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A Class II fructose-1,6-bisphosphate aldolase from a halophilic archaebacterium *Haloferax mediterranei*

Sandra E. D'Souza* and Wijaya Altekar

Radiation Biology and Biochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

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Fructose-1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13) was purified 97-fold from a halophilic archaebacterium *Haloferax mediterranei*, with a specific activity of 2.8. The enzyme was characterized as a Class II aldolase on the basis of its inhibition by EDTA and other metal chelators. The enzyme had a specific requirement for divalent metal Fe^{2+} for activity. Sulfhydryl compounds enhanced aldolase activity.

Key Words——Class II aldolase; divalent metal; EDTA; enzyme purification; fructose-1,6-bisphosphate; *Haloferax mediterranei*; halophilic; metal chelator

Fructose-1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13), a glycolytic enzyme, catalyzes the reversible cleavage of FBP to D-glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). FBP aldolases have been classified into two groups on the basis of their molecular properties and the mode of enzyme catalysis (Marsh and Lebherz, 1992; Perham, 1990; Rutter, 1964). The widely studied Class I aldolase, the prototype of which is the rabbit muscle enzyme, catalyzes the cleavage of FBP through a carbanion intermediate, stabilized by a Schiff base formed between the substrate and an active site lysine residue of the enzyme (Horecker et al., 1972; Morse and Horecker, 1968). In contrast, the Class II enzyme, typified by the yeast aldolase, utilizes a bound divalent metal ion that acts as an electron sink to catalyze a similar reaction (Mildvan et al., 1971). Although the Class I enzyme is usually a tetramer of molecular mass about 160 kDa, the Class II enzyme is a dimer of molecular mass of approximately 80 kDa and binds one Zn^{2+} ion per subunit, as in *Saccharomyces cerevisiae* (Harris et al., 1969). The Class I aldolase is found in animals, plants, and green algae, and the Class II enzyme is distributed in bacteria, blue green algae, yeast, and fungi. However, some organisms like *Escherichia coli* (Scamuffa and Caprioli, 1980) and *Euglena gracilis* (Pelzer-Reith et al., 1994) and others show the presence of both types of aldolases. Class I aldolases have been well studied—their sequence (Tsutsumi et al., 1984) and crystal structure have been analyzed (White et al., 1991). In comparison, little is known about the Class II enzymes because these have been relatively difficult to purify in the quantities necessary for structural and mechanistic studies. With the cloning of the enzyme gene in *E. coli* (Alefounder et al., 1989), it has been possible to have access to large amounts of the enzyme for structural analyses (Berry and Marshall, 1993; Naismith et al., 1992).

Although these studies have been carried out in eubacteria and eukaryotes, hardly any reports exist on FBP aldolase activity in the group archaebacteria. The first report on aldolase activity (Class II type) was in the halophilic archaebacterium *Halobacterium halobium* (D'Souza and Altekar, 1982). Subsequently, a Class I aldolase was found in *H. halobium* R113 (Dhar and Altekar, 1986a). Investigations among the different halobacteria revealed the distribution of both classes of the enzyme, viz., Class II FBP aldolase in *Halobacterium salinarium*, *Haloferax mediterranei*, and *Haloferax volcanii*, and Class I aldolase in *Halobacterium* R113, *Haloarcula vallismortis*, and *Halobacterium saccharovorum* (Dhar and Altekar, 1986a, b; D'Souza and Altekar, 1982). Halobacteria have an absolute requirement for high salt (>15% NaCl) for growth and survival (Kushner, 1985). The cells have adapted to the high salt in the medium by having an intracellular high concentration of KCl

* Address reprint requests to: Dr. Sandra E. D'Souza, Radiation Biology and Biochemistry Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India.

(Kushner, 1985). Consequently, halobacterial enzymes require high salt for activity and stability (Eisenberg et al., 1992; Kushner, 1985; Lanyi, 1974). Thus the purification of these enzymes needs the maintenance of high salt concentrations during the different stages. $(NH_4)_2SO_4$-mediated adsorption chromatography on Sepharose 4B and DEAE cellulose (Mevarech et al., 1976) has proved a useful technique in the purification of halobacterial enzymes (Eisenberg et al., 1992). Krishnan and Altekar (1991) purified and characterized a Class I aldolase from *Haloarcula vallismortis.* In the present paper we describe the purification of a halobacterial Class II aldolase from *H. mediterranei* and compare its properties with other known Class II aldolases.

