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EFFECT OF ENZYMIC ASSAY CONDITIONS ON SULFITE REDUCTION CATALYSED BY DESULFOVIRIDIN FROM *DESULFOVIBRIO GIGAS*

H.E. JONES and G.W. SKYRING

Baas Becking Geobiological Laboratory, P.O. Box 378, Canberra City, A.C.T. 2601 (Australia)

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Summary

The type and the amount of end products resulting from sulfite reduction catalysed by a single partially purified desulfoviridin preparation from *Desulfovibrio gigas* were shown to depend upon the enzymic assay conditions employed. Both manometric and spectrophotometric assays were used, with reduced methyl viologen serving as the electron donor in each system. Trithionate, thiosulfate, tetrathionate and sulfide were identified as possible end products. In the manometric assays, sulfide production was favoured by high reduced methyl viologen concentrations, low sulfite concentrations and a pH value of 7.0 as opposed to 6.0. In the spectrophotometric assays, results approaching the stoichiometric conversion of sulfite to sulfide were obtained only at high initial reduced methyl viologen concentrations.

Introduction

It has been established that desulfoviridin, a green pigment present in species of the dissimilatory sulfate-reducing bacterial genus *Desulfovibrio*, catalyses the reduction of sulfite [1—6]. Using manometric techniques, Suh and Akagi [1] found thiosulfate, and Lee and Peck [2] and Lee et al. [3] found trithionate as the sole end product of sulfite reduction. For optimum enzymic activity, Suh and Akagi [1] required the addition of another protein fraction. Using a spectrophotometric technique, Jones and Skyring [6] reported the formation of sulfide, but stoichiometric reduction to sulfide depended on the desulfoviridin preparation used. However, Kobayashi et al. [4,5] reported that during the reduction of sulfite, catalysed by desulfoviridin from *Desulfovibrio vulgaris*, mixtures of trithionate, thiosulfate and tetrathionate were formed, which varied with conditions prevailing in the manometric assay [5].

It is shown in the present paper that variations in the conditions of two

assay procedures also caused variations in the end products of sulfite reduction catalysed by desulfoviridin from *Desulfovibrio gigas*. A preliminary report of this work has appeared elsewhere [7].

Methods

Isolation of desulfoviridin

Cell extracts from *D. gigas* were prepared as previously described [6] but the trace elements content of the growth medium was omitted (H_3BO_3, $CuSO_4$, KI, $FeCl_3$, $MnSO_4$, Na_2MoO_4, $ZnSO_4$, $CoCl_2$). Desulfoviridin, purified to the G200 level, was also obtained by previous methods [6], except that it was passed only once through a Sephadex G200 column. This G200 preparation was sterilised by Millipore filtration and kept at 4°C. This method of storage avoids the degradation which can accompany freezing. Gel electrophoretic patterns and spectral characteristics of the G200 preparation were determined by previous methods [6,8].

Isolation of hydrogenase

Particulate material from *Desulfovibrio spp.* (NCIB 10455) prepared as previously described [9], and shown to contain hydrogenase activity, was washed five times in 0.1 M Tris—HCl buffer, pH 7, followed by four washes in 0.1 M potassium phosphate buffer, pH 6. At this stage the supernatant showed no fluorescence at 365 nm with or without NaOH, indicating that the preparation was not contaminated with the pink pigment or desulfoviridin also present in this strain of *Desulfovibrio* [9]. The particles were homogenised in 0.1 M potassium phosphate buffer. pH 6, at a protein concentration of 35 mg per ml and kept at −20°C. When the sulfite reductase manometric assays were performed at pH 7 the particulate hydrogenase preparation was washed twice more in 0.1 M Tris—HCl buffer, pH 7, and homogenised in the Tris—HCl buffer at the same protein concentration. Using manometric determination at 37°C (see below), the hydrogenase preparation (3.5 mg protein) reduced methyl viologen (10 mM) at rates of 0.013 μmoles H_2 per min in 0.1 M potassium phosphate, 0.001 M EDTA buffer at pH 6 and 0.024 μmoles H_2 per minute in 0.1 M Tris—HCl, 0.001 M EDTA buffer at pH 7. Determined spectrophotometrically in this Tris—HCl—EDTA buffer at 37°C and at 600 nm (see below), the same amount of hydrogenase oxidized 0.072 and 0.078 μmoles of reduced methyl viologen per min measured over absorbancies of 1.2 to 1.7 and 1.8 and 2.3, respectively. The spectrophotometric reactions were so fast that any turbidity differences due to unequal rates of settling of the particulate matter in the reference and sample cuvettes were negligible.

