Identification of Catalytically Important Residues in Yeast Transketolase†

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ABSTRACT: The possible roles of four histidine residues in the active site of yeast transketolase were examined by site-directed mutagenesis. Replacement of the invariant His69 with alanine yielded a mutant enzyme with 1.5% of the specific activity of the wild-type enzyme and with an increased K_M for the donor. This residue is located at the bottom of the substrate cleft close to the C1 hydroxyl group of the donor substrate, and the side chain of His69 might be required for recognition of this hydroxyl group and possibly for maintenance of the proper orientation of the reaction intermediate, (α,β-dihydroxyethyl)-thiamin diphosphate. Amino acid replacements of His481 by alanine, serine, and glutamine resulted in mutant enzymes with significantly increased K_M values for the donor substrate and specific activities of 4.4%, 1.9%, and 5.5% of the wild-type enzyme. The kinetic data suggest that this residue, although close to the C2 carbonyl oxygen of the substrate, is not absolutely required for stabilization of the negative charge that develops at this oxygen in the transition state. This points toward the 4′-NH_2 group of the pyrimidine ring of thiamin diphosphate as the major source of charge stabilization. Mutations at positions His30 and His263 result in mutant enzymes severely impaired in catalytic activity (1.5% and less of the activity of wild-type transketolase). The K_M value for the donor substrate was increased for the His30Ala mutant but remained unchanged in the His263Ala enzyme. The side chains of both residues interact with the C3 hydroxyl group of the donor substrate, and the results indicate that the two residues act in concert during proton abstraction of the C3 hydroxyl proton during catalysis.

Recent advances in the elucidation of the three-dimensional structures of thiamin diphosphate (ThDP)[1] dependent enzymes [for a review see (*1*)] have provided insights into the interactions of this cofactor with the protein and led to mechanistic proposals with respect to the molecular basis of enzymatic thiamin catalysis (*2*−*6*). Transketolase constitutes a particularly suitable model system to address such mechanistic issues; the three-dimensional structure of the yeast enzyme has been determined in the holo and apo forms (*2*, *7*, *8*) and in complex with the acceptor substrate erythrose 4-phosphate (*9*). Furthermore, expression systems have been described for the enzyme from yeast (*10*), *Escherichia coli* (*11*), and humans (*12*).

On the basis of the crystal structure of yeast holotransketolase (*2*, *7*), the functions of several active site residues have been probed by site-directed mutagenesis. The structure analysis identified Glu418, conserved in all ThDP utilizing enzymes, as the key residue responsible for the activation of the cofactor, by protonating the N1′ atom of the aminopyrimidine ring of ThDP (*2*). The proposed general function of this residue in ThDP-dependent enzymes was supported by site-directed mutagenesis of Glu418 in yeast transketolase (*10*) and the corresponding residues in *Zymomonas mobilis* (*13*) and yeast (*14*) pyruvate decarboxylase. NMR studies of wild-type and mutant pyruvate decarboxylase and transketolase provided further compelling support for this mechanistic proposal, as these studies revealed an involvment of the glutamic acid residue in proton exchange at the C2 atom of the thiazolium ring of ThDP (*15*). The conserved residue Glu162 in transketolase which interacts with Glu418 and Glu167 across the subunit−subunit interface is required for dimer stabilization (*16*). Amino acid replacement of Asp382, another conserved residue in the cofactor binding site of transketolase, revealed that this amino acid is required for cofactor binding and catalysis and suggested a role for this residue in compensation of the positive charge of the thiazolium ring of ThDP (*16*). The crystal structure analysis of a complex of holotransketolase and the acceptor substrate erythrose 4-phosphate combined with site-directed mutagenesis studies identified a number of conserved residues in the substrate channel of the enzyme that interact with the substrate (*9*).

While most studies so far have focused on invariant residues in the cofactor binding site or in the vicinity of the phosphate group of the substrate, only a few studies have addressed the role of residues in the immediate proximity of the catalytic center, the C2 carbon atom of the thiazolium ring of ThDP. The crystal structure of yeast transketolase (*2*, *7*) revealed a cluster of conserved histidine residues which surrounds this area of the active site. One of these residues, His481, is not conserved in transketolases from mammalian sources (*17*). The other four residues, His30, His69, His103,

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[1] Abbreviations: ThDP, thiamin diphosphate; P, phosphate; rms, root mean square.

