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Cloning, Overexpression and Isolation of the Type II FDP Aldolase from *E. coli* for Specificity Study and Synthetic Application

I. Henderson, E. Garcia-Junceda,[+] K. K.-C. Liu, Y.-L. Chen, G.-J. Shen and C.-H. Wong*
Department of Chemistry, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Abstract—A stable overexpression *E. coli* strain containing the plasmid pKEN 2 for the production of the Zn^{2+}-dependent FDP aldolase from *E. coli* has been developed. Approximately 14,000 U of the enzyme (specific activity = 23.3 U/mg) can be obtained from 4-L of growth medium. The enzyme was isolated, purified to homogeneity and used for the studies of stability, substrate specificity and metal ion replacement and dissociation. Crystals of the enzyme have been obtained for structural analysis. This *E. coli* strain was deposited with the American Type Culture Collection (ATCC #77472).

Introduction

Recently, aldolases have been used extensively in organic synthesis.[1] The most widely used aldolase is the metal-free, Schiff base forming fructose-1,6-diphosphate (FDP) aldolase from rabbit muscle.[2] This enzyme catalyzes the aldol reaction of dihydroxyacetone phosphate (DHAP) with a variety of aldehydes. The stereochemistry of the C–C bond formation is constant and completely controlled by the enzyme for all substrates tested so far. Although this enzyme is commercially available, it is isolated from a mammalian source and is relatively unstable (it is therefore not suitable for large-scale processes).[3] There are also Zn^{2+}-containing FDP aldolases found in many microorganisms, and the enzyme from *Escherichia coli* has been isolated and the corresponding gene (*fda* gene) has been sequenced, cloned and overexpressed.[1,4] Preliminary studies have shown this FDP aldolase to exhibit the same stereoselectivity as RAMA (rabbit muscle aldolase). The microbial enzyme is, however, significantly more stable ($t_{1/2}$= 60 d at room temperature compared to 2 d for RAMA). Figure 1 illustrates the mechanisms of Type I and Type II aldolase-catalyzed reactions.[5]

Although the sequencing, cloning and overexpression of the *E. coli* FDP aldolase was successfully achieved several years ago,[1] this system later proved to be unstable, with subsequent irretrievable loss of the clone. A new more stable and high yielding expression system for *E. coli* FDP aldolase has now been developed, utilizing the vector pKEN 2. The enzyme has subsequently been purified and its specific activity determined. Studies on the thermostability, substrate specificity and metal ion replacement and dissociation have also been carried out. In addition, crystals of the enzyme have been obtained and will be used in structural analysis.

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Results and Discussion

An initial attempt was made to clone the *fda* gene into the plasmid pKK 223-3. Unfortunately, a positive clone i.e. a colony of XL1-Blue containing the pKK 223-3 plasmid incorporating the *fda* insert, was not obtained. This was probably the result of the two selected restriction sites on the plasmid being too close together. It was therefore decided to change expression systems, and use the vector pKEN 2. Here the same two restriction sites (EcoR I and Sma I) as in the pKK 223-3 system were used, but they are now relatively far apart in the multiple cloning site (MCS) of the plasmid. The use of this vector avoided redesign of the DNA primers, and circumvented incomplete restriction enzyme cleavage of the plasmid.

Using the plasmid pKEN 2, a positive clone was quickly identified, and subsequent large overexpression of *fda* was achieved. This expression system (Figure 2) is very stable as it is under the control of the *tac* promoter of T7 RNA polymerase. The plasmid does not contain the gene for T7 RNA polymerase, and hence can only be induced by the addition of both IPTG and M13/T7 phage, which incorporates the T7 RNA polymerase gene. This strict double control of expression guarantees minimal leakage of the promoter, creating a very stable expression system, yet still allows large overexpression of the enzyme.

