtained in our laboratories.

To further support the deductions reached from pre-steady-state kinetic results, the rate constant for holoenzyme dissociation ($k_{-3}$, the loss of ThDP from the dimer) was also determined in an experiment where 1-lip PDHc was first saturated with ThDP (200 $\mu M$) and then diluted into a reaction medium containing no ThDP, and zero or 2 mM pyruvate (Fig. 4B). The $k_{-3}$ was calculated by fitting the initial rates for the overall reaction to Equation 5 (40).

\[
v_i = v_v \exp(-(k_{-3} \cdot t)) \quad \text{Eq. (5)}
\]

$v_v$ and $v_i$ are the initial rates at different times of preincubation $t$ and at time zero; $k_{-3}$ is the rate constant for holoenzyme dissociation. From the plot of $\log(v_v/v_i)$ versus $t$, the $k_{-3}$ was equal to $4.2 \times 10^{-2} \text{ s}^{-1}$ in the absence, and $3.8 \times 10^{-2} \text{ s}^{-1}$ in the presence of 2 mM pyruvate, as compared with the values of $0.921 \times 10^{-2} \text{ s}^{-1}$ and $0.311 \times 10^{-2} \text{ s}^{-1}$ calculated from Equation 4. With these values of $k_{-3}$ and $K_d$, we calculate $k_3 = 1.02 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ in the presence of 2 mM pyruvate, and $k_3 = 3.42 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ in its absence.

From these data it is clear that the presence of pyruvate has very little, if any, effect on ThDP binding under our conditions. Consequently, the lag phase observed is related to a slow activation process induced by ThDP binding, but precedes catalytic turnover. In contrast to our data, Biswanger (36) claimed that preincubation of the enzyme with ThDP and Mg(II) did not alter the duration of the lag phase, but that the lag phase became significantly shorter if the enzyme was preincubated with pyruvate or methylacetylphosphonate (a pyruvate analog that cannot be decarboxylated), in addition to ThDP,Mg(II) (33). It was proposed that the lag phase reflects the slow activation process for binding both ThDP,Mg(II) and pyruvate in the active center, but is not related to the catalytic turnover to 2-oxo-hydroxyethylidene ThDP, the intermediate resulting from decarboxylation.

ThDP Binding by Variant Enzymes—Site-directed substitutions were introduced into the E1 subunits of 1-lip PDHc in the region containing the proposed ThDP binding motif, GDG(X)$_{29}$NCN (23, 29). As can be seen in Table I, changes in the ThDP binding motif lead to significant loss of overall activity. In accordance with Russell et al. (1), replacing Gly-231 with alanine or serine abolished all but 0.97% and 0.03% (respectively) of the activity of the parental 1-lip PDH complex. However, these values may not represent the true activity of the variant enzymes, because the activity was measured under sub-saturating ThDP concentrations (0.2 mM). The activity of the variant complexes may be enhanced 2–5-fold once the ThDP concentration is elevated to 5 mM. It needs to be pointed out that the variant enzymes were correctly refolded according to several criteria. Russell et al. (1) showed that replacement of Gly-231 in E1 by serine, methionine, or alanine did not hinder assembly into a complex that was indistinguishable in its sedimentation characteristics from complex assembled from the wild-type E1 component, notwithstanding dramatically diminished E1 activities in the variant complexes. Similar conclusions can be drawn from the inhibition experiments performed in our laboratory (data not shown). It was found that the G231A, C259N, C259S, and N258G substitutions had no significant effect on the recognition of E1 by a monoclonal antibody 18A9 that was found to be 98% inhibitory against both E1 and the PDH complex (31). All variants and the parental 1-lip and 3-lip PDH complexes were inhibited to the same extent by both parental 1-lip PDH complex (31). All variants and the parental 1-lip and 3-lip PDH complexes were inhibited to the same extent by either antibody. Similarly, the activity of variants and 3-lip PDH complexes was diminished by the addition of GTP. Finally, it was found that fluoropyruvate inhibits all variants (G231A is too inactive to try in such an experiment) and both parental complexes to the same extent (see Ref. 15 for studies of this inactivation mechanism).
ThDP increased about 13 times compared with that of the parental 1-lip PDHc. The variant complexes with G231A, N258Q, and C259S substitutions were not saturated by concentrations of ThDP as high as 5–10 mM. Furthermore, the corresponding steady-state kinetic plots (Eadie-Scatchard and Lineweaver-Burk) exhibited negative cooperativity at all concentrations of ThDP studied. However, in the absence of saturation by ThDP, the $n_H$ values could not be reliably estimated from Hill plots. As Fig. 5A shows, the velocity curve for the G231A complex rises rapidly at low concentrations of ThDP, but the rate is not saturated at the highest levels of ThDP used. Replotting the data according to Eadie-Scatchard (Fig. 5A, inset) or Lineweaver-Burk (data not shown) confirmed the behavior, characteristic of negative cooperativity.

