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STUDIES ON YEAST SULFITE REDUCTASE

III. FURTHER CHARACTERIZATION

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SUMMARY

1. Acid ammonium sulfate treatment of purified yeast NADPH-sulfite reductase caused selective detachment of FMN from the protein, leaving FAD still attached to the precipitated enzyme. The NADPH-linked activities of the treated enzyme could be partly restored by the addition of FMN or FAD.

2. The NADPH-linked activities, but not the reduced methyl viologen (MVH)-sulfite reductase activity, of the enzyme were inactivated on exposure to low ionic strength. This inactivation was accompanied by a decrease in sedimentation coefficient for the enzyme protein. The NADPH-sulfite reductase activity thus inactivated could be restored, though to very limited extents, by the addition of a protein fraction from yeast mutants incapable of reducing sulfite.

3. The presence in yeast crude extracts of at least two types of NADPH-cytochrome *c* reductase activity was demonstrated; one type of activity was associated with the sulfite reductase and sensitive to low salt concentrations, whereas the other was stable to low ionic strength. The sensitive reductase activity was absent in all the mutant strains which are genetically blocked in the sulfite reduction step.

4. Heat treatment of the purified enzyme decreased the heights of absorption peaks at 587 and 386 mμ, and the loss of the MVH-sulfite reductase activity paralleled the decrease in the intensity of the 587-mμ peak.

5. The results of ultraviolet irradiation of the enzyme provided evidence that the 587-mμ chromophore also absorbs in the 386-mμ region.

6. The enzyme possessed two different fluorescent species; one seemed to be flavin(s) and the other was probably the 587-mμ chromophore.

7. The ESR spectrum of the reduced enzyme, but not of the oxidized enzyme, showed a sharp signal at $g = 2$.

INTRODUCTION

In a previous paper[1], we reported the purification of NADPH-sulfite reductase

Unauthorized abbreviations used: MVH, reduced methyl viologen; ESR, electron spin resonance.

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(hydrogen-sulfide:NADP oxidoreductase, EC 1.8.1.2) from baker's yeast to an almost homogeneous state and described some of its properties. Several genetically modified sulfite reductases, catalyzing the reduction of sulfite by reduced methyl viologen (MVH), but not by NADPH, were also partly purified from yeast mutant strains incapable of utilizing sulfate and sulfite as sulfur sources[2]. The enzyme purified from the wild-type yeast is a complex protein with a minimum molecular weight of 350 000, containing FMN, FAD, a chromophore absorbing at 587 mμ, essential sulfhydryl group(s), iron, and acid-labile sulfide[1,2]. Contrary to the reports by PRABHAKARARAO AND NICHOLAS[3] for the yeast enzyme, by SIEGEL AND KAMIN[4], and by KAMIN *et al.*[5] for *Escherichia coli* sulfite reductase, we could not obtain evidence for the presence of a heme in our preparation[1]. As reported for sulfite reductases from a variety of other sources[4–17] the yeast enzyme, though it behaves as a single protein, possesses multiple types of catalytic activities; it catalyzes the NADPH-linked reduction of sulfite, nitrite, hydroxylamine, cytochrome *c*, dyes, quinones, and oxygen, and the reduction of sulfite and $NADP^+$ by MVH[1]. This multiplicity of catalytic activities, as well as the finding that the synthesis of NADPH-sulfite reductase in *Salmonella typhimurium* is controlled by 6 discrete cistrons[18], suggests that the yeast enzyme protein consists of several subunits. This view seems to be supported by the fact that the genetically incomplete sulfite reductases isolated from yeast mutants have lower sedimentation coefficients than the native enzyme[2]. However, nothing is as yet known of the nature of postulated subunits.

Our previous reports[1,2] have provided evidence that the electron-transfer mechanism operating in the yeast sulfite reductase may be expressed as follows:

$$\text{NADPH} \rightleftharpoons \text{flavins} \rightleftharpoons \text{587-m}\mu\ \text{chromophore} \rightharpoonup SO_3^{2-},\ NO_2^-,\ NH_2OH$$

$$\text{flavins} \downarrow \qquad \text{chromophore} \Uparrow \text{MVH}$$

$$\downarrow \text{cytochrome } c, \text{ dyes, } etc.$$

However, more information concerning the properties of the enzyme is desired to reach a decisive conclusion on this problem.

The present study is concerned with further characterization of the purified yeast sulfite reductase. Most of the results described in this paper have been reported in a preliminary form[19].

EXPERIMENTAL PROCEDURE

Materials

The wild-type (haploid) strain of *Saccharomyces cerevisiae* and parathiotrophic mutants derived from it (strains 1, 6, 9, 11, 12, 14, 20, 21 and 24) were kindly supplied by Dr. N. NAIKI[20]. NADPH-sulfite reductase from commercial baker's yeast and an MVH-sulfite reductase from strain 21 were purified as described previously[1,2]. Rabbit liver microsomal NADPH-cytochrome *c* reductase was purified as described elsewhere[21]. Polyethyleneglycol (av. mol. wt., 6 000) and polyvinylpyrrolidone (av. mol. wt., 40 000) were purchased from Wako Pure Chemicals, Ltd. The sources of the other materials employed were cited in the previous papers[1,2].

Preparation of cell-free extracts

The wild-type strain and its mutants were grown with shaking at 30° for 18 h

in media described previously[22]. The cells were harvested by centrifugation, washed and frozen. The frozen cells were thawed, suspended in 3 vol. (v/w) of 0.3 M potassium phosphate–1 mM EDTA, pH 7.3, and sonicated (10 kC, 20 min). The sonicate was centrifuged at 10 000 × *g* for 10 min, and the supernatant was further centrifuged at 105 000 × *g* for 60 min. The supernatant obtained was used as crude cell-free extract. Extracts were also prepared from commercial baker's yeast essential as described above except that the cells were suspended in 1 vol. (v/w) of 0.5 M potassium phosphate-1 mM EDTA, pH 7.3, for the experiments of Fig. 7.

