Unexpected similarity in regulation between an archaeal inositol monophosphatase/fructose bisphosphatase and chloroplast fructose bisphosphatase

KIMBERLY A. STIEGLITZ,¹ BARBARA A. SEATON,² JAMES F. HEAD,²
BOGUSLAW STEC,³ AND MARY F. ROBERTS¹

¹Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02467, USA

²Department of Physiology and Biophysics, Boston University School of Medicine,
Boston, Massachusetts 02118, USA

³Department of Chemistry, University of Texas, El Paso, Texas 79968, USA

(RECEIVED October 14, 2002; FINAL REVISION December 21, 2002; ACCEPTED January 14, 2003)

Abstract

Hyperthermophilic archaea have an unusual phosphatase that exhibits activity toward both inositol-1-phosphate and fructose-1,6-bisphosphate, activities carried out by separate gene products in eukaryotes and bacteria. The structures of phosphatases from *Archaeoglobus fulgidus* (AF2372) and *Methanococcus jannaschii* (MJ0109), both anaerobic organisms, resemble the dimeric unit of the tetrameric pig kidney fructose bisphosphatase (FBPase). A striking feature of AF2372, but not of MJ0109, is that the sulfhydryl groups of two cysteines, Cys150 and Cys186, are in close proximity (4 Å). A similar arrangement of cysteines has been observed in chloroplast FBPases that are regulated by disulfide formation controlled by redox signaling pathways (ferredoxin/thioredoxin). This mode of regulation has not been detected in any other FBPase enzymes. Biochemical assays show that the AF2372 phosphatase activity can be abolished by incubation with O₂. Full activity is restored by incubation with thiol-containing compounds. Neither the C150S variant of AF2372 nor the equivalent phosphatase from *M. jannaschii* loses activity with oxidation. Oxidation experiments using *Escherichia coli* thioredoxin, in analogy with the chloroplast FBPase system, indicate an unexpected mode of regulation for AF2372, a key phosphatase in this anaerobic sulfate reducer.

Keywords: Inositol monophosphatase; fructose bisphosphatase; disulfide formation; archaea; thioredoxin

The dimeric *Archaeoglobus fulgidus* gene product AF2372, similar to the *Methanococcus jannaschii* gene product MJ0109, was initially identified as an inositol monophosphatase (IMPase) by sequence homology with mammalian IMPases (Chen and Roberts 1998). These enzymes were indicated to function in the biosynthesis of an unusual osmolyte, di-*myo*-inositol-1,1'-phosphate, that accumulates in anaerobic hyperthermophiles (Chen et al. 1998). However, recent three-dimensional structural analysis of MJ0109 (Stec et al. 2000) indicated that the archaeal enzyme has an even higher resemblance to the dimer unit of tetrameric pig

kidney fructose bisphosphatase (FBPase) than to IMPase enzymes. Recent crystallographic studies of AF2372 (Stieglitz et al. 2002) confirmed that the structure of this second dual-specificity phosphatase also resembles the dimer unit of FBPase. Both archaeal enzymes have FBPase and IMPase activity that requires divalent cations, Mg²⁺ or Mn²⁺ (Stec et al. 2000; Stieglitz et al. 2002). In eukaryotes and bacteria, IMPase and FBPase enzymes exhibit a variety of regulatory mechanisms. IMPases may be regulated by intracellular Mg²⁺, light, and pH (Pollack et al. 1993; Balmer et al. 2001), whereas mammalian cytosolic FBPase activity is regulated allosterically by adenosine monophosphate (AMP) and fructose-2,6-bisphosphate (F-2,6-BP; Muniyappa et al. 1983). Effector binding causes conformational changes that inhibit enzymatic activity (Liang et al. 1993; Zhang et al. 1994). The archaeal dual-function enzymes lack

Reprint requests to: Mary Roberts, Department of Chemistry, Boston College, Chestnut Hill, MA 02467, USA; e-mail: mary.roberts@bc.edu; fax: (617) 552-2705.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0236003>.

the allosteric AMP site (Johnson et al. 2001; Stieglitz et al. 2002), and their mode of regulation in vivo is unknown.

