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# STUDIES ON YEAST SULFITE REDUCTASE

## II. PARTIAL PURIFICATION AND PROPERTIES OF GENETICALLY INCOMPLETE SULFITE REDUCTASES

AKIO YOSHIMOTO* AND RYO SATO

*Institute for Protein Research, Osaka University, Osaka (Japan)*

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SUMMARY

Enzymes catalyzing the reduction of sulfite by reduced methyl viologen (MVH) were partially purified from four mutants of *Saccharomyces cerevisiae*, strains 6, 11, 20 and 21, which are genetically blocked in the sulfite reduction step in the sulfate assimilation pathway. Unlike NADPH-sulfite reductase from the wild-type strain, the enzymes from the mutants showed no activities coupled to NADPH oxidation. Sedimentation coefficients of these mutant enzymes ranged from 5.1 S to 6.6 S, values which are much smaller than the value of 14.8 S determined for yeast NADPH-sulfite reductase. All the mutant enzymes contained a chromophore or chromophores absorbing at 386 and 587 mμ. In contrast to the wild-type enzyme possessing both FMN and FAD, the enzymes from strains 6, 11 and 20 contained only FMN, and that from strain 21 lacked both flavins. Iron and acid-labile sulfide were detected in these mutant enzymes as well as in the wild-type enzyme.

## INTRODUCTION

NADPH-sulfite reductase (EC 1.8.1.2) is widely distributed in the microbial kingdom[1–6]. This enzyme catalyzes, besides the NADPH-linked reduction of sulfite, the reductions of nitrite, hydroxylamine, ferricyanide, cytochrome *c*, dyes, and quinones by NADPH[3,5,7–9,11–16], and the reduction of sulfite by reduced methyl viologen (MVH) and reduced benzyl viologen[12–14]. In previous papers[12–14], we reported the purification of this enzyme from baker's yeast to an almost homogeneous state and showed that it is a complex protein containing FMN, FAD and a chromophore absorbing at 587 mμ. SIEGEL AND KAMIN[15,16] and we[17] have also purified a similar enzyme from *Escherichia coli*.

NAIKI[3] has isolated several mutants of *Saccharomyces cerevisiae* incapable of reducing sulfite and reported that these mutants could be classified into two groups. Crude extracts of the mutants belonging to one group, though incapable of reducing sulfite by NADPH, still retained an activity to reduce sulfite by MVH, whereas those

Abbreviation: MVH, reduced methyl viologen.

* Present address: Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.

of the other group showed no sulfite-reducing activities with either NADPH or MVH as electron donor. Since these observations suggested the presence of a functionally incomplete sulfite reductase or reductases in the mutants of the first group, it seemed of interest to purify these enzymes and compare their properties with those of NADPH-sulfite reductase from the wild-type strain. This paper describes the partial purification and properties of genetically altered forms of sulfite reductase from four mutant strains of *S. cerevisiae*.

## EXPERIMENTAL PROCEDURE

### *Materials*

Four mutant strains of *S. cerevisiae* (haploid), strains 6, 11, 20 and 21, were kindly supplied by Dr. N. NAIKI; cell-free extracts of these strains could catalyze the reduction of sulfite by MVH, but were unable to reduce sulfite by NADPH[3]. These strains were grown at 30° for 18 h with vigorous aeration in the medium described by NAIKI[3]. The cells were harvested by filtration on Büchner funnels and thorougly washed with water.

The preparation of purified NADPH-sulfite reductase from pressed baker's yeast has been described previously[14]. Alcohol dehydrogenase was also crystallized from baker's yeast according to the procedure of RACKER[18]. The other materials employed have been described in a previous paper[14].

### *Enzyme assays*

MVH-sulfite reductase activity was measured by the manometric method as described previously[14], but for technical reasons the reaction mixture contained 0.2 M potassium phosphate buffer, pH 7.3, when crude extracts and ammonium sulfate fractions were used and the assay for more highly purified preparations was conducted in 0.05 M potassium phosphate buffer, pH 7.7. Since MVH-sulfite reductase activity was dependent on both ionic strength and pH (ref. 14), the activities determined under the above two sets of conditions were multiplied by 3.19 and 1.14, respectively, to convert them to the values under the standard assay conditions described in the previous paper[14], *i.e.* 0.02 M potassium phosphate buffer, pH 7.7. These conversion factors were determined experimentally. The activity of alcohol dehydrogenase was determined as described by RACKER[18], except that the final concentration of $NAD^+$ was 0.2 mM.

