Examination of Donor Substrate Conversion in Yeast Transketolase*

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The cleavage of the donor substrate D-xylulose 5-phosphate by wild-type and H263A mutant yeast transketolase was studied using enzyme kinetics and circular dichroism spectroscopy. The enzymes are able to catalyze the cleavage of donor substrates, the first half-reaction, even in the absence of any acceptor substrate yielding D-glyceraldehyde 3-phosphate as measured in the coupled optical test according to Kochetov (Kochetov, G. A. (1982) Methods Enzymol. 90, 209–223) and compared with the H263A variant. Overall, the H263A mutant enzyme is less active than the wild-type. However, an increase in the rate constant of the release of hydroxylactylthiamin diphosphate after incubation (of the enzyme-bound glycolyl moiety was observed and related to a stabilization of the “active glycoaldehyde” (α-carbanion) by histidine 263. Chemically synthesized DL-(α,β-dihydroxyethyl)thiamin diphosphate is bound to wild-type transketolase with an apparent KD of 4.3 ± 0.8 μM (racemate) calculated from titration experiments using circular dichroism spectroscopy. Both enantiomers are cleaved by the enzyme at different rates. In contrast to the enzyme-generated α-carbanion of (α,β-dihydroxyethyl)thiamin diphosphate formed by decarboxylation of hydroxylactylthiamin diphosphate after incubation of transketolase with β-hydroxypyruvate, the synthesized DL-(α,β-dihydroxyethyl)thiamin diphosphate did not work as donor substrate when erythrose 4-phosphate is used as acceptor substrate in the coupled enzymatic test according to Sprenger (Sprenger, G. A., Schörken, U., Sprenger, G., and Sahm, H. (1995) Eur. J. Biochem. 230, 525–532).

The pentose phosphate pathway is a major metabolic pathway in all cells. It consists of a dehydrogenase-decarboxylating system that converts D-glucose 6-phosphate to D-ribulose 5-phosphate, generating NADPH+H+ for use in reductive biosynthesis, an isomerizing system that interconverts β-ribulose 5-phosphate to D-xylulose 5-phosphate and D-ribose 5-phosphate, and a sugar rearrangement system that converts D-ribose 5-phosphate and D-xylulose 5-phosphate to the glycolytic intermediates D-fructose 6-phosphate and D-glyceraldehyde 3-phosphate. Transketolase (EC 2.2.1.1) plays an important part in the rearrangement system, since it creates, together with transaldolase, a reversible link between the pentose phosphate pathway and glycolysis.

The catalytic activity of transketolase is dependent on thiamin diphosphate (ThDP)¹ and divalent cations such as Mg²⁺ or Ca²⁺. The enzyme from Saccharomyces cerevisiae is composed of two identical subunits with a molecular mass of 74 kDa per monomer (1). The thiamin diphosphate molecule binds in a cleft at the interface between the two subunits and is completely inaccessible from the outer solution, except for the C2 atom of the thiazolium ring to which the donor substrate binds covalently (2). Transketolase catalyzes the cleavage of a carbon-carbon bond adjacent to a carbonyl group in ketosugars and transfers a two-carbon unit to aldoses. The reaction cycle of transketolase can be separated into two parts. The first half of the reaction consists of the cleavage of the donor substrate and formation of the first product, an aldose, and a covalently bound intermediate, the α-carbanion of α,β-dihydroxyethyl-ThDP (DHEThDP). In the second half of the reaction the α-carbanion reacts with the acceptor (aldose) substrate in a nucleophilic manner. The two-carbon unit from the first reaction is transferred to the acceptor substrate, and a ketose with its carbon skeleton extended by two carbon atoms is formed. The enzyme has a high specificity for donor ketoses with D-threo (C3-L, C4-D) configuration and for α-hydroxylated acceptor aldoses with C2-o configuration (3, 4).