In higher plants, there are two distinct FBPase enzymes. Cytosolic FBPase is involved in sucrose synthesis from triose phosphates exported from chloroplasts. This gluconeogenic enzyme is regulated like the mammalian FBPase, that is, through F-2,6-BP and AMP (Villeret et al. 1995). A second FBPase is involved in the generation of ribulose in the reductive pentose phosphate or Calvin cycle (Preiss et al. 1967). This FBPase enzyme is found sequestered in the chloroplast and is not regulated by AMP (Halliwell 1981), but by light via the ferredoxin/thioredoxin system (Schurmann and Jacquot 2000). Chloroplast FBPases are partially inactivated by oxidization of spatially close cysteines to form an intramolecular disulfide (Jacquot et al. 1997; Balmer et al. 2001). Reduction of the regulatory disulfide by thioredoxin f, which is itself reduced by photosynthetic electron flow through the ferredoxin/thioredoxin pathway, restores the FBPase activity (Schurmann and Jacquot 2000). The regulation of chloroplast FBPase by thioredoxin may be a response to photosynthetic oxidative stress (Ruelland and Miginiac-Maslow 1999). Disulfide formation as a mechanism to regulate FBPase activity has not been detected in any other FBPase thus far.

A unique feature of the *A. fulgidus* IMPase/FBPase structure (Stieglitz et al. 2002) is that the sulfhydryl groups of two of the four cysteines (Cys150 and Cys186) are separated by only 4 Å. Despite the close proximity, both sulfhydryls of Cys150 and Cys186 are fully reduced in the

refined model of the apo form of the enzyme (Fig. 1). If these cysteines are capable of reversible disulfide formation, they might indicate a mode of allosteric regulation through redox signaling not previously observed in archaea. The present work investigates the role of these two cysteine residues in *A. fulgidus* AF2372 and shows that the phosphatase activity of the enzyme can be abolished through oxidation and formation of an intramolecular disulfide bond.

Materials and methods

Chemicals

EDTA, sodium chloride, magnesium chloride, malachite green oxalate, ammonium molybdate, L-dithiothreitol (DTT), SDS-PAGE molecular weight markers, and Trizma base were obtained from Sigma. L-Inositol-1-phosphate (L-I-1-P) was synthesized enzymatically by using an I-1-P synthase cloned from *A. fulgidus* (Chen et al. 2000). *Escherichia coli* thioredoxin was purchased from Promega. All other chemicals were reagent grade.

Overexpression and purification of recombinant IMPase enzymes

Cloning of the AF2372 gene from *A. fulgidus*, overexpression of its gene product in *E. coli*, and purification of the recombinant enzyme were carried out as described else-

Figure 1. (A) Monomer unit of AF2372 dimer showing the location of the four cysteines. (B) The electron density of Cys150 and Cys186 of a single subunit superimposed on the model, showing that both cysteine residues are reduced in the structure.

where (Stieglitz et al. 2002). Fractions containing pure AF2372 (as measured by SDS-PAGE and silver staining of the gels) were concentrated to ~12 mg/mL and stored in 20 mM Tris HCl (pH 8.0) with 1.0 mM EDTA. For activity assays, the enzyme was diluted to 1 μ g/mL (36 μ M). MJ0109 from *M. jannaschii* was overexpressed and the recombinant protein purified as described previously (Chen and Roberts 1998).

Site-directed mutagenesis

Mutagenesis of AF2372 to replace Cys150 with a serine residue was performed with the Stratagene QuikChange kit. Wild-type plasmid was isolated with the QIA miniprep kit from QIAGEN and stored at 4°C until needed as a template for mutagenesis. Two primers for PCR were designed to change Cys150 to serine: 5'-GCAGAGGAGCTCTACTCG AACGCGATCATTTAC-3' and its complementary strand. The sequence of the variant gene was determined by the Molecular Medicine Unit, Beth Israel Deaconess Medical Center, by using T7 and P5 control primers for the plasmid containing the insert to ensure that the correct mutation had been prepared. Transformation of BI21-competent cells with the variant gene was used to overexpress the AF2372 C150S variant. Purification of the variant protein followed the protocol for wild-type protein, except that C150S required a slightly higher salt concentration for elution from the Q-Sepharose fast-flow column.