Extensive experiments identified conditions for maximum expression of *fda* (see Experimental section), whereby ~14,000 U of enzyme could be obtained from 4 L of growth medium. The enzyme was subsequently precipitated using $(NH_4)_2SO_4$, passed through an ion-exchange column, and finally purified by FPLC. After the Mono Q column, the active fraction showed a specific activity of 16.92 U/mg. A total of 8 mg of protein from this fraction were loaded in the chromatofocusing column. The FDP aldolase was eluted as a symmetric peak at pH 5.02. The specific activity of this fraction was 23.30 U/mg. The analysis of the purity of the enzyme after purification, revealed a single band in both SDS-PAGE and IEF (Figure 3). The enzyme is relatively stable from 25 °C

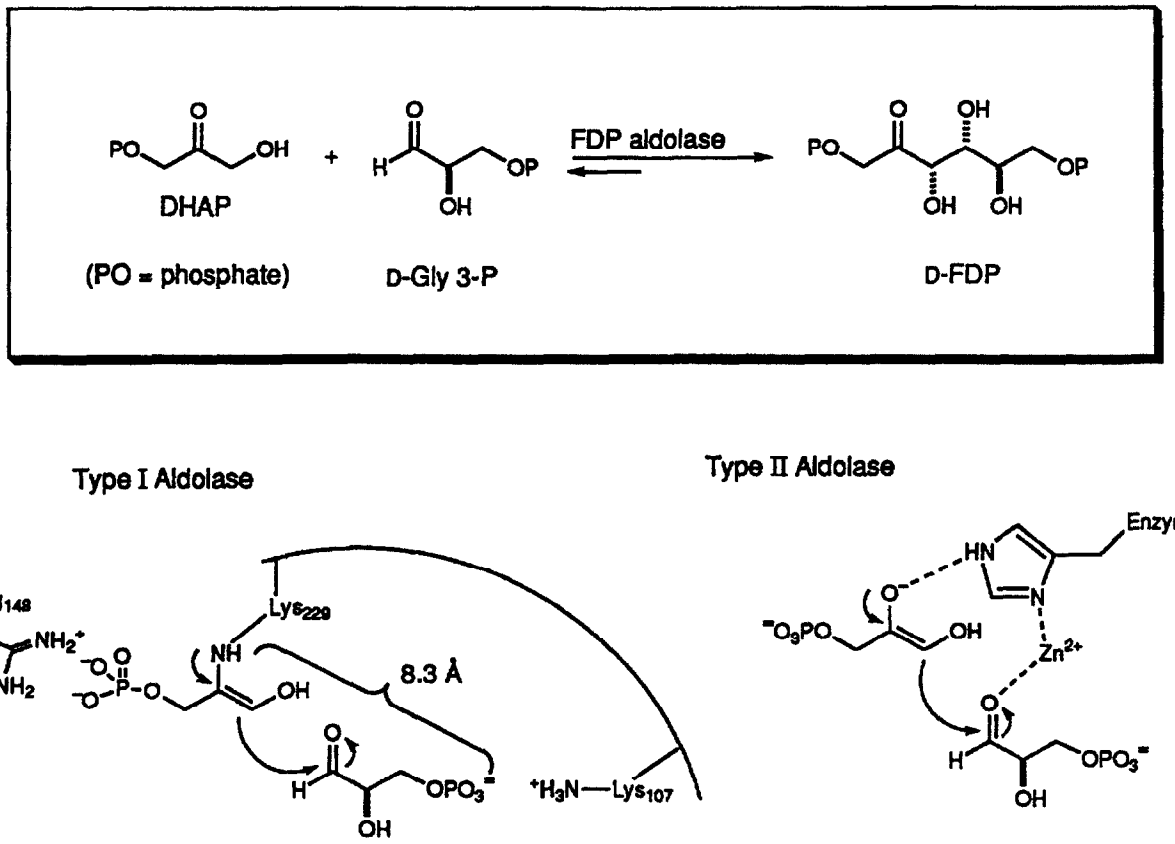

Figure 1. Mechanisms of Type I and Type II aldolases.