Detachment of FMN from enzyme protein

This was conducted in a room maintained at −5° to minimize the denaturation of protein. A 0.5 ml sample containing 6 mg protein of purified NADPH-sulfite reductase in 0.3 M potassium phosphate–1 mM EDTA, pH 7.3, was placed in a 10-ml plastic centrifuge tube kept in an icebath, and 2 ml of 70% saturated ammonium sulfate solution was added. The tube was then centrifuged at 10 000 × *g* for 10 min. The protein precipitated was mixed with 1.5 ml of 50% saturated ammonium sulfate that had been adjusted to either pH 2.7 with sulfuric acid or pH 3.5 with acetic acid. The mixture was stirred for 1 min and then centrifuged at 10 000 × *g* for 5 min. The supernatant containing detached FMN was removed by decantation as completely as possible. The residue was washed 5 times with the acid ammonium sulfate solution (pH 2.7 or 3.5), exactly as described above, to effect the removal of FMN as completely as possible. After the final washing, the protein precipitate was dissolved in 2.0 ml of 0.3 M potassium phosphate, pH 7.7, and then centrifuged at 10 000 × *g* for 10 min to remove a small amount of insoluble protein. The brown-coloured clear solution containing "aporeductase" was stored at −20° until use.

Heat treatment

The enzyme in a small test tube (7 mm inner diameter) was heated quickly in a water bath maintained at a desired temperature, kept at the temperature for a desired period of time, and cooled immediately in an icebath.

Ultraviolet irradiation

The enzyme solution placed in a quartz spectrophotometer cuvette (1 cm light path) was irradiated from the side with a Toshiba SHL-100UV lamp at a distance of 17 cm through a Toshiba UV-D2 filter. The filter had its maximal transmittance at 365 mμ, showing 10% of the maximal transmittance at 310 and 400 mμ.

Spectrophotometry

Spectrophotometric measurements were carried out in a Cary 14 spectrophotometer. The intensities of absorption peaks were calculated from the spectrum according to the following equations:

$$\text{Intensity of 386-m}\mu\text{ peak} = A_{386\ m\mu} - \tfrac{1}{2}(A_{330\ m\mu} + A_{442\ m\mu}),$$

$$\text{Intensity of 455-m}\mu\text{ peak} = A_{455\ m\mu} - A_{520\ m\mu},$$

$$\text{Intensity of 587-m}\mu\text{ peak} = A_{587\ m\mu} - \tfrac{1}{2}(A_{560\ m\mu} + A_{614\ m\mu}).$$

Density-gradient centrifugation

Sucrose density-gradient centrifugation was carried out in the swinging bucket

rotor (RPS 40) of the Hitachi 40P centrifuge according to the method of MARTIN AND AMES[23], as described previously[1]. Purified yeast alcohol dehydrogenase (EC 1.1.1.1) or beef liver catalase (EC 1.11.1.6) was employed as internal marker for the sedimentation coefficient. The sedimentation coefficients for alcohol dehydrogenase and catalase were assumed to be 7.4 S and 11.3 S, respectively[23].

Fluorescence measurements

An Aminco-Bowman spectrophotofluorimeter with a xenon arc lamp was used.

Electron spin resonance spectroscopy

ESR spectra were measured in a Varian V-4500 spectrometer equipped with a 100 kC field modulation unit and a V-4547 variable temperature attachment. Quartz sample tubes of 3 mm inner diameter were used at low temperatures. A field modulation amplitude of 15 gauss was used. For anaerobic measurements, quartz tubes (also 3 mm inner diameter) sealed with a stopper having two side arms were employed.

Other assay methods

All the other methods, including those for protein determination and for enzyme assays, were described in the previous papers[1,2].

RESULTS

Acid ammonium sulfate treatment

Yeast NADPH-sulfite reductase contains one mole each of FMN and FAD per 350 000 g of protein[1]. Quantitative detachment of both flavins could be effected by heat (100°, 3 min) or by trichloroacetic acid, but this resulted in complete denaturation of the enzyme protein. Treatment of the enzyme with 50% saturated ammonium sulfate at pH 2.7 or 3.5 (but not at pH's higher than 3.5), however, caused selective release of FMN, leaving most FAD still attached to the precipitated protein, which

TABLE I

SELECTIVE DETACHMENT OF FMN FROM NADPH-SULFITE REDUCTASE

Purified NADPH-sulfite reductase (specific activity, 1.85 unit/mg protein; FMN content, 2.84 mμmoles/mg protein; FAD content, 2.84 mμmoles/mg protein) was treated at indicated pH with ammonium sulfate as described in EXPERIMENTAL PROCEDURE, and flavin contents and NADPH-sulfite reductase activity were measured for the supernatant and precipitated protein.

Exp.	*pH*	*Fraction*	*FMN**	*FAD**	*NADPH-sulfite activity (unit/mg protein)*
1	2.7	Protein	0.02	2.47	0.002
		Supernatant	2.46	0.06	—
2	3.5	Protein	0.27	2.56	0.087
		Supernatant	2.45	0.46	—
3	3.5	Protein	0.29	3.08	0.197
		Supernatant	2.45	0.53	—

* Flavin contents in protein fractions were expressed as mμmoles per mg of protein, and their contents in supernatant as mμmoles per mg of original protein.

was called "aporeductase" (Table I). The aporeductase thus obtained still retained a low activity of NADPH-sulfite reductase, reflecting a small amount of FMN remaining in the preparation. SIEGEL AND KAMIN[4] have reported similar selective detachment of FMN by PCMB from purified *E. coli* sulfite reductase.

Reactivation of aporeductase by flavins

Although PRABHAKARARAO AND NICHOLAS[3] have reported the stimulation of the purified yeast enzyme by FMN, FAD and riboflavin, the NADPH-sulfite reductase activity of our enzyme was not affected by the addition of these flavins. The addition of FMN, however, could stimulate the NADPH-sulfite reductase and other NADPH-linked activities of the aporeductase obtained above (Table II). Because of consider-

TABLE II

REACTIVATION OF APOREDUCTASE BY FLAVINS

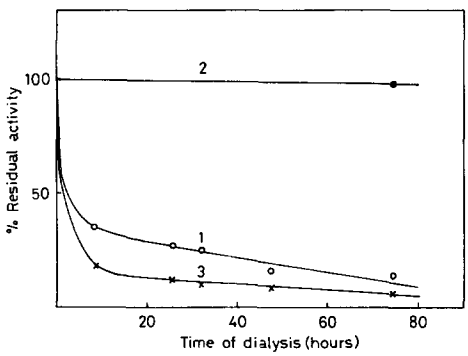
Preparations 1 and 2 were the precipitated protein fractions obtained in Experiments 1 and 2, respectively, of Table I. The NADPH-sulfite and NADPH-cytochrome *c* activities of Preparation 1 were 0.1 and 1.5%, respectively, of the native enzyme, whereas those of Preparation 2 were 4.7 and 20%, respectively. The aporeductase preparations were incubated in 0.2 M potassium phosphate buffer, pH 7.3, containing 1 μM FMN or FAD (final concentration) for 10 min at 25°, and the reductions of indicated acceptors by NADPH were measured under the standard assay conditions[1].

