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THE REDUCTION OF SULPHITE, NITRITE AND HYDROXYLAMINE BY AN ENZYME FROM BAKER'S YEAST

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SUMMARY

1. A 300-fold purified enzyme from yeast catalyzed the reduction of (a) SO_3^{2-} to S^{2-}, (b) NO_2^- or NH_2OH to NH_3 and (c) viologen dyes, when NADPH was the hydrogen donor.

2. The ratio of SO_3^{2-}/NO_2^- reduced was 1:3 and that of NO_2^-/NH_2OH reduced was 1:1.5. These ratios were constant throughout the purification of the enzyme.

3. The stoichiometry for NADPH, substrates and products has been determined for the purified enzyme as well as the Michaelis constants for SO_3^{2-}, NO_2^- and NH_2OH, and the inhibitor constants for the alternate substrates.

4. The effects of various inhibitors on the reductase enzymes as well as the effectiveness of electron donor systems for SO_3^{2-} reduction were also studied.

INTRODUCTION

Sulphite reductase preparations from various sources usually exhibit both nitrite and hydroxylamine reductase activities[1-7]. A 200-fold purified sulphite reductase from yeast (EC 1.8.1.2) catalyzed the reduction of NO_2^- and NH_2OH to NH_3 (ref. 8). A further purification and characterization of this enzyme system[9,10] and a comparison of its three reductase activities (SO_3^{2-}, NO_2^- and NH_2OH) was reported recently[11]. In this paper some properties of the enzyme which reduces SO_3^{2-}, NO_2^- and NH_2OH as well as methyl- and benzylviologens are presented.

MATERIALS AND METHODS

Enzyme preparations

Sulphite reductase enzyme fractions (I–V) from commercial baker's yeast were prepared as described earlier[10]. These fractions also contained nitrite and hydroxylamine reductase activities.

Assays for sulphite, nitrite and hydroxylamine reductases

The reductases were assayed in a standard reaction mixture (3 ml) containing

Abbreviations: PCMB, *p*-chloromercuribenzoate; PMS, phenazine methosulphate.

(μmoles): potassium phosphate buffer (pH 7.4), 600; Na_2SO_3, $NaNO_2$ or $NH_2OH \cdot HCl$, 3; enzyme (0.05–0.4 ml) and a NADPH-generating system consisting of $MgCl_2$, 3; $NADP^+$, 0.3; glucose 6-phosphate, 5; and glucose-6-phosphate dehydrogenase, 0.5 (I.U.). The reaction mixtures were incubated for 40 min at 30°, in Quickfit glass tubes (MF 24 type) sealed with rubber serum caps.

The sulphite reductase activity was assayed by determining the S^{2-} produced[10].

The residual SO_3^{2-} was measured as described previously[10].

The product of NO_2^- or NH_2OH reduction was NH_3. After 40 min incubation at 30°, 0.3 ml M acetic acid[8] was injected through the serum cap into the reaction mixture, then an aliquot (1.5 ml) was transferred to a Conway microdiffusion unit. The NH_3 produced after a 3-h incubation period at 37° was determined according to the method of RUSSELL[12]. Reaction mixtures without NO_2^- or NH_2OH were used as controls.

Residual NO_2^- was determined as follows: After 40 min incubation, 0.1 ml zinc acetate and 1.9 ml cold 95 % (v/v) ethanol were added to the reaction mixture to precipitate any residual NADPH which might otherwise interfere with the chemical determination of NO_2^- (ref. 13). The precipitated substances were removed by centrifugation and a suitable portion of the supernatant fraction was used to determine residual NO_2^-, according to the method of HEWITT AND NICHOLAS[14].

NH_2OH was determined by the colorimetric procedure of HAGEMAN *et al.*[15], and the absorbance at 540 nm was measured in an Optica spectrophotometer.

Assay of NADPH–methylviologen reductase

This activity was determined aerobically in a reaction mixture by following the oxidation of NADPH at 340 nm. The reaction mixture in a final volume of 3 ml contained in (μmoles): potassium phosphate buffer (pH 7.4), 600; NADPH, 0.3; methylviologen, 3 and 0.05 ml 300-fold purified enzyme (Fraction V, Table I). The reaction was started by adding 0.1 ml of the dye solution. The NADPH oxidation did not occur in the absence of the dye or if the enzyme was either omitted or boiled.