Activity assays

Phosphatase activities of reduced and oxidized wild-type AF2372, the C150S variant, and MJ0109 were measured toward 5 mM L-I-I-P with 30 mM MgCl₂ in 50 mM Tris (pH 8.0), conditions that represent $V_{\max}$ (Chen and Roberts 1998; Stec et al. 2000). Samples (containing ~1 μ g enzyme) and blanks (lacking enzyme) were heated for 1 min to 80°C. The inorganic phosphate generated was quantified by using a colorimetric assay (Itaya and Michio 1966). Phosphatase activities were also measured toward pNPP (15 mM) as a substrate (Stieglitz et al. 2002) by using the absorbance of the *p*-nitrophenylate product at 412 nm to measure product. This assay was used to test recovery of enzymatic activity from oxidized AF2372 before and after the addition of 1 mM DTT.

Oxidation of IMPase/FBPase enzymes

Ten milliliters of each enzyme, *A. fulgidus* AF2372, wild type and variant, and MJ0109 from *M. jannaschii*, in 0.05 M Tris-HCl (pH 8.0), were placed in a 50-mL Falcon tube and bubbled (1 to 2 L/h) with a gas mixture containing O₂/CO₂ (95 : 5) for up to 6 h at room temperature. The bubbling rate was chosen to minimize foaming (an antifoaming reagent

reduced enzyme activity so it was not used in the experiments). At intervals, an aliquot was removed and assayed for phosphatase activity, as well as intermolecular cross-linking (analyzed by nonreducing SDS-PAGE). At enzyme concentrations of 1.5 to 3.0 mg/mL, a small amount (5% to 10%) of a cross-linked dimer of AF2372 was observed. To prevent this aggregation and intermolecular cross-linking, the protein for all oxidation experiments was diluted to 0.1 mg/mL (3.6 μ M). Alternatively, the enzymes (2.5 μ M in 0.1 M Tris at pH 8.0, 1 mM EDTA) were incubated with up to 250 μ M oxidized thioredoxin (which contained 15% reduced thioredoxin) for 28 h at 37°C, and the solutions were tested for activity.

Quantitation of sulfhydryl groups

The concentration of cysteines in reduced and oxidized AF2372 and MJ0109 was measured by derivatizing the protein under denaturing conditions with DTNB and monitoring the absorbance at 412 nm (Ellman 1959). An aliquot (20 μ L) of a DTNB stock solution (3.96 mg/mL in 0.1 M phosphate at pH 7.0) was added to each 3.0 mL aliquot of protein (3.6 μ M in 0.1 M phosphate at pH 8.0), and SDS was added to a final concentration of 1% w/v. Oxidized samples and nonoxidized controls were incubated for 4 to 6 h or until A_{412} did not change. An extinction coefficient of 13,600 M⁻¹ cm⁻¹ was used to calculate the concentration of free sulfhydryl groups. In experiments in which *E. coli* thioredoxin was used to oxidize AF2372, the two proteins were separated using a Sephadex G150 sizing column (AF2372 is a dimer of 28-kD subunits, whereas *E. coli* thioredoxin is a 12-kD monomer) and analyzed separately as outlined above.

Results

Effect of O₂ on AF2372 and MJ0109 sulfhydryl content and phosphatase activity

Under normal protein expression and isolation conditions, the activity of AF2372 and MJ0109 were not sensitive to the presence of oxygen. If the proteins were denatured, 98.5% of the four cysteines in AF2372 and 99% of the three cysteines in MJ0109 could be derivatized with DTNB. To enhance the possibility of disulfide formation, protein samples were bubbled with O₂/CO₂ (95 : 5) and incubated for up to 6 h with aliquots withdrawn periodically to measure enzyme-specific activity and the sulfhydryl content. With this more rigorous oxidation, less cysteine was modified by DTNB over time, with the eventual loss of two of the four cysteines groups (for an example of an oxidation experiment, see Fig. 2A). With the same treatment and over the same time course (5 h), a much smaller fraction of cysteine was lost from MJ0109. Nonreducing SDS-PAGE confirmed

Figure 2. Loss of sulfhydryl groups detected with the DTNB assay (A) and decrease in activity of FBPase/IMPase enzymes (3.6 μM ; B) as a function of time bubbling in O₂ at 37°C: AF2372 (filled circles), C150S (empty circles), and MJ0109 (triangles). (C) Activity of the same three enzymes before and after addition of 1 mM DTT: AF2372 WT (black), C150S (white), and MJ0109 (gray).

that there was no intermolecular disulfide formation in AF2372, although a small amount of intermolecular cross-linking could be observed with MJ0109. For AF2372, there was a direct correlation between the amount of time O₂ was

bubbled through the solution and loss of free sulfhydryl content (Fig. 2A).

