The Function of the [4Fe-4S] Clusters and FAD in Bacterial and Archaeal Adenylylsulfate Reductases

EVIDENCE FOR FLAVIN-CATALYZED REDUCTION OF ADENOSINE 5'-PHOSPHOSULFATE*

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The iron-sulfur flavoenzyme adenylylsulfate (adenosine 5'-phosphosulfate, APS) reductase catalyzes reversibly the 2-electron reduction of APS to sulfite and AMP, a key step in the biological sulfur cycle. APS reductase from one archaea and three different bacteria has been purified, and the molecular and catalytic properties have been characterized. The EPR parameters and redox potentials (−60 and −520 mV versus NHE) have been assigned to the two [4Fe-4S] clusters I and II observed in the three-dimensional structure of the enzyme from Archaeoglobus fulgidus (Fritz, G., Roth, A., Schiffer, A., Büchert, T., Bourenkov, G., Bartunik, H. D., Huber, H., Stetter, K. O., Kroneck, P. M. H., and Ermler, U. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1836–1841). Sulfite binds to FAD to form a covalent FAD N(5)-sulfite adduct with characteristic UV/visible spectra, in accordance with the three-dimensional structure of crystalline enzyme soaked with APS. UV/visible monitored titrations reveal that the substrates AMP and APS dock closely to the FAD cofactor. These results clearly document that FAD is the site of the 2-electron reduction of APS to sulfite and AMP. Reaction of APS reductase enzyme with sulfite and AMP leads to partial reduction of the [4Fe-4S] centers and formation of the anionic FAD semiquinone. Thus, both [4Fe-4S] clusters function in electron transfer and guide two single electrons from the protein surface to the FAD catalytic site.

Adenosine 5'-phosphosulfate (APS) reductase is a key enzyme involved in the pathways of sulfate reduction and sulfide oxidation. In higher plants the so-called assimilatory pathway of sulfate reduction provides sulfide for the biosynthesis of sulfur containing amino acids and cofactors, whereas in the dissimilatory pathway sulfite serves as a terminal electron acceptor of an anaerobic respiratory electron transfer chain in bacteria and archaea (2). Prior to reduction the sulfite molecule has to be activated via ATP sulfurylase (E.C. 2.7.7.4) to APS [adenosine 5'-phosphosulfate (APS) reductase] catalyzes the 2-electron reduction of APS to sulfite and AMP in a flavin-dependent mechanism for APS reduction and formation of the anionic FAD semiquinone. Thus, both [4Fe-4S] clusters function in electron transfer and guide two single electrons from the protein surface to the FAD catalytic site.

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† The abbreviation used is: APS, adenosine 5'-phosphosulfate.

for a X–O–P bond in a biological molecule. This energy is utilized by APS reductase (E.C. 1.8.99.2) in the reductive transformation of APS to sulfite and AMP. The calculated standard reduction potential E°(APS/AMP+HSO3−) is −60 mV versus −516 mV for E°(SO42−/HSO3−), i.e. the potential is shifted by 450 mV (4). Sulfite is subsequently reduced by sulfite reductase (E.C. 1.8.7.1) to hydrogen sulfide. Although both pathways include the same intermediates the APS reductases involved differ with regard to molecular architecture and cofactor composition. Whereas the assimilatory APS reductase is built of two 50-kDa subunits forming a homodimer containing two iron-sulfur centers (5, 6), the dissimilatory enzyme is a heterodimer with one α-subunit (75 kDa, 1 FAD), and one β-subunit (15 kDa, 2 [4Fe-4S]) (7). The dissimilatory APS reductase also converts sulfite plus AMP to APS, providing electrons for anoxogenetic photosynthesis and denitrification (8). First reports on APS reductase appeared already several decades ago (9), nevertheless the role of its cofactors in the reaction is still not fully understood. The observation of a covalent flavin–sulfite adduct suggested that FAD might be involved in catalysis. However, many flavoproteins with different functions form such an adduct. Especially the flavin-dependent oxidases bind sulfite with high affinity (10). Therefore, the involvement of a FAD-sulfite adduct in catalysis remained questionable. The number and relative arrangement of the [4Fe-4S] clusters in APS reductase has also been debated. Whereas Lampreia et al. described APS reductase as an enzyme with two [4Fe-4S] clusters in close proximity (11), Verhagen et al. proposed just one iron-sulfur center containing more than four iron atoms (12). A recent analysis of the native molecular mass and the cofactor content clearly confirmed the presence of two [4Fe-4S] clusters (7).

