Role of Glu51 for Cofactor Binding and Catalytic Activity in Pyruvate Decarboxylase from Yeast Studied by Site-Directed Mutagenesis†

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ABSTRACT: We investigated the importance of the interaction between the N1′-atom of the cofactor thiamine diphosphate and glutamic acid residue 51 in pyruvate decarboxylase (EC 4.1.1.1). The yeast wild type gene *PDC1* and the respective mutant genes (E51Q and E51A) were expressed in *Escherichia coli*. The three enzymes were purified to homogeneity. They comigrated as a single band during silver-stained SDS/PAGE with a molecular mass of 60 000 Da. A molecular mass of 61 200 ± 200 Da was determined by mass spectrometry for the subunit. The native enzyme is a homotetramer as demonstrated by gel filtration experiments. Near- and far-UV CD spectra showed no significant differences for the apoenzyme of the wild type and the mutants. Slight differences in the rate of thiamine diphosphate binding to the apoprotein component were observed between the wild type and the E51Q PDC by CD spectroscopy. Compared to the wild type enzyme, thiamine diphosphate binding at the E51A mutant apoprotein is very slow. Only 0.04% of the catalytic activity of the wild type enzyme was observed for the E51Q mutant; the E51A mutant has no detectable catalytic activity. The $S_{0.5}$ value for the substrate pyruvate is increased 33-fold for the E51Q mutant. Substrate activation was observed for both the wild type and the E51Q mutant. The interaction between the N1′ atom of the coenzyme and glutamic acid 51 strongly influences the catalytic activity but only moderately the binding of the cofactor to the apoenzyme and the substrate activation rate.

Coenzymes exert their full catalytic activity exclusively after binding to a specific part of the enzyme protein. Therefore, it is crucial to find out how the reactivity of distinct groups of coenzymes is influenced via interactions with the amino acid residues at this binding site. Thiamine diphosphate (ThDP)[1] acts as a cofactor in a number of enzyme-catalyzed reactions, like the decarboxylation of α-keto acids, glycol transfer, and formation of α-ketols. For this reaction, the cofactor has to be activated by such specific interactions. Since the crystal structures of the ThDP-dependent enzymes pyruvate decarboxylase (PDC), transketolase, and pyruvate oxidase have been determined (Lindqvist et al., 1992; Dyda et al., 1993; Muller et al., 1993), the amino acid residues involved in the interaction with the cofactor are known. The cofactor ThDP is bound in the active site of the protein by polar interactions, including hydrogen bonds and hydrophobic interactions. In all three enzymes, a glutamate in the active site is a distance from the N1′ atom of the pyrimidine ring of ThDP which allows formation of a hydrogen bond. On the other hand, replacement of the N1′ atom of the cofactor by a carbon atom resulted in a loss of catalytic activity and a weaker binding of the cofactor to the protein (Golbik et al., 1991). From these results, we concluded that the interaction between the N1′ atom of the coenzyme and the glutamate is essential for catalysis. Here we investigated the influence of substitution of glutamate 51 by other amino acids on both catalysis and cofactor binding. For this, the yeast *PDC1* gene, one of the three known structural genes in *Saccharomyces cerevisiae* (*PDC1*, *PDC5*, and *PDC6*; Hohmann, 1991), as well as two mutants of *PDC1*, in which glutamate was replaced by glutamine and by alanine, were expressed in *Escherichia coli*.

MATERIALS AND METHODS

Strains, Plasmids, and Site-Directed Mutagenesis

Mutagenesis of E51 to Q Using PCR. PCR mutagenesis of E51 was performed according to Sarkar and Sammer (1990). In the first step, a 3.6 kb *Eco*RV/*Xho*I fragment of *PDC1* (Schaaff et al., 1989) was subcloned into the *Sma*I/*Xho*I-digested pUC19 (Yanisch-Perron et al., 1985), yielding pUC19:*PDC1*.

PCR was performed in a total volume of 100 μL, containing 100 ng of template DNA, 1 μg of each primer, 10 μL of Vent DNA polymerase buffer (provided with the enzyme), 0.25 mM dNTPs, and 2 units of Vent DNA polymerase (New England Biolabs). PCR cycles were as follows: one cycle at 95 °C for 5 min, and then polymerase was added, followed by 35 cycles at 92 °C for 1 min, 56 °C for 1 min, and 72 °C for 0.5 min. The reaction was completed by one cycle at 92 °C for 1 min, 56 °C for 1

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[1] Abbreviations: ADH, alcohol dehydrogenase (EC 1.1.1.1); BIS-TRIS, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NADH/NAD, nicotinamide adenine dinucleotide; PDC, pyruvate decarboxylase (EC 4.1.1.1); ThDP, thiamine diphosphate; TRICINE, *N*-tris(hydroxymethyl)methylglycine.

