Adenylylsulfate reductases from archaea and bacteria are 1:1 αβ-heterodimeric iron–sulfur flavoenzymes – high similarity of molecular properties emphasizes their central role in sulfur metabolism

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Abstract Highly active adenylylsulfate (APS) reductase was isolated under N_2/H_2 from sulfate-reducing and sulfide-oxidizing bacteria and archaea. It was a 1:1 αβ-heterodimer of molecular mass ≈95 kDa, and two subunits (α≈75, β≈20 kDa). The specific activity was 11–14 μmol (min mg)$^{-1}$; cofactor analysis revealed 0.96 ± 0.05 FAD, 7.5 ± 0.1 Fe and 7.9 ± 0.25 S^{2-}. The photochemically reduced enzyme had a multiline EPR spectrum resulting from two interacting [4Fe–4S] centers. The properties of the different APS reductases were remarkably similar, although the enzyme is involved in different metabolic pathways and was isolated from phylogenetically far separated organisms. A structural model is proposed, with FAD bound to the α-subunit, and two [4Fe–4S] centers located in close proximity on the β-subunit.

Key words: Adenylylsulfate reductase; Iron–sulfur flavoenzyme; Sulfur metabolism

1. Introduction

The use of sulfur compounds as electron donor or acceptor for energy conservation is characteristic of several bacterial lineages and hyperthermophilic archaea. The pathways of dissimilatory sulfate reduction and sulfide oxidation involve four enzymes: ATP sulfurylase, inorganic pyrophosphatase, adenylylsulfate reductase (EC 1.8.99.2), and sulfite reductase [1]. In bacteria which oxidize reduced sulfur compounds these three enzymes operate in the reverse direction, liberating electrons and ATP. Prior to reduction sulfate has to be activated to adenosine-5′-phosphosulfate (APS) via ATP sulfurylase [2], and E_0' for APS/AMP+HSO_3^- is shifted to −60 mV [3]. APS reductase is a Fe–S flavoprotein and catalyzes both the two-electron reduction of APS to sulfite and oxidation of sulfite and AMP to APS [4]. The molecular parameters of APS reductase, such as mass, subunit composition and cofactor stoichiometry, have been a matter of debate [5]. Analysis of the genes encoding the α- and β-subunits of the APS reductase from *Archaeoglobus fulgidus* and *Desulfovibrio vulgaris* revealed a putative FAD-binding domain on the α-subunit. On the β-subunit, the arrangement of seven cysteine residues suggested the presence of a [3Fe–4S] and a [4Fe–4S] cluster similar to 7Fe ferredoxins [6]. Those authors proposed an $\alpha_2\beta$-subunit composition, with one FAD located at the interface of two α-subunits. In contrast, APS reductase was described as an $\alpha_2\beta_2$ complex with one single iron–sulfur cluster/αβ heterodimer [7]. In this case, the iron–sulfur center was thought to consist of more than four iron atoms. APS reductase from *Thiobacillus* sp. was described as a 170–210 kDa protein with one FAD and 6–11 Fe per molecule [8–12]. The mechanism of action of APS reductase and the role of its cofactors in catalysis are still poorly understood. Michaels et al. [13] reported the formation of a sulfite adduct at the N(5) position of FAD, and proposed it as an intermediate during catalysis. Here we describe a new procedure to purify APS reductase and compare the molecular properties of the Fe–S flavoenzyme from four different microorganisms.

2. Materials and methods

2.1. Growth conditions

Desulfovibrio desulfuricans Essex (ATCC 29577) [14], *A. fulgidus* DSM 4304^T [15], and *T. denitrificans* [16] were cultivated as described. *D. vulgaris* Hildenborough (NCIB 8303) was grown on a modified Postgate medium [17,18].

2.2. Purification of APS reductase

All chromatographic steps were carried out with a Pharmacia FPLC system in a Coy anaerobe chamber (95% N_2, 5% H_2). Typically, 15 g cells (wet weight, 0.5–0.6 g ml^{-1}) were suspended in 10 mM potassium phosphate, pH 7.0, containing a few crystals of DNase and 1 mM $MgCl_2$. Cells were disrupted by two passages through a French press cell (138 MPa). APS reductase was purified from the soluble fraction by chromatography on DEAE (Whatman) followed by high-resolution Fractogel-TMAE (Merck), or ResourceQ 15 and Superdex 200 (Pharmacia-Amersham).

2.3. Analytical methods

FAD was determined photometrically (ε_{450nm} = 11 300 M^{-1} cm^{-1} for free FAD) after TCA precipitation. Fe was determined with 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine [19], S^{2-} by the methylene-blue method [20]. The native molecular mass was determined by dynamic light scattering and size exclusion chromatography. The subunit stoichiometry was determined by denaturing size exclusion chromatography on a Superose 12 column followed by integration of the absorption peaks at 280 nm. The molar ratio of the α-subunit and the β-subunit was obtained using the molar absorption coefficients at 280 nm which were calculated from the amino acid sequence of the enzymes from *D. vulgaris* (GenBank accession number Z69372), *D. desulfuricans* (AF226708) and *A. fulgidus* [6] (AAB89579) with the program WinPep [21].

