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STUDIES ON YEAST SULFITE REDUCTASE

I. PURIFICATION AND CHARACTERIZATION

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SUMMARY

1. NADPH-sulfite reductase, catalyzing the reduction of sulfite to sulfide by NADPH, was purified from baker's yeast to an almost homogeneous state. The purified enzyme showed a sedimentation coefficient of 14.8 S. A minimal molecular weight of 350000 was estimated from its flavin content.

2. The oxidized enzyme was greenish yellow in color, showing absorption peaks at 386, 455 and 587 mμ. The intensity of these peaks was lowered considerably by NADPH or dithionite.

3. The enzyme contained 1 mole each of FMN and FAD per 350000 g of protein. About 5 atoms of non-heme iron were detected per mole of FMN or FAD. The enzyme also contained a non-flavin chromophore (or chromophores) absorbing at 587 mμ and at about 386 mμ. The 587-mμ chromophore was unstable to treatment causing alterations in protein conformation. Five to six moles of *p*-chloromercuribenzoate combined with 350000 g of the enzyme protein.

4. The enzyme catalyzed, besides the NADPH-sulfite reductase activity, the reduction of sulfite by reduced viologen dyes, the reductions by NADPH of nitrite, hydroxylamine, ferricyanide, cytochrome *c*, quinones, and 2,6-dichlorophenolindophenol, and the reduction of $NADP^+$ by reduced methyl viologen.

5. All the NADPH-linked activities were inhibited by $NADP^+$, 2'-AMP and *p*-chloromercuribenzoate. $NADP^+$ and 2'-AMP, however, did not affect the reduction of sulfite by reduced viologen dyes.

6. Cyanide strongly inhibited the NADPH-sulfite reductase activity only when the reaction was started by adding sulfite to the enzyme which had been incubated with NADPH and cyanide. The addition of cyanide to the NADPH-reduced (but not the oxidized) enzyme caused an irreversible conversion to a reddish violet form. This conversion was prevented by the presence of sulfite.

INTRODUCTION

Enzymes possessing NADPH- and/or reduced methyl viologen (MVH)-sulfite reductase activities have been studied with and partially purified from various sources,

Abbreviations: MV, methyl viologen; MVH, reduced methyl viologen; DCIP, 2,6-dichlorophenolindophenol; PCMB, *p*-chloromercuribenzoate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

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such as yeast[1–4], *Escherichia coli*[5–11], *Salmonella typhimurium*[12,13], *Aspergillus nidulans*[14–17], *Neurospora crassa*[18–20], *Desulfovibrio desulfuricans*[21], *Desulfovibrio gigas*[22], *Clostridium nigrificans*[23], and higher plants[24–26].

WAINWRIGHT[2] has reported that the sulfite-reducing system from yeast could be resolved into six protein fractions. ASADA, TAMURA AND BANDURSKI[26] have also resolved partially purified spinach MVH-sulfite reductase into two components. Genetic analysis of *S. typhimurium* mutants has further suggested that synthesis of NADPH-sulfite reductase is controlled by six discrete cistrons[12,13,27]. Moreover, most of the sulfite reductases so far studied have been shown to catalyze not only the reduction of sulfite but also the reductions of nitrite, hydroxylamine, cytochrome *c*, and dyes[3,5,7,22,28,29].

Despite these findings, all the enzymes mentioned above, except for the system reported by WAINWRIGHT[2], have been shown to behave as single proteins during their partial purification, and no separation of the various activities has been noticed. It is, therefore, likely that all the activities are catalyzed by single proteins, though final conclusions must await more extensive purification of the enzymes.

NAIKI[3] has reported a 200-fold purification of yeast NADPH-sulfite reductase (EC 1.8.1.2), which also catalyzed MVH-sulfite reductase activity. He has also found that some of the mutant strains of yeast, incapable of assimilating sulfate and sulfite, contained the MVH-sulfite reductase activity, though they were devoid of the NADPH-sulfite reductase activity. Based on these observations, together with the data on the effects of inhibitors and low ionic strength, he has proposed a model for the enzyme consisting of two dissociable components. A similar conclusion has also been reached by us for *A. nidulans* sulfite reductase[16,17]. However, the nature of the postulated two components has not yet been elucidated because of insufficient purity of the preparations employed.

This paper describes the purification of NADPH-sulfite reductase from baker's yeast to an almost homogeneous state, and reports that this enzyme is a single protein possessing a sedimentation coefficient of 14.8 S. The purified enzyme contains FMN, FAD, non-heme iron, and an unidentified chromophore or chromophores. Some of the basic properties of the enzyme have also been characterized. The essential part of this work has already been published in preliminary communications[28,29].

EXPERIMENTAL PROCEDURE

Materials

Pressed baker's yeast (*Saccharomyces cerevisiae*) was obtained from the Oriental Yeast Company, Osaka. The following chemicals were obtained from commercial sources: DEAE-cellulose (Brown); Sephadex gels and DEAE-Sephadex A-50 (Pharmacia); NAD^+, $NADP^+$, NADH, NADPH, AMP, ADP, and ATP (Sigma); methyl viologen (MV) and benzyl viologen (British Drug Houses); *o*-nitrosoresorcinol monomethyl ether (Tokyo Kasei); 2,6-dichlorophenolindophenol (DCIP), menadione, riboflavin, FMN, and FAD (Wako). The flavins were purified chromatographically as described by HUENNEKENS AND FELTON[30]. 2′-AMP and 3′-AMP were kindly supplied from Dr. T. FUJITA of Takeda Chemical Industries, Ltd., Osaka, and ubiquinone Q_0 from Dr. T. SUGIMURA of the National Cancer Center, Tokyo. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) was prepared by the method of WILSON, ASAHI AND

BANDURSKI[31] and L-methionine sulfoxide by the method of ROBER AND MCILWAIN[32].

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was prepared from brewer's yeast according to the method of NOLTMANN, CLARK AND KUBY[33]; one unit of the enzyme was defined as the amount catalyzing the reduction of 1 μmole of $NADP^+$ per min at pH 8.0 (30°). Hydrogenase (EC 1.98.1.1) was prepared from *Desulfovibrio desulfuricans* according to the method of ISHIMOTO AND KOYAMA[34]; one unit of hydrogenase was defined as the amount catalyzing the production of 1 μmole of hydrogen from MVH per min at 30° under the conditions described by TAMIYA *et al.*[35]. Yeast alcohol dehydrogenase (EC 1.1.1.1) was crystallized and assayed according to the procedure of RACKER[36]. Crystalline beef-liver catalase (EC 1.11.1.6) was prepared by the method of SHIRAKAWA[37], and its activity was measured spectrophotometrically[38]. Purified D-amino acid oxidase (EC 1.4.3.3) and its apo-oxidase were prepared from hog kidney as described by NEGELEIN AND BRÖMEL[39]. Enzymes concerned in PAPS reduction (Enzymes A and C and Fraction C) were purified from baker's yeast as described by WILSON, ASAHI AND BANDURSKI[31]. Crystalline yeast cytochrome *c* was supplied by the Sankyo Company, Tokyo.

NADPH-sulfite reductase activity

This activity was routinely determined under aerobic conditions by following the oxidation of NADPH by sulfite spectrophotometrically at 340 mμ. The reaction mixture contained, in a final volume of 1.5 ml, 300 μmoles of potassium phosphate buffer (pH 7.3), 1.5 μmoles of EDTA, 0.23 μmole of NADPH, 1.5 μmoles of freshly dissolved sodium sulfite and a suitable amount of enzyme. The activity was determined from initial velocity and corrected for the slow oxidation of NADPH in the absence of sulfite. One unit of the enzyme was defined as the amount catalyzing the sulfite-dependent oxidation of 1 μmole of NADPH per min at 25° under the above conditions.

