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THE ASSOCIATION OF HYDROGENASE AND DITHIONITE REDUCTASE ACTIVITIES WITH THE NITRITE REDUCTASE OF DESULFOVIBRIO DESULFURICANS

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Summary: The membrane bound respiratory nitrite reductase from *Desulfovibrio desulfuricans* contains six c-type heme groups and catalyzes the six electron reduction of nitrite to ammonia. The purified enzyme required an excess of reducing equivalents for reduction relative to the amount of nitrite consumed in its reoxidation. The anomaly could be accounted for in terms of the presence of low levels of dithionite reductase and hydrogenase activity in the preparation. Dithionite reductase may be an alternate activity of nitrite reductase, whereas hydrogenase was shown to be a contaminant. The contaminating hydrogenase used nitrite reductase as electron acceptor in preference to cytochrome c_3 (M_r = 13,000) or benzyl viologen.

Growth of *Desulfovibrio desulfuricans* with nitrate as terminal electron acceptor was first demonstrated by Pichinoty (1) who ascribed the reduction of nitrite to ammonia to the presence of cytochrome c_3 (2). Recent studies in this laboratory (3), however, have shown the presence of a novel nitrite reductase, containing six c-type heme groups per subunit molecular weight of 66,000 daltons, in the cytoplasmic membrane of *D. desulfuricans*. Several aspects of this new kind of nitrite reductase, such as its topography relative to nitrate reductase in the cytoplasmic membrane, its natural electron donor and function in energy transduction are of interest and remain to be elucidated.

Nitrite reduction is also of interest from the point of view of comparative enzymology. Nitrite reduction by the nitrite reductases of denitrifying bacteria, which contain either two copper atoms (4,5) or c-and d-type hemes (6,7) as redox active centers, result primarily in the release of nitric oxide as a product, whereas nitrite reduction by the nitrite reductase of organisms such as *D. desulfuricans* or *E. coli* gives rise directly to ammonia without the formation of gaseous intermediates.

In the course of oxidation reduction titrations of *D. desulfuricans* nitrite reductase, which were undertaken as preliminary attempts to demonstrate the presence of enzyme bound intermediates, it was noticed that an excess of reducing equivalents is required for the reduction of the enzyme as compared to the amount of nitrite consumed in its reoxidation. An adequate explanation for this descripancy was considered mandatory in any further investigation of

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its reaction mechanism. The results reported here provide evidence for the existence of a dithionite reductase activity, in addition to the sulfite reductase activity often associated with nitrite reductases, in the preparation. A contaminating hydrogenase activity which uses the nitrite reductase, at least *in vitro*, preferentially as an electron acceptor is also described.

METHODS

Conditions for the growth of *D. desulfuricans* cells and isolation of the nitrite reductase have been described (3). The enzyme, which appeared pure on SDS-gel electrophoresis, was stored in 20% ethylene glycol at -15°C and was indefinitely stable. Nitrite reductase activity of crude preparations was determined by assaying for the production of ammonia (8). Enzyme activity may, however, be continuously monitored by recording the pH-change associated with nitrite reduction. Assay mixtures comprised 60 µmoles potassium phosphate buffer, pH 7.8 and 3 µmoles methyl viologen in a 3 ml volume contained in an oxygen electrode, the plunger of which had been modified to accommodate a Markson pH - microelectrode. After obtaining anaerobiosis by bubbling with purified argon, the methyl viologen (MV) was reduced by addition of a slight excess of dithionite. Nitrite (10 µmoles) was added and the reaction started by addition of a suitable quantity of nitrite reductase. The pH-change due to the reaction,

$NO_2^- + 6\ MV^{\cdot+} + 8\ H^+ \rightarrow NH_4^+ + 2H_2O + 6\ MV^{++}$, was recorded and quantitated by addition of standard hydrochloric acid. The temperature was 37°C.

In purified preparations of the enzyme the value of 659 $mM^{-1}\ cm^{-1}$ for the molar extinction of the oxidized enzyme at 410 nm, as determined by titration of dithionite reduced enzyme with nitrite, was used to estimate protein concentration. Hydrogen uptake due to hydrogenase activity was determined by following the reduction of benzyl viologen. Reaction mixtures contained 100 mM potassium phosphate, pH 7.8, 1 mM benzyl viologen and enzyme in a 1.0 ml volume. Sulfide was measured using a sulfide specific ion electrode (Orion Research).