Materials and Methods

Chemicals. FBP, reduced glutathione, dithiothreitol, mercaptoethanol (β-ME), phenylmethylsulfonyl fluoride (PMSF), DNase, acrylamide, SDS, DEAE-cellulose, and standard protein markers were from Sigma (St. Louis, MO, USA). Sepharose 4B and Phenyl Sepharose were products of Pharmacia (Sweden). Hydroxylapatite and ammonium per sulfate were purchased from Sisco Research Laboratories, India. All salts of analytical grade were used.

Growth conditions. H. mediterranei (ATCC 37500) was grown in the synthetic medium of Rodriguez-Valera et al. (1983) with fructose (0.5%) as carbon source. The conditions for growth, harvest, and preparation of cell extract have been described earlier (D'Souza et al., 1997a).

Enzyme assay. FBP aldolase activity was measured by the colorimetric method of Sibley and Lehninger (1949), as described earlier (D'Souza et al., 1997b). The reaction mixture (0.5 ml) contained 56 mM hydrazine sulfate, 1 mM cysteine hydrochloride, 0.5 mM $FeSO_4$, 2.5 M KCl, 50 mM Tris-HCl, pH 7.5, 5 mM FBP, and halophilic aldolase. One unit of aldolase activity is the amount of enzyme that cleaves 1 μmol FBP per min at 37°C.

Protein assay. Protein was determined by Lowry's modified method of Peterson (1984), using bovine serum albumin as the standard.

Purification of FBP aldolase. Aldolase from *H. mediterranei* was purified by the procedure described below. All steps for purification were carried out at 25°C, except for the preparation of cell extract and $(NH_4)_2SO_4$ precipitation, which were performed at 4°C. High salt (>2.0 M $(NH_4)_2SO_4$ or KCl) has been maintained in the buffers through all the steps.

Cell extract. H. mediterranei cells (40 g) were suspended in 1 M KCl-1 M $(NH_4)_2SO_4$-50 mM Tris-HCl, pH 7.5 (1 : 3, w/v) and sonicated in a Vibra Cell Sonicator for 2 min, using a 50% pulse mode. DNase and PMSF were added to the sonicate, stirred for 30 min, and centrifuged at 12,000×*g* for 15 min. The supernatant constituted the cell extract.

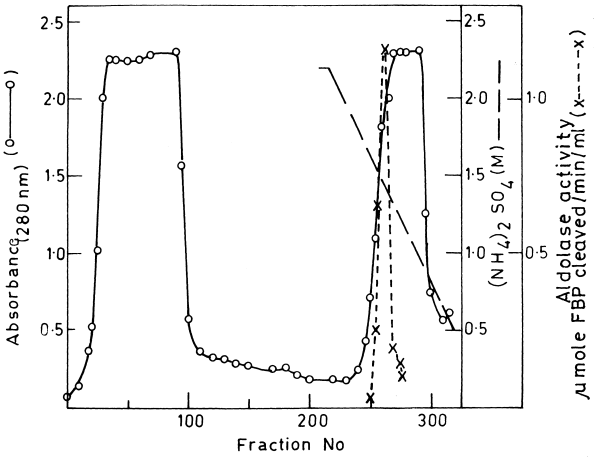

Fig. 1. Chromatography on Sepharose 4B.
Six hundred milliliters of 2.5 M $(NH_4)_2SO_4$ supernatant containing 1.7 g protein was loaded on the column. Details of the chromatography are given in MATERIALS AND METHODS.

$(NH_4)_2SO_4$ precipitation. To the cell extract was added, simultaneously with stirring, 1 volume of 50 mM Tris-HCl, pH 7.5, and solid $(NH_4)_2SO_4$ to make to 2.5 M. The extract was kept overnight at 4°C, then centrifuged at 12,000×*g* for 30 min. The supernatant containing aldolase activity was subjected to $(NH_4)_2SO_4$-mediated adsorption chromatography on Sepharose 4B, according to the procedure of Mevarech et al. (1976).

$(NH_4)_2SO_4$-mediated chromatography on Sepharose 4B. The supernatant was adsorbed on a column of Sepharose 4B (20×4 cm), equilibrated with 2.5 M $(NH_4)_2SO_4$-1 mM β-ME-50 mM Tris-HCl, pH 7.5 buffer (buffer A). The column was developed with a linear gradient of $(NH_4)_2SO_4$ (2.5–0.5 M) (Fig. 1). Fractions containing aldolase activity were pooled and solid $(NH_4)_2SO_4$ added to bring to a final concentration of 3.0 M.