The first crystal structure of a ThDP-dependent enzyme to be solved was that of holotransketolase from S. cerevisiae (2). This structure has been refined to 2.0-Å resolution (5). Beside transketolase, the crystal structures of other ThDP-dependent enzymes, such as pyruvate oxidase from Lactobacillus plantarum (6, 7), pyruvate decarboxylase from brewers’ yeast (8, 9), pyruvate decarboxylase from Zymomonas mobilis (10), pyruvamide-activated pyruvate decarboxylase from S. cerevisiae (11), benzoyleformate decarboxylase from Pseudomonas putida (12), and pyruvate ferredoxin oxidoreductase from Desulfovibrio africanus (13), have been solved and revealed the ThDP molecule to be bound in the V-conformation. The specificity of the coenzyme binding was extended to complexes of transketolase with ThDP analogues (14).

According to Scheme 1 several proton transfer steps occur in the catalytic cycle, which are likely mediated by the 4′-amino group of the pyrimidine moiety of the enzyme-bound ThDP as the major source of charge stabilization (2, 15, 16). On the basis of the x-ray structure of transketolase, catalytically important

* This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Swedish Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors supported by grants from the Graduiertenförderung of Sachsen-Anhalt.

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1 The abbreviations used are: ThDP, thiamin diphosphate; DHEThDP, 2-(α,β-dihydroxyethyl)thiamin diphosphate; HPA, β-hydroxypropionate.
residues in the reaction cycle have been suggested (2, 4, 5) and probed by site-directed mutagenesis (4, 15, 17–19). For instance, four histidine residues, located in the active site of transketolase from *S. cerevisiae*, have been replaced by alanine using site-directed mutagenesis and the enzymatic activity of the variants investigated (18). The overall crystal structure of the H263A variant is very similar to the wild-type holotransketolase structure, the amino acid replacement did not introduce significant structural changes in the mutant enzyme or in the apparent *Km* values for ThDP, the donor substrate d-xylulose 5-phosphate, and the acceptor substrate d-ribose 5-phosphate, respectively. The mutation is most deleterious in terms of *kcat* (0.23 s⁻¹, wild-type 46.3 s⁻¹; Ref. 18). The side chain of His263 forms a hydrogen bond to a phosphate oxygen of ThDP and thus, the second nitrogen atom of the imidazole ring, which is not protonated at the pH optimum of the enzyme (pH 7.6), points straight toward the C3 hydroxyl group of the donor substrate. In this way, it is perfectly positioned to abstract a proton from the donor substrate (Scheme 1) (20).

To shift the equilibrium established by transketolase, β-hydroxypyruvic acid (HPA) is used as the ketol donor (3). This strategy couples the formation of the glycolyl-ThDP complex (α-carbanion of DHEThDP) with the decarboxylation of HPA and renders the complete reaction practically irreversible.

Scheme 2 shows the catalytic cycle of the transketolase-mediated condensation of HPA and α-hydroxyaldehyde. Because of its strong stereospecificity, transketolase offers an enormous synthetic potential (21–23).

In this work we studied the cleavage of the donor substrate d-xylulose 5-phosphate in the absence of any acceptor substrate by wild-type and H263A mutant holotransketolase from *S. cerevisiae* by kinetics and circular dichroism spectroscopy. Furthermore, we have chemically synthesized racemic DHEThDP (dl-DHEThDP) and investigated its complex formation with wild-type apotransketolase and the cleavage of the respective complexes in the presence and absence of the acceptor erythrose 4-phosphate by kinetics. These results are compared with the formation of enzyme-bound α-carbanion of DHEThDP (glycolyl-ThDP, “active glycolaldehyde”; Ref. 24) according to Scheme 2 using HPA as substrate donor and the cleavage in the presence and absence of acceptor substrates.

