Generation and Characterization of Fumarase C Mutants

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ABSTRACT

Fumarase C is responsible for the reversible hydration/dehydration of S-malate to fumarate during the catabolic Kreb's cycle. Using PCR based site-directed mutagenesis; two active site mutants of fumarase C have been constructed. The recombinant enzymes, S139A and S140A, were purified using metal affinity chromatography and subjected to kinetic analysis and X-ray crystallization. The kinetic investigations indicate that serine 140 may play a role in the reversible conversion of S-malate to fumarate. Overall, this investigation aims to map the roles of serine 139 and 140 within the fumarase C active site.

INTRODUCTION

Fumarase is responsible for the reversible conversion of S-malate to fumarate during the ubiquitous Kreb's cycle. In eukaryotic organisms, there are both cytosolic and mitochondrial forms, each of which stems from a single gene (1). There are two classes of fumarase. Class I fumarases include fumarases A and B, and members of this classification are oxygen sensitive, Fe-dependent homdimers of 120,000 daltons. Class II fumarases include fumarase C (eFumC) from *Escherichia coli (E.coli)* and are oxygen insensitive, heat stable, and Fe-independent hometetramers of 200,000 daltons. Amino acid sequence studies have shown there is little homology between class I and class II fumarases. However, class II fumarases from different species share significant amino acid homology. In fact, this homology extends beyond class II fumarases into an entire super-family of enzymes, all of which evolve fumarate during their reaction mechanisms (1).

Figure 1 illustrates the eFumC tetramer where each subunit has been shaded independently. The twenty-helix structural core of the complete tetramer is formed by the contribution of a five-helix bundle from each of the four monomers. Fumarase C has four independent active sites located at the corners of the complete oligomer, and each is constructed from three of the four subunits within the tetramer. Each of the subunits donates one of three highly conserved regions of amino acids found within the fumarase family.

Figure 1. Fumarase tetramer

During the conversion of S-malate to fumarate, eFumC facilitates removal of the C3 proton from S-malate by lowering its effective pKa ~23 pH units. The resulting carboxyanion intermediate re-arranges into the aci-carboxylate transition state, which is further stabilized by groups within the active site. Figure 2 illustrates the proposed fumarase reaction mechanism. Next, the hydroxyl at C2 is protonated by an acidic group in the eFumC active site, leading to the formation of fumarate

and the loss of a water molecule (1). The putative mechanism requires three participating enzyme sites: (1) a group of sidechains responsible for coordinating the carboxylates of substrate, (2) a basic group for the removal of the proton, and (3) an acidic group for the removal of the hydroxyl group on C2.

Previous X-ray crystallographic investigations have provided details of the first two eFumC sites. Lysine 324 (Lys 324) and asparagine 326 (Asn 326) have been implicated in coordinating the carboxylates of the incoming substrate, while a highly coordinated free water has been proposed to be the catalytic base responsible for C3 proton abstraction from S-malate. Figure 3 illustrates some of the important sidechains within the eFumC active site. The dashed lines represent observed hydrogen bonds between various donor and acceptor atoms. Serine 139 (Ser 139) and 140 (Ser 140) have been identified as potentially important sidechains within the eFumC active site. Their respective proximity near the bound competitive inhibitor citrate has provided an indication that these sidechains donate groups, which either facilitate the chemical conversion or the binding of the incoming S-malate for attack by other

Figure 2. Fumarase catalyzed reaction
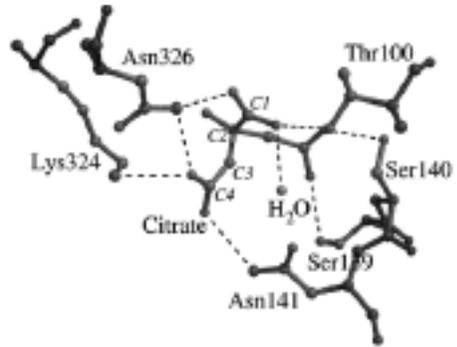
Figure 3. eFumC active site

groups on the enzyme (1). In order to better define the roles of these two residues, each serine sidechain has been replaced by an alanine. The resulting mutant form of serine 140 to alanine was the major focus of this investigation.

MATERIALS AND METHODS

Site-Directed Mutagenesis. QuickChange™ Site-Directed Mutagenesis kit by Stratagene was used to direct mutagenesis. Two unique sets of primers were used to construct the replacement of serine at positions 139 and 140 with alanine sidechains. The primers used during the PCR based site directed mutagenesis are illustrated below.

S139A Upper: **GTGAACAAAAGCCAAGCTTCCAACGATGTCTTT**
S139A Lower: **AAAGACATCGTTGGAAGCTTGGCTTTTGTTCAC**

S140A Upper: **AACAAAAGCCAAAGTGCGAACGATGTCTTTCCG**
S140A Lower: **CGGAAACAGATCGTTCGCACTTTGGCTTTTGTT**

All primers were obtained from Midland Certified Reagent Company and diluted to a final concentration of 10 ng/µl. PCR based site-directed mutagenesis was carried out utilizing the QuikChange™ Kit from Stratagene.

Transformation of pS139A and pS140A into JM105 cells. 1 µl of plasmid DNA (either pS139A or pS140A) was added to competent JM105 *E. coli* cells. Cells were put on ice for 20 minutes and then heat shocked for 90 seconds at 42 ºC. 450 µl of Luria Bertani (LB) broth was added to the cells and were incubated at 37ºC for 1 hour in a circulating water bath. 200 µL of each transformation mixture was plated onto LB-agar plates supplemented with 150 µg/ml of ampicillin.