Here we report on the assignment of the iron-sulfur centers observed by EPR spectroscopy to the two [4Fe-4S] clusters evident from the high resolution crystal structure (1). Furthermore the kinetic and substrate-binding properties of APS reductase from bacteria and archaea are described. These data clearly document that catalysis occurs at the FAD site. A novel flavin-dependent mechanism for APS reduction and formation is proposed.

EXPERIMENTAL PROCEDURES

Enzyme Purification and APS Synthesis—APS reductase from Desulfovibrio desulfuricans, Desulfovibrio vulgaris, Archaeoglobus fulgidus, and Thiobacillus denitrificans was purified as published previously (7). Adenosine 5'-phosphosulfate (APS) from Sigma served as a standard. Highly pure APS was synthesized according to Cooper and Trüper (13) with following modifications: the soluble fraction from Desulfovibrio was used. The reaction mixture contained 0.2 g of protein, 1.6 mmol of AMP, 3.2 mmol of Na2SO3, and 3.2 mmol of K3Fe(CN)6 in 50 mM Tris/HCl buffer and 10 mM EDTA, pH 7.6. The
formed APS was purified with Sephadex G-25, Q-Sepharose Fast Flow, and Resource Q column (Amersham Biosciences) with 50–800 mM NH₄HCO₃, pH 7.3, as buffer. The high purity APS (≥99.5%, high pressure liquid chromatography analysis) was freeze dried and stored at −20 °C.

Removal of Dioxygen—Experiments under the exclusion of dioxygen were performed as described by Beinert (14); after removal of dioxygen, solutions were stored in a Coy glove box (95%N₂/5%H₂).

Enzyme Activities—Reduction of APS was determined in Thunberg cuvettes in 80 mM potassium phosphate, pH 7.0, containing 50 μM APS and 0.75 mM methyl viologen as reductant. Methyl viologen was reduced photochemically in the presence of 24 μM 5-deazaflavin and 38 mM sodium oxalate (15). The reaction was started by the addition of APS reductase from the side arm, diluted in 6 mg/ml bovine serum albumin to minimize adsorption to the glassware. The oxidation of methyl viologen was followed at 662 nm (ε₆₆₂ = 7280 M⁻¹ cm⁻¹) or at 732 nm (ε₇₃₂ = 3150 M⁻¹ cm⁻¹) because the absorbance at the absorption maximum (ε₆₆₀ = 13600 M⁻¹ cm⁻¹) (16) was too high. Activity is expressed as μmol of APS reduced per min and mg of protein. APS oxidation was determined in 50 mM Tris/HCl buffer, pH 7.6, containing 2 mM sodium sulfite, 2 mM AMP, and 0.5 mM EDTA. The reaction was started with K₂Fe(CN)₆, final concentration 1 mM. The midpoint potential of FAD was determined according to Massey (19) using resorufin as a redox dye and the potential center II (left side) is partially exposed to the solvent, whereas center I (right side) is buried in the protein and close to FAD. r = Adenyl.

Redox Potentials—EPR-monitored potentiometric titrations of iron-sulfur centers were performed according to Dutton (18). Two titrations at pH 7.0 in 50 mM potassium phosphate, 150 mM KCl, 5% (v/v) glycerol and at pH 8.5 in 100 mM Tris/HCl were performed. APS reductase was 60–70 μM, and the redox mediators neutral red, phenazine methosulfate, methylene blue, 2-methyl-1,4-naphtoquinone, resorufin, indigo, anthraquinone-2-sulfonate, phenosafranin, safranin T, benzyl viologen, and methyl viologen were added to a final concentration of 50 μM. The potential was adjusted by addition of 0.1 mM sodium dithionite or potassium ferricyanide; hereafter 300 μl of sample was transferred with a gas-tight syringe to a sealed EPR tube flushed with argon. The samples were quickly frozen in an isopentane bath (−150 °C) and stored in liquid nitrogen. The midpoint potential of FAD was determined according to Massey (19) using resorufin as a redox dye and the xanthine/xanthine oxidase system as reductant.

