CLEAVAGE OF SULFITE REDUCTASE INTO TWO NON-DIALYZABLE COMPONENTS AND STIMULATION OF REDUCTASE ACTIVITY BY NUCLEOTIDES.*

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 November 2, 1966

The sulfite reductase of micro-organisms and of higher plants catalyzes the reduction of sulfite to sulfide, utilizing pyridine nucleotide (cf. Broquist and Trupin, 1966), methyl viologen (Naiki, 1965) or ferredoxin (Akagi, 1965; Le Gall and Dragoni, 1966) as reductant. In at least two cases (Yoshimoto and Sato, 1965; Tamura, 1965) the enzyme has been purified to homogenity. The pure protein catalyzes this six electron reduction. Genetically the reductase maps as being under the control of six cistrons (Dreyfuss and Monty, 1963) and thus it should be possible to cleave it into its component parts. In the present work we have demonstrated the presence of, at least, two catalytically active sites. One site is pCBM[1] inhibitable and this inhibition is prevented by sulfite ion. The other site is cyanide sensitive and is not protected by sulfite. The enzyme becomes sensitive to both inhibitors only after it has been reduced by reduced methyl viologen. A further fractionation of the reductase resulted in separation into two non-dialyzable components, both of which are required for sulfide formation. In addition, we have observed that the reductase is stimulated by nucleotides and by pyrophosphate.

---

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

** On leave from the Research Institute for Food Science, Kyoto University, Kyoto, Japan.

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

[1] Abbreviations used are: MV, oxidized methyl viologen, MV·, reduced methyl viologen, tricine, N-tris-(hydroxymethyl)-methylglycine and pCBM, p-chloromercuribenzoate.

MATERIALS AND METHODS

Enzymatic activity was assayed by the initial rate of bleaching of MV· upon the addition of sulfite. MV· was introduced anaerobically into a Thunberg cuvette containing the reaction mixture. The molecular extinction coefficient used for MV· at 604 mμ was 1.40×10^4. One unit of sulfite reductase is defined as that amount of enzyme, which oxidized one μmole of MV· per min, under the assay conditions of Table I.

A partially purified enzyme preparation was obtained from spinach leaves by ammonium sulfate and acetone fractionation, DEAE-cellulose chromatography, gel-filtration using Bio-gel, P-150 yielding a 188 fold purification (Stage I). Where indicated, a more highly purified two component preparation was obtained utilizing the above method except that acetone fractionation was employed prior to ammonium sulfate and without gel filtration (Stage II). Final resolution of the two components was attained with calcium phosphate gel adsorption and sequential elution with potassium phosphate buffer over the concentration range of 10 to 300 mM. The enzyme catalyzes the reduction of sulfite to sulfide using MV· as the electron donor. A stoichiometry of six moles of MV· oxidized per mole of sulfide formed was confirmed.

RESULTS AND DISCUSSION

Sensitivity to inhibitors: The spinach MV··-linked sulfite reductase (Stage I) was inhibited by cyanide and sulfhydryl reagents. Carbonyl reagents, including semicarbazide, dimedon, isonicotinyl hydrazide and sodium borohydride did not inhibit enzymatic activity, suggesting that the cyanide sensitive site is not a carbonyl group. Arsenite and Cd^{++} ions are not as effective inhibitors as pCMB and p-mercuryacetyl aniline and the enzyme is only slightly, or not at all, inhibited by most heavy metal reagents.

The data of Table I demonstrates that the enzyme was sensitive to cyanide and pCMB only after reduction of the enzyme by MV··. The enzyme was reacted with the inhibitors either in the absence of MV· or after reduction by MV·, or after reduction and reoxidation. After treatment, the unbound inhibitors were removed and enzymatic activity assayed. Sensitivity of the enzyme to pCMB and cyanide is found only when the enzyme was treated with the inhibitors in the presence of MV··. Treatment of the inhibited enzyme with 20 mM reduced glutathione or 1 mM 1, 4-dithiothreitol did not restore activity.

Table I

Effects of pCMB and cyanide on reduced sulfite reductase.