Expression and Purification of mutant fumarase. Three overnight cultures of the recombinant *E. coli* were grown and used to inoculate three fernbach flasks with 1.4 liters LB broth supplemented with 150 µg/ml of ampicillin. The cultures were grown at 37ºC under continuous shaking at 175 rpm until an OD (600 nm) of 0.8. Over-expression of the mutant forms of eFumC was induced through the addition of 1 mM IPTG. Induction of mutant eFumC was carried out at 37 ºC for 16 hours. After induction, cells were harvested via centrifugation at 5,000 x g for 20 minutes.

The harvested recombinant *E. coli* were mixed with 30 ml of Buffer A (50mM NaPhosphate pH 7.5, 300mM NaCl) and lysed via sonication. Nucleic acid material was removed through the addition of polyethyleneimine (PEI) to a final concentration of 0.5% and subsequent centrifugation at 15,000 x g for 30 minutes. Each cell free extract was applied to a metal-chelate column. After the initial loading of the cell free extract, the column was washed with Buffer A until baseline was re-established. Contaminants were removed by washing the column with Buffer B (50mM NaPhosphate pH 7.5, 300mM NaCl, 60 mM imidazole). The mutant forms of eFumC were eluted with Buffer C (50mM NaPhosphate pH 7.5, 300mM NaCl, 300 mM imidazole). Buffer C was exchanged for Buffer D (25 mM Tris pH 7.5) using dialysis. Finally, each mutant form of eFumC was concentrated to between 10 – 12 mg/ml. SDS-PAGE analysis confirmed the purity of the mutant eFumC preparations.

Crystallization and Kinetic assays. S139A and S140A were crystallized using the hanging-drop vapor diffusion method. Crystallization conditions consisted of 300mM citrate pH 6.0 and 13-15% PEG 3350. Enzymatic activity assays were performed in the forward and reverse directions with varying concentrations of S-malate and fumarate at 30ºC using a Shimadzu UV-Vis spectrophotometer. .

RESULTS

Each mutated plasmid was subjected to restriction digest and agarose gel separation. Figure 4 (left hand side) represents the agarose gel results. The S139A mutant gene was subjected to a Hind III digest and the resulting two bands in lane 2 of figure 4 indicate the incorporation of the alanine replacement at position 139. The S1410A mutant gene was subjected to a Xba I/Hind III digest. The two bands in lane 3 of figure 4 confirm the incorporation of an alanine replacement at position 140 with the eFumC gene sequence.

Purification of S140A was determined to be successful by the presence
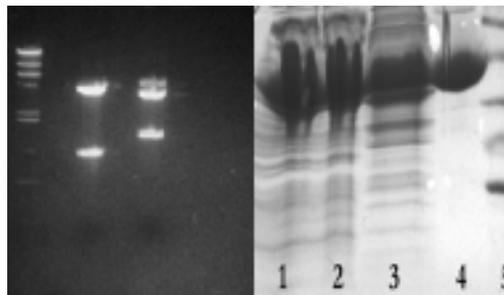
Figure 4. Agarose and SDS-PAGE analysis

of a purified band on the SDS-PAGE gel as illustrated in figure 4 (right hand side). Lane 1 represents the starting cell free extract, lane 2 post Buffer A wash, lane 3 post Buffer B wash, lane 4 purified S140A post Buffer C wash, and lane 5 molecular weight markers. Following purification, S140A was subjected to both steady state kinetic analysis and crystallization. Kinetic data for S140A is shown in Table 1. The K_m showed no significant change when compared to native eFumC, while the kcat lowered by a factor of 10^6. A lowering of the rate constant by a million times is of biological significance. Additional crystallization trials performed at the University of Minnesota have been able to grow larger crystals, but an X-ray diffraction dataset has not yet been collected.

Table 1. Native and S140A Kinetic Data

Sample	Native	S140A
$K_m(\mu M)(f{\rightarrow}m)^\dagger$	1.6E-1	4E-2
$k_{cat}(sec^{-1})(f{\rightarrow}m)$	1.4E7	1.2E1
$K_m(\mu M)(m{\rightarrow}f)^\dagger$	1.9E3	5E3
$k_{cat}t(sec-1)(m{\rightarrow}f)$	2E7	1.4E1

† (f→m) represents the eFumC hydration of fumarate to S-malate,
while (m→f) represents the dehydration of S-malate to fumarate.

DISCUSSION

The effective replacement of serine 140 for an alanine sidechain removes a single hydroxyl from the eFumC active site. The modest change in K_m clearly indicates that this alteration has little effect upon substrate binding (either S-malate or fumarate). The alteration of k_{cat} indicates that serine 140 may be critical for the reversible conversion of S-malate to fumarate. The k_{cat} of S140A is lowered by six orders of magnitude. The one million-fold reduction in catalytic efficiency is of biological significance and seems to implicate Ser 140 as the third essential group (acidic group) within the eFumC active site capable of protonating the hydroxyl at C2 of S-malate. Further study to support this hypothesis would involve determining the three dimensional structure of S140A using X-ray diffraction data, in addition to mutating other surrounding sidechains.

ACKNOWLEDGEMENTS

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