The only variant that exhibited an activation lag phase was C259N, and its extent varied inversely with the concentration of ThDP. With an increase in concentration of ThDP from 0.001 mM to 0.1 mM the lag phase diminished and disappeared, as was detected for the 1-lip PDHc. At lower concentrations of ThDP (<1 µM) the kinetic data for the lag phase were too inaccurate for treatment. For a concentration range of ThDP of 0.005–0.1 mM, a plot of $1/t$ versus [ThDP] was linear (data not shown). This enabled us to calculate the kinetic parameters from Equation 3, presumably, for binding the second ThDP to the E1 dimer (Table II). The $K_d$ calculated from the rate constants was $1.24 \times 10^{-5}$ M for 1-lip PDHc and $5.16 \times 10^{-5}$ M for the C259N variant, i.e. an approximately 4-fold reduction in affinity. For all other variant 1-lip PDH complexes the lag phase was not observed at any concentrations of ThDP. In Fig. 5B are presented data for the G231A variant PDH complex.

The G231A and G231S variants were preincubated with ThDP, Mg(II) for different times, as was described for the parental 1-lip PDHc. Measurement of the overall rates for NADH production exhibited no lag phase for either variant. A rapid initial rate increase was observed in the first 10–20 s of incubation. However, any further increase in the preincubation time to even 5–20 min did not significantly change the rate, suggesting that the slow, time-dependent steps for ThDP binding are eliminated in the case of the G231A and G231S variants.
Activation of PDHc from E. coli by Cofactors and Substrate

Circumstantial Studies of ThDP Binding—Fig. 6 presents near-UV CD spectra for apo-E1 isolated from 1-lip PDHc. A broad negative CD band is observed in the 260–288 nm region, similar to that reported for E1 isolated from pigeon breast muscle (43), bovine heart (44), and human recombinant E1 (45). This band is likely due to tyrosine and/or tryptophan and/or phenylalanine. A smaller positive band is observed with a maximum at 293 nm and may be due to tryptophan. Addition of increments of ThDP leads to a significant increase in the negative CD band centered near 280 nm, and a concomitant decrease in the positive band at 293 nm. In addition, there is evidence for a very weak negative CD band near 330 nm (Fig. 6, inset), that is believed to result from a charge transfer origin in the active center has been altered by the substitution.

Regulation of PDHc and Its E1 Subunit by Pyruvate—Fig. 8A shows the dependence of the steady-state rates for 1-lip PDHc on pyruvate concentration (using the NADH assay for the complex). Both 3-lip and 1-lip parental complexes could be saturated by substrate. The $K_m$ for pyruvate is 0.21 mM and 0.22 mM for the 3-lip PDHc and 1-lip PDHc, respectively. The Eadie–Scatchard plots revealed weak positive cooperativity for both complexes (Fig. 8A; inset presents the data for 1-lip PDHc), with $n_H = 1.13$ for 3-lip PDHc and $n_H = 1.24$ for 1-lip PDHc. Bisswanger (12) reported values of $n_H = 1.7–1.85$ at low, and $n_H = 1.1$ at higher pyruvate concentrations for wild-type enzyme.

Using the E1-specific assay with E1 resolved from the 1-lip PDHc, the values of $K_m = 0.11$ mM and $n_H = 1.0$ were obtained with pyruvate. Apparently, pyruvate is bound to resolved E1 without cooperativity. On the other hand, the NADH assay for the overall reaction showed positive cooperativity over the same range of pyruvate concentrations. This suggests that the cooperativity is caused by additional factors, or that the combination of E1 is altered when it is resolved from the complex.

The C259S variant complex exhibited similar cooperativity with respect to pyruvate binding, although the substitution caused a 4-fold reduction in activity and a 10-fold increase increase in $S_{0.5}$ relative to the parental complex, 1-lip PDHc (Table I). At lower pyruvate concentrations, similar weak positive cooperativity is observed for both variant and parental complexes. This result provides further evidence that Cys-259 is not of critical regulatory importance.