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min, and 72 °C for 20 min. The oligonucleotide 5′-CGTTCAATTGGTTGGCGTTAC-3′ was used as a PCR primer to change glutamic acid in position 51 to glutamine by mutating codon 51 from GAA to CAA. As a second primer in this first PCR, the oligonucleotide 5′-CAC-CGTTTTCGGTTTGCC-3′ (nucleotide positions 60−77) was used, resulting in a 0.1 kb megaprimer. In the second PCR, the megaprimer and primer 5′-CAAGATGGTGTCAAT-GACTTCCTT-3′ (nucleotide positions 592−617) were used, giving a 0.56 kb fragment. In this reaction, the primers were allowed to anneal for 2 min at 65 °C instead of 1 min at 56 °C. The PCR product was purified using the DNA gel extraction kit from Quiagen. After purification, the DNA fragment was digested with *Bgl*II and *Eco*RI and subcloned into the *Bgl*II/*Eco*RI-digested pUC19:*PDC1*. The mutation was confirmed by sequencing with the T7 sequencing kit (Pharmacia), using the oligonucleotide 5′-AAGGATATT-TCACTCTCC-3′ (nucleotide positions −118 to −135) as the sequencing primer.

Mutagenesis of E51 to A Using the Altered Sites in Vitro Mutagenesis System (Promega). Mutagenesis of E51 to A with the Altered Sites in vitro Mutagenesis System (Promega) was performed using the instructions of the manufacturer. A 3.4 kb *Sph*I/*Xho*I fragment of *PDC1* (Schaaff et al., 1988) was subcloned into the *Sph*I/*Sal*I-digested vector pALTER1. The oligonucleotide 5′-AGCGTTCAATGCGTTGGCGTT-3′ was used to exchange glutamic acid at position 51 to alanine by mutating codon 51 from GAA to GCA. After second-strand synthesis, ampicillin resistant colonies were checked by sequence analysis using the same sequencing primer as above.

Cloning of Mutagenized Alleles

Cloning of the mutant *PDC1* alleles into the *E. coli* expression vector was done in three steps. First, a 1.8 kb *Nco*I/*Xba*I fragment of the pET22b:*PDC1* vector (a gift from F. Jordan) was subcloned into the *Nco*I/*Xba*I-digested YIplac 211 (Gietz & Sugino, 1988), resulting in YIplac211:*PDC1*. Subsequently, a 0.8 kb *Eco*RI/*Bgl*II fragment from pUC19:*PDC1* containing either mutation was used to replace a 0.8 kb *Bgl*II/*Eco*RI fragment of YIplac 211:*PDC1*. Finally, a 1.8 kb *Nco*I/*Xba*I fragment fromYIplac211:*PDC1* was ligated into *Nco*I/*Xba*I-digested pET22b:*PDC1*. The *E. coli* BL21 (DE) strain was used for the expression of the recombinant PDC protein. PDC was induced as described by Barburina et al. (1994).

