A FLAVIN-SULFITE ADDUCT AS AN INTERMEDIATE IN THE REACTION CATALYZED BY ADENYLYL SULFATE REDUCTASE FROM DESULFOVIBRIO VULGARIS

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A mechanism is postulated for adenylyl sulfate (APS) reductase, considered as the oxidation of sulfite to the level of sulfate (APS), which involves three steps in the absence of an added electron acceptor. The first step involves the reversible association of sulfite with enzyme-bound FAD to form a flavin-sulfite adduct; the second, a transfer of the sulfur moiety from FAD to a mononucleotide acceptor to yield the corresponding nucleotide 5'-phosphosulfate; and the third, the internal reduction of an as yet unknown chromophore to yield oxidized FAD.

The enzyme adenylyl sulfate (APS) reductase catalyzes the reduction of APS to sulfite and AMP in the presence of reduced methyl viologen (MVH) (Eq. 1) and the oxidation of sulfite to APS in the presence of AMP and $Fe(CN)_6^{3-}$ (Eq. 2).[1,2]

$$APS + 2\ MVH \longrightarrow SO_3^{2-} + AMP + 2\ MV \quad (1)$$

$$SO_3^{2-} + AMP + 2\ Fe(CN)_6^{3-} \longrightarrow APS + 2\ Fe(CN)_6^{4-} \quad (2)$$

APS reductase is found in the dissimilatory sulfate reducing bacteria, species of Desulfovibrio and Desulfotomaculum;[3,4] in certain Thiobacilli, organisms which oxidize reduced sulfur compounds to the level of sulfate;[5,6] and in the sulfur-purple photosynthetic bacteria, where the enzyme appears to be involved in the oxidation of reduced sulfur compounds to sulfate during photosynthetic growth.[7] It is a metalloflavoprotein enzyme, which in Desulfovibrio vulgaris has a molecular weight of 220,000 and contains 1 mole of FAD plus 6-8 gram atoms of non-heme iron per mole of enzyme.

The mechanism of APS reductase has been of particular interest because the oxidation of sulfite in the presence of AMP results in the formation of a phosphosulfate bond with a ΔF^o of 18-19 Kcal/mole.[8] Two possible reaction mechanisms have been postulated for the oxidative formation of APS from AMP and sulfite.[2] The first is the direct oxidation of AMP and sulfite to APS

through the intermediate formation of adenosine phosphosulfite. The second is sequential binding and oxidation of enzyme-bound sulfite to the level of sulfate followed by transfer of the high energy sulfate to a mononucleotide acceptor. Evidence has now been obtained which supports the second mechanism and indicates a covalent bonding of sulfite to the flavin moiety of the enzyme during the enzymatic reaction.

MATERIALS AND METHODS

APS reductase was purified as described by Peck et al.[2] Enzyme for spectro photometric studies was freed of nucleotides by dialysis for 16 hrs against 0.05 M tris-maleate buffer, pH 7.5, containing 5 gms of acid washed Norite per liter. Spectra were obtained using a Cary Model 15 Recording Spectrophotometer.