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and His263, are, however, invariant in all transketolase sequences known so far. The function of His103 (and its equivalent in the human enzyme, His110) has been probed by site-directed mutagenesis, and a role in binding the donor substrate (*18*) and in catalysis (*12*, *18*) has been proposed.

Here we report the results of a mutagenesis study which focuses on the role of the remaining invariant histidine residues in the active site of transketolase. Although the active site residue His481 is not strictly conserved, it has nevertheless been included in this study since it was proposed that this residue might be involved in proton abstraction of the positively charged 4′-imino group of the cofactor, resulting in the 4′-iminopyrimidine (*3*).

MATERIALS AND METHODS

Materials. Xylulose-5-P, ribose-5-P, and ThDP were purchased from Sigma, glyceraldehyde-3-P dehydrogenase was from Boehringer Mannheim, and hydroxypyruvate and glycolaldehyde were from Fluka. Restriction enzymes, DNA modifying enzymes, ATP, and premixed deoxyribonucleotides were from Pharmacia Biotech. All plasmid preparations were made with the Wizard kits (Promega). DNA sequencing was performed with the T7 Sequencing kit (Pharmacia Biotech), using Redivue [^{35}S]dATP (Amersham) and Sequagel RAPID gels (National Diagnostics).

Mutagenesis, Expression, and Purification. Unless otherwise indicated, standard molecular biology procedures (*19*) were used. All manipulations of the mutagenesis/expression plasmid pTKL1 (*10*) in *E. coli* were made in the strain JM109 (*20*). Site-directed mutagenesis of TKL1 was performed by the unique site elimination technique (*21*). Transformation of the *Saccharomyces cerevisiae* tkl1::HIS3 strain H402 (*10*) with mutated pTKL1 was done according to procedures described elsewhere (*22*). All mutants were sequenced throughout their entire open reading frames. Recombinant mutant transketolase was expressed from the TKL1 wild-type promoter in cultures growing in leucine free medium, and the mutant enzymes were purified as described earlier (*10*, *23*).

Activity Measurements and Determination of Kinetic Parameters. The assay for transketolase activity, based on a coupled system with glyceraldehyde-3-P dehydrogenase and NAD^+, was performed as described by Kochetov (*24*). Determination of protein concentration was done spectrophotometrically using the absorption coefficient $A^{1\%}(280\ nm) = 14.5$ (*25*). All kinetic measurements were made at 25 °C in duplicate. The dependence of transketolase activity on pH was measured as described before (*10*).

Kinetic steady-state parameters were determined from initial rate measurements in 50 mM glycylglycine buffer, pH 7.6, at varying ThDP and substrate concentrations. Xylulose-5-P was used as donor substrate and ribose-5-P as acceptor substrate. When the parameters for the substrates were measured, the ThDP concentration was 100 μM (150 μM for H69A) and the Mg^{2+} concentration 2.5 mM. Whenever possible, the concentration of the cosubstrate was about 5−10 times its K_M value, while the concentration of the investigated substrate was varied in a series from approximately 0.1 to 10 times its K_M value. Transketolase can cleave the donor substrate, with release of glyceraldehyde-3-P even in the absence of acceptor substrate, which gives rise to a background reaction, and this background was subtracted from the rate of the complete reaction.