Reductase reactions

All reactions were carried out at 37°C. The spectrophotometric sulfite reductase assays were conducted in anaerobic cuvettes at pH 7.0 by the method of Jones and Skyring [6] and rates of reactions determined over absorbancies of 1.2 to 2.1 [6]. For manometric assays three buffer systems were used, 0.1 M potassium phosphate, pH 6.0 and pH 7.0 and 0.1 M Tris—HCl, pH 7.0. The Warburg flasks contained methyl viologen (1—20 μmoles) particulate hydro-

genase (0.7—14 mg protein), G200 desulfoviridin preparation (0.6 mg protein) and 0.1 ml sodium sulfite (1 and 5 μmoles) in the appropriate buffer containing 0.01 M EDTA in the side arm. The total volume after addition of the sulfite was 1.05 ml. The centre well contained 0.1 ml of 20% aqueous $CdCl_2$ plus a strip of fluted ashless filter paper. Using a Gilson differential respirometer, each sample flask was balanced against a similar reference flask which did not contain desulfoviridin. After gassing with O_2-free H_2 for 20 min and 10 min equilibration the sulfite was tipped into both flasks. 10 min after H_2 uptake stopped, the $CdCl_2$ papers were removed, and the contents of each flask frozen at —20° C for subsequent analysis.

Analysis of products

Sulfide formed in the cuvettes was estimated as previously described [10]. Sulfide trapped on the $CdCl_2$ papers was estimated with the reagent of Gilboa-Garber [11] which was purified by filtration through activated charcoal to lower the absorbance of the blank. The reaction was carried out at 50° C for 15 min prior to the addition of ferric chloride. Strips of filter paper containing known amounts of CdS served as standards. Trithionate, thiosulfate and tetrathionate were determined by the method of Kobayashi et al. [4]. The presence of these compounds in the contents of four Warburg flasks was confirmed by one-dimensional chromatography on Whatman No. 1 paper. Three solvent systems were used, *n*-butanol—methylethyl ketone—water—ammonia (40 : 40 : 20 : 1, by vol.); *n*-butanol—pyridine—water—acetic acid (60 : 40 : 30 : 3, by vol.); *t*-butanol—methylethyl ketone—formic acid—water (40 : 30 : 15 : 15, by vol.) and positions of the sulfur compounds located by staining with a solution of 5% (w/v) silver nitrate in 90% (v/v) aqueous acetone.

Protein estimations

The biuret method of Gornall et al. [12] was used with bovine serum albumin as the reference protein.

Results

The desulfoviridin preparation

Apart from a weak band of desulfoviridin with a low E_f value (0.11), presumably indicating aggregation, the electrophoretic pattern of the G200 desulfoviridin preparation was similar to that described previously [6] with the main proteins occurring as two bands, the major and minor desulfoviridin bands. As before, formation of sulfide from sulfite in the gels was found only at the major and minor desulfoviridin bands, while none of the bands catalysed the reduction of thiosulfate to sulfide [6,8]. In difference spectra, a CO-binding reaction after reduction with cysteine and borohydride [3] could not be demonstrated. This, in conjunction with the observation that in polyacrylamide gels only protein bands identified as desulfoviridin showed sulfite reductase activity [8], suggested that the preparation was not contaminated with an assimilatory sulfite reductase of the type described by Lee et al. [3].

The major peaks in the visible spectrum of the G200 preparation were at 627, 581 and 409 nm, with absorbance ratios 2 : 1 : 4.8, respectively; a very

slight broad peak occurred around 490 nm. The absorbance ratios, Δ627 − 700 nm : Δ581 − 700 nm : Δ409 − 700 nm were 2.37 : 1 : 6.25, respectively. At a protein concentration of 12 mg/ml the absorbance of the peak at 627 nm was 2.96 and the absorbance difference 627 − 700 nm was 2.56. These results are almost identical with those previously reported for purified preparations of desulfoviridin from *D. gigas* [2].

Reductase reactions

In the spectrophotometric assays, the G200 preparation catalysed the reduction of sulfite, but not of thiosulfate or trithionate. The ratios of reduced methyl viologen oxidized versus sulfide produced in experiments with different initial concentrations of reduced methyl viologen, sulfite and desulfoviridin are given in Table I. At higher initial reduced methyl viologen concentrations (1.08—1.14 μmoles) the ratios approach the stoichiometric value of 6 : 1 (see also ref. 6). At the lower initial reduced methyl viologen concentrations (0.44—0.55 μmoles) ratios were around 10 : 1 indicating the existence of products in addition to sulfide. Estimations for trithionate, thiosulfate and tetrathionate were made in comparable systems, and slight reactions for thiosulfate and trithionate were found. However, the amounts present were too small to allow reliable determinations or chromatographic identification.

