METHYL VIOLOGEN-LINKED SULFITE REDUCTASE FROM SPINACH LEAVES: A HEMOPROTEIN.*

Kozi Asada**, Goro Tamura*** and Robert S. Bandurski
Department of Botany and Plant Pathology, Michigan State University
East Lansing, Michigan 48823, U.S.A.

Received February 7, 1968

The pyridine nucleotide-linked sulfite reductase from yeast (Yoshimoto and Sato, 1965) and E. coli (Siegel and Kamin, 1967) have an absorption maximum at 587 mμ, in addition to the maxima due to flavins. The methyl viologen-linked sulfite reductase from Allium odorum also has an absorption maximum at 587 mμ (Tamura, 1965). These observations suggest the occurrence of a functional group in sulfite reductase common to a number of organisms and irrespective of the electron donor employed. This communication deals with the chromogen of the methyl viologen-linked sulfite reductase from spinach leaves. From the absorption spectrum, metal analysis, carbon monoxide sensitivity, and light reversibility of CO inhibition, we conclude that the spinach sulfite reductase is an unusual hemoprotein and that the heme is involved in catalysis of the reduction of sulfite to sulfide.

MATERIALS AND METHODS

Enzymatic activity was assayed by the initial rate of bleaching of reduced methyl viologen at 604 mμ following addition of sulfite (Asada, 1967). For simplicity the "a" fraction (cf. Table III of Asada, et al, 1966) was replaced with bovine serum albumin, thiols or disulfides. Data from this laboratory (unpublished) have shown that the "a" fraction can be supplanted by these compounds, although with low efficiency.

---

* This investigation was supported by a grant from the National Science Foundation (GB 3408).

** Present address: The Research Institute for Food Science, Kyoto University, Kyoto, Japan.

*** On leave from Faculty of Horticulture, Chiba University, Chiba Japan.

Purification of the sulfite reductase from spinach leaves was attained by acetone and ammonium sulfate fractionations, DEAE-cellulose chromatography, calcium phosphate gel fractionation, gel-filtration using Sephadex G-200 and hydroxylapatite chromatography. The final preparation represents a 500 fold purification over the acetone fraction. The enzyme thus obtained (about 500 mg from 120 kg of leaves) appears homogeneous in the ultracentrifuge and in its gel elution pattern. However, electrophoresis using polyacrylamide gel or cellulose acetate at pH 8.1 and 9.0 disclosed heterogeneity or dissociation. The molecular weight of the enzyme is 83,000 to 85,000 as determined with Sephadex G-200 according to Andrews (1965). Sulfite and hydroxylamine, but not nitrite, can induce oxidation of reduced methyl viologen with the purified enzyme. The separation of sulfite and nitrite reductase activity will be the subject of a separate report.

RESULTS AND DISCUSSION

The absorption spectrum of the purified enzyme is shown in Fig. 1. Maxima occur at 279, 404 and 589 mμ with a shoulder at 385 mμ. Unlike all previous preparations of sulfite reductase the enzyme does not contain flavins detectable by fluorometric analysis. Thus, the shoulder at 385 mμ of Fig. 1 is not due to flavins. Addition of cysteine does not effect the absorption spectrum. After reduction of the enzyme with sodium borohydride or sodium dithionate, the absorption maximum at 589 mμ disappears and the Soret band shifts to 417 mμ, with a decrease of absorbancy. Anaerobic addition of sulfite to enzyme reduced with sodium borohydride causes a shift of the Soret band to 403 from 417 mμ and a reappearance of a small peak near 589 mμ (Fig. 2). The addition of cyanide (3mM) to the oxidized enzyme does not affect the peak at 589 mμ. However, the addition of cyanide to enzyme reduced with methyl viologen, followed by subsequent oxidation of reduced methyl viologen with air, shifts the peak at 589 to 578 mμ. This is consistent with our prior observation of irrever sible inactivation of reduced enzyme by cyanide (Asada, 1967).

Using 15.9 mg of the purified enzyme, a spectrographic analysis for metals was conducted in the laboratory of Dr. B. Vallee and disclosed the occurrence of 506 μg of iron per g of the purified enzyme. Based on a molecular weight of 84,000, this would correspond to 0.76 atom of iron per molecule of the enzyme. Assuming a molecular extinction coefficient at the Soret band of 10^5, porphyrin-iron would account for about one-half of the iron found. Manganese, molybdenum, magnesium and copper were not detected.