$(NH_4)_2SO_4$-mediated DEAE-cellulose chromatography. The pooled aldolase fraction from Sepharose 4B was adsorbed to a DEAE-cellulose column (10×2.5 cm) equilibrated with buffer A. The enzyme was eluted by 2 M KCl-1 mM β-ME-50 mM Tris-HCl, pH 7.5. This step helped to achieve an exchange of KCl for ammonium sulfate.

Phenyl Sepharose chromatography. The DEAE-cellulose fraction containing aldolase activity was brought to 4 M KCl with solid KCl and loaded on Phenyl Sepharose column (20×2.5 cm), equilibrated with the same buffer. The chromatogram was developed by batchwise elution with decreasing concentra-

Table 1. Summary of the purification of Class II aldolase from *H. mediterranei.*

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Cell extract	55	1,870	0.03	1.0	100
$(NH_4)_2SO_4$ supernatant	51	1,707	0.03	1.0	93
Sepharose 4B	51	511	0.10	3.6	94
DEAE cellulose	51	434	0.12	4.0	94
Phenyl Sepharose	29	38	0.76	27	54
Hydroxylapatite	17	6	2.8	97	30

Details of purification are given in MATERIALS AND METHODS.

tion of KCl, from 4 to 1 M. Aldolase did not bind to Phenyl Sepharose, but it was purified from other proteins that remained bound to the gel.

Hydroxylapatite chromatography. The pooled aldolase fraction was diluted to 2.0 M KCl with 50 mM Tris-HCl, pH 7.5, and loaded on a hydroxylapatite column (5×2.0 cm), equilibrated with the same buffer. The proteins were eluted batchwise by increasing the concentration of K_2HPO_4/KH_2PO_4 (20 to 300 mM), pH 7.5, in 2.0 M KCl-1 mM β-ME. Aldolase eluted at 20–40 mM phosphate concentration.

SDS-PAGE. SDS-PAGE was performed in tube gels by using 7.5% acrylamide according to the method of Weber and Osborn (1969) after the removal of salt by dialysis. Standard protein markers such as bovine serum albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa) were used.

Molecular mass determination. Sucrose density gradient ultracentrifugation: The molecular mass of the native enzyme was determined by sucrose density ultracentrifugation (Martin and Ames, 1961). The enzyme (0.5 ml) was layered on 11 ml of sucrose gradient (5 to 20%, w/v) in 2.5 M KCl-50 mM Tris-HCl, pH 7.5, and centrifuged (4°C) for 19 h at 152,000×*g* in a Beckman SW41 rotor in a Beckman L8-M ultracentrifuge. Fractions (0.5 ml) were collected and assayed for enzyme activity. To estimate the size of the native enzyme, standard proteins were run under identical conditions and the proteins identified in the different fractions by absorbance at 280 nm.

Results

Purification and molecular mass

FBP aldolase was purified 97-fold with a specific activity of 2.8 units (Table 1). The halobacterial enzyme has been purified by techniques involving hydrophobic interactions between the gel and proteins under high salt concentration, viz., $(NH_4)_2SO_4$-mediated chromatography on Sepharose 4B and DEAE cellulose, Phenyl Sepharose chromatography in 4 M KCl, and hydroxylapatite chromatography in 2.0 M KCl.

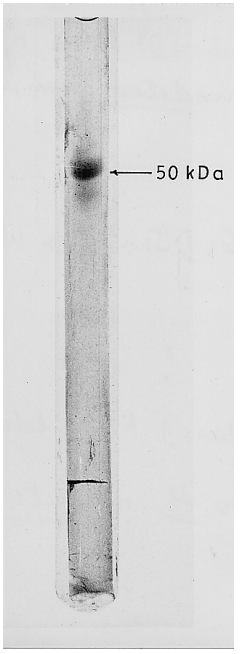

Fig. 2. SDS-PAGE of *H. mediterranei* aldolase.

SDS-PAGE analysis of this preparation revealed a heavy protein band corresponding to a molecular weight of 50±2 kDa and a minor band of 40±2 kDa (Fig. 2). During purification, a comparative SDS-PAGE revealed an increase in 50±2 kDa at each step of purification commensurate with the increase in specific activity of aldolase. Attempts to purify the enzyme further resulted in loss of activity. This purified aldolase preparation has been used in the following studies.