### *Chemical analyses*

Determinations of protein, FMN, FAD and total iron were performed as described previously[14]. Acid-labile sulfide was estimated by a modification of the method of ST. LORANT[19]. A suitably diluted sample (1.13 ml containing 0.5–4.0 mg of protein) was mixed with 0.15 ml of 0.27 M zinc acetate containing 0.12 M sodium acetate and 0.086 M NaCl in a test tube (0.8 cm × 11 cm). To the mixture were quickly added 0.2 ml of 1.35 mM *N,N'*-dimethyl-*p*-phenylenediamine sulfate in 3.6 M $H_2SO_4$ and 0.02 ml of 0.13 M $Fe(NH)_4(SO_4)_2$ in 0.49 M $H_2SO_4$. The tube was immediately sealed with a sheet of Parafilm and the contents were well mixed. After standing at 25° for 30 min, the mixture was centrifuged and the absorbance of the clear supernatant was read at 670 mμ against a reagent blank. A known amount of sodium sulfide

solution, which had been standardized iodometrically, was mixed with a suitable amount of bovine serum albumin and the mixture was treated as above to obtain the standard curve.

*Sucrose density gradient centrifugation*

Sedimentation coefficients were determined by sucrose density gradient centrifugation as described previously[14], using crystalline yeast alcohol dehydrogenase as internal marker. The results were expressed in terms of the distance of movement of sulfite reductase relative to that of alcohol dehydrogenase ($R_{ADH}$) according to the method of LEINWEBER, SIEGEL AND MONTY[20]. For calculation of sedimentation coefficients, a value of 7.4 S was assumed for yeast alcohol dehydrogenase[21].

## RESULTS

*1. Purification of mutant sulfite reductases*

All manipulations, except for autolysis, were carried out at about 4°. Centrifugation was always performed at 10000 × *g* for 15 min, and dialysis was conducted against 0.3 M potassium phosphate buffer, pH 7.3, containing 1 mM EDTA. Potassium phosphate buffers, pH 7.3, containing 1 mM EDTA were always used.

*Enzymes from strains 6, 11 and 20.* MVH-sulfite reductases from these three strains were purified by the same method. The washed cells (200–400 g, wet weight) were autolyzed at 30° in the presence of $K_2HPO_4$ and toluene as described for NADPH-sulfite reductase from the wild-type strain[14], and insoluble materials were removed by centrifugation (crude extract). The extract was brought to 30 % saturation with respect to $(NH_4)_2SO_4$ at pH 7.0, and the precipitate formed was removed by centrifugation. The proteins, which were precipitated by increasing the $(NH_4)_2SO_4$ concentration to 60 % saturation at pH 7.0, were collected by centrifugation and dialyzed overnight (ammonium sulfate fraction).

This fraction was diluted 3-fold with water (to 0.10 M buffer), adsorbed immediately on a DEAE-cellulose column (4 cm × 20 cm) equilibrated with 0.1 M buffer, and then eluted with 0.3 M buffer. The fractions possessing the MVH-sulfite reductase activity were combined (DEAE-cellulose eluate). The combined fractions were diluted with water to a buffer concentration of 0.18 M, and applied to a DEAE-Sephadex A-50 column (2 cm × 15 cm) equilibrated with 0.2 M buffer. Elution was then conducted with a linear concentration gradient of buffer from 0.2 to 0.5 M, and the eluates containing the sulfite reductase activity were combined. From the combined eluates the enzyme was recovered by ammonium sulfate precipitation and dialyzed overnight (first DEAE-Sephadex eluate). The preparation thus obtained was further purified by a second DEAE-Sephadex column chromatography as described above.

In the second DEAE-Sephadex column chromatography, almost all the enzyme activities from strains 6 and 11 were eluted at a buffer concentration range from 0.20 to 0.25 M, yet small but definite portions of the activity were eluted even at a buffer concentration as high as 0.3 M. This latter activity may correspond to Peak B enzyme from strain 20, which will be described below. Only the main fractions eluted between 0.20 and 0.25 M were collected. When the preparation from strain 20 was subjected to the first DEAE-Sephadex column chromatography, the enzyme was

eluted over a wide range of buffer concentration with two broad peaks at about 0.20 and 0.28 M. In the second chromatography, however, the enzyme was separated more clearly into two fractions which were eluted at 0.20 and 0.26–0.29 M (Fig. 1). These two fractions were called Peak A and Peak B enzymes, respectively, and collected separately.