Cytochrome c_3 (M_r = 13,000) free of hydrogenase and with a $A_{552\,(red)}/A_{280\,(oxi}$ ratio of 2.23 (see Ref. 9) was prepared from *D. desulfuricans* as follows: *D. desulfuricans* cells were disrupted as described (3). The broken cells were centrifuged for 30 min at 13200g and the pellet discarded. The supernatant was centrifuged at 160000g for 1h. The pellet was used for the preparation of nitrite reductase. The supernatant was adjusted to 85% saturation using solid $(NH_4)_2SO_4$, precipitated proteins removed by centrifugation and the supernatant applied to a DEAE-cellulose DE-52 column (40 X 2.5 cm) equilibrated was 85% saturated $(NH_4)_2SO_4$, the pH of which had been adjusted to 8.0 with 2M Tris-base. The adsorbed proteins were eluted with a one-liter gradient using the latter solution as starting and 50 mM Tris-HCl, pH 8.0 as limiting buffer. The cytochrome emerged from the column as a red band well separated from the bulk of the protein. After concentration by ultrafiltration, the cytochrome was applied to a Sephadex G-50 column and eluted with 50 mM potassium phosphate, pH 7.6. All preparative procedures were carried out at 0-5°C and the pH of Tris-buffers refers to 0°C.

RESULTS

The spectral course of the reoxidation of dithionite reduced nitrite reductase by nitrite is shown in Fig. 1. The titration is a mere reversal of the reduction of the enzyme and no unusual spectral features, which might

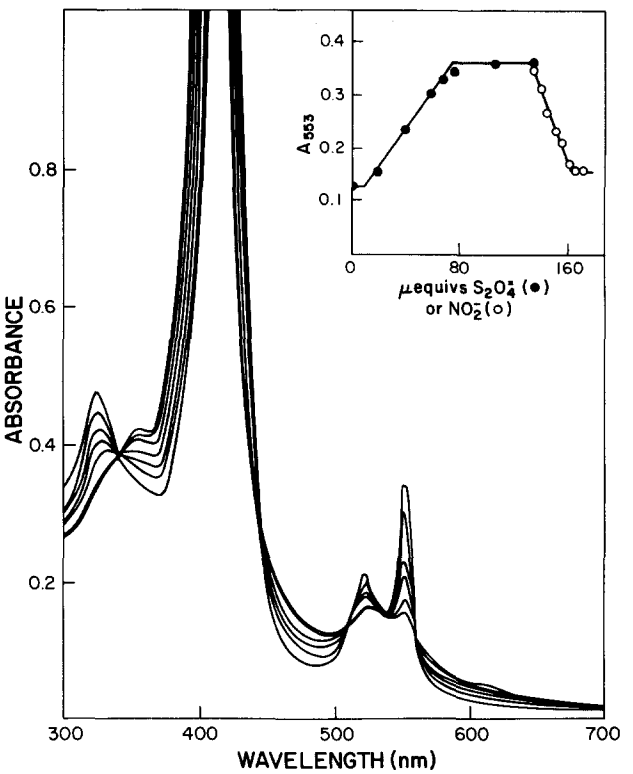

Figure 1: Reoxidation of dithionite reduced nitrite reductase with nitrite. The enzyme in 100 mM K-phosphate, pH 7.6, under an atmosphere of argon was reduced with dithionite as shown in the inset and subsequently reoxidized with nitrite. The absorbance spectrum corresponding to the penultimate data point in the inset is not shown. Dithionite was standardized by titration of cytochrome c for which a difference molar extinction of 21 $mM^{-1}cm^{-1}$ at 550 nm (10) was assumed.

have indicated the formation of enzyme-bound nitrogenous intermediates are evident. However, the difference molar extinction coefficients calculated for the reductive as opposed to oxidative phases of the titration are 9.04 mM cm^{-1} and 17.6 $mM^{-1}cm^{-1}$ at 553 nm respectively (inset of Fig. 1) and therefore differ by a factor of about two. The fact that complete reoxidation of the enzyme was not obtained with nitrite suggest the presence of some inactive enzyme. It is of interest that excess dithionite added at the end of the titration was unaccounted for in the reoxidation with nitrite. Spectra obtained at intermediate stages of the reductive titration were stable suggesting that the descripancy does not arise from reoxidation of the enzyme by sulfite generated from dithionite, in agreement with the findings of Liu (3) that sulfite does not reoxidize the reduced enzyme. Reduction of the enzyme by reduced methyl viologen proceeded linearly, but indicated a molar extinction of only about

12.9 $mM^{-1}cm^{-1}$ at 553 nm. Moreover, reduced methyl viologen did also not accumulate when added in excess of the amount required to reduce the enzyme.

The latter observation suggested the presence of hydrogenase in the preparation and, indeed, the reoxidation of the methyl viologen radical, generated from a somewhat less that stoichiometric amount of dithionite, by catalytic amounts of enzyme, could readily be demonstrated. This "methyl viologen oxidizing" activity co-chromatographed with the heme Soret band on DEAE-Biogel A and its heat inactivation at 70°C followed similar kinetics to that of the nitrite reductase activity, in contrast to the behaviour of the hydrogenase activity described below. The "methyl viologen oxidizing" activity in the presence of sulfite presumably involves the reduction of sulfite. However, in view of difficulties in its precise quantitation, its discrimination from hydrogenase activity and its low level as compared to the dithionite reductase, this activity is not further considered here.