The native molecular weight of FBP aldolase as determined by sucrose density gradient ultracentrifugation was approximately 110±10 kDa (Fig. 3). Because the monomeric molecular mass is approximately 50±2 kDa, the enzyme can be considered to be a homomeric dimer.

Catalytic properties

Metal ion dependence. FBP aldolase activity was inhibited by metal chelators EDTA, *o*-phenanthroline,

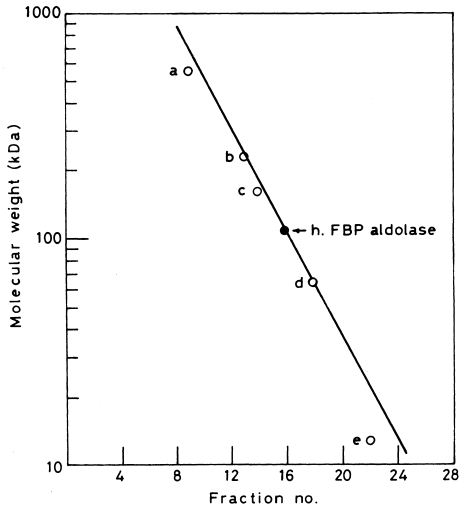

Fig. 3. Estimation of molecular mass of halobacterial aldolase by sucrose density gradient centrifugation.

The standards used were: a, apoferritin (440 kDa); b, bovine liver catalase (230 kDa); c, rabbit muscle aldolase (160 kDa); d, bovine serum hemoglobin (64.5 kDa), and e, horse heart cytochrome (12.5 kDa). ○, *H. mediterranei* aldolase.

and α,α'-bipyridyl at a concentration of 2 mM (Table 2). Although EDTA inhibited enzyme activity completely, there was partial activity in the presence of *o*-phenanthroline and α,α'-bipyridyl, viz., 15–25%.

An addition of excess divalent metal (10 mM) to the inhibited enzyme (i.e., enzyme+EDTA (2 mM)) revealed that activity could be completely restored only by Fe^{2+}. Other divalent metal ions such as Mn^{2+}, Mg^{2+}, and Zn^{2+} had no effect. Co^{2+} and Ca^{2+} could restore 20% and 10%, respectively, of enzyme activity (Table 3).

Optimum aldolase activity (137%) was observed with 1.0 mM Fe^{2+} concentration; higher concentrations (>1.0 mM), however, inhibited enzyme activity and also interfered with the colorimetric estimation of the triose phosphates.

Effect of sulfhydryl compounds on aldolase activity. Sulfhydryl compounds enhanced enzyme activity (Table 4). Aldolase activity increased with increasing concentration of the sulfhydryl compound—a 4-fold increase was observed with 10 mM cysteine and 30 mM glutathione. Dithiothreitol, up to 5 mM, enhanced enzyme activity twofold, higher concentrations interfered with the colorimetric assay of the triose phosphates. Mercaptoethanol alone did not activate the enzyme.

pH optimum. The pH optimum for aldolase activity was 7.5 in Tris-HCl buffer; in buffers like phosphate, glycylglycine, and imidazole, the pH optimum was broader, 7–8. The buffer constituent also affected enzyme activity, which was maximum in imidazole buffer (200%) and minimum in Tris-HCl buffer (100%).

Effect of KCl on enzyme activity. Aldolase activity increased with an increasing concentration of KCl (Fig. 4). At 4.0 M KCl concentration, activity was 2.5 times that at 0.5 M KCl concentration. Enzyme activity was appreciable even in the absence of added salt.

Kinetic constant. The K_m of *H. mediterranei* aldolase for FBP was 2 mM (Fig. 5).

Table 2. Effect of metal chelators on aldolase activity.

Addition	Aldolase activity (%)
Enzyme	100
Enzyme+EDTA	0
Enzyme+*o*-phenanthroline	15
Enzyme+α,α'-bipyridyl	24

The enzyme was preincubated with the inhibitor (2 mM) for 10 min before addition of the substrate.

Table 3. Reversal of EDTA inhibition by divalent metal ions.