Fig. 8B presents data for the G231A variant. Relative to the parental complex, it has a lower affinity for pyruvate: $S_{0.5}$ is about 2.3 mM; the Hill coefficient is 0.84 reflecting weak negative cooperativity. These dramatically altered properties indicate that substitution in the highly conserved GDG triplet of the ThDP binding motif affects the homotropic regulation by substrate, as well as regulation by ThDP. The results also provide strong corroboration for regulation of PDHc by both substrate and ThDP.

DISCUSSION

It is apparent that even after deletion of two of the three lipoyl domains, the kinetic behavior of the 1-lip PDHc is very similar to that of the 3-lip PDHc. The two complexes exhibit similar activities (1), activation lag phases and regulation by ThDP and substrate. However, single amino acid substitutions in the ThDP binding motif significantly diminish the affinity of the enzyme for ThDP, and dramatically influence regulation by both ThDP and pyruvate acid. Residues Gly-231 and Asn-258 are
 Activation of PDHc from E. coli by Cofactors and Substrate

FIG. 8. A, Michaelis-Menten plot for 1-lip PDHc. The reaction mixture (0.25 ml) contained: 2.5 mM NAD\(^+\), 2.6 mM DTT, 0.47 mM CoA, 0.2 mM ThDP, 1.0 mM Mg(II). The reaction was initiated by the enzyme (3.5 \(\mu\)g). Inset, Eadie-Scatchard plot. B, dependence of the steady-state rates on pyruvate concentration for the G231A variant 1-lip PDHc. Reaction conditions were the same as in A. The reaction was initiated by the enzyme (140 \(\mu\)g). Inset, Eadie-Scatchard plot.

important in the mechanism of activation by ThDP, while Cys-259 appears to be less critical. The C259N substituted complex exhibits kinetic behavior more similar to that of the parental complex, unlike those with Gly-231 and Asn-258 substitutions. Perhaps the altered complexes retain the unactivated conformation ((E1)\(_2\)) (ThDP, Mg(II)). The reaction was initiated by the enzyme (3.5 \(\mu\)g). Inset, Eadie-Scatchard plot.

The 1-lip PDHc shows no cooperativity over a ThDP concentration range of 10\(^{-6}\) to 10\(^{-7}\) \(\mu\)M and weak negative cooperativity with \(n_H = 0.79\) above that concentration range. However, the G231A, G231S, N258Q, and C259S variants exhibit strong negative cooperativity over and above the same range of ThDP concentrations.

Although the C259S substituted complex has a higher activity than the G231A, G231S, and N258Q variants (Table I), it still shows strong negative cooperativity at low ThDP concentration (data not shown), while the C259N complex exhibits an activation lag phase and regulation by ThDP, very similar to that of the parental complex.

We considered several models to interpret the observations concerning the existence of the activation lag phase in the parental complexes, and its absence in four less active variants.

1) The lag phase is due to association of E1 with E2-E3 subcomplex. Bisswanger (36) had reported that there was a ThDP-independent lag phase in NADH production when the PDHc was reconstituted from E1 and E2-E3 subcomplex. In our experiments, the ThDP-dependent lag phase was evident in experiments carried out with either PDHc or resolved E1. Therefore, our observations cannot be the result of reassociation of the E1 with the E2-E3 subcomplex.

2) The lag phase is due to ThDP induced slow subunit association. In the case of transketolase, it was proposed that the lag phase observed in the presence of ThDP and metal ions is due to a slow association of subunits to form dimers (47), since preincubation with ThDP-Mg(II) decreased the duration of the lag phase. There was also a lag phase found in product formation with the D440E variant (Asp-440 is the aspartate in the conserved GDG triplet) of PDC from Zymomonas mobilis (29), but not with the wild-type enzyme, or with several other variants of the ThDP motif. The duration of the lag phase diminished with increasing ThDP concentration, and the lag phase was attributed to slow formation of the functional tetramer from inactive subunit dimers. According to our results (size exclusion high performance liquid chromatography), E1 is a dimer both in the absence and in the presence of ThDP-Mg(II), we therefore rule out dimerization as being the source of the ThDP-dependent lag phase in PDHc from E. coli.

3) The duration of the lag phase, and indeed the association and dissociation rate constants (as well as their ratio, i.e. the \(K_c\) for ThDP binding depend on pyruvate concentration. This was reported for PDHc from pig heart (40). Our results clearly ruled this out.