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Abbreviations: APS reductase, adenylylsulfate reductase; APS, adenosine-5′-phosphosulfate

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2.4. Activity and spectroscopy

APS reductase activity was determined as formation of APS [10] in 50 mM Tris–Cl, pH 7.6, 2 mM Na_2SO_3, 2 mM AMP, 1 mM $K_3Fe(CN)_6$, 25°C. UV-visible spectra were recorded on a Perkin-Elmer Lambda 16 instrument equipped with temperature-controlled cuvette holders. EPR spectra at X-band (9.6 GHz) were recorded on a Bruker ESP300 spectrometer as described [22].

2.5. Cloning and sequencing of the apr operon from D. desulfuricans

Primers were derived from N-terminal sequences of the β-subunit of APS reductase from *D. desulfuricans* and from the nucleotide sequence of the *apr* operon from *D. vulgaris*. Double strand sequencing was performed by GATC GmbH Konstanz.

3. Results

3.1. Purification and activity of APS reductase

Four APS reductases were purified to homogeneity under the exclusion of dioxygen by a new three-step chromatographic procedure, with a 5–10% yield as outlined for APS reductase from *Desulfovibrio* sp. (Table 1). The specific activity of pure APS reductase was 10–14 μmol min^{-1} mg^{-1} at 25°C which is the highest published so far [8,10,11,23].

3.2. The apr operon of D. desulfuricans

The operon structure of the genes encoding APS reductase of *D. desulfuricans* was the same as previously found in *D. vulgaris*, *A. fulgidus*, and *Allochromatium vinosum* [6,24]. The deduced amino acid sequences showed a high homology to the corresponding gene products from *D. vulgaris*, *A. fulgidus*, and *A. vinosum*. The homology was highest for the gene products of *D. vulgaris* (α-subunit, 91% similarity; β-subunit, 88% similarity), followed by that for the archaeon *A. fulgidus* (α-subunit, 55% similarity; β-subunit, 73% similarity), and sulfide-oxidizing *A. vinosum* (α-subunit, 58% similarity; β-subunit, 56% similarity). The AprB gene product contained eight conserved cysteine residues, with four of them arranged in a conventional Cys-x^1-x^2-Cys-x^3-x^4-Cys-...-Cys-Pro (x^n = variable amino acid) binding motif. The other four cysteine residues were arranged in a modified Cys-x^1-x^2-Cys-x^3-...-x^7-Cys-...-Cys-Pro motif, where five additional residues were inserted [6].

3.3. Molecular properties

The native molecular mass was 90–124 kDa (dynamic light scattering) compared to 128–154 kDa (size exclusion chromatography). On SDS–PAGE all four APS reductases showed two bands around 75 kDa (α-subunit) and 20 kDa (β-subunit), respectively. APS reductase was a 1:1 αβ-heterodimer with a mass of 95 kDa, in accordance with the amino acid sequences and results from SDS–PAGE. Cofactor analysis gave 0.96 ± 0.05 FAD, 7.5 ± 0.1 Fe and 7.9 ± 0.25 S^{2-}/95 kDa for the four enzymes.

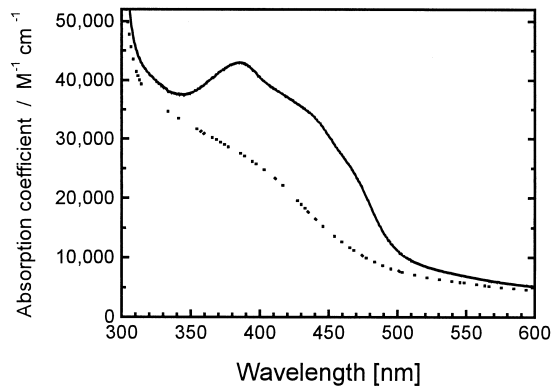

Fig. 1. UV-vis spectra of APS reductase from *D. desulfuricans*. Solid line, oxidized enzyme; dotted line, reduced with 2 mM Na_2SO_3 and 2 mM AMP.

3.4. UV-vis spectra

The UV-vis spectra of the APS reductases were almost identical with maxima at 390 and 278 nm (*D. desulfuricans*, *D. vulgaris* and *T. denitrificans*), and 388 and 278 nm (*A. fulgidus*) in the oxidized state (Fig. 1). The molar absorption coefficients at 390 nm (43 000 M^{-1} cm^{-1}) confirm the presence of one FAD ($\varepsilon_{380\ \mathrm{enzyme-bound\ flavin}} \approx 12\,000$ M^{-1} cm^{-1}) [25] per αβ-heterodimer, and two [4Fe–4S] centers ($\varepsilon_{400} = 16\,000$ M^{-1} cm^{-1} per center) [26]. APS reductase formed a sulfite adduct at the N(5) position of FAD. After addition of AMP to the sulfite adduct, formation of a transient flavin radical anion was observed at 380 nm followed by full reduction of FAD and the Fe–S centers [27]. The formation of a N(5)-sulfite adduct as well as of the flavin radical anion suggests a positive charge close to the N(1)–C(2) position of the isoalloxazine ring of FAD, as in glycolate oxidase [28].