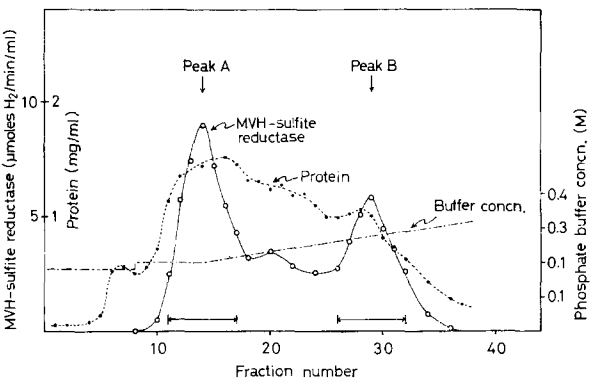


Fig. 1. Second DEAE-Sephadex A-50 column chromatography of MVH-sulfite reductase from strain 20. The preparation (244 mg protein) obtained from the first DEAE-Sephadex chromatography was applied to another column of DEAE-Sephadex A-50 (2 cm × 15 cm) equilibrated with 0.15 M phosphate (pH 7.3)–1 mM EDTA. Elution was effected by a linear concentration gradient of potassium phosphate buffer (pH 7.3, containing 1 mM EDTA) ranging from 0.20 to 0.50 M, and 5-ml fractions were collected. Combined fractions of Tube Nos. 11 through 18 and those of Tube No. 26 through No. 32 were employed as Peak A and Peak B enzymes, respectively.

The preparations obtained in the second DEAE-Sephadex chromatography were concentrated as follows. The solution was first diluted with water to a buffer concentration of 0.07 M and immediately adsorbed on a small DEAE-cellulose column (usually 1 cm × 10 cm) equilibrated with 0.05 M buffer. The greenish yellow band of the enzyme on the top of the column was eluted with 0.3 M buffer. To avoid possible inactivation of the enzyme due to low ionic strength (especially below 0.2 M buffer)[2,3,14], the adsorption and elution were carried out as quickly as possible. The preparations thus concentrated (second DEAE-Sephadex eluate) were used as the final products. The purifications of MVH-sulfite reductases from strains 6, 11 and 20 are summarized in Table IA–C.

*Enzyme from strain 21.* The cells of strain 21 (370 g, wet weight) were autolyzed and the extract was fractionated with $(NH_4)_2SO_4$ as described above. However, the enzyme at this stage of purification could not be adsorbed to DEAE-cellulose equilibrated with 0.1 M buffer. Therefore, the purification procedure was modified as follows. The dialyzed $(NH_4)_2SO_4$ fraction was diluted with water to a buffer concentration of 0.15 M, and immediately adsorbed on a DEAE-Sephadex A-50 column (3 cm × 20 cm) equilibrated with 0.15 M buffer. Elution was then performed with a linear concentration gradient of buffer ranging from 0.15 to 0.45 M. To minimize possible inactivation due to buffer concentrations lower than 0.2 M, 2 ml of 1 M buffer per 10 ml of eluate were placed in the collector tubes before the start of chromatography. The fractions containing the enzyme activity, emerging at buffer concentrations from 0.16 and 0.22 M, were combined and made 70% saturated with

TABLE I

PURIFICATION OF MVH-SULFITE REDUCTASES FROM YEAST MUTANT STRAINS

The MVH-sulfite reductase activity was measured by the manometric (hydrogenase) method and converted to the value in 0.02 M phosphate buffer, pH 7.7, as described in EXPERIMENTAL PROCEDURE. The activity is expressed as μmoles of hydrogen absorbed per min. Specific activity is calculated on a mg protein basis.

A: Purification from strain 6 (240 g, wet weight).

| *Fraction* | *Total protein (mg)* | *Total activity* | *Specific activity* | *Yield (%)* |
|---|---|---|---|---|
| Crude extract | 5 280 | 220 | 0.042 | 100 |
| Ammonium sulfate fraction | 2 220 | 148 | 0.067 | 67 |
| DEAE-cellulose eluate | 490 | 148 | 0.30 | 67 |
| 1st DEAE-Sephadex eluate | 44 | 127 | 2.89 | 58 |
| 2nd DEAE-Sephadex eluate | 7.4 | 43.4 | 5.87 | 20 |

B: Purification from strain 11 (300 g, wet weight).

| *Fraction* | *Total protein (mg)* | *Total activity* | *Specific activity* | *Yield (%)* |
|---|---|---|---|---|
| Crude extract | 12 400 | 622 | 0.050 | 100 |
| Ammonium sulfate fraction | 5 110 | 517 | 0.10 | 83 |
| DEAE-cellulose eluate | 590 | 330 | 0.56 | 53 |
| 1st DEAE-Sephadex eluate | 235 | 292 | 1.24 | 47 |
| 2nd DEAE-Sephadex eluate | 32.9 | 89.8 | 2.73 | 14 |

C: Purification from strain 20 (290 g, wet weight).

| *Fraction* | *Total protein (mg)* | *Total activity* | *Specific activity* | *Yield (%)* |
|---|---|---|---|---|
| Crude extract | 11 800 | 685 | 0.058 | 100 |
| Ammonium sulfate fraction | 9 050 | 527 | 0.058 | 77 |
| DEAE-cellulose eluate | 582 | 507 | 0.87 | 74 |
| 1st DEAE-Sephadex eluate | 224 | 375 | 1.67 | 55 |
| 2nd DEAE-Sephadex eluates | | | | |
| Peak A | 27.4 | 117 | 4.27 | 17 |
| Peak B | 19.2 | 56.1 | 2.92 | 8 |

