# Molecular Mechanism of Regulation of the Pyruvate Dehydrogenase Complex from *E. coli*†

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ABSTRACT: The pyruvate dehydrogenase multienzyme complex from *E. coli* shows a sigmoidal dependency of the reaction rate on the substrate concentration when product formation is followed in the presence of physiological concentrations of the cofactor thiamin diphosphate. To elucidate the molecular mechanism of this regulation, the influence of the substrate pyruvate on the coenzyme−protein interaction has been investigated using several coenzyme analogues. The observed binding constants of all coenzymatically active analogues are increased in the presence of the substrate pyruvate, whereas those of all coenzymatically inactive analogues are not altered in the presence of pyruvate. This points to an increased binding affinity of a reaction-intermediate−coenzyme complex to the protein. Since cofactor binding and dissociation at physiological concentrations of thiamin diphosphate are slow compared to the catalytic reaction, a slow transition to the active state of the enzyme occurs. After lowering the pyruvate concentration, the opposite effect, a dissociation of the thiamin diphosphate from the enzyme is observed. This slow substrate dependent enhancement of cofactor binding enables efficient regulation of the pyruvate dehydrogenase complex by its substrate pyruvate.

The pyruvate dehydrogenase multienzyme complex (PDH-complex)[1] contains three different enzymes that catalyze the conversion of pyruvate, coenzyme A (CoA), and $NAD^+$ into acetyl-CoA, $CO_2$, and NADH (*4*). The initial reaction, the decarboxylation of pyruvate, is catalyzed by the thiamin diphosphate (ThDP) dependent decarboxylating component of the complex (EC 1.2.4.1; E1). In a consecutive step, the hydroxyethyl group is transferred to the dihydrolipoamide transacetylase (EC 2.3.1.12; E2) of the PDH complex which contains covalently bound lipoyl groups. Then the acetyl group reacts with CoA to produce acetyl-coenzyme A and E2-dihydrolipoamide. The reactions catalyzed by the third enzyme of the PDH-complex, the lipoamide dehydrogenase (EC 1.8.1.4; E3), comprise the reduction of its tightly bound $FAD^+$ to $FADH_2$ and the subsequent reduction of $NAD^+$ to NADH (*2*−*4*). E1 catalyzes the rate-limiting step of the overall reaction (*5*, *6*) and therefore presents an ideal target for regulatation.

The *E. coli* PDH-complex shows a regulation by the substrate pyruvate whereas the mammalian enzyme is regulated by phosphorylation of E1 (*7*, *8*). Interestingly, the regulation by pyruvate in *E.coli* is only observed at non-saturating, physiological concentrations of the cofactor ThDP (*9*). Therefore ThDP binding to E1 appears to be the target in this regulatory process. To elucidate the molecular mechanism of this regulation, the influence of the substrate pyruvate on the coenzyme−protein interaction has been investigated by kinetic (activity) and thermodynamic (fluorescence) measurements using active and inactive coenzyme analogues. The ThDP-cofactor analogues contained substitutions at positions that are known from other ThDP dependend enzymes to be important for binding and/or catalysis (for summary see ref 10).

## MATERIALS AND METHODS

*Materials:* The PDH-complex was isolated from the wild type strain Ymel of *E. coli* K 12 and purified as described (*11*). E1 [EC 1.2.4.1] was dissociated from the PDH complex using the protocol of Graupe et al. (*12*).

The synthesis of ThDP-analogues (Figure 7) DAThDP (*13*), N1ThDP (*14*) and N3ThDP (*15*) was performed as described earlier. OThDP was synthesized according to Rydon (*16*). The synthesis of acetylphosphinate was performed as described by Baillie et al. (*17*).

ThDP and enzyme substrates were purchased from Sigma. All substances used were of the highest commercially available grade of purity.

*Enzyme Assays:* The overall enzymatic reaction of the PDH-complex was measured at 25 °C in a Uvicon 941 (Kontron Instruments) at 340 nm according to Schwartz and Reed (*18*). The assay mixture contained 0.14-13.2 $\mu$g of the purified PDH-complex in 1 mL of 50 mM potassium phosphate buffer, pH 7.6, containing 2.5 mM NAD, 2.5 mM dithioerythritol, 1 mM magnesium sulfate, 0.1 mM coenzyme

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[1] Abbreviations: PDH-complex: pyruvate dehydrogenase complex; ThDP: thiamin diphosphate; N1ThDP: *N*-1′-pyridylthiamin diphosphate; N3ThDP: *N*-3′-pyridylthiamin diphosphate; DAThDP: 4'-deaminothiamin diphosphate; OThDP: 4′-hydroxy-4′-deaminothiamin diphosphate; $NAD^+$: nicotinamidedinucleotide (oxidized); NADH: nicotinamidedinucleotide (reduced); CoA: coenzyme A; E1: decarboxylating component of the PDH complex.

