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SULPHITE REDUCTASE FROM BAKERS' YEAST: A HAEMOFLAVOPROTEIN

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SUMMARY

1. The NADPH-linked sulphite reductase (EC 1.8.1.2) has been purified 300-fold.

2. The stoichiometry for NADPH/S^{2-} is 3:1 and for SO_3^{2-}/S^{2-} 1:1.

3. Inhibition studies indicate a requirement for thiol groups, flavin and iron for enzyme activity. A CO inhibition which was reversed by light, suggests the participation of a haemprotein.

4. The enzyme is stimulated by riboflavin, FMN or FAD and the K_m values are 4.6, 6.3 and $8.3 \cdot 10^{-5}$ M, respectively.

5. The participation of flavin and haemprotein for the reaction is further confirmed by a difference spectrum obtained by adding sulphite to the enzyme. Difference spectra of oxidized *versus* reduced enzyme are typical of a haemoflavoprotein. Moreover, in a difference spectrum reduced *versus* reduced *plus* CO, a Soret peak (416 mμ) and trough at 432 mμ, is similar to that of cytochrome *o*.

6. During enzyme action, gaseous products were not detected in a mass spectrometer, nor were any free sulphur intermediates found when [^{35}S]sulphite was used.

7. A spectrophotometric method for determining SO_3^{2-} using 5,5'-dithiobis-(2-nitrobenzoic acid) has been developed.

8. A model for the electron transfer sequence for sulphite reductase is presented

INTRODUCTION

Sulphate is reduced to thiols by microorganisms and plants, prior to their incorporation into amino acids and proteins. The enzyme, sulphite reductase (EC 1.8.1.2), catalyses the 6 electron reduction of SO_3^{2-} to S^{2-} provided a suitable hydrogen donor is added. Although the enzyme has been found in a variety of microorganisms[1-8] and in plants[9,10], it has been purified from a few of them only[11-16].

Sulphite reductase from yeast or bacteria utilize either NADPH[1,5,16-20] or reduced methyl viologen[2,12,20] as an electron donor, in contrast to the plant enzyme which utilizes the reduced dye only[13-16].

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene; PPO, 2,5-diphenyloxazole; PCMB, *p*-chloromercuribenzoate; PAPS, 3'-phosphoryl-5'-adenosinophosphosulphate; DCIP, 2,6-dichlorophenolindophenol.

Although WAINWRIGHT[6] had earlier reported that the NADPH-linked sulphite reductase in yeast is composed of 6 protein fractions *viz.* α, β, γ, *etc.*, he[21] subsequently found that the enzyme corresponds to a single α-protein. Meanwhile NAIKI[20] purified the enzyme from bakers' yeast and found that it contained two protein moieties *viz.* an NADPH–dye reducing system coupled to the reduced methyl viologen sulphite reducing component. Although a flavin requirement has been reported for a purified enzyme from bakers' yeast[22], the enzyme has not been fully characterized.

MATERIALS AND METHODS

Organism

Bakers' yeast was obtained locally from Mauri Bros. and Thompson, Adelaide, South Australia.

Reagents

$NADP^+$, NADPH, DTNB glucose-6-phosphate dehydrogenase (A-grade) and 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) were obtained from Calbiochem, Calif., U.S.A.; glucose 6-phosphate, FMN and GSH were from Sigma Chemical Co., St. Louis, U.S.A.; riboflavin was supplied by British Drug Houses, Poole, England. Sephadex G-25 was purchased from Pharmacia, Sweden; DEAE-cellulose (DE 11) was from Whatman Co., England and ^{35}S-labelled sodium sulphite (specific activity 2.2 mC/mmole) was purchased from Radiochemical Centre, Amersham, England; the 2,5-diphenyloxazole (PPO) was from Nuclear-Chicago, Des Plains, Ill., U.S.A.; carbon monoxide was obtained from Townson and Mercer (Dist.) Pty., Adelaide.

All inorganic reagents were of analytical grade.

Assay of sulphite reductase activity

The reaction mixture, contained in addition to the enzyme, the following reagents in μmoles, in a final volume of 3 ml: phosphate buffer (pH 7.4), 600; $MgCl_2$, 3; Na_2SO_3, 3; $NADP^+$, 0.3; glucose 6-phosphate, 5; and glucose-6-phosphate dehydrogenase, 0.5 (I.U.) to generate NADPH.