The activity of the enzyme at each time point during the incubation with O₂ was also measured (Fig. 2B). Only AF2372 lost significant activity as the solution was bubbled with O₂. There was little decrease in MJ0109 activity over time, despite a small drop in the number of free sulfhydryl groups. In contrast, AF2372 showed a decrease in activity that correlated with the loss of two free sulfhydryl groups, which presumably formed a disulfide bond on oxidation. After 5-h incubation with O₂, AF2372 activity was <2% of the original activity. This loss of activity was correlated with a loss of 1.95 sulfhydryl groups per molecule of enzyme.

Cys150 in AF2372 aligns with a serine residue in the *M. jannaschii* IMPase/FBPase. This comparison prompted us to generate a variant of AF2372 with the cysteine at position 150 replaced with a serine residue. If the loss of activity of AF2372 on incubation with O₂ is owing to disulfide bond formation between Cys150 and Cys186, then the C150S variant should retain activity under oxidative conditions. The C150S enzyme had V_{max} ($43.1 \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and K_m (4.1 mM) values with pNPP as a substrate that were similar to those for the wild-type AF2372 ($V_{\text{max}} = 30.1 \pm 0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $K_m = 3.8 \text{ mM}$). Thus, substitution of the serine for cysteine did not affect the catalytic machinery. When a solution of AF2372 C150S was bubbled with O₂ in parallel to native AF2372 for several hours, there was no loss of activity or cysteine content (Fig. 2A,B). The lack of O₂ sensitivity of the variant is consistent with formation of the intramolecular disulfide between Cys150 and Cys186 in native AF2372.

The loss of activity of AF2372 on O₂ treatment could be reversed by the addition of 1 mM DTT (Fig. 2C). For comparison, DTT had no effect on activity of the C150S variant or MJ0109 after exposure to oxidizing conditions. Repeating oxidation experiments multiple times over varied time courses showed that the activity of AF2372 decreased well before two of the four cysteines were lost (Fig. 3). This nonlinear relationship between loss of activity and loss of free cysteines indicates there is some level of cooperativity between the two monomeric units of the dimer.

Selective oxidation of AF2372 by thioredoxin

A commercially available *E. coli* thioredoxin was tested for its ability to oxidize (Pigiet and Schuster 1986) wild-type AF2372, the C150S variant, and MJ0109, because no thioredoxins have been isolated from *A. fulgidus*. Prior to the experiment, thioredoxin was tested for free cysteine, and it was determined that ~10% was already in a reduced form. AF2372, the C150S variant, and MJ0109 (2.5 μM) were incubated with various concentrations of thioredoxin for 28 h at 37°C (Fig. 4A). AF2372, and not C150S or MJ0109,

lost activity when the *E. coli* thioredoxin was present. The extent of enzyme inactivation was enhanced with higher concentrations of thioredoxin. The same incubation conditions in the absence of thioredoxin and without actively bubbling the solution with O_2 had no effect on the activity of AF2372.

The time dependence of the thioredoxin-catalyzed inactivation of AF2372 was also monitored. Enzyme (125 μ M) was incubated with a 1 : 1.1 ratio of thioredoxin at 37°C. Over the 30-h time course of the experiment, there was a direct correlation between the decrease in DTNB-modifiable cysteines in AF2372 and the increase in DTNB-modifiable cysteines of thioredoxin (Fig. 4B). This corresponded to the loss of two of the four cysteines at the end of the experiment.

Discussion

The IMPase superfamily of proteins consists of bacterial and eukaryotic enzymes with highly conserved structural motifs of alternating α -helices and β -sheets connected by loops (York et al. 1995). AF2372 and MJ0109, archaeal members of this family, share the same molecular fold (Johnson et al. 2001; Stieglitz et al. 2002). Their tertiary structures are organized as two domains that are reminiscent of the AMP- and FBP-binding domains in eukaryotic FBPs, although the archaeal enzymes are not regulated by AMP or FBP binding (Chen and Roberts 1998; Stieglitz et al. 2002). Although they carry out FBPase chemistry, AF2372 and MJ0109 have very low sequence homology with other FBPs and, in particular, with plant (pea and spinach chloroplast) FBPase. Nevertheless, there is an intriguing analogy regarding the spatial positioning of cysteines in one of the archaeal proteins, AF2372 (Stieglitz

Figure 3. Relative activity of AF2372 as a function of sulfhydryl (reduced cysteine) content.