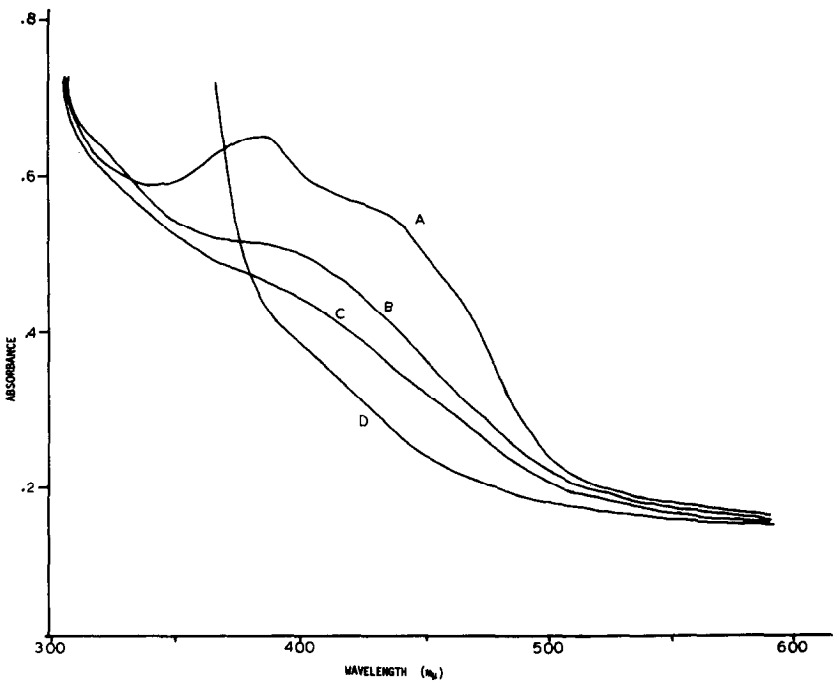


Fig. 1. Spectral changes resulting from the sequential addition of sulfite, AMP and dithionite. Each cuvette contained 3 mg APS reductase dialyzed for 16 hrs against 0.05 M tris-maleate buffer, pH 7.5, containing 5 gm of acid washed Norite per liter. Curve A, no additions; Curve B, Na_2SO_3, 3 X 10^{-2} M; Curve C, AMP, 2 X 10^{-3} M (plus Na_2SO_3); Curve D, several crystals of $Na_2S_2O_4$ (plus Na_2SO_3 and AMP).

RESULTS AND DISCUSSION

Preliminary evidence for the second mechanism was provided by the observation that sulfite causes a partial bleaching of the absorption spectrum of purified APS reductase in the absence of added AMP. The spectral change induced by the addition of sulfite is shown in Fig. 1. The oxidized spectrum (Curve A) shows a broad shoulder from 420-460 nm with an absorption maximum at 385 nm. Sulfite causes an incomplete bleaching of the enzyme (Curve B), and a plot of the difference spectrum exhibits maxima at 385 and 440 nm (Fig. 2A). The change in absorbance at 440 nm can be accounted for by the amount of FAD released from the enzyme by boiling or by precipitation with trichloroacetic acid. The unique feature of the absorption spectrum in the presence of sulfite (Fig. 1, (Curve B) is the increase of absorbance around 320 nm above that of the oxidized enzyme (Curve A). These observations were initially interpreted as indicating that enzyme-bound FAD is reduced by sulfite and that FAD is probably the major contributor to this observed spectral change.

It was important to determine whether any APS was formed under the conditions of the experiment described above in order to establish that the enzyme-bound FAD was being reduced without concomitant APS formation. As indicated in Table I, APS formation was compared with the reduction of enzyme-bound FAD by sulfite in undialyzed and Norite-dialyzed enzyme. With the undialyzed enzyme, the amount of enzyme-bound FAD reduced is approximately equal to the amount of APS formed; however, in the case of the Norite-dialyzed enzyme, flavin is reduced but very little APS formation is observed. When each of the enzyme preparations is supplemented with AMP, APS is formed in considerable excess over the amount of enzyme-bound FAD reduced due to the reactivity of the enzyme with O_2.

The addition of AMP to the sulfite-reduced enzyme causes a further broad decrease in the absorption spectrum (Fig. 1, Curve C) including the range around 320 nm. Whereas the difference spectrum of the sulfite-induced reduction (Fig. 2A) exhibits maxima at 385 and 445 nm, the difference spectrum of the AMP-induced reduction (Fig. 2B) shows a main absorption band from 400-430 nm

