Analysis of an Invariant Cofactor-Protein Interaction in Thiamin Diphosphate-dependent Enzymes by Site-directed Mutagenesis

GLUTAMIC ACID 418 IN TRANSKETOLASE IS ESSENTIAL FOR CATALYSIS*

(Received for publication, August 16, 1994, and in revised form, October 20, 1994)

Christer Wikner, Ludmila Meshalkina‡, Ulrika Nilsson, Matti Nikkola, Ylva Lindqvist, Michael Sundström§, and Gunter Schneider¶

From the Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Center, Box 590, S-751 24 Uppsala, Sweden

A homologous expression system and a purification protocol for pure, highly active recombinant yeast transketolase have been developed. The invariant transketolase residue Glu418, which forms a hydrogen bond to the N-1' nitrogen atom of the pyrimidine ring of the cofactor thiamine diphosphate has been replaced by glutamine and alanine. Crystallographic analyses of the mutants show that these amino acid substitutions do not induce structural changes beyond the site of mutation. In both cases, the cofactor binds in a manner identical to the wild-type enzyme. Significant differences in the CD spectra of the mutant transketolases are observed with the exception of wild-type enzyme indicate differences in the electron distribution of the aminopyrimidine ring of the cofactor. The E418Q mutant shows 2% and the E418A mutant shows about 0.1% of the catalytic activity of wild-type enzyme. The affinities of the mutant enzymes for thiamine diphosphate are comparable with wild-type transketolase. The hydrogen bond between the coenzyme and the side chain of Glu418 is thus not required for coenzyme binding but essential for catalytic activity. The results demonstrate the functional importance of this interaction and support the molecular model for cofactor deprotonation, the first step in enzymatic thiamin catalysis.

Thiamin diphosphate (ThDP) is the cofactor in a number of enzymatic reactions, where a carbon-carbon bond adjacent to a carbonyl group is cleaved. Irrespective of the particular reaction catalyzed, enzymatic thiamin catalysis proceeds through two common intermediates. One of these intermediates is the ylide form of ThDP, resulting from the deprotonation of the C-2 atom of the thiazolium ring in the initial step of the catalytic cycle. The second common intermediate is an α-carbanion, which is formed after the nucleophilic attack of the ylide on the substrate (for a review see Kluger (1987)).

ThDP-dependent transketolase catalyzes the interconversion of monosaccharides by transferring glycoaldehyde groups from ketones to aldehydes (for a review see Kochetov (1982)) (Lindqvist and Schneider, 1993). The crystallographic analyses of transketolase in complex with the coenzyme ThDP or the coenzyme analogue thiamin thiazolene diphosphate, respectively, have provided detailed information on the mode of coenzyme binding and the interactions of ThDP with the enzyme (Lindqvist et al., 1992, Nilsson et al., 1993, Nikkola et al., 1994). In the crystal structure of transketolase, there is no enzymic base in a position suitable for proton abstraction at the C-2 atom of the thiazolium ring of ThDP. Instead, a molecular mechanism for proton abstraction has been proposed that suggests a cofactor-assisted deprotonation (Lindqvist et al., 1992, Schneider and Lindqvist, 1993). Essential to this model is the observed hydrogen bond between the side chain of the invariant residue Glu418 and the N-1' nitrogen atom of ThDP (Fig. 1). This interaction has also been found in other ThDP-dependent enzymes (Müller and Schulz, 1993; Dyda et al., 1993), and it has been proposed that the mechanism for generating the C-2 carbanion, the first step in enzymatic thiamin catalysis, is common to all ThDP-dependent enzymes (Müller et al., 1993, Schneider and Lindqvist, 1993).

Chemical modification of ThDP through replacement of the N1' nitrogen by a carbon atom has provided evidence that the N-1' nitrogen atom of the pyrimidine ring is indeed required for catalytic activity (Schellenberger, 1967; Golbik et al., 1991). Subsequently it was shown that thebinding mode of this cofactor analogue to transketolase is identical to the binding of ThDP ( König et al., 1994), and structural differences in cofactor binding can therefore be ruled out as being responsible for the loss of catalytic function. Replacement of the N-3' nitrogen atom of the pyrimidine ring results in a cofactor analogue that is catalytically active.