The reaction mixtures were incubated for 40 min at 30°, in 1 cm × 10 cm test tubes, covered with rubber caps and the enzymically produced sulphide was determined according to SIEGEL[23], except that 0.3 ml each of the colour reagents (*N*,*N*-dimethyl-*p*-phenylene diamine sulphate and ferric chloride), were injected through the rubber caps.

The calibration graph prepared with standards of $Na_2S \cdot 9\ H_2O$ in the phosphate buffer was linear over the range, 10–200 mμmoles.

Protein was determined by the Folin method as modified by COWGILL AND PARDEE[24], with bovine serum albumin as a standard.

The unit of enzyme activity is defined as μmoles sulphide produced per ml of enzyme in 40 min at 30° and the specific activity as these units per mg protein.

Purification

NAIKI's[20] method has been modified to attain a 300-fold purification as follows: All operations were carried out a 2° using 0.2 M potassium phosphate buffer (pH 7.4) and centrifuging was done at 30000 × *g* for 20 min. Enzyme fractions were dialysed in Visking cellulose tubing, against the same buffer.

Yeast cells suspended in an equal volume of the buffer (w/v) were broken in an Aminco French pressure cell (20000 lb/inch^2). The homogenate was centrifuged and then dialyzed (Fraction I). The enzyme protein was collected between 38% and 48% saturation of ammonium sulphate, dissolved in and dialyzed against the buffer (Fraction II). The resulting fraction, diluted to a final phosphate concentration of 0.15 M was applied to a DEAE-cellulose column and a stepwise elution was made with 0.15 M and 0.25 M buffer. The enzyme was eluted finally with 0.25 M buffer and was concentrated by ammonium sulphate fractionation (0–60% saturation) and was then dialyzed (Fraction III). Fraction III was applied to another DEAE-cellulose column and this time a linear gradient elution was made with 200 ml each of 0.1 M and 0.3 M buffer, collecting 5-ml fractions. The enzyme was eluted between 0.22 M and 0.25 M buffer and the active portions were pooled (Fraction IV). Finally Fraction IV was further purified and was concentrated to about one-tenth of its volume by adding dry Sephadex G-25 beads and filtering the supernatant fraction through Whatman No. 1 paper under mild suction. This process was repeated 3 times (Fraction V). The dimensions of the DEAE-cellulose column were 2.2 cm × 33 cm.

Sulphite determination

Sulphite was determined by the following colorimetric method: 0.1 ml 1% (w/v) 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)[25] in 0.1 M phosphate buffer (pH 7.0) was added to a series of samples containing 20–200 mμmoles sulphite, in a final volume of 3.0 ml and mixed well. The colour which developed after 5 min was determined at 412 mμ in a spectrophotometer. Sulphate did not interfere even at 10^{-2} M. The interference due to reduced sulphur compounds or flavin is negligible if their concentration is kept below 10^{-5} M. The DTNB-reagent is stable as is the colour produced with sulphite in contrast to the GRANT's basic fuchsin method[26].

Residual sulphite was determined in open tubes. The reaction was stopped by adding 2 ml absolute alcohol, and the precipitated protein was centrifuged. A suitable portion of the supernatant fraction was used for the sulphite determination. The results are comparable with those obtained with the GRANT's method[26]. The sensitivity of the method can be increased by decreasing the final volume.

Inhibitor and kinetic studies

Studies with inhibitors and the determination of K_m values for flavin compounds were done with the Unicam SP 700 recording spectrophotometer by following the oxidation of NADPH at 340 mμ.

Carbon monoxide inhibition and light reversibility studies were carried out as follows: about 5 mg of NADPH were added to 0.1 mg of the purified enzyme (Fraction V) in 1 ml phosphate buffer (pH 7.4). CO was bubbled through the solution for 3 min at 4°. Portions of this pretreated enzyme were used to assay S^{2-} produced under assay conditions given in METHODS except that 0.3 μmole of NADPH was used without the NADPH-generating system. The CO-treated enzyme in a duplicate sample of the reaction mixture was exposed to a bright tungsten lamp for 10 min.

Spectral studies

All the spectra were determined in the Unicam SP 800 spectrophotometer, using an expansion recorder (Servoscribe type RE 511) at 20 mV.

