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Catalytically Important Residues in *E. coli* 1-Deoxy-D-Xylulose 5-Phosphate Synthase

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Abstract

1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the initial step of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway consisting in the condensation of (hydroxiethyl)thiamin derived from pyruvate with D-glyceraldehyde 3-phosphate (GAP) to yield 1-deoxy-D-xylulose 5-phosphate (DXP). The role of the conserved residues H49, E370, D427 and H431 of *E. coli* DXS was examined by site-directed mutagenesis and kinetic analysis of the purified recombinant enzyme mutants. Mutants at position H49 showed a severe reduction in their specific activities with a decrease of the k_{cat}/K_M ratio by two orders of magnitude lower than the wild-type DXS. According to available structural data residue H49 is perfectly positioned to abstract a proton from the donor substrate. Mutations in DXS E370 showed that this residue is also essential for catalytic activity. Three-dimensional structure supports its involvement in cofactor deprotonation, the first step in enzymatic thiamin catalysis. Results obtained with H431 mutant enzymes indicate that this residue plays a role contributing to transition state stabilization. Finally, mutants at position D427 also showed a severe specific activity decrease with a reduction of the k_{cat}/K_M ratio. A role in binding the substrate and selecting the stereoisomer is proposed for D427.

Keywords

Active Site, 1-Deoxy-D-Xylulose 5-Phosphate Synthase, Isoprenoid Biosynthesis, Kinetic Parameters, MEP Pathway, Methylerythritol Phosphate, Mutagenesis

1. Introduction

Isoprenoids, the most diverse group of natural products with thousands of compounds identified [1] derive from two common C5 units: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In most eubacteria IPP and DMAPP biosynthesis proceed exclusively through the methylerythritol 4-phosphate (MEP) pathway [2] [3]. In the first reaction pyruvate and D-glyceraldehyde 3-phosphate (GAP) are condensed to yield deoxyxylulose 5-phosphate (DXP). This involves the decarboxylation of pyruvate and the transfer of the resulting C2 unit to the aldehyde group of GAP (**Figure 1**) in a reaction dependent on thiamin diphosphate (TDP).

This reaction is catalyzed by DXP synthase (DXS) [4]-[6], an enzyme belonging to the superfamily of TDP-dependent enzymes including transketolases and the E1 subunit of pyruvate dehydrogenase. Then, DXP is con-

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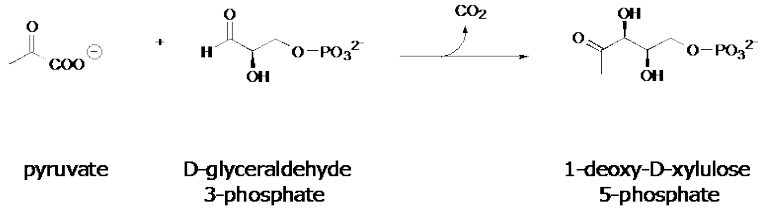


Figure 1. Reaction catalyzed by deoxyxylulose 5-phosphate synthase (DXS).

verted by intramolecular rearrangement and reduction into 2-C-methyl-D-erythritol 4-phosphate (MEP) in a reaction catalyzed by DXP reductoisomerase (DXR) [7]. Since DXP is also the substrate for the synthesis of vitamins B1 (thiamin) and B6 (pyridoxol) [8] [9], MEP is the first specific intermediate of the pathway and it is accepted to name it as MEP pathway. Many pathogenic microorganisms, such as *M. tuberculosis* [10] and the malaria parasite *P. falciparum* [11], synthesize IPP and DMAPP through the MEP pathway. Given the essential role of isoprenoids in these organisms, and the fact that the MEP pathway is absent in mammals, where these precursors are exclusively synthesized via the mevalonate pathway, all the enzymes participating in the MEP pathway are potential targets for the design of inhibitors that could be used as antibiotic or antimalarial drugs [10] [11]. In this context, DXS is one of the most attractive targets, because of its regulatory role in the biosynthesis of isoprenoids in various systems [12] [13], indicating that this enzyme catalyzes a rate-limiting step in the MEP pathway. Identification of catalytically essential amino acid residues is necessary in order to design effective drugs. In the present work we analyzed the role of other conserved residues in DXS activity by site-directed mutagenesis. Nine recombinant proteins (wild-type, H49Q, H49A, E370Q, E370A, D427N, D427A, H431Q and H431A) were purified to apparent homogeneity using Ni^{2+}-affinity chromatography and kinetic analysis of the mutant enzymes were performed in order to examine their catalytic role in *E. coli* DXS.