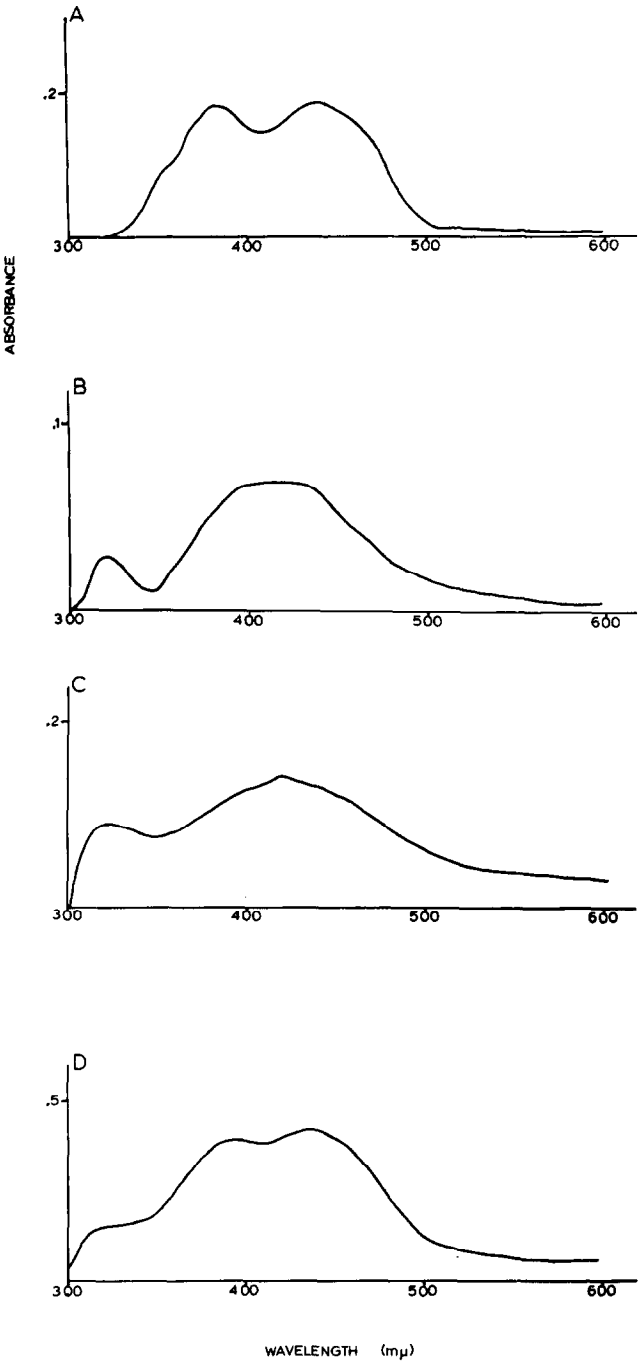


Fig. 2. Difference spectra obtained upon the sequential addition of sulfite, AMP and borohydride to APS reductase. Enzyme, same as in Fig. 1. A - oxidized minus Na_2SO_3, 3 X 10^{-2} M; B - Na_2SO_3 minus AMP, 2 X 10^{-3} M; C - Na_2SO_3 minus sodium borohydride (several crystals); D - oxidized minus borohydride.

and a smaller broad peak at 320 nm. Further additions of sulfite or AMP at these concentrations cause no further reduction in the absorption spectrum, although dithionite or sodium borohydride do reduce absorption at 440 nm by an additional 30-40%. The "AMP minus borohydride" difference spectrum (Fig. 2C) is qualitatively similar to the "sulfite minus AMP" spectrum and suggests that the same chromophore is reduced by both AMP and borohydride after the addition of sulfite. The "oxidized minus borohydride" difference spectrum is shown in Fig. 4D for comparison. Failure to observe more extensive reduction of the second chromophore with AMP may be closely related to the catalytic activity of the enzyme, as the bleaching of enzyme-bound FAD is usually complete and does not correlate with losses in catalytic activity.

The addition of AMP to the oxidized enzyme produces no significant spectral changes; however, the subsequent addition of sulfite causes a reduction of the absorbance equivalent to that resulting from the addition of sulfite followed by AMP (Fig. 1, Curve C).

These results were tentatively interpreted to suggest the formation of a

TABLE I

THE REDUCTION OF ENZYME-BOUND FAD IN THE ABSENCE OF APS FORMATION

Each reaction mixture contained purified APS reductase (5.0 mg) in 1 ml of 0.05 M tris-maleate buffer, pH 7.0; $Na_2{}^{35}SO_3$, 4 µmoles (7.8 X 10^6 cpm/µmole); tris-maleate buffer, pH 7.0, 100 µmoles; and where indicated, AMP, 5 µmoles. Reaction was stopped by boiling and five µmoles of unlabeled APS added to facilitate isolation of the nucleotide from Norite.

Treatment of APS reductase	Additions	FAD reduced (mµmoles)	APS formed (mµmoles)
NONE	NONE	16.3	16.0
NONE	AMP	16.3	261
Norite-dialyzed	NONE	13.8	0.89
Norite-dialyzed	AMP	17.0	100

flavin-sulfite adduct similar to that recently reported for model compounds,[9] glucose oxidase[10] and other flavoproteins by Massey and coworkers.[11] These flavin adducts are formed by the binding of sulfite at the N-5 position of the isoalloxazine ring: characteristically the visible absorption spectrum, in contrast to that of 1,5-dihydroflavin, completely disappears, and a new broad absorption band in the region of 310-330 nm is observed. It was further postulated that AMP facilitates the reduction of a chromophore having a major absorption band in the region of 400-430 nm and a minor band at 310-330 nm. This spectral change may be related to the non-heme iron content of the enzyme and would be qualitatively consistent with spectral changes observed upon the reduction of ferredoxin.[12]

As spectral changes at 320 nm using purified enzyme were not conclusive with regard to adduct formation, an attempt was made to obtain direct spectrophotometric evidence for the adduct. In these experiments, oxidized enzyme or enzyme reduced at 20° with sulfite or sulfite plus AMP was chilled in an ice bath and cold trichloroacetic acid added to 0.244 M. This treatment liberates the flavin and eliminates the absorption due to non-heme iron and protein. After centrifugation, the supernatant fluids were examined in the spectrophotometer for the characteristic absorption of FAD and the sulfite adduct as shown in Fig. 3. The spectrum of the oxidized enzyme (Curve A) shows only the two absorption peaks at 445 and 375 nm characteristic of oxidized flavin. The absorption spectrum of the sulfite-reduced enzyme (Curve B) shows no absorption at 445 or 375 nm but exhibits the broad peak at 320 nm which is characteristic of the flavin-sulfite adducts. After fifteen minutes incubation at room temperature, approximately 80% of the flavin absorbance at 445 and 375 nm had reappeared and most of the 320 nm absorbance had disappeared. This change was essentially complete after 30 min (Curve C). When the enzyme preparation was reduced with borohydride, there was no indication of absorption at 320 nm. These observations constitute direct evidence for the formation of a flavin-sulfite adduct and indicate that the increase in absorbance at 320 nm is probably due to the adduct formation.