Reduction Experiments—APS reductase was reduced under exclusion of dioxygen with sodium dithionite for 30 min or photochemically with 5–deazaflavin. For EPR measurements, 300 μl of 70 μM APS reductase in 80 mM Tris/HCl, pH 8.0, 25 mM sodium oxalate, and 5 μM 5-deazaflavin were transferred in the anaerobe chamber into EPR tubes that were subsequently sealed and illuminated for 20 min in a modified slide projector equipped with a thermostatted cell holder. The redox state of the samples was checked spectrophotometrically.

EPR Spectroscopy—EPR spectra were recorded on a Bruker ESP300 spectrometer with peripheral equipment and data handling as described elsewhere (20). Spectra were simulated using the program EPR (21) and quantified by comparison with a CuSO₄ standard.

RESULTS AND DISCUSSION

EPR and Redox Properties of Iron-Sulfur Centers—The three-dimensional structure of APS reductase from A. fulgidus (1) showed the presence of two [4Fe–4S] clusters in close proximity harbored by the β-subunit. Whereas one cluster is deeply buried in the protein matrix (Fe–S center I), center II is located at the surface of the protein and partially exposed to the solvent. The FAD is located nearby the buried center I, pointing with its C–S methyl group to the iron-sulfur center. The distances of 9.8 Å between the two [4Fe–4S] clusters and of 15.4 Å between the FAD N-5 atom and center I are close enough to allow magnetic interaction between the individual redox cofactors, i.e. if the [4Fe–4S] centers are reduced and the FAD is in the semiquinone state (Fig. 1.).

Dithionite reduction of APS reductase from all four microorganisms at pH 7.0, resulted in a rhombic EPR spectrum with gₓ,ᵧ≈ 2.08, 1.94, and 1.90. One single [4Fe–4S] cluster, center I, became reduced, with the signal integrating to −0.9–1.0 spin/mol (7). Under these conditions, there were no other EPR signals that could have been assigned to the second iron-sulfur center. The standard reduction potentials of center I in
APS reductase of *D. desulfuricans* and of *A. fulgidus* were determined at pH 7 to −59 ± 12 mV and −57 ± 5 mV (Fig. 2, A and C), respectively, by an EPR-monitored redox titration. These values are close to those reported for the Fe-S center I in *D. vulgaris* (−19 mV) (12) and of *Desulfovibrio gigas* (−50 mV) (22). The midpoint potentials of center I are unusually high for [4Fe-4S] clusters, which normally range between −200 and −500 mV (23). In the titration at pH 7.0 a potential of −440 mV was reached. At this potential no further EPR signals were observed, suggesting that the midpoint potential of Fe-S center II is below that value. A second redox titration was performed at pH 8.5 and a potential of −560 mV was obtained. The midpoint potentials of center II of the enzymes of *D. desulfuricans* and *A. fulgidus* were calculated to −540 ± 15 mV to −520 ± 10 mV, respectively (Fig. 2, B and D).

The line shape and the parameters of the EPR spectra of center I in all four APS reductases were independent of the ionic strength of the buffer, confirming that Fe-S center I is shielded from the solvent. At pH 8, however, reduction of the APS reductases with dithionite gave EPR spectra characteristics for interacting [4Fe-4S] clusters (Fig. 3). At half-field the so-called g ~ 4 signal was observed, which originates from the dipolar interaction of two S = 1/2 systems (Fig. 4). Spin quantitation yielded only 1.2–1.5 spins/mol. Even photochemical reduction with 5-deazaflavin/oxalate generating electrons at −650 mV resulted in the same spin concentrations. Therefore

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**Fig. 3.** EPR spectra of APS reductases from *D. desulfuricans* (A), *D. vulgaris* (B), *A. fulgidus* (C), and *T. denitrificans* (D) and dependence on ionic strength. The lower trace in A, B, C, and D shows the spectrum of each enzyme reduced with sodium dithionite in 10 mM Tris/HCl, pH 8.0. The spectra change significantly at higher ionic strength. The upper trace shows the enzyme reduced with sodium dithionite in 10 mM Tris/HCl, 1.0 M NaCl, pH 8.0. EPR parameters were: microwave frequency, 9.65 GHz; microwave power, 10 mW; modulation amplitude, 10 millitesla; temperature, 10 K.