Table 1: Data Collection Statistics and Crystallographic Refinement Parameters for Mutant His263Ala

parameter	
resolution (Å)	2.6
no. of measured reflections	380321
no. of unique reflections	36959
completeness of X-ray data (%)	82.7 (65.1 highest resolution shell)
R-merge (%)	9.7 (21.5 highest resolution shell)
R-free (%)	25.2
R-factor (%)	19.6
rms bond angle deviations (deg)	1.4
rms bond length deviations (Å)	0.007
rms deviation between subunits (Å)	0.033
Ramachandran plot	
residues in most favored regions (%)	87.7
no. of non-glycine residues in unfavorable regions	0

The combination of very high K_M values for xylulose-5-P and very low specific activities in some of the mutants (His30Ala and His69Ala) resulted in initial rates for the background reaction comparable to rates of the complete reaction at low ribose-5-P concentrations and saturating concentrations of xylulose-5-P. This prevented a reliable determination of initial rates at low ribose 5-phosphate concentrations and, thus, a reliable determination of the K_M for ribose 5-phosphate. In these cases, the concentration of the cosubstrate xylulose 5-phosphate was kept at subsaturating concentrations (approximately 1 mM) in order to minimize the background reaction. The apparent K_M for ribose-5-P was then calculated according to Bisswanger (*26*).

The apparent K_M for ThDP was determined by incubating apotransketolase with 2.5 mM $MgCl_2$ and different concentrations of ThDP, in a series from 50 nM to 100 μM, for 30−90 min before the start of the reaction by addition of substrates. The program ULTRAFIT (Biosoft) was used for nonlinear regression analysis of the kinetic data and calculation of K_M, V_{max}, and standard errors.

Circular Dichroism Measurements. The near-ultraviolet CD spectra of all mutants except those at position His481 were recorded with an Aviv 62DS instrument, using the Aviv software with standard parameters for smoothing the spectra. The spectra of wild-type transketolase and the His481 mutants were recorded on a Jasco J-41 A spectropolarimeter. The protein concentration was 0.5−0.8 mg/mL (10 mm pathlength cuvette) or 1.0−1.6 mg/mL (5 mm path length) in 50 mM glycylglycine buffer, pH 7.6.

Crystallization and X-ray Crystallography. Mutant transketolase was crystallized by the vapor diffusion technique in hanging drops as described for wild-type holotransketolase (*27*). The X-ray data set for the mutant His263Ala was collected with a MAR image plate mounted on a Rigaku rotating anode at 4 °C, and data frames were processed with DENZO (*28*). Statistics of the X-ray data collection are given in Table 1.

Crystallographic computing was carried out with the CCP4 suite of programs (*29*). The model of holotransketolase, refined to 2.0 Å resolution (*7*), was used as a source of initial phase information for the calculation of electron density maps. Refinement was performed with the program package X-PLOR (*30*) using the Engh and Huber bond distance and angle parameters (*31*). A randomly selected subset of the data was omitted in the refinement and used for calculation of the free *R*-value (*32*). The *B*-factors were not refined but

Table 2: Kinetic Parameters for Wild-Type and Mutant Transketolase

enzyme	k_{cat} (s^{-1})	pH optimum	apparent K_M (μM)		
			ThDP	xylulose-5-P	ribose-5-P
wild type	46.3	7.6−8.2	0.6 ± 0.1	70 ± 10	146 ± 21
H30A	0.69	6.8−8.0	0.8 ± 0.1	1010 ± 170	290 ± 100
H30N	0.09	nd[a]	0.5 ± 0.1	nd[a]	nd[a]
H69A	0.69	7.5−7.8	1.8 ± 0.7	400 ± 98	193 ± 40
H263A	0.23	7.6−8.0	1.2 ± 0.2	23 ± 5	93 ± 10
H481A	2.04	7.0−7.8	0.3 ± 0.1	1240 ± 90	150 ± 50
H481Q	0.88	6.6−7.4	0.7 ± 0.1	4080 ± 510	nd
H481S	2.54	nd	0.5 ± 0.6	3350 ± 370	nd

[a] nd, not determined because of too low activity of the mutant.