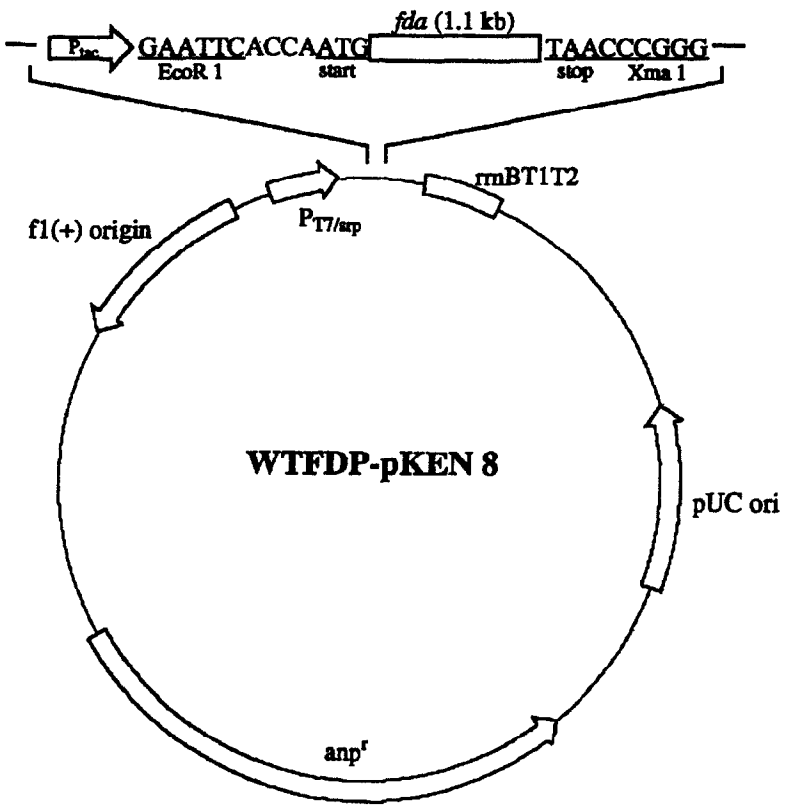

Figure 2. Construction of plasmid WTFDP–pKEN 8 containing *fda* gene from *E. coli.*

to 37 °C. At 50 °C, the enzyme is, however, inactivated rapidly (Table 1). Replacement of the active-site Zn^{2+} with Co^{2+} resulted in a decrease of activity (~85 % that of the Zn^{2+} enzyme) and stability ($t_{1/2}$ at 50 °C is ~4 min). The dissociation constants determined for Zn^{2+} are $K_1 = 10^{-8.9}$ M, $K_2 = 10^{-11.8}$ M and that for Co^{2+} are $K_1 = 10^{-7.62}$ M, $K_2 = 10^{-10.9}$. The relatively lower stability of the Co^{2+} enzyme may attribute to the lower metal affinity.

Several aldehydes were then tested as substrates for the Zn^{2+} aldolase and the results are shown in Table 2. It appears that the Zn^{2+} enzyme also exhibits a broad substrate specificity comparable to RAMA.[2b] Although this enzyme has only been used in the synthesis of deoxynojirimycin and deoxymannojirimycin,[1] the broad substrate specificity suggests that the enzyme will be useful for the synthesis of compounds that have been prepared based on RAMA.

Experimental

Reagents

The plasmid pKEN 2 was obtained from Professor G. L. Verdine, Department of Chemistry, Harvard University, Cambridge, MA 02138. Commercially available M13/T7 phage was used (Invitrogen, San Diego), and a stock solution was prepared and titered as directed. Protein contents were measured using the BCA Protein Assay Kit (Pierce). All the chemicals were purchased from commercial sources as molecular biology grade reagents.

