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Mutagenic Characterization of a Conserved Functional Amino Acid in Fuculose-1-Phosphate Aldolase from *Methanococcus jannaschii*, a Hyperthermophic Archaea

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Abstract To elucidate the putative role of the amido group in the metal binding of the fuculose-1-phosphate aldolase from *Methanococcus jannaschii*, we have examined a potential target using site-directed mutagenesis. The replacement of asparagine 25 with leucine or threonine was shown to have a negative effect, not only on catalytic efficiency, but also on substrate recognition as well. The Hill coefficient values yielded a value of ≈1. All metals used with the wild-type aldolases exhibited higher activity than that of the mutants. The spectra of the mutants were quite different from the wild-type aldolase. A highly conserved amino acid of asparagine 25 in a related family of aldolase does not appear to provide sufficient evidence for evolution.

Key words: Aldolase, site-directed mutagenesis, *Methanococcus jannaschii*

L-Fuculose-1-phosphate aldolase (FucA) from *Methanococcus jannaschii*, a hyperthermophic archaea [2, 10, 11, 12, 16], catalyzes the cleavage of L-fuculose-1-phosphate to dihydroxyacetone phosphate (DHAP) and L-lactaldehyde, i.e. the conversion of six- to three-carbon units, which is a central step in bacteria fucose metabolism [6]. Besides their importance in major metabolic pathways, FucA and other DHAP-dependent aldolases have gained much attention as catalysts for aldol additions, where they offer an effective method for stereospecific carbon-carbon bond formation [1, 13, 14]. In particular, these aldolases carry a great potential for the synthesis of rare sugars which are hardly accessible via classic organic syntheses. A common feature of all aldolases studied is their high specificity for DHAP and their comparatively low specificity for the aldehyde component [5, 15].

There is about a 30% overall sequence identity in the aldolase enzyme family and a much higher degree of homology when including conservative substitutions. In addition, there are several well-defined regions of high sequential identity [3]. This sequential homology has assisted in the selection of potential targets which are examined by site-directed mutagenesis. Asparagine 25 was chosen as a target for mutagenesis, because it is highly conserved and it has a putative role in metal binding as revealed by previous structural studies [4, 8, 9]. Among the changes which have been engineered into the amino acid sequence of the aldolase are conservative changes that preserve the properties of the original amino acid (asparagine → threonine), and changes that preserve the side chain volume but eliminate the amino acid charge (asparagine → leucine). These specific amino acid substitutions have been carried out in order to examine the role of the conserved functional amino acid in aldolase.

The wild-type enzyme and mutant enzymes were characterized with respect to the saturation curves for the substrates. Substrate curves for the wild-type enzyme and mutants N25T and N25L were hyperbolic (data not shown). The kinetic data of the wild-type and mutant enzymes are compared in Table 1. The V_{max}/E_t values of the mutants are about 5 times less than that of the wild-type enzyme. No significant difference was observed between the V_{max}/E_t values of the N25T and N25L mutants. The K_m of the mutant N25T for DHAP was approximately 5-fold higher than that of the wild-type, indicating that asparagine 25 is probably involved in direct interactions with DHAP, and that there probably is a different electrostatic effect between asparagine and threonine. However, the K_m of the mutant N25L for DHAP was 3-fold lower than that of the wild-type, indicating that leucine 25 binding with a metal ion probably induces

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Table 1. Kinetic parameters of wild-type and mutant *M.J* aldolases.

Enzyme	V_{max}/E_t (sec^{-1})	K_m (DHAP, mM)	K_m (DL-glyceraldehyde)	K_{cat}/K_{DHAP}	$K_{cat}/K_{DL\text{-}glyceraldehyde}$
Native	7.6×10^4	1.01	7.8	7.5×10^4	9.7×10^3
N25T	1.3×10^4	5.0	8.2	2.6×10^3	1.6×10^3
N25L	1.4×10^4	0.36	23.1	3.9×10^4	6.1×10^2

the affinity of DHAP by a proper structural alternation. The K_m of the mutant N25T for D,L-glyceraldehyde was similar to that of the wild-type, indicating that asparagine 25 is probably not involved in direct interactions with D,L-glyceraldehyde. However, the K_m of the mutant N25L for D,L-glyceraldehyde was approximately 3-fold higher than that of the wild-type, indicating that the substitution of asparagine 25 with leucine probably causes minor structural alterations.

The k_{cat}/K_{DHAP} value of the mutant N25T and N25L decreased by a factor of twenty-nine and two, respectively. The $k_{cat}/K_{D,L\text{-}glyceraldehyde}$ value of the mutant N25T and N25L decreased by a factor of six and sixteen, respectively. A Hill plot corresponding to the activity data is shown in Fig 1. The Hill coefficients (*n* value) for the mutants were similar to the wild-type enzymes. Determination of the Hill coefficient *n* for DHAP concentrations between 0.25–2.5 mM yielded a value of ≈1, indicating that all active sites are saturated. Replacement of asparagine 25 with leucine and threonine was found to have a negative effect on catalytic efficiency and on substrate recognition. The reduction in catalytic activity can most likely be attributed to minor structural alterations. This view is supported by the small changes that have been observed in the circular dichroism spectrum of this mutant (*vide infra*).