Expression and Enzyme Purification

BL-21 cells were pregrown overnight in LB medium with ampicillin (100 μg/mL) at 37 °C in 10 mL of preculture, used to incubate a 1 L shaking culture growing for 5 h at 30 °C in ampicillin/LB medium containing 0.5 mM ThDP. PDC expression was induced in late log phase by 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 10000*g* at 4 °C for 8 min (Centricon T-324, Kontron Instruments) and stored at −20 °C. Frozen cells were thawed at 4 °C and suspended in 2-fold volume of 0.1 M sodium phosphate (pH 6.1) containing 5 mM DTE, 1 mM ThDP, 1 mM $MgSO_4$, 1 mM EDTA, and 10% (v/v) glycerol. The cells were disrupted in a French press apparatus (SLM Instruments, Inc.) and centrifuged at 96000*g* at 4 °C for 40 min (L860M, Beckman Instruments). Ammonium sulfate was added to a concentration of 25% (w/v) under continuous stirring at 4 °C within 30 min. The solution was centrifuged at 96000*g* at 4 °C for 20 min. The precipitate was discarded, and 7% (w/v) ammonium sulfate was added to the supernatant. The solution was stirred for at least 30 min and then centrifuged at 96000*g* at 4 °C for 30 min, and the supernatant was discarded. The precipitate containing the crude enzyme could be stored at 4 °C for more than 3 months without considerable loss of activity. It was dissolved in 10 mL of 20 mM BISTRIS at pH 6.0 (containing 1 mM DTE, 0.2 mM ThDP, 1 mM $MgSO_4$, and 1 mM EDTA) and dialyzed against the same buffer overnight. The desalted enzyme solution was applied to a Fractogel EMD TMAE-650 M column (Merck; 1.0 × 10 cm, flow rate of 1 mL/min) equilibrated with 20 mM BISTRIS at pH 6.0 (containing 1 mM DTE and 0.1 mM ThDP). The protein was eluted with an ammonium sulfate gradient (100 mM, 100 mL). Subsequently, the PDC was purified by rechromatography using the same buffer and gradient at pH 6.6. Eluted PDC was applied on a Superdex 200 HR column (Pharmacia; 1.0 × 30 cm, flow rate of 0.5 mL/min) equilibrated with 50 mM sodium phosphate at pH 6.0. The protein solution was stored at −80 °C in elution buffer.

Enzyme Assay and Protein Determination

Enzymatic activity was measured in 0.1 M Mes/NaOH or 0.1 M sodium citrate at pH 6.1 at 340 nm and 30 °C (DU70, Beckman Instruments) with a NADH/ADH-coupled optical test (Holzer et al., 1956).

Substrate activation measurements were performed with a SX 18 MV stopped-flow spectrophotometer (Applied Photophysics), with one syringe containing the substrate pyruvate and the other PDC and the auxiliary enzyme system ADH/NADH (ADH at 0.066 mg/mL and NADH at 0.2 mg/mL) (Hübner et al., 1978).

The protein concentration was determined in the crude extract according to Bradford (1976) with bovine serum albumin as the standard in all other cases from the UV spectra at 280 nm using the molar extinction coefficient of PDC from brewer's yeast (281 000 M^{-1} cm^{-1}; Hübner et al., 1986).

Preparation of Apoenzyme

The apoenzyme was prepared according to Sieber et al. (1983). PDC (1 mg) was incubated in 0.5 mL of 0.1 M TRICINE/NaOH at pH 8.9 for 10 min at room temperature. The protein was precipitated with ammonium sulfate. After centrifugation, the pellet was resuspended in 0.2 mL of 0.02 M TRICINE/NaOH at pH 8.0. The protein component was separated from ThDP by gel filtration on a Superdex 200 column (Pharmacia; 1.0 × 30 cm) equilibrated with 0.05 M sodium phosphate at pH 6.0.

Polyacrylamide Gel Electrophoresis

SDS/PAGE (7% T) was carried out according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue G 250 or with silver according to Nesterenko et al. (1994). Calibration proteins for SDS gel electrophoresis (Boehringer Mannheim) were used as molecular mass markers. Western blot analysis was carried out as described by Mücke et al. (1995).

Table 1: Purification Procedure for Recombinant Wild Type PDC

	activity (u)	protein (mg)	specific activity (u/mg)	yield (%)
crude extract	2800	528	5.3	100
ammonium sulfate precipitate	2440	177	13.8	87
dialysis	2330	151	15.4	83
Fractogel TMAE (pH 6.0)	2080	65	32	74
Fractogel TMAE (pH 6.6)	1920	35	54	68
Superdex 200 HR	970	12.6	77	35

Circular Dichroism Measurements

CD spectra were obtained in a Jasco J710 spectropolarimeter at a path length of 0.1 cm and at 20 °C.

Mass Spectrometry

Homogeneous recombinant wild type PDC was desalted with 10 mM ammonium acetate at pH 6.3 in a microcon cell (exclusion volume of 10 000 Da). Enzyme solution (1 μL, 1.5 mg/mL) was mixed with 1 μL of 90 mM 3,5-dimethoxy-4-hydroxycinnamic acid solution. The molecular mass was detected with a REFLEX (Bruker-Franzen Analytik GmbH) time of flight (TOF) mass spectrometer with matrix-assisted laser desorption ionization (MALDI) using a nitrogen laser at 337 nm and an acceleration voltage of 30 kV. Bovine serum albumin (Merck) was used as the standard.

