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Pyruvate decarboxylase from *Zymomonas mobilis*

Structure and re-activation of apoenzyme by the cofactors thiamin diphosphate and magnesium ion

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To study the mechanism of re-activation of *Zymomonas mobilis* pyruvate decarboxylase apoenzyme by its cofactors thiamin diphosphate and Mg^{2+}, cofactor-free enzyme was prepared by dialysis against 1 mM-dipicolinic acid at pH 8.2. This apoenzyme was then used in a series of experiments that included determination of: (*a*) the affinity towards one cofactor when the other was present at saturating concentrations; (*b*) cofactor-binding rates by measuring the quenching of tryptophan fluorescence on the apoenzyme; (*c*) the effect of replacement of cofactors with various analogues; (*d*) the stoichiometry of bound cofactors in holoenzyme; and (*e*) the molecular mass of apoenzyme by gel filtration. The results of these experiments form the basis for a proposed model for the re-activation of *Z. mobilis* pyruvate decarboxylase apoenzyme by its cofactors. In this model there exists two alterative but equivalent pathways for cofactor binding. In each pathway the first step is an independent reversible binding of either thiamin diphosphate (K_d 187 μM) or Mg^{2+} (K_d 1.31 mM) to free apoenzyme. When both cofactors are present, the second cofactor-binding step to form active holoenzyme is a slow quasi-irreversible step. This second binding step is a co-operative process for both thiamin diphosphate (K_d 0.353 μM) and Mg^{2+} (K_d 2.47 μM). Both the apo- and the holo-enzyme have a tetrameric subunit structure, with cofactors binding in a 1:1 ratio with each subunit.

INTRODUCTION

Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyses the non-oxidative decarboxylation of pyruvate to produce acetaldehyde and CO_2. Enzyme activity is dependent on the cofactors thiamin diphosphate (ThDP) and Mg^{2+}. Mg^{2+} is preferred in Nature, but can be replaced by alternative bivalent cations such as Mn^{2+}, Co^{2+} and Ca^{2+} without loss of catalytic power [1]. The enzymes from yeast [1–6], wheat germ [5–7] and the bacterium *Zymomonas mobilis* [8,9] have received particular attention.

The kinetics of, and structural changes associated with, cofactor binding in yeast PDC have been well documented [1,10–17], but only preliminary information has been presented for the *Z. mobilis* enzyme [8,11]. In both yeast and *Z. mobilis* PDC the cofactors dissociate from holoenzyme at pH values above 8.0, and recombine tightly with apoenzyme at pH 6.5 in a time-dependent manner to yield active enzyme.

Yeast PDC exists as a heterotetramer ($\alpha_2\beta_2$) with a molecular mass of 240 kDa [2,4]. At alkaline pH, where cofactors are released, this tetrameric enzyme structure dissociates into two dimeric halves [14]. The re-association of these dimeric halves to form tetramers at pH 6.5 does not require the cofactors [17].

The mechanism for cofactor binding in yeast PDC has been suggested to follow a quasi-irreversible pathway [1,10]. In such a mechanism, either cofactor can interact separately with apoenzyme in a reversible manner. Only when both cofactors are present is binding irreversible. The rate of cofactor recombination is independent of the protein-association step.

In this report we investigate PDC from *Z. mobilis* to determine the mechanism for re-activation of apoenzyme by cofactors and to describe the re-activation pathway in quantitative terms. As the enzyme from *Z. mobilis* is a homotetramer with a molecular mass of 240 kDa [9], the possibility of subunit association in cofactor recombination is also investigated.

Our overall aim with this enzyme from *Z. mobilis* is to attempt to identify those structural elements of PDC which are involved in its catalytic properties, by using a combination of site-directed mutagenesis and chemical modification. Such studies are made possible by the successful cloning and expression of the gene for *Z. mobilis* PDC into *Escherichia coli* [9]. Therefore, to assess fully the effects of any planned amino acid substitutions it was necessary to characterize the kinetic properties of this enzyme.

Before undertaking studies on *Z. mobilis* PDC apoenzyme, a method was required to produce metal-free apoenzyme. To date, the methods for preparing PDC apoenzyme from various sources [11,12] have been successful in removing ThDP, but have failed to completely remove bivalent cations. An indication of bivalent-cation levels can be obtained from the residual activity after prepared apoenzyme is preincubated with excess ThDP in the absence of Mg^{2+}. This residual activity has been reported to be as high as 41–45% of fully reconstituted enzyme activity [11,12].

We therefore developed a method for preparing metal-free apoenzyme that was based on maintaining high protein concentrations (0.1–1.0 mM) during preparation and storage of apoenzyme. This decreases possible re-activation of apoenzyme by adventitious metals. Apoenzyme prepared by this method was then used in all subsequent structural and cofactor-binding studies.