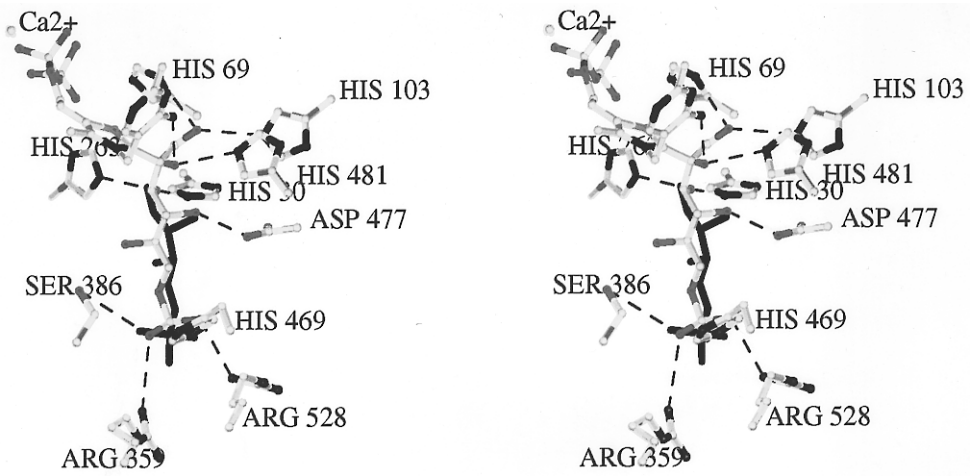

FIGURE 1: Stereo diagram showing a model of the adduct between the donor substrate fructose-6-P (yellow) and ThDP in the active site of transketolase. The position of the acceptor substrate erythrose-4-P as observed in the crystal structure is also shown (black). Hydrogen bonds are indicated as dashed lines. The figure was generated with the program O (33).

were taken from the refined model of the holoenzyme. Tight noncrystallographic symmetry restraints were kept throughout the refinement to compensate for the moderate observation/parameter ratio due to the limited resolution of the X-ray data. The mutated residue was replaced with the proper side chain after the first round of positional refinement, and the model was then subjected to another round of positional refinement. All model building, structural comparisons, and inspection of electron density maps were performed using the program O (*33*). PROCHECK (*34*) was used for the analysis of the final protein model. Details of the refinement procedure and final model statistics are given in Table 1. The atomic coordinates for the mutant transketolase have been deposited with the Brookhaven Protein Data Bank (file name 1ayo).

Model Building. On the basis of the crystal structure of the holotransketolase−acceptor substrate complex (*9*), the fructose-6-P−ThDP adduct was modeled in the active site of transketolase using the program O (33) and energy minimized with X-PLOR (*30*).

RESULTS

Mutagenesis and Expression of the Mutant Enzyme. DNA sequencing confirmed that no unintended mutation had been introduced during the course of mutagenesis. All mutant enzymes were expressed in similar amounts as reported for recombinant wild-type transketolase and could be purified to homogeneity using the same purification protocol (*10*).

Kinetic Properties of Mutants. All side chain replacements caused significant decreases in k_{cat} of the mutant enzymes and in most cases increases in the apparent K_M values for the donor substrates. The apparent K_M values for the acceptor substrate and the coenzyme were, however, not or only moderately affected. The pH optima for the mutant enzymes are within pH intervals similar to those of the wild-type enzyme (Table 2), and the observed decreases in k_{cat} at pH 7.6, therefore, do not simply reflect large shifts of the pH optima as a consequence of the side chain replacement.

The His69Ala mutant retained 1.5% specific activity with a minor increase in the apparent K_M for the cofactor. The K_M for the donor substrate is increased about six times. All mutants at position 481 have phenotypes very similar to each other. They show unaltered ThDP binding properties, decrease in specific activities, and, in particular, considerable increases in the K_M values for the donor substrate.

The side chain of His30 was replaced by alanine and asparagine. For both mutants the apparent K_M for ThDP was similar to the value for the wild-type enzyme. Due to the very low specific activity for His30Asn, the apparent K_M values for donor and acceptor substrates could not be determined reliably. For the His30Ala mutant the apparent K_M for xylulose-5-P was increased by approximately 15 times while the apparent K_M for the acceptor substrate was in the same range as for wild-type enzyme.

Substitution of the side chain of His263 by alanine did not influence the apparent K_M for ThDP nor the K_M values for the substrates. However, this mutation was one of the most deleterious in terms of k_{cat}, with only 0.5% residual activity.