RESULTS

Expression and Purification of Yeast PDC. The yeast wild type *PDC1* gene was expressed in *E. coli* BL21; about 10% of the total cell protein was PDC. We found the expression product in the soluble fraction after cell disruption. The purification procedure started with 6 g of wet *E. coli* cells arising from 1 L of shaking culture. After five purification steps (Table 1) 10−13 mg of enzyme was obtained (yield of 35−45%). The maximum activity of the final purification product was 77 u/mg. As judged by silver-stained SDS/PAGE, the protein has been purified to homogeneity and migrated as a single band with a molecular mass of 60 000 Da. An approximate molecular mass of 61 200 ± 200 Da was determined by mass spectrometry (Figure 1). Size exclusion chromatography of the pure enzyme gave a distinct single peak with a molecular mass of about 200 000 Da (not shown). The N-terminal amino acid sequence of the pure enzyme was determined as Ser-Glu-Ile-Thr-Leu which fits with the sequence predicted from the known nucleotide sequence of *PDC1* (Hohmann & Cederberg, 1990) at these positions.

Site-Directed Mutagenesis. Derivatives of *PDC1* were constructed by site-directed mutagenesis in which glutamic acid 51 in the active site of the enzyme was replaced by glutamine (E51Q) and by alanine (E51A). Determinations of the protein levels showed that the mutant enzymes were expressed in *E. coli* in similar amounts as the recombinant wild type. The E51Q and E51A mutants were successfully purified according to the procedure described above.

Circular Dichroism Measurements. ThDP binding can be monitored by characteristic changes in the near-UV region using CD spectroscopy (Ullrich and Wollmer, 1971). The CD spectra of the apoprotein of the three enzymes were almost identical (Figure 2). Addition of ThDP resulted in subsequent occurrence of two distinct peaks between 260 and 290 nm. These changes are typical for ThDP binding in PDC (Ullrich & Wollmer, 1971). Both mutant enzymes showed differences in the spectra compared to those of the wild type. The minimum at 283 nm shifted to 291 nm. The