2. Materials and Methods

2.1. Materials

Materials from commercial sources included NADPH, DL-glyceraldehyde 3-phosphate (DL-GAP) and TDP from Sigma. Hi-Trap Chelating columns were from GE Healthcare. The protease inhibitors Pefabloc SC Plus and the protease inhibitor cocktail Complete EDTA-free were obtained from Roche. All other reagents were of analytical grade. Recombinant *E. coli* DXR was obtained as described in [14].

2.2. Construction of the *E. coli* DXS Expression Vector pET-23-DXS

The coding region of the *E. coli* DXS, cloned into the expression vector pT7-7 [15], was amplified by PCR using *Pfu* DNA polymerase and primers T7 (5'-TAATACGACTCACTATAGG-3') and pET-23-*XhoI* (5'-CGCTCGAGTCCTGCCAGCCAGGCCTTGATTTTGGC-3'). After digestion with *NdeI* and *XhoI*, the amplified DNA fragment was ligated into the same sites of the expression vector pET-23(b). Strain DH5*α* (Promega) was used as the recipient during this transformation. Positive clones were identified by DNA sequencing. The resulting plasmid, pET-23-DXS, produces a C-terminal histidine-tagged enzyme (DXS-cHis).

2.3. Site-Directed Mutagenesis

Point mutations H49Q, H49A, E370Q, E370A, D427N, D427A, H431Q and H431A were introduced into the *E. coli* DXS coding sequence by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) as described [15]. Primers used for introducing these point mutations are summarized in **Table 1**. The underlined letters represent the substituted nucleotides. Presence of the desired mutations and absence of other mutations that could be introduced during PCR were verified by DNA sequencing.

2.4. Overexpression and Purification of Cloned DXS Proteins

BL21 (DE3) *pLysS* cells carrying pET-23-DXS or the corresponding mutants were grown in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 22˚C to an absorbance at 600 nm of 0.3 - 0.4 and then induced with 0.3 mM isopropyl *β*-D-thiogalactoside for 18 - 20 h. Bacterial cells were recovered by

Table 1. Primers used in this study for site-directed mutagenesis[a].

name	Sequence
H49Q	5'-CCAGCGGGCAGTTCGCCTCC-3'
H49A	5'-TCCAGCGGGGCCTTCGCCTC-3'
E370Q	5'-GCAATTGCCCAGCAACACGCGG-3'
E370A	5'-GCAATTGCCGCGCAACACGCG-3'
D427N	5'-TGTTGGTGCTAACGGTCAAACCC-3'
D427A	5'-GTTGGTGCTGCCGGTCAAACCC-3'
H431Q	5'-GGTCAAACCCAGCAGGGTGCTT-3'
H431A	5'-GGTCAAACCGCTCAGGGTGCTT-3'

[a]The underlined letters represent the substituted nucleotides.

centrifugation and the cell pellet was resuspended in Buffer 1 (40 mM Tris-HCl, pH 8.5, 100 mM NaCl, 10 mM imidazole, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol) containing 1 mM TDP, lysozyme (1 mg/ml), Pefabloc SC Plus (1 mM) and one tablet of protease inhibitor cocktail Complete EDTA-free. After incubation at 4˚C for 30 min and brief sonication (3 pulses, 30 w, 20 s), lysate was centrifuged at 12,000 ×g for 45 min at 4˚C. Recombinant DXSs were purified by Ni^{2+} affinity chromatography (1ml Hi-Trap Chelating column, GE Healthcare) with a linear gradient of 10 to 500 mM imidazole. Fractions containing DXS were pooled, dialyzed by ultrafiltration (Ultrafree-0.5 and Ultrafree-4 centrifugal filter Biomax-5K, Millipore) against 50 mM glycylglycine buffer (pH 7.8), 1 mM $MgCl_2$, 1 mM TDP, 5 mM 2-mercaptoethanol and stored at −20˚C in 50% glycerol. Purified fractions were analyzed by SDS-PAGE. After dialyzing the samples against (1) glycylglycine buffer 10 mM, pH 7.8, 1 mM DTT, 50 mM EDTA and (2) glycylglycine buffer 10mM, pH 7.8, 1 mM DTT, protein concentration was determined by measuring the absorbance at 280 nm using an Abs 1% = 6.25.