Electron donor systems

The following electron donor systems were tested in place of the NADPH-generating system in the standard reaction mixture, described above.

(1) NADH-generating system consisted of 0.3 μmole NAD^+, crystalline lactate dehydrogenase (25 μg) and 12 μmoles sodium lactate.

(2) $FADH_2$ was continuously generated under an atmosphere of H_2 in the reaction tube, using 0.3 μmole FAD, 5 mg of D-amino-acid oxidase and 15 μmoles (+)-alanine. Rubber serum caps which closed the reaction tubes ensured complete anaerobic conditions, after the tube had been repeatedly evacuated and flushed with H_2. A hypodermic syringe needle inserted through the serum cap was used to sparge H_2 through the reaction mixture. The reduction reaction was started on injecting a hydrogenated solution (0.5 ml) of (+)-alanine (15 μmoles) and SO_3^{2-} (3 μmoles).

(3) Cytochrome *c* from horse heart (0.3 μmole) was reduced with 12 μmoles sodium lactate and crystalline lactate dehydrogenase cytochrome b_2 from yeast (25 μg protein). The reduction was carried out under H_2 gas as for the $FADH_2$ assay. The reaction was started by injecting a solution of Na_2SO_3 (3 μmoles) previously sparged with H_2, into the reaction mixture.

(4) Benzyl- or methylviologen (0.01 M) was reduced chemically in Quickfit tubes (MF 24) sealed with rubber serum caps, by adding palladized asbestos and sparging with H_2. An appropriate amount (0.1–0.2 ml) of reduced dye solution was injected into the reaction mixture with a hypodermic syringe needle. The residual reduced dye in the reaction tube after a 40-min incubation was autooxidised on adding the reactants used to determine S^{2-} (ref. 10). Reaction mixtures with boiled enzymes were used as controls.

(5) Phenazine methosulphate (PMS) *plus* NADH system contained 0.3 μmole PMS and 3 μmoles NADH. After thoroughly flushing the reaction mixture with H_2, 0.2 ml of a solution containing 3 μmoles each of NADH and Na_2SO_3 similarly sparged with H_2 was injected into the tube.

When other electron donors such as succinate, citrate, glutamate, *etc.* were used, 3 μmoles of either replaced the NADPH-generating system in the standard reaction mixture. When pyruvate was the electron donor, ATP (5 μmoles) and $MgCl_2$ (3 μmoles) were also added.

Cofactors and other reagents

ATP, $NADP^+$, NADPH, were from Calbiochem, Calif., U.S.A.; glucose 6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.40) as crystalline suspension in Na_2SO_4, D-amino-acid oxidase, cytochrome *c* (horse heart Type II), crystalline lactate dehydrogenase Type I (EC 1.1.1.27), and cytochrome b_2 Type IV (EC 1.1.2.3), FMN, FAD, NAD^+, NADH and GSH were obtained from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals were purchased either from British Drug Houses, Poole, Great Britain, or from May and Baker, Degenham, Great Britain. Hydrogen of high purity (99.995%) was supplied by Commonwealth Industrial Gases, Torrensville, Queensland, Australia. Quickfit glass tubes (MF-24 type) were from Quickfit and Quartz, Stone, Staffordshire, Great Britain.

RESULTS

The results in Table I show that the ratio of the enzyme activities for SO_3^{2-}, NO_2^- and NH_2OH was fairly constant throughout a 300-fold purification. The average ratio of the activities of nitrite reductase and sulphite reductase was 3:1, whereas for nitrite/hydroxylamine reductase it was 1:1.5.

Stoichiometry

3 moles NADPH were utilized by the purified enzyme to reduce 1 mole SO_3^{2-} to S^{2-} or NO_2^- to NH_3, while only 1 mole of NADPH was required to reduce NH_2OH to NH_3 (Table II).