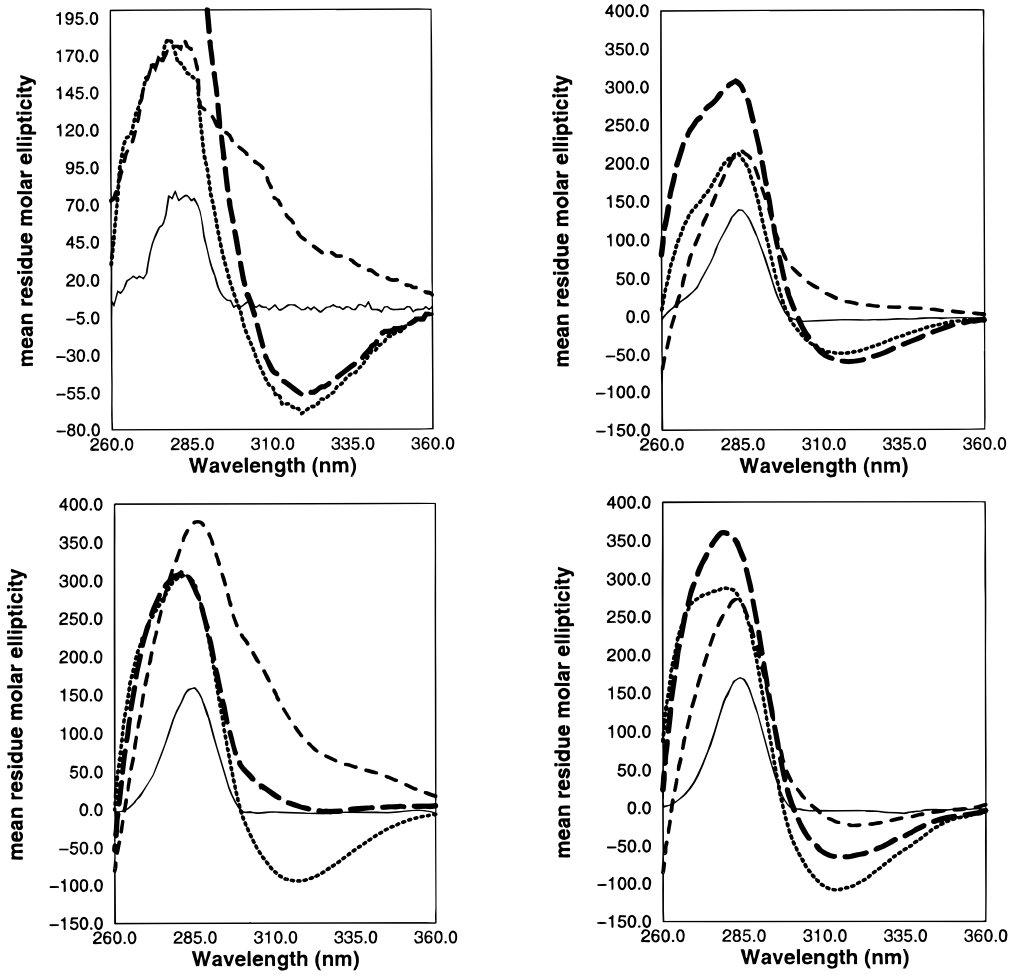

FIGURE 2: Near-ultraviolet CD spectra for wild-type transketolase (upper left) and mutants His69Ala (upper right), His30Asn (lower left), and His263Ala (lower right). The spectra of the apoenzymes are shown as thin lines. The spectra of the holoenzymes (dotted line) were recorded after addition of 30−60 μM ThDP to the apoenzyme. The donor substrate hydroxypyruvate was added to a concentration of 2 mM (thin dashed lines), and finally the acceptor substrate glycolaldehyde was added to a concentration of 20 mM to the holoenzyme/hydroxypyruvate mixture (bold dashed lines).