In this paper, we describe an efficient expression system for yeast transketolase with high yields of pure and highly active recombinant enzyme. We also report on the results of a mutagenesis study to probe the interaction between ThDP and the invariant residue Glu418 in transketolase and discuss general implications of these results for the enzymatic reaction mechanism of thiamin catalysis.

EXPERIMENTAL PROCEDURES

Materials—Commonly used chemicals and reagents were of the highest purity readily available. Glyceraldehyde-3-phosphate dehydrogenase was purified from rabbit muscle as described by Eldén and Skörengi (1956). Glycerol-3-phosphate dehydrogenase and triosephosphate isomerase from rabbit muscle were obtained from Boehringer. Restriction enzymes were purchased from Promega; T4 DNA ligase, T4 polynucleotide kinase rATP, and the T7 sequencing kit were from Pharmacia; Redview [32P]ATP was from Amersham; and Sequagel RAPID.
Site-directed Mutagenesis of Transketolase

was from National Diagnostics. Oligonucleotides used for mutagenesis and DNA sequencing were provided by Dr. Steve Guttridge (Central Research and Development, Du Pont).

Yeast and Bacterial Strains and Plasmids—The yeast-<em>E. coli</em> shuttle vector pTKL1, used for both site-directed mutagenesis and expression of transketolase, was derived from the genomic library made in the laboratory of T. Kihara (Shibata et al., 1988; Hendrickson et al., 1993). pTKL1 consists of a 2664-bp fragment inserted into the BamHI site of pBR322. This fragment contains the 2040-bp-long TKL1, flanked by an upstream sequence of 472 bp and a sequence of 152 bp downstream. <em>E. coli</em> strain BMH 71-18 mutS was used in the mutagenesis step. All other manipulations of pTKL1 in <em>E. coli</em> were done using the strain JM109 (Yanisch-Perron et al., 1985). The yeast strain H402, with a disrupted TKL1, is a tklZ::HIS3 derivative of W303-1A (Sundstrom et al., 1989; Thomas and Rothstein, 1989). H402 was transformed with pTKL1 using the method described by Soni et al. (1993), selecting for URA3 in pTKL1.

Site-directed Mutagenesis—Unless otherwise indicated, standard molecular biology procedures (Maniatis et al., 1982; Sambrook et al., 1989) were used. Single- and double-stranded plasmid DNA was prepared with the Wizard kits (Promega). Mutagenesis was performed using the reagents in the Altered Sites kit (Promega) using single- and double-stranded plasmid DNA prepared with 20 mM Tris. Transketolase was eluted at a flow rate of 100 ml/min with a 0.0-75 mM linear gradient of NaCl in 20 mM Tris buffer. Transketolase was detected in two major peaks. Purified homogeneous transketolase was found in the first peak at about 22-33 mM NaCl. The second peak of transketolase appeared immediately after the first but was not homogeneous and was therefore discarded. Transketolase was precipitated by addition of solid ammonium sulfate to 70% saturation and centrifuged by centrifugation at 25,000 x g for 30 min. The pellet was dissolved in 25 mM glycy1-glycerine (pH 7.6) to a final concentration of transketolase of approximately 20 mg/ml. The enzyme can be stored as ammonium sulfate precipitate at 4°C without loss of enzymic activity. For long term storage, samples of transketolase were frozen in liquid nitrogen and stored at -80°C.

**Gel Electrophoresis and Western Blots**—Polyacrylamide gel electrophoresis was run under denaturing conditions in the presence of sodium dodecyl sulfate in 10% (w/v) slab gels in the Tris-glycine system (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R. PhastTransfer (Pharmacia) and Protoblot IIAP System (Promega) were used to transfer proteins to PVDF PLUS membranes according to the manufacturer's recommendations.

**Antiserum against Transketolase**—Polyclonal antiserum against transketolase was obtained from rabbits immunized and boosted with wild-type transketolase. A partial purification of the antiserum was performed using a DEAE-Sepharose CL-6B ion exchange column. A sample of antibodies against transketolase was also provided by Dr. N. Tkhamirova (Moscow).