D: Purification from strain 21 (370 g, wet weight).

| *Fraction* | *Total protein (mg)* | *Total activity* | *Specific activity* | *Yield (%)* |
|---|---|---|---|---|
| Crude extract | 15 600 | 578 | 0.037 | 100 |
| Ammonium sulfate fraction | 9 380 | 468 | 0.050 | 81 |
| 1st DEAE-Sephadex eluate | 872 | 452 | 0.52 | 78 |
| 1st Sephadex G-100 fraction | 189 | 274 | 1.45 | 47 |
| 2nd DEAE-Sephadex eluate | 32.9 | 255 | 7.75 | 44 |
| 2nd Sephadex G-100 fraction | 12.8 | 151 | 11.8 | 26 |

$(NH_4)_2SO_4$. The resultant precipitate was collected by centrifugation and dialyzed (first DEAE-Sephadex eluate). The dialyzed solution was then passed through a Sephadex G-100 column (5 cm × 30 cm) equilibrated with 0.3 M buffer, and the fractions possessing the enzyme activity were pooled (first Sephadex G-100 fraction). After dilution to a buffer concentration of 0.15 M, the pooled fractions were subjected to a second DEAE-Sephadex A-50 column chromatography as described above. The active fractions were combined, diluted to reduce the buffer concentration to 0.05 M, and concentrated by means of a small DEAE-cellulose column as in the cases of the enzymes from the other strains (second DEAE-Sephadex eluate). It should be mentioned that the enzyme from strain 21 could be adsorbed to DEAE-cellulose when the buffer concentration was lowered to 0.05 M. The preparation was finally subjected to gel filtration through a second Sephadex G-100 column (3 cm × 30 cm) equilibrated with 0.3 M buffer (second Sephadex G-100 fraction). The purification of the enzyme from strain 21 is summarized in Table ID.

*Purity of enzymes.* No attempts were made to assess the purity of the MVH-sulfite reductase preparations obtained above. However, they all seemed to be still rather impure, as can be seen, for example, from Fig. 1 which shows the elution pattern of Peak A and Peak B enzymes from strain 20 in the final column chromatography. If we could assume that the wild-type and mutant strains contained the same concentration of sulfite reductase, the purification ratio of each enzyme would give a measure of its purity (although the assumption is very unlikely). While NADPH-sulfite reductase of almost 100 % purity could be obtained from the wild-type strain by 385-fold purification over crude extract (*cf.* Table I of ref. 14), the purification ratios attained in the present study were 140, 55, 74, 50 and 318 for the enzymes from strains 6, 11, 20 (Peak A), 20 (Peak B), and 21, respectively. Another criterion of the purity would be the specific enzyme activity finally attained. Although the purest NADPH-sulfite reductase preparation from the wild-type strain consumed 21.1 μmoles of hydrogen per min per mg of protein under the standard conditions for the manometric assay of the MVH-sulfite reductase activity[14], the values for the mutant enzymes from strains 6, 11, 20 (Peak A), 20 (Peak B) and 21 were 5.87, 2.73, 4.27, 2.92 and 11.8 μmoles per min per mg of protein, respectively. However, these values may not be proportional to the purities, because of the possibility that these enzymes possess different specific activities even when purified to homogeneity. At any rate, the figures mentioned above suggest that the preparations obtained here were quite impure and that the enzyme from strain 21 might have been much purer than the other enzymes.

*2. Properties of mutant sulfite reductases*

*Catalytic properties.* Although NADPH-sulfite reductase from wild-type yeast could catalyze the NADPH-linked reductions of sulfite, nitrite, hydroxylamine, ferricyanide, cytochrome *c*, 2,6-dichlorophenolindophenol and menadione[12–14], none of the mutant sulfite reductases could catalyze these reactions at significant rates. However, they catalyzed the reduction of sulfite by MVH. While the sulfite-reducing activity of wild-type NADPH-sulfite reductase was inactivated by treatment with both NADPH and cyanide and the lost activity was not restored by dialysis[14], no inactivation was observed when the mutant enzymes were treated with NADPH and cyanide and then thoroughly dialyzed. No reduction of sulfite by NADPH was

detected when any of these mutant enzymes were added to the purified wild-type enzyme which had been treated with NADPH and cyanide and then dialyzed.