**MATERIALS AND METHODS**

**Chemicals and Enzymes**—Thiamin diphosphate and formaldehyde were purchased from Merck Eurolab GmbH (Darmstadt, Germany) and d-ribose 5-phosphate, d-xylulose 5-phosphate, d-erythrose 4-phosphate, d-glyceraldehyde 3-phosphate from Sigma Aldrich Chemie GmbH (Deisenhofen, Germany). The auxiliary enzymes glycerol-3-phosphate dehydrogenase, triose phosphate isomerase, phosphoglucone isomerase,
and glucose-6-phosphate dehydrogenase were obtained from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals used were commercially available.

Protein Expression and Purification—H402 (tk11::HIS3 derivative of W303-1A) yeast cells carrying wild-type plasmid pTKL1 (1) or the H263A mutant (18) were cultured in a leucine-deficient medium, containing galactose as a carbon source to obtain a high copy number of the plasmid (25). Cells were collected by centrifugation and disrupted in a beat beater. The protein purification was performed according to the established protocol of Wikner et al. (15) with some modifications. Sephacryl S-200 HR was used instead of Sephacryl S-300 HR and Source 15Q instead of Mono Q (Amersham Pharmacia Biotech, Europe GmbH). About 90 mg of pure transketolase having a specific catalytic activity of 33–40 units/mg for the wild-type and 0.2 unit/mg for the H263A variant could be obtained from 5 liters of cell culture.

The protein concentration of pure transketolase was determined spectrophotometrically at 280 nm using a molar absorption coefficient of 157,600 M⁻¹·cm⁻¹ according to the method of Gill and von Hippel (26).

Activity Measurements—The specific activities of wild-type transketolase and of the H263A variant were measured spectrophotometrically according to Kochetov (27), but without the acceptor substrate D-ribose 5-phosphate. The concentration of D-erythrose 4-phosphate was 2 mM. The other syringe was filled with the same reaction mixture containing 4 mM D-fructose 6-phosphate. The concentration of D-xylulose 5-phosphate was 2 mM. The binding of the donor substrate HPA was directly followed by the absorbance change at 300 nm and 25 °C in a stopped-flow machine but without the acceptor substrate D-ribose 5-phosphate. The concentration of D-erythrose 4-phosphate was 4 mM. The other syringe was filled with the same reaction mixture containing 4 mM D-fructose 6-phosphate. The concentration of D-xylulose 5-phosphate was 0.37 mM. The assay mixture contained 1 mM DHETDP, 2.5 mM CaCl₂, 5 units of triose phosphate isomerase, 5 units of glyceraldehyde 3-phosphate dehydrogenase, and 1–100 mM ThDP (corresponding to 1 μmol of n-glucuronate 5-lactone)/min. The assay mixture contained 0.6 mM D-erythrose 4-phosphate, 4 mM β-hydroxypropionate, 0.37 mM NAD⁻, 0.1 mM ThDP, 2.5 mM CaCl₂, 4 units of each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase.

The specific activity of wild-type transketolase and of the H263A variant were measured spectrophotometrically at 300 nm using a molar absorption coefficient of 157,600 M⁻¹·cm⁻¹ according to the method of Gill and von Hippel (26).

The entire cleavage of the donor substrate without the acceptor was followed by the absorbance change at 340 nm and 25 °C in a stopped-flow machine (SX.18MV, Applied Photophysics, Leatherhead, Great Britain) working in the single mixing mode. The two syringes had the same volume, resulting in a 1:2 dilution (1:1 mixing) of the solutions. One syringe contained the reaction mixture mentioned for the assay according to Kochetov (27), but without the acceptor substrate D-ribose 5-phosphate. The concentration of D-xylulose 4-phosphate was 2 mM. The other syringe was filled with the same reaction mixture containing different amounts of transketolase and no substrate. Furthermore, the activity of the auxiliary enzymes was increased to 1550 units/ml by desalting on HiTrap G25 columns (5 × 5 ml) and subsequent concentration using Pallfiltron concentration tubes (cutoff: 5000 Da). The programs SigmaPlot® (Jandel Scientific Software) and Dynafit (29) were used for nonlinear regression analyses of the kinetics.