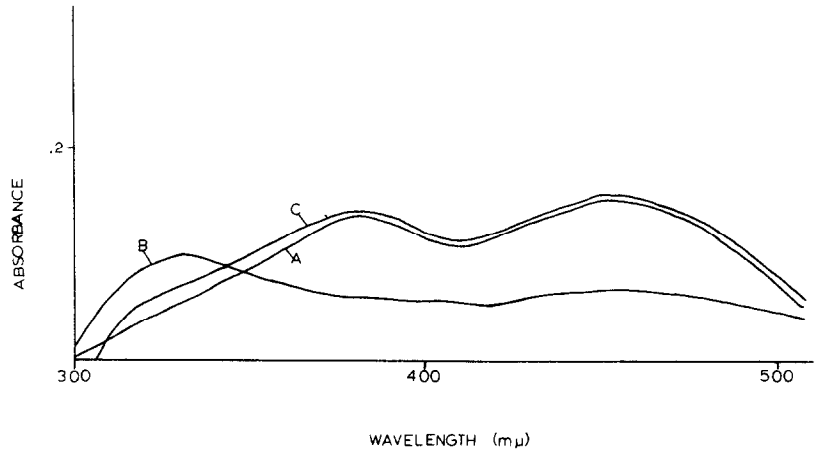


Fig. 3. The absorption spectrum of the sulfite-reduced flavin liberated from APS reductase by trichloroacetic acid. The Norite-dialyzed enzyme, 3 mg, was incubated with sulfite or sulfite plus AMP, chilled, and TCA added (0.244 M) to precipitate the protein which was removed by centrifugation. Curve A, no additions; Curve B, Na_2SO_3, 3 X 10^{-2} M, or Na_2SO_3, 3 X 10^{-2} M, plus AMP, 1 X 10^{-3} M; Curve C, Na_2SO_3, 3 X 10^{-2} M, after incubation for 30 min at 25°.

The mechanism of APS reductase, considered as the oxidation of sulfite to the level of sulfate (APS) is now postulated to include at least three steps in the absence of an electron acceptor. The first step involves the reversible association of sulfite with enzyme-bound FAD to form the N-5 adduct (Eq. 3).

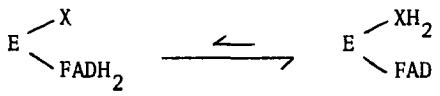
$$E\begin{matrix}\diagup X(H_2) \\ \diagdown FAD\end{matrix} + SO_3^{2-} \rightleftharpoons E\begin{matrix}\diagup X(H_2) \\ \diagdown FAD\text{-}SO_3^-\end{matrix} \qquad (3)$$

X represents the unknown chromophore, probably non-heme iron, either in the oxidized or reduced (H_2) state. The second step is the transfer of the sulfur moiety from FAD to a mononucleotide acceptor, shown here as AMP (Eq. 4).

$$E\begin{matrix}\diagup X \\ \diagdown FAD\text{-}SO_3^-\end{matrix} + AMP \rightleftharpoons E\begin{matrix}\diagup X \\ \diagdown FADH_2\end{matrix} + APS \qquad (4)$$

Because transfer of the sulfur moiety to acceptor has only been observed concurrently with chromophore reduction, it is postulated that the equilibrium of

reaction (4) lies in the direction of adduct formation and that detectable net reaction requires reduction of the chromophore, reaction (5).



(5)

The evidence presented indicates that the sulfite adduct of FAD is an intermediate in the mechanism of action of APS reductase. Although this enzyme appears to be a flavoprotein dehydrogenase based on its preference for one electron acceptors such as ferricyanide as opposed to oxygen, its reactivity with sulfite, one of its substrates, does not necessarily contradict the observations of Massey et al[11] correlating sulfite reactivity with the oxygen reactivity of flavoprotein oxidases where the sulfite adduct is catalytically inactive.

The energetics of the oxidation are such that there must be a change in the electronic configuration of the adduct so that the nitrogen-sulfur bond acquires sufficient energy to account for the synthesis of the phosphosulfate linkage in APS.

ACKNOWLEDGEMENT

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