*Sedimentation coefficients.* Fig. 2 shows the results of sucrose density gradient centrifugation of mutant sulfite reductases. By comparing the positions of peaks of the sulfite reductases with that of yeast alcohol dehydrogenase (7.4 S) included as an internal marker, sedimentation coefficients of the enzymes from strains 6, 11, 20 (Peak A), 20 (Peak B) and 21 were determined to be 6.6, 6.5, 6.6, 5.5 and 5.1 S, respectively. These values were much smaller than 14.8 S as determined for NADPH-sulfite reductase from the wild-type strain[14]. The sedimentation pattern of the first DEAE-Sephadex eluate from strain 20 showed a symmetric peak of MVH-sulfite reductase activity with a sedimentation coefficient of 5.9 S. This result was somewhat unexpected, because this preparation should have contained both Peak A and Peak B enzymes having sedimentation coefficients of 6.6 and 5.5 S, respectively. Although this preparation could be separated into the two different components on the second DEAE-Sephadex chromatography, the possibility could not yet be excluded that one of the two components was a modified product of the other. The detection of minor components, eluted at a buffer concentration of about 0.3 M, in the second DEAE-Sephadex column chromatography of the enzymes from strains 6 and 11 might also support this possibility.

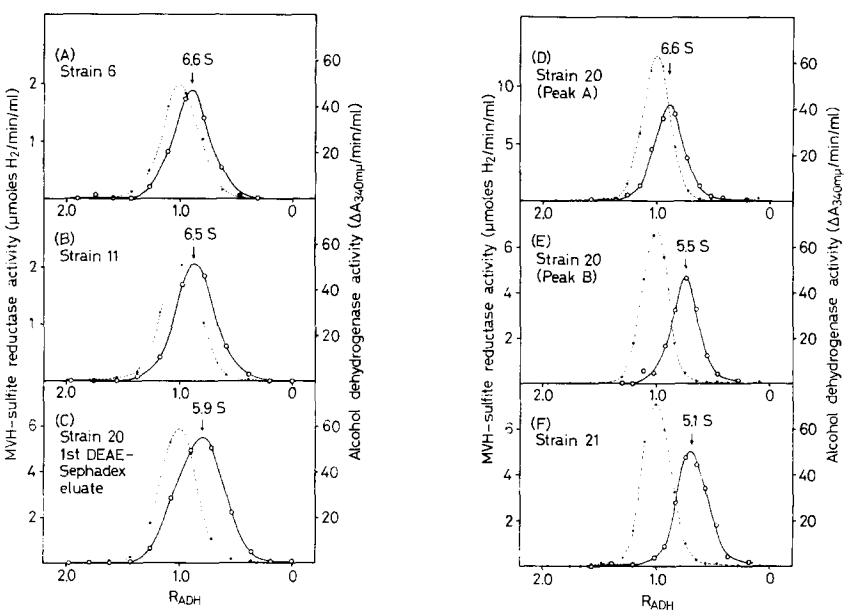


Fig. 2. Sucrose density gradient centrifugation of MVH-sulfite reductases from various strains. The enzyme preparation (0.1–0.2 ml) was mixed with 14 μg of crystalline yeast alcohol dehydrogenase (0.01–0.05 ml) and the mixture was layered over 4.3 ml of a linear gradient of sucrose (5–20% in 0.3 M potassium phosphate–1 mM EDTA, pH 7.3). After centrifugation for 13–15 h at 35000 rev./min in a Hitachi RPS 40 swinging bucket rotor, 17–34 fractions were collected by puncturing the bottom of the tube. The MVH-sulfite reductase and alcohol dehydrogenase (ADH) activities of each fraction were determined and plotted against $R_{ADH}$. (A) Enzyme from strain 6, 0.23 mg. (B) Enzyme from strain 11, 0.53 mg. (C) Preparation of enzyme from strain 20 after first DEAE-Sephadex column chromatography, 4.88 mg. (D) Peak A enzyme from strain 20, 1.03 mg. (E) Peak B enzyme from strain 20, 0.77 mg. (F) Enzyme from strain 21, 0.194 mg.

*Absorption spectra.* As can be seen from Figs. 3 and 4, the absorption spectra of MVH-sulfite reductases from strains 6 and 11 and the two components of the enzyme from strain 20 (Peak A and Peak B) were similar to that of wild-type NADPH-sulfite reductase (shown in Fig. 3, Curve A). All the preparations showed absorption peaks