Prep.	*Acceptor*	*Concentration (mM)*	*Fold increase in activity with*	
			FMN	*FAD*
1	Na_2SO_3	1	ca. 30*	ca. 20*
	Cytochrome *c*	0.0024	41	27
	Na_2SO_4	1	4.0	2.4
	$NaNO_2$	20	5.8	2.2
	Cytochrome *c*	0.0024	5.6	3.3
2	DCPI	0.1	4.0	2.7
	Menadione	0.1	3.0	2.1
	Ubiquinone Q_0	0.2	4.9	3.2
	Oxygen	0.24**	3.9	2.8

* The NADP-sulfite reductase activity of this preparation in the absence of flavins could not be measured accurately because of the very low activity.

** Saturated with air.

able denaturation of the enzyme protein during the acid ammonium sulfate treatment, the reactivation was by no means complete. The addition of FAD, though less effective than FMN, could also stimulate the activities of the aporeductase. Simultaneous addition of both FMN and FAD caused the same effect as in the case of the addition of FMN alone, indicating the preferential utilization of FMN for reactivation. The concentrations of flavins required for reactivation were very low; half-maximal reactivation was attained at 0.03 μM FMN or FAD. The MVH-sulfite and -NADP$^+$ reductase activities of the aporeductase were also restored by FMN or FAD, but the extents of restoration were much smaller than the NADPH-linked activities. Riboflavin failed to stimulate the NADPH-sulfite reductase activity, but enhanced the NADPH-cytochrome *c* reductase activity slightly.



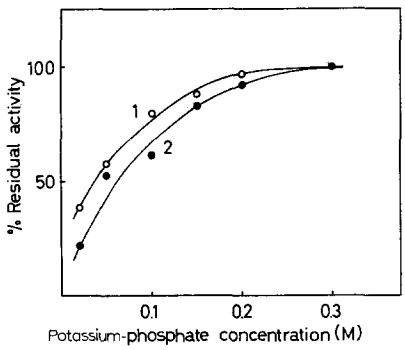


Fig. 1. Decay of catalytic activities of purified yeast NADPH-sulfite reductase on exposure to a low salt concentration. A solution containing 13.8 mg of the purified enzyme (specific NADPH-sulfite reductase activity, 1.85 unit per mg) per ml of 0.3 M K-phosphate (pH 7.3)-1 mM EDTA was dialyzed at 0° against 0.01 M K-phosphate (pH 7.3)-1 mM EDTA for the indicated period of time, and the residual NADPH-sulfite reductase (*Curve 1*, ○—○), MVH-sulfite reductase (*Curve 2*, ●—●) and NADPH-cytochrome *c* reductase (*Curve 3*, ×—×) activities were measured

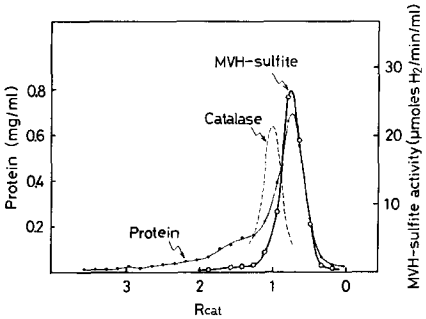
Fig. 2. Effect of exposure to various phosphate concentrations on catalytic activities of yeast NADPH-sulfite reductase. A solution containing 0.81 mg of the enzyme (specific NADPH-sulfite reductase activity, 1.01 unit per mg) per ml of the indicated concentration of K-phosphate buffer (pH 7.3) containing 1 mM EDTA was allowed to stand at 0° for 18 h, and the residual NADPH-sulfite reductase (*Curve 1*, ○—○) and NADPH-cytochrome *c* reductase (*Curve 2*, ●—●) activities were measured.

Inactivation at low ionic strength

The unusual instability of the NADPH-sulfite reductase activity of the yeast enzyme to low salt concentrations has been noticed by several workers[1,22,24,25]. Exposure of the purified yeast sulfite reductase to 10 mM potassium phosphate-1 mM EDTA, pH 7.3, at 0° did cause the inactivation of both the NADPH-sulfite and -cytochrome *c* reductase activities, which decayed according to first-order kinetics (Fig. 1). In confirmation of NAIKI's finding[22], the MVH-sulfite reductase activity was quite stable to this treatment. The lability of the NADPH-linked activities could also be shown in an experiment in which the enzyme was kept at 0° for 18 h in various concentrations of potassium phosphate buffers, pH 7.3, containing 1 mM EDTA (Fig. 2). It will be seen that complete protection of the enzyme could be attained in 0.3 M phosphate buffer. Low salt concentrations also inactivated the MVH-$NADP^+$ reductase activity completely. Despite such inactivation, the absorption spectrum of the enzyme was not appreciably altered by the treatment. Sucrose (1 M) and polyethyleneglycol (50 mg/ml) showed only slight stabilizing effects at low phosphate concentrations; polyvinylpyrrolidone (50 mg/ml) and bovine serum albumin (50 mg/ml) rather stimulated the inactivation.

Sedimentation behavior after low ionic strength treatment

When the enzyme whose NADPH-linked activities had been inactivated by prolonged exposure to low ionic strength was subjected to sucrose density gradient centrifugation, using bovine liver catalase as internal marker, it was found that the remaining MVH-sulfite reductase activity as well as the bulk of the enzyme protein showed a sedimentation coefficient of about 7 S (Fig. 3). This value was considerably



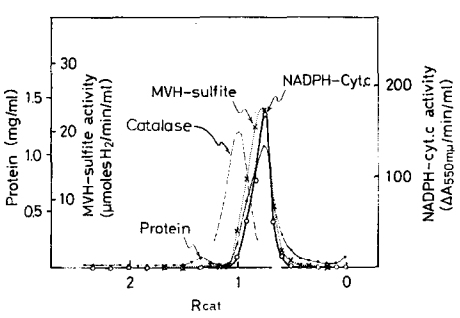


Fig. 3. Sucrose density gradient sedimentation pattern of NADPH-sulfite reductase after prolonged exposure to a low salt concentration. The enzyme was dialyzed at 4° for 72 h against 0.01 M K-phosphate (pH 7.3)-1 mM EDTA before being subjected to density gradient centrifugation. A 0.11 ml solution (in 0.3 M K-phosphate-1 mM EDTA, pH 7.3) containing 1.46 mg of the treated enzyme and 12.8 μg of beef liver catalase was layered over 4.3 ml of a linear sucrose gradient (5–20%, w/v) containing 0.3 M K-phosphate (pH 7.3)-1 mM EDTA. The tube was centrifuged at 36 000 rev./min for 6 h in a swinging bucket rotor of a Hitachi 40P centrifuge. The bottom of the tube was punctured, and 24 fractions were collected. The fractions were assayed for protein, MVH-sulfite reductase activity, and catalase activity.