With cruder preparations containing powerful NADPH oxidase activities, an NADPH-generating system was employed and the reaction was conducted under nitrogen at 30° in Warburg manometric vessels equipped with two side arms. The main compartment of the vessel received 1.9 ml of a mixture containing 400 μmoles of potassium phosphate buffer (pH 7.3), 2 μmoles of EDTA, 0.2 μmole of $NADP^+$, 5 μmoles of glucose 6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase and 0.005 to 0.04 unit of sulfite reductase. The use of more than 0.04 unit of the reductase was avoided because of the impairment of the linearity between sulfide production and the enzyme concentration. One of the side arms contained 0.1 ml of 20 mM sodium sulfite, whereas 0.1 ml of 3 M H_2SO_4 was placed in the second side arm. The center well contained 0.2 ml of 2 M KOH and a strip of filter paper. The reaction was started by adding the sulfite by tipping and continued for 15–60 min under nitrogen at 30°. The reaction was stopped by adding the H_2SO_4, and the sulfide absorbed by the alkali in the center well was determined colorimetrically[40].

MVH-sulfite reductase activity

A modification of the method of ISHIMOTO, KOYAMA AND NAGAI[41] was employed to determine this activity. The main compartment of a Warburg vessel received 1.9 ml of a mixture containing 40 μmoles of potassium phosphate buffer (pH 7.7), 4 μmoles of MV, 8 units of hydrogenase, and sulfite reductase. The side arm received

0.1 ml of 0.1 M sodium sulfite, and the center well 0.2 ml of 2 M KOH and a strip of filter paper. The gas phase was pure hydrogen. After preincubation for 15 min to reduce MV completely, the reaction was started by tipping and hydrogen uptake was followed at 30°.

NADPH-cytochrome c reductase activity

This activity was assayed spectrophotometrically by following the increase in absorbance at 550 mμ at 25°. The activity was calculated from initial velocity. The reaction mixture contained, in a final volume of 1.5 ml, 300 μmoles of sodium phosphate buffer (pH 7.3), 1.5 μmoles of EDTA, 0.23 μmole of NADPH, 0.035 μmole of yeast cytochrome *c*, and sulfite reductase.

Other NADPH-linked activities

The oxidation of NADPH by acceptors other than sulfite and cytochrome *c* was measured spectrophotometrically under the conditions for spectrophotometric assay of the NADPH-sulfite reductase activity, except that sulfite was replaced by the acceptor to be tested. When nitrite, hydroxylamine, menadione (0.1 mM), ubiquinone Q_0 (0.2 mM) and MV (2 mM) were used as acceptors, the oxidation of NADPH was measured at 340 mμ. When DCIP (0.1 mM) and ferricyanide (1.5 mM) were used, the wavelength was set at 600 and 420 mμ, respectively. The concentrations of nitrite and hydroxylamine were varied. All the measurements were carried out aerobically, because the aerobic oxidation of NADPH without acceptor was negligible as compared with that in the presence of acceptors.

Reduction of $NADP^+$ by MVH

This reaction was assayed by measuring the initial rate of hydrogen uptake in a system consisting of hydrogen, hydrogenase, MV, $NADP^+$ and enzyme. The main compartment of a Warburg vessel contained 1.9 ml of a mixture containing 400 μmoles of potassium phosphate buffer (pH 7.3), 4 μmoles of MV, 8 units of hydrogenase, and sulfite reductase. The side arm received 0.1 ml of 40 mM $NADP^+$ and the center well, 0.2 ml of 2 M KOH and a strip of filter paper. The gas phase was pure hydrogen. After preincubation for about 15 min, the reaction was started by tipping and hydrogen uptake was followed manometrically at 30°.

Sedimentation coefficient

Sedimentation analysis was conducted in a Spinco Model E centrifuge at 56100 rev./min. The sample was dissolved in 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA at concentrations of 3.4 to 13.5 mg of protein per ml. The sedimentation coefficient in water at 20° ($s^\circ_{20,w}$) was calculated by extrapolating to zero protein concentration.

The sedimentation coefficient was also estimated by sucrose density gradient centrifugation according to the procedure of MARTIN AND AMES[42]; a swinging bucket rotor of a Hitachi 40P centrifuge was used. A solution of sulfite reductase (0.1–0.2 ml) containing a marker enzyme (crystalline yeast alcohol dehydrogenase or beef-liver catalase) was layered over 4.3 ml of a linear, continuous sucrose gradient, from 5 to 20 % (w/v), in 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA, and centrifuged at 40000 rev./min for 6–15 h. By puncturing the bottom of the tube, 25–33 fractions

(3 drops each) were collected and assayed for enzyme activities and for protein. The sedimentation coefficients of alcohol dehydrogenase and catalase were assumed to be 7.4 S and 11.3 S, respectively[42].

Absorption spectra

Absorption spectra were measured at 25° in a Cary Model 14 spectrophotometer.

Identification and determination of flavins

The flavins were released from purified NADPH-sufite reductase by boiling the preparation, and identified by paper chromatography as described by HUENNEKENS AND FELTON[30]. The released flavins were also tested for their capacity to replace FAD as cofactor for D-amino acid apo-oxidase according to the procedure of NEGELEIN AND BRÖMEL[39]. Quantitative determination of FMN and FAD was performed fluorimetrically as described by BURCH[43]; an Aminco Bowman spectrofluorimeter equipped with a xenon light source was used.

Other analytical procedures

The binding of *p*-chloromercuribenzoate (PCMB) by the sulfhydryl groups of the enzyme protein was determined spectrophotometrically as described by BOYER[44].

For analysis of iron and copper, the enzyme was digested with 0.1 ml of conc. H_2SO_4 and then treated with 0.4–0.6 ml of 30 % hydrogen peroxide. The digest was then neutralized with ammonia. After treatment of the neutralized digest with hydroxylamine hydrochloride to reduce the iron, the ferrous iron was determined colorimetrically with *o*-nitrosoresorcinol monomethyl ether as reagent[45]. Copper content was also determined colorimetrically in the neutralized digest[46].

Protein was determined by the method of LOWRY *et al.*[47].

RESULTS

1. Purification of yeast NADPH-sulfite reductase

As has been reported previously[1–3], yeast NADPH-sulfite reductase was unstable at low ionic strength and was inactivated fairly rapidly at phosphate concentrations lower than 0.2 M. This property of the enzyme had to be taken into consideration in its purification. Potassium phosphate buffers (pH 7.3) containing 1 mM EDTA were used, unless otherwise stated.

Step 1: *Extraction.* Pressed baker's yeast (30 kg) was crumbled, mixed with 1.05 kg of K_2HPO_4 and 3 l of toluene, and stirred for 30 min at 30°. To this mixture were added 1.57 kg of K_2HPO_4 and 30 l of water, and autolysis was further continued for 4.5 h without stirring; the pH was maintained at 8.0–8.3 by occasional addition of 4 M ammonia. All the subsequent steps were conducted at 4°. Finely powdered $(NH_4)_2SO_4$ was added to the autolysate to 30 % saturation (pH 7.0), and insoluble material was removed by filtration.

Step 2: *First ammonium sulfate fractionation.* The filtrate (crude extract) was brought to 50 % saturation (pH 7.0) with $(NH_4)_2SO_4$. The precipitate was collected by filtration and dialyzed for 40 h against 0.3 M buffer.