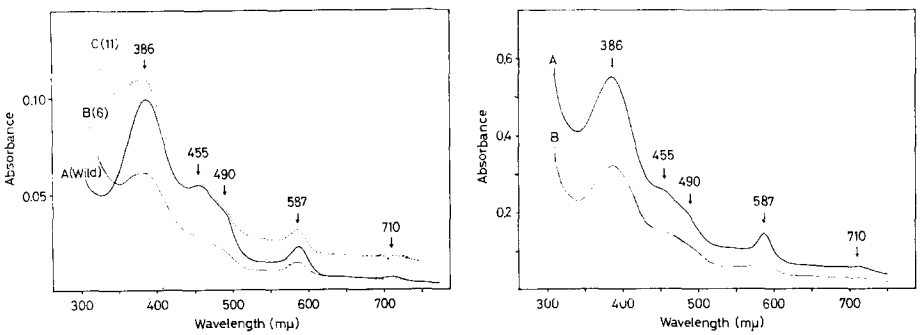


Fig. 3. Absorption spectra of NADPH-sulfite reductase from the wild-type strain and MVH-sulfite reductases from strains 6 and 11. All the preparations were dissolved in 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA. Curve A: NADPH-sulfite reductase from wild-type strain, 0.422 mg/ml. Curve B: MVH-sulfite reductase from strain 6, 1.15 mg/ml. Curve C; MVH-sulfite reductase from strain 11, 2.65 mg/ml.

Fig. 4. Absorption spectra of Peak A and Peak B MVH-sulfite reductases from strain 20. The preparations were dissolved in 0.3 M potassium phosphate (pH 7.3)–1 mM EDTA. Curve A: Peak A enzyme, 10.3 mg/ml. Curve B: Peak B enzyme, 7.70 mg/ml.

at 385–386 and 587 mμ and shoulders at about 455 and 490 mμ. In agreement with recent observations of SIEGEL AND KAMIN[15] on purified *E. coli* NADPH-sulfite reductase, a very small peak was also observed in the 710-mμ region of the spectra of all the above preparations. The shoulders at about 455 and 490 mμ in the spectra of these mutant enzymes were, however, much less distinct than those of the wild-type enzyme, suggesting lower flavin contents in the genetically altered sulfite reductases. The spectrum of the enzyme from strain 21, shown in Fig. 5, was markedly different from the other ones in that it had practically no shoulders in the wavelength region from 400 to 500 mμ. It was, therefore, inferred that this enzyme was devoid of any flavin prosthetic groups. This enzyme, however, showed the peaks at 386, 587 and

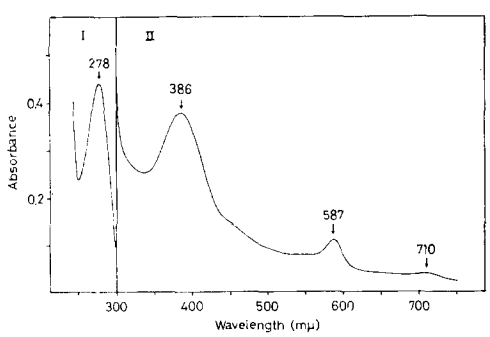


Fig. 5. Absorption spectrum of MVH-sulfite reductase from strain 21 in 0.3 M phosphate (pH 7.3)–1 mM EDTA. I; 0.388 mg/ml. II; 1.94 mg/ml.

710 mμ. In contrast to the wild-type sulfite reductase[14], addition of NADPH to these mutant enzymes caused no bleaching of their spectra.

*Flavins, iron and acid-labile sulfide.* We have reported that NADPH-sulfite reductase purified from baker's yeast contained one mole each of FMN and FAD and about 5 atoms of iron per 350000 g of protein[13,14]. SIEGEL *et al.*[15,16] have also reported that purified *E. coli* NADPH-sulfite reductase (molecular weight, 700000) contained 7–8 moles of flavins (FMN and FAD in equal amounts) and 12–16 gram-atoms of iron per mole of enzyme. Therefore, it seemed of interest to examine the presence of these components in the mutant enzymes. Although the enzyme preparations obtained in the present study seemed to be rather impure as discussed above, they were analyzed for FMN, FAD and total iron and the results are recorded in Table II together with the data for the purified wild-type enzyme. It will be seen that the enzymes from strains 6 and 11 contained appreciable amounts of FMN, but the contents of FAD were negligible. The FMN contents in both Peak A and Peak B enzymes from strain 20 were considerably higher than those in the above two, but again only a negligible amount of FAD could be detected in the preparation. Although it is premature to draw any conclusions from these data in view of the insufficient purity of the preparations, it may be plausible to assume that these four enzymes contained only FMN as flavin prosthetic group. A fascinating finding was that neither FMN nor FAD was present in significant quantities in the preparation obtained from strain 21. This fact, together with the aforementioned lack of shoulders in the 400–500-mμ region of the spectrum, indicated that this mutant enzyme was in fact devoid of any flavin prosthetic groups. All the preparations from the mutant strains contained sizable amounts of iron, but the contents were considerably lower than that in the purified wild-type enzyme.