Direct Measurement of Donor Substrate Binding to Transketolase—According to Kochetov et al. (30) the binding of the donor substrate D-fructose 6-phosphate was directly followed by the absorbance change at 300 nm and 25 °C in a stopped-flow machine with the same arrangement mentioned above. One syringe contained the wild-type enzyme (12 μM), 100 μM ThDP, 2.5 mM CaCl₂ in 50 mM glycyglycine (pH 7.6). The other syringe contained the donor substrate D-fructose 6-phosphate at various concentrations (0.05–1 mM) and 100 μM ThDP, 2.5 mM CaCl₂ in 50 mM glycyglycine (pH 7.6). The kinetics were analyzed using the program Dynafit (29).

Direct Measurement of the Transketolase Catalyzed Reaction of β-Hydroxypropionate and β-Glyceraldehyde 3-Phosphate with Transketolase—The binding of the donor substrate HPA was directly followed by the absorbance change at 300 nm and 25 °C in a stopped-flow machine but with the double mixing mode. One syringe contained the wild-type

![Diagram](image-url)
enzyme (8.25 μm), 30 μm ThDP, 2.5 mm CaCl2 in 50 mm glycy glycine (pH 7.6). The other syringe contained the donor substrate HPA (8.25 μm) and 30 μm ThDP, 2.5 mm CaCl2 in 50 mm glycy glycine (pH 7.6). After a reaction time of 8 s, D-glyceraldehyde 3-phosphate (8.25 μm) was added.

**Circular Dichroism Measurements**—Near-UV circular dichroism spectra of transketolase were recorded on a Jasco J710 circular dichroism spectrophotometer using cuvettes with an optical path length of 1 cm at a temperature of 25 °C. All samples were measured in 50 mm glycy glycine (pH 7.6) containing 2.5 mm CaCl2.

To determine the κ2, value of chemically synthesized racemic DHETThDP (DL-DHETThDP) to apotransketolase, the titration was followed by the change in ellipticity at an analytical wavelength of 320 nm until binding kinetics were finished (about 3 min).

**H/D-exchange Measurements on Wild-type and H263A Mutant Enzyme**—The kinetics of the H/D-exchange of the C2-H of ThDP in the transketolase proteins were measured by rapid quenched flow and 1H NMR as described previously (16).

**Chemical Synthesis of DL-DHETThDP**—DL-DHETThDP was synthesized according to Krampitz and Votaw (31). To separate DL-DHETThDP from nonconverted ThDP, the reaction mixture was supplied to an anion exchange chromatography on QAE-Sephadex A25 (Amersham Pharmacia Biochemicals, Uppsala, Sweden). The components were eluted by an acetic acid gradient (0–50 mM). DL-DHETThDP eluted in the front of ThDP. Afterward, the samples were lyophilized and analyzed by mass spectroscopy (VG BIO Q Tripel-Quadrupol mass spectrometer) and 1H NMR (Bruker ARX Avance 500). Synthesis and chromatography yielded about 40–50 mg of pure DL-DHETThDP. The stability of chemically synthesized DL-DHETThDP was followed by 1H NMR in 100 mM sodium phosphate (pH 7.6) at 10 °C. After 2 weeks only 7% DL-DHETThDP converted to ThDP under these conditions.