Crystal Structure of the Mutant His263Ala. The mutants His30Asn, His30Ala, His263Ala, His69Ala, and His481Gln crystallized isomorphously to the wild-type enzyme in space group $P2_12_12_1$. However, only the mutant His263Ala yielded crystals of sufficient size and quality for structure determination. The structure of this mutant was refined to 2.6 Å resolution (Table 1). There is well-defined electron density for ThDP and the metal ion in the electron density maps, and the positions of ThDP and the Ca^{2+} ion in the mutant are identical to those in wild-type transketolase. The overall root-mean-square deviations from the positions of the Cα atoms in the wild-type enzyme are 0.20 Å for the mutant structure, and these differences are evenly distributed along the polypeptide chain. Thus, the overall structure is very similar to the wild-type holotransketolase model, and the amino acid replacement did not introduce changes in the structure of the mutant enzyme.

Model of the Donor Substrate−ThDP Adduct. On the basis of the crystal structure of the complex between holotransketolase and the acceptor substrate erythrose-4-P (*9*), a model of the donor substrate fructose-6-P was built in the active site as an adduct with the C2 atom of ThDP (Figure 1). The adduct could be modeled without any unfavorable interactions with enzyme or cofactor groups and with a binding pattern for the hydroxyl groups and phosphate group similar to that observed for erythrose-4-P. Energy minimization did not change the position of any side chain significantly.

In this model, there is an ambiguity as to the possible interactions of the substrate C1 hydroxyl group with protein residues. It can be positioned such that it forms a hydrogen bond to the side chain of either His103 or His69 or to both of these residues. The C2 oxygen of the substrate is within hydrogen bond distance to both the 4′-NH_2 group of the cofactor and the side chain of His481. Residues His30 and His263 form hydrogen bonds to the C3 hydroxyl group of the substrate. As in the holoenzyme−erythrose-4-P complex, the C4 hydroxyl group (corresponding to the C2 hydroxyl in erythrose-4-P) points toward Asp477. The phosphate group interacts with the conserved residues Arg359, Ser386, His469, and Arg528, located at the entrance of the substrate channel.

Circular Dichroism Measurements. The transketolase reaction can be monitored by near-ultraviolet CD spectroscopy (*35*, *36*). For all mutants the apo- and holoenzyme (formed after addition of 30−60 μM ThDP to the apoenzyme) spectra were qualitatively very similar to the wild-type spectrum, indicating that ThDP binding was not disturbed by the amino acid substitutions (Figure 2). The CD spectra of mutants at positions 263 and 481 are very

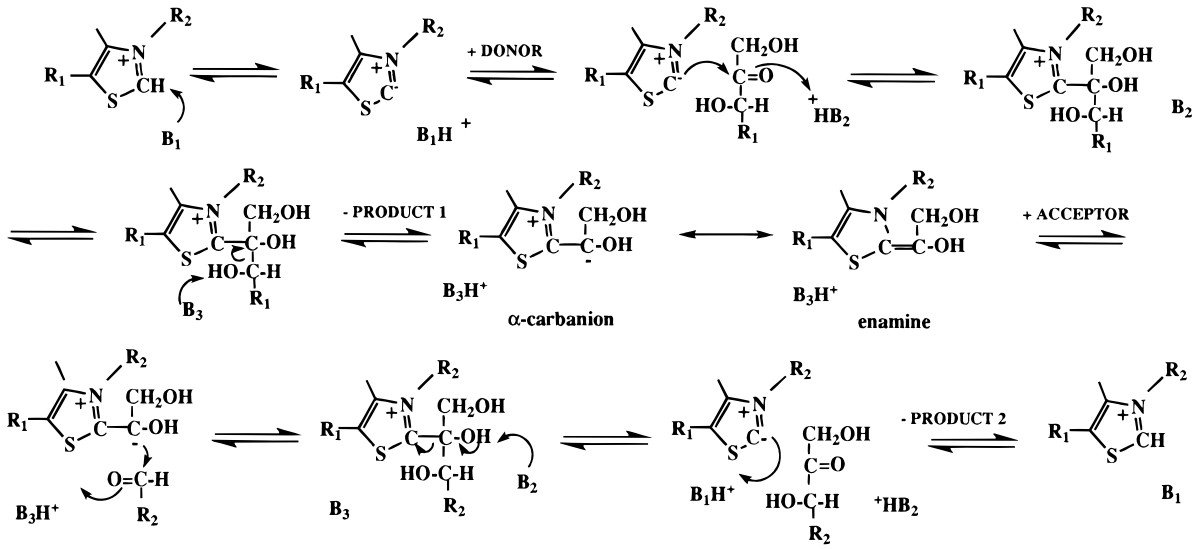

FIGURE 3: Scheme of the transketolase reaction. B1−B3 denote catalytic groups involved in possible proton transfer steps during the reaction.

similar; therefore, only the spectrum for His263Ala is shown.