Figure 4. (A) Effect of *Escherichia coli* thioredoxin concentration on the activity of AF2372 (filled circles), C150S (empty circles), and MJ0109 (triangles). Samples (2.5 μ M phosphatase) were incubated at 37°C with the indicated concentration of thioredoxin for 28 h. (B) Correlation of DTNB-modifiable cysteine (as measured by A_{412}) in AF2372 (circles) and thioredoxin (crosses) as a function of time on incubation of a 1 : 1.1 AF2372 (125 μ M)/thioredoxin mixture.

et al. 2002) and the two plant chloroplast FBPase structures (Villeret et al. 1995; Chiadmi et al. 1999).

Pig kidney and chloroplast FBPs contain six to seven cysteine residues, whereas the archaeal enzymes contain half that number (Table 1). In the mammalian enzyme, none of the cysteines are close enough to form intramolecular disulfides. However, in chloroplast FBPs, there is an insertion of ~15 amino acids, forming an elongated and flexible connector loop containing cysteine residues that

Table 1. Cysteine content and disulfide formation in FBPsases

Source	Cysteines	Disulfide formation	Cysteine pair	Protein Data Bank file	Reference
Methanococcus jannaschii	3	No		1DK4	Stec et al. 2000
Archaeoglobus fulgidus	4	Yes (in vitro)	150/186	1LBV	Stieglitz et al. 2002
Pea	7	Yes	153/173	1DBZ	Chiadmi et al. 1999
Spinach	7	Yes	155/174	1SPI	Balmer et al. 2001 Villeret et al. 1995
Pig kidney	6	No		1CNQ	Choe et al. 2000

undergo reversible disulfide formation as a mechanism to regulate FBPAse activity. The archaeal enzymes MJ0109 and AF2372, contain only three and four cysteines each, respectively. Their positions are not conserved between the archaeal species, nor are they conserved when compared with other FBPsases. All the cysteines in MJ0109 are separated from each other by >6 Å and could not form an intramolecular disulfide bond (Stec et al. 2000; Johnson et al. 2001). However, in AF2372 two of the cysteine residues, Cys150 and Cys186, are in sufficiently close proximity to form a disulfide bond. Their sulfur atoms are only 3.90 Å apart in monomer A and 3.96 Å apart in monomer B. The electron density (Fig. 1B) clearly shows that these two cysteine residues are in the reduced form (Stieglitz et al. 2002). These two cysteine residues are located close to the connecting loop between the domains.

The present studies clearly demonstrate that in vitro oxidation of AF2372 (but not MJ0109) causes intramolecular disulfide formation and loss of catalytic activity. The correlation between the loss of two of the four cysteine sulfhydryl groups in AF2372 with decreased enzymatic activity, clearly shows that AF2372 activity is regulated by disulfide formation in vitro. The lack of effect on the C150S variant of AF2372 provides a confirmation that those two spatially close Cys residues are involved. Furthermore, the observation that a thioredoxin (even one from the mesophilic *E. coli*) can catalyze disulfide formation is important because *A. fulgidus* is an anaerobic hyperthermophile, and free O_2 is not available in its growth medium. These data indicate a disulfide-mediated mechanism for regulating the FBPAse activity of AF2372 by thioredoxin system.

How could disulfide bond formation in AF2372 regulate activity?

In absence of the crystal structure of the fully oxidized form of AF2372, it is difficult to delineate the exact mechanism for the enzyme deactivation. However, the clues to understand the underlying mechanism are provided by studies of chloroplast FBPAse enzymes. It has been proposed in the case of spinach FBPAse, that a simple side-chain rotation could reduce the distance between the sulfur atoms from 4 to 2.4 to 2.7 Å to accommodate disulfide formation (Villeret et al. 1995). In pea chloroplast FBPAse, this process was

visualized under physiological conditions, and a crystal structure shows a clear disulfide bond between Cys152 and Cys173, which inactivated the enzyme (Chiadmi et al. 1999). A comparison of the oxidized to the reduced pea FBPAse as exemplified by the structure C153S variant, which cannot form the disulfide bond, showed very few structural differences. However, small structural variations were detected at the loop containing Glu105, a residue critical for binding Mg^{2+} . It is believed that those changes could cause a diminished binding of metal ions to the oxidized enzyme.