2.5. DXS Activity Measurements and Determination of Kinetic Parameters

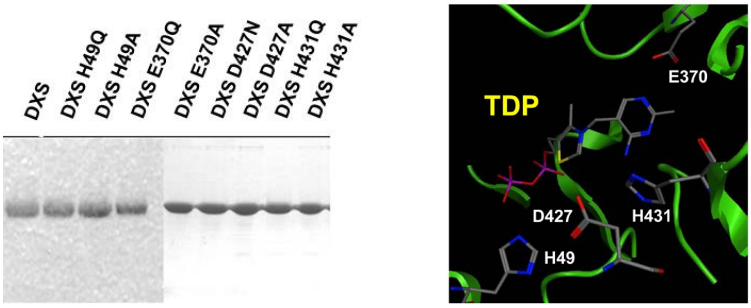
Specific activity for wild-type and mutant DXS proteins was monitored by a coupled system using purified *E. coli* DXR [16]. All kinetic measurements were made at 37˚C in triplicate. The standard reaction mixture consisted in 50 mM glycylglycine buffer pH 7.8, 1 mM TDP, 1 mM $MgCl_2$, 0.05 mM $MnCl_2$, 1 mM DTT, 0.15 mM NADPH, 1 mM pyruvate, 50 μg DXR and variable amounts (10 μg to 160 μg) of DXS or the corresponding mutants in a final volume of 1 ml. Mixtures were incubated at 37˚C for 10 min and the reaction was initiated by adding 1 mM DL-GAP. Reactions were conducted at 37˚C and initial velocities were monitored at 340 nm due to the oxidation of NADPH in a UNICAM UV-3 spectrophotometer. Specific activities were determined from the slope of a plot of initial velocities versus the enzyme amount.

Steady-state kinetic parameters for the wild-type and mutant enzymes were determined at saturating concentrations of the second substrate, 0.05 mM to 1.6 mM pyruvate and 25 μM to 800 μM DL-GAP, by measuring initial rates. K_M values for pyruvate and GAP were calculated by fitting the data to the Michaelis-Menten equation using the program GRAFIT. For mutants H49Q, H49A, D427N, D427A and H431Q, k_{cat}/K_M values for pyruvate were calculated from initial velocities at unsaturating concentrations of this substrate (30 μM).

3. Results and Discussion

3.1. Expression of DXS-cHis Mutants in *E. coli*

The gene encoding *E. coli* DXS was cloned into the expression vector pET-23. Mutations at positions H49, E370, D427 and H431 were introduced by site-directed mutagenesis. DNA sequencing confirmed that undesired mutations were not introduced during the course of mutagenesis. Over expression of the wild-type and mutant enzymes was carried out in *E. coli* BL21 (DE3) *pLysS*. Purification of the recombinant proteins, containing a His-tag at the C-terminus, was performed in a single chromatographic step (Hi-Trap Chelating, GE Healthcare). Wild-type and mutant DXS proteins were obtained in similar amounts (≥10 mg of pure DXS/litre) and purified (>95%) as shown by SDS-PAGE and Coomassie-blue staining (**Figure 2(a)**). The enzyme purity was comparable for the wild-type and the mutant proteins.



(a) (b)

Figure 2. (a) SDS-PAGE analysis of purified E. coli DXS mutants. Purified DXS mutants obtained by using a Hi-Trap Chelating column were analyzed by SDS-PAGE. Proteins were stained with Coomassie brilliant blue R-250. Purified wild type and mutant proteins are indicated in the top of the gel; (b) Pose of the mutated residues and the coenzyme TDP at the catalytic site of E. coli DXS based on structural data reported at [17].

3.2. Kinetic Studies

E370Q and E370A mutations resulted in a completely inactive DXS and the H431A mutant showed only a moderate decrease in its specific activity compared to the wild-type enzyme (0.84 and 1.46 μmol pyruvate/min μg, respectively). The greatest decrease in activity levels were observed with the H49Q, H49A, D427N and D427A mutants which showed only 2%, 6%, 3% and 10% of the activity of the wild-type DXS. H431Q also exhibited a reduced catalytic activity (4% of the wild-type) while H431A mutant retained about 57% activity.

For mutants at positions H49, D427 and H431Q k_{cat} values were obtained from the plot of initial velocities versus the enzyme amount. k_{cat}/K_M values for pyruvate were determined by measuring the initial velocities at subsaturating concentrations of pyruvate (**Table 2**).