Step 3: *DEAE-cellulose column chromatography.* The dialyzed solution was divided into 10 portions. Each portion was diluted 3-fold with water, and adsorbed

immediately to a DEAE-cellulose column (7 cm × 35 cm), equilibrated with 0.1 M buffer. The enzyme, which could be recognized by its greenish brown color, was eluted with 0.3 M buffer. This step should be carried out as rapidly as possible to minimize the enzyme inactivation due to dilution. The eluted enzyme solutions from 10 separate runs of chromatography were combined.

Step 4: *Second ammonium sulfate fractionation.* The enzyme in the combined eluates was precipitated at 50 % saturation of $(NH_4)_2SO_4$. The precipitate was washed several times in the centrifuge tube with 40 %-saturated $(NH_4)_2SO_4$ containing 0.3 M buffer (pH 7.0) containing no EDTA, and the washed residue was extracted repeatedly with 35 %-saturated $(NH_4)_2SO_4$ containing 0.3 M buffer (pH 7.0) containing no EDTA. The $(NH_4)_2SO_4$ concentration of the extract was then raised to 50 % saturation, and the resultant precipitate was collected and dissolved in a minimal volume of 0.3 M buffer and dialyzed against the same buffer.

Step 5: *First Sephadex G-200 gel filtration.* The dialyzed solution was divided into 4 portions, and each portion was passed through a Sephadex G-200 column (7 cm × 40 cm) equilibrated with 0.3 M buffer. The eluates containing the sulfite-reducing activity were combined, and made 50 % saturated with respect to $(NH_4)_2SO_4$. The precipitate formed was collected by centrifugation, dissolved in 0.3 M buffer, and dialyzed against the same buffer.

Step 6: *First DEAE-Sephadex A-50 column chromatography.* The dialyzed solution was divided into 3 portions, and the phosphate concentration of each portion was reduced to 0.2 M with 1 mM EDTA. The diluted solution was adsorbed to a DEAE-Sephadex A-50 column (4 cm × 30 cm) equilibrated with 0.2 M buffer. Elution was performed with a linear buffer concentration gradient ranging from 0.2 to 0.5 M. The enzyme, which was eluted at a buffer concentration range of 0.30–0.35 M, was recovered by $(NH_4)_2SO_4$ precipitation, and dialyzed as described above.

Step 7: *Second Sephadex G-200 gel filtration.* The enzyme solution was then passed through a Sephadex G-200 column (5 cm × 55 cm) equilibrated with 0.3 M buffer. The eluates containing the sulfite reductase activity were combined. The enzyme was precipitated by $(NH_4)_2SO_4$ and dialyzed as above.

Steps 8 and 9: *Second and third DEAE-Sephadex A-50 column chromatography.* The preparation was further purified by two cycles of DEAE-Sephadex A-50 column

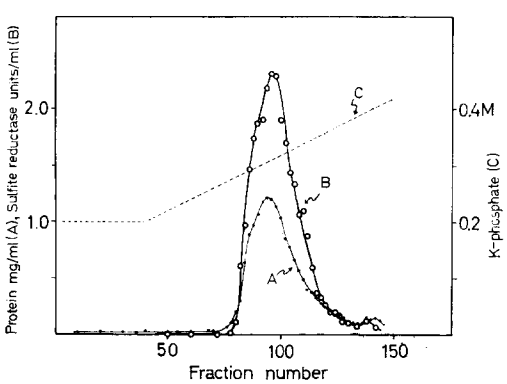


Fig. 1. Elution pattern of NADPH-sulfite reductase in the third DEAE-Sephadex A-50 column chromatography. 388 mg of enzyme protein (1.70 unit/mg) were applied. Flow rate was 0.5 ml per min, and 10-ml fractions were collected.

chromatography, using columns of 4 cm × 30 cm and 4 cm × 28 cm, under conditions similar to those described for the first chromatography. Fig. 1 shows the elution pattern obtained in the third chromatography. The fractions from No. 85 to No. 114 in this pattern were combined and used as purified NADPH-sulfite reductase.

Summary of purification. Table I shows a summary of the purification experiment. The final product (220 mg of protein) represented a 400-fold purification over the crude extract, and the recovery was 12 %. The purified enzyme dissolved in 0.3 M buffer could be stored at −20° for a year without any loss of activity.

TABLE I

A SUMMARY OF PURIFICATION OF NADPH-SULFITE REDUCTASE FROM BAKER'S YEAST

The NADPH-sulfite reductase activity was measured by determining H_2S production up to Step 5; in Steps 6 through 9 it was assayed by the spectrophotometric method. 30 kg of yeast were used as starting material. For detailed procedures, see text.

Purification step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*
1. Crude extract	694 000	3350	0.0048	100
2. 1st $(NH_4)_2SO_4$ (30–50 %)	410 000	2720	0.0066	81
3. DEAE-cellulose	88 000	2470	0.028	74
4. 2nd $(NH_4)_2SO_4$ (35–40 %)	25 400	1970	0.078	59
5. 1st Sephadex G-200	17 100	1630	0.095	49
6. 1st DEAE-Sephadex A-50	2 170	930	0.44	28
7. 2nd Sephadex G-200	1 450	770	0.53	23
8. 2nd DEAE-Sephadex A-50	357	607	1.70	18
9. 3rd DEAE-Sephadex A-50	220	407	1.85	12

2. Characterization of purified enzyme

Homogeneity and sedimentation behavior. As shown in Fig. 2, the purified preparation of yeast NADPH-sulfite reductase was almost homogeneous in centrifugal analysis. The only impurity detected was a minute amount of a slower-sedimenting

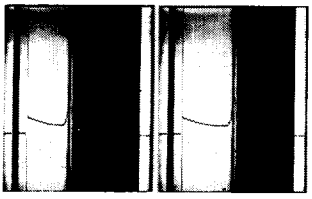


Fig. 2. Ultracentrifugal patterns of purified NADPH-sulfite reductase. 9.0 mg of enzyme protein per ml of 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA. Sedimentation was observed at 56 100 rev./min, and photographs were taken after 38 min (left) and 47 min (right).

substance, and the main component was associated with a greenish yellow color. The sedimentation coefficient ($s°_{20,w}$) was determined to be 14.8 S from a series of centrifugal runs with varying protein concentrations. This sedimentation coefficient was of the same order of magnitude as that (17 S) determined for partially purified *A. nidulans* NADPH-sulfite reductase[16,17] and that (16 S) for *E. coli* enzyme[48]. Electro-

phoresis of the enzyme in a Tiselius-type apparatus also yielded patterns displaying a single boundary.

Fig. 3 shows the result of sucrose density gradient centrifugation of the purified enzyme, in which crystalline beef-liver catalase (11.3 S) was included as marker. In another experiment, yeast alcohol dehydrogenase was used as marker. In both experiments, the peak of protein coincided with that of the NADPH-sulfite reductase activity. Its sedimentation coefficient was estimated to be 14.6 S, a value which was in excellent agreement with that determined above. As described below, this enzyme possessed multiple catalytic activities. Two of these activities, *i.e.* the MVH-sulfite

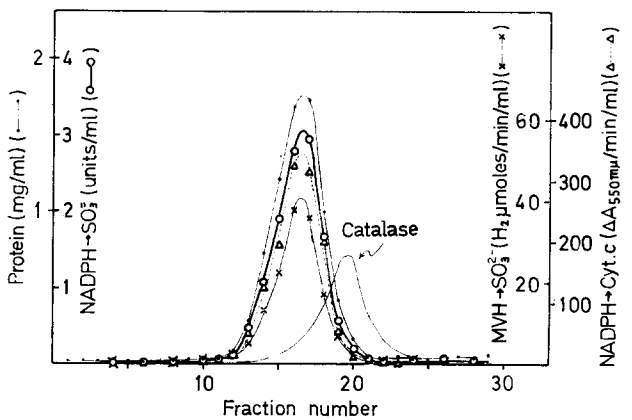


Fig. 3. Sucrose density gradient centrifugation of purified NADPH-sulfite reductase. 0.11 ml of a solution containing 1.35 mg of purified enzyme and 12.8 μg of crystalline catalase in 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA was layered over the sucrose gradient, and centrifuged at 36000 rev./min for 6 h. ●—●, Protein; ○—○, NADPH-sulfite reductase activity; ×—×, MVH-sulfite reductase activity; △—△, NADPH-cytochrome *c* reductase activity.