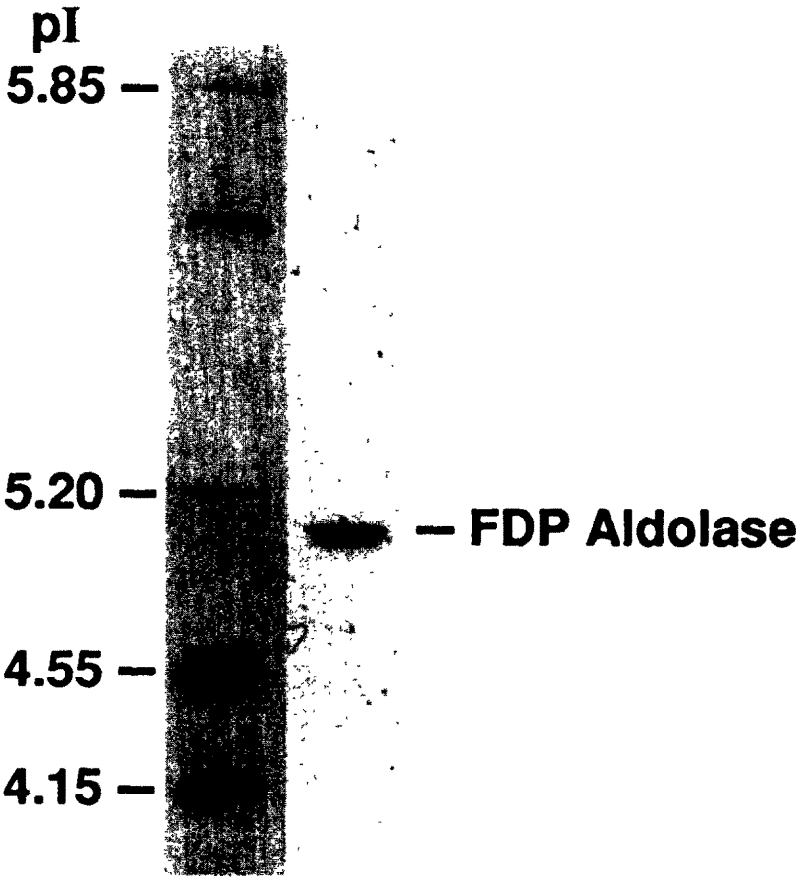

Figure 3. IEF (pH 6.5–4) of the enzyme purified after chromatofocusing (pH 5.5–4.5); 8 mg of proteins were loaded in the column. The gels were stained with silver using the Pharmacia PhastSilver Kit, as described in the Experimental. The isoelectric point of the FDPA is 5.02.

Table 1. Thermostability of the zinc-dependent FDP aldolase at pH 7.5 (0.1 M triethanolamine buffer) containing 0.3 mM $ZnCl_2$

Temperature (°C)	Half-life[a]
25	60 days
37	6 days
50	10 min

[a]The remaining enzyme activity was measured in different periods of time for the determination of half-life. Since the decrease of activity was not first order, the half-life represented the period of time when 50 % of the original enzyme activity disappeared.

Table 2. Relative rates of aldehydes (RCHO) reacting with DHAP in *E. coli* aldolase-catalyzed aldol condensations

R	V_{rel}	cosolvent
$PiOCH_2CH(OH)$	100%	
CH_3	25.1%	
CH_3CH_2	19.5%	
$ClCH_2$	24.5%	
$(CH_3)_2CHCH_2$	4.5%	40% DMSO
$CF_3CONHCH_2CH_2$	1.9%	40% DMSO
$CH_3SCH_2CH_2$	<1%	40% DMSO
$HOCH_2CH(CH_2CH{=}CH_2)$	<1%	40% DMSO
$HOCH_2$	6.1%	
$HOCH_2CH_2$	6.2%	
$HOCH_2CH(OH)$	7.7%	
$CH_3CH(OH)$	18.2%	
$CH_3CH(OH)CH^2$	9.0%	
$CH_3OCH_2CH(OH)$	7.0%	
$N_3CH_2CH(OH)$	10.2%	
$ClCH_2CH(OH)$	8.8%	
$BrCH_2CH(OH)$	5.9%	
$FCH_2CH(OH)$	7.2%	
$BzlOCH_2CH(OH)$	2.1%	40% DMSO
OCH	2.9%	
$OCH(CH_2)_3CH(OH)$	<1%	
CH_3CO	18.6%	
RCHO = D-ribose	1.1%	
RCHO = D-ribose-5-P_i	2.1%	
RCHO = D-glucose	<1%	
RCHO = D-glucose-6-P_i	1.4%	
$HOCH_2(CH_2)_3$	1.8%	
$CH_2{=}CH$	--- (inactivator)	
$CH_3CH{=}CH$	--- (inactivator)	
$(CH_3CH_2O)_2C$	<1%	40% DMSO
$(CH_3)_3$	---	40% DMSO
Br_3C	---	40% DMSO
Ph	---	40% DMSO
RCHO = 3,4,5-trihydroxybenzaldehyde	---	40% DMSO
2-pyrrolyl	---	40% DMSO
2-furanyl	---	40% DMSO
2-pyridyl	---	40% DMSO

The rate was determined by monitoring the consumption of DHAP. To 970 μL of 0.2 M TEA buffer (triethanolamine buffer, pH 7, 25 °C) containing DHAP (55 mM), $ZnCl_2$ (0.3 mM) and the appropriate substrate (55 mM) was added 30 μL of a solution containing *E. coli* FDP aldolase (10 U/mL of the TEA buffer). Every 3 min during the course of 20–30 min, 100 μL of the assay solution was withdrawn, quenched with 30 μL of 7 % perchloric acid solution, neutralized with 20 μL of 1 N NaOH, and diluted with 0.5 mL of 0.2 M TEA buffer. This diluted solution (30 μL) was subsequently assayed for DHAP. A control reaction was run without aldolase and assayed under the same condition for reference. In the case where the aldehyde is not readily soluble in the buffer, DMSO was added.