TABLE I

SPECTROPHOTOMETRIC ASSAYS FOR SULFITE REDUCTION CATALYSED BY A G200 DESULFOVIRIDIN PREPARATION

Variation in complete system*	Incubation (min)	Initial reduced methyl viologen** (μmoles)	Reduced methyl viologen oxidised (μmoles)	nmoles reduced methyl viologen oxidized per min***	Sulfide produced (μmoles)	Ratio of reduced methyl viologen oxidized to sulfide produced
—	42	1.14	1.12	52.5	0.2	5.6
—	50	1.08	1.05	39.1	0.17	6.17
—	40	0.95	0.93	47.2	0.13	7.15
—	40	0.73	0.71	35.7	0.10	7.1
—	35	0.55	0.53	23.6	0.05	10.6
—	35	0.49	0.47	22.4	0.05	9.4
0.5 μmoles Na_2SO_3 as substrate	60	0.76	0.75	23.6	0.10	7.5
plus 10 mg bovine serum albumin	38	0.73	0.72	33.8	0.10	7.2
Alkaline glucose reduction of methyl viologen, final pH 7.17	60	1.00	0.97	26.8	0.13	7.46
Alkaline glucose reduction of methyl viologen, final pH 7.73	90	1.22	0.97	9.6 (14.7)	0.12	8.08

* Complete system consisted of 3.35 ml of 0.1 M Tris—HCl—0.01 M EDTA buffer, pH 7.0, containing 5 μmoles of Na_2SO_3, 0.6 mg of desulfoviridin preparation and 0.3 ml of 0.3% (w/v) Zn-reduced methyl viologen [6].

** Determined from the absorbance at 600 nm immediately after addition of enzyme.

*** Determined between absorbancies of 1.2 to 2.1. Parentheses indicate that fastest rate in that system occured between absorbancies of 2.8 to 4.0 rather than 1.2 to 2.1.

To determine if Zn, which was used to reduce the methyl viologen, had any influence on the stoichiometry between reduced methyl viologen oxidized and sulfide produced, spectrophotometric experiments were performed in which the methyl viologen was reduced by the alkaline glucose method [13]. The methyl viologen was 0.3% (w/v) and the reduction was carried out with 0.1 M and 0.05 M Na_2CO_3. The rates of oxidation of reduced methyl viologen were slower than with Zn-reduced methyl viologen, possibly due, in part, to a slightly higher pH (7.37 and 7.17) caused by Na_2CO_3. Ratios of reduced methyl viologen oxidized versus sulfide produced suggested slightly less than stoichiometric conversion (Table I).

Asada et al. [14] reported that the assimilatory sulfite reductase (b fraction) from spinach required the presence of other proteins, notably bovine serum albumin, for activity. The addition of bovine serum albumin (10 mg) which might also be considered a replacement for the protein afforded by the hydrogenase in the manometric assays, did not affect the stoichiometry obtained in the spectrophotometric experiments (Table I).

In the manometric assays, thiosulfate, trithionate and tetrathionate were identified as end products in the contents of four Warburg flasks by chromatography in the three solvent systems. The flasks chosen contained different initial concentrations of methyl viologen and sulfite and were representative of each buffer system. Thiosulfate, trithionate, tetrathionate and sulfide were formed in amounts which varied according to the assay conditions.

In Tables II, III and IV, amounts of products shown are contents of sample flask minus contents of reference flask; figures in brackets represent molar ratios relative to sulfide fixed as unity. Sulfide was the only sulfur compound found in zero time controls and this never exceeded 0.007 μmoles. Sodium sulfite prepared in the presence of 0.001 M EDTA was stable for several hours at 37°C. Table II shows the amounts of products obtained when only the reduced methyl viologen content in each system was altered by increasing the methyl viologen concentration. Figures for the amounts of reduced methyl viologen present cannot be given, but the colour of the reaction mixture, which varied from intense blue to almost colourless, showed that there were large differences in the reduced methyl viologen content. The molar ratio between trithionate (see Lee and Peck [2], Lee et al. [3]) and sulfide (see Jones and Skyring [6]) varied from approximately 16 : 1 to 1 : 1. At the higher methyl viologen concentrations at pH 7, giving a blue colour like that initially present in the high absorption spectrophotometric assays, the ratio favoured sulfide.

Table III shows the results obtained by varying both the hydrogenase and the methyl viologen concentrations. The trithionate : sulfide molar ratios were much greater in the system with the low reduced methyl viologen content than in the system where a high content of reduced methyl viologen was present. A similar situation occurred with the trithionate : thiosulfate ratios. Tetrathionate was a minor product of both systems.