**Activity Measurements and Protein Determination**—The specific activity of transketolase was measured spectrophotometrically in two different ways (de la Haba et al., 1986; Racker, 1961; Kochetov, 1983). In both assays, 1 unit is defined as the formation of 1 umol of glyceraldehyde-3-phosphate per minute. In one assay, the reaction is followed by the rate of NAD+ reduction in a coupled system with glyceraldehyde-3-phosphate dehydrogenase (Racker, 1961). In the second assay, activity is measured by the rate of NADH oxidation in a coupled system with triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (de la Haba et al., 1955). The first method has been used for all of the kinetic experiments, and the second assay was used for activity measurements in the crude extract and during the purification procedure.

The dependence of transketolase activity on pH has been determined in the pH interval 6.6-8.6 using MOPS buffer in the pH interval 6.6-7.6 and glycy1-glycerine buffer in the pH interval 7.6-8.6. The rate of the reaction was followed spectrophotometrically (Racker, 1961) and by formation of sedoheptulose 7-phosphate. The concentration of sedoheptulose 7-phosphate was determined colorimetrically (Villafranca et al., 1980). The enzyme was also used in a coupled assay with fructose-1,6-bisphosphate and aldehyde dehydrogenase as acceptor substrate. Before the start of the reaction by the addition of substrates, transketolase was incubated with ThDP and metal ions for 15 min at 25°C. All measurements were performed at 25°C in triplicate. The data were analyzed by nonlinear regression analysis resulting in values of K<sub>M</sub> and α<sub>MAX</sub> and standard errors with the program ULTRAFIT (Biosoft).

**Determination of Kinetic Parameters**—For the recombinant enzyme and the mutant E418Q, kinetic parameters were determined from measurements of initial rates at different ThDP or substrate concentrations with xylulose 5-phosphate as donor and D-ribose 5-phosphate as acceptor substrate. Before the start of the reaction by the addition of substrates, transketolase was incubated with ThDP and metal ions for 15 min at 25°C. All measurements were performed at 25°C in triplicate. The data were analyzed by nonlinear regression analysis resulting in values of K<sub>M</sub> and α<sub>MAX</sub> and standard errors with the program ULTRAFIT (Biosoft).

**Circular Dichroism**—Near-UV CD spectra were measured with a Jasco J-710 spectropolarimeter using a path length of 1.0 cm at 25°C.

**Crystallography**—Mutant transketolase was crystallized by vapor diffusion under conditions similar to those used for holotransketolase (Schneider et al., 1989). 7.5 µl of a 12 mg/ml protein solution were mixed with the same amount of mother liquid, 15-17% (w/v) of polyethylene glycol.
**TABLE I**

| Resolution (Å) | E418Q | E418A |
|----------------|-------|-------|
| No. of unique reflections measured | 44,920 | 20,670 |
| Completeness of x-ray data (%) | 70% | 65% |
| Model R-factor | 16.6% | 14.8% |
| No. of solvent molecules | 971 | 0 |
| Overall temperature factor (Å²) | 18.9 | 16.0 |
| r.m.s. bond deviations (Å) | 0.017 | 0.020 |
| r.m.s. angle deviations (%) | 3.3 | 3.7 |

* R-factor: $R_{c,ref} = \frac{\sum |I| - |<I>|}{\sum |I|}$, where values of $I$ are the intensity measurements for a reflection and $<I>$ is the mean value for this reflection.

The R-factor for the E418Q mutant is calculated on all data in the resolution interval 6.5–2.3 Å and for the E418A mutant in the interval 6.5–2.9 Å.

