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# Spectroscopic Studies on APS Reductase isolated from the hyperthermophilic sulfate-reducing archaebacterium *Archaeglobus fulgidus*

Jorge Lampreia[1], Guy Fauque[2], Norbert Speich[3], Christiane Dahl[3]
Isabel Moura[1], Hans G. Trüper[3] and José J. G. Moura[1]

[1] Centro de Tecnologia Química e Biológica (CTQB) and Universidade Nova de Lisboa, Rua da Quinta Grande 6, Apartado 127, P-2780, PORTUGAL

[2] Centre d'Océanologie de Marseille, CNRS UA 41, Faculté des Sciences de Luminy, Case 901, 13288 Marseille, Cedex 9, FRANCE

[3] Institut für Mikrobiologie & Biotechnologie, Rheinische Friedrich-Wilhelms-Universität Meckenheimer Allee 168, D-5300 Bonn 1, GERMANY

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Adenylyl sulfate (APS) reductase, the key enzyme of the dissimilatory sulfate respiration, catalyzes the reduction of APS (the activated form of sulfate) to sulfite with release of AMP. A spectroscopic study was carried out with the APS reductase purified from the extremely thermophilic sulfate-reducing archaebacterium *Archaeoglobus fulgidus* DSM 4304.

Combined ultraviolet/visible spectroscopy and low temperature electron paramagnetic resonance (EPR) studies were used in order to characterize the active centers and the reactivity towards AMP and sulfite of this enzyme. The *A. fulgidus* APS reductase is an iron-sulfur flavoprotein containing two distinct [4Fe-4S] clusters (Centers I and II) very similar to the homologous enzyme from *Desulfovibrio gigas*. Center I, which has a high redox potential, is reduced by AMP and sulfite, and Center II has a very negative redox potential.


Sulfate-reducing bacteria are anaerobic microorganisms utilizing sulfate as terminal electron acceptor in a "respiratory" like process called dissimilatory sulfate reduction (1,2). This process requires the activation of the rather inert sulfate molecule. A general mechanism for the activation and reduction of sulfate to sulfide has been proposed involving at least three key enzymes: ATP sulfurylase (also called sulfate adenyltransferase) (EC 2. 7. 7. 4), APS reductase (EC 1. 8. 99. 2) and bisulfite reductase (EC 1. 8. 99. 1) (3). Until recently all sulfate-reducing bacteria were supposed to belong to the eubacteria (4). However, in 1987, Stetter *et al* isolated an archaebacterial member of this physiological group (5), a marine hyperthermophile, described as *Archaeoglobus (A.) fulgidus* VC-16 (6) [another species, *A. profundus*, has been isolated very recently (7)]. Speich and Trüper purified and characterized APS reductase from *A. fulgidus* (8). It was described as an iron-sulfur flavoprotein containing 8 non-heme iron atoms, 6 labile sulfur atoms and 1 FAD moiety per 160 kDa. It is composed of two subunits of 80 kDa and the optimal activity is at pH 8.0 and 85° C.

***Abbreviations:*** **APS**, Adenylyl sulfate; ***(A.)***, *Archaeoglobus*; ***(D.)***, *Desulfovibrio*; **Da**, Dalton; **EPR**, Electron paramagnetic resonance; **DSM**, German Collection of Microorganisms, Braunschweig.

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The isoelectric point is 4.8 and the Km values for ferricyanide and AMP are 0.4 mM, and 1 mM, respectively. The aim of this report is to describe the characterization of the active centers of APS reductase isolated from *A. fulgidus* and to compare them with the data obtained for APS reductases involved in the metabolism of sulfate-reducing eubacteria of the genus *Desulfovibrio*. To accomplish this goal, visible and EPR studies were carried out in native and different redox states of the APS reductase, obtained after reaction of the enzyme with AMP and sulfite (substrates) and chemical reductants.

## MATERIALS AND METHODS

***Organism/Growth Medium/EnzymePurification.*** *Archaeoglobus fulgidus* VC-16 (DSM 4304) was grown anaerobically at 85° C on a lactate sulfate medium in 300 l enamel-protected fermenters (HTE Bioengineering). The preparation of cell-free extracts and enzyme purification were carried out as previously described (8). An additional final LKB/HPLC purification step was introduced using a DEAE - 5PW column. All chemicals and reagents were of analytical grade and obtained commercially.