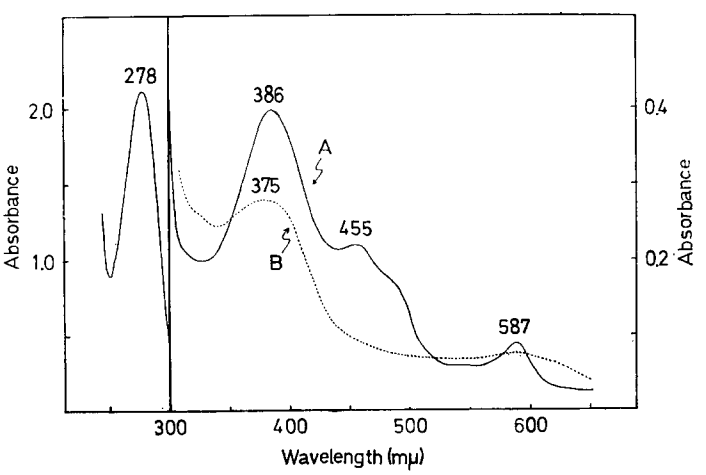


Fig. 4. Absorption spectra of purified NADPH-sulfite reductase. Curve A: 3.38 mg of enzyme protein (specific activity, 1.85) in 2.0 ml of 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA. Curve B: a mixture containing 3.38 mg of enzyme protein, 0.2 μmole of $NADP^+$, 10 μmoles of glucose 6-phosphate, 8 units of glucose-6-phosphate dehydrogenase, 0.3 M potassium phosphate buffer (pH 7.3) and 1 mM EDTA in a final volume of 2.0 ml was incubated anaerobically for 60 min. The reference cell contained all the components except sulfite reductase.

and NADPH-cytochrome *c* reductase activities, were also associated with the protein peak in the density gradient centrifugation.

Absorption spectra. The purified enzyme was greenish yellow in color and showed the absorption spectrum illustrated in Fig. 4. The spectrum had peaks at 386, 455, and 587 mμ and a shoulder at 490 mμ, in addition to an intense protein peak at 278 mμ. The ratio of absorbances at 278, 386, 455, and 587 mμ was 23.8:4.48:2.36:1.00. This spectrum was essentially identical with those reported for partially purified yeast NADPH-sulfite reductase[3], purified *E. coli* NADPH-sulfite reductase[48], and purified *A. nidulans* MVH-sulfite reductase[16,17]. It was also similar to the spectrum of MVH-sulfite reductase from a green plant[25]. The greenish yellow color of the purified enzyme faded to pale green on addition of NADPH or dithionite, both under aerobic and anaerobic conditions. As shown in Fig. 4, this change was mainly due to the disappearance of the 455-mμ peak, suggesting the reduction of a flavin. The treatment also caused decreases in absorbance in the 386- and 587-mμ regions, indicating that the chromophore or chromophores responsible for these peaks were also reducible. The spectrum of the oxidized enzyme was not affected by sulfite.

Nature of flavin prosthetic groups. Since the spectral properties of the enzyme suggested its flavoprotein nature, the purified preparation was analyzed for flavins. Paper chromatography[30] of a boiled supernatant of the enzyme indeed demonstrated the presence of both FMN and FAD. The FAD released into the boiled supernatant was determined by the D-amino acid oxidase method[39] to be 3.05 mμmoles per mg of enzyme protein. On the other hand, fluorimetric determination[45] showed that the enzyme contained 2.84 mμmoles of FMN and 2.84 mμmoles of FAD per mg of protein. Digestion of the boiled enzyme with trypsin did not cause any increment of the flavin liberation. The fluorimetrically determined content of FMN or FAD corresponded to a minimal molecular weight of 350000 for the enzyme. As the sedimentation coefficient of the enzyme (14.8 S) suggested a molecular weight of this order of magnitude, it may be tentatively concluded that yeast sulfite reductase contains one mole each of FMN and FAD per mole of enzyme. As shown in Table II, similar contents of FMN and FAD were also obtained with the enzyme purified from crude extracts prepared by mechanically disrupting the yeast cells with quartz sand (instead of

TABLE II

CONTENTS OF FLAVINS IN YEAST NADPH-SULFITE REDUCTASE PURIFIED BY TWO DIFFERENT PROCEDURES

Preparation A was purified from an autolysate as described in the text. Preparation B was purified from crude extracts obtained by mechanically disrupting the cells as follows: Pressed yeast (460 g) was mixed with 460 g of quartz sand, 40 g of K_2HPO_4, and 8 ml of 0.1 M EDTA, and the mixture was mechanically ground for 4 h at 4°. 450 ml of water were then added, and the mixture was centrifuged at 10000 × *g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 30% saturation, and the resultant precipitate was removed by centrifugation. The preparation thus obtained was then processed exactly as for Preparation A.

Preparation	*NADPH-sulfite reductase activity (units/mg protein)*	*Flavin content (mμmoles/mg protein)*	
		FMN	*FAD*
A	1.85	2.84	2.84
B	1.76	3.02	2.77

autolysis), suggesting that the FMN detected was not an artificial product of FAD formed during autolysis. The NADPH-sulfite reductase activity of the purified enzyme was not stimulated by exogenous flavins, in contrast to the findings reported for sulfite reductases from *E. coli*[5] and *N. crassa*[18]. This lack of response to added flavins indicated that no flavins had been lost during the purification. It seemed certain, therefore, that both FMN and FAD are inherent prosthetic groups of yeast NADPH-sulfite reductase. The two flavins could also be detected in purified *E. coli* NADPH-sulfite reductase[48].

The bleaching of the enzyme by NADPH or dithionite, mentioned above, could thus be accounted for at least partly by the reduction of the flavins. In fact, the decrease in absorbance at 455 mμ reached a level of full reduction of the flavins expected from the fluorimetrically determined contents of FMN and FAD. Evidence for the involvement of both FMN and FAD in the NADPH-sulfite reductase activity will be reported in a later communication.

Nature of non-flavin chromophore(s). The greenish yellow color of the enzyme could thus be explained partly by its flavin content, which seemed to be responsible for the absorption peak at 455 mμ and part of the 386-mμ peak. However, the spectrum showed another peak at 587 mμ, which could not be ascribed to flavin. Furthermore, for a typical flavoprotein the intensity of the peak at 386 mμ was unusually high relative to that at 455 mμ. It was, therefore, likely that a non-flavin chromophore or chromophores were also present.