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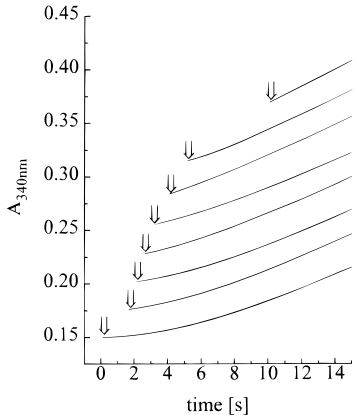


FIGURE 1: Influence of the preincubation time of the PDH-complex (13.2 $\mu$g/mL) with 100 $\mu$M ThDP on the progress curves for NADH production in the overall PDH-reaction. The assay mixtures were completed by pyruvate at different time intervals (indicated by arrows) except for the 0 s incubation time where the reaction was started by addition of the PDH-complex to a complete assay mixture. The different curves are shifted in OD units for better visualization.

A, 2 mM pyruvate, ThDP, and analogues in concentrations as described in Results. The reaction was initiated either by adding ThDP, pyruvate, CoA, or the PDH-complex.

The fast kinetic measurements were performed on a Mikro Volume Stopped-flow Reaction Analyzer SX-17MV with a sequential-flow option (Applied Photophysics).

*Fluorescence Measurements:* The tryptophan fluorescence of the PDH-complex and of E1 was measured with a fluorescence spectrophotometer F 3010 (Hitachi) at 25 °C. The decrease in tryptophan fluorescence of both the PDH-complex and E1 upon ThDP or ThDP-analogues binding was used to determine the dissociation constants. Excitation was set at 280 nm and emission followed at 330 nm and corrected for inner filter effects. Respective experimental conditions were 35 $\mu$g/mL PDH-complex or 15 $\mu$g/mL E1 in 50 mM potassium phosphate buffer, pH 7.6, 1 mM $MgSO_4$ in the presence of 0 and 2 mM pyruvate, or 2 $\mu$M acetylphosphinate and variable concentrations of ThDP.

*Modification of the Histidine Residues of the PDH-Complex by Diethyl Pyrocarbonate.* The reaction medium contained 50 mM potassium phosphate buffer, pH 7.6, 1 mg/mL ThDP-free PDH-complex, 8 $\mu$M diethylpyrocarbonate and various concentrations of pyruvate. Aliquots of 2 $\mu$L of this mixture were taken after different incubation times for activity measurements in a complete assay mixture. The rate constants of inactivation were calculated from the dependence of the steady state rates on the preincubation time at the respective pyruvate concentration.

## RESULTS

*Activation of the PDH-Complex with ThDP. 1. High ThDP Concentrations.* The influence of pyruvate as well as the time of preincubation of the PDH-complex with ThDP on the lag phase was examined using stopped flow and multimixing stopped flow techniques (the dead time of the instrument was 3 ms).

If the enzyme reaction is initiated by addition of the PDH-complex to a complete assay mixture containing high ThDP concentrations (100 $\mu$M), a lag phase is observed (Figure 1, curve starting at 0 min incubation time). From this progress curve a pseudo first-order rate constant of PDH-complex activation by ThDP ($k_{obs}$ = 0.13 $s^{-1}$) in the presence of 2 mM pyruvate can be calculated by fitting the time dependence of the product accumulation to the following equation:

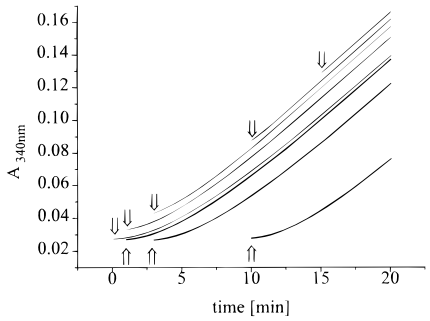


FIGURE 2: Influence of the preincubation time (indicated by arrows) of the PDH-complex (0.26 $\mu$g/mL) with 1 $\mu$M ThDP in the absence of pyruvate (thick lines) and presence of 2 mM pyruvate (thin lines) on the progress curves for NADH production in the overall PDH-reaction. The reactions were started by addition of either pyruvate (⇑) or CoA (⇓) after different time intervals. The different sets of curves are shifted in OD units for better visualization.

$$P(t) = v_{ss}t - (v_{ss} - v_0)/k_{obs}(1 - e^{(-k_{obs}t)})$$

where $P(t)$ is the product concentration at time $t$, $v_{ss}$ the steady-state reaction rate, $v_0$ the initial rate, and $k_{obs}$ the observed pseudo first-order rate constant.

In order to determine the activation kinetics of the PDH-complex at high ThDP concentrations but in the absence of pyruvate, a sequential stopped-flow technique was used. In a first step, the PDH-complex was mixed with a ThDP solution to a final ThDP concentration of 100 $\mu$M. After different delay times (indicated by arrows in Figure 1), a second mixing completed the assay mixture and therefore initiated the enzyme reaction. The obtained progress curves show initial rates that increase with the delay time (Figure 1) in the same way as the rate of the progress curve, obtained in the case where pyruvate was present from the beginning of the reaction. Therefore the activation process of the PDH-complex at high ThDP concentrations occurs even in the absence of pyruvate with a rate comparable to that measured in the presence of pyruvate. This indicates that the activation process is the recombination of ThDP with the PDH complex.