TABLE II

CONTENTS OF FLAVINS, IRON AND ACID-LABILE SULFIDE IN VARIOUS PREPARATIONS OF YEAST SULFITE REDUCTASES

| *Sulfite reductase purified from* | *Flavins (mμmoles/mg)* | | *Iron (mμatoms/mg)* | *Acid-labile sulfide (mμmoles/mg)* |
|---|---|---|---|---|
| | *FMN* | *FAD* | | |
| Wild-type | 2.84 | 2.84 | 15.4 | 7.5 |
| Strain 6 | 0.75 | 0.02 | 6.6 | 2.9 |
| Strain 11 | 0.36 | 0.06 | 4.3 | 2.8 |
| Strain 20 (Peak A) | 1.53 | 0.03 | 4.3 | 3.1 |
| Strain 20 (Peak B) | 1.16 | 0.03 | 4.1 | 2.2 |
| Strain 21 | 0.07 | 0.07 | 7.2 | 11.0 |

In a previous paper[14] we concluded that yeast NADPH-sulfite reductase was free from labile sulfide, based on the observation that *p*-chloromercuribenzoate did not affect the absorption spectrum of the enzyme. However, SIEGEL *et al.*[15,16] have reported that NADPH-sulfite reductase purified from *E. coli* released 12–16 moles of sulfide per mole of enzyme on strong acidification. Therefore, the acidification method of labile sulfide determination (see EXPERIMENTAL PROCEDURE) was applied to the purified yeast NADPH-sulfite reductase and the five preparations of mutant enzymes. It was thus found that significant quantities of acid-labile sulfide were actually present

in all the preparations examined (Table II). The data obtained indicate that purified NADPH-sulfite reductase contained 5–6 gramatoms of iron and 2–3 moles of acid-labile sulfide per mole of FMN or FAD (iron/acid-labile sulfide, *ca.* 2). On the other hand, the data of SIEGEL *et al.*[15,16] show that the *E. coli* enzyme contained about 1 gramatom of iron per mole of acid-labile sulfide.

*Non-flavin chromophore.* As mentioned above, the spectra of the five mutant enzymes invariably showed absorption peaks at 386, 587 and 710 mμ, indicating the presence in these enzymes of the same non-flavin chromophore or chromophores as has been described for the wild-type enzyme[14]. The fact that the enzyme from strain 21, though lacking flavins, also showed a definite peak at 386 mμ indicated that this peak in the other preparations was also mainly due to the "587-mμ chromophore(s)" as concluded previously[14].

## DISCUSSION

Based on studies on cysteine-requiring mutants of *Salmonella typhimurium*, DREYFUSS and MONTY[22] have concluded that synthesis of NADPH-sulfite reductase in this organism is controlled by six cistrons, *i.e.*, a cluster of three ($B_a$, $B_b$, $B_c$), a cluster of two (I, J) and a separate cistron (G). Furthermore, evidence has been presented that the three B cistrons are also involved in the synthesis of 3'-phosphoadenosine-5'-phosphosulfate reductase and thiosulfate reductase[22,23]. Similarly, it has been reported that synthesis of NADPH-nitrate reductase in *Neurospora crassa*[24] and *Aspergillus nidulans*[25] is also under the control of at least four and six genes, respectively.

These findings suggested that NADPH-sulfite reductase is a system consisting of several, probably six, different proteins or protein subunits. WAINWRIGHT[2] has in fact reported that yeast NADPH-sulfite reductase can be resolved into six different protein fractions. However, except for this enzyme, all the microbial NADPH-sulfite reductases so far studied have been shown to behave as single proteins during their partial purification[1,4,7,9–11], and the enzymes from yeast[12–14] and *E. coli*[15–17] have actually been isolated as highly purified single proteins. Furthermore, separation of these purified enzymes into different subunits has not yet been reported.

SIEGEL, CLICK AND MONTY[8] have shown that mutants of *S. typhimurium* incapable of reducing sulfite can be divided into two groups; one having lost the ability to catalyze the NADPH-linked reductions of sulfite, hydroxylamine, cytochrome *c* and FAD, and the other still retaining the activities for cytochrome *c* and FAD, but not for sulfite and hydroxylamine. As mentioned above, NAIKI[3] has also noticed the presence of two groups in yeast mutants which cannot utilize sulfate because of genetic defects in the sulfite-reducing step; although both groups of mutants lack the NADPH-sulfite reductase activity, strains of one group (but not the other) possess an activity to reduce sulfite with MVH as electron donor. As discussed by NAIKI[3], these and other observations suggest that NADPH-sulfite reductase consists of at least two components; one is a protein possessing an MVH-sulfite reductase activity and capable of reacting directly with sulfite, nitrite and hydroxylamine, and the other reacts with NADPH and reduces cytochrome *c*, *etc.* Association of these two components seems to be required for the reductions of sulfite, nitrite and hydroxylamine by NADPH. A similar conclusion has also been reached by us for *A. nidulans* sulfite reductase[10,26].