Fig. 4. Sucrose density-gradient centrifugation of purified NADPH-sulfite reductase in the presence of a low salt concentration. A 0.1 ml solution (in 0.3 M K-phosphate-1 mM EDTA, pH 7.3) containing 0.67 mg of NADPH-sulfite reductase and 6.4 μg of catalase was layered over 4.3 ml of a linear sucrose gradient (5–20%, w/v) containing 0.01 M K-phosphate (pH 7.3)-1 mM EDTA. The tube was centrifuged for 8 h as described in Fig. 3. The fractions were immediately made 0.3 M with respect to K-phosphate to prevent further inactivation, and assayed for protein, catalase activity, MVH-sulfite reductase activity and NADPH-cytochrome *c* reductase activity.

lower than 14.8 S for the native enzyme[1]. A similar reduction in the sedimentation coefficient (to about 9 S) was also observed when the native enzyme (without previous treatment at low ionic strength) was directly subjected to sucrose density gradient centrifugation in the presence of a low concentration of phosphate (Fig. 4). Since the exposure time of the enzyme to low ionic strength was much shorter under these conditions, the enzyme still retained considerable NADPH-cytochrome *c* reductase activity, in addition to the stable MVH-sulfite reductase activity. It is interesting to note that both the MVH-sulfite and NADPH-cytochrome *c* reductase activities were associated with the protein having a lowered sedimentation coefficient.

Restoration of NADPH-sulfite reductase activity after low ionic strength treatment

Since the low ionic strength treatment inactivated the NADPH-cytochrome *c* reductase activity but gave no effect on the MVH-sulfite reductase activity, it seemed of interest to see if the NADPH-sulfite reductase activity could be reconstituted by adding NADPH-cytochrome *c* reductase to the enzyme inactivated by low ionic strength. However, no restoration of the activity was observed when the inactivated enzyme was mixed with NADPH-cytochrome *c* reductase purified from rabbit liver microsomes[21], or with the yeast sulfite reductase that had been treated with KCN in the presence of NADPH to abolish reactivity with sulfite[1].

In the course of these attempts, it was found that extracts of a yeast mutant, strain 11, contained a factor which could partly restore the lost NADPH-sulfite reductase activity of the enzyme that had been exposed to low ionic strength. Strain 11 has been shown to contain only the MVH-sulfite, but not the NADPH-sulfite reductase

TABLE III

EFFECTS OF FRACTIONS FROM STRAIN 11 ON NADPH-SULFITE REDUCTASE ACTIVITY OF THE ENZYME EXPOSED TO LOW IONIC STRENGTH

Partially purified yeast NADPH-sulfite reductase (specific NADPH-sulfite activity, 0.30 unit/mg protein) was dialyzed for 48 h at 4° against 10 mM potassium phosphate buffer-1 mM EDTA, pH 7.3, to inactivate its NADPH-linked activities almost completely (NADPH-cytochrome *c* reductase activity, $\Delta A_{550m\mu}$/min/mg protein = 0.080; MVH-sulfite reductase activity, 3.6 μmoles H_2/min/mg protein). The 0.1 M and 0.3 M phosphate eluates from DEAE-cellulose were prepared from crude extracts of strain 11 as described previously[2]. The NADPH-sulfite reductase activity was measured by determining sulfide production as described previously[1]. The reaction was carried out at 30° for 60 min.

Inactivated sulfite reductase added (mg)	*0.1 M phosphate eluate added (mg)*	*0.3 M phosphate eluate added (mg)*	*H_2S produced (mμmole)*
4.1	0	0	10
0	30	0	8
0	0	5.0	0
4.1	30	0	68
4.1	0	5.0	18
0	30	5.0	11
4.1	30	5.0	66

activity[2,20]. The active fraction, isolated from strain 11 as a 0.1 M phosphate eluate from DEAE-cellulose as described previously[2], possessed by itself little activities of MVH-sulfite and NADPH-cytochrome *c* reductase. Nevertheless, supplementation of the low salt-inactivated enzyme with this fraction led to partial restoration of the

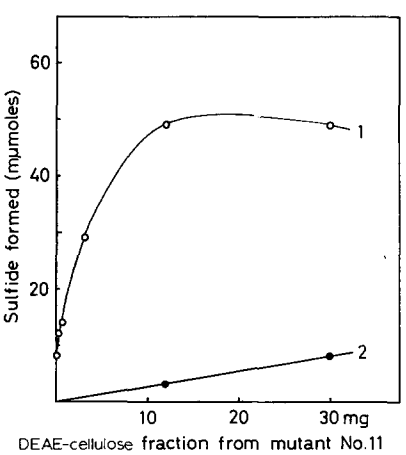


Fig. 5. Restoration of NADPH-sulfite reductase activity of the yeast enzyme exposed to low ionic strength by a fraction from mutant strain 11. The yeast enzyme was inactivated by dialyzing at 4° for 48 h against 0.01 M K-phosphate (pH 7.3)-1 mM EDTA. The 0.1 M DEAE fraction from strain 11 was prepared as described in the text. The NADPH-sulfite reductase activity was measured by determining the sulfide production at 30° for 60 min, as described previously[1]. Each manometric vessel contained 4.1 mg of the inactivated sulfite reductase and the indicated amount of 0.1 M DEAE fraction. *Curve 1* (○—○), activity with inactivated enzyme plus 0.1 M DEAE fraction; *Curve 2* (●—●), activity with 0.1 M DEAE fraction alone.