Microorganisms

The host *E. coli* strain Epicurian coli XL1-Blue was purchased from Stratagene Co. (San Diego, CA) and was maintained on LB (Luria-Bertani) medium. The *E. coli* XL-1 Blue containing plasmids derived from pKEN 2 were grown in either LB or SOB medium containing 10 mM $MgCl_2$ and 100 μg/mL ampicillin. Stock cultures were kept as cell suspensions at –70 °C in 10–20 % glycerol solution.

DNA manipulations

The DNA of *E. coli* strain K-12 was extracted according to the method of Perbal.[6] Primers were obtained from Genosys (Houston, TX). The sequences of the primers WTFDP 5' and WTFDP 3' are shown in Figure 4.

5'- ATATTGAATTCACCAATGTCTAAGATTTTTGATTTCGTA
EcoR I start

Primer WTFDP 3'
5'- AAAACCCGGGTTACAGAACGTCGATCGCGTTCAG
Sma I stop

Figure 4. The designed primer sequences for the amplification of the FDP aldolase gene.

Amplification of the fructose diphosphate aldolase gene

PCR amplification was performed in a 100 µL reaction mixture containing 1 µL (1 µg) of *E. coli* strain K-12 DNA, 400 nmol each of primers WTFDP 5' and WTFDP 3', 200 µM of different dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01 % gelatin, 0.1 % Triton X-100 and 2 U of *Thermus aquaticus* DNA polymerase. The reaction was overlayed with 100 µL of mineral oil and subjected to 30 cycles of amplification. The cycle conditions were set as denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min and elongation at 72 °C for 1.5 min.

Construction of fructose diphosphate aldolase expression vector

The DNA incorporating the *fda* gene obtained from PCR amplification was purified on a 0.8 % agarose gel. The DNA band corresponding to 1.1 Kb was separated from the agarose using the Geneclean II kit obtained from Bio 101, Inc. (La Jolla, CA) and precipitated from *i*-PrOH. The DNA was then dissolved in restriction enzyme buffer A supplied by Boehringer Mannheim Biochemical Co. (Indianapolis, IN) and digested with Sma I at 25 °C for 2 h, then digested with EcoR I at 37 °C for 2 h. The digested DNA was then recovered by treatment with Strataclean resin obtained from Stratagene Co. (San Diego, CA) followed by ethanol precipitation, and resuspension in TE buffer (pH 7.7).

The plasmid pKEN 2 was also digested with EcoR I and Sma I under the same conditions as the insert, and dephosphorylated with HK Phosphatase obtained from Epicenter Technologies (Madison, WI). The digested plasmid DNA was then recovered by treatment with Strataclean resin followed by ethanol precipitation, and resuspension in water. It was subsequently purified on a 0.8 % agarose gel, separated from the agarose using the Geneclean II kit, precipitated from *i*-PrOH and resuspended in water.

The digested *fda* DNA fragment was then ligated into the digested dephosphorylated pKEN 2 vector in which the expression of recombinant enzyme was under the control of the T7 RNA polymerase promoter (the ribosome binding site present in the plasmid was also utilized). The new plasmid designated WTFDP–pKEN 8 (Figure 2) was then transformed into Epicurian coli XL-1 Blue and plated onto LB agar plates containing 50 µg/mL ampicillin.

Screening for positive clones

A positive clone was identified by direct PCR on a random selection of the transformants using the WTFDP 5' and WTFDP 3' primers. Using this protocol allows selection of the most intense *fda* DNA bands as possible clones, as *fda* is a wild-type *E. coli* gene and PCR performed directly on an *E. coli* colony will always give amplification. Unambiguous proof of the presence of the *fda* insert in the plasmid was obtained by isolation of the plasmid and subsequent analysis of it's DNA using the restriction enzymes EcoR I and Sma I and/or PCR using the WTFDP 5' and WTFDP 3' primers.

