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FSAB: A new fructose-6-phosphate aldolase from *Escherichia coli*. Cloning, over-expression and comparative kinetic characterization with FSAA

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ABSTRACT

Fructose-6-phosphate aldolase B (FSAB) from *Escherichia coli* was successfully over-expressed as His-tagged recombinant protein. A decameric protein was observed as for FSAA. Unlike FSAA, FSAB is not thermally stable at temperatures higher than 60 °C. The 70% identity between the two aldolases has allowed the generation of a 3D structure which has shown a high similarity of the two active sites. Full kinetic studies towards several substrates have revealed that FSAB catalytic activity is very close to FSAA activity, corroborated by the similarity of their active sites. FSAB has been able to react with three known donors (dihydroxyacetone, hydroxyacetone and glycolaldehyde) but always slightly slower than FSAA.

1. Introduction

Aldol reaction is one of the most useful tools for the synthetic chemist. This reaction offers powerful strategies towards the synthesis of both enantiopure natural and novel poly-hydroxylated compounds. Aldolases have been used in the synthesis of chiral compounds by stereoselectively catalysing the reversible formation of C—C bonds. These enzymes catalyse aldol additions between nucleophilic donors (ketones or aldehydes) and electrophilic acceptors (aldehydes) [1]. One of the most important recent discoveries in the aldolase family was fructose-6-phosphate aldolase (FSAA, gene *mipB* from *Escherichia coli*) which was first reported by Sprenger in 2001 [2]. This enzyme has the striking advantage of accepting unphosphorylated dihydroxyacetone (DHA) as donor instead of the expensive and unstable dihydroxyacetone phosphate (DHAP) strictly used by the well-known DHAP-dependent aldolases. Moreover, FSAA presents the unprecedented advantage to react with four different unphosphorylated donors (DHA, hydroxyacetone, 1-hydroxybutan-2-one and glycolaldehyde) [3] providing an access to a large variety of sugars and analogues (Scheme 1).

Thus, FSAA has been efficiently used to synthesize different polyhydroxylated compounds such as iminocyclitols and carbohydrates [3a,b,4]. To broaden the scope of such biocatalyst for synthetic applications, mutants were constructed by rational design. Indeed, A129S mutant [5] conferred a more hydrophilic active site to FSAA, increasing the enzyme affinity for DHA. Then the double mutant A129S/A165G [6] was designed to better accommodate C-α substituted aldehydes in the active site. It should also be noticed that using the scaffold of a transaldolase (TALB, gene *talB* from *E. coli*) a new efficient DHA-dependent aldolase was designed by a single amino acid replacement, F178Y [7]. This work together with some FSAA results has been recently reviewed [1b]. Another approach to provide organic chemists with useful biocatalysts, different from protein engineering, is to study aldolases that can be found in nature. Notably, at the same period as Sprenger's group discovered FSAA, another protein codified in the genome of *E. coli* K-12 (gene *talC*) was identified as a new FSA (named FSAB) [2b]. Due to its lower specific activity towards DHA compared to FSAA, this

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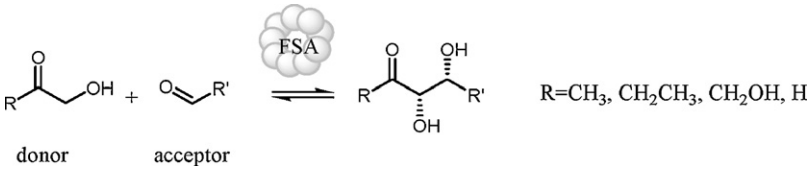

Scheme 1. Aldol reaction catalysed by fructose-6-phosphate aldolase (FSA).

enzyme was not further investigated. Since then, other donors like hydroxyacetone and glycolaldehyde have been described as much better donors than DHA [3c]. In this way, a complete study of FSAB is of interest, in particular with the above cited donor substrates.

This work presents the biochemical characterization of FSAB and a comparison of kinetic parameters between FSAA and B for several substrates.

2. Materials and methods

2.1. General

Expression E. coli strains BL21(DE3)pLysE and BL21(DE3)pLysS cells were from Invitrogen. Plasmids pET22b(+) and pET11a were from Novagen. Plasmid pET22b(+) (Novagen) modified for ligation independent cloning [8] was done by Génoscope [8b]. D-Fructose-6-phosphate dipotassium salt and D,L-glyceraldehyde 3-phosphate diethyl acetal barium salt were purchased from Sigma–Aldrich. D,L-Glyceraldehyde 3-phosphate diethyl acetal barium salt was deprotected according to the protocole provided by Sigma–Aldrich. The enzymes phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, α-glycerophosphate dehydrogenase-triosephosphate isomerase from rabbit muscle, and glycerol dehydrogenase from *Cellulomonas* sp. were also purchased from Sigma–Aldrich.