**Fig. 4.** EPR spectra of APS reductase at half-field. Because of the magnetic interaction between both [4Fe-4S] clusters a signal around g ~ 4 appears. EPR conditions were as in Fig. 2.
the apparent low spin count is probably due not only to incomplete reduction of the Fe-S center I and II. The spin-coupled system relaxes very fast and is very sensitive to variations in temperature and microwave power (data not shown). Therefore strong line broadening might already occur at low temperature resulting in a poorer spin count upon double integration.

An increase in ionic strength clearly influenced the line shape of the spectra of the interacting [4Fe-4S] centers (Figs. 3 and 4). Because the ionic strength did not influence the EPR spectra of center I, it was concluded that the magnetic properties of center II must be affected. This can be explained by the partial exposure of Fe-S center II to the solvent. Consequently, center I was assigned to the [4Fe-4S] cluster in close proximity to FAD. If this assignment is correct a magnetic interaction between Fe-S center I in the reduced state and the FAD in the flavosemiquinone state should become detectable by EPR spectroscopy (24).

Oxidized APS reductase (enzyme as isolated) showed only minor EPR signals of a [3Fe-4S] center, which integrated to 0.03 spin/mol showing that this species originated from degradation of one [4Fe-4S] cluster. The addition of just AMP to oxidized APS reductase under the exclusion of dioxygen gave no additional EPR signal. Addition of just sulfite led to the formation of a very weak signal of Fe-S center I and radical signal at $g = 2.004$, with a line width of 1.75 millitesla. Such a line broadening suggests magnetic interaction of the flavosemiquinone with another paramagnet (24), in APS reductase most likely Fe-S center I. Further support arises from the slight shift of $g$ values
of center I upon reduction by AMP and sulfite as described above. This effect was already described by Lampreia et al. (11), who also reported that the relaxation behavior of center I was different in the AMP/sulfite-reduced enzyme compared with the dithionite-reduced enzyme. Such a change in relaxation behavior again is evidence for the interaction between two paramagnets. However, the authors did not observe a radical signal, which is most likely due to the fact that EPR spectra were recorded only at low temperature (10–20 K), where the radical signal is covered by the [3Fe-4S] signal. Note that the flavin radical is the only component in the EPR spectra recorded at higher temperature (70 K) (Fig. 5, A and B).

The assignment of the high potential center I to the buried [4Fe-4S] cluster and the low potential center II to the surface exposed [4Fe-4S] receives strong support by features in the crystal structure of APS reductase from A. fulgidus (1). A different number of backbone amides interact with the sulfur atoms of the two Fe-S centers. Local dipoles formed by the amide groups stabilize the additional negative charge upon reduction of center I, thus the reduction potential is shifted to a more positive value (26–28). Fewer interactions are observed for the Fe-S cluster at the surface of APS reductase, which leads to a lower reduction potential than that of the buried cluster.

**Electronic Spectra and Binding of AMP, APS, and Sulfite—**

The UV-visible spectra of APS reductase (enzyme as isolated) from the four different microorganisms were very much identical. APS reductase purified from D. desulfuricans, D. vulgaris, and T. denitrificans exhibited absorption maxima at 390 and 278 nm in the oxidized state, versus 388 and 278 nm for the enzyme from A. fulgidus. The extinction coefficients were calculated based on a molecular mass of 95 kDa, with ε390 = 43,000 M⁻¹ cm⁻¹ and ε278 = 94,000 M⁻¹ cm⁻¹ for the enzymes from D. desulfuricans and D. vulgaris, ε390 = 43,000 M⁻¹ cm⁻¹ and ε278 = 206,000 M⁻¹ cm⁻¹ for the enzyme from T. denitrificans, and ε390 = 42,000 M⁻¹ cm⁻¹ and ε278 = 216,000 M⁻¹ cm⁻¹ for the enzyme from A. fulgidus. Shoulders were observed around 432 and 462 nm in the spectra of all four enzymes (Fig. 6). The absorption coefficients and the analytical data documented the presence of one FAD (ε380 enzyme-bound flavin ~12,000 M⁻¹ cm⁻¹) (10) and two [4Fe-4S] centers (ε390 = 16,000 M⁻¹ cm⁻¹/Fe-S center) (29). The reduction potential of FAD in APS reductase from D. desulfuricans was determined to E°′ = −45 ± 10 mV (n = 5). No formation of a radical was observed.