---

**RESULTS**

**Gene Disruption and Background Activity in the Expression Strain**—The yeast strain H402, which has a disrupted TKL1 gene, cannot grow on the culture medium used in this work (Sundström et al., 1993). As a control of the TKL1 gene disruption experiments, a 2467-bp SacI fragment, containing the promoter region and the complete transketolase gene (with the exception of the last 60 bp) was removed from pTKL1. The plasmid was religated, yielding pTKΔ. After transformation of the TKL1-disrupted yeast strain H402 with the plasmid pTKΔ, it is possible to analyze strain H402 under identical expression conditions as when producing recombinant enzyme. In cell extracts of H402 and H402pTKΔ, no trace of transketolase activity was found. SDS-PAGE gels of the crude extract revealed that transketolase is absent (Fig. 2). Western blots of crude extract verified the absence of a protein able to cross-react with polyclonal antibodies raised against wild-type transketolase (data not shown).

**Expression and Purification of Recombinant Transketolase**—Yeast strain H402pTKL1 expresses recombinant transketolase at high levels, about 15% of total cell protein. A summary of the purification procedure starting from 50 g of wet yeast cells is shown in Table II. The specific activity of purified transketolase was about 37 units/mg at 25 °C. The purification protocol results in about 60–80 mg of pure transketolase from 5 liters of cell culture. SDS-PAGE electrophoresis (Fig. 2) shows that purified recombinant transketolase was homogenous and electrophoretically indistinguishable from wild-type enzyme.

**Site-directed Mutagenesis and Properties of Mutant Enzyme**—The invariant residue Glu418 was replaced by glutamine and alanine, respectively. As judged from polyclonal antibodies raised against wild-type transketolase, the levels of mutant transketolase expressed in H402pTKL1 cells are similar to the levels of wild-type transketolase. Both mutants, E418A and E418Q could be readily purified according to the protocol for the wild-type recombinant enzyme with similar final yields of pure protein (Fig. 2).

The E418Q mutant retained only 2% of the wild-type catalytic activity (Table III), but the apparent $K_m$ for ThDP (0.86 µM) is similar to the $K_m$ for wild-type enzyme (0.47 µM). The $K_m$ value for the donor substrate xylulose 5-phosphate is about twice the value for the wild-type enzyme, whereas the $K_m$ value for the acceptor substrate ribose 5-phosphate is, within the error limits of the experiment, identical to that of wild-type transketolase. The E418Q mutant enzyme has the same pH optimum (pH 7.6–8.2) as wild-type transketolase.

Substitution of glutamic acid 418 with alanine has a more profound effect on catalytic activity. This mutant shows a 1000-fold decrease in activity (Table III). The affinity of the mutant for the cofactor ThDP is, however, essentially unchanged as can be seen from the similarities in binding constants (Table III). The residual catalytic activity remains constant in the pH range from 7.6 to 8.6, and there is no decrease in activity at high pH, as observed in wild type and the E418Q mutant. Because of the very low activity of this mutant, $K_m$ values for substrates could not be determined.

**Circular Dichroism Measurements**—Binding of ThDP to transketolase can be monitored by characteristic changes in the near-UV region of the CD spectrum. Addition of ThDP to the enzyme leads to an increase of the maximum at 280 nm and the appearance of a negative broad band with a minimum at 320 nm (Kochetov and Usmanov, 1970). The CD spectra of the recombinant enzyme and the E418A and E418Q mutants in the absence of ThDP are very similar to the spectrum of apo-transketolase. Upon addition of ThDP, recombinant transketolase (Fig. 3) shows the same spectral change as nonrecombinant enzyme. The CD spectra of the mutants in the presence of ThDP are qualitatively similar to each other but are very different from the spectrum of wild-type transketolase. In the mutant spectra, the maxima at 280 nm and 320 nm are shifted toward shorter wavelengths at 265 and 295 nm, respectively (Fig. 3).
Site-directed Mutagenesis of Transketolase

### Table II
**Summary of the purification protocol**

| Step               | Volume | Protein | Total activity | Specific activity | Yield  |
|--------------------|--------|---------|----------------|------------------|--------|
| Crude extract      | ml     | mg      | units          | units/mg         | %      |
| Ammonium sulfate   |        |         |                |                  |        |
| Precipitate 70% HR |        |         |                |                  |        |
| Mono Q HR 10/10    |        |         |                |                  |        |

*One unit is defined as 1.0 pmol of glyceraldehyde 3-phosphate produced per min in a coupled assay with ribose 5-phosphate and xylulose 5-phosphate as substrates.*