**Preparation of "Acceptor-free" D-Xylulose 5-Phosphate**—The progress curve of transketolase action using commercially available D-xylulose 5-phosphate in the absence of an acceptor substrate shows a background reaction of d-glyceraldehyde 3-phosphate formation with 6% of that rate measured in the presence of the acceptor substrate in performing the test according to Kochetov (27). Furthermore, an initial burst phase could be detected, the amplitude of which was dependent on the initial concentration of D-xylulose 5-phosphate. The jump could be repeated by additional D-xylulose 5-phosphate, but not by additional transketolase and must, therefore, be caused by acceptor compound(s) present in the commercially available donor substrate (Fig. 1). To remove these impurities in all measurements, the D-xylulose 5-phosphate was first preincubated with 20 μg/ml wild-type or 50 μg/ml H263A mutant holotransketolase for a time period of 5 or 20 min. This

**Fig. 1.** Background reaction of wild-type holotransketolase with the commercially available donor substrate D-xylulose 5-phosphate indicating acceptor substrate impurities. The assay was performed according to Kochetov (27) but in the absence of the acceptor substrate D-ribose 5-phosphate. The initial burst phase was repeatedly detectable after addition of D-xylulose 5-phosphate (dashed-dotted line) in the course of the enzymatic reaction but not by additional enzyme (full solid line). The concentration of D-xylulose 5-phosphate was 1 mM, and 2 mM after the second addition, respectively. The activity of the enzyme used was 100 milliunits/ml. The reaction was run at a temperature of 25 °C.

**Fig. 2.** Stopped-flow kinetics of the holotransketolase reaction with the acceptor-free donor substrate D-xylulose 5-phosphate. The assay was performed according to Kochetov (27) but in the absence of the acceptor substrate D-ribose 5-phosphate. A, progress curves measured with wild-type holotransketolase at different donor substrate concentrations (35 μM (inverted open triangles), 50 μM (open diamonds), 70 μM (open circles), and 1000 μM (open triangles)) D-xylulose 5-phosphate, respectively) and at a protein concentration of 7.4 μM. B, progress curves measured with wild-type holotransketolase at different protein concentrations (from top to bottom: 4.8, 9.7, 19.4, and 35 μM, respectively) at a concentration of D-xylulose 5-phosphate of 1 mM. C, progress curves measured with H263A mutant holotransketolase at different donor substrate concentrations (50 μM (inverted open triangles), 500 μM (open triangles), 1000 μM (open circles)) D-xylulose 5-phosphate, respectively) and at a protein concentration of 20.4 μM. The progress curves in all figures are displaced by an offset in absorbance for better comparison, and the experimental data sets are reduced for better visualization. The lines in A and C represent the fits of the data according to Scheme 3 with the program Dynafit. All the reactions were run at a temperature of 25 °C.
procedure yielded acceptor-free donor substrate that was used in the kinetic experiments.

Determination of dl-DHETdDP Turnover by Transketolase—Transketolase (75 μM) was incubated with an equimolar concentration of racemic DHETdDP in 0.02 m sodium phosphate (pH 7.6) at 25 °C. The samples were quenched with 200 μl of 12.5% (w/v) trichloroacetic acid, 1 m DCl. After separation of the denatured protein by centrifugation, the supernatant containing DHETdDP and ThDP was analyzed by 1H NMR spectroscopy. The formation of ThDP due to the enzymatic cleavage of DHETdDP by transketolase was monitored by the decrease of the relative integral of the C6-H signal (singlet) of DHETdDP at 7.30 ppm and the increase of the relative integral of the C6-H signal of ThDP (singlet) at 8.01 ppm.

Chromatographic Determination of dl-DHETdDP Turnover by Transketolase—The aenzyme of wild-type transketolase was incubated with equimolar amounts of chemically synthesized dl-DHETdDP in 50 mM glycolaldehyde and its high 9-phosphate, rate constants were only calculated for the -H signal (singlet) of DHETdDP. The enzymatic test reaction of the transketolase proteins was performed according to Kochetov (27). The respective rate constants were derived from fitting the progress curves of the enzymatic reaction using the program Dynafit (29). The equilibrium constants K were calculated from the rate constants k1 and k2. These values are considered as estimated constants only.