EXPERIMENTAL

Strain and cultivation

Cultures of *Z. mobilis* strain ZM6 (A.T.C.C. 29191) were obtained from Dr. Lindsay Sly (Culture Collection, Microbiology Department, University of Queensland). Cells were grown at pH 5.0 and 30 °C in a medium containing 10% (w/v) glucose, 0.5% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.1% (w/v) $MgSO_4,7H_2O$, 0.15% (w/v) $(NH_4)_2SO_4$, 1 mg of biotin/l, 2 mg of calcium pantothenate/l and 20 mg of $Fe(NH_4)_2(SO_4)_2,6H_2O$/l.

Abbreviations used: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; NEM, *N*-ethylmaleimide.

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The pH during growth was maintained by addition of NaOH. Cells were harvested as described by Neale *et al.* [9].

Purification of enzyme

Cells were lysed and enzyme was purified as described by Neale *et al.* [9], but with some modifications. Lysis buffer was 100 mM-KH_2PO_4/KOH, pH 6.8, with the additional components 0.1 mM-ThDP and 0.1% (v/v) toluene. In the purification, the two dye-absorbent columns of Neale *et al.* [9] were replaced by a batch procedure using hydroxyapatite (Merck). PDC binds to hydroxyapatite in 100 mM-KH_2PO_4/KOH, pH 6.8, and is eluted with 400 mM-KH_2PO_4/KOH, pH 6.8. This step gave a 4-fold increase in specific activity and 80% yield. Pure enzyme (specific activity 115 units/mg) was stored in 50% (v/v) glycerol at −20 °C. All reagents and other enzymes were obtained from commercial sources.

Enzyme assay

PDC activity was assayed by measuring the rate of production of acetaldehyde, determined by measuring the oxidation of NADH in the presence of alcohol dehydrogenase (ADH). The rate of change of A_{340} at 30 °C in 50 mM-Mes/KOH, pH 6.5 was recorded. Unless otherwise stated, final assay mixtures contained 5 mM-$MgCl_2$, 0.1 mM-ThDP, 0.15 mM-NADH, 10 units of ADH/ml and 5 mM-pyruvate. For preincubation experiments, apoenzyme was exposed to various cofactor combinations for 15 min at 30 °C before initiation of the assay by addition of a pyruvate/NADH/ADH mixture. In preincubation experiments, saturating ThDP was 0.1 mM and saturating Mg^{2+} was 5 mM. One unit of activity is defined as the quantity of enzyme that catalyses the formation of 1 μmol of product/min.

Preparation of PDC apoenzyme

All labware was cleaned with 20% (v/v) HNO_3 and rinsed several times with metal-free water (14 MΩ·cm) obtained from an Elgastat Spectrum water purification system. All buffers and ThDP solutions used for binding studies were passed through a column of Bio-Rad Chelex 100 cation-exchange resin (Na^+ form) to remove bivalent metal. Dialysis tubing was prepared by heating in four changes of metal-free water at 70–80 °C for periods of 2 h each.

PDC native enzyme (10 mg/ml) was dialysed at room temperature against three changes of 50 mM-Hepes/KOH, pH 8.2, which contained 1 mM-dipicolinic acid as chelating agent. To remove chelating agent, the apoenzyme was further dialysed against several changes of 50 mM-Mes/KOH, pH 6.5, at 4 °C. This apoenzyme preparation was stored at 4 °C under a N_2 atmosphere, where it was stable for several weeks.

Protein measurements

During purification, protein was measured with a bicinchoninic acid protein-determination kit from Sigma Chemical Co. The method was based on that described by Smith *et al.* [18]. Protein in pure preparations was measured at pH 6.5 by using an A_{280} of 1.28 for a 1 mg/ml solution [9]. For cofactor-stoichiometry measurements, concentrations were determined on the basis of the known tryptophan and tyrosine contents [19] from absorbance measurements at 280 and 294 nm in 0.1 M-NaOH. The absorption coefficients for tryptophan and tyrosine were taken from Mihalyi [20].

Mg^{2+} measurements

Mg^{2+} was measured with a Varian Techtron model AA-6 atomic-absorption spectrophotometer equipped with an air–acetylene flame. Measurements were made at 285.2 nm with a slit width of 0.5 nm. Standards in the range 0.1–1.0 μg of Mg^{2+}/ml were made up in 10% HNO_3. Absorbance values were obtained by integration over 3 s.

ThDP measurements

For stoichiometry determinations ThDP was measured by oxidation to the fluorescent derivative thiochrome diphosphate. The procedure involved addition of 50 μl of a sample containing ThDP to 3 ml of 50% (v/v) ethanol, which was left for 3 min before addition of 500 μl of oxidizing reagent [0.04% (w/v) $K_3Fe(CN)_6$ in 15% (w/v) NaOH]. After 2 min the reaction was stopped with 10 μl of 30% (v/v) H_2O_2.