2.2. Bacterial strains and vectors

Genes encoding FSAA (*mipB*, GeneBank Accesion number BAA13552.1) and FSAB (*talC*, GeneBank Accesion number P32669.1) enzymes from *E. coli* K12 (strain MC4100) were amplified by PCR using specific primers (P*fsa*A_5PRIM: 5′-aaagaaggagataggatcatgcatcatcaccatcaccatgaactgtatctggatacttcagacg-3′; P*fsa*A_3PRIM 5′-gtgtaatggatagtgatcttaaatcgacgttctg cc-3′; P*fsa*B_5PRIM: 5′-aaagaaggagataggatcatgcatcatcaccatca ccatgaactgtatctggacacc-3′ P*fsa*B_3PRIM 5′-gtgtaatggatagtga tcttagagatgagtagtgccaaatg-3′). The amplified sequences were inserted into an expression vector pET22b(+) modified [9]. The forward primers introduced a hexahistidine sequence in the proteins after the initial methionine for purification purposes. The sequences of the resulting plasmids, named pET22-*fsa*A and pET22-*fsa*B, were verified.

For expression of an untagged version of FSAB, the gene was amplified by PCR from chromosomal DNA of *E. coli* K12 (strain W3110) using specific primers (*fsa*B up 5′-ttttgctagcgaactgtatctggacaccgc-3′; *fsa*B down 5′-ttttaagcttttaga gatgagtagtgccaaatgcg-3′). The amplified sequence was cloned into an expression vector pET11a using the restriction sites *Nhe*I and *Hind*III. The pET16-*fsa*A construct was used for expression of untagged FSAA as described previously [2,7].

2.3. Expression and purification

E. coli colonies (expression strain) containing the plasmids pET22-*fsa*A and pET22-*fsa*B were cultured in Luria–Bertani (LB) broth in presence of selection antibiotic at 37 °C with shaking. When the culture reached an $OD_{600\,nm}$ of 0.5, protein expression was induced with IPTG (0.5 mM) and the temperature was dropped to 30 °C. The culture was incubated for a further period of 12 h. Cells were harvested by centrifugation, washed twice and resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Cell suspension was disrupted by sonication and the cell lysate was centrifuged at 10,000 × *g* for 20 min. Clear supernatant was loaded onto a Ni^{2+}–NTA–agarose column (Qiagen, *h* = 1.5 cm; Ø = 2.5 cm) pre-equilibrated with buffer A containing imidazole 20 mM (buffer B). The column was washed with buffer B, and the retained proteins were eluted with the same buffer containing 250 mM of imidazole. The purity of fractions was assessed by SDS-PAGE. Eluted fraction containing pure protein was dialysed against buffer for desalting and imidazole removal. The protein was dialysed against water (pH close to 8.0) to remove buffer salts before the lyophilization process. This was also used when protein freeze-drying was necessary without loss of activity. Untagged FSAA was expressed and purified as previously described [5] and untagged FSAB as described for FSAA by Schneider et al. [7] but omitting the heat treatment.

2.4. Biochemical characterization

SDS-PAGE was performed as described by Laemmli [10] using 15% polyacrylamide gels. Electrophoresis samples were always run under reducing conditions and gels were stained with Coomassie brilliant blue R-250. Protein gels were quantified by densitometry using the image analysis software *GeneTools* (Syngene). Apparent proteins molecular weight in their native state was determined by gel filtration on a Superdex 200 column (Amersham Biosciences) as previously described [9].

2.4.1. Optimum pH

FSAB optimum pH was calculated assaying its retroaldol activity at pH values between 5.0 and 9.0. Activity was determined by measuring the formation of dihydroxyacetone (DHA) and D-glyceraldehyde-3-phosphate (D-G3P) from 40 mM D-fructose-6-phosphate (F6P) by spectrophotometric monitoring in reaction mixtures of 2 mL. Each reaction contained 50 mM glycylglycine buffer and 0.18 U pure FSAB. pH was adjusted before enzyme addition and it was checked at the end of the reaction. Standard spectrophotometric assays for DHA/D-G3P detection were performed in 1 mL of 50 mM glycylglycine buffer containing NADH 0.4 mM, glycerol dehydrogenase (1.5 U)/α-glycerophosphate dehydrogenase (1.3 U) plus triose phosphate isomerase (12.6 U), and reaction aliquots of 30–60 μL. Variations of $A_{340\,nm}$ were proportional to DHA/D-G3P concentration in reaction ($\varepsilon^{NADH} = 6220\,cm^{-1}\,M^{-1}$).

2.4.2. Enzyme thermostability

Thermal stability of FSA enzymes was determined by monitoring the remaining retroaldol activity of purified enzymes (5–10 mg mL^{-1} in glycylglycine buffer 50 mM pH 8.5) along the time at three different incubation temperatures: 50 °C, 60 °C and 70 °C. Retroaldol activity was assayed using the previously reported FSA activity assay from 45 mM F6P [2].

2.4.3. D,L-G3P stability

Stability of D,L-G3P was assayed at different reaction conditions, such as in presence of FSA's purified by IMAC or heat treatment. All