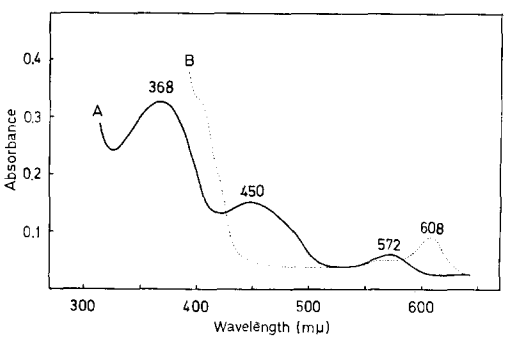


Fig. 5. Effect of alkali on absorption spectra of NADPH-sulfite reductase. Curve A: 0.1 ml of enzyme solution (0.83 mg protein) was added to 0.5 ml of 1 M NaOH. Curve B: a small amount of solid $Na_2S_2O_4$ was added to the alkali-treated enzyme.

Treatment of the enzyme with alkali caused a change in its absorption spectrum; the new spectrum showed peaks at 368, 450, and 572 mμ, as illustrated in Fig. 5. When dithionite was added to the alkali-treated enzyme, the peaks at 450 and 572 mμ disappeared and new peaks at 608 mμ and shoulders at 405, 415 and 560 mμ could be observed. The appearance of a peak at 608 mμ in the reduced form of alkali-treated enzyme and the presence of a peak at 587 mμ in the native, oxidized enzyme suggested that the unknown chromophore might be a heme *a*-like pigment. However, no heme could be detected in the enzyme by the pyridine hemochromogen test.

The peak at 587 mμ was lowered or even abolished by treatment of the enzyme with 3 M urea, 3 M KSCN, 3 M LiCl, or 0.5 % sodium dodecyl sulfate. Treatment of the enzyme at 60° for 50 min or prolonged digestion with a bacterial protease (Na-

garse) also had similar effects. It seemed, therefore, that a special conformation of the enzyme protein is necessary for the appearance of the 587-mμ peak. In a later communication, evidence will be presented for the involvement of the 587-mμ chromophore in both the NADPH- and MVH-sulfite reductase activities.

The purified enzyme was found to contain 14 mμatoms of iron per mg of protein. This corresponded to an iron content of about 5 atoms per mole of enzyme, assuming a molecular weight of 350000 for the enzyme. Although no evidence has yet been obtained, it seems probable that this iron is involved in the non-flavin chromophore. The enzyme did not contain any significant amount of copper; the copper content determined was about 0.5 mμatom per mg of protein or less than 0.2 atom per mole of protein.

Substrate specificity and reactions catalyzed. As reported previously for partially purified sulfite reductases from various sources[3,5,7,22,28,29], the purified yeast enzyme catalyzed not only the reduction of sulfite by NADPH but also the NADPH-linked reductions of nitrite, hydroxylamine, cytochrome *c*, ferricyanide, menadione, ubiquinone Q_0, and DCIP. Even molecular oxygen could serve as acceptor at a very slow rate. Relative rates of the reductions of sulfite, nitrite, hydroxylamine, and cytochrome *c* at their respective optimal concentrations were 1, 3, 4.5, and 13, respectively, when calculated per one-electron transfer. NADH could not replace NADPH in any of these reactions. No oxidation of NADPH could be observed with tetrathionate, cystine, oxidized glutathione, L-methionine sulfoxide, and nitrate as acceptors.

The enzyme also catalyzed the reduction of sulfite by MVH or reduced benzyl viologen; the efficiency of reduced benzyl viologen as electron donor was 14% of that of MVH. It was found further that MVH, continuously generated by hydrogen and hydrogenase, could reduce $NADP^+$ in the presence of purified sulfite reductase, probably by a reversal of electron flow. This reaction proceeded linearly only for a short period of time; its initial activity was about 30 μmoles of hydrogen consumed per min per mg of protein under the conditions described in EXPERIMENTAL PROCEDURE. No reduction of cytochrome *c* could be observed with sulfite, nitrite, or hydroxylamine as electron donor.

DREYFUSS AND MONTY[27] have reported evidence that synthesis of NADPH-sulfite reductase in *S. typhimurium* is controlled by six cistrons and that three of them are also involved in synthesis of the PAPS- and thiosulfate-reducing systems. However, purified yeast NADPH-sulfite reductase could not catalyze the reduction by NADPH of PAPS and thiosulfate, even when the system was fortified with any one or two of the three protein fractions (Enzymes A and B and Fraction C) which have been reported to constitute the PAPS-reducing system[49].

Stoichiometry of NADPH-linked reduction of sulfite. Table III shows the stoichiometry of the NADPH-linked reduction of sulfite by purified yeast sulfite reductase. In these experiments, the enzyme was incubated for 60 min with an excess amount of sulfite and limited amounts of NADPH, and the amount of sulfide produced was correlated with that of NADPH added. As may be seen, the data obtained were in good agreement with the stoichiometry expected from the following equation:

$$3\ NADPH + 3\ H^+ + SO_3^{2-} \rightarrow 3\ NADP^+ + 3\ H_2O + S^{2-}.$$

Effects of pH and substrate concentration. Fig. 6 shows the effects of pH on the NADPH-sulfite, MVH-sulfite, and NADPH-cytochrome *c* reductase activities of the

TABLE III

STOICHIOMETRY OF NADPH-SULFITE REDUCTASE REACTION

The reaction conditions were as described in EXPERIMENTAL PROCEDURE except that the amount of NADPH was varied as indicated and 350 μg of purified NADPH-sulfite reductase were used. The reaction was allowed to proceed at 30° for 60 min, and sulfide formed was determined colorimetrically. The reaction had stopped before the end of incubation.

NADPH added (μmole)	*Sulfide formed* (μmole)	*NADPH/sulfide*
0.943	0.300	3.1
0.471	0.149	3.2

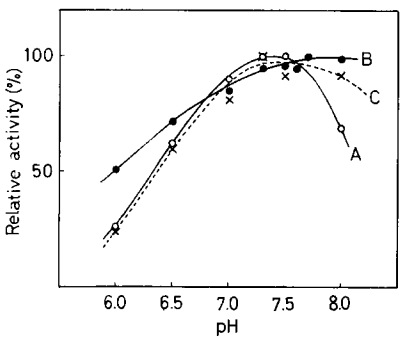


Fig. 6. Effects of pH on enzyme activities. Curve A: NADPH-sulfite. Curve B; MVH-sulfite. Curve C: NADPH-cytochrome *c*.

purified enzyme. While the two NADPH-linked activities were measured in 0.2 M phosphate buffer, 0.02 M buffer was used for the assay of the MVH-sulfite reductase activity. The buffer of low ionic strength was preferred for the latter activity, because the activity in 0.02 M buffer was about 3 times as high as that in 0.2 M buffer; the optimum pH was unaffected by the buffer concentration. The optimal pH values determined were 7.0–7.5 for the NADPH-sulfite activity and 7.5–8.0 for the NADPH-cytochrome *c* activity. The MVH-sulfite activity was highest at pH 7.7 and remained constant up to pH 8.5.

The rates of NADPH-linked reductions of sulfite, nitrite, hydroxylamine, and cytochrome *c* were dependent on the acceptor concentrations, following the general form assumed in the Michaelis–Menten treatment. Sulfite at concentrations higher than 2 mM was, however, inhibitory. The Michaelis constants determined for these four acceptors were 14 μM (sulfite), 1 mM (nitrite), 4.5 mM (hydroxylamine), and 60 μM (cytochrome *c*). Although these values were rather different from those reported for a cruder preparation of the yeast enzyme[3], both data were in agreement in that the Michaelis constant for sulfite was by far the lowest. The Michaelis constant for sulfite and the maximal velocity of the NADPH-sulfite reductase reaction were unaffected by the addition of PAPS, cystine, serine, DL-allocystathionine, and L-methionine.