NADPH-sulfite reductase activity (Table III). Although the 0.3 M phosphate eluate of strain 11 extracts showed much higher activities of MVH-sulfite and NADPH-cytochrome *c* reductase than the 0.1 M eluate, the former fraction could not restore the activity significantly. The NADPH-sulfite reductase activity of the treated enzyme (4.1 mg of protein) increased, by the addition of increasing amounts of the 0.1 M eluate, up to 10 mg of protein, where the activity became saturated (Fig. 5).

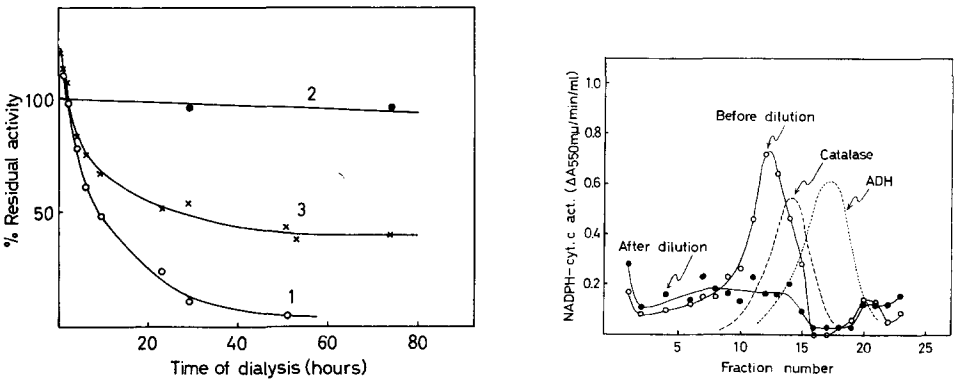


Fig. 6. Decay of catalytic activities of cell-free extract from commercial baker's yeast on exposure to a low salt concentration. A cell-free extract prepared from commercial baker's yeast as described in EXPERIMENTAL PROCEDURE (7.48 mg protein/ml; NADPH-sulfite reductase activity, 0.73 mμ-moles H_2S/min/mg protein) was dialyzed at 0° against 0.01 M K-phosphate (pH 7.3)-1 mM EDTA for the indicated period of time, and the residual NADPH-sulfite reductase (assayed by determining the sulfide production[1]), MVH-sulfite reductase activity and NADPH-cytochrome *c* reductase activity (assayed in the presence of 1 mM KCN) were determined. *Curve 1* (○—○), NADPH-sulfite reductase activity; *Curve 2* (●—●), MVH-sulfite reductase activity; *Curve 3* (×—×), NADPH-cytochrome *c* reductase activity.

Fig. 7. Sucrose density-gradient centrifugation of cell-free extract and effect of low ionic strength on NADPH-cytochrome *c* reductase activity. A cell-free extract from commercial baker's yeast was dialyzed at 0° for 18 h against 0.3 M K-phosphate (pH 7.3)-1 mM EDTA. The dialyzed extract (0.2 ml, 6.1 mg of protein) was layered over 4.3 ml of a linear sucrose gradient (5–20%, w/v) containing 0.3 M K-phosphate (pH 7.3)-1 mM EDTA. After centrifugation for 7 h at 40 000 rev./min, 23 fractions were collected. Each fraction was divided into 2 portions. One portion was stored at 4° for 48 h, and assayed for NADPH-cytochrome *c* reductase, catalase and alcohol dehydrogenase activities. The other portion was diluted 30-fold with water to a phosphate concentration of 0.01 M, stored at 4° for 48 h and assayed for NADPH-cytochrome *c* reductase activity. NADPH-cytochrome *c* reductase activity was measured in the presence of 1 mM KCN. — — —, catalase activity; – – – –, alcohol dehydrogenase (ADH) activity; ○—○, NADPH-cytochrome *c* reductase activity before dilution; ●—●, NADPH-cytochrome *c* reductase activity after dilution.

Similar restoration could also be observed with the 0.1 M eluate obtained from another mutant, strain 6. It should, however, be emphasized that the observed restoration was only to very limited extents; the maximally restored activity was 0.5% or less of that of the native enzyme.

Low-ionic-strength treatment of cell-free extracts

The inactivation of NADPH-sulfite reductase could also be observed when a crude cell-free extract of the wild-type strain was exposed to a low salt concentration (Fig. 6). The data further indicate that two types of NADPH-cytochrome *c* reductase

activity were present in the extract, one unstable and the other stable to low ionic strength. It seemed that the unstable activity was due to the action of the sulfite reductase, which constituted more than half the total NADPH-cytochrome *c* reductase in the extract. Sucrose density gradient centrifugation of a crude extract from commercial baker's yeast in the presence of a high concentration of phosphate revealed that the NADPH-cytochrome *c* reductase activity was widely distributed through the gradient with one major peak corresponding to a sedimentation coefficient of about 14 S (Fig. 7). This major peak was, however, sensitive to low ionic strength and disappeared when stored at a low phosphate concentration. From its sedimentation coefficient (about 14 S)[1] and sensitivity to low ionic strength, it was evident that the major peak represented the NADPH-cytochrome *c* reductase activity of the sulfite reductase. It was also noticed that the stable activity represented more than one enzymatic entity.

Lack of sensitive NADPH-cytochrome c reductase activity in mutants

Table IV shows the results of low-ionic-strength treatment of crude cell-free extracts from the wild-type and nine parathiotrophic mutant strains isolated by NAIKI[20]. About 50% or more of the total NADPH-cytochrome *c* reductase activity was labile to exposure to 0.01 M phosphate buffer in extracts from the wild-type yeast, commercial baker's yeast and mutant strain 12, all of which contained active NADPH-sulfite reductase activity. However, the low-ionic-strength treatment of extracts from 8 mutant strains lacking the NADPH-sulfite reductase activity resulted

TABLE IV

STABILITY OF NADPH-CYTOCHROME *c* REDUCTASE ACTIVITY TO LOW IONIC STRENGTH IN EXTRACTS FROM VARIOUS YEAST STRAINS

Crude cell-free extracts (3–8 mg protein/ml) from various yeast strains were dialyzed at 0° for 48 h against 0.3 M or 0.01 M potassium phosphate buffer, pH 7.3, containing 1 mM EDTA. NADPH-sulfite reductase activity was assayed by determining the production of H_2S (ref. 1) and expressed as mμmoles H_2S/min/mg protein. NADPH-cytochrome *c* reductase activity was measured by the standard method[1] except that 1 mM KCN was added to the reaction mixture; this was expressed as ΔA (550 mμ)/min/mg protein.