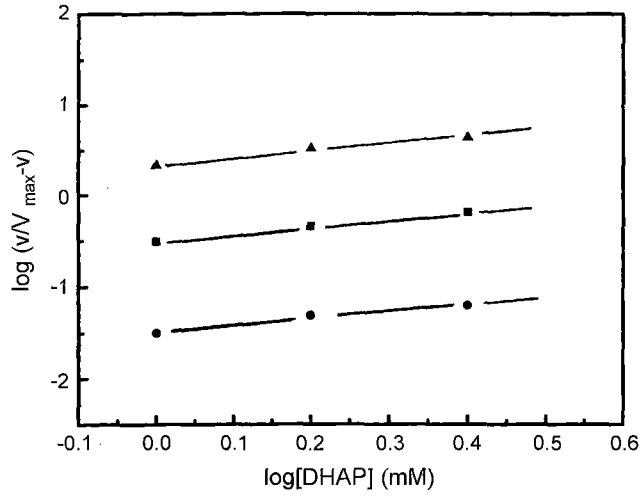

Fig. 1. Hill plots for the binding of DHAP to *Methanococcus jannaschii* aldolase.

The aldolase activity was measured by determining the remaining DHAP, as described by Ghalambor *et al.* [7]. The reaction mixtures at pH 8.0 contained 100 mM of Tris-HCl, 0.1 mM of $ZnCl_2$, 2.5 mM of DL-glyceraldehyde, and various concentrations of DHAP. The lines represent the wild-type (▲), N25L (■), and N25T (●).

Most aldolases from microorganisms are type II aldolases that require metal ions as cofactors. The structure of L-fuculose-1-phosphate aldolase showed that DHAP ligates the Zn^{2+} of this metal-dependent class II aldolase with its hydroxyl and keto oxygen atoms, shifting Glu 73 away from the zinc coordination sphere to a nonpolar environment [4]. The metal ion in type II aldolase serves as the Lewis acid in the active site.

Metal ion dependency activity of the wild-type and mutant enzymes was tested for several divalent cations. Prior to extensive dialysis, the enzymes were incubated for 30 min in the presence of 0.1 M EDTA. The activities of Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+} metal ions were examined (Table 2). At low concentrations (0.2 mM), Zn^{2+} was found to be the most potent activator among the examined divalent cations, indicating that the zinc ion consisted of the most pertinent geometry for a catalytic structure. At low concentrations, the most potent activators were as follows: in order of Co^{2+}, Mn^{2+}, Mg^{2+}, and Cu^{2+}. In fact, these results agree with that of Choi *et al.* [3]. However, Mg^{2+} at a high concentration (2 mM) was found to be the most potent activator among the divalent cations examined, indicating that the magnesium ion has the most pertinent geometry for a catalytic structure. At high concentrations, Co^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} followed in order of the next most potent activators (data not shown). The activity for the mutant N25T and N25L was 2 to 6 times lower, depending on the concentration. The activity order of N25T at low and high metal concentrations was the same as the wild-type enzyme. Regardless of high or low concentration level, all metals used with the wild-type aldolase revealed a higher activity than the N25T and N25L mutants.

The CD spectra were measured in order to compare the structure of the mutant enzymes with that of the wild-type enzyme (Fig. 2). The wild-type aldolase exhibited negative cotton effects from 206 through 218 nm. From these values, the helical content of the wild-type aldolase was estimated to be at least 30.3%, using poly-L-lysine as a standard (100%). The mutants N25T and N25L exhibited negative

Table 2. Metal effect of aldolase activity at 0.2 mM concentration.

	Wild-type	N25T	N25L
Zn^{2+}	0.022	0.012	0.006
Cu^{2+}	0.015	0.004	0.004
Co^{2+}	0.019	0.005	0.008
Mn^{2+}	0.019	0.008	0.006
Mg^{2+}	0.017	0.008	0.005

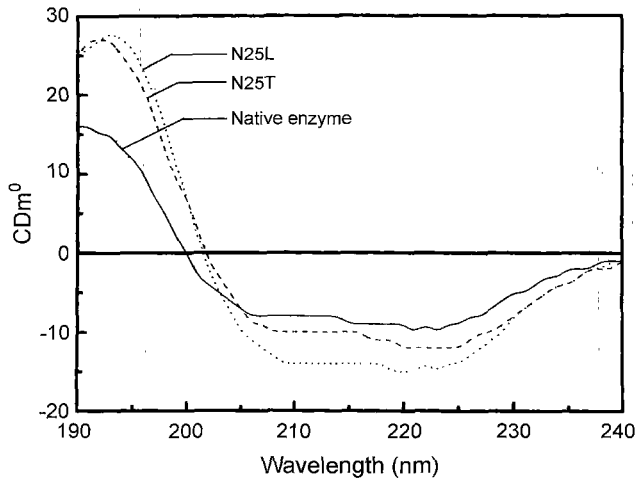

Fig. 2. CD spectra of the wild-type (——), N25T (-----), and N25L (·····) aldolase.
CD spectra were obtained at 25°C on a Jasco J-715CD spectropolarimeter. Samples were held in 50 µl quartz cuvettes. All far-UV spectra were scanned from 260 to 190 nm at intervals of 1 nm with a protein concentration of 0.1 mg/ml. The sample buffer was potassium phosphate buffer, pH 8.0, and each spectrum made up an average of three scans. The sample spectra were corrected by subtracting the appropriate buffer blank.

cotton effects from 208 to 224 nm and from 208 to 222 nm, respectively. The helical contents of the N25T and N25L mutants were 68.3% and 38.8%, respectively. The percentage of helical structure of the mutant N25T (68.3%) was more than twice as much as that of the wild-type aldolase, although the percentage (7.1%) of its sheet structure was 5 times less than that of the wild-type aldolase (35.3%). The percentages of the helical and sheet structures of the mutant N25L were very similar to those of the wild-type aldolase.

Sequence homology studies related to the protein families could frequently serve to identify potentially important amino acid residues. However, the conserved asparagine 25 residue has been shown to play no direct role in the mechanism of action for this aldolase. Although it is necessary to preserve the functionality of amino acid residues that play an essential role in DHAP binding, an identification of a highly conserved amino acid of this asparagine 25 in a related family of aldolase does not appear to provide sufficient evidence for evolution.

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