### Table III
**Kinetic parameters for wild-type and mutant transketolase**

| Enzyme | Activity | pH optimum | K<sub>v</sub> THDP | K<sub>a</sub> X-5-P | K<sub>a</sub> R-5-P |
|--------|----------|------------|---------------------|---------------------|---------------------|
| Wild type | 100 | 7.6-8.2 | 0.5 ± 0.1 | 162 ± 15 | 146 ± 21 |
| E418Q | 2 | 7.6-8.2 | 0.9 ± 0.1 | 354 ± 47 | 131 ± 13 |
| E418A | 0.1 | 7.2-8.6 | 0.9 ± 0.2 | ND | ND |

*K<sub>a</sub> values were determined for xylulose 5-phosphate as donor and ribose 5-phosphate as acceptor substrate.

*Mean value of five independent measurements.*

*ND, not determined because of low catalytic activity.*

---

**FIG. 3.** Near-UV CD spectra of recombinant wild-type and mutant transketolase. All spectra were recorded in 50 mM glycyl-glycine buffer, pH 7.6, containing 2.5 mM CaCl<sub>2</sub>. Enzyme concentration in all cases was 4.6 μM. ---, apotransketolase; thick line, holotransketolase, formed after addition of 30 μM ThDP; ..., E418Q transketolase in the presence of 30 μM ThDP; thin line, E418A transketolase in the presence of 30 μM ThDP.

Crystal Structures of the E418Q and E418A Mutant Transketolases—The crystal structures of the E418Q and E418A mutants have been determined and refined to 2.3- and 2.9-Å resolution, respectively. The overall structures of the two mutants are very similar to the crystal structure of the holoenzyme. Superposition of the structure of a subunit of E418Q with holoenzyme gives a r.m.s. deviation of 0.25 Å for 678 C-α atoms; a similar superposition of the E418A mutant with holotransketolase results in a r.m.s. deviation of 0.44 Å. The superposition also shows that no significant local structural changes are found in the two mutants when compared with the structure of wild-type transketolase (Fig. 4). Similarly, the positions of the Ca<sup>2+</sup> ion and ThDP are, within the error limits of the electron density maps, identical.

Fig. 5 shows a view of the electron density maps at the site of mutation. In the E418Q mutant, the observed electron density at position 418 is identical to the one found in wild-type transketolase. Indeed, at 2.3-Å resolution, a crystallographic distinction between glutamate and glutamine side chains is not possible. In the refined model of the E418Q mutant, the glutamine side chain forms a hydrogen bond to the N-1’ atom of the pyrimidine ring. Other interactions of the cofactor with the enzyme are unchanged upon amino acid substitution.

### DISCUSSION

Transketolase catalyzes two reactions of the nonreductive pentose phosphate pathway and is found in all organisms. To avoid misinterpretations of mutagenesis studies caused by co-purification of endogenous enzyme, we have produced the yeast strain H402 where the TKL1 gene has been disrupted (Sundström et al., 1993). The present study provides evidence by activity measurements and gel electrophoresis that cell extracts from this TKL1-disrupted yeast strain indeed do not contain any transketolase. Using similar gene disruption experiments with the TKL1 gene, Zimmermann and co-workers (Schaff-Gerstenschläger et al., 1993) also reported a yeast strain without endogenous transketolase.

Transketolase from *Saccharomyces cerevisiae* has been purified to homogeneity earlier, however, with low yields (de la Haba et al., 1955; Saitou et al., 1974). The expression system described here produces considerable amounts of recombinant enzyme from modest amounts of yeast cells. The recombinant enzyme is highly active, with a specific activity between 35 and 40 units/mg. This value is higher than for most transketolase preparations described earlier, which had specific activities...
Site-directed Mutagenesis of Transketolase

![Graph](image)

Fig. 4. Distance between corresponding C-α atoms of mutant and wild-type transketolase after superposition of the atomic coordinates of (a) E418Q and (b) E418A with the coordinates of wild-type transketolase using the graphics program O (Jones et al., 1991).

ranging from 12 to 20 units/mg. Tikhomirova et al. (1991) described a purification protocol using affinity chromatography resulting in a transketolase with specific activities between 12 and 60 units/mg dependent on the yeast source, with activities typically around 30 units/mg.