We previously reported that mutation of residue H49 by glutamine in *E. coli* DXS resulted in a mutant enzyme with undetectable activity [15]. Here we have analyzed the effect of H49Q and H49A point mutations by measuring their kinetic parameters k_{cat} and k_{cat}/K_M. Both mutations result in severally impaired catalytic activities. k_{cat}/K_M values for mutant H49A was two orders of magnitude lower than the wild-type enzyme. No kinetic parameters could be determined for mutant H49Q due to its reduced specific activity. The H431A mutant retained 57% specific activity but increased K_M for pyruvate (one order of magnitude compared to the wild-type). No significant change in the K_M for GAP was observed for this mutant. When residue H431 was replaced by glutamine, k_{cat} was reduced by two orders of magnitude. Kinetic analysis of the purified D427N and D427A mutants showed reduced k_{cat} values (3.3 min^{-1} and 10 min^{-1} respectively, compared to the value of 100.3 min^{-1} for the wild-type enzyme) (**Table 2**).

Site-directed mutagenesis was used to confirm the essential role of selected residues for *E. coli* DXS activity. Kinetic parameters of the mutant enzymes H49Q and H49A obtained in this work corroborate that this residue is essential for DXS activity. The structure of the catalytic center of *E. coli* DXS [17] confirms that residue H49 is perfectly positioned to abstract a proton from the donor substrate.

Kinetic analysis of the purified DXS mutant enzymes at the corresponding positions (E370, D427 and H431) were carried out to establish the role of these conserved residues for *E. coli* DXS catalytic activity. Residue E418, equivalent to E370 in DXS, has been reported to be essential for TK catalytic activity since its implication in cofactor deprotonation, the first step in enzymatic thiamin catalysis [18]. Analysis of mutations in DXS E370 demonstrated that this residue is also essential for catalytic activity. *E. coli* DXS 3D-structure [17] shows a direct hydrogen bond to the pyrimidine of thiamin supporting the catalytic role suggested by Wikner *et al*. [19].

Site-directed mutagenesis studies of residue H481 in yeast transketolase indicate that this residue contributes to transition state stabilization but is not absolutely required for catalysis [19]. When the *E. coli* DXS histidine residue at position 431 was replaced by glutamine, a mutant DXS with a severe reduction in enzyme activity (4% of the wild-type) was obtained. In contrast, moderated effects in terms of specific activity and k_{cat} were observed when H431 was replaced by alanine. Only K_M for pyruvate was increased one order of magnitude (**Table 2**). Similar results have been obtained for mutants at residue H481 in yeast transketolase [19] or the correspond-

Table 2. Comparison of kinetic parameters of wild-type (DXSwt) and mutant DXS enzymes.

Enzyme	K_{Mpyr} (μM)	K_{MGAP} (μM)	k_{cat} (min^{-1})	k_{cat}/K_{Mpyr} ($min^{-1}\cdot\mu M^{-1}$)
DXSwt	134±8	41±6	100.3	0.7480
H49Q	n.d.	n.d.	n.d.	n.d.
H49A	n.d.	n.d.	6.6	0.0091
D427N	n.d.	n.d.	3.3	0.0022
D427A	n.d.	n.d.	10.0	0.0095
H431Q	n.d.	n.d.	4.4	0.1600
H431A	1623±81	62±6	57.4	0.0350
E370Q	n.d.	n.d.	n.d.	n.d.
E370A	n.d.	n.d.	n.d.	n.d.

ing residue in human transketolase Q428 [20]. Site-directed mutagenesis studies of H481 in yeast transketolase suggested that this residue contributes to transition state stabilization but is not required for catalysis [20]. Our results would indicate that TK H481 and DXS H431 are conserved equivalent residues playing similar roles. The fact that H431Q mutant shows a markedly reduced specific activity could be explained by considering that the increase in side chain size may result in steric clashes with the substrate.

Substitution of aspartic acid residue at position 427 by glutamine or alanine yielded a DXS with highly decreased specific activity with k_{cat}/K_M ratio reduced by two orders of magnitude. The hydrogen at the side chain of the corresponding residue, D477, in yeast TK is proposed to be the determinant of the enantioselectivity of the enzyme [21].

In this study we describe the active site architecture of 1-deoxy-D-xylulose 5-phosphate synthase, a target for antimicrobial agents, herbicides, and antimalarial drugs [22] [23]. A role in catalysis has been assigned to conserved amino acid residues and a model of the catalytic site has been generated which can be of great interest to predict drug-enzyme interactions.

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