*2. Low ThDP Concentrations.* However preincubation of the PDH-complex with 1 $\mu$M ThDP in the absence of pyruvate and completion of the assay mixture by addition of pyruvate produces progress curves that do not depend on the preincubation time (Figure 2, bold lines). Even preincubation for 10 min did not yield any detectable initial activity. This shows that in the absence of pyruvate and low ThDP concentrations the equilibrium is strongly shifted toward the ThDP-free form of the PDH-complex. Interestingly, if the PDH-complex is preincubated with ThDP under identical conditions but in the presence of pyruvate and the reaction is started by addition of CoA, the initial rate of the progress curves increases with the preincubation time (Figure 2, fine lines). This indicates that in the presence of pyruvate, the cofactor ThDP binds to the PDH-complex even at low ThDP concentrations. In the range between 0.5 and 1000 $\mu$M ThDP a plot of the pseudo first-order rate constant for ThDP-binding in the presence of 2 mM pyruvate versus the concentration of ThDP is linear (Figure 3).

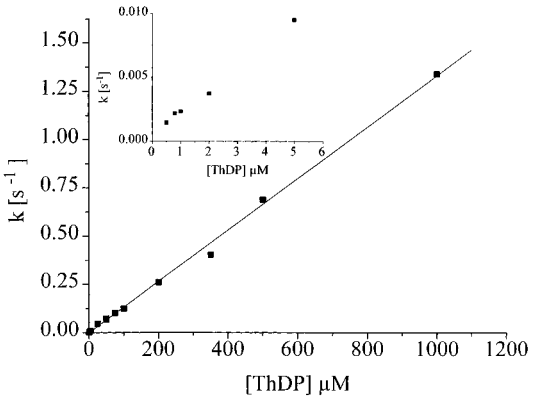


FIGURE 3: Dependence of the pseudo first-order rate constant of ThDP-binding to the PDH-complex on the ThDP concentration in the presence of 2 mM pyruvate. For experimental details see Material and Methods.

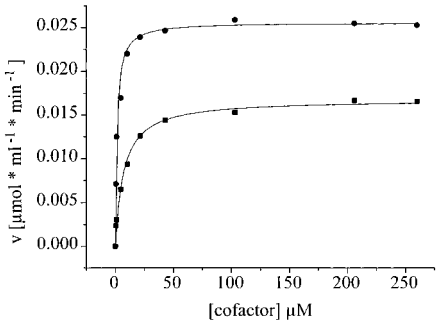


FIGURE 4: Influence of the coenzyme concentration (ThDP ●, N1ThDP ■) on the steady-state activity of the PDH-complex (1.4 $\mu$g/mL) in the overall enzyme reaction. 2 mM pyruvate was present in the preincubation mixture.

Table 1: Dissociation Constants for ThDP and ThDP-Analogues from the PDH-Complex Measured in 50 mM Potassium Phosphate, pH 7.6, containing 1 mM $MgSO_4$ in the Absence and Presence of 2 mM Pyruvate at 25 °C; the Dissociation Constants Were Determined by Kinetic Measurements (§) or Fluorescence Measurements (*), Respectively. Values for E1 Alone Are Given in Parentheses

| | dissociation constant in the absence of pyruvate ($\mu$M) | apparent dissociation constant in the presence of pyruvate ($\mu$M) |
|---|---|---|
| ThDP | $17 \pm 2$§ $18 \pm 3$* ($22 \pm 3$) | $1.4 \pm 0.1$§ $1.5 \pm 0.2$* ($1.4 \pm 0.1$) |
| N1ThDP | $91 \pm 5$§ | $7.3 \pm 0.4$§ |
| N3ThDP | >100§ | >100§ |
| DAThDP | $2.3 \pm 0.2$§ $2.0 \pm 0.3$* | $2.2 \pm 0.1$§ $2.0 \pm 0.3$* |
| OThDP | $1.5 \pm 0.1$§ $1.2 \pm 0.2$* | $1.0 \pm 0.1$§ |

*Effect of Pyruvate on the Coenzyme Dissociation from the PDH-Holoenzyme.* At saturating pyruvate concentrations (2 mM) the dissociation constants of ThDP was determined from the linear part of the progress curves (steady state conditions) at various ThDP concentrations. From the hyperbolic dependence of the activity on the coenzyme concentration (Figure 4), an apparent dissociation constant of 1.4 $\mu$M in the presence of 2 mM pyruvate was calculated (Table 1).

In order to investigate the role of pyruvate in cofactor binding, first the PDH-complex was preincubated with ThDP (200 $\mu$M) in the absence of pyruvate. Second this mixture was 1:200 diluted into an activity assay mixture lacking pyruvate. In a third step pyruvate (2 mM) was added after different times, and progress curves were recorded. Their initial rate depends on the length of the time between the

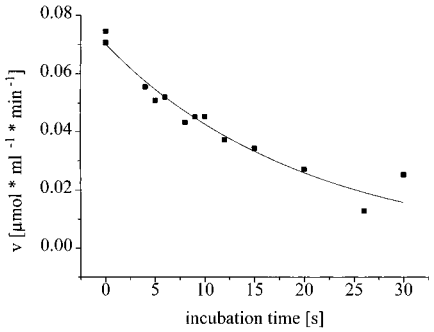


FIGURE 5: Dissociation of ThDP from the PDH-complex in the absence of pyruvate. An incubation mixture containing both the PDH-complex (1760 $\mu$g/mL) and ThDP (200 $\mu$M) was diluted 200 fold into an assay mixture lacking pyruvate. At the indicated time intervals (■) 2 mM pyruvate was added and the residual activity determined from the initial rate. The line represents a fit according to a first-order reaction ($k = 0.05$ s$^{-1}$).