Addition	Aldolase activity (%)
Enzyme	100
Enzyme+EDTA	0
Enzyme+EDTA+Fe^{2+}	99.5
Enzyme+EDTA+Ca^{2+}	10
Enzyme+EDTA+Mn^{2+}	0
Enzyme+EDTA+Mg^{2+}	0
Enzyme+EDTA+Zn^{2+}	0
Enzyme+EDTA+Co^{2+}	22

The enzyme was preincubated with EDTA (2 mM) for 10 min. Divalent metal ions (10 mM) were added to the inhibited enzyme.

Table 4. Effect of sulfhydryl compounds on aldolase activity.

Addition	Aldolase activity (%)
Enzyme	100
Enzyme+cysteine (1 mM)	119
Enzyme+cysteine (5 mM)	250
Enzyme+cysteine (10 mM)	388
Enzyme+dithiothreitol (1 mM)	150
Enzyme+dithiothreitol (5 mM)	200
Enzyme+dithiothreitol (10 mM)[a]	—
Enzyme+glutathione (1 mM)	156
Enzyme+glutathione (5 mM)	156
Enzyme+glutathione (10 mM)	178
Enzyme+glutathione (30 mM)	428
Enzyme+mercaptoethanol (5 mM)	100
Enzyme+mercaptoethanol (10 mM)	100
Enzyme+mercaptoethanol (30 mM)	100

[a] Dithiothreitol at concentrations of 10 mM and higher interfered with the colorimetric assay of enzyme activity.

Discussion

Of the many Class II aldolases that have been purified and characterized, the yeast enzyme has yielded a wealth of information on the function of the divalent

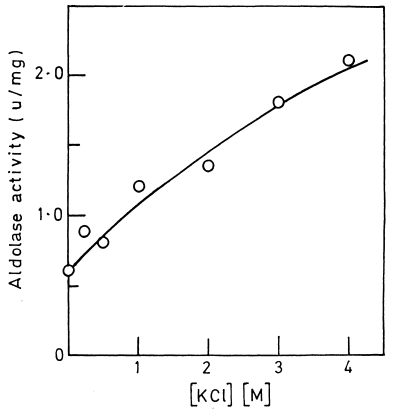

Fig. 4. Effect of KCl concentration on aldolase activity.

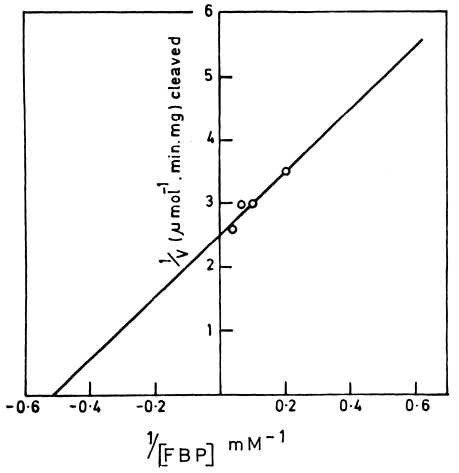

Fig. 5. Lineweaver-Burk plot of *H. mediterranei* aldolase for FBP.

metal ion and its involvement in the catalysis of the reaction (Mildvan et al., 1971). Subsequently, with the cloning of the *fda* gene of *E. coli*, structural and sequencing studies have added to the information of Class II aldolases (Alefounder et al., 1989; Berry and Marshall, 1993; Naismith et al., 1992). The Class II aldolases that have been purified to date, either from a eubacterium, e.g., *E. coli* (Baldwin et al., 1978) and *Bacillus stearothermophilus* (Hill et al., 1976), or from a eukaryote, viz., *S. cerevisiae* (Harris et al., 1969), show a similarity in their requirement for a divalent metal ion for activity and in their inhibition by EDTA and other metal chelators.

The present studies describe for the first time a Class II FBP aldolase that has been purified from a halophilic archaebacterium, *H. mediterranei.* The enzyme exhibited a subunit molecular mass of 50±2 kDa on SDS-PAGE and a native mass of 110±10 kDa by the sucrose density ultracentrifugation method. This suggests that the enzyme is a homomeric dimer, a feature shared by other Class II aldolases derived from a variety of bacteria and fungi.

The subunit molecular mass of most Class II aldolases is 40 kDa, as in yeast (Harris et al., 1969), *E. coli* (Baldwin et al., 1978), and *E. gracilis* (Pelzer-Reith et al., 1994). However, in some bacilli, e.g., *B. stearothermophilus* (Sugimoto and Nosoh, 1971) and *Bacillus subtilis* (Ujita, 1978), the subunit mass is 30 kDa. The eukaryotic Class I aldolase, on the other hand, is a tetramer with the subunit molecular mass of 40 kDa (Rutter, 1964). From our studies, the molecular mass of 50±2 kDa for the *H. mediterranei* Class II aldolase subunit seems slightly bigger than the reported subunit mass of other Class II aldolases.