Overexpression of FDP aldolase

The overexpression of *fda* was induced from WTFDP–pKEN 8 by addition of both IPTG and M13/T7 phage containing the T7 RNA polymerase gene. Extensive screening experiments were carried out to optimize the conditions for overexpression of *fda*. A large overexpression was obtained using SOB medium containing 10 mM $MgCl_2$ and 100 µg/mL ampicillin. Addition of IPTG was optimized to a final concentration of 1 mM. Addition of M13 phage was initiated 1 h after addition of IPTG to give 10–40 pfu/cell. Cells were then harvested 13 h after addition of M13 phage (OD 2.1 for 1 L culture) at 30 °C to give the largest yield of FDP aldolase.

Isolation and purification of FDP aldolase

Cells were harvested by centrifugation (15,000 *g*) at 0 °C and resuspended in 0.1 M TEA buffer (pH 7.5) containing 3.0 mM $ZnCl_2$ (50 mL for 4 × 1 L cultures). (Note: unless otherwise stated, buffer refers to 0.1 M TEA buffer (pH 7.5) containing 3.0 mM $ZnCl_2$). After lysing cells using the French Press, debris was removed by centrifugation at 0 °C (30,000 *g* for 1 h) and the supernatant collected. Enzymatic assay showed 17,000 U of activities from 4 L of culture. Protein precipitated at 0 °C, using $(NH_4)_2SO_4$ between 45 % and 80 % saturation, was then collected by centrifugation at 0 °C (30,000 *g* for 30 min), and resuspended in buffer (50 mL for 4 × 1 L cultures). After dialysis against 4 L of buffer at 4 °C for 24 h, enzymatic assay showed most of the enzyme was recovered. Specific activity of the enzyme was 3.3 U/mg at this stage (note: the enzyme activity and concentration were measured via enzymatic assay, and the BCA Protein Assay Kit (Pierce) respectively). The enzyme was then purified by ion-exchange filtration on DEAE cellulose (14"/1.25" column) equilibrated with the same buffer at 4 °C. After loading the enzyme, 300 mL of buffer was passed through the column (collected in one fraction), followed by 500 mL of a 0–1 M gradient of KCl in buffer (7 mL fractions). Fraction 33 was found to have the highest activity, and a specific activity of 4.9 U/mg was obtained. This crude enzyme preparation is suitable for synthesis.

Anion exchange chromatography. For further analysis of the enzyme, a portion of the enzyme was further purified by FPLC. The anion exchange chromatography was

performed on a Mono Q column 10/10 (Pharmacia). The sample was eluted with a gradient between 0 and 0.5 M NaCl in 200 mL of 50 mM Tris–HCl buffer, pH 7.5. The elution of the proteins was monitored by absorbance at 280 nm. Fractions of 4 mL were collected. After each run the column was washed with 2 M NaCl. The active fractions were pooled and the buffer exchanged with the initial buffer of the chromatofocusing using Centriprep tubes.

Chromatofocusing. The chromatofocusing was performed on a Mono P column 5/20 (Pharmacia). The sample was eluted with a pH gradient between 5.5 and 4.5. The initial buffer was piperazine (25 mM pH 6.3). The elution buffer was Polybuffer 7–4 diluted by a factor of 10 with distilled water. The pH was adjusted to 4.5 with HCl (2 M). Before loading the sample a pregradient was made by washing the column with 3 mL of the elution buffer. The elution was monitored by absorbance at 280 nm, and 0.5 mL fractions were collected. After each run the column was washed with 2 M NaCl.

Determination of the enzyme purity. The purity of the fractions obtained from the different columns was determined by SDS-PAGE (Polyacrylamide Gel Electrophoresis) and isoelectrofocusing (IEF), using a Pharmacia PhastSystem instrument. The SDS-PAGE preformulated gels were used with a gradient of polyacrylamide in the separating zone between 8–25 %. Prior to electrophoresis, the samples were incubated at 100 °C for 3 min in a solution containing 0.5 % sodium dodecyl sulfate and 5 % 2-mercaptoethanol. The gels were stained with Coomasie Blue. The IEF was performed in preformulated gels in a pH range of 4.5–6.5. In both cases the markers used were from Pharmacia. The gels were stained using the Pharmacia PhastSilver Kit, with a modification in the method to provide a higher sensitivity. The average sensitivity of this technique is between 0.05 and 0.1 ng of protein per band.[7]