Michaelis constants

The K_m values for SO_3^{2-}, NO_2^- and NH_2OH, determined by Lineweaver–Burk plots[16], were 38 μM, 180 μM and 4.5 mM, respectively.

Electron donors

NADPH and reduced viologen dyes were the most effective hydrogen donors (Table III). NADH or reduced PMS, however, were ineffective for SO_3^{2-} reduction.

TABLE I

RELATIVE REDUCTASE ACTIVITIES OF ENZYME DURING PURIFICATION

Sulphite, nitrite and hydroxylamine reductases were assayed by determining the products, S^{2-} and NH_3, as described in MATERIALS AND METHODS. The reaction mixtures contained potassium phosphate buffer (pH 7.4), NADPH-generating system, the enzyme and optimum amounts of substrates (see K_m values). Equal amounts of enzyme (0.05–0.4 ml) were used for assaying the three reductases for any given fraction. Fractions I–V contained 25, 20, 4, 0.84 and 2.0 mg protein per ml, respectively. Reaction mixtures without the substrates were used as controls.

Fraction	*Sulphite reductase* (S^{2-}, *nmoles*) (*A*)	*Nitrite reductase* (NH_3, *nmoles*) (*B*)	*Hydroxylamine reductase* (NH_3, *nmoles*) (*C*)	*Fold purification*	*Ratios*	
					B/A	*C/B*
I. Cell-free extract (dialysed against 0.2 M potassium phosphate buffer (pH 7.4))	147	456	662	1	3.1	1.5
II. The enzyme protein collected between 38 and 48% $(NH_4)_2SO_4$ satn. and dialysed against the buffer for 24 h	128	410	575	7	3.2	1.4
III. Fraction II applied to a DEAE-cellulose column. Stepwise elution with 0.15 and 0.25 M potassium phosphate buffer (pH 7.4). Concentrated by $(NH_4)_2SO_4$ fractionation (0–60% satn.) and dialysed against 0.2 M buffer for 24 h	108	313	470	73	2.9	1.5
IV. Fraction III applied to another DEAE-cellulose column. Linear gradient elution made with 200 ml of 0.1 and 0.3 M phosphate buffer, respectively	180	514	806	192	2.8	1.6
V. Dry Sephadex G-25 beads added to Fraction IV. Activity of the supernatant fraction filtered under suction	150	450	680	306	3.0	1.5

Reduced forms of FAD, cytochrome *c* and the citric acid cycle substrates were not readily utilized.

Inhibition studies

Table IV shows the effect of various inhibitors on the activities of sulphite, nitrite and hydroxylamine reductases. All three activities were retarded to the same extent by inhibitors of thiol, flavin and iron, and the first two effects were reversed by GSH and FAD, respectively.

CN^- and iron-chelating agents inhibited the activities of sulphite, nitrite and

TABLE II

STOICHIOMETRY OF SO_3^{2-}, NO_2^- AND NH_2OH REDUCTION BY THE PURIFIED ENZYME

Duplicate reaction mixtures, containing 600 μmoles phosphate buffer (pH 7.4), 300-fold purified enzyme (1 unit)[10], NADPH and appropriate substrate (3 μmoles), were incubated at 30°. After the incubation period (40 min), the residual substrates (SO_3^{2-}, NO_2^-, NH_2OH) and end products (S^{2-} or NH_3) were assayed, as described in MATERIALS AND METHODS.

Reductase activity	*NADPH added (nmoles)*	*Substrate utilized (nmoles)*	*Product formed (nmoles)*	*Stoichiometry*		
				Hydrogen donor	: *Substrate*	: *Product*
Sulphite	300	107	106	NADPH	: SO_3^{2-}	: S^{2-}
	600	215	204	3	: 1	: 1
Nitrite	300	116	101	NADPH	: NO_2^-	: NH_3
	600	211	198	3	: 1	: 1
Hydroxylamine	300	309	305	NADPH	: NH_2OH	: NH_3
	600	610	603	1	: 1	: 1

TABLE III

ELECTRON DONORS FOR SULPHITE REDUCTASE

The sulphite reductase (Fraction I, Table I) was assayed by determining the S^{2-} produced as described in MATERIALS AND METHODS. Benzyl- and methylviologens were reduced with palladized asbestos and H_2. PMS was reduced chemically with NADH. NADPH, NADH and $FADH_2$ were continuously generated (see MATERIALS AND METHODS). When sodium pyruvate was used, ATP and Mg^{2+} were included in the reaction mixture (MATERIALS AND METHODS).