Mass spectrometry

The enzyme mixture was incubated either in Rittenberg tubes or in a double arm Warburg flask. Sulphite solution was kept in one arm and the reaction mixture was in the other limb of the Rittenberg tube or when a Warburg flask was used, the flask contained the reaction mixture and the sidearm the sulphite. After the Rittenberg tubes or the Warburg flasks were rigorously evacuated to 10^{-2} mm Hg, the reaction was started by adding the sulphite solution. The products of the reaction were passed through a solid CO_2 Dewar trap to freeze out moisture and then into a mass spectrometer (AEI MS-2). Mass peak readings were recorded at intervals (0–5 min) for 30 min. When the Warburg flask was used, the reaction was terminated after 5 min by adding 0.3 ml of a mixture of 20% (w/v) $HgCl_2$ and 6% (w/v) $ZnCl_2$ from the second sidearm.

Experiments with [^{35}S]sulphite

The reaction mixture in a final volume of 0.2 ml, contained: 0.3 μmole of NADPH, 50 mμC of $Na_2{}^{35}SO_3$ labelling 22 mμmoles Na_2SO_3, and 20 milliunits of enzyme (Fraction III). These reagents were dissolved in 0.2 M phosphate buffer (pH 7.4). The samples were incubated at 30° for 5, 10, 15 and 20 min, respectively. Then 0.02 ml of each sample was spotted on a 15 cm × 58 cm DEAE-cellulose (DE 81) paper which was first dipped in 0.1 M citrate buffer (pH 5.0) and then dried. The electrophoresis was carried out immediately at 1.5 kV for 70 min using the citrate buffer and a carbon tetrachloride solvent cooling system.

After the electrophoresis, the paper was cut into 2.5 cm × 2.0 cm strips and the radioactivity was counted in a Packard Tri-carb spectrometer (model 3375) using a scintillation fluid, with toluene base, containing PPO and POPOP. When iodine was used in the enzyme mixtures, a small crystal of iodine was dissolved in the duplicate sample before spotting onto the DEAE-paper.

RESULTS

The results in Table I show that the specific activity of the enzyme was increased 300-fold by ammonium sulphate fractionation, DEAE and Sephadex procedures.

The enzyme is stable at −15° for at least 4 weeks.

Stoichiometry

When 300 milliunits of the purified enzyme which normally produces 300 mμmoles of S^{2-} in the standard assay system (see METHODS), were incubated with 300 mμmoles NADPH, without the generating system, the reduced nucleotide was completely oxidized within 40 min as measured at 340 mμ. Under these conditions, the amounts of $SO_3{}^{2-}$ utilized and the S^{2-} formed were about 100 mμmoles each; thus giving a stoichiometry of NADPH/S^{2-} 3:1 and $SO_3{}^{2-}/S^{2-}$ 1:1 (Table II).

Inhibitory studies

Mepacrine and *p*-chloromercuribenzoate (PCMB) each at 10^{-1} M inhibited the enzyme activity as shown in Table III and these effects were reversed by $4 \cdot 10^{-4}$ M FMN and GSH, respectively, indicating a requirement for thiol groups and flavin for

TABLE I

PURIFICATION OF SULPHITE REDUCTASE

Phosphate buffer (pH 7.4) was used throughout.

Fraction	*Total protein (mg)*	*Total units (μmoles of S^{2-} produced in 40 min)*	*Specific activity (units/mg protein)*	*Fold purification*	*Recovery (%)*
I. Cell free extract (dialysed against the buffer)	23000	284	0.012	—	100
II. The enzyme protein collected between 38 and 48% $(NH_4)_2SO_4$ saturation and dialysed	2095	176	0.084	7	62
III. Fraction II applied to a DEAE-cellulose column. Stepwise elution with 0.15 M and 0.25 M buffers. Enzyme eluted with 0.25 M buffer. Concentrated by $(NH_4)_2SO_4$ fractionation (0–60% saturation) and dialysed	180	159	0.89	73	56
IV. Fraction III applied to another DEAE-cellulose column. Linear gradient elution made with 200 ml of 0.1 M and 0.3 M buffers respectively	37	85	2.30	192	30
V. Dry Sephadex G-25 beads added to Fraction IV. Activity of the supernatant fraction filtered under suction	12	44	3.67	306	15.5

TABLE II

STOICHIOMETRY OF SULPHITE REDUCTION

Sulphite and sulphide were determined as described in the METHODS (0.3 unit of the 300-fold purified enzyme fraction used in the assay). The reaction was conducted for 40 min.