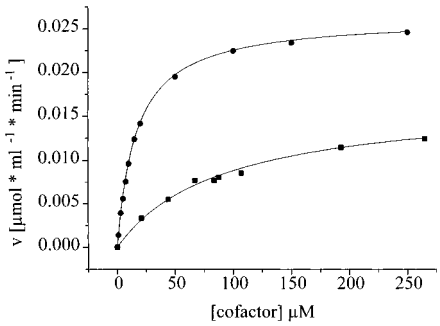


FIGURE 6: Influence of the coenzyme concentration (ThDP ●, N1ThDP ■) on the initial rate of the PDH-reaction determined from progress curves as described for Figure 5. No pyruvate was present in the preincubation mixture.

dilution and addition of pyruvate (Figure 5) since ThDP dissociates from the complex during this time. Addition of pyruvate stops the dissociation and therefore allows the detection of the residual holoenzyme concentration by activity measurements. The residual activity after long dissociation times (>25 s) mirrors the amount of holoenzyme at equilibrium in the presence of 1 $\mu$M ThDP but in the absence of pyruvate. The dissociation process fits to a first-order reaction with a rate constant of $k = 0.05$ s$^{-1}$. As expected for a first order reaction, protein concentration has no influence on the rate constant of ThDP dissociation.

However, if the enzyme reaction is started in step two by addition of pyruvate (final concentration 2 mM), linear progress curves are observed, showing that no dissociation in the presence of pyruvate occurs within 10 min. The slope of theses curves depends exclusively on the concentration of ThDP in the preincubation mixture. From this dependence the $K_D$-value for ThDP in the absence of pyruvate was calculated (17 $\mu$M) (Figure 6, Table 1). This value is about one order of magnitude higher than that determined in the presence of pyruvate (Figure 4). If pyruvate has no effect on the cofactor binding in this experiment, the activity should decrease over time in the same way as in the experiment described before due to a dilution induced dissociation of the cofactor.

*Coenzymatic Activity of ThDP-Analogues.* In order to elucidate the molecular basis of the increased ThDP affinity in the presence of the substrate pyruvate the above described experiments were performed with ThDP analogues (Figure 7).

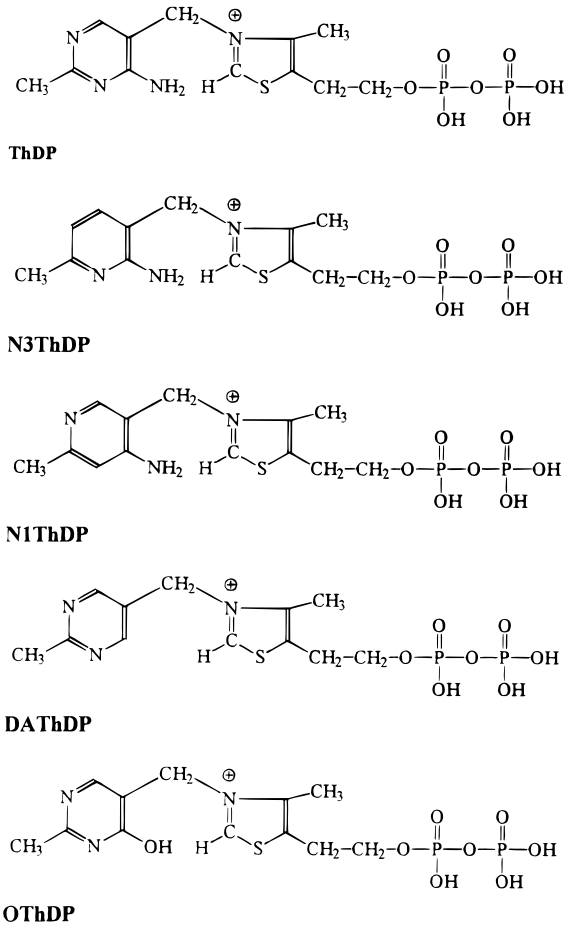


FIGURE 7: Structures of ThDP and the ThDP analogues N3ThDP, N1ThDP, DAThDP, and OThDP.

Measurements of the overall PDH reaction in the presence of the ThDP-analogues show coenzymatic activity for N1ThDP, whereas N3ThDP, DAThDP, and OThDP are coenzymatically inactive. At saturating concentrations of N1ThDP, the PDH-complex reaches 70% of the activity of the ThDP-containing complex.

*Dissociation Constants of the ThDP-Analogues. Active Analogue.* The N1ThDP coenzyme has an increased $K_D$-value of 91 $\mu$M or 7.3 $\mu$M in the absence or presence of pyruvate (Table 1) compared to the natural cofactor ThDP (Figure 6, Figure 4). However, the dissociation constant is again decreased in the presence of the substrate pyruvate as already observed for ThDP.

*Inactive Analogues.* The dissociation constants of the coenzymatically inactive analogues in the presence of pyruvate were determined by a competition experiment where the assay was started with coenzyme A after simultaneous preincubation of the PDH-complex with both the ThDP-analogue and ThDP.