From the dependence of the activities on the NADPH concentration, the

Michaelis constants for NADPH were determined to be 21 and 18 μM for the reductions of sulfite and cytochrome *c*, respectively.

Inhibition by PCMB and NADP$^+$. Inhibition of yeast NADPH-sulfite reductase by PCMB has already been reported[3]. In the present study, the purified enzyme was found to combine with PCMB. By the spectrophotometric method of BOYER[44] it was estimated that 1 mole of PCMB combines with 66000 g of enzyme protein. This corresponded to the binding of 5–6 moles of PCMB to 1 mole of enzyme, assuming a molecular weight of 350000 for the enzyme. The binding of PCMB led to complete inactivation of all the NADPH-linked activities. Since this inhibition could be reversed by reduced glutathione, the involvement of sulfhydryl groups in the NADPH-linked activities was suggested. The MVH-sulfite reductase activity was not inhibited by PCMB, but non-participation of sulfhydryl groups in the MVH-linked activity could not be concluded, because the hydrogenase preparation employed in the assay contained appreciable amounts of sulfide and the NADPH-linked activities of the PCMB-treated enzyme was restored by incubation with the hydrogenase preparation. The PCMB treatment did not affect the absorption spectrum of the enzyme in the wavelength range from 350 to 650 mμ. The decrease in absorbance at 455 mμ on addition of NADPH was much slower in the PCMB-treated enzyme, though the bleaching finally reached the level attainable with the untreated enzyme.

The NADPH-linked activities were also inhibited by NADP$^+$, though not so strongly as by PCMB. This inhibition was competitive with respect to NADPH. The NADPH oxidation by sulfite under the standard assay conditions was, therefore, slowed down gradually, despite the presence of the reactants at saturating levels. The inhibition constant for NADP$^+$ was determined to be 20 μM, and 0.2 mM NADP$^+$ inhibited the NADPH-sulfite, -nitrite, -hydroxylamine, and -cytochrome *c* reductase activities by 70–80 % under the standard assay conditions.

2′-AMP, at 1 mM, also inhibited the NADPH-sulfite and -cytochrome *c* reductase activities by 40–50 %. Both activities were, however, not significantly affected by 3′-AMP, 5′-AMP, ATP, NAD$^+$, and NADH. On the other hand, the MVH-sulfite reductase activity was not inhibited by NADP$^+$, 2′-AMP, 3′-AMP, and NADPH. The MVH-NADP$^+$ activity was inhibited (47 %) by 0.5 mM NADPH. It was suggested that both the adenine and 2′-phosphate moieties of NADPH were required for its combination with the enzyme.

Interaction with cyanide. Cyanide has been shown to inhibit yeast NADPH-sulfite reductase[3]. In the present study, it was found that this inhibition of the NADPH-sulfite reductase activity was observable only when the reaction was started by the addition of sulfite to the reaction mixture consisting of the enzyme, NADPH, and cyanide (Table IV). The enzyme treated with cyanide in the presence of NADPH was completely inactive not only in the NADPH-sulfite reaction but also in the MVH-sulfite reaction, even after thorough dialysis. On the other hand, the enzyme treated with cyanide in the absence of NADPH could regain these activities after dialysis. The NADPH-nitrite and -hydroxylamine reductase activities were also completely inhibited by treatment of the enzyme with both cyanide and NADPH. Half maximal inhibition of the NADPH-sulfite reductase activity in the presence of 0.15 mM NADPH was attained at a cyanide concentration of about 50 μM, and 1 mM cyanide inhibited the reaction completely.

In contrast to the above-mentioned activities, the NADPH-linked diaphorase-

TABLE IV

EFFECT OF ORDER OF ADDITION OF REACTANTS ON CYANIDE INHIBITION OF NADPH-SULFITE REDUCTASE ACTIVITY

The final reaction mixture contained 1 mM KCN in addition to the components of the standard spectrophotometric assay system (35 μg of purified enzyme). In the control experiments, in which KCN was omitted, the activity was not influenced by the order of addition. To the primary mixture, the first addition was made and, after 1 min, the second addition was made.

Primary mixture	*First addition*	*Second addition*	% *Inhibition*
Enzyme + NADPH	KCN	Sulfite	100
Enzyme + sulfite	KCN	NADPH	4
Enzyme + NADPH	Sulfite	KCN	4
Enzyme + KCN	NADPH	Sulfite	100
Enzyme + KCN	Sulfite	NADPH	12
NADPH + KCN	Sulfite	Enzyme	5
NADPH + KCN	Enzyme	Sulfite	100
Sulfite + KCN	Enzyme	NADPH	13

like activities using cytochrome *c*, ferricyanide, quinones, *etc.* as acceptors, as well as the MVH-$NADP^+$ reductase activity, were inhibited by cyanide to a much lesser extent both in the presence and absence of NADPH. For example, in the enzyme which had been treated with 1 mM KCN and 0.15 mM NADPH and then dialyzed against 0.3 M phosphate buffer (pH 7.3), the NADPH-cytochrome *c* and MVH-$NADP^+$ reductase activities were inhibited only by 20 and 32 %, respectively, as compared to the 100 and 94 % inhibitions of the NADPH-sulfite and MVH-sulfite reductase activities, respectively. These weak inhibitions of the diaphorase-like activities, except for the NADPH-cytochrome *c* reductase activity, were almost completely prevented when the acceptor was added prior to cyanide, as in the case of the strong inhibition of the NADPH-sulfite reductase activity. The reason for the exceptional behavior of cytochrome *c* is not yet clear.

The addition of cyanide to the enzyme in the absence of NADPH caused no changes in the absorption spectrum. However, when cyanide was added to the NADPH-treated enzyme, the color of the solution turned from pale green to reddish violet, and simultaneously two new peaks emerged at 411 and 397 mμ, as shown in Fig. 7. On dialysis of this cyanide-treated preparation under aerobic conditions, the color again became greenish yellow. This form of the enzyme, though similar in color to the native, oxidized form, was inactive in the NADPH- and MVH-sulfite reductase activities. The spectrum of this form of the enzyme (Fig. 7, Curve D) showed a peak at 578 mμ (but not 587 mμ) and an asymmetric peak at about 390 mμ, in addition to the typical flavin peak at 455 mμ. All these peaks disappeared again on addition of NADPH and the reddish violet color was restored concomitant with the appearance of the peaks at 411 and 397 mμ. These observations suggested the occurrence of the following series of reactions:

$$\underset{\text{greenish yellow}}{\text{Enz(ox)}} \underset{\text{sulfite, oxygen}}{\overset{\text{NADPH}}{\rightleftharpoons}} \underset{\text{pale green}}{\text{Enz(red)}} \xrightarrow{\text{KCN}} \underset{\text{reddish violet}}{\text{Enz(red)-CN}} \underset{\text{NADPH}}{\overset{\text{oxygen}}{\rightleftharpoons}} \underset{\text{greenish yellow}}{\text{Enz(ox)-CN}}$$

Here, Enz(ox) and Enz(red) represent the oxidized and reduced forms of the enzyme, respectively.

The conversion of the reduced enzyme, Enz(red), to the reddish violet form, Enz(red)-CN, in the presence of 1 mM KCN was prevented completely by the addition of 1 mM sulfite, which also prevented the inactivation of the NADPH-sulfite reductase activity. This effect of sulfite was less pronounced as the sulfite concentration was decreased, suggesting that sulfite inhibited competitively the cyanide binding by the reduced enzyme. The conversion to the Enz(red)-CN form seemed to be irreversible; once converted to this form, even an excess of sulfite could not reconvert it to the Enz(red) form and restore the NADPH-sulfite reductase activity.