Electron donors	*Relative activity of SO_3^{2-} reduction (nmoles S^{2-} produced per 40 min)*	*Redox potential E_0' (pH 7.0) (V)*
NADPH	100	−0.324
NADH	5	−0.320
Reduced benzylviologen	113	−0.314
PMS + NADH	0	+0.080
$FADH_2$	18	−0.209
Reduced methylviologen	148	−0.40
Succinate	13	+0.03
Citrate	15	−0.03
Glutamate	13	−0.121
Pyruvate	10	−0.63
Lactate	5	−0.185
Reduced cytochrome *c*	12	+0.26
Acetate	10	−0.246
Butyrate	12	−0.025

hydroxylamine reductases only when the enzymes and inhibitor had been preincubated in the reaction mixture containing NADPH, without SO_3^{2-}, NO_2^- or NH_2OH. Mixing of substrate with the enzyme prior to adding the inhibitors offset this effect.

A similar substrate protection of the enzyme against a mepacrine inhibition was observed for the NADPH–methylviologen reductase activity of the enzyme (Table V).

TABLE IV

EFFECT OF INHIBITORS ON REDUCTASE ACTIVITIES

NADPH-linked sulphite, nitrite and hydroxylamine reductase activities were assayed by following the oxidation of NADPH at 340 nm in a Unicam SP700 recording spectrophotometer. Reaction was started by adding 0.1 ml containing 3 μmoles Na_2SO_3, $NaNO_2$ or NH_2OH to 600 μmoles buffer (pH 7.4), 0.3 μmole NADPH, enzyme (Fraction V) and inhibitor, in 2.9 ml in a 1-cm quartz cuvette. The absolute activities for NADPH oxidised per 5 min on adding SO_3^{2-}, NO_2^- and NH_2OH without inhibitor are 68, 82 and 48 nmoles.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*		
		Sulphite reductase	*Nitrite reductase*	*Hydroxylamine reductase*
PCMB*	0.1	100	100	98
KCN***	1.0	100	100	100
Mepacrine**	0.4	86	88	80
Quinine sulphate	1.0	30	33	25
8-Hydroxyquinoline	3.0	90	85	85
α,α'-Dipyridyl***	10.0	62	65	63
o-Phenanthroline***	10.0	80	78	81

* Inhibition reversed with GSH.

** Inhibition reversed with FAD.

*** Inhibition was not observed if any one of these compounds was added subsequent to the addition of SO_3^{2-}, NO_2^- or NH_2OH to the enzyme.

TABLE V

EFFECT OF VARIOUS INHIBITORS ON NADPH–METHYLVIOLOGEN REDUCTASE ACTIVITY

The enzyme was assayed by following oxidation of NADPH at 340 nm. The reaction mixture (2.9 ml) contained 600 μmoles phosphate buffer (pH 7.4), 0.3 μmole NADPH and inhibitor. When SO_3^{2-}, NO_2^- or NH_2OH was tested as an inhibitor of the dye reduction, the enzyme was first preincubated with KCN (1 mM) in the reaction mixture for at least 1 min prior to the addition of 3 μmoles SO_3^{2-}, NO_2^- or NH_2OH. This ensured that the three substrates would not be enzymically reduced. The dye reduction was not inhibited by CN^-. Finally, the NADPH–dye reduction reaction was started by adding 0.1 ml containing 3 μmoles of methylviologen.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*
N-Ethylmaleimide	1.0	50
PCMB*	0.1	96
$NaAsO_2$	2.0	0
Mepacrine**, ***	1.0	100
KCN	1.0	0
	5.0	0
Iodoacetic acid	2.0	9
SO_3^{2-}, NO_2^- or NH_2OH	1.0	0
o-Phenanthroline	5.0	0
α,α-Dipyridyl	5.0	0

* Inhibition was observed irrespective of the order of adding PCMB and methylviologen to the reaction mixture.