High resolution crystallographic studies of recombinant enzyme (Konig et al., 1994) did not detect any structural differences between wild-type and recombinant transketolase. The binding constant of the cofactor ThDP to recombinant enzyme is, within the error limit of the experiment, identical to the value found using wild-type enzyme (Heinrich et al., 1972b). CD spectra of recombinant apo- and holotransketolase are identical to the corresponding spectra obtained from wild-type enzyme. We conclude from these data that recombinant yeast transketolase is structurally and functionally indistinguishable from the endogenous enzyme.

Substitution of the invariant residue Glu^{418} with a glutamine or an alanine residue has little effect on the affinity for substrate and, surprisingly, ThDP (Table III). From the three-dimensional structure, it is obvious that the side chain of Glu^{418} is shielded from the substrate by the cofactor ThDP and cannot form any direct contacts to the substrate. As anticipated from the three-dimensional structure, mutations at position 418 do indeed have little effect on the affinities of the enzyme for the substrates. The side chain of Glu^{418} forms, however, a hydrogen bond to the N-1’ nitrogen atom of the cofactor, an interaction found in all ThDP-dependent enzymes (Muller et al., 1993). Nevertheless, removal of this hydrogen bond by either chemical modification of the cofactor (Golik et al., 1991; König et al., 1994) or site-directed mutagenesis of the enzyme has little effect on the binding constants of ThDP or its analogues and does not contribute to coenzyme binding in a significant manner.

Removal of this hydrogen bond results, however, in a drastic drop in catalytic activity. Structural effects caused by the amino acid substitution can be excluded as revealed by the crystallographic analysis of the mutants. The results of the mutagenesis study therefore must reflect functional deficiencies in the mutant enzymes, and we conclude that the interaction of the side chain of E418 with the cofactor is not required for cofactor binding but for catalytic activity.

The appearance of the strong absorbance band in the CD spectrum upon binding of the coenzyme has been interpreted as being caused by the formation of a charge-transfer complex between ThDP and an aromatic residue at the coenzyme binding site of the enzyme (Kochetov and Usmanov, 1970). The crystal structure analysis of holotransketolase (Lindqvist et al., 1992; Nikkola et al., 1994) shows that the pyrimidine ring of the cofactor binds in a hydrophobic pocket and interacts tightly with side chains of conserved aromatic residues, in particular Phe^{445} and Tyr^{446}, which could give rise to the formation of a charge-transfer complex. If this interpretation of the near-UV CD spectra is correct, then they will provide a sensitive probe to analyze the microenvironment of the pyrimidine ring, since the charge-transfer band will depend strongly on the electron distribution in the pyrimidine ring.

The replacement of Glu^{418} by alanine or glutamine shifts the absorption maximum in the near-UV region of the CD spectrum by 25 nm, indicating a change in the distribution of the π-electron system of the pyrimidine ring. The spectral changes are largest in the E418A mutant, where the hydrogen bond to the N-1’ nitrogen atom has been removed completely, and least in the E418Q mutant, where this hydrogen bond, albeit weaker, is still present.

Based on the crystal structure of transketolase, we have suggested a scheme for cofactor-assisted deprotonation of the C-2 atom of the thiazolium ring, the first step of enzymatic thiamin catalysis (Fig. 6), and the chemical arguments behind this model have been discussed at length elsewhere (Lindqvist et al., 1992; Schneider and Lindqvist, 1993). In this model, the base abstracting the proton from the C-2 carbon atom is the 4-imino group of the pyrimidine ring. The hydrogen bond between the N-1’ nitrogen atom and the side chain of Glu^{418} is...
Site-directed Mutagenesis of Transketolase

essential for the conversion of the 4-amino group to the 4-imino group during the catalytic cycle. It is known from experiments with model compounds that protonation of the N-1' nitrogen in pyrimidine derivatives will shift the pKₐ of the 4-amino group (Jordan et al., 1982). Protonation of the N-1' atom will also perturb the distribution of π-electrons in the ring by promoting resonance form II (see Fig. 6). This change in electron distribution will certainly influence the charge-transfer complex of the cofactor with the aromatic side chain(s) in the hydrophobic pocket at the coenzyme binding site and might be the cause for the observed changes in the CD spectrum of the mutants. In this context, it is interesting to note that mutations in transketolase at positions not directly interfering with the interactions of the aminopyrimidine ring and the protein have CD spectra very similar to wild-type enzyme.²