Expt No.	Inhibitor	Enzyme treatment	MV· oxidation rate as percent of control
1	None	None	100
2	pCMB	None	100
	KCN	None	98
3	pCMB	MV·, inhibitor added aerobically	100
	KCN	MV·, inhibitor added aerobically	98
4	pCMB	MV·, inhibitor added anaerobically	0
	KCN	MV·, inhibitor added anaerobically	7

The enzyme (Stage I), 173 munit, was treated with inhibitors in 0.1 M potassium phosphate buffer, pH 7.7, in a total volume of 1.5 ml. Concentrations of pCMB and KCN were 0.66 and 1 mM, respectively. Exp. 2: without treatment of enzyme with MV·. Exp. 3: The enzyme was reduced by 0.27 mM MV·, in a Thunberg cuvette for 10 min, then air was introduced and after bleaching of MV·, the inhibitors were added immediately. Exp. 4: The enzyme was reduced as in Exp. 3, and the inhibitors added anaerobically, from a side arm. Control enzyme (Exp. 1) was obtained by the above treatment, however, without MV· and the inhibitors. The enzymes were dialyzed against 5 mM potassium phosphate buffer, pH 7.7 and an aliquot of the dialyzed enzyme assayed using the following reaction mixture; in mMolar concentrations, potassium phosphate buffer, (pH 7.75), 100; potassium sulfite, 1; MV·, 0.27 (about 40 to 50% in reduced form). Total volume was 1.5 ml and incubation was at 25°.

The experiment of Table II was conducted to determine whether either of the inhibitor sensitive sites were protected by sulfite. As can been seen, sulfite completely protects the pCMB site but does not affect cyanide sensitivity.

These experiments suggest the occurrence of two active sites in sulfite reductase, a thiol grouping and a cyanide sensitive site. Both sites bind pCMB and cyanide only after reduction of the enzyme. Comparison of Exps. 3 and 4 of Table I demonstrate that the reduced forms of both sites are autooxidizable. Thus sulfite reductase appear to have a functional protein disulfide-thiol "prosthetic" group, as in the following enzymes: lipoyl dehydrogenase, glutathione reductase, fraction c protein in the reduction of 3'-phosphoadenosine-5'-phosphosulfate, the enzyme system for the reduc-

tion of methionine sulfoxide (cf. Black, 1963), thioredoxin in the reduction of ribonucleotides (Laurent et al, 1964; Moore et al, 1964) and fraction P_2 protein in the decarboxylation of glycine (Baginsky and Huennekens, 1966). Decreased sensitivity to pCMB of the reduced enzyme in the presence of sulfite indicates that sulfite binds to the pCMB sensitive site. A cyanide sensitive site may be related to the "587 mμ pigment", observed in yeast sulfite reductase (Yoshimoto and Sato, 1965), which is also affected by cyanide and the oxidation-reduction state of the enzyme. The enzyme from *Alium odorum* has an absorption peak in the same region as that of yeast (Tamura, 1965).

Table II

Effect of sulfite on pCMB and cyanide sensitivity of reduced sulfite reductase.

Inhibitor	Sulfite	MV· oxidation rate
None	+	100
pCMB	+	101
KCN	+	0
pCMB	-	0
KCN	-	0

The enzyme (Stage I), 173 munit, was reduced with 2.7 mM MV·, in 0.1 M potassium phosphate buffer, pH 7.7 and in the presence of 1 mM potassium sulfite, where indicated, for 1 min, using a Thunberg cuvette. Total volume was 1.5 ml. Then pCMB (0.66 mM) or KCN (1 mM) was added anaerobically from a side arm. After 1 min of contact with the inhibitors, air was introduced. The treated enzyme was dialysed and assayed as in Table I. Sulfide formation during the 1 min preincubation with MV· and sulfite was 0.076 μmoles, so the effect of sulfite was not due to a trapping of pCMB by sulfide.

Cleavage of the reductase into two fractions: A more direct proof for multiple catalytic sites involved in sulfite reductase activity is shown in the data of Table III. In these experiments, employing a further purification of the reductase with calcium phosphate gel, it was possible to show that, at least, two fractions are required for the oxidation of MV· by sulfite and for the formation of sulfide. Both fractions are non-dialyzable (36 hrs, against 10 mM potassium phosphate buffer, pH 7.7). The 10 to 20 mM calcium phosphate gel eluate fraction is pCMB sensitive and cyanide insensitive. It is inactivated 80 per cent when heated for 5 min at 70°, but the remaining activity is stable to boiling for as long as 30 min. The 120 to 160 mM fraction is heat labile being totally inactivated - 70°, 5 min.