In the case of the wild-type enzyme, the addition of a donor substrate leads to an inversion of the band at 320 nm, probably due to formation of the intermediate (α,*β*-dihydroxyethyl)-ThDP (*35*). Subsequent addition of acceptor substrate restores the band at 320 nm to that of the holoenzyme.

Qualitatively, all mutants show similar responses to addition of donor and acceptor substrates; they differ however among each other and from wild-type enzyme in the extent to which these spectral changes are realized (Figure 2).

DISCUSSION

The substitution of four histidine residues at the active site of transketolase resulted in mutant enzymes severely impaired in their kinetic behavior. The CD spectra for all mutants indicated that binding of the cofactor was not perturbed, consistent with the small changes in the apparent K_M values for ThDP. This conclusion is further supported by the crystallographic analysis of the His263Ala mutant, which confirms that the three-dimensional structure of this mutant is, within the error limits of the analysis, identical to that of the wild-type enzyme. The responses of the CD spectra of the mutant enzymes towards addition of substrates showed that qualitatively, similar spectral changes as in the wild-type enzyme were found; the observed differences might reflect changes in the distribution of Michaelis complexes and reaction intermediates rather than structural defects.

At the bottom of the substrate channel, close to the cofactor, there is a small hydrophilic pocket, and the walls of this pocket are formed in part by the conserved residues His69 and His103. The former residue is forming a hydrogen bond to one of the phosphate groups of ThDP. In the model of the fructose-6-P−ThDP adduct at the active site (Figure 1), the side chain of His69 can also interact with the C1 hydroxyl group of the substrate, in a fashion similar to that of His103. The properties of the His69Ala mutant are in fact very similar to those found for mutants of His103 (*18*), an increase in the K_M for the donor substrate and a reduction of the specific activity to about 1−2% of the wild-type enzyme. This suggests that these residues might play similar roles in binding the C1 hydroxyl group of the donor substrate and/or stabilization of subsequent reaction intermediates. It is surprising that substitution of His69 does not disturb binding of ThDP more seriously, since this residue is interacting with a phosphate group of ThDP. It might well be that the large number of protein−cofactor interactions (*7*) makes the affinity of the enzyme for its cofactor less sensitive to the removal of one of these interactions at a time.

The crystal structure of yeast transketolase (*2*) revealed that the side chain of His481 is very close to the 4′-NH_2 group of ThDP, making it a potentially suitable group for proton abstraction. However, the subsequent determination of the nucleotide sequences of mammalian transketolase genes (*17*) showed that His481 is not conserved, which suggests that this residue might not be an essential catalytic base in the reaction. Singleton and co-workers replaced the corresponding residue in human transketolase (Gln428) with various side chains, with modest effect on the specific activity and kinetic constants (*12*). Measurements of the C2 deprotonation rate of ThDP in the His481Ala mutant of the yeast enzyme also strongly argue against a function of this residue for proton abstraction (*15*).

The mutants at position 481 all have ThDP binding properties similar to that of wild-type. Interestingly, the K_M for the donor substrate is considerably increased for all mutants at that position. The residual specific activity of about 5% indicates that this residue very likely is not involved in acid−base catalysis.

We note, however, that the substitution of His481 with glutamine, the residue found in mammalian transketolases, does result in a mutant enzyme with impaired activity. The corresponding inverse change in the human enzyme, Gln428His, has less severe consequences (*12*). The amino acid sequence downstream of this residue in mammalian transketolases deviates significantly from the sequences of other transketolases, which might indicate structural differences that could account for these different phenotypes.