On the other hand, crystallographic studies of cytosolic FBPsases with AMP bound indicated that structural changes are small with allosteric regulators, and indicate that it is a global accumulation of these small changes that causes the misalignment of catalytically important residues that weakens binding of the metal ions (Choe et al. 2000; Nelson et al. 2000). It seems that the main effect caused by AMP binding (cytosolic FBPsases) or disulfide formation (chloroplast FBPAse) is at the level of the β -sheet that houses the active site. A conformational strain imposed on this β -sheet is most likely responsible for the small displacements needed for inhibition of catalytic activity.

To investigate whether a similar type of mechanism is responsible for inactivation of AF2372, a model with a disulfide between Cys150 and Cys186 was built in Quanta (Brooks et al. 1983; K. Stieglitz, unpubl.). The oxidized model superimposed on to the apoenzyme with a root mean square derivation of 1.14 Å for all 504 residues in the dimer. As expected, there were no gross changes observed, but a pattern of small changes emerged that was similar in nature to that observed in pea FBPAse when comparing oxidized and variant C153S structures. For example, the orientation of side-chain carboxylates of Asp38, Glu67, and Asp85 were altered in the oxidized model. These are all metal ion ligands and indicate that disulfide formation reduces metal ion affinity of the protein. Because divalent cations are necessary for activity, any changes that alter their affinity for the enzyme would reduce activity.

Role of redox regulation in an anaerobic archaeon

FBPsases, critical enzymes in gluconeogenesis, are highly regulated in many species. In plants within the same organism, there is a difference in the way the two FBPsases are

regulated. The cytosolic variety is controlled by AMP and F-2,6-BP, whereas the chloroplast variety is regulated by disulfide reduction. The selection of cysteine oxidation/reduction as a regulatory mechanism for FBPase activity is quite surprising. The chloroplast FBPase has evolved such that redox regulation either replaced or remained superior to regulation of phosphatase activity by AMP. Therefore, this mechanism must reflect an unknown evolutionary reason for selecting one type of regulation over the other. The analog of the AMP-binding site does not necessarily guarantee that the enzyme will be sensitive to AMP. For example, an AMP-like site exists on the spinach FBPase (Ke et al. 1990); yet, the plant enzyme is insensitive to AMP (Villeret et al. 1995).

The two anaerobic archaeal hyperthermophiles are not regulated by AMP or F-2,6-BP (in fact, F-2,6-BP is a substrate for these archaeal enzymes; Chen and Roberts 1998; Stieglitz et al. 2002). This is a puzzling feature because some form of control of FBPase activity is needed. Although Mg^{2+} could contribute to in vivo regulation of AF2372, the high K_D for this ion (15 to 30 mM; Stieglitz et al. 2002) and the variation of intracellular levels of this ion may not be sufficient to turn off the activity when necessary.

M. jannaschii, a methanogen, derives all its carbon compounds from CO_2 . It does not use glucose. If ^{14}C -glucose is added to the medium, it is not incorporated into macromolecules (M.F. Roberts, unpubl.). Thus, it needs an active and robust gluconeogenesis pathway. This organism contains no inositol lipids and does not synthesize di-myoinositol-1,1'-phosphate (DIP) as an osmolyte (H. Meekins and M.F. Roberts, unpubl.) and would not need to control an IMPase activity. In contrast, *A. fulgidus* is usually grown heterotrophically with a variety of carbon sources (most often lactate) as the electron donor and SO_4^{2-} or $S_2O_3^{2-}$ as the acceptor (Robb and Place 1995). This organism also uses DIP as an osmolyte, particularly at supraoptimal growth temperatures (Martins et al. 1995), and AF2372 has been proposed as the second enzyme required for DIP synthesis (Chen et al. 1998). Neither AMP (FBPase inhibitor) nor Mg^{2+} (often shown to inhibit IMPase activities) inhibit AF2372; hence, there must be another mechanism to regulate both FBPase and IMPase activities. Clearly, our structural and functional studies of AF2372 strongly indicate that cysteine oxidation/reduction is a candidate to regulate the IMPase/FBPase enzyme from the anaerobe *A. fulgidus*.