The dissociation constants of the analogues in the absence of pyruvate were determined from the dependence of the initial rates on the concentration of the respective coenzyme analogue. Here the assay was started by addition of pyruvate. In both cases the data were analyzed according to Dixon (*19*) (Figure 8). As shown in Table 1 the $K_D$-values for the inactive analogues N3ThDP, DAThDP, and OThDP are not influenced by the substrate pyruvate. Whereas both DAThDP and OThDP bind with high affinity to the PDH-complex, the N3ThDP analogue shows weak binding (Table 1).

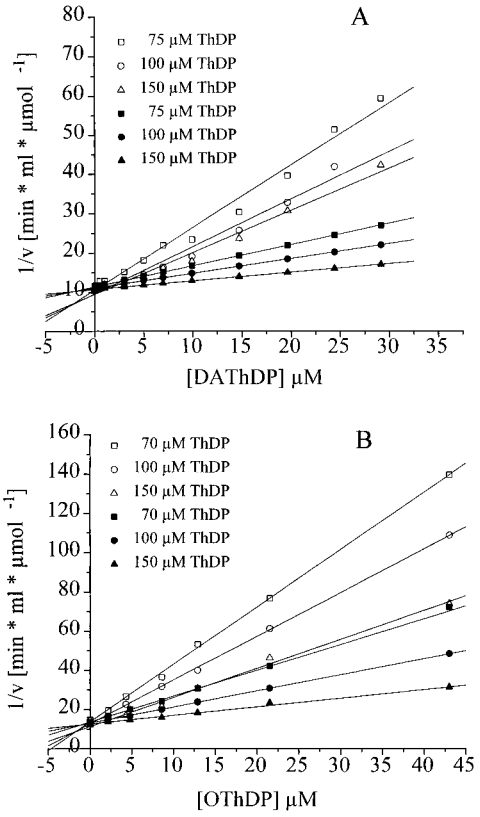


FIGURE 8: Dixon plot of the inhibition of the PDH-complex (5 $\mu$g/mL) by DAThDP (A) and OThDP (B) in the presence (full symbols) and in the absence (open symbols) of 2 mM pyruvate. Activities were determined from the initial reaction rates, respectively.

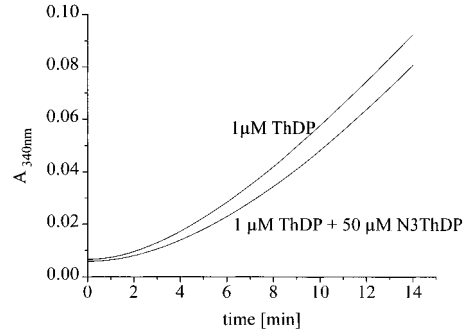


FIGURE 9: Progress curves for NADH production in the overall PDH-reaction (0.26 $\mu$g PDH-complex/mL) at 1 $\mu$M ThDP in the presence and absence of 50 $\mu$M N3ThDP. The reaction was started after preincubation of the enzyme with the coenzymes by addition of pyruvate.

However, if the reaction is started with pyruvate in the presence of 50 $\mu$M N3ThDP at a non saturating ThDP-concentration (1 $\mu$M), the rate constant for ThDP binding decreases from 0.16 $min^{-1}$ to 0.092 $min^{-1}$, but the steady state rate remains unaltered (Figure 9).

*Determination of the Dissociation Constants for ThDP and for Analogues by Fluorescence Measurements.* We could confirm the data for cofactor binding obtained by activity measurements using a different method. Coenzyme binding to the PDH-complex results in a decrease of the intrinsic protein fluorescence with a saturation behavior (Figure 10), which can be fitted to a hyperbolic function yielding the apparent dissociation constants. As shown in Table 1, both the fluorescence and the kinetic measurements yield identical values within the experimental error. A comparison of the

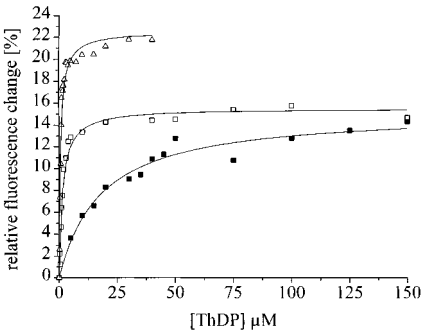


FIGURE 10: Dependence of the relative fluorescence decrease of the PDH-complex (35 $\mu$g/mL) on the concentration of ThDP in the absence (■) and presence of 2 mM pyruvate (□), or 2 $\mu$M acetylphosphinate (Δ), respectively. $\lambda_{exc} = 280$ nm, $\lambda_{em} = 330$ nm, slit width = 5 nm.

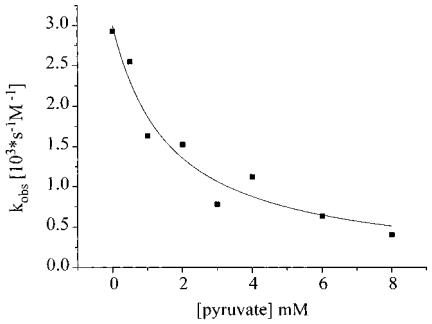


FIGURE 11: Dependence of the second-order rate constant of inactivation of the PDH-complex (1 mg/mL) by diethyl pyrocarbonate (8 $\mu$M) on the pyruvate concentration in 50 mM potassium phosphate buffer, pH 7.6. The line represents a fit according to a competitive mechanism with a dissociation constant of 1.65 mM for pyruvate.

apparent dissociation constants, measured in the presence and absence of pyruvate, shows that the apparent dissociation constants for the coenzymatically active analogues, ThDP and N1ThDP, are clearly decreased in the presence of pyruvate whereas those of the coenzymatically inactive analogues are not influenced by this substrate.

