Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1710 (2005) 103 – 112
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

The interaction of 5′-adenylylsulfate reductase from *Pseudomonas aeruginosa* with its substrates ☆

Sung-Kun Kim [a,*], Afroza Rahman [c], Jeremy T. Mason [a], Masakazu Hirasawa [a], Richard C. Conover [d], Michael K. Johnson [d], Myroslawa Miginiac-Maslow [e], Eliane Keryer [e], David B. Knaff [a,b], Thomas Leustek [c]

[a] *Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, USA*
[b] *Center for Biotechnology and Genomics, Texas Tech University, Lubbock, Texas 79409-1061, USA*
[c] *Biotechnology Center for Agricultural and the Environment, Department of Plant Biology and Pathology, Rutgers University, New Brunswick, New Jersey 08901-8520, USA*
[d] *Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, USA*
[e] *Institut de Biotechnologie des Plantes, UMR CNRS 8618, Bâtiment 630, Université Paris-Sud, 91405 Orsay Cedex, France*

Received 17 June 2005; received in revised form 2 August 2005; accepted 21 September 2005
Available online 7 October 2005

Abstract

APS reductase from *Pseudomonas aeruginosa* has been shown to form a disulfide-linked adduct with mono-cysteine variants of *Escherichia coli* thioredoxin and *Chlamydomonas reinhardtii* thioredoxin *h*1. These adducts presumably represent trapped versions of the intermediates formed during the catalytic cycle of this thioredoxin-dependent enzyme. The oxidation–reduction midpoint potential of the disulfide bond in the *P. aeruginosa* APS reductase/*C. reinhardtii* thioredoxin *h1* adduct is −280 mV. Site-directed mutagenesis and mass spectrometry have identified Cys256 as the *P. aeruginosa* APS reductase residue that forms a disulfide bond with Cys36 of *C. reinhardtii* TRX *h*1 and Cys32 of *E. coli* thioredoxin in these adducts. Spectral perturbation measurements indicate that *P. aeruginosa* APS reductase can also form a non-covalent complex with *E. coli* thioredoxin and with *C. reinhardtii* thioredoxin *h*1. Perturbation of the resonance Raman and visible-region absorbance spectra of the APS reductase [4Fe–4S] center by either APS or the competitive inhibitor 5′-AMP indicates that both the substrate and product bind in close proximity to the cluster. These results have been interpreted in terms of a scheme in which one of the redox-active cysteine residues serves as the initial reductant for APS bound at or in close proximity to the [4Fe–4S] cluster.

Keywords: *Pseudomonas aeruginosa* APS reductase; Thioredoxin; Iron–sulfur cluster; Disulfide-linked protein complex

1. Introduction

The assimilation of sulfate into cysteine is a hallmark of aerobic bacteria, archaea, and photosynthetic eukaryotes. The process requires the conversion of +6 sulfur to −2 sulfur, a reduction that requires 8 electrons. In all cases sulfate is first activated by ATP sulfurylase to 5′-adenylylsulfate (APS). In some species APS is modified by phosphorylation at the 3′ position to produce 3′-phospho-5′-adenylylsulfate (PAPS). The sulfur in APS or PAPS is then reduced in two steps to sulfide. The first step adds two electrons and the second step, catalyzed by sulfite reductase, adds 6 electrons. The two-electron transfer step is catalyzed in different species by significantly diverged, but related, enzymes referred to as APS or PAPS reductases.

Abbreviations: APS, 5′-adenylylsulfate; BsAPR, *Bacillus subtilis* APS reductase; CNBr, cyanogen bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GRX, glutaredoxin; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization-Time Of Flight; PaAPR, *Pseudomonas aeruginosa* APS reductase; PAGE, polyacrylamide gel electrophoresis; RR, resonance Raman; SDS, sodium dodecylsulfate; TRX, thioredoxin; TRX C35A, the C35A mono-cysteine variant of *Escherichia coli* thioredoxin; TRX *h*1C39S, the C39S mono-cysteine variant of *Chlamydomonas reinhardtii* thioredoxin *h*1; WT, wild type

☆ Supported by grants from the U.S. Department of Agriculture (2002-35318-12503 to T.L. and D.B.K.) and from NIH (GM62524 to M.K.J.).

* Corresponding author. Tel.: +1 806 742 3051; fax: +1 806 742 1289.
E-mail address: sung-kun.kim@ttu.edu (S.-K. Kim).

0005-2728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2005.09.004

The PAPS reductase of *Escherichia coli*, encoded by the *cysH* gene, was the first member of this group of enzymes to be studied and remains the most extensively characterized enzyme in the group. *E. coli* CysH uses PAPS exclusively, does not catalyze APS reduction and does not contain any prosthetic groups. The protein has a single cysteine at the active site and relies on thioredoxin (TRX) or glutaredoxin (GRX) as an electron donor [1,2]. Until recently it was thought that most sulfate assimilating organisms use the PAPS reductase pathway for sulfate assimilation, but this hypothesis has recently been modified [3]. It is now clear that PAPS reductases have only a limited taxonomic range and most sulfate assimilators use an APS-dependent reductase for assimilatory sulfate reduction.