Strain	*MVH-sulfite activity*	*in 0.3 M buffer*		*in 0.01 M buffer*	*B/A*
		NADPH-sulfite activity	*NADPH-cyt. c activity (A)*	*NADPH-cyt. c activity (B)*	
Wild-type Commercial baker's yeast	+	0.45	0.390	0.176	0.45
12*	+	0.64	0.334	0.141	0.42
6†	+	0.36	0.177	0.081	0.46
11†	+	0.01	0.230	0.272	1.18
20†	+	0.00	0.132	0.147	1.11
21†	+	0.01	0.087	0.104	1.20
1†	+	0.00	0.080	0.085	1.06
9†	−	0.00	0.182	0.207	1.14
14†	−	0.00	0.161	0.191	1.19
24†	−	0.00	0.088	0.087	0.99
			0.067	0.086	1.28

* Mutant blocked between PAPS and sulfite.
† Mutants blocked between sulfite and sulfide.

in no appreciable inactivation of the NADPH-cytochrome *c* reductase activity. It was of special interest that even the mutants lacking the MVH-sulfite reductase activity (strains 1, 9, 14 and 24) contained no sensitive NADPH-cytochrome *c* reductase activity. It was concluded that all the yeast mutants incapable of reducing sulfite by NADPH contained no NADPH-cytochrome *c* reductase activity associated with NADPH-sulfite reductase or its genetically modified forms.

Heat inactivation

Heat treatment of the purified yeast sulfite reductase resulted in decreases in absorption peaks at 587 and 386 mμ as well as a deformation of spectral shape due to flavins. It was found that there was a good correlation between the decrease in the intensity of the 587-mμ peak and the loss of the MVH-sulfite reductase activity on treatment of the enzyme at various temperatures (Fig. 8). The time-course of inacti-

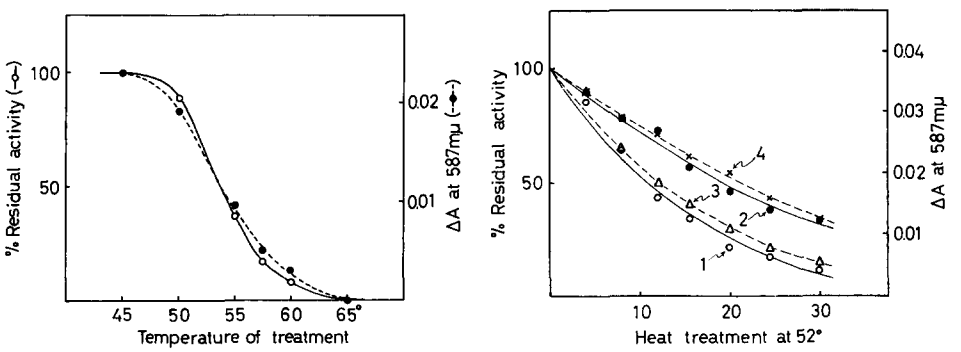


Fig. 8. Inactivation of MVH-sulfite reductase activity and disappearance of 587-mμ peak by heat treatment. A purified enzyme preparation, having a specific NADPH-sulfite reductase activity of 1.21 unit per mg, was exposed to 0.01 M K-phosphate (pH 7.3)-1 mM EDTA at 4° for 72 h to inactivate the NADPH-linked activities. The treated enzyme (1.14 mg/ml) was maintained at the indicated temperature for 3 min, cooled immediately and assayed for MVH-sulfite reductase activity (○—○) and for intensity of the 587-mμ peak (●— — —●). For the definition of the intensity of the 587-mμ peak, see EXPERIMENTAL PROCEDURE.

Fig. 9. Time-courses of inactivation of catalytic activities and disappearance of 587-mμ peak on treatment of the enzyme at 52°. A purified enzyme preparation, having a specific NADPH-sulfite reductase activity of 1.21 unit per mg, dissolved in 0.2 M K-phosphate (pH 7.3)-1 mM EDTA at a concentration of 1.7 mg per ml was maintained at 52° for the indicated period of time and immediately cooled. NADPH-sulfite reductase activity (*Curve 1*, ○—○), MVH-sulfite reductase activity (*Curve 2*, ●—●), NADPH-cytochrome *c* reductase activity (*Curve 3*, △— — —△) and the intensity of 587-mμ peak (*Curve 4*, ×— — —×) of the heat-treated sample were measured.

vation at 52° could also confirm the close parallelism between the 587-mμ peak and the MVH-sulfite reductase activity (Fig. 9). The data also indicate that both the NADPH-sulfite and -cytochrome *c* reductase activities decayed in parallel; these activities were somewhat more sensitive to heat than the MVH-sulfite reductase activity. These results provided further support to the view that the MVH-sulfite reductase activity is closely related to the 587-mμ chromophore which seems to be the site of sulfite interaction with the enzyme[1,2]. It was also concluded that a factor which is more heat-labile than the chromophore is required for the NADPH-linked activities, but not for the MVH-sulfite reaction. It is still to be elucidated whether or

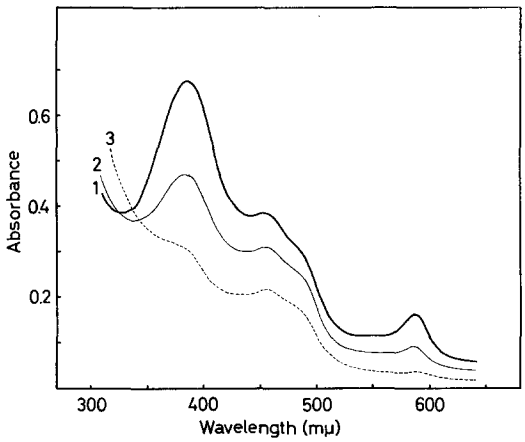


Fig. 10. Spectral deformation of NADPH-sulfite reductase by near-ultraviolet irradiation. A solution containing 3.38 mg of purified enzyme (specific NADPH-sulfite reductase activity, 1.85 unit/mg) per ml of 0.2 M K-phosphate (pH 7.3)-1 mM EDTA was irradiated with light having maximum intensity at 365 mμ, as described in EXPERIMENTAL PROCEDURE. *Curve 1*, before irradiation; *Curve 2*, after 6-h irradiation; *Curve 3*, after 16-h irradiation.