**FIG. 8.** Sulfite adduct of APS reductase from D. desulfuricans (A), D. vulgaris (B), A. fulgidus (C), and T. denitrificans (D). The inset shows the change in absorption at 445 nm versus [Na₂SO₃]. The solid line represents a hyperbolic fit through the data. The lower parts show the difference spectra.
Addition of sulfite to oxidized APS reductase caused bleaching of the FAD chromophore between 300 and 500 nm as documented by the difference spectra in Fig. 8. An increase in absorbance around 320 nm is characteristic for a sulfite adduct in the N-5 position of the isoalloxazine ring (10, 30). From the change in the spectra $K_d$ values in the range 0.14–31 $\mu$M were calculated for the APS reductase-sulfite complex (Fig. 8, Table I).

In the absence of dioxygen, further addition of AMP to the enzyme-sulfite complex caused the band at 320 nm to disappear and produce a slight increase around 380 nm and a decrease around 445 nm (Fig. 9, A and C). The data are consistent with the decay of the FAD (N-5)-sulfite adduct and reduction of the flavin. The band at 380 nm is indicative for an anionic flavosemiquinone. Based on the absorption coefficient of anionic flavin radicals ($\epsilon_{380} = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$) (31) a radical content of 8% of total flavin in APS reductase of $D. desulfuricans$ was calculated in excellent agreement with the radical content determined from the EPR spectra. The absorption spectra of anionic flavosemiquinones are characterized by two major absorption maxima centered at 380 and 470 nm (31). In the difference spectra of AMP/sulfite-reacted minus sulfite-reacted APS reductase only the maximum at 380 nm is visible, whereas the maximum at 470 nm is hidden in the spectrum by the contribution of fully reduced FAD. In addition, the absorption in this region is decreased by the binding of AMP and APS and by the reduction of iron-sulfur center I.

The flavosemiquinone was detected only in the enzymes of the three sulfate-reducing organisms $D. desulfuricans$, $D. vulgaris$, and $A. fulgidus$ under exclusion of dioxygen. Access of dioxygen to the samples resulted in the immediate decay of the radical, and the iron-sulfur center I became further reduced (Fig. 9D). Integration of the signal gave $\sim 0.2$ spin/mol. In contrast, the enzyme isolated from sulfur-oxidizing $T. denitrificans$ did not form a stable flavosemiquinone under the same conditions, and the FAD and iron-sulfur center I became reduced immediately. Interestingly, an anionic flavin radical was observed as a transient species in fast kinetic measurements with the homologous enzyme from $T. thioparus$ (32).

From these results we conclude that FAD becomes reduced first during the oxidation of sulfite and AMP by two electrons, which are subsequently transferred one-by-one to the iron-sulfur centers. The stabilization of a small portion of the flavin radical in the enzymes from the sulfate-reducing organisms, in contrast to the enzyme from the sulfur-oxidizing organism, might reflect a preferential direction of the catalysis.

**Catalytic Properties of APS Reductase**—The reactivity of APS reductase from $D. desulfuricans$ was investigated under different conditions of enzyme and substrate concentration and ionic strength. The specific activity of the oxidative reaction, $\text{AMP} + \text{HSO}_3^- \rightarrow \text{APS} + 2 \text{e}^- + 2\text{H}^+$, as well as the reductive reaction, $\text{APS} + 2 \text{e}^- + 2\text{H}^+ \rightarrow \text{AMP} + \text{SO}_3^-$, did not depend on the enzyme concentration (data not shown). High concentration (0.5–1.0 $\mu$M) of salt (KCl, NaCl, K$_2$PO$_4$) or Tris buffer did inhibit the enzyme (data not shown). At low salt concentrations (20 mM Tris/Cl, pH 7.6) the maximum activity was observed, which was about 20% higher than under standard conditions (100 mM Tris/Cl, pH 7.6).