The 2% residual activity observed in the E418Q mutant fits rather well into this model since, as revealed by the crystal structure analysis, this side chain still forms the hydrogen bond to the N-1' nitrogen atom. However, for chemical reasons this hydrogen bond is expected to be weaker than the corresponding interaction in the wild-type enzyme, possibly resulting in a smaller pKₐ shift for the 4-amino group and, therefore, lower activity. The low activity of the E418A mutant is then readily explained by the complete absence of this interaction in this mutant. One might have anticipated that the activity in the E418A mutant caused by the complete absence of this interaction should be even lower than 0.1% of wild-type activity. The crystal structure of this mutant offers a possible explanation. Despite the moderate resolution of the electron density map, clear indications of a water molecule close to the N-1' atom were found. It is conceivable that this solvent molecule can act to a certain extent as a hydrogen bond donor and thus be responsible for the residual activity of this mutant.

In conclusion, the functional significance of the invariant cofactor-enzyme interaction between a glutamate side chain and the N-1' nitrogen atom of the cofactor has been analyzed by chemical replacement of the nitrogen atom by a carbon atom (Golbik et al., 1991; König et al., 1994) and site-directed mutagenesis reported here. The results from both studies show that this interaction is of functional importance and support the general model for cofactor-assisted deprotonation as the first step in enzymatic thiamine catalysis.

² C. Wikner, S. Bäckström, T. Kostikowa, L. Meshalkina, Y. Lindqvist, and G. Schneider, unpublished results.

Fig. 5. Stereo views of the final 2 |Fᵣ| - |Fᵣ'\rangle electron density maps of the E418Q (a) and E418A (b) mutants at the site of mutation. The refined protein model of the mutant is superimposed. The contour level of the electron density is at the standard deviation of the electron density map. Hydrogen bonds are indicated by dashed lines. The position of the putative solvent molecule close to the N-1' atom of the coenzyme in the E418A mutant is indicated by a sphere.
Acknowledgments—We thank Dr. Steve Gutteridge, Central Research & Development, DuPont for the syntheses of the oligonucleotides and Dr. N. Tikhomirova for antibodies against transketolase. We also thank Prof. Ingmar Bjljrk, Department of Veterinary Medical Chemistry, Uppsala, for access to the CD instrument.