*ThDP-Bound Acetylphosphinate, a Model for the Tight Binding ThDP-Substrate Intermediate.* Acetylphosphinate is a tight binding inhibitor of the PDH-complex (*20*) since it mimics a noncleavable reaction intermediate. Interestingly, the observed decrease of the apparent dissociation constant of ThDP in the presence of pyruvate can also be observed in the presence of acetylphosphinate (Figure 10), whereas the apparent dissociation constant of the coenzymatically inactive analogue OThDP is not influenced by acetylphosphinate. In the presence of 2 $\mu$M inhibitor, the apparent dissociation constant for ThDP decreases to a value of 0.7 $\mu$M.

*Interaction of Pyruvate with the ThDP-Free PDH-Complex.* As shown above there is a slow transition from an inactive to an active state of the PDH-complex induced by slow binding of the cofactor ThDP. Such a transition results in a substrate regulation if the substrate can bind to both of these states. Therefore we investigated the interaction of the substrate pyruvate with the ThDP-free PDH-complex by an indirect method. The histidine residues of the ThDP-free PDH-complex were modified by diethyl pyrocarbonate. This leads to a decrease in catalytic activity of both the PDH-complex and E1. In the presence of the substrate pyruvate the rate of this modification is decelerated. This shows that pyruvate interacts even with the ThDP-free PDH-complex. A dissociation constant of 1.65 mM for this interaction can be calculated from the hyperbolic plot of the rate constant of inactivation by diethyl pyrocarbonate versus the pyruvate concentration (Figure 11). This observation confirms qualitatively previous results obtained by ultrafiltration experiments (*21*).

## DISCUSSION

The aim of this study was to elucidate the molecular mechanism of regulation for the *E. coli* PDH-complex. The ThDP affinity to the PDH-complex is increased in the presence of the substrate pyruvate. This suggests formation of a ThDP-substrate reaction intermediate that is tightly bound to the protein. To confirm these results and to test whether formation of this intermediate establishes a possible regulation mechanism of the PDH-complex activity, binding experiments were performed with the natural cofactor ThDP as well as with a number of active and inactive cofactor analogues.

The activity studies with the PDH-complex recombined with these coenzymes (Figure 7) clearly show the requirement of both the N-1′atom and the 4′-amino group for catalytic activity.

Furthermore kinetic as well as fluorescence measurements (Table 1) show that the nitrogen in position 1' of the pyrimidine ring is required for both efficient binding and catalytic activity of the cofactor whereas the N-3′ of the aminopyridine ring and the 4'-amino group are not essential for the binding of the cofactor analogue in the PDH-complex. These functional studies correlate well with cofactor binding studies performed on other ThDP dependend enzymes (*10*) and in addition to a sequence alignment (*22*) provide further evidence for a ThDP binding motif of E1 that is similar to that of other ThDP binding enzymes (*23*−*28*).

Interestingly the affinity to the PDH-complex is exclusively increased for the active coenzymes ThDP and N1ThDP by the substrate pyruvate while the dissociation constants for the coenzymatically inactive analogues N3ThDP, DAThDP and OThDP remain unaltered (Table 1).

Our experiments, using two independent methods, clearly show that the affinity of the coenzymatically active coenzymes ThDP and N1ThDP is increased in the presence of pyruvate (Table 1). This suggests that formation of at least one reaction intermediate, that is exclusively formed between an active coenzyme and the substrate, causes the apparent increased coenzyme affinity to the protein. Binding of pyruvate to a ThDP-independent pyruvate binding site, as has been discussed for the PDH-complex from pig heart (*29*) can therefore be ruled out to cause the apparent increased coenzyme affinity in the *E. coli* PDH-complex.

The stabilizing effect of pyruvate on ThDP binding can be mimicked by acetylphosphinate. This substrate surrogate interacts with the PDH-complex under formation of a ThDP-acetylphosphinate adduct and mimics the normal reaction intermediate, 2-(1-carboxy-1-hydroxyethyl)-ThDP. This result suggests that 2-(1-carboxy-1-hydroxyethyl)-ThDP is a tight binding reaction intermediate. Decarboxylation of this natural intermediate leads to the enzyme bound 2-(1-hydroxyethyl)-ThDP. Gel filtration experiments performed with E1 using $^{32}$P-labeled ThDP and $^{14}$C-labeled 2-$^{14}$C- and 1-$^{14}$C-pyruvate, respectively, have shown that this intermedi-

ate or its carbanion is also stably bound to E1 (*30*). Experiments performed by Gutowski and Lienhard (*31*) provide evidence that the stable binding of the 2-carbanion of 2-(1-hydroxyethyl)-ThDP is a result of a reduced repulsive charge interaction resulting from the partial neutralisation of the positive charge of the nitrogen in the thiazolium ring of this intermediate. They determined a high affinity of the ThDP-analogue thiamin thiazolon diphosphate, which lacks the positive charge in the thiazole ring to the PDH-complex. In the 2-(1-carboxy-1-hydroxyethyl)-ThDP as well as in the acetylphosphinate adduct, a favorable charge interaction between the negative charge of the carboxylate or phosphinate residue and the protein, could be responsible for the stable binding of these intermediates to the protein.