**Function of [4Fe-4S] and FAD in Adenylsulfate Reductase**

![Graph](http://www.jbc.org/)

**Fig. 9.** UV-visible and difference spectra of APS reductase after addition of sulfite and AMP. A, enzyme, fully oxidized (solid line), enzyme plus 10 mM sulfite, 5 mM AMP (open diamond line) (difference spectrum in B); enzyme plus 10 mM sulfite, 5 mM AMP (closed circle line) (difference spectrum in C); after exposure to dioxygen (closed diamond line) (difference spectrum in D); B, [APSR + 10 mM sulfite] minus [APSR fully oxidized]; C, [APSR + 10 mM sulfite + 5 mM AMP] minus [APSR + 10 mM sulfite]; D, [APSR + 10 mM sulfite + 5 mM AMP + O$_2$] minus [APSR + 10 mM sulfite + 5 mM AMP].

during the redox titration. The spectrophotometric determination of the redox potential of FAD was disturbed by the contribution from Fe-S center I of APS reductase (~60 mV), producing a rather large error. The potential of FAD in APS reductase appears rather high compared with the value reported for free FAD (~207 mV) or the value for other flavoproteins carrying non-covalently bound FAD (25, 26). The high potential of FAD in APS reductases can be attributed to the binding of the isoalloxazine moiety of FAD in APS reductase (1). For the first time it was shown that both AMP and APS bind to the oxidized enzyme in a defined mode close to FAD moiety. Addition of AMP or APS to oxidized APS reductase induced a blue-shift of the absorption maxima between 300 and 500 nm. The difference spectra recorded for the four enzymes revealed a high degree of similarity, demonstrating the conservation of the substrate-binding site. From the change in absorbance, $K_d$ values for the binding of AMP and APS were derived (Fig. 7, Table I). The affinity of the four APS reductases for APS ($K_d = 0.1–0.2$ $\mu$M) was about 5–10 times higher than the affinity for AMP ($K_d = 0.6–1.5$ $\mu$M). This difference in affinity could arise from the additional negative charge present in the APS molecule enabling it to more polar interactions with the protein matrix.
Variation of substrate concentrations in the oxidative reaction (AMP + HSO$_3^-$ → APS + 2 e$^-$) yielded the typical pattern by Michaelis (33). A $K_m$ for AMP = 0.17 mM and a $K_m$ for HSO$_3^-$ = 0.18 mM were obtained with an apparent $V_{max}$ = 25 μmol min$^{-1}$ mg$^{-1}$. In contrast, variation of the APS concentration in the reductive reaction (APS + 2e$^-$ → AMP + HSO$_3^-$) did not result in Michaelis type kinetics. At APS concentrations higher than 0.3 mM a 50% inhibition of the activity was observed. Below 0.3 mM APS the APS reductase activity (6.0 μmol min$^{-1}$ mg$^{-1}$) was not affected by the APS concentration, the lowest being 20 μM. Below 20 μM APS no reliable kinetic data could be obtained. The reductive reaction was effectively inhibited by AMP, with a half-maximum inhibition at 0.28 mM AMP. At AMP concentrations higher than 1.8 mM, the reduction of APS was completely inhibited.

Similar $K_m$ values for AMP and sulfite were reported for the APS reductase from $D$. vulgaris (12) (Table II). The behavior of APS reductase from $T$. denitrificans regarding the kinetics in the oxidative assay was different. Increase of the AMP concentration from 0.01 mM up to 0.2 mM gave a typical Michaelis type kinetic behavior, with $K_m$ = 0.02 mM for AMP, and $V_{max}$ = 21 μmol min$^{-1}$ mg$^{-1}$. At AMP concentrations higher than 0.2 mM the enzyme became inhibited (data not shown). The $K_m$ for sulfite with 1.2 mM was higher as in APS reductases from $D$. desulfuricans or $D$. vulgaris.