Enzyme assay

The FDP aldolase was assayed on the basis of the cleavage of FDP coupled with glycerophosphate dehydrogenase-catalyzed reduction of dihydroxyacetone phosphate (DHAP) in the presence of NADH as described by Boehringer Mannheim.[8] A typical assay uses 910 μL of α-glycerophosphate dehydrogenase–triosephosphate isomerase, and 20 μL of sample (diluted, such that Abs at 340 nm was measured between 0.2 and 2.0, after zeroing to air). All results were adjusted for background rate, i.e. assay was run initially without sample to determine background. Activity was calculated from the equation: Activity (U per vol. of sample used) = Rate of absorbance change (ΔA/min)/(2 × 6.22).

Preparation of Zn^{2+}- and Co^{2+}-aldolase and determination of the metal dissociation constants

For determination of the dissociation constant of Zn^{2+}–enzyme complex and the replacement of Zn^{2+} with Co^{2+}, all the buffer solutions were prepared from deionized distilled water, followed by passing through the chelating resin Chelex-100 and further extraction with 0.01 % dithiozone solution in CCl_4 to remove trace metal ion contaminations. The pH was adjusted with 1 N of metal free HCl solution. The dissociation constant was determined based on the metal ion buffer technique,[9] in which the enzyme activity was measured after equilibration of the enzyme with different known concentration of Zn^{2+} or Co^{2+} maintained by metal ion buffers. The free Zn^{2+} or Co^{2+} ion concentration was determined using nitrilotriacetic acid (NTA)[9] which forms a stable 1:1 complex with the metal ion. At pH 8.0 the dissociation contant[9] for $Zn{\cdot}NTA^-$ is $10^{-10.66}$ M and that for $Co{\cdot}NTA^-$ is $10^{-10.40}$ M. At pH 8, the free nitrilotriacetic acid is a mixture of doubly and triply ionized forms. The dissociation constant of $Zn{\cdot}NTA^-$ can be expressed as

$$K_x' = [Zn^{2+}]\,[NTA^{3-} + NTA^{2-}]/[Zn{\cdot}NTA^-]$$
$$= K_x\,(1 + 10^{-pH}/K_{a3}) \text{ where}$$
$$K_{a3} = 10^{-pH}\,[NTA^{3-}]/[NTA^{2-}]$$

Since the $Zn{\cdot}NTA^-$ complex is so stable that with a stoichiometric excess of NTA the concentration of $Zn{\cdot}NTA^-$ is approximately equal to the total concentration of Zn^{2+}. Therefore, when K_x' is known ($10^{-8.427}$ at 20 °C),[9] the concentration of free Zn^{2+} can be calculated from $K_x' \simeq [Zn^{2+}]([NTA]_O - [Zn_O)/[Zn]_O$ and $[Zn^{2+}] \simeq [Zn]_O \cdot K_x'/([NTA]_O - [Zn]_O)$.

To prepare the metal free enzyme, the enzyme in Tris buffer (0.05 M, pH 8.0) was dialyzed for 24 h against three changes of 50 mM EDTA in Tris (50 mM, pH 8.0) at 4 °C, followed by dialysis against the same buffer without the chelating reagent (the residual enzyme activity after metal removal is <5 %). A solution of $ZnSO_4$ or $CoSO_4$ (99.999 % purity from Aldrich Co.) was then added to restore the enzyme activity. The equations indicating the dissociation of Zn^{2+} from the dimeric aldolase and the interaction of free Zn^{2+} with NTA^- are indicated in the following:

$$Enz{\cdot}Zn_2 \overset{K_1}{\rightleftharpoons} Enz{\cdot}Zn + Zn^{2+}$$
$$Enz{\cdot}Zn \overset{K_2}{\rightleftharpoons} Enz + Zn^{2+}$$
$$Zn^{2+} + NTA^- \overset{K_x}{\rightleftharpoons} ZnNTA^-$$

K_1 and K_2 are the first and second dissociation constants, and K_x is the dissociation constant of the $Zn{\cdot}NTA^-$ complex. To determine the dissociation constant of the enzyme–metal complex, the fraction of enzyme activity at a given metal concentration was determined, which is a function of the free metal ion concentration. For detailed analysis and experimental procedure, please refer to the Thesis report described previously.[10]

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