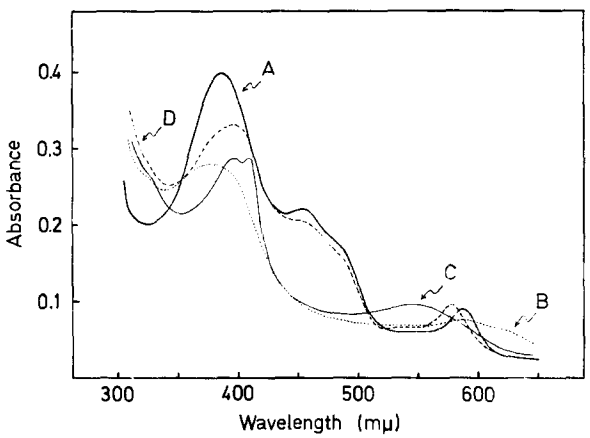


Fig. 7. Effects of KCN on absorption spectra of NADPH-sulfite reductase. Curve A: spectrum of oxidized enzyme (same as Curve A of Fig. 4). Curve B: spectrum of NADPH-reduced enzyme (same as Curve B of Fig. 4) Curve C: spectrum of KCN-treated, reduced enzyme (1 mM KCN was added to the NADPH-reduced enzyme and incubated anaerobically for 5 min). Curve D: KCN-treated enzyme after dialysis (the sample treated as in Curve C was dialyzed aerobically for 24 h against 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA; correction was made for dilution by dialysis).

These properties of the cyanide–enzyme interaction were employed to determine the time course of cyanide inactivation of the NADPH-sulfite reductase activity. Thus, the enzyme was added to a mixture of NADPH and KCN to start the inactivation reaction. After incubation for a desired period of time, an excess of sulfite was added to stop the inactivation reaction and to initiate the NADPH-sulfite reductase reaction. Assuming that the inactivation-preventing effect of sulfite is instantaneous, the NADPH-sulfite reductase activity thus measured could be regarded as proportional to the enzyme remaining in the form of Enz(red) at the time of sulfite addition. The result of this experiment is illustrated in Fig. 8. As may be seen, the inactivation in the presence of 1 mM KCN and 0.15 mM NADPH at pH 7.3 (25°) took place according to first-order kinetics with a velocity constant of 0.2 sec^{-1}. The inactivation under these conditions was complete after 30 sec of incubation.

When the MVH-sulfite reductase activity of the enzyme treated with cyanide and NADPH was assayed under the standard conditions after removal of cyanide and NADPH by dialysis, the rate of initial hydrogen uptake was almost negligible.

However, the rate increased slowly with time. An adequate explanation for this finding is not yet available.

ASADA, TAMURA AND BANDURSKI[26] have reported that both cyanide and PCMB are inhibitory to the MVH-sulfite reductase activity of an enzyme from spinach leaves and that this enzyme binds cyanide and PCMB only after the reduction.

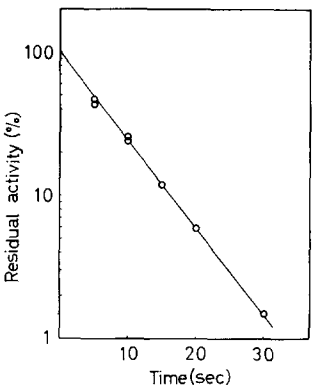


Fig. 8. Time course of KCN inactivation of the NADPH-sulfite reductase activity. 35 μg of purified enzyme were added to a mixture (final vol., 1.50 ml) containing 300 μmoles of potassium phosphate buffer (pH 7.3), 1.5 μmoles of EDTA, 0.23 μmole of NADPH, and 1.5 μmoles of KCN. After incubation at 25° for an indicated period of time, 1.5 μmoles of Na_2SO_3 were added to arrest the inactivation and at the same time to start the reaction. The activity thus measured was regarded as that remaining at the time of sulfite addition (residual activity).

Competition between substrates. In agreement with previous findings[4,13], the addition of nitrite and hydroxylamine to the enzyme resulted in competitive inhibition of the NADPH-cytochrome *c* reductase activity measured at 550 mμ. The inhibition constants for nitrite and hydroxylamine were estimated to be 1.5 and 4 mM, respectively. These values were in good agreement with the Michaelis constants of 1 and 2.5 mM determined above for nitrite and hydroxylamine, respectively. Sulfite was not appreciably inhibitory to the cytochrome *c* reduction, probably because this reaction was much faster than the sulfite reduction in the standard assay system for cytochrome *c* reduction. Table V, however, shows that sulfite inhibited competi-

TABLE V

COMPETITIVE INHIBITION OF NADPH-NITRITE AND -HYDROXYLAMINE REDUCTASE ACTIVITIES BY SULFITE

The reaction was followed spectrophotometrically under the standard assay conditions, using 72 μg of purified enzyme per vessel, except that the indicated acceptors were used.

Acceptors	*Concentration (mM)*	$\Delta A_{340\,m\mu}/min$
Na_2SO_3	1	0.377
$NaNO_2$	1	0.560
NH_2OH	1	0.338
$Na_2SO_3 + NaNO_2$	1 + 1	0.430
$Na_2SO_3 + NH_2OH$	1 + 1	0.333

tively the reductions of nitrite and hydroxylamine, as reported previously for the enzymes from yeast[3], *S. typhimurium*[13], and *A. nidulans*[16].

Effects of other inhibitors. Arsenite inhibited the NADPH-sulfite reductase activity progressively, but not the NADPH-cytochrome *c* reaction. The MVH-sulfite reductase activity was also inhibited by arsenite. Sulfide had some inhibitory effect on the NADPH-sulfite reductase activity. No inhibition of the NADPH-sulfite reductase activity was observed after preincubation for 20 min with 0.01 M Tiron, 0.01 M sulfosalicylate, 3.3 mM thiourea, 0.033 M diethyl dithiocarbamate, 0.01 M α,α'-dipyridyl, 3.3 mM *o*-phenanthroline, 1.7 mM cystine, 5 μM dicumarol, 1 mM amytal, Antimycin A (6.7 μg/ml), and rotenone (10 μg/ml).

DISCUSSION

Although enzymes catalyzing the reduction of sulfite to sulfide have been studied in a variety of organisms, none of them has been obtained in a sufficiently purified state to permit its detailed characterization. The present work has provided, for the first time, an almost homogeneous preparation of NADPH-sulfite reductase from baker's yeast. The purified enzyme has a sedimentation coefficient of 14.8 S, and a minimal molecular weight of 350000 has been estimated from its flavin content.

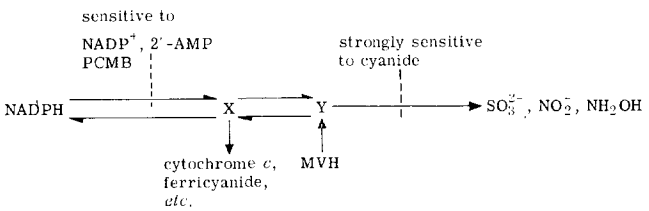
The enzyme contains one mole each of FMN and FAD per 350000 g of protein. The presence of two different flavins in a single protein is rather unusual, though crystalline dihydroorotate dehydrogenase (EC 1.3.3.1) from *Zymobacterium oroticum* has been shown to contain one mole each of FMN and FAD, together with 2 atoms of iron, per mole of enzyme[50]. Partially purified NADH oxidase from *Clostridium perfringens* has also been shown to contain FAD and an unidentified flavin[51].