3.5. EPR spectra

The EPR spectrum of APS reductase from *D. desulfuricans*, isolated in the presence of dioxygen, was typical for a [3Fe–4S] cluster with *g*-values at 2.03 and 2.00, and integrated to 0.05–0.1 spin/molecule (data not shown). This signal was absent in the spectrum of the enzyme prepared under N_2/H_2. With just sulfite added, or AMP, no signals from reduced Fe–S centers were observed. Reaction of APS reductase with sulfite and AMP led to a rhombic signal with $g_{z,y,x}$ = 2.082, 1.941, 1.902, characteristic for a reduced [4Fe–4S] cluster [29] (Fig. 2). With dithionite, the rhombic signal persisted with close to 1.0 spin/mol indicating full reduction of this cluster (center I). The EPR properties of the other APS reductases were practically identical. There was no EPR signal from the second iron–sulfur center (center II) with sulfite and AMP, or of dithionite, as reducing agents. It could be reduced photochemically with 5-deazaflavin/oxalate which generates

Table 1
Purification of APS reductase from *D. desulfuricans*

Purification step	Protein (mg)	Activity (μmol min^{-1})	Sp. activity (μmol min^{-1} mg^{-1})	A_{278nm}/A_{390nm}	Yield (%)
Crude extract	4092	5872	1.45	n.d.	100
Soluble fraction	2450	3710	1.50	n.d.	63
Membrane fraction	1420	1220	0.85	n.d.	21
DEAE	800	2510	3.15	7.3	43
TMAE	150	810	5.35	5.6	13
Superdex 200	78	677	13	4.6	11

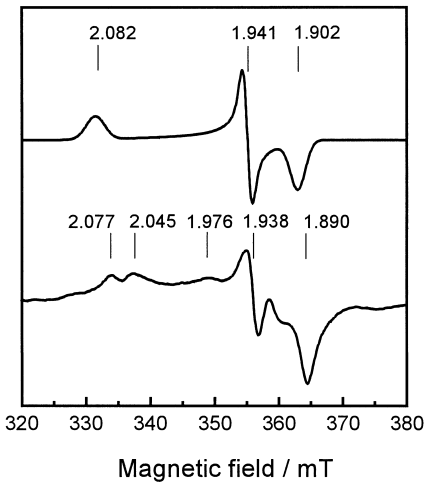

Fig. 2. Electron paramagnetic resonance spectra of APS reductase from *D. desulfuricans*. Upper curve: enzyme reduced with sulfite and AMP; lower curve: enzyme reduced photochemically with 5-deazaflavin and oxalate. Instrument settings: microwave frequency, 9.63 GHz; microwave power, 2 mW; modulation amplitude, 1 mT; temperature, 5–10 K; data handling [22].

electrons at a potential around −650 mV [30]. The resulting multiline signal (Fig. 2) points towards a magnetic interaction between the two [4Fe–4S] clusters as described for 2[4Fe–4S] ferredoxins [31]. Such interactions between [4Fe–4S] clusters are only observed for clusters at a distance of less than 15 Å [32].

3.6. Structural model of APS reductase

Based on the data it is proposed that bacterial APS reductase comprises one α-subunit with one FAD, and one β-subunit with two [4Fe–4S] clusters in close vicinity (Fig. 3). Presumably, formation of APS from sulfite and AMP occurs at the FAD site followed by electron transfer to [4Fe–4S] center I. Consistent with this assumption a transient flavin semiquinone radical anion was observed [27]. Although the enzymes originate from different bacterial lineages and even domains of life, their molecular and spectroscopic properties are remarkably similar suggesting similar active site structures. The comparative analysis of the sequences [24,33] of dissimilatory APS reductases and sulfite reductases strongly suggests that these enzymes were most probably present in a last common ancestor of archaea, bacteria and eukarya [24,33,34]. Note that the dissimilatory sulfite reductases show more variations of their molecular parameters by comparison to dissimilatory APS reductase [33,35–40].

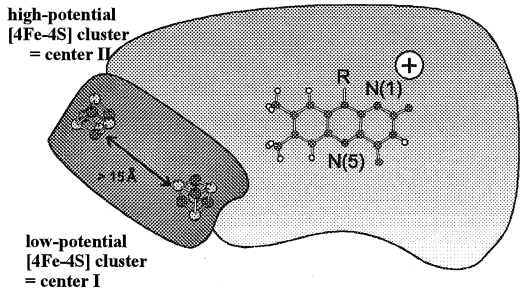

Fig. 3. Structural model of the APS reductase 1:1 αβ-heterodimer. FAD is non-covalently bound to the α-subunit, [4Fe–4S] centers I and II are located on the β-subunit in a distance of less than 15 Å. Center I accepts electrons from FAD. A positive charge located close to the N(1)–C(2)O position of the isoalloxazine ring stabilizes a flavin radical anion.

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