Fluorescence was determined on an Aminco SPF 500 spectrofluorimeter at an excitation wavelength of 375 nm (bandwidth 2 nm) and an emission wavelength of 430 nm (bandwidth 8 nm). The range of standards containing 1–18 μg of ThDP/ml were made up in 50 mM-Mes/KOH, pH 6.5. All final solutions were protected from light, and all labware was washed with 8 M-HNO_3.

For binding studies the concentrations of stock ThDP solutions were measured in 50 mM-Mes/KOH, pH 6.5, by using an ϵ_{267} of 8520 $M^{-1} \cdot cm^{-1}$.

Measurement of cofactor binding

The time course for cofactor binding was determined by monitoring the quenching of tryptophan fluorescence on the PDC enzyme. Measurements were done on an Aminco SPF 500 spectrofluorimeter with an excitation wavelength of 300 nm (bandwidth 5 nm) and an emission wavelength of 340 nm (bandwidth 5 nm). This unusually high excitation wavelength was chosen so as to avoid excessive absorption of the incident light by ThDP.

The conditions for binding of cofactors were 50 mM-Mes/KOH, pH 6.5, and 30 °C. Unless stated otherwise, reactions were initiated by addition of apoenzyme (final concn. 0.112 mg/ml) to a mixture of various cofactor concentrations.

Inactivation by *N*-ethylmaleimide (NEM)

Apoenzyme (1 mg/ml) was preincubated for 90 min at 30 °C with various ThDP or Mg^{2+} concentrations in 50 mM-Mes/KOH, pH 6.5. NEM (2 mM) was added at the end of this period, and 5 μl samples were subsequently removed for up to 1 h after addition of NEM. Each sample was added to a 2.06 ml assay mixture that contained 0.1 mM-ThDP, 5 mM-Mg^{2+} and 0.03 mM-dithiothreitol and preincubated for 15 min at room temperature before assay. The function of dithiothreitol was to react with the excess NEM and so stop any further reaction with enzyme during the preincubation step required for activity measurements.

Synthesis of thiochrome diphosphate

For binding studies, thiochrome diphosphate was prepared from ThDP by using commercially obtained CNBr (Sigma). The method for preparation was the procedure of Nishimune *et al.* [21], who established the optimum concentrations for oxidation as 0.04 M-CNBr and 0.16% (w/v) NaOH. After oxidation, the reaction mixture was adjusted to pH 4 with HCl and applied to a column of DEAE-Sephacel (Pharmacia; acetate form) and washed with water. Bound thiochrome diphosphate was eluted with a gradient of 0–0.2 M-sodium acetate, pH 4.5. Fractions containing thiochrome diphosphate were pooled and adjusted to pH 6.5 with NaOH.

Thiochrome diphosphate was characterized on the basis of its fluorescence spectrum, and the concentration was determined by

using an ϵ_{367} of 20600 $M^{-1} \cdot cm^{-1}$ [22]. Determination of phosphate by the procedure of Bartlett [23] using Fiske and Subbarow reagent verified that the product was in the diphosphate form.

On the basis of activity measurements with PDC the product was also deemed to be free of ThDP, since no activity was observed after apoenzyme was preincubated with Mg^{2+} and prepared thiochrome diphosphate.

Molecular mass of apoenzyme

The molecular mass of apoenzyme was determined by gel filtration on a column of Sephacryl S-300 HR (Pharmacia). The column dimensions were 13.9 $cm^2 \times 82$ cm and the equilibration buffer was 50 mM-Hepes/KOH, pH 8.2, which also contained 1 mM-dipicolinic acid. Flow rate was 1.05 ml/min, and the column was calibrated with catalase (240 kDa) and bovine serum albumin (66 kDa). Apoenzyme (5 ml) was loaded in the same equilibration buffer, and its elution position was determined by activity measurements on collected fractions.

Data analysis

Experimental data were analysed by non-linear regression using either the DNRP53 [24] or GraphPAD computer programs. The fitted equations are described below.

Cofactor-saturation curves. The rate of reaction (v) as a function of cofactor concentration ([C]) was described by eqn. (1):

$$v = V_m[C]^h/(K^h + [C]^h) \quad (1)$$

where the fitted parameters are V_m (the rate at saturating cofactor), K (the half-saturating concentration of cofactor) and h (the Hill slope).