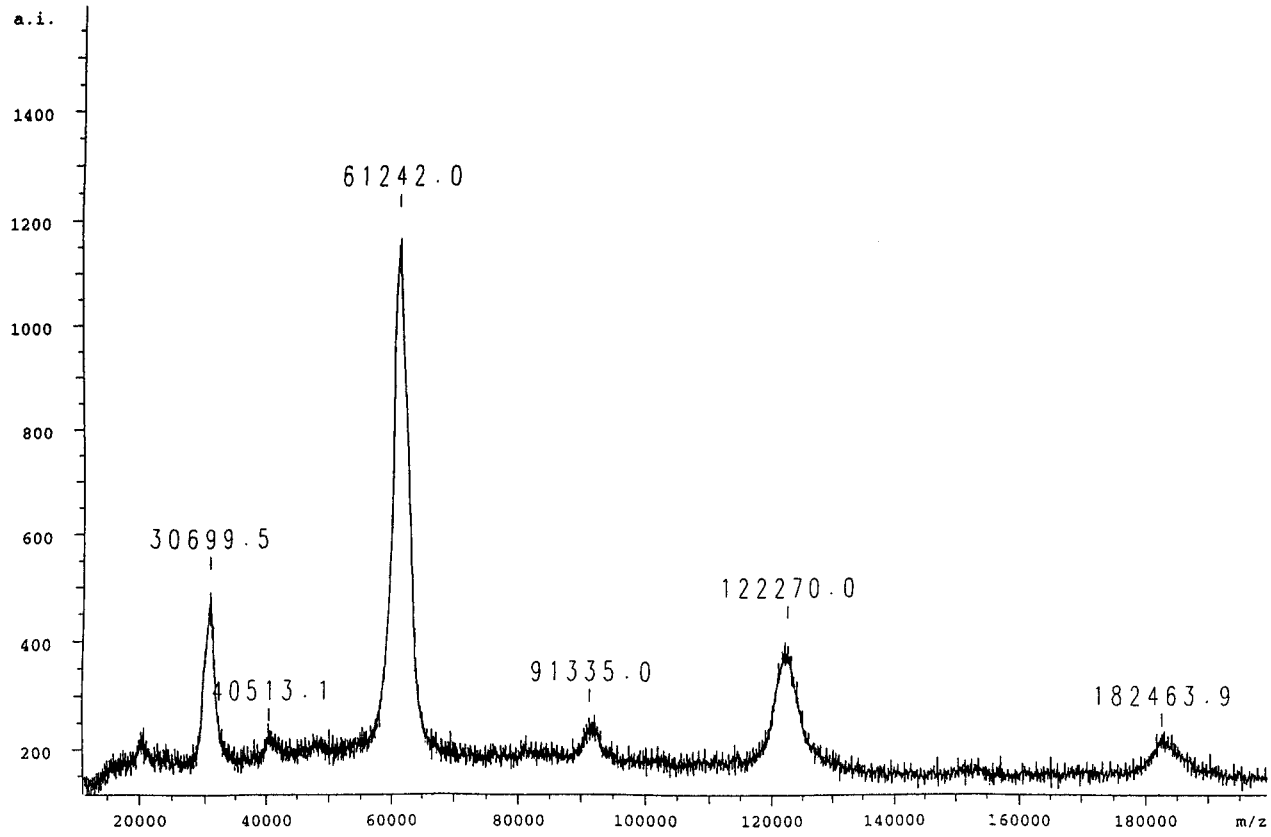

FIGURE 1: Mass spectrum of recombinant wild type PDC. [PDC] = 1.5 mg/mL.

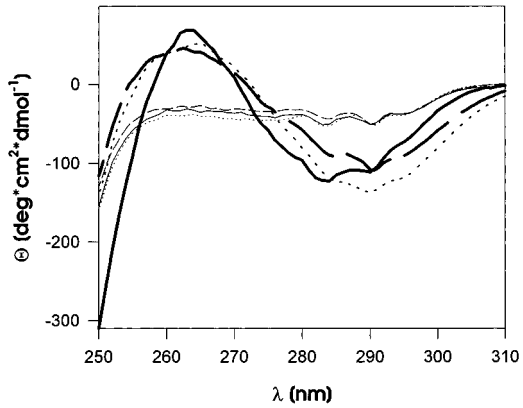

FIGURE 2: Near-UV CD spectra of recombinant yeast PDC. Spectra were recorded in 50 mM sodium phosphate at pH 6.0, with a path length of 0.1 cm. The subunit concentration was 160 μM in all cases: (—) wild type enzyme, (− − −) E51Q mutant, and (▪▪▪) E51A mutant (thin lines, apoprotein; thick lines, holoprotein in the presence of 0.33 mM ThDP).

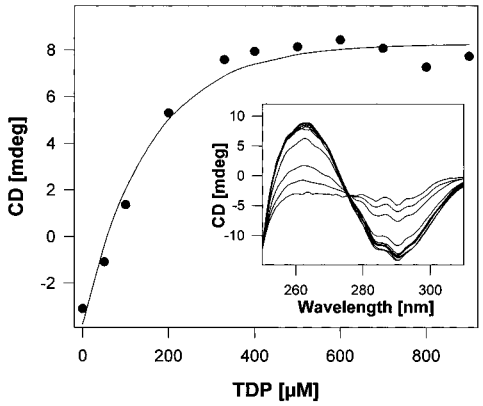

FIGURE 3: Dependence of the CD signal of the E51A mutant PDC at 265 nm on the concentration of ThDP. The subunit concentration was 136 μM in 50 mM sodium phosphate at pH 6.0, with $T = 30$ °C and a path length of 0.1 cm. (insert) Dependence of near-UV CD spectra on the ThDP concentration.

maximum at 263 nm showed a distinct widening of the CD signal in the case of the mutant enzymes. The signal for the holoenzyme of the E51A mutant could only be observed in the presence of additional ThDP. A dissociation constant for the coenzyme in the E51A mutant of 120 μM was calculated from the dependence of the CD signal on the concentration of the coenzyme ThDP (Figure 3). In the absence of the cofactor, the spectrum is similar to that of the apoenzyme. The cofactor could be separated by gel filtration chromatography at pH 6.0; hence, E51A was purified as apoenzyme. The CD spectra of both the wild type and the E51Q mutant showed the typical signals for the holoenzyme (Figure 2) also in the absence of additional ThDP, indicating a tight binding of the coenzyme in these enzymes with a dissociation constant below 1 nM. There were no differences in far-UV CD spectra for the three enzyme species (data not shown).