In addition to the flavins, yeast sulfite reductase also contains a non-flavin chromophore (or chromophores) which is responsible for the absorption peak at 587 mμ and also contributes to the unusually intense absorption in the 386-mμ region. This chromophore, tentatively called "587-mμ chromophore", is not a heme *a*-like pigment. The possibility that it is a bile pigment or other heme degradation product has not yet been checked experimentally. The absorption peak at 587 mμ is lowered or even abolished by various treatments which cause protein denaturation, suggesting that a special conformation of the enzyme protein is required for the integrity of the chromophore. Although it is likely that non-heme iron, which is present in the enzyme at a concentration of 5 atoms per 350000 g of protein, is a component of this chromophore, no experimental support for this view is as yet available.

Sulfite reductases from *D. gigas*[22] and *C. nigrificans*[23] have been shown to utilize reduced ferredoxin as electron donors for sulfite reduction. These findings, together with the presence of non-heme iron in the yeast enzyme, suggest the possibility that yeast sulfite reductase may contain a built-in ferredoxin-type pigment. However, the absorption spectrum of the yeast enzyme suffers no change on treatment with PCMB, a treatment which causes decolorization of ferredoxin[52]. It is, therefore, likely that the yeast enzyme is free from so-called "labile sulfide", which is characteristic of ferredoxin[52]. The presence in yeast sulfite reductase of a rubredoxin-type pigment is another possibility to be studied further, because rubredoxin lacks labile sulfide[53] and the spectrum of the yeast enzyme is similar in some respects to those of rubredoxin and ferredoxin.

Like sulfite reductases from other sources, the yeast enzyme possesses multiple catalytic activities. The reactions catalyzed by this enzyme may be classified into the following four groups: (1) reductions of sulfite, nitrite and hydroxylamine by NADPH; (2) diaphorase-like reactions in which cytochrome *c*, ferricyanide, quinones and DCIP are reduced by NADPH; (3) reduction of sulfite by MVH or reduced benzyl viologen; and (4) reduction of $NADP^+$ by MVH. Of these reactions, all the NADPH-linked activities (Groups 1 and 2) are sensitive to PCMB, $NADP^+$ and 2'-AMP. While Group 1 reactions are strongly inhibited by cyanide, those belonging to Group 2 are only weakly sensitive to cyanide. It seems that the site at which electrons are passed to sulfite, nitrite, and hydroxylamine is different from the site where cytochrome *c*, ferricyanide, *etc.* interact with the enzyme. In contrast to the NADPH-linked activities, the reduction of sulfite by MVH (Group 3) is not affected by $NADP^+$ and 2'-AMP; it is not yet certain if this reaction is sensitive to PCMB. This indicates that NADPH and MVH are interacting with the enzyme at different sites. The reduction of $NADP^+$ by MVH (Group 4) is insensitive to $NADP^+$ and 2'-AMP, but is inhibited by NADPH. This reaction seems to involve a reversal of electron flow.

These findings suggest the operation of a rather complicated intramolecular electron-transfer pathway consisting of several carriers or reactive sites. This pathway may tentatively be expressed by the following scheme:



X and Y are hypothetical electron carriers. This mechanism is similar to those proposed for partially purified sulfite reductases from yeast[3] and *A. nidulans*[16]. SIEGEL AND MONTY[13] have also concluded that in *S. typhimurium* sulfite reductase an intermediate carrier participates in the NADPH-sulfite reductase reaction and this intermediate is able to pass electrons either directly to cytochrome *c* or indirectly to sulfite or hydroxylamine. However, no information has so far been obtained concerning the nature of the postulated intermediate carriers.

From the discovery of both FMN and FAD in yeast sulfite reductase, it is likely that these flavins are functioning as the intermediate electron carriers. In fact, both flavins are fully reducible by NADPH. It does not seem unreasonable to assume that either FMN or FAD or both are involved in the electron transfer from NADPH to cytochrome *c*, ferricyanide, *etc*, in view of the diaphorase-like nature of these reactions. Detailed evidence for the participation of both flavins in the catalytic activities and their sequence in the electron-transfer pathway will be reported in a later communication.

The 587-mμ chromophore is also reducible by NADPH. This chromophore seems to be involved in the reduction of sulfite (and also of nitrite and hydroxylamine) by NADPH as well as the reduction of sulfite by MVH. These reactions, unlike the

diaphorase-like reactions, are strongly inactivated when cyanide is added to the reduced enzyme. This inactivation is accompanied by the appearance of a new absorption peak at 578 mμ, which is only about 10 mμ apart from the 587-mμ peak observable in the oxidized enzyme. It may, therefore, be concluded that the 587-mμ chromophore, when it is in the reduced state, combines irreversibly with cyanide, leading to the inactivation of the above-mentioned activities. The cyanide binding to the reduced chromophore as well as the cyanide-induced inactivation can be prevented by sulfite in apparent competition with cyanide. This finding suggests that the 587-mμ chromophore is the site where sulfite, and probably also nitrite and hydroxylamine, combines with the enzyme. In a later publication the function of this chromophore will be dealt with in more detail.

Working with partially purified nitrite reductase (sulfite reductase), KEMP *et al.*[10] have reported that cyanide inhibits reversibly the reduction of nitrite and sulfite. With the present enzyme, however, cyanide causes irreversible inactivation of the sulfite-, nitrite-, and hydroxylamine-reducing activities, and even the addition of an excess of sulfite to the inactivated enzyme does not restore these activites. The same results have also been obtained with spinach sulfite reductase[26].

Cyanide has a common property with sulfite and hydroxylamine in that it combines chemically with carbonyl groups. This fact, together with the report that *N. crassa* hydroxylamine reductase is strongly inhibited by sulfite and cyanide[54], may suggest that a carbonyl group is the site of interaction of sulfite in the yeast enzyme. However, nitrite is not a carbonyl reagent, and our unpublished experiments showed that the NADPH-sulfite reductase activity of the yeast enzyme was not inhibited by preincubation with sufficient concentrations of hydrazine and phenylhydrazine.

Two different FMN-containing NADPH-cytochrome *c* reductases have so far been isolated from yeast, *i.e.*, "old yellow enzyme" of WARBURG AND CHRISTIAN[55] and NADPH-cytochrome *c* reductase of HAAS, HORECKER AND HOGNESS[56]. Furthermore, an FAD-containing enzyme called "new yellow enzyme" has also been prepared from yeast[57]. The FMN-containing enzyme of HAAS, HORECKER AND HOGNESS[56] is fairly unstable to dialysis against water. However, none of these enzymes shows the absorption peak at 587 mμ. Therefore, yeast NADPH-sulfite reductase, though possessing the NADPH-cytochrome *c* reductase activity, appears to differ from the above-mentioned enzymes. The possibility, however, still exists that these NADPH-linked enzymes are derived from the sulfite reductase during isolation and purification.

Finally, it should be mentioned that an entirely different type of sulfite reductase has been isolated from *D. desulfuricans*, an organism which uses sulfate and sulfite as the oxidants in energy metabolism[21]. Although the nature of this system has not yet been elucidated, evidence has been presented that this system consists of at least four proteins[21].

ADDENDUM

After completion of the manuscript, we were informed of the work by KAMIN *et al.*[58], who purified NADPH-sulfite reductase from *E. coli*. This enzyme was reported to have a molecular weight of 700000 and contain 7–8 moles of flavins per mole of

enzyme (FAD and FMN in equal quantities) and 12–16 atoms of iron per mole. It was also reported that a heme-like pigment and labile sulfur are present in the purified enzyme.

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