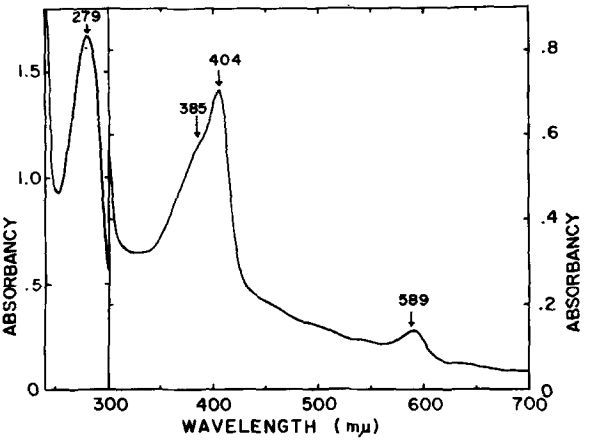

Figure 1. Absorption spectrum of spinach sulfite reductase. Protein concentration was 1.73 mg per ml in 10 mM potassium phosphate at pH 7.7.

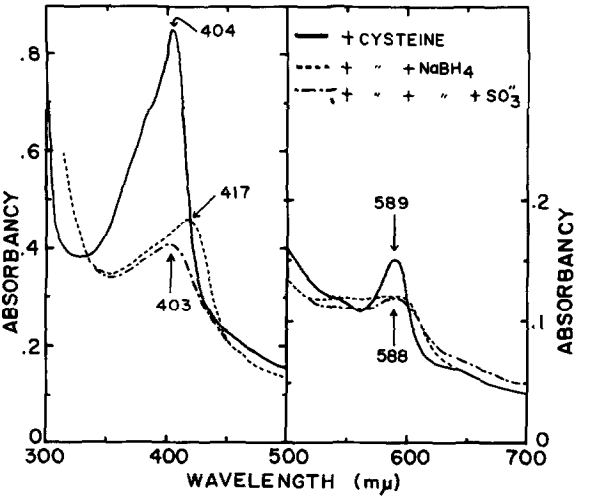

Figure 2. Effects of cysteine, sodium borohydride and sulfite on the spectrum of spinach sulfite reductase. Protein concentration was 2.54 mg per ml in 200 mM potassium phosphate, pH 7.7, in the presence of 0.1 mM cysteine (A), after addition of 1 mg of sodium borohydride to (A) under anaerobic conditions (B), and, (C) after subsequent anaerobic addition of sulfite (43 mM, final concentration) to (B).

The purified sulfite reductase is sensitive to cyanide and p-chloromercuribenzoate. Sensitivity to both reagents is found only after the enzyme has been reduced by reduced methyl viologen, as is the case for crude preparations (Asada, 1967). The hydroxylamine reducing activity of the enzyme is also inhibited by both poisons.

Sulfite reductase activity was inhibited by carbon monoxide under our usual assay conditions i. e., starting the reaction by the addition of sulfite to a mixture of enzyme and reduced methyl viologen. However, when reduced methyl viologen was added to a mixture of the enzyme and sulfite, the oxidation of reduced methyl viologen and the formation of sulfide were not affected by carbon monoxide. Thus, sensitivity of the enzyme to carbon monoxide is prevented by sulfite. The data of Fig. 3 demonstrate that the inhibition due to carbon monoxide is reversed by light. Illumination of enzyme in the presence of sulfite resulted in continued oxidation of reduced methyl viologen, even after turning off