The time-dependent change of the CD signal at 265 nm after addition of ThDP to the apoprotein was used to determine the reconstitution rate of apoprotein and coenzyme. The time courses of the CD signals at 265 nm (Figure 4) were fitted to a second-order reaction to calculate the reconstitution rate constants for all enzyme species. Table 2 demonstrates that substitution of glutamate by glutamine resulted in a 2-fold decrease of this rate constant; the

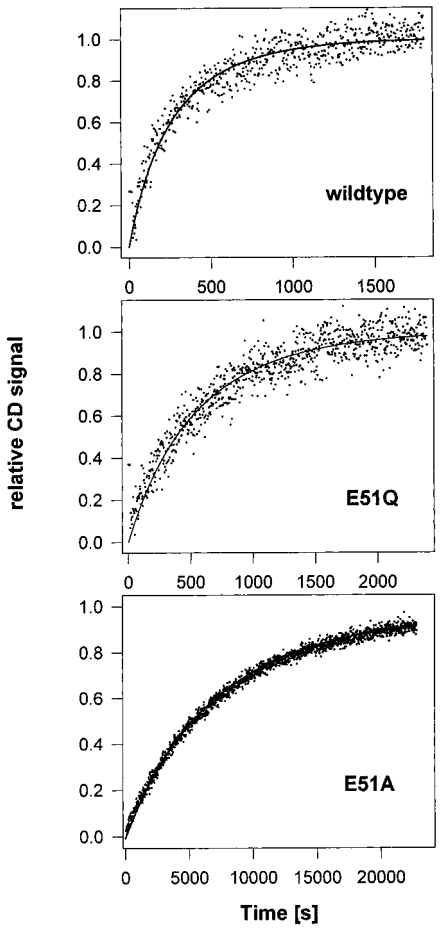

FIGURE 4: Dependence of the CD signal at 265 nm on the reconstitution time. Reconstitution of wild type apoprotein, E51Q mutant apoprotein, and E51A mutant apoprotein (protein concentration of 160 μM) with 330 μM ThDP in 50 mM sodium phosphate at pH 6.0 and 20 °C, with a path length of 0.1 cm. The data were fitted to a second-order reaction in the case of the wild type and the E51Q mutant enzyme. In the case of the E51A mutant enzyme, the data were fitted to a reversible reaction of reconstitution according to the equation $x = {}^1/_2\{[(B + \sqrt{C})De^{-\sqrt{C}k_1t} + \sqrt{C} - B]/(1 - De^{-\sqrt{C}k_1t})\} + mt$ with $B = b - a + K$, $C = B^2 + 4K$, and $D = (2a + B - \sqrt{C})/(2a + B + \sqrt{C})$, with the following parameters: a, initial protein concentration; b, initial TDP concentration; x, holoenzyme concentration at time t; K, dissociation constant; k_1, second order rate constant of the forward reaction. The data were fitted with a linear drift due to photochemical degradation and/or aggregation ($m = 1.6 \times 10^{-4}$ s^{-1}) because of the long time scale of the measurement.

Table 2: Kinetic Data for the Recombinant Wild Type and the Mutants E51Q and E51A

enzyme	specific activity (u/mg)	$S_{0.5}$ (mM pyurvate)	k_{ThDP}[b] (μM^{-1} s^{-1})	k_+[c] (s^{-1})
recombinant WT	77	1.65 ± 0.15	1.28×10^{-5}	0.53 ± 0.11
E51Q	0.03	54 ± 5	5.93×10^{-6}	0.86 ± 0.18
E51A	nd[a]	nd	3.09×10^{-7}	nd

[a] nd, not detectable. [b] Second-order rate constant of reconstitution of the apoprotein (136 μM) with ThDP (330 μM) measured by CD spectroscopy at 265 nm in 50 mM sodium phosphate at pH 6.0 and 20 °C. [c] Pseudo-first-order rate constant of enzyme activation by pyruvate according to Hübner et al. (1978) in sodium citrate at pH 6.0 at saturating concentrations of pyruvate at 30 °C.

reconstitution rate of the alanine mutant is reduced 40-fold.