A comparison of ThDP binding to the complete PDH-complex and to E1 alone (Table 1) excludes that enhanced binding of the cofactor ThDP is triggered by the subunit interaction in the complex, since the observed dissociation constants were identical.

The rate of the overall reaction catalyzed by the PDH-complex is limited by the ThDP dependent E1 *(5,6)*. Therefore, the overall reaction is strongly influenced by the concentration of the cofactor ThDP as well as by the substrate pyruvate.

At nonsaturating cofactor concentrations tight binding of the reaction intermediates, should increase the amount of enzyme-bound cofactor. This shift of the equilibrium towards a more ThDP-saturated state is observed as a lag phase in product formation (Figure 2). In agreement with this mechanism, preincubation of the PDH complex with low ThDP concentrations in the absence of pyruvate has no influence on the lag phase (Figure 2).

In contrast to our results, Yi and co-workers (*32*) report no significant effect of pyruvate on the cofactor binding for a mutant PDH complex containing one lipoyl domain per dihydrolipoamide transacetylase chain. In addition they report that the lag phase in product formation decreases with increasing preincubation time of this mutant with ThDP even in the absence of pyruvate. In contrast to our model where the binding of the substrate pyruvate initiates an increase in cofactor binding, they conclude that the activation process in the PDH reaction precedes catalytic turnover. Although they verify our previously published results that the $K_D$ value for ThDP is decreased in the presence of pyruvate (*33*) with $K_D$ values for the mutant of 12.3 $\mu$M in the absence and 3.7 $\mu$M in the presence of pyruvate, their conclusion is contradictory that at 11 $\mu$M ThDP, pyruvate has no influence on the ThDP binding. Under these conditions the enzyme should be approximately 50% saturated with cofactor according to their reported $K_D$ values and not be near saturation as stated. Under their conditions the pyruvate enhanced cofactor binding should be well detectable also for this mutant enzyme. Unfortunately they do not provide any information about the behavior of the wild type enzyme.

The following points support that the lag phase in product formation at physiological ThDP concentrations ($\leq$1 $\mu$M) (*34*) is indeed the result of a slow recombination of E1 with ThDP.

1. Due to its structural similarity to the natural cofactor, the coenzymatically inactive N3ThDP-analogue competes with ThDP for the diphosphate−Mg binding site in E1 in the absence of pyruvate. In the preincubation mixture only weak and reversible binding of the analogue to the enzyme occurs under the experimental conditions. Upon addition of pyruvate, the apparent affinity of ThDP increases and leads to an increased binding of the natural cofactor. However, this recombination process is slowed down by the competition for the diphosphate binding site of ThDP with the N3ThDP if both cofactors are present at nonsaturating concentrations before the addition of pyruvate. This leads to an increase in the lag phase, i.e. the establishment of the thermodynamic equilibrium for the ThDP binding in the presence of pyruvate is slowed down. Since the $K_D$ value for the natural cofactor ThDP is at least 50 fold smaller compared to that for N3ThDP in the presence of pyruvate, no influence on the final activity is observed in the presence of both pyruvate and this analogue (Figure 9). This effect is not observed for the other inactive ThDP analogues due to their tight binding properties which results in a strong and rapid inhibition during the preincubation period in the absence of pyruvate. In the presence of pyruvate, the new equilibrium between the PDH-complex, ThDP, and the tight binding analogue is established only after prolonged incubation times (data not shown).

2. The rate of the activation measured at high ThDP concentrations is comparable in the presence and absence of pyruvate (Figure 1). This shows that the same process is observed in the absence and presence of pyruvate. We suggest that the recombination of the cofactor with the enzyme is this rate-limiting step in the activation process. At high ThDP concentrations the enzyme is saturated with the cofactor even in the absence of pyruvate. Therefore, a preincubation of the enzyme with ThDP before initiation of the reaction by addition of pyruvate leads to a disappearance of the lag phase (Figure 1). From these experiments it can be ruled out that the lag phase is the result of a slow isomerization of an enzyme−substrate-complex to an active state as discussed earlier (*9*).

3. At low ThDP concentrations, ThDP dissociates from the complex in the absence of pyruvate. Both the binding to (pseudo first-order rate constant of 0.0013 $s^{-1}$ at a ThDP concentration of 1 $\mu$M) and the dissociation of the cofactor from the PDH-complex (first-order rate constant of 0.05 $s^{-1}$) are slow compared to the catalytic steps ($k_{cat}$ = 50 $s^{-1}$). Such a mechanism with a slow transition between two enzyme states (in case of the PDH-complex a ThDP-free and ThDP-bound state) that interact with the substrate results in sigmoidal progress curves for the substrate if the substrate shifts the equilibrium toward the active (more ThDP-saturated) state (*35*). The mechanism of substrate regulation of the PDH-complex from *E. coli* described here, which is triggered by the formation of a tightly bound reaction-intermediate−cofactor complex, represents a new molecular aspect of substrate regulation according to the slow transition model and pronounces not only the kinetic cooperativity for the substrate pyruvate, but also for the cofactor ThDP, as shown by the rate equation derived in the appendix. This regulation mechanism allows a fine and slow tuning of the PDH-complex activity by its substrate pyruvate at physiological concentrations of ThDP and contradicts a mechanism where activation precedes catalytic turnover (*32*).