Fluorescence quenching. In the presence of both cofactors, PDC apoenzyme shows a progressive decrease in tryptophan fluorescence lasting for a period of several minutes. For most experiments of this type, data (fluorescence, Y_t, versus time, t) were analysed by fitting eqn. (2):

$$Y_t = Y_0 - (Y_0 - Y_\infty)[1 - \exp(-k't)] \quad (2)$$

to the data, where the fitted parameters are Y_0 and Y_∞ (the initial and final fluorescence respectively) and k' (an apparent first-order rate constant).

In some experiments, the initial concentrations of enzyme ($[E]_0$) and cofactor ($[C]_0$) were comparable, whereupon depletion of cofactor had to be taken into account. The data follow eqn. (3):

$$Y_t = Y_0 - (Y_0 - Y_\infty)(1 - x_1)/(1 - x_1x_2) \quad (3)$$

if $[E]_0 < [C]_0$ then	if $[C]_0 < [E]_0$ then
$x_1 = \exp(k\{[E]_0 - [C]_0\}t)$	$x_1 = \exp(k\{[C]_0 - [E]_0\}t)$
$x_2 = [E]_0/[C]_0$	$x_2 = [C]_0/[E]_0$

where the fitted parameters are Y_0 and Y_∞ and k (the second-order rate constant); the apparent first-order rate constant ($k' = k[C]_0$) was then calculated.

Cofactor-protection experiments. In the presence of limiting cofactor, an equilibrium is set up between free enzyme (E) and the enzyme–cofactor complex (EC). When this mixture is treated with an inactivating reagent which preferentially reacts with free enzyme, activity (Y_t) will decline from the initial value (Y_0) in a first-order manner, with a rate constant of k'. The final (Y_∞) will reflect the fraction of EC in the mixture. The first-order decay curve (eqn. 2) was fitted to the data consisting of measurements of activity versus time, to obtain values for k', Y_0 and Y_∞; the latter value was then expressed as a percentage of Y_0.

RESULTS

Analysis of cofactor levels

Cofactor levels were assessed on the basis of activity measurements in the presence of various cofactor combinations and also by direct determination of ThDP and Mg^{2+}. Determinations were carried out on apo- and holo-enzyme, which included both native and reconstituted enzyme (Table 1).

For apoenzyme, indications are that the preparation procedure is successful in removing both cofactors. A value of only 2% activity (relative to fully reconstituted enzyme) was obtained when apoenzyme was preincubated with ThDP alone. This value, in conjunction with direct determinations on Mg^{2+}, indicate that substantially lower levels of bivalent cations are present in this apoenzyme preparation than in previous preparations [11,12].

The values obtained from stoichiometry measurements on holoenzyme (Table 1) show a cofactor/subunit ratio close to 1 for both ThDP and Mg^{2+}. The reason why the values are in general less than 1 is due to some loss of bound cofactors during gel filtration. This is supported by activity measurements on holoenzyme samples after gel filtration (Table 1). If 100% activity represents fully reconstituted enzyme (after preincubation with both cofactors), then a decrease in activity, as seen for both native and reconstituted holoenzyme samples when assayed without preincubation, indicates some loss of bound cofactors during gel filtration. A further small decrease in activity was apparent for each holoenzyme sample when assayed with ThDP or Mg^{2+} alone or with no added cofactors at all.

The preparation of PDC apoenzyme does result in some loss of catalytic activity, since reconstituted enzyme formed from apoenzyme has only 80% of the activity of native enzyme (result not shown). This loss of catalytic activity is not reflected as a decrease in cofactor content, as the values for stoichiometry measurements are similar for both holoenzyme samples (Table 1).

Binding constants in the presence of both cofactors

The affinity for each cofactor was determined from activity measurements on apoenzyme preincubated with one cofactor limiting and the other saturating. The saturation curves showed some deviation from a rectangular hyperbola (results not shown). The sigmoidal saturation curve obtained for Mg^{2+} (Fig. 1*a*) gives a half-saturating concentration of 2.931 ± 0.162 μM and a Hill slope (h) of 1.401 ± 0.107. The sigmoidal saturation curve for ThDP (Fig. 1*b*) gives a half-saturating concentration of 0.449 ± 0.036 μM and a h value of 1.424 ± 0.146.

Previous studies on yeast PDC have used protein fluorescence and fluorescent probes to measure cofactor binding [11,12]. Some preliminary experiments were performed on *Z. mobilis* PDC to determine if fluorescence was useful in our work, and it was found that, as with yeast PDC, ThDP binding to apoenzyme results in quenching of tryptophan fluorescence of the protein. This finding formed the basis for direct time-course measurements of cofactor binding to PDC apoenzyme. A typical example of such an experiment is illustrated in Fig. 2. This highlights that under the experimental conditions, where one cofactor is saturating and the other limiting, binding follows pseudo-first-order kinetics.