RESULTS

Cleavage of the Donor Substrate D-xylulose 5-phosphate in the Absence of an Acceptor Substrate by Wild-type and H263A Mutant Transketolase—The cleavage of the acceptor-free donor substrate by wild-type transketolase was measured according to Kochetov (27) using a stopped-flow machine. The progress curves are shown in Fig. 2A. The initial burst phase observed corresponds to a single turnover of the donor substrate per active site and is directly proportional to the enzyme concentration used (Fig. 2B). A turnover number of 0.65 per active site has been measured at a specific transketolase activity of 26 units/mg. An extrapolated turnover number of 1 was calculated for an enzyme having a specific activity of 40 units/mg. The following slow steady state phase is indicative for the cleavage of the enzyme-bound glycolyl moiety (α-carbanion/enamine state of DHETdDP) to ThDP and glycolaldehyde and/or a replacement of the DHETdDP by ThDP and its further reaction with D-xylulose 5-phosphate. The product D-glyceraldehyde 3-phosphate is converted by the auxiliary enzymes in the test system according to Kochetov (27), making the reaction practically irreversible. As a control, the rate of the complete transketolase reaction could be regained after addition of the donor substrate D-fructose 6-phosphate, or D-fructose 6-phosphate, full solid line; holoenzyme and 1 mM D-fructose 6-phosphate, dashed line). The measurements were started after 10-min incubation time.

The binding of D-fructose 6-phosphate to the wild-type of holotransketolase (Table I).

H/D-exchange Measurements on Wild-type and H263A Mutant Enzyme—The rate constant of the H/D-exchange of the C2-H atom of the enzyme-bound cofactor in H263A mutant transketolase was estimated to 50 ± 5 s⁻¹ and seems, therefore, to be unaltered with respect to the wild-type enzyme (61 ± 5 s⁻¹; Ref. 16) within the experimental error.

Near-UV Circular Dichroism Spectroscopy—The binding of the cofactor ThDP to apotransketolase, as well as the binding of the artificial donor substrate HPA and the acceptor substrate glycolaldehyde to holotransketolase, can be monitored by near-UV circular dichroism (32, 33). This approach has already been used for the spectroscopic characterization of the H263A mutant enzyme (18).

On incubation of wild-type holotransketolase with only the donor substrates D-xylulose 5-phosphate or D-fructose 6-phos-
Reconstitution and Enzymatic Activity of Wild-type Apotransketolase with \( \text{DL-DHEThDP} \)—Chemically synthesized racemic DHEThDP (\( \text{DL-DHEThDP} \)) shows no circular dichroism spectrum in the wavelength range between 240 and 380 nm. On incubation of \( \text{DL-DHEThDP} \) with wild-type apotransketolase, a near-UV circular dichroism spectrum showing a negative ellipticity with an extremum at 320 nm was measured, which is identical to that of holotransketolase. The ellipticity was used as an analytical tool in a titration experiment of \( \text{DL-DHEThDP} \) to apotransketolase (Fig. 5), yielding a \( K_D \) value of 4.3 ± 0.8 \( \mu \)M for the racemate (for comparison, \( K_D \) value of ThDP was determined to 0.6 \( \mu \)M). The near-UV circular dichroism spectra, however, do not prove the cleavage of the synthesized \( \text{DL-DHEThDP} \) by transketolase, because they are identical to those of ThDP reconstituted enzyme.

Therefore, the determination of DHEThDP and ThDP from incubation mixtures of wild-type apotransketolase and \( \text{DL-DHEThDP} \) was performed by NMR via the changes of the C6'-H signal (singlet) of DHEThDP at 7.30 ppm, and of ThDP at 8.01 ppm, respectively, as described under “Materials and Methods.” As illustrated in Fig. 6, both enantiomers were accepted by the enzyme, but converted with different rates \( (k = 0.0012 \text{ s}^{-1} \text{ and } k' = 0.0002 \text{ s}^{-1}) \). The preparative separation of the intermediate DHEThDP and cofactor ThDP after 10- and 30-min incubation is shown in the insets of Fig. 6. A circular dichroism signal of the DHEThDP fraction could be detected in the wavelength range between 260 and 280 nm (not shown) and the degree of the signal, which depends on the incubation time, confirms the different reaction rates calculated from the NMR measurements.