4) The ThDP-dependent lag phase is shortened by pyruvate. This was claimed for PDHc from E. coli by Horn and Bisswanger (33) at ThDP concentrations< 1 \(\mu\)M. Since methylyactylphosphonate (a pyruvate analog, which is not decarboxylated to the 2-hydroxyethylidene ThDP intermediate) shortened the lag phase for PDHc from E. coli in the presence of limiting amount of ThDP (<1 \(\mu\)M), just as pyruvate did, it was suggested that the ThDP-dependent lag phase pertains to the binding of both ThDP-Mg(II) and pyruvate, rather than to formation of the enzyme-bound hydroxymethylideneThDP intermediate. While we did not investigate the influence of pyruvate on the duration of the lag phase at ThDP concentrations <1 \(\mu\)M, above this concentration we showed that the duration of the lag phase is independent of pyruvate, and pyruvate does not significantly change the binding constants for ThDP.

We conclude that in E. coli PDHc the ThDP-dependent lag phase is not directly related to the catalytic transformation of pyruvate in the active sites of the enzyme; this activation is not stimulated by pyruvate, rather it is a result of slow activation of E1 specifically by ThDP binding.

The complex dependence of the lag phase on ThDP concentration clearly signals that the slow binding/activation process involves multiple steps. A reasonable explanation of the data in the entire ThDP concentration range involves stepwise binding of two molecules of ThDP-Mg(II) to the E1 dimer. The first molecule is bound with a \(K_c\) of micromolar magnitude, that binding is then followed by a slow conformational equilibrium step leading to the half-saturated E1 dimer. This conformational change is likely related to the activation process, its forward and reverse rate constants account for the first stage of the lag phase observed at very low ThDP concentrations. The second stage of the lag phase observed at higher ThDP concentrations is adequately accounted for by a bimolecular association step, again with relatively slow forward and reverse rate constants (note that the apparent rate constant for addition of
the second ThDP is orders of magnitude lower than that expected for a diffusion controlled reaction, and produces the fully activated enzyme required for catalytic turnover. The cooperativity reported by Bisswanger (36) already anticipated interaction of reactive centers in the E1 dimer of PDHc.

The variant complexes fall into two classes. Variant C259N still exhibits the activation lag phase, but only the region observed at high ThDP concentration for the parental complex is measurable in this case. Because of the linear dependence of $k_2$ on ThDP concentration, and because of the relatively uncompromised $K_m$ and specific activities of this variant, we assign that lag phase to the slow binding of the second ThDP molecule (as for the parental complex). This variant still exhibits its positive cooperativity with respect to pyruvate (data not shown), as does the parental complex.

The remaining four variants do not exhibit any lag phases at any of the ThDP concentrations studied. For these complexes, there is evidence of not only abolition of cooperativity, but rather of negative cooperativity for ThDP binding. A reasonable explanation of these observations is that the binding of the first ThDP is already impaired, but binding of the second ThDP molecule is no longer feasible and turnover proceeds from the E1 dimer half-saturated with ThDP.

The CD experiments provided important structural insight. First, they showed that addition of ThDP to the apo-E1 produces a large enhancement of the negative band at 280 nm, and that this enhancement is reproduced in the ThDP fold variants. This constitutes important evidence for similar tertiary structural changes being induced by ThDP binding in parental and variant (G231A) enzymes. One notes in this regard that this is a rather global observation that need not be related to events taking place at the active center. On the other hand, it is generally accepted that the low extinction negative CD band induced by ThDP binding to its enzymes at 330 nm describes more local events at the active center. It is in this light that the presence of this CD band in the parental, and its absence in the G231A variant E1 needs interpretation. It is tempting to conclude that the distortion experienced by the ThDP bound to the variant E1 eliminates the charge-transfer interaction that is believed to give rise to this band, and this difference may be related to lack of lag phase in the same variant complex.

In an attempt to explain both the CD and the binding kinetic data according to Reaction 8, we suggest that the large change induced in the negative CD band centered around 280 nm is due to a fast conformational change immediately following formation of the encounter complex between E1 and the first ThDP molecule. In other words, it is a rather global observation that need not be related to events taking place at the active center. On the other hand, it is generally accepted that the low extinction negative CD band induced by ThDP binding to its enzymes at 330 nm describes more local events at the active center. It is in this light that the presence of this CD band in the parental, and its absence in the G231A variant E1 needs interpretation. It is tempting to conclude that the distortion experienced by the ThDP bound to the variant E1 eliminates the charge-transfer interaction that is believed to give rise to this band, and this difference may be related to lack of lag phase in the same variant complex.

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