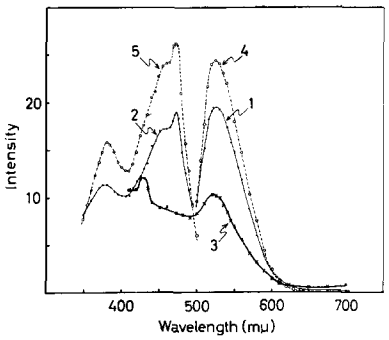
not this heat-labile factor is related to the sensitivity of the NADPH-linked activities toward low salt concentrations.

Ultraviolet irradiation

Near-ultraviolet irradiation (maximum intensity at 365 mμ) of the purified enzyme for 16 h inactivated the NADPH-sulfite, NADPH-cytochrome *c* and MVH-sulfite reductase activities completely. The irradiation also caused decreases in the heights of absorption peaks (Fig. 10). When the activities and absorption peaks were followed at 1–2 h intervals, it was found that the inactivation proceeded in parallel to the decreases in the intensities of the two peaks at 587 and 386 mμ, but no clear correlation was found between the activities and the 455-mμ peak. On the other hand, the irradiation resulted in a 42% loss of FMN, but only a 2% loss of FAD. Both the NADPH-sulfite and -cytochrome *c* reductase activities of the irradiated enzyme could be significantly restored by addition of FMN. A MVH-sulfite reductase from a yeast mutant, strain 21, has been shown to lack flavins[2]. When this mutant enzyme was irradiated, it was more clearly shown that the decrease in the height of the 587 mμ peak was accompanied by a corresponding decrease in the 386-mμ peak. The inactivation of the MVH-sulfite reductase activity was a linear function of the decreases in the heights of these peaks. In confirmation of the previous suggestion[1,2], it was concluded that the 587-mμ chromophore absorbs also in the 386-mμ region.

Fluorescence spectra

Fluorescence spectra of the enzyme revealed the presence of at least two different fluorescent species. These two species could be distinguished from each other not only by emission spectrophotometry but also by excitation spectrophotometry. The excitation spectrum for emission at 525 mμ was closely similar to that for flavin (Fig. 11). Assuming that this was due to the prosthetic FMN alone, it was estimated that



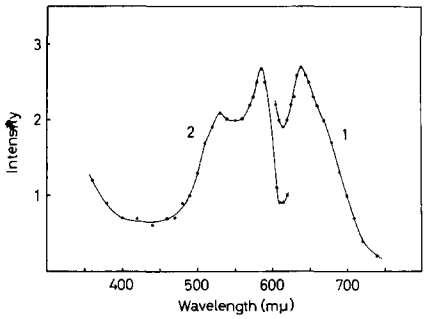


Fig. 11. Fluorescence spectra of NADPH-sulfite reductase and riboflavin. Spectra were measured with a purified preparation of NADPH-sulfite reductase in 0.2 M K-phosphate (pH 7.3)-1 mM EDTA (1.39 mg/ml) or with an aqueous solution of riboflavin (5.2×10^{-8} M). *Curve 1*, emission spectrum of the enzyme activated at 525 mμ; *Curve 2*, excitation spectrum of the enzyme for emission at 525 mμ; *Curve 3*, emission spectrum of the enzyme activated at 378 mμ; *Curve 4*, emission spectrum of riboflavin activated at 470 mμ; *Curve 5*, excitation spectrum of riboflavin for emission at 525 mμ. Light intensity is given in an arbitrary unit.

Fig. 12. Fluorescence spectra of NADPH-sulfite reductase (continued). The same enzyme solution as in Fig. 11 was used. *Curve 1*, emission spectrum activated at 585 mμ; *Curve 2*, excitation spectrum for emission at 640 mμ.

the fluorescence intensity of this FMN was about 1% of that for free flavin. The excitation spectrum obtained for emission at 460 mμ was characteristically different from that at 525 mμ, though the former was weaker than the latter. The excitation spectrum for emission at 640 mμ showed a peak at 585–590 mμ with a shoulder around 530 mμ (Fig. 12), suggesting that the emission at 640 mμ was related to the 587-mμ chromophore.

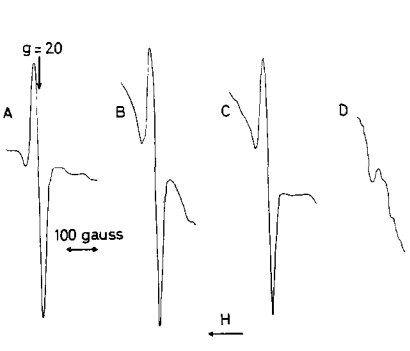


Fig. 13. ESR spectra of sulfite reductases. *A*, 2.7 mg of purified NADPH-sulfite reductase dissolved in 0.2 ml of 0.3 M K-phosphate (pH 7.3)-1 mM EDTA was mixed with 1 μmole of glucose 6-phosphate, 1.7 unit of glucose 6-phosphate dehydrogenase and 0.1 μmole of $NADP^+$ in a final volume of 2.05 ml; the sample was incubated for 5 min at 20° and then frozen. *B*, Sample A was evacuated, incubated for 10 min at 20° and then frozen. *C*, 0.05 ml of 10% sodium dithionite (in 5% sodium bicarbonate) was added to Sample B which was then frozen. *D*, 0.39 mg of purified MVH-sulfite reductase from mutant strain 21 (ref. 2), dissolved in 0.2 ml of 0.3 M K-phosphate (pH 7.3)-1 mM EDTA, was mixed with 0.05 ml of 10% sodium dithionite (in 5% sodium bicarbonate) and then frozen. The measurements were carried out at 121°K. Modulation amplitude was 15 gauss. The arrow at the bottom shows the direction of increasing magnetic field.

ESR spectroscopy

The oxidized form of yeast NADPH-sulfite reductase in the frozen state showed no electron-spin-resonance (ESR) signals in the g value region from 1–7. On reduction of the enzyme with NADPH, however, a sharp, symmetrical signal appeared with an absorption maximum (g_m) value of 2, which seemed to be due to the formation of a free radical (Fig. 13, Curve A). On evacuation or addition of dithionite, no significant changes were observed in the signal amplitude of the NADPH-reduced enzyme (Fig. 13, Curves B and C). The addition of KCN to the reduced enzyme also failed to affect the signal appreciably. Although the mutant MVH-sulfite reductase from strain 21 has been shown to be devoid of significant amounts of flavins[2], this enzyme showed a small but significant signal at $g = 2$ when reduced by dithionite (Fig. 13, Curve D). The saturation effect of microwave power on the ESR signal of the reduced native enzyme was also measured. Saturation of the signal became noticeable at high microwave power. The temperature dependence was determined according to the method of EHRENBERG[26]. The constant of temperature dependency (α) thus obtained was 154° K.