Catalytic Properties. Strikingly, the replacement of glutamic acid 51 by glutamine caused a drastic decrease of

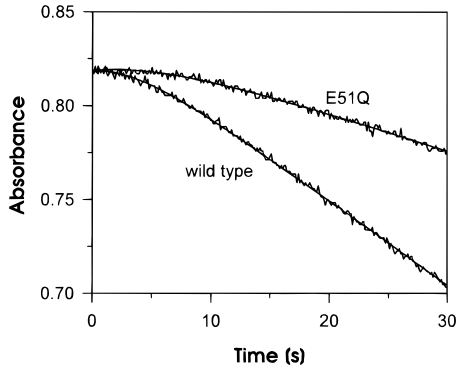

FIGURE 5: Progress curves of the catalytic activity of wild type and E51Q mutant of PDC [original data and fitted curves according to the equation absorbance $= A + Bt + C \exp(-kt)$]. Measurements were carried out in the optical test (Holzer et al., 1956) in 100 mM sodium citrate buffer at pH 6.1 and 30 °C and 33 mM pyruvate, with a subunit concentration of 24 nM wild type PDC and 16 μM E51Q mutant enzyme, respectively, and a path length of 1 cm.

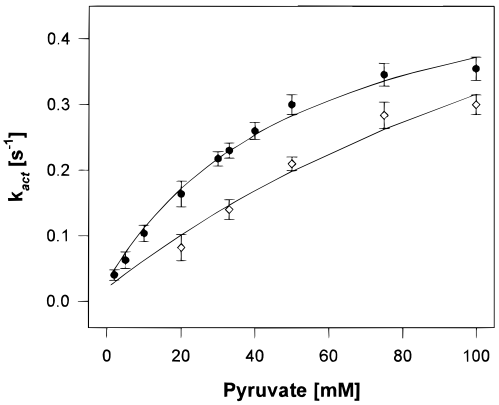

FIGURE 6: Dependence of the activation rate constant of the wild type PDC (●) and of the E51Q mutant PDC (◇) on the substrate concentration. Measurements were carried out in 100 mM sodium citrate buffer at pH 6.1; $T = 30$ °C. Solid lines are fits to the mechanism of activation with the following parameters for the wild type PDC and the E51Q mutant PDC, respectively: $K_a = 54$ mM, $k_+ = 0.53$ s^{-1}, and $k_- = 0.03$ s^{-1} and $K_a = 192$ mM, $k_+ = 0.86$ s^{-1}, and $k_- = 0.02$ s^{-1}.

catalytic activity to 0.04 ± 0.008% of that of the wild type enzyme. The E51A mutant was totally inactive (Table 2). Due to slow ThDP binding, the enzyme was preincubated with 5 mM ThDP for 4 h at 20 °C. However, no catalytic activity could be detected.

Steady state measurements indicated significant differences between both active enzymes (wild type and E51Q). The weaker hydrogen bond between Q51 and the N1′ atom of the ThDP resulted in a 33-fold-increased $S_{0.5}$ value (54 mM; Table 2). It was difficult to demonstrate substrate activation by measurements of steady state velocities because of the high $S_{0.5}$ value for pyruvate and the low catalytic activity. Therefore, we have investigated the time course of substrate activation by stopped-flow measurements. Both enzymes showed lag phases in product formation which is typical for substrate activation (Figure 5). From the dependence of the observed pseudo-first-order rate constant on the substrate concentration (Figure 6), which is similar to that of the native enzyme from brewer's yeast, a minimal mechanism was derived where the substrate binds to a regulatory site before the active form of the enzyme (SE_{act} in Scheme 1) is formed in a rate-limiting reaction. The rate equation derived from

$$E + S \underset{}{\overset{K_a}{\rightleftharpoons}} SE \underset{k_-}{\overset{k_+}{\rightleftharpoons}} SE_{act} \qquad \text{(Scheme 1)}$$

this mechanism provides $k_{act} = (k_+S)/(S + K_a) + k_-$ as the observed pseudo-first-order rate constant of activation (Table 2).