NADPH oxidized (mμmoles)	*Sulphite utilized (mμmoles)*	*Sulphide formed (mμmoles)*	*Ratio*	
			$NADPH/S^{2-}$	*SO_3^{2-}/S^{2-}*
300	107	106	2.9	1.0
300	112	102	2.9	1.1

enzyme activity. The PCMB inhibition was observed within 10 sec (mixing time), after finally starting the reduction reaction by adding SO_3^{2-}, NADPH or enzyme to the remainder of the reaction mixture.

The enzyme was also inhibited by metal chelating agents including 8-hydroxyquinoline, α,α′-dipyridyl, *o*-phenanthroline, and KCNS.

Carbon monoxide inhibited the enzyme after it was first reduced with excess of NADPH. The sulphide produced was taken as measure of enzyme activity. This effect was reversed by exposing the inhibited enzyme to a bright tungsten light.

TABLE III

EFFECT OF INHIBITORS ON SULPHITE REDUCTASE

$NADPH–SO_3^{2-}$ reducing activity was assayed by following the oxidation of NADPH at 340 mμ in a Unicam SP 700 recording spectrophotometer. Reaction was started by adding 0.1 ml containing 3 μmoles of Na_2SO_3 solution to 600 μmoles phosphate buffer (pH 7.4), 0.3 μmole NADPH, enzyme (Fraction III) and inhibitor contained in 2.9 ml in a 1-cm quartz cuvette.

Inhibitor	*Final concentration (M)*	*Inhibition (%)*
PCMB*	10^{-4}	100
Mepacrine**	10^{-4}	75
8-Hydroxyquinoline	10^{-3}	70
α,α'-Dipyridyl	$5 \cdot 10^{-3}$	56
o-Phenanthroline	$3 \cdot 10^{-3}$	50
KCNS	$5 \cdot 10^{-3}$	37
Quinine sulphate	10^{-4}	30
Antimycin A	10^{-4}	5
Sodium azide	10^{-3}	0
Amytal	10^{-3}	0

* Inhibition reversed with $4 \cdot 10^{-4}$ M GSH.
** Inhibition reversed with $4 \cdot 10^{-4}$ M FMN.

Flavin requirement

Riboflavin, FMN and FAD stimulated the activity of the purified enzyme when determined by the NADPH-oxidation method (Fig. 1).

The K_m values for the flavin compounds were determined as 4.6, 6.3 and $8.3 \cdot 10^{-5}$ M, respectively, for riboflavin, FMN and FAD, showing a somewhat smaller dependence of rate on flavin concentration with riboflavin than with FMN and FAD (Fig. 1).

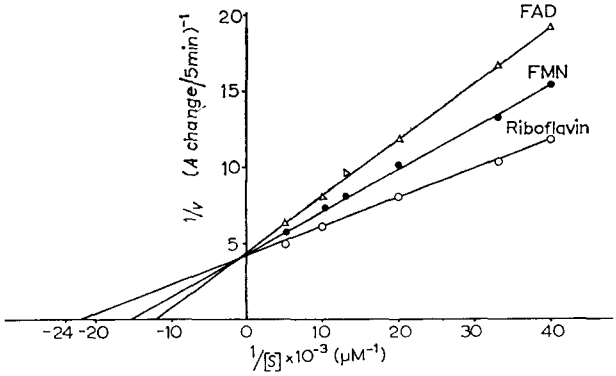

Fig. 1. K_m values for FAD, FMN and riboflavin in the $NADPH–SO_3^{2-}$ reducing system (Fraction V). The oxidation of NADPH was followed at 340 mμ in a Unicam SP 700 recording spectrophotometer. The reaction mixture contained 600 μmoles phosphate buffer (pH 7.4); NADPH, 0.3 μmole; Na_2SO_3, 3 μmoles; enzyme 25 milliunits and varying amounts of added FAD, FMN or riboflavin, in 3 ml in a 1-cm quartz cuvette. The increased rate of NADPH-oxidation with added flavin (S) after correcting for enzyme activity without added flavin is plotted according to the method of Lineweaver and Burk. The enhanced rate observed did not result from a chemical reaction, since NADPH-oxidation did not occur when the enzyme was either omitted or boiled.