DISCUSSION

The data presented here provide a variety of information concerning the properties of yeast NADPH-sulfite reductase, which is a unique, multifunctional enzyme containing FMN, FAD, iron, acid-labile sulfide and a chromophore absorbing at 587 mμ[1,2].

One of the remarkable properties of the yeast enzyme is the unusual instability of its NADPH-linked activities, but not the MVH-sulfite reductase activity, toward low salt concentrations. The inactivation of NADPH-linked activities at low ionic strength is accompanied by a significant decrease in the sedimentation coefficient for the enzyme protein from 14.8 S to about 7 S (Fig. 3), suggesting the occurrence of a marked change in protein conformation or dissociation into smaller subunits. The possibility of dissociation, however, can not yet be stated with certainty, because only one major peak (about 7 S) is seen in sucrose density gradient centrifugation of the inactivated enzyme (Fig. 3). Furthermore, after brief exposure of the enzyme to low ionic strength, both the MVH-sulfite reductase activity and the still-remaining NADPH-cytochrome *c* reductase activity are associated with the protein having a lowered sedimentation coefficient of about 9 S (Fig. 4). It appears that a factor sensitive to low ionic strength is involved in the NADPH-linked activities, but not in the MVH-sulfite reductase activity. It is also likely that destruction of this factor by low salt concentrations results in a profound conformational change (or fragmentation into smaller units) of the enzyme protein. The nature of the sensitive factor still remains to be elucidated, but the finding that a protein fraction from mutant strains 11 and 6 restores the NADPH-sulfite reductase activity of the low-salt inactivated enzyme to limited extents (Table III, Fig. 5) seems to be promising in this respect. It is also interesting that the NADPH-linked activities are more heat-labile than the MVH-sulfite reductase (Fig. 9).

Taking advantage of the lability of the enzyme to low ionic strength, it is possible to demonstrate the presence in crude yeast extracts of 2 types of NADPH-cytochrome *c* reductase activity: one associated with the sulfite reductase and sensitive

to low ionic strength, and the other stable to low salt concentrations (Fig. 6). The stable activity does not seem to represent a single enzymatic entity; it shows a wide distribution in sucrose density gradient centrifugation (Fig. 7).

NAIKI[22] has reported that yeast mutants genetically blocked between sulfite and sulfide in the sulfate assimilation pathway can be divided into 2 groups; although both groups of mutants lack the NADPH-sulfite reductase activity, strains of one group (but not of the other) possess an activity to reduce sulfite by MVH. SIEGEL *et al.*[10], on the other hand, have also described two groups of *S. typhimurium* mutants incapable of reducing sulfite; those which have lost the ability to reduce sulfite by NADPH, and those which still retain the NADPH-cytochrome *c* reductase activity. These observations suggest that some of the yeast mutants lacking the MVH-sulfite reductase activity may contain an NADPH-cytochrome *c* reductase activity which is labile to low ionic strength. However, all the mutants having defects in the sulfite-reducing step have been shown to be devoid of such a labile activity (Table IV). This might suggest that the NADPH-cytochrome *c* reductase portion of the enzyme synthesized in some of the mutants is labile even *in vivo* unless it combines with the portion carrying the 587-mμ chromophore and the MVH-sulfite reductase activity.

Our previous findings, including the KCN inhibition of sulfite reduction by both NADPH and MVH, the protection from this inhibition by sulfite, the spectral interaction of the enzyme with KCN in the presence of NADPH, and the invariable presence of the 587-mμ absorption peak in all the enzymes possessing the MVH-sulfite reductase activity from several mutants, suggest that the 587-mμ chromophore is involved in both the NADPH- and MVH-sulfite reductase activities[1,2]. It seems also likely that the chromophore is the terminal component of the electron-transfer system in the native enzyme and is directly responsible for the interaction of the enzyme with sulfite and probably also with nitrite and hydroxylamine. The finding in the present study that heat inactivation of the MVH-sulfite reductase activity parallels the decrease in the height of 587-mμ peak (Figs. 8 and 9) lends further support to the close correlation between the chromophore and the MVH-linked activity. The fact that the NADPH-linked activities are more heat labile than the MVH-sulfite activity (Fig. 9) is also consistent with the terminal location of the 587-mμ chromophore.

The parallel destruction of the 587- and 386-mμ peaks by ultraviolet irradiation (Fig. 11) confirms the previous suggestion that the 587-mμ chromophore also absorbs in the 386-mμ region[1,2]. The chromophore also seems to have a small absorption peak at 710 mμ[2]. Furthermore, fluorescence studies show that the emission at 640 mμ is due to the chromophore (Fig. 13). The spectral properties of the chromophore together with its reactivity with KCN may suggest its heme nature as reported by other workers for the yeast[3] and *E. coli* enzymes[4,5] as well as for an MVH-sulfite reductase from spinach[16]. However, it has been impossible for us to detect a heme component in the enzyme by the pyridine hemochromogen test[1]. It, therefore, seems that if the 587-mμ chromophore actually contains a heme, it must be a rather atypical one, as has been stated for the *E. coli* enzyme[4].

The reduced, but not oxidized, form of the yeast enzyme shows a sharp signal at $g = 2$ in the ESR spectrum (Fig. 14). Although it seems likely that this signal represents half-reduced flavin prosthetic groups, it can not be solely accounted for by the flavin radicals, because an MVH-sulfite reductase from strain 21, which is deficient in flavins[2], shows a significant signal at the same g value. Working with a

purified *E. coli* sulfite reductase containing flavins, iron, labile sulfide and two heme-like chromophores absorbing at 570 and 620 mμ, SIEGEL AND KAMIN[4] have shown that the oxidized enzyme exhibits a doublet signal at g = 5.27 and 6.7 at 35° K but not at 100° K; they have assigned the species responsible for this signal to a high-spin ferrous iron in the heme-like chromophore absorbing at 620 mμ. It is desirable to measure the ESR spectrum of our enzyme at 35°K. SIEGEL AND KAMIN[4] have also observed that the reduced *E. coli* enzyme shows a g = 2 signal, which is associated with a broad absorbance increase in the 520–700 mμ region, and suggested that this signal is due to the flavin semiquinone.

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