With APS reductase from hyperthermophilic $A$. fulgidus the measurement of the oxidative reaction used earlier with K$_3$Fe(CN)$_6$ as electron acceptor (17) at temperatures higher than 75 °C proved to be rather difficult (see “Experimental Procedures”). Here we report for the first time activities of APS reductase from $A$. fulgidus determined as APS reduction in an assay that worked highly reproducibly at temperatures up to 83 °C. APS reduction was catalyzed with a $K_m$ value of 15 μM and a $V_{max}$ of 10.3 μmol min$^{-1}$ mg$^{-1}$.

Reaction Mechanism—Although APS reductase from different organisms has been studied intensively over the past 30 years not much is known on the mechanism of APS reduction.
The most striking property of APS reductases was the formation of a FAD N(5)-sulfite adduct. It was proposed that the sulfite adduct represents an intermediate in the catalytic cycle (34, 35), although a wide variety of flavoenzymes show unspecific high affinity binding of sulfite to FAD (10). However, in contrast to those flavoenzymes the sulfite adduct does not inhibit APS reductase. Furthermore, upon soaking crystals containing reduced APS reductase with APS a covalent FAD N(5)-sulfite adduct was observed in the crystal structure (1). Both AMP and FAD bind close to FAD, as shown by the changes in the UV-visible spectra of oxidized APS reductase with AMP or APS. The 5–10-fold lower \( K_m \) values for AMP and FAD suggest that the affinity for the nucleotides is strongly enhanced under turnover conditions. The structure of the APS molecule points toward the involvement of a nucleophile in catalysis, which should cleave the APS molecule accompanied by the release of the stored energy. In the oxidative reaction sulfite and AMP have to be combined, most likely via a nucleophilic attack by one substrate on the other.

In summary, these observations strongly suggest a role in catalysis for FAD. In view of the homology of APS reductase to fumarate reductase and other oxido-reductases (1) a hydride transfer from reduced FAD to closely bound APS might be the mechanism for the reduction of APS. The S–O bond would be cleaved producing AMP and sulfite. Consequently, for the back reaction one should expect that sulfite becomes oxidized by FAD producing reduced FAD and sulfate. Note that the formation of the N(5)-sulfite adduct is not evident from such a mechanism. Therefore we propose the nucleophilic mechanism outlined in Fig. 10, which satisfies all experimental observations. FAD is reduced first, and subsequently APS is bound. N5 of reduced FAD undergoes a nucleophilic attack on the sulfur atom of APS and a covalent FAD-APS intermediate is formed (Fig. 10A). Rearrangements of electrons result in the release of AMP, which is a good leaving group, accompanied by the formation of the N(5)-sulfite adduct. Finally, sulfite dissociates and oxidized FAD is regained. The dissociation of sulfite might be triggered by the protonation of one sulfite oxygen to form bisulfite, which does not bind to FAD. Such a nucleophilic attack by N-5 of reduced FAD has not been formulated so far in the literature, mainly because the N-5 atom is a rather weak nucleophile. However, the APS molecule is perfectly suited for a nucleophilic attack. The sulfur atom of APS is a good electrophile and the energy liberated upon cleavage of the mixed anhydride bond of APS is rather high. Further support comes from the fact that the affinity of APS toward the reduced enzyme is much higher compared with that toward the oxidized enzyme.

Concerning the formation of APS from sulfite and AMP it is assumed that the nucleophile sulfite adds to the N-5 atom of oxidized FAD, which is the most electrophilic position of the molecule (Fig. 10B). The negative charge on the sulfite sulfur is shifted toward the flavin ring, which acts as a sink. This enables a nucleophilic attack of the phosphate moiety of AMP on the sulfur atom of the FAD-sulfite adduct. After rearrangement of electrons APS is eliminated and FAD becomes reduced by two electrons, which are subsequently transferred to the iron-sulfur centers.

Further proof for this mechanism will arise from future structural and stopped-flow kinetic measurements that are currently underway in our laboratory.

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The Function of the [4Fe-4S] Clusters and FAD in Bacterial and Archaeal Adenyllysulfate Reductases: EVIDENCE FOR FLAVIN-CATALYZED REDUCTION OF ADENOSINE 5'-PHOSPHOSULFATE

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