According to SORGER[27], NADPH-nitrate reductase of *A. nidulans* also appears to be an aggregate of two proteins, one possessing the NADPH-cytochrome *c* reductase activity and the other having an ability to reduce nitrate by reduced benzyl viologen. However, none of the postulated components have been isolated and characterized.

The data described in this paper indicate that certain alterations in a cistron or cistrons involved in the synthesis of yeast NADPH-sulfite reductase results in the production of genetically incomplete enzymes which reduce sulfite by MVH, but are devoid of any activities coupled to NADPH oxidation. These maimed enzymes are much smaller in molecular size than NADPH-sulfite reductase from the wild-type strain. From the sedimentation coefficients, the mutant enzymes seem to fall into at least two categories: (1) 6.6-S enzymes from strains 6, 11 and 20 (Peak A), and (2) 5.1-S enzyme from strain 21. Peak B enzyme from strain 20 has an intermediate sedimentation coefficient of 5.5 S. However, in view of the above-mentioned possibility that this enzyme is a modification product, it is not yet certain if this represents a third category. Corresponding to the difference in molecular size, the enzymes of the first category seem to contain FMN, whereas that of the second category is free from flavins. A chromophore or chromophores absorbing at 587 m$\mu$ is present in all the mutant enzymes as well as in the wild-type enzyme which has a sedimentation coefficient of 14.8 S and contains both FMN and FAD[12–14]. The relationship between the wild-type enzyme and the two categories of genetically altered enzymes is tentatively illustrated in Fig. 6.

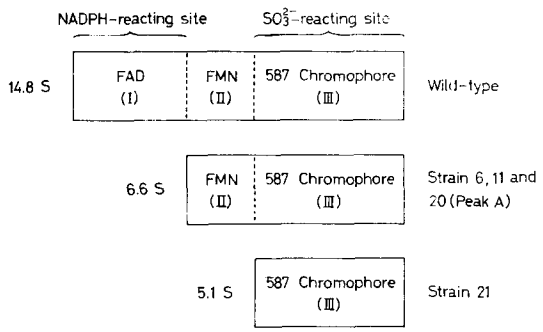


Fig. 6. Schematic illustration of a tentative relationship between NADPH-sulfite reductase from the wild-type strain and two categories of MVH-sulfite reductases from various mutant strains.

It seems that either the 6.6-S or the 5.1-S enzyme corresponds to one of the components of NADPH-sulfite reductase postulated by NAIKI[3]. However, the models shown in Fig. 6 indicate that the situation is somewhat more complicated than suggested by NAIKI[3], because intact NADPH-sulfite reductase appears to have at least three components, *i.e.*, a FAD-containing component (I), a FMN-containing component (II), and a component containing the "587-m$\mu$ chromophore" (III). It is, therefore, expected that some of the mutants lacking the MVH-sulfite reductase activity contain incomplete enzymes consisting of Components I and II, Component I alone, and Component II alone. As will be reported in a later communication, the NADPH-linked reductions of cytochrome *c*, quinones, and dyes by intact yeast sulfite reductase involve both FAD and FMN. Therefore, only the enzyme consisting of both

Components I and II will show these NADPH-linked activities. If Peak B enzyme from strain 20, which has a sedimentation coefficient of 5.5 S, can be established as a separate category of the altered enzyme, then we have to postulate the presence of a fourth component lacking both flavins and the 587-m$\mu$ chromophore. Further studies are needed to settle these questions and to correlate these components to the cistrons controlling the synthesis of sulfite reductase.

Since the 6.6-S enzyme (consisting of Components II and III) and the 5.1-S enzyme (Component III) possess no activities coupled to NADPH oxidation, it is reasonable to conclude that the intact enzyme reacts with NADPH at a site residing on Component I. On the other hand, since these two types of mutant enzymes can catalyze the reduction of sulfite by MVH, the site where sulfite interacts with sulfite reductase can be located on Component III. Evidence has been presented[14], and will be reported in detail in a later communication, which indicates that the 587-m$\mu$ chromophore, present in Component III, is actually involved in this interaction. Furthermore, all the other enzymes capable of the MVH-sulfite reaction, isolated from *A. nidulans*[10,26], *E. coli*[15–17], and higher plants[28], have been reported to contain this chromophore. Although the enzyme from strain 21 catalyzes the reduction of sulfite by MVH, this enzyme does not contain any flavins. Therefore, FMN which is found in the other mutant enzymes, is not essential for the MVH-sulfite reductase activity. However, the MVH-sulfite reductase activities of various preparations are not proportional to the content of the 587-m$\mu$ chromophore. In particular, the activity to the chromophore ratio is appreciably lower in the enzyme from strain 21, which lacks FMN. It seems that the presence of FMN increases the MVH-sulfite reductase activity. In a later communication, it will be reported that the electrons from MVH enter the sulfite reductase not only at the 587-m$\mu$ chromophore but also at the FMN site.

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