***Protein Measurement.*** Protein concentration was determined according to the method of Lowry *et.al* (9) using bovine serum albumin as a standard.

***Spectroscopic Methods.*** Visible and ultraviolet spectra were recorded with a Shimadzu model 265 spectrophotometer. EPR measurements were made with a Bruker ER - 200tt spectrometer fitted with an Oxford Instruments ESR-9 continuous flow helium cryostat and connected to an IBM-AT computer using a Data Translation DT-2801 board for acquisition of experimental data and further data manipulation.

***Preparation of EPR Samples.*** EPR samples were prepared under anaerobic conditions (argon atmosphere). Substrates (AMP and sulfite) and chemical reductants (sodium dithionite, zinc-reduced methyl viologen and ascorbate, sodium salt) were added directly to the calibrated quartz EPR tubes using gas tight Hamilton syringes. Sodium dithionite (1g/50 ml dearated 0.2 M Tris-HCl buffer, pH 9.0) and zinc-reduced methyl viologen were prepared at pH 9.0 (0.2 M Tris-HCl). After reaction for the adequate time lengths, samples were anaerobically frozen in liquid nitrogen. Spin quantitations were determined against CuEDTA standards and calculated numerically with baseline and the Aasa and Vanngard corrections (10).

## RESULTS

### 1. Ultraviolet/Visible Spectroscopy

The ultraviolet/visible spectrum of the oxidized form of purified A. *fulgidus* APS reductase (**Figure 1-A**) shows a broad maximum around 394 nm with shoulders at 445 and 475 nm and a protein absorption peak at 278 nm. The overall spectrum indicates the presence of iron-sulfur clusters as well as flavin. The addition of sulfite to the native enzyme (**Figure 1-B**) causes a decrease in absorbance between 500 nm and 340 nm and then a slight increase in absorbance appears around 320 nm, consistent with the formation of an adduct between the FAD and sulfite, as previously described (11). Further addition of AMP (**Figure 1-C**) causes a minor bleaching of the spectrum. Reduction of the enzyme by dithionite (**Figure 1-D**) leads to maximal bleaching of the overall spectrum. Difference spectra were obtained in order to characterize the chromophores reacting with AMP, sulfite and dithionite. A flavin-like spectrum (**Figure 1, Insert**) was obtained when the spectrum of the native APS reductase was