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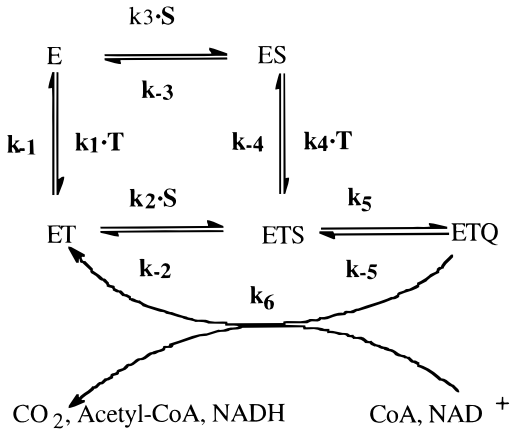
## APPENDIX

The discussed slow transition of the PDH-complex to an active state by reconstitution with ThDP, induced by tight binding of a reaction intermediate, can be described by the following minimal reaction scheme:




where E is the ThDP-free enzyme complex, T is the cofactor ThDP, S is the substrate pyruvate, Q is the carboxyethyl residue bound to T. The step assigned with $k_6$ summarizes all reactions necessary to regenerate ET from ETQ by product release, including formation of the hydroxyethyl ThDP intermediate. For this mechanism, the following steady-state rate equation for the formation of product can be derived

$$v = \frac{k_6 E_0(a\mathrm{TS} + b\mathrm{T^2S} + c\mathrm{TS^2})}{d + e\mathrm{S} + f\mathrm{S^2} + g\mathrm{TS} + h\mathrm{TS^2} + i\mathrm{T^2S} + j\mathrm{T^2} + k\mathrm{T}}$$

where

$$a = k_5(k_1k_2k_{-3} + k_3k_4k_{-1})$$

$$b = k_5k_2k_4k_1$$

$$c = k_5k_2k_3k_4$$

$$d = (k_{-5} + k_6)(k_{-1}k_{-2}k_{-3} + k_{-1}k_{-3}k_{-4}) + k_5k_6k_{-1}k_{-3}$$

$$e = (k_{-5} + k_6)(k_2k_{-3}k_{-4} + k_3k_{-1}k_{-2} + k_3k_{-1}k_{-4}) + k_6k_3k_{-1}k_{-5}$$

$$f = (k_{-5} + k_6)k_2k_3k_{-4}$$

$$g = (k_{-5} + k_6)(k_{-2}k_3k_4 + k_1k_2k_{-4} + k_3k_4k_{-1} + k_1k_2k_{-3}) + k_5(k_3k_{-1}k_4 + k_1k_2k_{-3})$$

$$h = (k_{-5} + k_6)k_2k_3k_4 + k_5k_2k_4k_3$$

$$i = (k_{-5} + k_6)k_1k_2k_4 + k_5k_1k_2k_4$$

$$j = (k_{-5} + k_6)k_1k_{-2}k_4 + k_6k_5k_1k_4$$

$$k = (k_{-5} + k_6)(k_4k_{-1}k_{-2} + k_1k_{-3}k_{-2} + k_1k_{-4}k_{-3}) + k_5k_6(k_4k_{-1} + k_1k_{-3})$$

This rate equation contains terms in $T^2$ as well as in $S^2$ showing cooperativity for S, which depends on the concentration of T and cooperativity for T which depends on the concentration of S. At high concentrations of T the dominance of the quadratic term in T results in noncooperativity with respect to S. In the same way, the dominance in $S^2$ at high concentrations of S results in a loss of cooperativity with respect to T. If the binding and releasing step of pyruvate to the ThDP-free complex is assumed to be fast compared to all other steps, then the cooperativity for T vanishes, whereas the cooperativity for S remains intact. In this case the steady state equation reduces to

$$v = \frac{k_6 E_0(a\mathrm{TS} + c\mathrm{TS^2})}{d + e\mathrm{S} + f\mathrm{S^2} + g\mathrm{TS} + h\mathrm{TS^2} + k\mathrm{T}}$$

where

$$a = k_5(k_1k_2k_{-1} + k_4K_3)$$

$$c = k_5k_4k_2K_3$$

$$d = (k_{-5} + k_6)(k_{-1}k_{-2} + k_{-1}k_{-4}) + k_6k_{-1}k_5$$

$$e = (k_{-5} + k_6)(k_2k_{-4} + k_{-1}k_{-2}K_3 + k_{-1}k_{-4}K_3) + k_6k_{-1}k_5K_3$$

$$f = (k_{-5} + k_6)k_2k_{-4}K_3$$

$$g = (k_{-5} + k_6)(K_3k_4k_{-2} + K_3k_4k_{-1} + k_1k_2) + k_5(K_3k_{-1}k_4 + k_1k_2)$$

$$h = (k_{-5} + k_6)k_2k_4K_3 + k_5k_2k_4K_3$$

$$k = (k_{-5} + k_6)(k_1k_{-2}k_1) + k_5k_6k_1$$

$$K_3 = \frac{k_3}{k_{-3}}$$

All experimental data of the present work, as well as the data concerning the cooperativity for S and T published earlier (*32*, *36*), are consistent with the proposed model.

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