The two conserved residues, His30 and His263, are both within hydrogen-bonding distance to the carbonyl oxygen of the acceptor substrate (*9*) and the C3 hydroxyl group of the model of the reaction intermediate (Figure 1). Both residues are therefore candidates for an acid−base catalyst in the reaction. Mutants at both positions are severely

impaired in specific activity, with unperturbed cofactor binding capabilities. For the mutants at position 30, changes in the K_M for xylulose-5-P are observed, consistent with a role for this residue in binding of donor substrate and reaction intermediate. The mutant at position 263 shows a different behavior. The specific activity is severely disturbed (0.5% of wild type), but the K_M values for the substrates are not changed, suggesting a dominantly catalytic role for His263.

The Reaction Mechanism. Figure 3 gives a scheme of the transketolase reaction. The first half of the reaction consists of the cleavage of the donor substrate and formation of the first product, an aldose and a covalent intermediate, the α-carbanion of (α,*β*-dihydroxyethyl)-ThDP. In the second half of the reaction, a nucleophilic attack of the α-carbanion on the acceptor substrate occurs, and the second product, a ketose with its carbon skeleton extended by two carbon atoms, is formed. The formation of the Michaelis complex of holotransketolase with the donor substrate involves a number of conserved amino acid side chains in the substrate channel that interact with the substrate through hydrogen bonds (*9*).

During the first half-cycle of catalysis, at least two proton transfer steps have to occur; the C2 carbon atom of the thiazolium ring of ThDP has to be deprotonated, and upon cleavage of the donor−ThDP product, the C3 hydroxyl group of the substrate will have to be deprotonated to yield the aldose product. In addition, the negative charge developing at the C2 carbonyl oxygen of the donor substrate will have to be stabilized electrostatically.

The first proton transfer is common to all ThDP-dependent enzymes, and compelling evidence has been assembled that this step is catalyzed by ThDP itself. This step is facilitated by the enzyme in two ways: maintenance of the V-conformation, which positions the 4-NH_2 group in the vicinity of the C2 carbon of the thiazolium ring, and protonation of the N1′ nitrogen atom of the pyrimidine ring, which generates the 4-imino group. The 4′-imino group (B_1 in the reaction scheme) can act as a sufficiently basic group to abstract the C2 atom of the thiazolium ring (*2*, *10*, *15*, *37*, *38*).

Once formed, the C2 carbanion of the cofactor is able to attack the carbonyl carbon of the donor substrate. For covalent bond formation between these two atoms to occur, stabilization of the negative charge developing at the carbonyl oxygen is required. In the model we find two possible candidates which might act in transition state stabilization (B_2 in Figure 3), His481 and the charged 4′-imino group of ThDP. The contribution of His481 to transition state stabilization can be estimated to about 3.7 kcal/mol, based on the ratio of the k_{cat}/K_M values for the donor substrate for the wild-type enzyme and the His481 mutants. Another important contribution to the stabilization of the negative charge at this oxygen comes from the 4′-imino group, as has been suggested previously by Schellenberger (*39*). In conclusion, the functions of B_1 and B_2 in the catalytic cycle of transketolase might thus be carried out by the same group, the 4′-imino group of ThDP.

For steric reasons, proton abstraction at the C3 atom of the donor substrate cannot be catalyzed by the group(s) involved in steps 1 and 2. The mutagenesis and crystallographic results strongly point toward His30 or His263 as the proton acceptor and donor in the reaction (B_3 in Figure 3). In fact, a scenario can be described in which both residues act in concert during this catalytic step. The side chain of His263 forms a hydrogen bond to a phosphate oxygen of ThDP, and thus, the second nitrogen atom, which is not protonated at the pH optimum (pH 7.6), points straight toward the C3 hydroxyl group and is perfectly positioned to abstract a proton from the donor substrate. His30 could facilitate proton abstraction by His263 through a hydrogen bond to the oxygen atom of the C3 hydroxyl group, thus (a) binding and positioning this group in the proper orientation and (b) stabilizing the negative charge developing in the transition state during proton abstraction.

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