A series of such curves were generated (results not shown) by varying the concentration of one cofactor with the other saturating. Apoenzyme alone, or apoenzyme plus either cofactor

Table 1. PDC apo- and holo-enzyme: specific activity and cofactor content

Determination of cofactors and protein concentration was as described in the Experimental section. Before stoichiometry and activity measurements on native and reconstituted enzyme samples (native enzyme was that directly obtained by purification, stored in saturating cofactors; reconstituted enzyme was that formed from preincubation of apoenzyme with saturating cofactors), excess cofactors were removed by gel filtration on a column of Bio-Gel P-6DG (Bio-Rad) equilibrated with 50 mM-Mes/KOH, pH 6.5. The activity of all enzyme samples was determined in the presence of various cofactor combinations, as indicated. For apoenzyme these assays were initiated after preincubation with the various cofactor combinations, whereas holoenzyme samples were assayed without preincubation. Results are expressed as % activity, where 100% is the activity of each enzyme sample preincubated with both cofactors.

	Ratio cofactor/subunit		Activity (%)			
Enzyme sample	Mg^{2+}	ThDP	No cofactors	Mg^{2+} alone	ThDP alone	Both cofactors
Native	1.10	0.76	93	91	92	96
Apoenzyme	0.05	0.01	0	0	2	100
Reconstituted	0.80	0.76	76	75	75	84

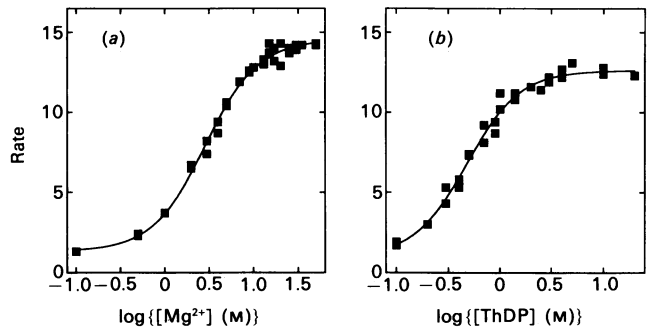


Fig. 1. Cofactor-saturation curves for PDC apoenzyme as measured by changes in activity

(*a*) Mg^{2+} binding to apoenzyme in the presence of saturating ThDP. Apoenzyme was preincubated with Mg^{2+} as indicated and 0.1 mM-ThDP before assay. Rates are expressed as change in absorbance per 10^3 s. Duplicate determinations are both plotted. The line represents the best fit of eqn. (1) to the data. (*b*) ThDP binding to apoenzyme in the presence of saturating Mg^{2+}. Apoenzyme was preincubated with ThDP as indicated and 5 mM-Mg^{2+} before assay. Rates are expressed as change in absorbance per 10^3 s. Duplicate determinations are both plotted. The line represents the best fit of eqn. (1) to the data.

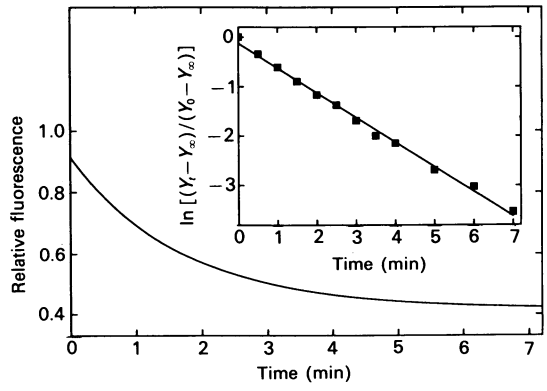


Fig. 2. Example of a fluorescence time course for binding of cofactors

Apoenzyme (1.85 μM subunits) was added at zero time to a mixture of 0.15 mM-Mg^{2+} and 0.1 mM-ThDP, and tryptophan fluorescence was measured as a function of time. Apparent first-order rate constants (k') were obtained by fitting eqn. (2) to such curves. The inset shows a first-order plot of these data.

alone, was found to produce no appreciable fluorescence change (results not shown). From each curve an apparent first-order rate constant (k') was obtained and replotted against cofactor concentration (Fig. 3). The slopes of these lines give the second-order rate constants of 2.79 ($\pm$0.19) $\times 10^{-3}$ $\mu M^{-1} \cdot min^{-1}$ for Mg^{2+} and 3.69 ($\pm$0.14) $\times 10^{-2}$ $\mu M^{-1} \cdot min^{-1}$ for ThDP.

Preincubation of apoenzyme with one cofactor saturating for 1 h at 30 °C before initiation of time-course experiments with addition of limiting concentration of second cofactor produced similar second-order rate constants. The values determined were 3.57 ($\pm$0.93) $\times 10^{-3}$ $\mu M^{-1} \cdot min^{-1}$ for Mg^{2+} and 3.30 ($\pm$0.24) $\times$ 10^{-2} $\mu M^{-1} \cdot min^{-1}$ for ThDP.