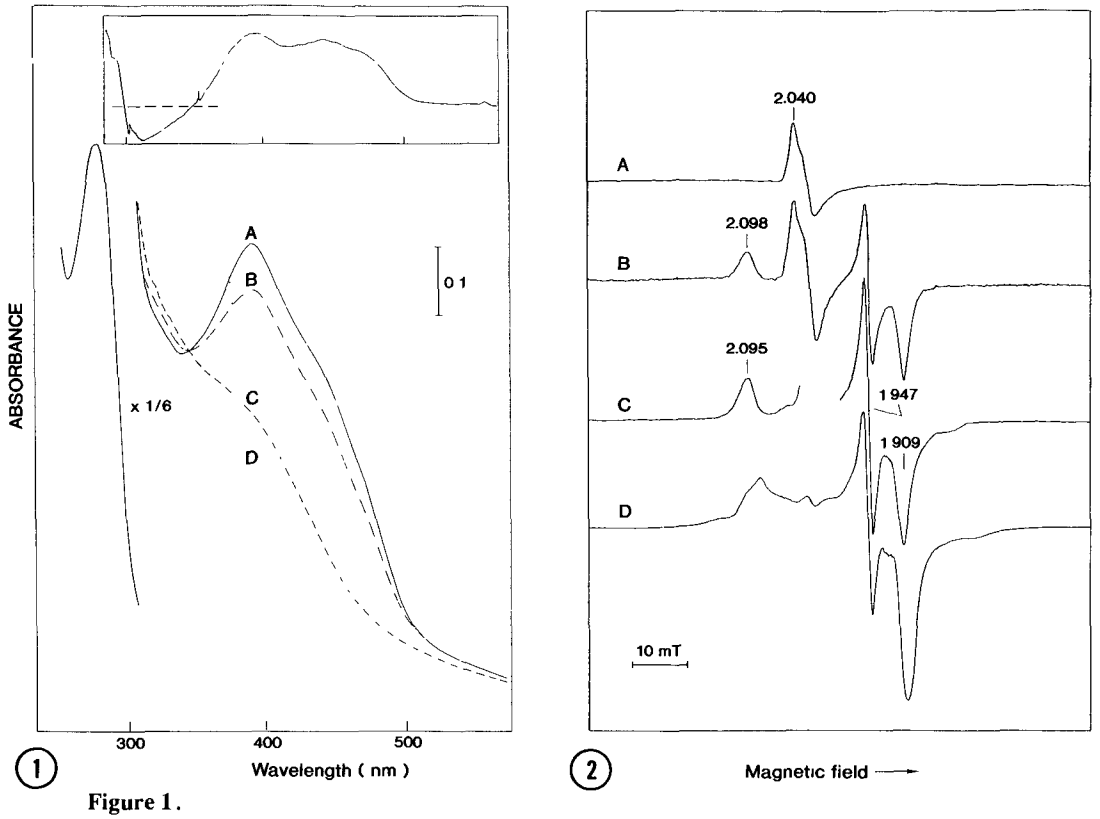


**Figure 1.**

Visible/ultraviolet spectrum of *A. fulgidus* APS reductase. **A** - native enzyme; **B** - after sulfite addition; **C**- B after AMP addition and **D** - C after dithionite addition. **Insert** - Difference spectrum between native (**A**) and sulfite-reacted enzyme (**B**).

**Figure 2.**

X-band EPR spectra of *A. fulgidus* APS reductase. **A** - native enzyme; **B** - sulfite plus AMP reacted enzyme; **C** - methyl viologen reduced enzyme (Center I); **D** - dithionite (15 minutes) "fully reduced" enzyme. Experimental conditions: temperature 8 K; modulation amplitude, 1 mT, microwave power, 2 mW. Protein concentration: 90 μm.

run against the sulfite-reacted enzyme, suggesting again the formation of a flavin-sulfite adduct in the sulfite-reacted enzyme (11). Also a broad peak in the 400-450 nm region was found in the difference spectrum between AMP plus sulfite-reacted enzyme and the sulfite-reacted enzyme (not shown). This spectral change is related to the reduction of the iron-sulfur centers of the enzyme, indicating that AMP induces the reduction of the iron-sulfur centers in the presence of sulfite. Most of these observations are supported by the results shown in the following EPR section.

## 2. EPR Spectroscopy

### *i) Native APS reductase*

As isolated (native) APS reductase from A. fulgidus shows a weak and almost isotropic signal centered around g=2.04, detectable at temperatures below 36 K (Figure 2-A). Double

integration of this signal accounts for less than 0.01 spin/mole. The spectral shape and temperature dependence of the EPR spectrum of this minor paramagnetic species are reminiscent of those observed for [3Fe-4S] clusters (12).

***ii) AMP/sulfite reacted APS reductase***

Reaction of the enzyme with AMP causes no change in the native EPR spectrum. Addition of sulfite to the native reductase (not shown) produces the appearance of a weak "g=1.94" type EPR signal due to reduced iron-sulfur centers. The sequential addition of AMP to the sulfite reacted enzyme increases the intensity of the previously observed rhombic EPR signal (**Center I**) and concomitant disappearance of the isotropic signal. This rhombic EPR signal has well defined g-values at 2.098, 1.948 and 1.910, accounting for a maximal intensity of approximately 0.2 spin/mole (**Figure 2-B**).

***iii) Partially reduced APS reductase***

The rhombic EPR signal described above can be also generated (as a minor species) after reacting the native enzyme with sodium ascorbate. The reaction of the native enzyme with reduced methyl viologen or sodium dithionite for a very short time (15 seconds) gave rise to the full development of the so called **Center I** with g-values of 2.095, 1.947 and 1.909 (**Figure 2-C**) slightly different from those observed for the AMP plus sulfite-reacted sample. At this redox state the isotropic signal is not observed due to its complete reduction. Spin quantitation of the EPR signal of reduced **Center I** obtained by chemical reduction indicates a value of 0.85-0.95 spin/mole. The general shape, g-values and intensity of this spectrum are independent of the reducing agent.