Inhibition by metal chelators indicates the dependency of *H. mediterranei* aldolase on divalent metal for its activity. This confirms our earlier studies, in vitro (Dhar and Altekar, 1986b) and in situ (using permeabilized and stabilized whole cells) (D'Souza et al., 1992), that aldolase of *H. mediterranei* is of the Class II type. Of all the divalent metals tested, only Fe^{2+} could reverse EDTA inhibition completely, indicating that the metal requirement for enzyme activity was specific for Fe^{2+}. Co^{2+} could restore only 20% activity. In most Class II aldolases, viz., *Aspergillus* (Jaganathan, 1956), *S. cerevisiae* (Kobes et al., 1969), *B. stearothermophilus* (Hill et al., 1976), *E. coli* (Baldwin et al., 1978), and *E. gracilis* (cytosol enzyme) (Pelzer-Reith et al., 1994) the naturally occurring metal ion, Zn^{2+}, can be replaced by other divalent ions such as Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+} to yield a holoenzyme of lower activity.

The divalent metal in most Class II aldolases is Zn^{2+}, as in *S. cerevisiae* (Kobes et al., 1969), *Aspergillus niger* (Jagannathan, 1956), *B. stearothermophillus* (Hill et al., 1976), and *E. coli* (Berry and Marshall, 1993). In some other aldolases, viz., *Clostridium perfringens* (Groves et al., 1966) and *Vibrio marinus* (Jones et al., 1979), the divalent metal is Co^{2+}, and in cyanobacteria (Willard and Gibbs, 1975) and *Cl. perfringens* (Groves et al., 1966), it is Fe^{2+}. The Class II aldolases of halophilic archaebacteria, viz., *H. halobium* (D'Souza and Altekar, 1982) and *H. mediterranei*, have been found to be Fe^{2+} dependent.

H. mediterranei aldolase activity was enhanced by sulfhydryl compounds, suggesting a requirement of -SH groups for activity. The requirement of exogenous thiol compounds for optimal activity has been observed in yeast and other Class II aldolases (Rutter et al., 1966; Ujita, 1978; Willard and Gibbs, 1975). The halophilic enzyme showed a pH optimum of 7.5, in keeping with other Class II aldolases (Rutter et al., 1966).

The K_m of the enzyme for FBP is 2.0 mM, a value quite close to that observed in other Class II aldolases viz., *Bacillus* enzyme (Ujita, 1978) and *Aspergillus* en-

zyme (Jagannathan et al., 1956). The aldolases of yeast (Harris et al., 1969), *Clostridium* (Groves et al., 1966), and *Vibrio* (Jones et al., 1979) have a lower K_m of about 0.3 mM, and the cytosol enzyme of *E. gracilis*, a K_m of 0.175 mM (Pelzer-Reith et al., 1994).

As with halobacterial enzymes (Eisenberg et al., 1992; Kushner, 1985; Lanyi, 1974), *H. mediterranei* aldolase activity and stability was enhanced in the presence of increasing concentration of KCl.

Thus from these studies it is clear that aldolase from *H. mediterranei* behaves like other Class II aldolases in its mode of catalysis, viz., requirement of divalent metal, Fe^{2+}, for enzyme activity and inhibition by EDTA and other metal chelators. Further in-depth studies on the structure and sequence of the enzyme would help shed light on the relationship of this halobacterial enzyme with other known eubacterial and eukaryotic Class II aldolases from an evolutionary point of view. The *E. coli* Class II FBP aldolase obviously shows no sequence homology with any of the Class I aldolases so far known (Alefounder et al., 1989). There is, however, sizable homology of the *E. coli* enzyme with the Class II enzyme in yeast (Schwelberger et al., 1989) and comparatively less homology with the aldolases of *Corynebacterium glutamicum* (von der Osten et al., 1989) and *Rhodobacter sphaeroides* (Chen et al., 1991). The amino acid sequences of Class II aldolases of *S. cerevisiae* and *Schizosaccharomyces cerevisiae* show the most homology among all the aldolases, probably because of their taxonomic relationship (Mutoh and Hayashi, 1994).

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