Table III

Requirement of two non-dialyzable components for sulfite reductase activity.

System	MV· oxidation rate, mμmoles/min
10-20 mM Ca-phosphate gel eluate (a)	0
120-160 mM " (b)	2.1
(a) + (b)	14.1

Stage II enzyme was subjected to calcium phosphate gel fractionation and the fractions shown above were assayed as in Table I.

Stimulation of the reaction by nucleotides: As shown in Fig. 1, ADP and ATP stimulate the bleaching of MV· in the presences of sulfite and the enzyme. Other nucleoside diphosphates (UDP, CDP, GDP, IDP, deoxy ADP) and triphosphates (UTP, CTP, GTP, ITP, deoxy ATP) also stimulate 1.4 - 1.7 and 2 - 2.6 fold, respectively, at 2 mM, under the same condition as Fig. 1. Pyrophosphate gave the same stimulation as nucleoside-diphosphates. Nucleoside monophosphates and orthophosphate had little or no effect under the same conditions. The stimulation observed in tricine buffer is not observed in phosphate buffer. The stoichiometry of the sulfite reductase reaction was maintained, regardless of the presence or absence of ATP, so this stimulation is not due to a side reaction. The K_m for ATP obtained from the reciprocal

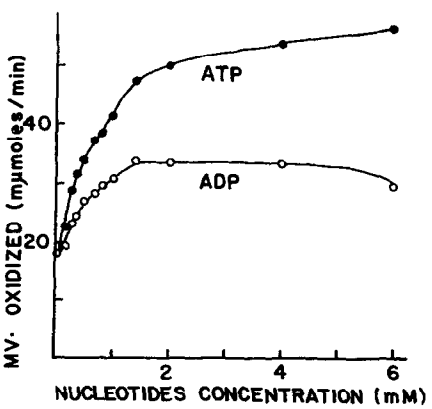

Fig. 1
Effects of ADP and ATP on sulfite reductase activity. Reaction mixture contained in 1.5 ml (in mMolar concentrations): tricine-NaOH buffer, pH 7.2, 50; EDTA, 2; potassium sulfite, 0.5; MV·, 0.27; enzyme (Stage I), 14.7 munit and nucleotides as indicated. Nucleotides were added before the addition of MV· and sulfite.

plot of ATP concentration and reaction rate is 0.44 mM. The K_m for sulfite (2.1×10^{-5} M) was not appreciably affected by the addition of 2 mM ATP (3.6×10^{-5} M). Stimulation was thus due to an increase in V_{max}.

Determination of the function of the two fractions of sulfite reductase awaits the availability of homogeneous fractions. As a working hypothesis however, it seems reasonable to suppose that the 20 mM eluate fraction is the sulfite binding site. Since, at least the Bc locus of the 3'-phosphoadenosine-5'-phosphosulfate reductase of Salmonella (Dreyfuss and Monty, 1963) is also involved in sulfite reductase it is possible that the 20 mM fraction is related to the yeast sulfite binding protein (Torii and Bandurski, 1964) of the sulfate reductase. Thus a mechanism would be available to reduce sulfate to sulfide without free intermediates.

REFERENCES

Akagi, J.M., Biochem. Biophys. Res. Commun., 21, 72 (1965)
Baginsky, M.L., and Hunnekens, F.M., Biochem. Biophys. Res. Commun., 23, 600 (1966)
Broquist, H.P., and Trupin, J.S., Ann. Rev. Biochem., 35, 231 (1966)
Dreyfuss, J., and Monty, K.J., J. Biol. Chem., 238, 1019 (1963)
Laurent, T.C., Moore, E.C., and Reichard, P., J. Biol. Chem., 239, 3436 (1964)
Le Gall, J., and Dragoni, N., Biochem. Biophys. Res. Commun., 23, 145 (1966)
Moore, E.C., Reichard, P., and Thelander, L., J. Biol. Chem., 239, 3445 (1964)
Naiki, N., Plant Cell Physiol. (Tokyo), 6, 179 (1965)
Tamura, G., J. Biochem. (Tokyo), 57, 207 (1965)
Torii, K., and Bandurski, R.S., Biochem. Biophys. Res. Commun., 14, 537 (1964)
Yoshimoto, A., and Sato, R., Proceedings of Symposium of Enzyme Chemistry of Japan, 19, 65 (1965)