***iv) The "fully" reduced APS reductase***

This state is obtained after an extensive reduction (>15 minutes) with sodium dithionite (pH=9.0). The EPR spectrum of the "fully" reduced state is complex (**Figure 2-D**) and shows characteristics of interacting iron-sulfur centers (13) indicating the presence of at least one more iron-sulfur center, designated as **Center II**. Integration of the EPR spectra of the "fully" reduced APS reductase gives 1.75 spin/mole.

The relaxation behavior of the described EPR signals (partially and "fully" reduced APS reductase) indicates that the relaxation of center I is enhanced by the development of a second paramagnet in the sample, when a further reduced state is attained (not shown).

## DISCUSSION

The central role of APS reductase in dissimilatory sulfur metabolism has been discussed in many different genera of microorganisms. This enzyme was shown to be present in all dissimilatory sulfate-reducing eubacteria, in some thiobacilli and in most phototrophic sulfur bacteria, and APS reductase was purified from *Desulfovibrio (D.) vulgaris* Hildenborough

(14,15), *Thiobacillus (T.) denitrificans* (16), *T. thioparus* (17), *Thiocapsa roseopersicina* (18), *Chlorobium (C.) limicola* (19), *C. limicola* f.sp. *thiosulfatophilum* (20), *Desulfobulbus propionicus* (21), *D. thermophilus* (22) [now classified as *Thermodesulfobacterium mobile* (23)] and *D. gigas* (24,25).

The isolation and preliminary characterization of APS reductase from a hyperthermophilic sulfate-reducing archaebacterium, previously reported (8), already indicated a high degree of homology and identical physiological properties to the APS reductases isolated from sulfate-reducing eubacteria (14,15,22,24,25). In addition, the EPR study presented in this manuscript demonstrates the similar spectroscopic features of the *A. fulgidus* APS reductase when compared with the *D. gigas* enzyme (24,25).

The *A. fulgidus* APS reductase presents in the native state a weak signal with properties reminiscent of a [3Fe-4S] center which accounts for a very small value of spin concentration. The g-values of this almost isotropic signal (g=2.04) are slightly different from the ones found in *D. gigas* APS reductase (g=2.02) (24). As explained, the origin of this isotropic signal may be due to an *in vitro* destruction/conversion of a small quantity of one of the 4Fe centers present in the APS reductase.

Upon interaction with both substrates, AMP and sulfite, a reduced [4Fe-4S] center (named Center I) develops. In the presence of the natural substrates, the isotropic signal is observed concomitantly with reduced Center I suggesting that this center has an unusual high redox potential. This assumption is also supported by the observation that Center I is partially reduced by ascorbate.

The chemical reduction of the *A. fulgidus* APS reductase with methyl viologen or dithionite (short reduction time) gives raise to the full development of Center I (intensity close to 1 spin/mole) with g-values (and linewidths) slightly different from those observed after reaction with AMP and sulfite.

The extensive reduction with sodium dithionite (>15 minutes) produces a very complex EPR spectrum, identical with the one observed for *D. gigas* APS reductase. In this last case, Moessbauer spectroscopic studies showed that the "fully" reduced APS reductase contained two interacting [4Fe-4S] centers antiferromagnetically coupled and the interaction could be broken by the application of a magnetic field of 1 Tesla (24). The reactivity towards dithionite and the spin integration of the "fully" reduced sample of *A. fulgidus* APS reductase (1.75 spin/mole) suggests that Center II has a very negative mid-point redox potential and might not be fully reduced under the experimental conditions used.

The general properties described so far for APS reductase in sulfate-reducing eubacteria indicate that this enzyme is highly conserved in terms of its physiological properties as well as active site composition. The APS reductase isolated from *A. fulgidus* is described as a flavin-[iron-sulfur]-containing protein. Most probably, the iron-sulfur centers are arranged as two distinct [4Fe-4S] clusters what is supported by comparison with the data

obtained for the *D. gigas* enzyme as well as some other *Desulfovibrio* species (our unpublished results). One of the [4Fe-4S] clusters, Center I, has atypical characteristics, in particular, its high redox potential (the center is partially reduced by ascorbate) and its reactivity towards AMP and sulfite.