Although the rates of AF2372 oxidation in vitro were low at 37°C (either by bubbling O_2 or by adding an *E. coli* thioredoxin), an *A. fulgidus* thioredoxin is likely to be much more effective (and possibly specific) near the growth temperature of the organism in catalyzing cysteine oxidation. Based on the known sequence of pea thioredoxin, a BLAST search of the *A. fulgidus* genome yielded three putative thioredoxins: AF1284, AF2144, and AF0769, with identities and positives of 49% and 66%, 38% and 68%, and 24%

and 50%, respectively. Similar searches with the *E. coli* thioredoxin also highlighted the first two of these putative proteins in *A. fulgidus*. This strongly indicates that the hyperthermophilic anaerobic *A. fulgidus* has thioredoxin-like proteins, and one or more of these could be responsible for regulating FBPase/IMPase activity. Changes in the physiological state of this anaerobic cell could be relayed by various stressors that either directly or indirectly control the oxidation of AF2372 by a thioredoxin-like protein. In addition, several protein disulfide oxidoreductases discovered in archaea show structural features similar to glutaredoxin and thioredoxin (Cave et al. 2001), and perhaps, these could be involved in regulating AF2372 activity as well.

Acknowledgments

This work has been supported by grant MCB-9978250 (to M.F.R.) from the National Science Foundation grant DE-FG 02-91ER20025 (to M.F.R.) from the Department of Energy Biosciences, and from the NIH grant (1R01 GM64481) to B.S.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Balmer, Y., Stritt-Etter, A., Hirasawa, M., Jacquot, J.P., Keryer, E., Knaff, D.B., and Schurmann, P. 2001. Oxidation-reduction and activation properties of chloroplast fructose 1,6-bisphosphatase with mutated regulatory site. *Biochemistry* **40**: 15444–15450.
- Brooks, B.R., Brucoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., and Karplus, M. CHARMm: A program for macromolecular energy, minimization, and dynamics calculations. *J. Comp. Chem.* **4**:187–217.
- Cave, J.W., Cho, H.S., Batchelder, A.M., Yokota, H., Kim, R., and Wemmer, D.E. 2001. Solution nuclear magnetic resonance structure of a protein disulfide oxidoreductase from *Methanococcus jannaschii*. *Protein Sci.* **10**: 384–396.
- Chen, L. and Roberts, M.F. 1998. Cloning and expression of inositol monophosphatase gene from *Methanococcus jannaschii* and characterization of the enzyme. *Appl. Environ. Microbiol.* **64**: 2609–2615.
- Chen, L., Spiliotis, E.T., and Roberts, M.F. 1998. Biosynthesis of di-myoinositol-1,1'-phosphate: A novel osmolyte in hyperthermophilic archaea. *J. Bacteriol.* **180**: 3785–3792.
- Chen, L., Zhou, C., Yang, H., and Roberts, M.F. 2000. Inositol-1-phosphate synthase from *Archaeoglobus fulgidus* is a class II aldolase. *Biochemistry* **39**: 12415–12423.
- Chiadmi, M., Navaza, A., Miginiac-Maslow, M., Jacquot, J.P., and Cherfils, J. 1999. Redox signalling in the chloroplast: Structure of oxidized pea fructose-1,6-bisphosphate phosphatase. *EMBO J.* **18**: 6809–6815.
- Choe, J.Y., Fromm, H.J., and Honzatko, R.B. 2000. Crystal structures of fructose 1,6-bisphosphatase: Mechanism of catalysis and allosteric inhibition revealed in product complexes. *Biochemistry* **39**: 8565–8574.
- Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**: 70–77.
- Halliwell, B. 1981. *Chloroplast metabolism*. Oxford University Press, New York.
- Itaya, K. and Michio, U. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta* **14**: 361–366.
- Jacquot, J.P., Lopez-Jaramillo, J., Miginiac-Maslow, M., Lemaire, S., Cherfils, J., Chueca, A., and Lopez, J. 1997. Cysteine-153 is required for redox regulation of pea chloroplast fructose-1,6-bisphosphatase. *FEBS Lett.* **401**: 143–147.
- Johnson, K.A., Chen, L., Yang, H., Roberts, M.F., and Stec, B. 2001. Crystal structure and catalytic mechanism of the MJ0109 gene product: A bifunc-