The activity of the wild-type apotransketolase reconstituted on incubation with \( \text{DL-DHEThDP} \) was measured either by the complete assay according to Kochetov (27) or the modified assay according to Sprenger et al. (28). The data are summarized in Table II and are related to the wild-type holotransketolase. In the presence of the natural substrates \( \text{D-xylulose-5-phosphate} \) and \( \text{D-ribose-5-phosphate} \), the enzyme reconstituted with \( \text{DL-DHEThDP} \) established full catalytic activity as found for holotransketolase. On the other hand, no catalytic activity could be detected for the wild-type apotransketolase-DHEThDP complex using the acceptor substrate \( \text{D-erythrose-4-phosphate} \), which is one of the best acceptor substrates known for transketolase having a \( K_m \) value of about 41 \( \mu \)M. The concentration of \( \text{DL-DHEThDP} \) in these experiments was between 0.5 and 1 mm and far above the \( K_D \) value determined.

DISCUSSION

Cleavage of Donor Substrates by Wild-type and H263A Mutant Transketolase—According to Scheme 1 the kinetic mechanism of action of transketolase comprising two reaction sequences follows a ping-pong mechanism. A donor substrate (naturally a ketosugar) is cleaved, and a glycolyl moiety covalently bound to ThDP in the holoenzyme yields enzyme-bound \( \alpha \)-carbanion of DHEThDP (24). The two-carbon unit is transferred to an acceptor substrate (naturally an aldose sugar) in the second reaction under reformation of the holoenzyme. It was unknown whether transketolase is able to cleave a donor substrate in the absence of the acceptor substrates as well. We could prove that the enzyme is indeed able to cleave the donor substrate \( \text{D-xylulose-5-phosphate} \) in a single turnover reaction. The release of the product \( \text{D-glyceraldehyde-3-phosphate} \) could be monitored by a coupled kinetic test system using triose phosphate isomerase and glycerol-3-phosphate dehydrogenase as auxiliary enzymes.
Donor Substrate Conversion in Transketolase

The cleavage of d-xylulose 5-phosphate can be divided into two phases, a burst phase, the amplitude of which corresponds to a single turnover, and a slower second phase proceeding continuously. The latter phase could be related to the production of d-glyceraldehyde 3-phosphate from d-xylulose 5-phosphate. This continuous production of d-glyceraldehyde 3-phosphate could result either from a replacement of the enzyme-bound α-carbanion of DHEThDP by ThDP present in excess in the incubation mixture or from a cleavage of enzyme-bound α-carbanion of DHETHDP to enzyme-bound ThDP and glycolaldehyde. Both of these reactions would result in the formation of holotransketolase being available for a next cycle with donor substrate. However, the reaction of chemically synthesized DL-DHETHDP with the apoenzyme of transketolase, which shows a cleavage of this intermediate (Fig. 6) at a low rate, should result from the cleavage of enzyme-bound α-carbanion of DHETHDP.