Sulfide replaced trithionate as the major product when the sulfite content of the system was decreased to 1 μmole (Table IV). The H_2 uptake with 1 μmole of sulfite as substrate was approximately 1 μmole more than was required for formation of the quantities of sulfur compounds detected. Further

TABLE II

MANOMETRIC ASSAYS FOR SULFITE REDUCTION CATALYSED BY A G200 DESULFOVIRIDIN PREPARATION*

System	μl H_2 utilised per min**	Products (μmoles)†				μmoles H_2 utilised	
		H_2S	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$S_3O_6^{2-}$	Actual	Expected
Phosphate buffer pH 6.0							
Methyl viologen 1 μmole	0.9	0.1 (1)***	0.13 (1.25)	0.03 (0.25)	1.35 (13.5)	1.79	1.97
Methyl viologen 10 μmoles	1.8	0.25 (1)	0.3 (1.2)	0.08 (0.3)	1.0 (4)	2.96	2.65
Methyl viologen 1 μmole	0.85	0.09 (1)	0.15 (1.66)	0.08 (0.83)	1.43 (15.8)	1.99	2.21
Methyl viologen 10 μmoles	2.6	0.27 (1)	0.43 (1.58)	0.05 (0.19)	1.13 (4.2)	2.96	2.93
Phosphate buffer pH 7.0							
Methyl viologen 1 μmole	0.66	0.23 (1)	0.05 (0.2)	0.09 (0.39)	1.35 (5.9)	2.75	2.46
Methyl viologen 10 μmoles	1.3	0.59 (1)	0.1 (0.17)	0.08 (0.13)	1.08 (1.82)	3.78	3.27
Tris buffer pH 7.0							
Methyl viologen 1 μmole	0.5	0.31 (1)	0.15—0.38 (0.48—1.21)	0	1.28 (4.1)	2.76	2.51—2.96
Methyl viologen 10 μmoles	1.2	1.06 (1)	0.2 —0.43 (0.19—0.4)	0	0.95 (0.9)	5.44	4.53—4.98

* For method see text. Amounts of desulfoviridin preparation, sulfite and hydrogenase were initially constant at 0.6 mg protein, 5 μmoles and 3.5 mg protein, respectively.

** Measured over the linear part of reaction curve.

*** Figures in brackets are ratios equivalent to H_2S content fixed as unity.

† In the reference flasks the quantities of sulfur compounds estimated ranged between the following values: sulfide, 0.01—0.18 μM; trithionate, 0.00—0.10 μM, thiosulfate; 0.00—0.15 μM; tetrathionate, 0.00—0.10 μM.

TABLE III

MANOMETRIC ASSAYS FOR SULFITE REDUCTION CATALYSED BY A G200 DESULFOVIRIDIN PREPARATION*

System	μl H_2 utilised per min**	Products (μmoles)				μmoles H_2 utilised	
		H_2S	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$S_3O_6^{2-}$	Actual	Expected
Methyl viologen 1 μmole and hydrogenase 0.7 mg protein	0.4	0.07 (1)***	0.14 (2.15)	0	1.49 (21)	2.35	1.99
Methyl viologen 10 μmoles and hydrogenase 14 mg protein	3.2	0.2 (1)	0.35 (1.75)	0.08 (0.38)	0.85 (4.25)	2.77	2.44

* For method see text. Both systems contained phosphate buffer, pH 6.0. Amounts of desulfoviridin preparation and sulfite were initially constant at 0.6 mg protein and 5 μmoles, respectively.
** Measured over the linear part of reaction curve.
*** Figures in brackets are ratios equivalent to H_2S fixed as unity.

manometric experiments showed that this discrepancy could not be accounted for by remaining sulfide which would not have been trapped by the $CdCl_2$ papers. If the hydrogen taken up was used only in the reduction of sulfite, the lack of stoichiometry could be due to the presence of reduced sulfur compounds not detected or interaction between the products of reduction and the reaction mixture components.