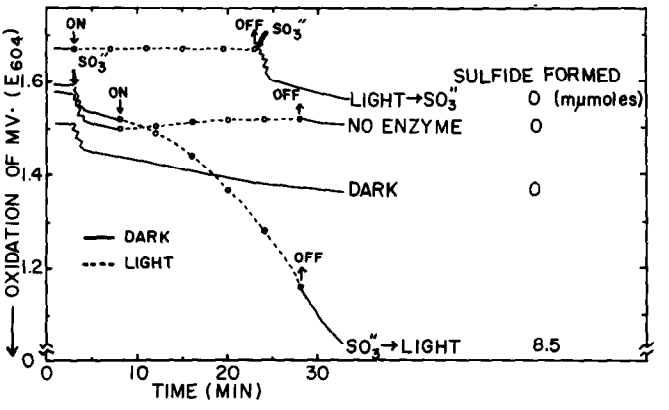

Figure 3. Effect of light on inhibition of sulfite reductase by carbon monoxide. The reaction mixture, in a final volume of 1.5 ml in a Thunberg photocell, contained 150 μmoles of potassium phosphate, pH 7.7, 1.5 μmoles of potassium sulfite, 0.4 μmoles of reduced methyl viologen (about 40 to 50% reduced), 2 mg of crystalline bovine serum albumin, 0.4 ml of a saturated solution of carbon monoxide and 6 μg of sulfite reductase. Before introduction of reduced methyl viologen, the reaction mixture was made anaerobic by repeating evacuation and flushing with carbon monoxide. Sulfite was added at the points shown by arrows. Illumination of the reaction mixture was carried out with 200,000 lux of light from a projector lamp. Measurement of absorbancy at 604 mμ was made during a 20 to 30 sec. interruption of illumination. In the case of light followed by sulfite, the sulfite was added 30 sec. after turning off of the light. Sulfide was determined using an aliquot of the reaction mixture (Asada, 1967).

the light. This was not a photochemical oxidation since oxidation of reduced methyl viologen was not observed in the absence of enzyme. By contrast, illumination of a mixture of carbon monoxide and enzyme, followed by sulfite addition 30 sec. after turning off the light does not give activity, suggesting that in the dark, carbon monoxide and the enzyme recombine rapidly. Sulfide formation was found only in the case of illumination in the presence of sulfite. Hydroxylamine reducing activity is also inhibited by carbon monoxide.

Although the present preparation of sulfite reductase is not homogeneous, its absorption spectrum, the occurrence of iron, carbon monoxide sensitivity, and light reversal of CO inhibition indicate that the spinach sulfite reductase is a hemoprotein. Moreover, sulfite protection against carbon monoxide suggests that the sulfite combines with an heme moiety. On the other hand, the following properties of the reductase are different from those of a typical hemoprotein. Reduction of the enzyme does not give typical α and β bands and cyanide inhibition is found only after reduction of the enzyme with reduced methyl viologen. Analogously however, catalase does not show a reduced spectrum with reducing agents and again cyanide binds with the ferroperoxidase (Keilin and Hartree, 1955; Yamazaki et al, 1967). Thus, it is proposed, that the spinach sulfite reductase is a hemoprotein. In contrast to the yeast and E. coli sulfite reductases, the spinach enzyme has a low molecular weight, does not contain flavins, and can not utilize NADPH as an electron donor (Asada, 1967). These results suggest that the active site of the spinach sulfite reductase is the same as the sulfite reducing moiety of the yeast and E. coli enzymes except that the spinach enzyme has been cleaved from its NADPH coupling site and the flavin moiety. Demonstration that a partially purified spinach sulfite reductase can be coupled to ferredoxin indicates that reconstruction of the reductase system can be attained (Tamura et al, 1967).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. B.L. Vallee and Dr. K. Fuwa for metal analysis of the enzyme and Dr. Y. Morita for his suggestions. We are also indebted to Mr. D. Bishop for his skillful technical assistance and to Mr. W. Jacob of the Bowman Cattle Ranch, Uvalde, Texas, for donation of the spinach.

REFERENCES

Andrews, P., Biochem. J., 96 595 (1965).
Asada, K., J. Biol. Chem., 242, 3646 (1967).
Asada, K., Tamura, G., & Bandurski, R.S., Biochem. Biophys. Res. Commun., 25, 529 (1966).
Keilin, D. & Hartree, E.F., Biochem. J., 61, 153 (1955).
Siegel, L.M. & Kamin, H., a paper presented at "The Conference on Flavins and Flavin Enzymes" at Nagoya, Japan, Aug., 1967.
Tamura, G., J. Biochem. (Tokyo), 57, 207 (1965).
Tamura, G., Asada, K., & Bandurski, R.S., Plant Physiol, 42:S36 (1967).
Yamazaki, I., Nakajima, R., Honma, H. & Tamura, M., Biochem. Biophys. Res. Commun., 27, 53 (1967).
Yoshimoto, A. & Sato, R., Koso Kagaku Shimpoziumu, 17, 65 (1965).