** Inhibition was not observed if mepacrine was added, subsequent to the addition of methylviologen.

*** Inhibition was reversed by FMN.

This reduction was also strongly retarded by *p*-chloromercuribenzoate (PCMB), but it was not prevented by premixing the substrate with the enzyme. The dye reductase activity, however, was not affected either by iron inhibitors or by KCN, SO_3^{2-}, NO_2^- and NH_2OH.

Substrate inhibition

According to the double-reciprocal plots[17] the sulphite, nitrite and hydroxylamine reductase activities were competitively inhibited by alternate substrates. However, when the SO_3^{2-} concentration was increased to 1 mM, the NH_2OH inhibition of SO_3^{2-} reduction was not observed. Inhibition of sulphite reductase activity by NH_2OH (K_m = 4.5 mM) was achieved, when the concentration of SO_3^{2-} (K_m = 38 μM) was 0.33 mM or less.

The inhibition constants for SO_3^{2-} (Fig. 1A), NO_2^- (Fig. 1B) and NH_2OH (Fig. 1C), determined according to the method of DIXON[18], were 12 μM, 188 μM and 5 mM, respectively. These values are in good agreement with Michaelis constants for the three substrates.

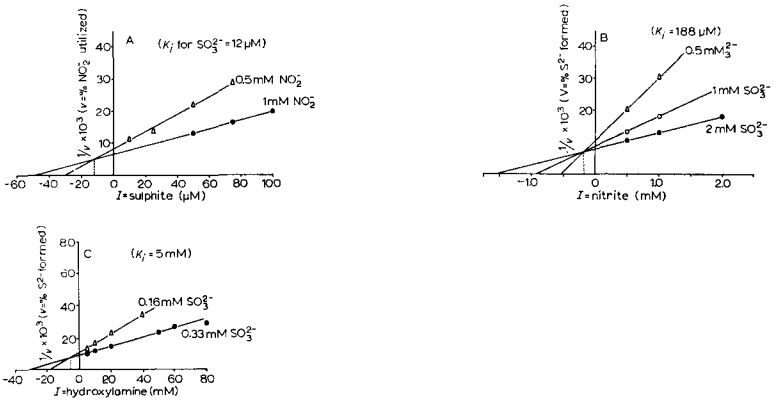

Fig. 1. Inhibition constants. A. K_i value for SO_3^{2-}. Nitrite reductase activity in a standard reaction mixture (MATERIALS AND METHODS) was competitively inhibited by fairly low concentrations of SO_3^{2-}. The results were plotted according to the method of DIXON[18]. B. K_i value for NO_2^-. Enzymic reduction of SO_3^{2-} was inhibited by varying amounts of NO_2^-. The amount of S^{2-} produced was determined as described in MATERIALS AND METHODS. C. K_i value for NH_2OH. Sulphite reductase activity was inhibited by fairly high concentrations of NH_2OH. The smaller the SO_3^{2-} concentration, the greater was the inhibition by NH_2OH. The reaction mixture (3 ml) contained phosphate buffer, 600 μmoles; $MgCl_2$, 3 μmoles; glucose 6-phosphate, 5 μmoles; $NADP^+$, 0.3 μmoles; glucose-6-phosphate dehydrogenase, 0.5 I.U.; Na_2SO_3, 0.5 or 1 μmole; and varying amounts of NH_2OH. The yeast enzyme (Fraction V, Table I) was finally added to start the reaction.

DISCUSSION

Although a separation of nitrite and hydroxylamine reductase (EC 1.6.6.4 and EC 1.7.99.1) has been attained in plants[19–22], our observations with the yeast enzyme indicate that the reductases of SO_3^{2-}, NO_2^-, and NH_2OH are probably associated with one enzyme as is the case in *Escherichia coli*[1–3]. This interpretation is based on the following facts: (a) the ratios of the reduction of $SO_3^{2-}/NO_2^-/NH_2OH$ were constant throughout the 300-fold purification of the enzyme, (b) the inhibitors

which affected the sulphite reductase activity also affected the NO_2^- and NH_2OH reductions to the same extent and (c) in all three cases, the inhibitions by KCN and iron-chelating agents were prevented by premixing the enzyme and substrate.