Binding constants in the presence of only one cofactor

Since neither cofactor alone gives a change in protein fluorescence, the use of various analogues was pursued in the hope of producing a measurable spectral change as a result of binding to apoenzyme. Mg^{2+} binding was probed firstly with Co^{2+}, which can replace Mg^{2+} without loss of catalytic power and has a half-saturating concentration of 7.8 μM, and secondly by the lanthanide elements terbium and europium, which both inhibit reconstitution of apoenzyme at high concentrations (5 mM). ThDP binding was explored with the fluorescent analogue thiochrome diphosphate, which displays competitive inhibition (K_i = 3.93 μM) towards ThDP binding in the presence of Mg^{2+}. In all cases we were unable to detect any spectral change (results not shown).

The possibility of ThDP binding to PDC apoenzyme in the absence of Mg^{2+} was subsequently investigated by using the thiol-modifying reagent NEM. In a series of protection experiments, apoenzyme was preincubated with various ThDP concentrations before addition of NEM.

The protection of ThDP against NEM inactivation is displayed in Fig. 4. For the sake of clarity in the Figure, not all ThDP concentrations used have been shown. Under the same conditions holoenzyme is not inactivated by NEM.

Since the form of ThDP used in these experiments was the hydrochloride and relatively high ThDP concentrations were required for protection, there existed the possibility that protection was due to chloride ions and not ThDP binding. A protection experiment with 20 mM-NaCl instead of ThDP was found to give the same degree of inactivation as with NEM alone (result not shown). This indicates ThDP binding to apoenzyme was responsible for protection against NEM inactivation.

The degree of protection [final activity (%)] for each ThDP concentration was then used to determine a K_d of 187 $\pm$ 55 μM

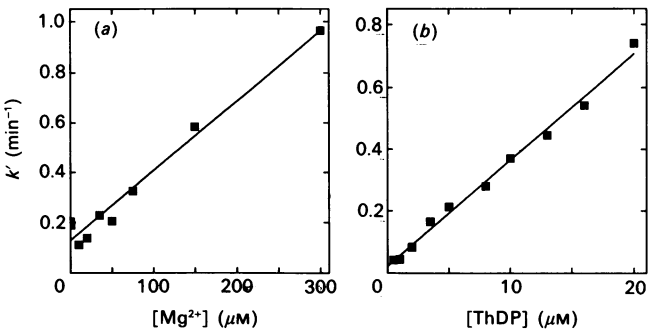


Fig. 3. Concentration-dependence of the apparent first-order rate constant for cofactor binding

Apparent first-order rate constants (k') were obtained from time-course experiments as illustrated in Fig. 2. (*a*) Mg^{2+} binding. (*b*) ThDP binding. Rate constants obtained from ThDP concentrations of 5 μM or less were corrected for changes in free ThDP as a result of binding to apoenzyme (subunit concentration of 1.85 μM) by fitting eqn. (3) to the data.

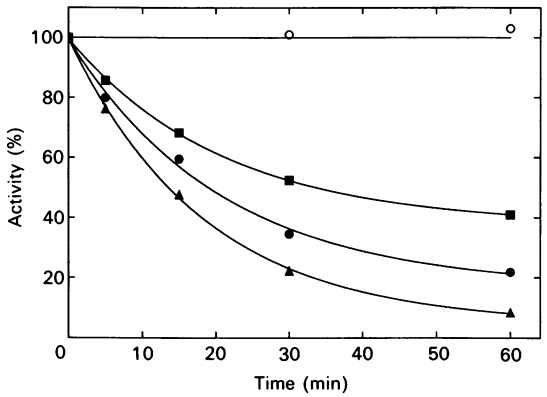


Fig. 4. Inactivation of apoenzyme by NEM

Apoenzyme (1 mg/ml) was preincubated without ThDP (▲) or with 0.1 mM-(●) or 10 mM-(■) ThDP for 90 min at 30 °C before addition of 2 mM-NEM. Preincubation of apoenzyme without addition of NEM served as a control (○). Results are expressed as a percentage of the control. Each set of data points (including ThDP concentrations not shown) was analysed by using eqn. (2) to determine final activity values.

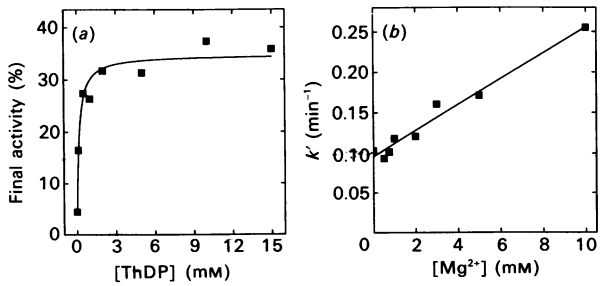


Fig. 5. Effect of cofactors on inactivation of PDC by NEM

(*a*) ThDP: the results represent the degree of protection [final activity (%)] afforded by varying ThDP after exposure to NEM as determined from Fig. 4. (*b*) Mg^{2+}: first-order rate constants for NEM inactivation of apoenzyme were determined in experiments similar to those depicted in Fig. 4 while varying Mg^{2+} concentrations in the absence of ThDP.