The addition of ferredoxin prepared from *Clostridium pasteurianum*[27] did not have any effect on the enzyme.

Difference spectra

After obtaining a straight base line with the 70-fold purified enzyme in both the cuvettes, the addition of a crystal of $Na_2S_2O_4$ to the sample cuvette resulted in a pronounced peak at 428 mμ and comparatively small ones at 558 and 589 mμ in addition to a broad trough around 458 mμ (Fig. 2).

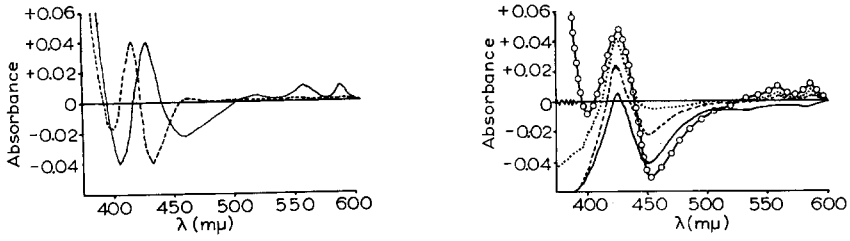

Fig. 2. Difference spectra for (a) oxidized *versus* $Na_2S_2O_4$ reduced and (b) $Na_2S_2O_4$ reduced *versus* $Na_2S_2O_4$ reduced *plus* CO. Straight base line was obtained with a 70-fold purified enzyme (Fraction III) in phosphate buffer (pH 7.4) in both cuvettes (1 cm) in a final volume of 2.5 ml. The enzyme in the sample cuvette was then reduced with solid $Na_2S_2O_4$ and the difference spectrum (———) was recorded. Then $Na_2S_2O_4$ was added to both cuvettes and CO bubbled through the sample cuvette (– – – –).

Fig. 3. Difference spectra under anaerobic conditions. Each Thunberg cuvette contained phosphate buffer (pH 7.4), 600 μmoles; $MgCl_2$, 12 μmoles; glucose 6-phosphate, 10 μmoles; NADP, 0.6 μmole; glucose-6-phosphate dehydrogenase, 10 I.U.; yeast enzyme, 4.5 units (Fraction III) in 3 ml. Solid Na_2SO_3 was in the sidearm of the sample cuvette. After a rigorous evacuation of the Thunberg cuvettes a base line was recorded. The reaction was started by mixing sulphite into the enzyme solution and difference spectra were recorded at intervals of 2 min (———); 10 min (– – – –); and 20 min (· · · · ·). After 30 min the enzyme in the sample cuvette was completely reduced with $Na_2S_2O_4$ (O—O).

When the enzyme in both the cuvettes was reduced with $Na_2S_2O_4$ and then CO was bubbled through the sample cuvette, the 428-mμ peak shifted to 416, the 558- and 589-mμ peaks were eliminated, and the trough at 458 mμ shifted to 432 mμ (Fig. 2). This spectrum is characteristic of cytochrome *o* as observed by CHANCE and coll.[28,29] and others[30,31] in microorganisms.

Difference spectra (Fig. 3) were obtained on adding solid sodium sulphite (about 5 mg) from sidearm into the Thunberg cuvette containing NADPH-generating system, $MgCl_2$, phosphate buffer (pH 7.4) and the enzyme. A trough around 450 mμ appeared within 1 min, followed by the gradual appearance of a Soret peak at 426 mμ. A decrease in the trough at 450 mμ was paralleled by an increase in the Soret peak at 426 mμ, suggesting that the haem component was accepting electrons from the reduced flavin in the normal course of sulphite reduction. Subsequently two small peaks developed in the vicinities of 558 and 589 mμ. The addition of $Na_2S_2O_4$ to the sample cuvette after a 30-min incubation reduced both the flavin and the haem components completely.

Experiments with mass spectrometer and $Na_2{}^{35}SO_3$

Gaseous sulphur compounds, *e.g.* SO_2, SO or S_2O, were not detected, in the mass spectrometer, during the enzymic reduction of sulphite.

The peak, which was oxidised by iodine, observed in the middle of the electrophoretogram (Fig. 4) after 5, 10 and 15 min incubation was identified as free sulphide, by the use of a $Na_2{}^{35}S$ marker.