DISCUSSION

The described expression system allows production of high amounts of soluble PDC in the cytoplasm of the *E. coli* cells. The recombinant enzyme purified to homogeneity had a specific activity comparable to that of PDC purified from brewer's yeast (Sieber et al., 1983). However, in contrast to the yeast enzyme with an $\alpha_2\beta_2$ structure (with molecular masses of 59 000 and 61 000 Da for the subunits α and β, respectively; Sieber et al., 1983), the recombinant enzyme gave only a single band after the SDS/PAGE with a molecular mass of 60 000 Da. This value and that determined by mass spectrometry (61 500 ± 200 Da) correspond to the molecular mass calculated from the nucleotide sequence of the *PDC1* gene (61 480 Da). Determination of the molecular mass by small-angle X-ray scattering (König et al., 1995) and size exclusion chromatography (not shown) indicated mainly tetramers at pH 6 for the recombinant yeast PDC. The same homotetrameric state was described earlier for the haploid yeast PDC (Ullrich et al., 1991; Killenberg-Jabs et al., 1996). In contrast to that for all yeast PDC isoforms, the N terminus of the recombinant enzyme was not blocked. This might be due to different post-translational processing in the native host yeast, which is a eukaryote, and the prokaryotic expression host. The sequence of the first five amino acids of the N terminus confirmed the nucleotide sequence of the *PDC1*.

The crystal structure of PDC shows that the carboxyl group of the E51 might interact with the N1′ atom of the coenzyme ThDP (Arjunan et al., 1996). Experiments using structural analogues of the ThDP indicated the important role of the interaction of the N1′ atom of the ThDP and the protein component (Golbik et al., 1991). Although crystal structures of the mutant PDC enzymes have not been available, the CD spectra indicating ThDP binding (Ullrich & Wollmer, 1971) showed only small differences between the mutants and the wild type enzyme. Similar small differences in the CD spectra were observed in the ThDP-dependent enzyme transketolase and its mutants. For this enzyme, crystallographic analysis of the mutants shows that mutation of the invariant glutamate does not induce structural changes that may result in differences of the cofactor binding (Wikner et al., 1994). From these results, we concluded that binding and orientation of the coenzyme ThDP in the wild type PDC and the mutant PDCs should also be similar. Structural changes can be ruled out as a reason for both the drastically reduced catalytic activity of the E51Q mutant and the inactivity of the E51A mutant (Table 2). This indicates the importance of the interaction between the N1′ atom of the coenzyme and the glutamic acid in the catalytic mechanism of PDC.

However, there are differences in the reconstitution rate of the coenzyme with the apoenzyme between the wild type enzyme and the mutant enzymes determined by CD spectroscopy (Table 2) and in the stability of the coenzyme binding in the holoenzyme (E51A). Although in the E51Q

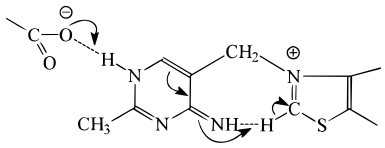

FIGURE 7: Possible mechanism of the deprotonation of C2 of the enzyme-bound ThDP.

mutant the coenzyme is as tightly bound as in the wild type enzyme, there are small differences in the reconstitution rate. This is not surprising for a substitution of one of the polar groups contributing to the cofactor binding by another one. In contrast to that for the E51Q mutant, the substitution of glutamate 51 by the nonpolar alanine resulted in a drastically decreased stability of the cofactor binding. In this case, the loss of binding interaction goes in parallel with the loss of catalytic activity. The specific interaction starting from the glutamate to the N1′ atom of the coenzyme dramatically influences the catalytic power of PDC. This result and the information from the crystal structure of PDC, showing the N4′ atom and the C2 atom of the coenzyme a distance excluding the probability of having two hydrogen atoms at N4′ and one at C2 simultaneously (Arjunan et al., 1996), support a mechanism for catalysis in which the initial step, the deprotonation of C2 of the enzyme-bound ThDP, is influenced by the N1′−glutamate interaction (Figure 7). In this model, the deprotonation starts from the iminotautomeric form stabilized by the interaction of the coenzyme with the protein component. This iminotautomeric form could be deprotonated by the conserved glutamate residue. Finally, the imino group acts as a general base catalyst in the deprotonation of the C2 atom.

The substitution of glutamic acid residue by glutamine in the α domain, which is involved in ThDP binding together with the γ domain, leads to an enzyme showing the phenomenon of substrate activation like the wild type. This confirms the hypothesis of Baburina et al. (1994) that substrate activation originates from the binding of a substrate molecule in the regulatory site located in the β domain (not involved in the cofactor binding).

Although H/D exchange experiments of the proton bound to C2 of ThDP in PDC and mutants have shown that substrate activation triggers the fast dissociation of this proton, yielding the reactive C2-ylid intermediate (Kern et al., 1997), the structural changes responsible for this process are still unknown. Therefore, further investigations are necessary to interpret the slight differences in the rates of substrate activation of the wild type enzyme and the E51Q mutant.

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