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## REFERENCES

1. Widdel, F. (1988), in Biology of Anaerobic Microorganisms (A.J.B.Zehnder, Ed.), pp.469-585, John Wiley and Sons, New York.
2. Fauque, G., LeGall, J., and Barton, L.L. (1991), in **Variations in Autotrophic Life**, (J. M. Shively and L. L. Barton Eds.), pp.271-337 Academic Press Limited, London.
3. Peck, H. D., Jr. (1962), *Bact. Rev.*,**26**, 67-94.
4. Fowler, V. J., Widdel, F., Pfennig, N., Woese, C. R. and Stackebrandt, E., (1986), *System. Appl. Microbiol.* , **8**, 32-41.
5. Stetter, K. O., Lauerer, G., Thomm, M. and Neuner, A., (1987), *Science*, **236**, 822-824.
6. Stetter, K. O., (1988), *System. Appl. Microbiol.*, **10**, 172-173.
7. Stetter, K. O., Fiala, G., Huber, G., Huber, H., and Segerer, A., (1990), *FEMS Microbiol. Rev.*,**75**, 117-124.
8. Speich, N., and Trüper, H.G., (1988), *J. Gen. Microbiol.*,**134**, 1419-1425.
9. Lowry, O.H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., (1951), *J. Biol. Chem.*,**193**, 265-275.
10. Aasa, R., and Vanngard, T., (1975), *J. Magn. Reson.* ,**13**, 548-560.
11. Müller, F., and Massey, V. , (1969), *J. Biol. Chem.* ,**244**, 4007-4016.
12. Huynh, B. H., Moura, J. J. G., Moura, I., Kent,T. A., LeGall, J., Xavier, A. V. and Münck, E. (1980), *J. Biol. Chem.*, **255**, 3242-3244.
13. Cammack, R., Dickinson, D. P. E. and Johnson, C. E., (1977), in **Iron-Sulfur Proteins**, Vol. III, (W. Lovenberg, Ed.), pp. 283-330, Academic Press, New York.
14. Michaels, G.B., Davidson, J. T. and Peck, H. D. Jr., (1970), *Biochem. Biophys. Res. Commun.*,**39**, 321-328.
15. Bramlett, R. N. and Peck, H. D. Jr. , (1975), *J. Biol. Chem.* ,**250**, 2979-2986.
16. Bowen, T. J., Happold, F. C. and Taylor, B. F., (1966), *Biochim. Biophys. Acta* , **118**, 566-576.
17. Adachi, K. and Suzuki, I., (1977), *Can. J. Biochem.*, **55**, 91-98.
18. Trüper, H. G. and Rogers, L. A., (1971), *J. Bacteriol.*,**108**, 1112-1121.
19. Kirchhoff, J., Trüper, H. G., (1974), *Arch. Microbiol.*, **100**, 115-120.
20. Khanna, S. K. and Nichols, D. J. D., (1983), *J.Gen. Microbiol.*,**129**, 1365-1369.
21. Stille, W. and Trüper, H. G., (1984), *Arch. Microbiol.*,**137**, 145-150.
22. Kremer, D.R., Veenhuis, M., Fauque, G., Peck, H. D., Jr., LeGall, J., Lampreia, J., Moura, J. J. G. and Hansen, T. A., (1988), *Arch. Microbiol.*,**150**, 296-301.
23. Rozanova, E. P. and Pivovarova, T. A., (1988), *Mikrobiologiya*,**57**, 102-106.
24. Lampreia, J., Moura, I., Teixeira, M., Peck, H. D., Jr., LeGall, J., Huynh, B. H. and Moura, J. J. G., (1990), *Eur. J. Biochem.*, **188**, 653-664.
25. Lampreia, J., Moura, I., Fauque, G., Xavier, A. V., LeGall, J., Peck, H. D., Jr. and Moura, J. J. G., (1987), *Rec. Trav. Chim.Pays-Bas* ,**106**, 234.