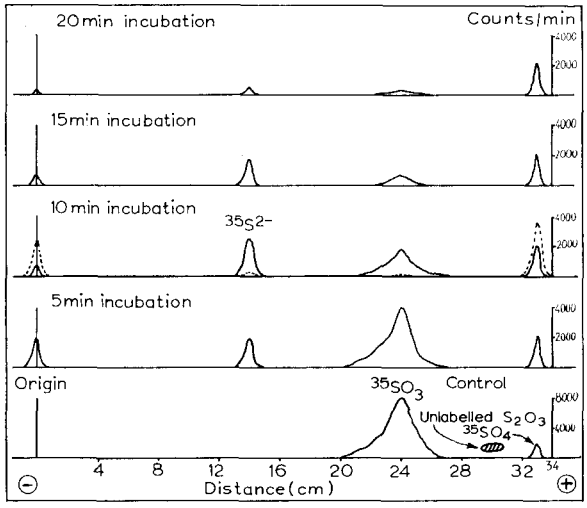

Fig. 4. Electrophoretograms of the reaction products of [^{35}S]sulphite reduction. Reaction mixture and experimental conditions are described in METHODS. Radioactivity was counted in a Packard Tri-carb Spectrophotometer (Model 3375). A small crystal of iodine dissolved in the duplicate sample after 10 min incubation, prior to electrophoresis (-----).

DISCUSSION

The stoichiometry of the enzymic reduction of sulphite can be written as follows:

$$3\ NADPH_2 + SO_3{}^{2-} \longrightarrow 3\ NADP^+ + S^{2-} + 3\ H_2O$$

Thus 3 moles $NADPH_2$ are oxidized per mole S^{2-} produced as observed also by ELLIS[32] in *E. coli*. This is equivalent to the utilization of 6 moles of reduced methyl viologen by sulphite reductases prepared from spinach[14] and Aspergillus[11].

There has been, thus far, no experimental evidence for the formation of free intermediates during the enzymic reduction of sulphite. However, there are suggestions based on the transfer of the sulphonyl group of 3'-phosphoryl-5'-adenosinophosphosulphate (PAPS) to a thiol group of a protein, that all intermediates between $SO_3{}^{2-}$ and S^{2-} are bound to proteins[33]. On the other hand, ALEXANDER[34] postulated a mechanism for H_2S formation from sulphate, which involves the stepwise reduction *via* sulphite, sulphoxylate (H_2SO_2) and sulphur hydrate (H_4SO_2); while IVERSON[35] has recently reported that disulphur monoxide (S_2O) gas is formed by *Desulphovibrio desulphuricans*.

In our experiments using the mass spectrometer and tracer techniques with [^{35}S]sulphite, no free intermediates were detected during the enzymic reduction of sulphite in yeast. These results are supported by our observed stoichiometry of $SO_3{}^{2-}/S^{2-}$ 1:1 as well as by the data of ELLIS[36] with intact cells of *E. coli*.

Inhibition of enzyme activity by chelating agents indicate that there may be

an iron component in the system. Similarly, KIKUCHI observed that the production of H_2S in yeast cells was depressed either by iron inhibitors[37] or by iron deficiency[38].

The CO inhibition of the enzyme which is light reversible indicates that the iron component is likely to be a haem protein and its participation in enzyme action was further confirmed by the SO_3-difference spectrum (Fig. 3).

Sulphite reductase is also dependent on flavin for its activity, since the addition of riboflavin, FMN or FAD stimulated the reaction whereas flavin inhibitors depressed it. MAGER[1] has also reported a similar effect of FAD in the *E. coli* enzyme. The difference spectra with dithionite or sulphite also confirm that flavin acts as an electron carrier during enzyme action. A similar conclusion was made by others for the enzyme from yeast[19] and *E. coli*[12].

Since the results reported herein suggest the participation of a flavin component as well as a haem compound, we conclude that the yeast sulphite reductase is a haemoflavoprotein. The haem component is likely to be cytochrome *o* (refs. 28–31), based on the characteristic CO-difference spectrum for the $Na_2S_2O_4$-reduced enzyme.