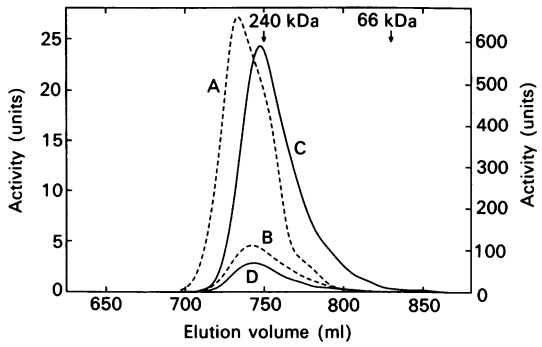


Fig. 6. Determination of apoenzyme molecular mass

Apoenzyme was passed through a column of Sephacryl S-300 HR equilibrated with 50 mM-Hepes/KOH, pH 8.2, which also contained 1 mM-dipicolinic acid. Enzyme was loaded at protein concentrations of 9, 0.76, 0.13 and 0.03 mg/ml. The elution profile was determined from measurements of PDC activity. The right ordinate corresponds to the broken-line curves labelled A (9 mg/ml) and B (0.76 mg/ml). The left ordinate corresponds to the continuous-line curves labelled C (0.13 mg/ml) and D (0.03 mg/ml). Appropriate molecular-mass markers are eluted at the positions indicated by arrows.

(Fig. 5*a*) for ThDP binding to apoenzyme in the absence of Mg^{2+}.

Mg^{2+} binding to apoenzyme in the absence of ThDP was also investigated by the same method. Instead of protecting apoenzyme against inactivation, Mg^{2+} was found to increase the rate of NEM inactivation. The residual activity after NEM inactivation was the same in the absence and presence of Mg^{2+} but the rate constant for inactivation increased in a linear fashion over the range 0–10 mM-Mg^{2+} (Fig. 5*b*). The slope of this linear increase was 1.6 ($\pm$0.1) $\times 10^{-2}$ $mM^{-1} \cdot min^{-1}$.

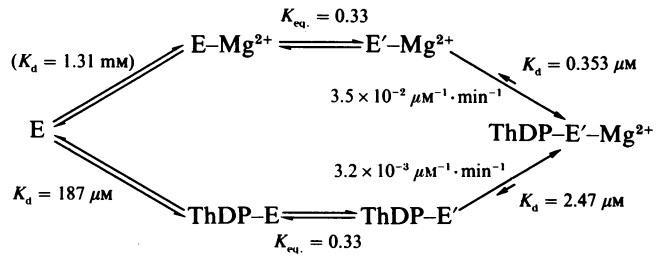
Molecular mass of apoenzyme

To investigate possible subunit dissociation upon cofactor removal, apoenzyme was passed through a gel filtration column of Sephacryl S-300 HR equilibrated with 50 mM-Hepes/KOH, pH 8.2, which also contained 1 mM-dipicolinic acid. Enzyme loadings ranged from 9 down to 0.03 mg/ml, the lower concentrations being employed so that any tendency for the subunits to dissociate would be accentuated. Each protein loading was found to be eluted at a position close to the tetrameric molecular mass for holoenzyme of 240 kDa (Fig. 6). A shift in the elution profile towards a higher molecular mass with the highest protein loading of 9 mg/ml may reflect aggregation of PDC tetramers.

DISCUSSION

PDC, as normally isolated, contains its two cofactors (Mg^{2+} and ThDP) in near-stoichiometric amounts (Table 1). However, the fact that they remain bound during purification is no measure of how tightly the cofactors bind, since both are present in all buffers used in the procedure. Nevertheless, removal of Mg^{2+} has proved difficult in the past [11,12], as measured by the residual activity with only ThDP added. In this report we describe an improved preparation of apoenzyme which contains only 0.05 Mg^{2+} ion per subunit, and has very little (2%) activity with no added metal (Table 1).

Previous work on yeast PDC has shown that removal of cofactors results in dissociation of the heterotetramer ($\alpha_2\beta_2$) into two dimers [14], although it has not been established whether these are identical $\alpha\beta$ dimers. By contrast, no evidence was found for dissociation of the homotetrameric *Z. mobilis* PDC apoenzyme (Fig. 6) into subunits.