In addition to the difference in substrate specificity, APS and PAPS reductases differ in prosthetic group and cysteine contents. APS reductases contain a [4Fe–4S] center and four conserved cysteine residues that are not present in the PAPS reductases. Three of these cysteines serve as ligands for the [4Fe–4S] cluster [3–5]. The [4Fe–4S] cluster of APS reductase has been proposed to be a structural determinant of the specificity for APS over PAPS, although the form of the enzyme found in *Bacillus subtilis* (BsAPR) is able to catalyze reduction of both APS and PAPS despite having a [4Fe–4S] cluster and its three conserved cysteine ligands [4].

The APS reductases are classified as belonging to one of two types: one that utilizes reduced TRX as an electron donor [6–9]; and another that is specific for reduced glutathione as an electron donor [10]. The glutathione-dependence of the later enzyme can be explained by its structure, which includes two domains, a reductase domain and a TRX/GRX-like domain [10–12].

The catalytic mechanism(s) of APS and PAPS reductases have not yet been clearly elucidated. More is known about PAPS reductase, for which kinetic data is consistent with the operation of a Ping-Pong mechanism. It was proposed that the single cysteine residue of PAPS reductase, present in a (KRT)ECG(LI)H motif, functions as the acceptor of electrons from reduced TRX. The catalytic cycle begins with a homo-dimeric, oxidized form of PAPS reductase linked by a disulfide bridge. Reduced TRX reduces the disulfide bridge, producing two cysteine thiols. One thiol binds PAPS, reducing the sulfur of the substrate to a thiosulfonate, with the second thiol completing the two-electron transfer to produce sulfite and regenerate the disulfide-linked oxidized form of the enzyme [1]. The APS reductase from *Pseudomonas aeruginosa* (PaAPR) also exhibits Ping-Pong kinetics with respect to TRX and APS [7]. Unlike the case for PAPS reductase, the entry of reducing equivalents into oxidized PaAPR appears to involve the reduction of an intramolecular disulfide, formed between Cys140 and Cys256, the two cysteines that are not ligands to the [4Fe–4S] cluster [5]. Both Cys256, which corresponds to the active site cysteine of PAPS reductase, and Cys140, which is conserved among APS reductases, appear to be essential for the enzymatic activity of PaAPR [5].

The question of how APS reductases that use reduced TRX as an electron donor interact with this substrate has not been studied in detail. However, it is known that the reduction of protein disulfides to dithiols in reactions utilizing reduced thioredoxins as the electron donor generally involves a transient, mixed-disulfide between one cysteine of the target protein and the TRX active-site cysteine closest to the N-terminus [13–15,17]. The reaction is completed when the thiol group of the second cysteine in the TRX active site makes a nucleophilic attack on the disulfide, producing a dithiol on the enzyme and a disulfide on TRX. It was thus of interest to examine whether a similar disulfide-linked intermolecular intermediate is formed during the reaction between PaAPR and TRX.

Given our previous demonstration that the [4Fe–4S] cluster present in PaAPR is not redox-active [5], and faced with formulating a role for the cluster, we have devised a scheme (see Fig. 1) that proposes that the [4Fe–4S] cluster serves to bind APS (and its competitive inhibitor 5′-AMP) and that all of the redox-related functions of the enzyme involve only the redox-active dithiol/disulfide couple. Evidence has been obtained, consistent with the mechanism of Fig. 1, for the formation of a mixed disulfide between PaAPR and two thioredoxins that serve as effective electron donors for the PaAPR-catalyzed reduction of APS, *E. coli* TRX and TRX *h1* from the green alga *Chlamydomonas reinhardtii* and these intermediates have been characterized. Evidence has been obtained, also consistent with the scheme shown in Fig. 1, that the [4Fe–4S] cluster present in PaAPR may play a role in anchoring or activating the substrate for thiol-mediated reduction rather than having a direct role in electron transfer per se.

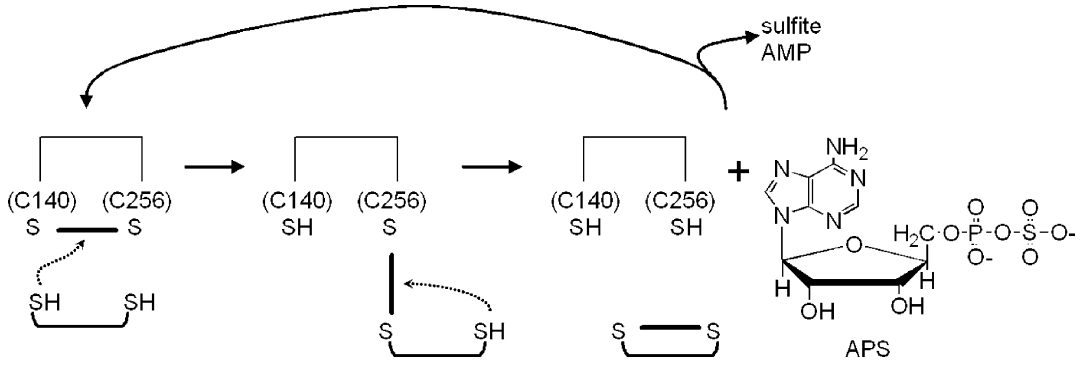

Fig. 1. A scheme for the catalytic cycle of PaAPR. The angular bracket represents PaAPR and the curved bracket represents TRX.