The cleavage of the donor substrate d-xylulose 5-phosphate was measured both with wild-type and H263A mutant transketolase. Investigation of the progress curves at various donor substrate concentrations yielded at least four kinetic constants, which are related to the catalytic steps illustrated in Scheme 3. A comparison of the corresponding rate constants between the two proteins offers differences in the catalytic action studied. It was impossible to calculate precisely equilibrium constants (K in Table I, reflecting the binding and dissociation of the donor substrate according to Scheme 3) from the progress curves, but the nearly identical pH/D-exchange rate constants show that the lower overall catalytic activity of the H263A variant cannot be due to the decelerated deprotonation reaction of the enzyme-bound ThDP and precludes a possible involvement of His263 in the acid-base catalysis of the initial proton abstraction at the C2 atom. Consequently, the His263 is either directly involved in subsequent elementary steps of the catalytic cycle or in the stabilization of reaction intermediates. The wild-type enzyme, on one hand, displays a higher rate constant of the donor substrate cleavage (k3) in Table I) than the H263A variant. On the other hand, the cleavage of the glycolyl moiety (k4) in the H263A mutant enzyme is surprisingly faster than in the wild-type transketolase. These observations indicate that His263 is involved in the reaction leading to the cleavage of the donor substrate, probably by acting as a catalytic base abstracting a proton from the C3-hydroxy group of the donor substrate (18) and by stabilizing the formed α-carbanion intermediate through its positive charge. The loss of charge stabilization in the H263A variant may then explain why the continuous production of d-glyceraldehyde 3-phosphate is much faster in this mutant enzyme than in the wild-type. Similar rate constants k3 and k4 were obtained for the wild-type reaction by substituting the directly calculated rate constants for the primary binding (k1 and k2) with the separately determined values from the measurement of the direct binding of the donor substrate d-fructose 6-phosphate (Table I).

**Near-UV Circular Dichroism Spectroscopy**—The negative extremum at 320 nm appearing in the near-UV circular dichroism spectrum of holotransketolase disappeared after addition of the donor substrates d-xylulose 5-phosphate and d-fructose 6-phosphate and a new positive band with an extremum at about 300 nm appears in both cases (Fig. 3). This is indicative for the same asymmetry (state) of the reactants in the futile cycle reaction with the products(s) originating from the first half of the reaction being the reactant(s) of the condensation reaction (second half-reaction). After incubation of holotransketolase with HPA, an inversion of the circular dichroism spectrum occurs (Fig. 4), which is very likely related to enzyme-bound hydroxylactyl-ThDP or the α-carbanion of DHETHDP. Simultaneous incubation of holotransketolase with HPA as donor and d-glyceraldehyde 3-phosphate as acceptor substrate results in the production of d-xylulose 5-phosphate, which is itself a substrate of the first half-reaction as described above. The circular dichroism spectrum of holotransketolase after incubation with HPA and d-glyceraldehyde 3-phosphate in equimolar amounts showed the same spectrum as measured for d-xylulose 5-phosphate (Figs. 3 and 4). The corresponding progress curve and the calculated rate constant of this reaction (3.5 s⁻¹) are illustrated in the inset of Fig. 4.

**Interaction of Wild-type Apotransketolase with DL-DHETHDP**—Chemically synthesized DL-DHETHDP was incubated with the apoenzyme of transketolase, revealing a Kₚ value of 4 μM (racemate) similar to that of ThDP (0.6 μM). Both enantiomers of the analogue are cleaved by transketolase at rather low rates as shown by ¹H NMR spectroscopy (Fig. 6). The enzyme incubated with DL-DHETHDP was unable to transfer the glycolyl moiety to the acceptor substrate d-erythrose 4-phosphate. This was in contrast to the control experiment performed with HPA as glycolyl donor and d-erythrose 4-phosphate as acceptor substrate, where the formation of d-fructose 6-phosphate was detected in the coupled enzymatic test system (Table II). The failure of DHETHDP to act as the precursor of reaction intermediate in the transketolase reaction at catalytically relevant rates may be due to the lack of a suitably positioned enzymic group able to abstract the proton at the α-carbon of DHETHDP, a prerequisite for the binding of acceptor substrate. The presence of such a group might in fact counteract catalytic deficiency, because of possible protonation of the donor substrate.
α-carbanion intermediate, giving rise to an unwanted side reaction.

Acknowledgment—We thank Christer Wikner for providing the plasmid of the mutant H263A and Georg Wille for his help in investigating the enzyme kinetics using the program Dynafit.

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J. Biol. Chem. 2001, 276:16051-16058.
doi: 10.1074/jbc.M007936200 originally published online February 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M007936200

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