Since the three reductase activities were inhibited to the same extent by PCMB, mepacrine, α,α'-dipyridyl and *o*-phenanthroline, it is possible that the electrons from NADPH were transported along the same sequence suggested earlier for SO_3^{2-} reduction[10].

The stoichiometry of the three reduction reactions is as follows:

$$3\ NADPH + H^+ + SO_3^{2-} \longrightarrow 3\ NADP^+ + S^{2-} + 3\ H_2O \quad (1)$$

$$3\ NADPH + H^+ + HNO_2 \longrightarrow 3\ NADP^+ + NH_3 + 2\ H_2O \quad (2)$$

$$NADPH + H^+ + NH_2OH \longrightarrow NADP^+ + NH_3 + H_2O \quad (3)$$

Because the sulphite, nitrite and hydroxylamine reductase activities were competitively inhibited by the alternate substrates, it is possible that the three substrates were competing for a common site in the enzyme.

The purified enzyme also catalyses the reduction of benzyl- or methylviologen by NADPH. This reaction which is inhibited by mepacrine is reversed by FMN, indicating a flavin requirement; this was proposed earlier[10].

Based on the K_m and K_i values for SO_3^{2-}, NO_2^- and NH_2OH, it is suggested that SO_3^{2-} reduction is the physiological function of the enzyme.

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REFERENCES

1 J. MAGER, *Biochim. Biophys. Acta*, 41 (1960) 553.
2 R. A. LAZZARANI AND D. E. ATKINSON, *J. Biol. Chem.*, 236 (1961) 3330.
3 J. D. KEMP, D. E. ATKINSON, A. EHRET AND R. A. LAZZARANI, *J. Biol. Chem.*, 238 (1963) 3466.
4 L. M. SIEGEL AND K. J. MONTY, *Biochem. Biophys. Res. Commun.*, 17 (1964) 201.
5 A. YOSHIMOTO, T. NAKAMURA AND R. SATO, *J. Biochem. Tokyo*, 62 (1967) 756.
6 L. M. SIEGEL, K. J. MONTY AND H. KAMIN, *Federation Proc.*, 26 (1967) 858.
7 K. ASADA, *J. Biol. Chem.*, 242 (1967) 3646.
8 N. NAIKI, *Plant Cell Physiol., Tokyo*, 6 (1965) 179.
9 K. PRABHAKARARAO AND D. J. D. NICHOLAS, *Proc. Australian Biochem. Soc., Canberra, 1968*, p. 32.
10 K. PRABHAKARARAO AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 180 (1969) 253.
11 K. PRABHAKARARAO AND D. J. D. NICHOLAS, *Proc. Australian Biochem. Soc., Adelaide, 1969*, p. 60.
12 J. A. RUSSELL, *J. Biol. Chem.*, 156 (1944) 457.
13 A. MEDINA AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 23 (1957) 440.
14 E. J. HEWITT AND D. J. D. NICHOLAS, in H. F. LINSKENS, B. D. SANWAL AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. 7, Springer, Berlin, 1964, p. 83.
15 R. H. HAGEMAN, C. F. CRESSWELL AND E. J. HEWITT, *Nature*, 193 (1962) 247.
16 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
17 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, Green and Co., London, 1966, pp. 326.
18 M. DIXON, *Biochem. J.*, 55 (1953) 170.
19 G. G. ROUSSOS AND A. NASON, *J. Biol. Chem.*, 235 (1960) 2997.
20 G. F. BETTS AND E. J. HEWITT, *Nature*, 210 (1966) 1327.
21 H. HUZISIGE, K. SATOH, K. TANAKA AND T. HAYASHIDA, *Plant Cell Physiol. Tokyo*, 4 (1963) 307.
22 J. M. RAMIREZ, F. F. DEL CAMPO, A. PANEQUE AND M. LOSADA, *Biochim. Biophys. Acta*, 118 (1966) 58.