Scheme 1. Proposed model for the re-activation of *Z. mobilis* PDC apoenzyme by its cofactors ThDP and Mg^{2+}

The second-order rate constants shown are averages of the two values given in the text, determined with or without preincubation. The dissociation constant for Mg^{2+} binding in the absence of ThDP is shown in parentheses, as it was not experimentally determined.

PDC from *Z. mobilis* [9], unlike its eukaryotic counterparts [25–28], exhibits Michaelis–Menten kinetics towards its substrate, pyruvate. However, in the experiments reported here on cofactor binding (Fig. 1), a small but unmistakable degree of positive co-operativity was observed, with Hill coefficients of 1.401 (Mg^{2+}) and 1.424 (ThDP). The half-saturating concentrations of cofactor determined from these data therefore do not represent true dissociation constants.

If we assume a classical Monod model [29] with two conformational forms of tetramer in equilibrium, only one of which binds cofactor, the data should follow eqn. (4):

$$v = V_m([C]/K_c)(1+[C]/K_c)^3/\{L+(1+[C]/K_c)^4\} \quad (4)$$

where K_c is the true dissociation constant and L is the equilibrium constant for the two conformations. The data are described well by this equation, and gave values for K_c of 2.472 ± 0.283 μM for Mg^{2+} and 0.353 ± 0.057 μM for ThDP. The conformational equilibrium constant was approx. 3 in both cases.

From the results of cofactor-binding studies we have proposed a kinetic model to explain the re-activation of *Z. mobilis* PDC apoenzyme by its cofactors (Scheme 1). In this scheme there is no ordered cofactor binding, but instead there exist two alternative, equivalent, pathways. In each pathway the first step is an independent reversible binding of either ThDP or Mg^{2+} to free apoenzyme. When both cofactors are present, the second cofactor-binding step to form active holoenzyme is a slow quasi-irreversible step. This proposed model is similar to that for yeast PDC [1,10].

This model is supported by the following observations. First, if cofactor binding was an ordered process, then the rate of cofactor binding to apoenzyme would become independent of that cofactor, at saturating levels, which bound first. For example, if Mg^{2+} bound first, then at saturating levels all enzyme would be in the form of PDC–Mg^{2+} complex and the rate of cofactor binding would only be dependent on ThDP concentration. We found that in the presence of both cofactors, even at saturating levels of each, the rate of cofactor binding did not become independent of either cofactor (Fig. 3). This also illustrates that the second binding step is the slow, rate-limiting, factor in cofactor binding to PDC apoenzyme.

The possibility that a complex of ThDP and Mg^{2+} is formed in solution and binds directly to apoenzyme is discounted by the 6-fold difference between the half-saturating concentrations (Fig. 1) and the 13-fold difference between second-order rate constants (Fig. 3) measured for each cofactor. Therefore there must exist two alternative pathways for cofactor binding to apoenzyme (Scheme 1).

For the proposed model to hold, we had to demonstrate that either cofactor binds independently to apoenzyme. Evidence is presented for ThDP binding in the absence of Mg^{2+} (Figs. 4 and 5*a*), but as yet no direct measurement of Mg^{2+} binding in the absence of ThDP has been possible. The method of determining ThDP binding was based on protection by ThDP against NEM inactivation of apoenzyme. Under the same conditions Mg^{2+} was found to enhance NEM inactivation (Fig. 5*b*) in a non-saturable fashion, so we were unable to measure a K_d for Mg^{2+} by this method.

For ThDP, the binding affinity in the presence of Mg^{2+} (K_d = 0.353 μM) is 530-fold greater than in the absence of Mg^{2+} (K_d = 187 μM). Such a difference in binding affinities shows that the first step of cofactor binding (Scheme 1) is readily reversible, whereas the second step results in tight, quasi-irreversible, binding.

Quasi-irreversible binding is also supported by the results presented in Table 1. If binding is truly irreversible, then gel filtration should not decrease the activity or cofactor content of holoenzyme. Thus, although the ternary ThDP–PDC–Mg^{2+} complex is very stable (as judged by the fact that it retains > 80% of its activity after gel filtration), it can dissociate slowly (as judged by the fact that this procedure does diminish activity and cofactor content to some extent). This heterogeneous cofactor binding is not the result of subunit association, as *Z. mobilis* PDC apoenzyme was shown not to dissociate into subunits (Fig. 6).

In conclusion, the two alternative pathways for cofactor re-activation are proposed to be equivalent, and therefore the products of the binding constants should be the same for each pathway. As we have no method for detecting Mg^{2+} binding in the absence of ThDP, we cannot directly demonstrate this step, but an estimation of K_d for Mg^{2+} can be obtained. Based on the three dissociation constants so far determined, the value for Mg^{2+} binding should be in the order of 1.31 mM.

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