By contrast, although the reduced methyl viologen-linked sulphite reductase from spinach is a haemprotein[15], it did not contain flavin nor did it utilize NADPH. This observation, together with the fact that the NADPH-linked sulphite reductases from yeast[19,22] and *E. coli*[12] contain flavin and utilize either NADPH or reduced methyl viologen as an electron donor[2,20], suggests that (a) the bacterial enzyme is composed of two protein moieties[12,20], *viz.* NADPH-coupling and reduced methyl viologen sulphite reducing system; and (b) the reduced methyl viologen-sulphite reductases from Aspergillus[11] and spinach[15] are similar to the bacterial counterpart. According to this interpretation, it is the NADPH-coupling protein moiety which contains the flavin. Further, when SO_3^{2-} and enzyme were pre-mixed, CO did not inhibit the reduced methyl viologen-[15] and NADPH-linked sulphite reductase activities. This type of sulphite protection against the binding of CO, suggests that CO and SO_3^{2-} bind to the same site. Since a PCMB inhibition (which was not reversed by GSH) was also observed after the enzyme was first reduced with reduced methyl viologen[14] and since this inhibition was prevented by sulphite, it is likely that the binding site is a thiol in the haemprotein. Such protein-bound *S*-sulphonyl compounds are not uncommon during sulphate reduction; *e.g.* the bound sulphite, intermediate in PAPS-reduction, liberates inorganic sulphite to a protein disulphide acceptor to form an *S*-sulphonyl derivative[39].

In addition to this, the flavoprotein moiety of NADPH-linked sulphite reductase apparently contains another protein disulphide, which accepts the reducing power directly from NADPH. This view is supported by our experiments wherein, (a) NADPH-oxidation, the first step in enzymic sulphite reduction, was inhibited by PCMB; and this inhibition was not prevented by the presence of SO_3^{2-}, (b) the PCMB inhibition was reversed by GSH and (c) the inhibition was observed within 10 sec (mixing time) after finally starting the reduction reaction by adding either SO_3^{2-}, NADPH or enzyme to the remainder of the reaction mixture. Moreover during the normal course of sulphite reduction (Fig. 3) a definite interval was observed between the reduction of flavin and haem components of the enzyme system (50 sec and 100 sec respectively) after starting the reaction.

In addition to these observations, it has been shown that the yeast enzyme has a diaphorase activity when NADPH (but not reduced methyl viologen) was the

electron donor, and this activity was also inhibited by PCMB[20]. Thus it seems that the NADPH-coupling moiety which appears to be a flavoprotein, contains another disulphide bond in addition to the one in the haemprotein moiety. Accordingly, a tentative model for the electron transfer sequence in yeast sulphite reductase is given in Fig. 5. The direct participation of protein disulphide and thiol groups in a redox reaction, as shown in the model, has also been observed in several other cases, *e.g.* Fraction C protein in the reduction of PAPS[33,39,40], the enzyme system for the reduction of methionine sulphoxide[41], thioredoxin in the reduction of ribonucleotides[42,43], glutathione reductase[44–46], lipoyl-dehydrogenase[47–49] and Fraction P_2 protein in the decarboxylation of glycine[50].

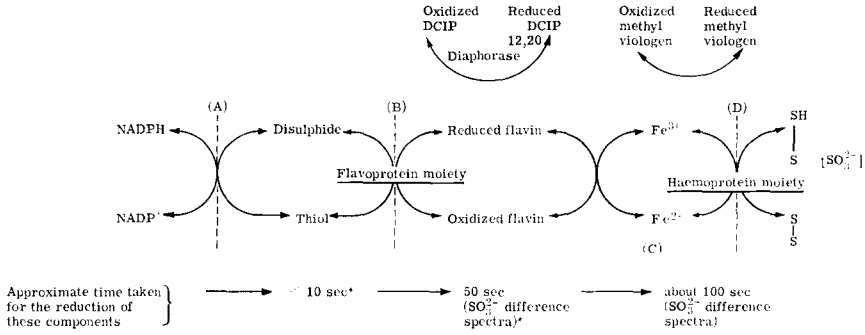

Fig. 5. A tentative model for the electron transfer sequence in yeast sulphite reductase: (A) GSH-reversed PCMB inhibition* (not prevented by SO_3^{2-}). (B) Mepacrine inhibition reversed by flavin*. (C) Inhibition by KCN (ref. 20) and iron inhibitors*. KCN inhibition not due to its reaction with carbonyl groups[14]. (D) PCMB inhibition (not reversed by GSH) offset by preincubating enzyme with reduced methyl viologen (inhibition prevented by SO_3^{2-})[13,14,15].

* Results reported in this paper; and in refs. 12–15 and 20 cited.

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