REFERENCES

Blum, M., Metcalf, P., Harrison, S. C., and Wiley, D. C. (1987) J. Appl. Crystallogr. 20, 235–242
Brünger, A. T., Karplus, M., and Petsko, G. A. (1989) Acta Crystallogr. Sect. A 45, 50–61
Caldwell, G. A., and Becker, J. M. (1993) Progr. Nucleic Acid Res. Mol. Biol. 44, 6–9
de la Haba, G., Lester, I. G., and Racke, E. (1965) J. Biol. Chem. 241, 409–426
Deng, W., and Nickoloff, J. A. (1992) Anal. Biochem. 200, 81
Dyda, F., Furuy, W., Swaminathan, S., Sax, M., Farrenkopf, B., and Jordan, F. (1995) Biochemistry 34, 6155–6170
Eldes, P., and Storreg, E. (1993) Acta Physiol. Acad. Sci. Hung. 9, 339–346
Ehara, E., and Hollenberg, C. P. (1963) J. Bacteriol. 113, 265–265
Golbik, R., Neeb, H., Hübner, G., König, S., Seliger, H., and Kochetov, G. A., and Schellenberger, A. (1996) Bioorg. Chem. 19, 10–17
Heinrich, P. C., Neuk, K., and Wise, O. (1972a) Biochem. Biophys. Res. Commun. 49, 1427–1432
Heinrich, P. C., Stoffen, H., Jansen, F., and Wise, O. (1972b) Eur. J. Biochem. 30, 533–541
Jones, T. A., Zou, J.-Y., and Cowan, S. W. (1991) Acta Crystallogr. Sect. A 47, 110–119
Jordan, F., Chen, G., Ishikawa, S., and Sundoro, W. B. (1982) Ann. N.Y. Acad. Sci. 378, 14–29
Kluger, R. (1987) Chem. Rev. 87, 863–876
Kochetov, G. A. (1982) Meth. Enzymol. 90, 209–223
Kochetov, G. A., and Umezawa, R. A. (1970) Biochem. Biophys. Res. Commun. 41, 1134–1140
König, S., Schellenberger, A., Neef, H., and Schneider, G. (1994) J. Biol. Chem. 269, 10879–10882
Lawson, U. K. (1970) Nature 227, 690–695
Lindqvist, Y., and Schneider, G. (1993) Curr. Opin. Struct. Biol. 3, 891–901
Lindqvist, Y., Schneider, G., Ernster, U., and Sundström, M. (1992) EMBO J. 11, 2078–2089
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–276
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Muller, Y. A., and Schultz, G. E. (1993) Science 259, 965–967
Muller, Y. A., Lindqvist, Y., Jurey, W., Schultz, G. E., Jordan, F., and Schneider, G. (1993) Structure 1, 95–103
Nehlin, J. O., Carlberg, M., and Rone H. (1989) Gene (Amst.) 5, 313–319
Nikola, M., Lindqvist, Y., and Schneider, G. (1984) J. Mol. Biol. 238, 287–404
Nilsson, U., Lindqvist, Y., Kluger, R., and Schneider, G. (1993) FEBS Lett. 335, 145–146
Nowa, T., Saion, S., and Tminta, T. (1972) Chem. Pharmoc. Biol. (Tokyo) 20, 2715–2720
Racker, E. (1961) in The Enzymes (Boy, P. D., Lardy, H., and Myrback, K., eds) Vol. 5, pp. 547–568, Academic Press, New York
Russel, M., Kidd, S., and Kelley, M. R. (1989) Gene (Amst.) 5, 333–338
Saitou, S., Ozawa, T., and Tomita, T. (1974) FEBS Lett. 40, 114–118
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sato, R., Yamamoto, M., Imada, K., and Kutsue, Y. (1992) J. Appl. Crystallogr. 25, 342–347
Schaff-Gerstenschlager, I., Mannau, G., Vetter, I., Zimmermann, F. K., and Fldmann, H. (1993) Eur. J. Biochem. 217, 487–492
Scheißenberger, A. (1997) Angew. Chem. Int. Ed. Engl. 6, 1024–1035
Scheißenberger, A., and Hübner, G. (1986) Hoppe-Seyler's Z. Phys. Chem. 343, 189–192
Schneider, G., and Lindqvist, Y. (1993) Bioorg. Chem. 21, 169–177
Schneider, G., Sundström, M., and Lindqvist, Y. (1989) J. Biol. Chem. 264, 21619–21620
SERC (1979) Collaborating Computing Project 4, Daresbury Laboratory, Warrington, U. K.
Sherman, F., Fink, G. R., and Hics, J. B. (1986) Methods in Yeast Genetics, pp. 164–166, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Soni, R., Carmichael, J. F., and Murray, J. A. H. (1993) Curr. Genet. 34, 455–459
Sundström, M., Lindqvist, Y., Schneider, G., Hellman, U., and Rone H. (1993) J. Biol. Chem. 268, 24346–24355
Thomas, B. J., and Rothstein, R. (1989) Cell 58, 619–630
Tikhomirova, N. K., and Kochetov, G. A. (1990) Biochemistry Int. 22, 31–36
Villafranca, J. J., and Axelrod, B. (1971) J. Biol. Chem. 246, 3126–3131
Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 105–119

Fig. 6. Proposed mechanism of proton abstraction from the C-2 carbon atom of ThDP, the first step in enzymatic thiamin catalysis.