- tional enzyme with inositol monophosphatase and fructose 1,6-bisphosphatase activities. *Biochemistry* **40**: 618–630.
- Ke, H., Zhang, Y., and Lipscomb, W.N. 1990. Crystal structure of fructose-1,6-bisphosphatase complexed with fructose 6-phosphate, AMP, and magnesium. *Proc. Natl. Acad. Sci.* **87**: 5243–5247.
- Kelley, N., Giroux, E.L., Lu, G., and Kantrowitz, E.R. 1996. Glutamic acid residue 98 is critical for catalysis in pig kidney fructose-1,6-bisphosphatase. *Biochem. Biophys. Res. Commun.* **219**: 848–852.
- Liang, J.Y., Zhang, Y., Huang, S., and Lipscomb, W.N. 1993. Allosteric transition of fructose-1,6-bisphosphatase. *Proc. Natl. Acad. Sci.* **90**: 2132–2136.
- Martins, L.O., Huber, R., Huber, H., Stetter, K.O., da Costa, M.S., and Santos, H. 1995. Organic solutes in hyperthermophilic archaea. *Appl. Environ. Microbiol.* **63**: 896–902.
- Muniyappa, K., Leibach, F.H., and Mendicino, J. 1983. Reciprocal regulation of fructose 1,6-bisphosphatase and phosphofructokinase by fructose 2,6-bisphosphate in swine kidney. *Life Sci.* **32**: 271–278.
- Nelson, S.W., Iancu, C.V., Choe, J.Y., Honzatko, R.B., and Fromm, H.J. 2000. Tryptophan fluorescence reveals the conformational state of a dynamic loop in recombinant porcine fructose-1,6-bisphosphatase. *Biochemistry* **39**: 11100–11106.
- Pigiet, V.P. and Schuster, B.J. 1986. Thioredoxin-catalyzed refolding of disulfide-containing proteins. *Proc. Natl. Acad. Sci.* **83**: 7643–7647.
- Pollack, S.J., Knowles, M.R., Atack, J.R., Broughton, H.B., Ragan, C.I., Osborne, S., and McAllister, G. 1993. Probing the role of metal ions in the mechanism of inositol monophosphatase by site-directed mutagenesis. *Eur. J. Biochem.* **217**: 281–287.
- Preiss, J., Biggs, M.L., and Greenberg, E. 1967. The effect of magnesium ion concentration on the pH optimum of the spinach leaf alkaline fructose diphosphatase. *J. Biol. Chem.* **242**: 2292–2294.
- Robb, F.T. and Place, A.R. 1995. *Archaea: A laboratory manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.
- Ruelland, E. and Miginiac-Maslow, M. 1999. Regulation of chloroplast enzyme activities by thioredoxins: Activation or relief from inhibition? *Trends Plant Sci.* **4**: 136–141.
- Schurmann, P. and Jacquot, J.P. 2000. Plant thioredoxin systems revisited. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**: 371–400.
- Stec, B., Yang, H., Johnson, K.A., Chen, L., and Roberts, M.F. 2000. MJ0109 is an enzyme that is both an inositol monophosphatase and the “missing” archaeal fructose-1,6-bisphosphatase. *Nat. Struct. Biol.* **7**: 1046–1050.
- Stieglitz, K.A., Johnson, K.A., Yang, H., Roberts, M.F., Seaton, B.A., Head, J.F., and Stec, B. 2002. Crystal structure of a dual activity IMPase/FBPase (AF2372) from *Archaeoglobus fulgidus*: The story of a mobile loop. *J. Biol. Chem.* **277**: 22863–22874.
- Villeret, V., Huang, S., Zhang, Y., Xue, Y., and Lipscomb, W.N. 1995. Crystal structure of spinach chloroplast fructose-1,6-bisphosphatase at 2.8 Å resolution. *Biochemistry* **34**: 4299–4306.
- York, J.D., Ponder, J.W., and Majerus, P.W. 1995. Definition of a metal-dependent/Li⁺ inhibited phosphomonoesterase protein family based on a conserved three-dimensional core structure. *Proc. Natl. Acad. Sci.* **92**: 5149–5153.
- Zhang, Y., Liang, J.Y., Huang, S., and Lipscomb, W.N. 1994. Toward a mechanism for the allosteric transition of pig kidney fructose-1,6-bisphosphatase. *J. Mol. Biol.* **244**: 609–624.