TABLE IV

MANOMETRIC ASSAYS FOR SULFITE REDUCTION CATALYSED BY A G200 DESULFOVIRIDIN PREPARATION*

System	μl H_2 utilised per min**	Products (μmoles)				μmoles H_2 utilised	
		H_2S	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$S_3O_6^{2-}$	Actual	Expected
Phosphate buffer pH 6.0, methyl viologen 20 μmoles and hydrogenase 7 mg protein	2.0	0.12 (1)***	0	0.08 (0.62)	0.03 (0.21)	1.52	0.62
Phosphate buffer pH 7.0, methyl viologen 10 μmoles and hydrogenase 3.5 mg protein	0.9	0.35 (1)	0.08 (0.21)	0	0.05 (0.15)	2.21	1.25

* For method see text. Amounts of desulfoviridin preparation and sulfite were initially constant at 0.6 mg protein and 1 μmole, respectively.
** Measured over the linear part of reaction curve.
*** Figures in brackets are ratios equivalent to H_2S fixed as unity.

Discussion

The results show that both the type and the amount of end products of sulfite reduction catalysed by a single desulfoviridin preparation are dependent upon the enzyme assay conditions employed. Kobayashi et al. [5] also made similar observations with preparations of desulfoviridin from *D. vulgaris.*

In the assays reported here neither thiosulfate [1] nor trithionate [2,3] was ever obtained as the sole end product, and sulfide was always at least a partial product (see also refs 4 and 6).

Although in a multi-enzyme system different assay conditions could be considered to affect each enzyme selectively, the desulfoviridin preparation contained no detectable thiosulfate or trithionate reductase activities, or an assimilatory sulfite reductase [3]. The formation of sulfide in the present experiments thus appears to be due to desulfoviridin.

In manometric assays, the reduced methyl viologen concentrations, the higher sulfite concentrations (5 μmoles) and the lower pH value (6.0) were less favourable to sulfide production, while the higher reduced methyl viologen concentrations, the lower sulfite concentration (1 μmole) and the higher pH value (7.0) were more favorable to sulfide production. Kobayashi et al. [4] also commented that the ratios of end products were affected by the sulfite and methyl viologen concentrations and subsequently [5] showed that at lower sulfite and higher reduced methyl viologen concentrations, conditions were more favourable for sulfide production. Although the activities of the hydrogenase preparations, and therefore of the electron-donating systems used by other workers cannot be directly compared, in our experiments the concentrations of methyl viologen (1—1.5 μmoles/ml), sulfite (5—10 μmoles/ml) and the pH value (6.0) used by these workers were unfavourable for formation of sulfide as the major product.

In the spectrophotometric experiments (Table I) the reduced methyl viologen concentration again affected the reaction perhaps indicating a redox effect. Results approaching the stoichiometric conversion of sulfite were obtained only at the higher initial reduced methyl viologen concentrations.

Conditions in the spectrophotometric and manometric assays are different. In the former, the reduced methyl viologen disappears completely while in the latter the reduced methyl viologen is kept at a constant level by the hydrogenase and it is the sulfite which disappears. Further, in the spectrophotometric assays it is continuously removed from the reaction mixtures by the $CdCl_2$ papers. However, the trends with respect to the initial concentrations of reduced methyl viologen appeared coincident in both assay procedures.

The reasons for the effects of pH, and sulfite and reduced methyl viologen concentrations are not yet apparent. Certainly the rate of reaction is altered by these conditions. Low sulfite concentrations and high reduced methyl viologen concentrations, conditions which favour sulfide production in the manometric assays, caused increased rates of reactions. However, high pH, which also favours sulfide production, slows the rate of reaction.

In some present experiments tetrathionate was formed in more than trace amounts. Kobayashi et al. [4] also reported the formation of small quantities of tetrathionate in their experiments, but its presence is not accounted for in

their model for sulfite reduction. It is possible that tetrathionate is formed by chemical disproportionation of sulfur compounds generated in the reaction mixtures.

In its spectral characteristics before and after addition of sodium dithionite, the desulfoviridin preparation used here was similar to that of Lee and Peck [2]. Jones and Skyring [6] using a desulfoviridin preparation with atypical spectral characteristics also showed stoichiometric conversion of sulfite to sulfide in spectrophotometric assays in which only high concentrations of methyl viologen were used. Thus the results previously obtained [6] were not related to the atypical spectral characteristics. Unexplained variations in spectra of desulfoviridin are apparent from the literature, the middle absorption peak varying from 580 nm [2,3] to 583 nm [15] to 585 nm [4].

The variability of the end products under different assay conditions eventually may be of use in determining the mechanism of the electron transfer taking place at the active centre(s) of desulfoviridin. For example on the basis of similar observations Kobayashi et al. [4,5] suggested that intermediate compounds (designated X, SO_2^{2-}? and Y, S?) are formed when sulfite is reduced to sulfide in the presence of desulfoviridin, and that these intermediates react with sulfite to form trithionate and thiosulfate. An important question is whether or not compounds such as thiosulfate, trithionate and tetrathionate are true enzymic products or the products of chemical reactions.

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