In the presence of excess dithionite the enzyme catalyzed the formation of sulfide. Two approaches were adopted to ascertain whether this activity derives from the presence of sulfite or from dithionite itself. In a reaction mixture containing methyl viologen and excess dithionite the generation of sulfide from sulfite is described by equations 1 and 2.

$$3S_2O_4^{=} + 3H_2O \rightarrow 0.9HS^- + 0.1H_2S + 4.64\ SO_3^{=} + 0.35\ HSO_3^- + 4.55\ H^+ \quad \text{(1)}$$

$$0.93\ SO_3^{=} + 0.07\ HSO_3^- + 7.03\ H^+ + 6\ MV^+ \rightarrow 0.9HS^- + 0.1\ H_2S + 3H_2O + 6\ MV^{++} \quad \text{(2)}$$

Equation 1 holds in the presence of excess dithionite with methyl viologen in a steady state and equation 2 describes the process after the depletion of dithionite.

An initial acid production of 4.55 H^+ per mole of sulfide formed should therefore be followed by a consumption of 7.03 H^+ per mole of sulfide. Values of 6.9 and 7.04 for the pKa's of bisulfite and hydrogen sulfide are assumed. Independent investigation of the biphasic pH-change resulting from the enzyme catalyzed reduction of nitrite in the presence of excess dithionite with methyl viologen as immediate electron donor, showed that this approach is essentially sound.

In the case of sulfide production, however, a value of 0.22 for the ratio

$$\frac{\text{rate of proton consumption after depletion of dithionite}}{\text{rate of proton release in presence of excess dithionite}}$$

was found (Fig. 2), rather than the ratio of 1.55 predicted from equations 1 and 2. Since the rate of nitrite reduction in the presence of dithionite was only about 6% of the rate obtained in the presence of reduced methyl viologen

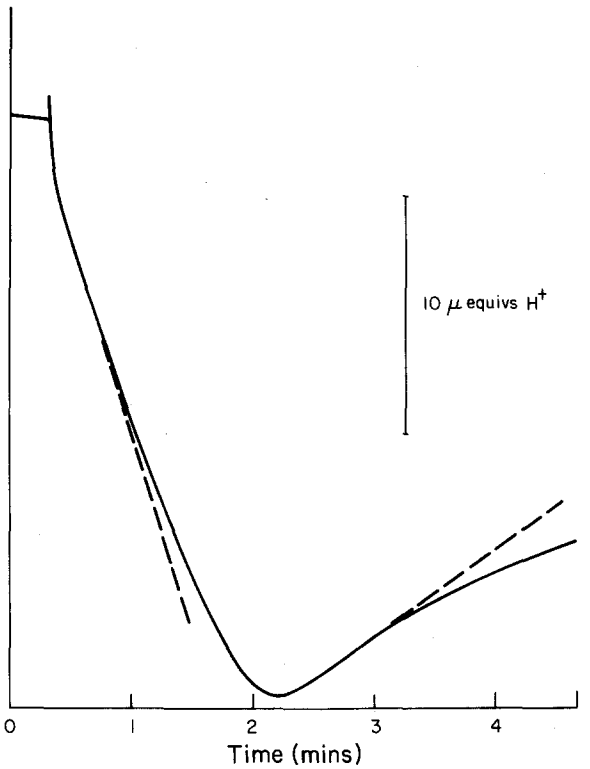

Figure 2: Change in pH accompanying dithionite reductase activity. 3.0 ml 20 mM K-phosphate, pH 8.0, containing 3 μmoles methyl viologen was made anaerobic by bubbling with argon. The dye was reduced by adding an approximately 3-fold excess of dithionite, the pH adjusted to 8.0 with 0.5 M NaOH and the reaction started by addition of 93 μg nitrite reductase (indicated by an arrow) after stabilization of the pH. The rate of acid production is about 17.3 μequiv. H^+ min^{-1} mg^{-1} and the rate of alkalinization about 3.85 μequiv. H^+ min^{-1} mg^{-1} after depletion of dithionite. The initial rapid drop in pH reflects pH-equilibration of the enzyme solution with the assay mixture.

the descripancy cannot readily be rationalized in terms of a poorer efficiency of methyl viologen as electron donor. The data, therefore, suggest that dithionite or its dissociation product, the SO_2^- radical, is a substrate for sulfide formation. This possibility was further examined in an experiment the results of which are shown in Fig. 3. In a series of reaction mixtures containing a constant amount of methyl viologen and increasing amounts of dithionite, sulfide production increased markedly when dithionite was added in excess over methyl viologen, but below the equivalence point sulfide production in assay mixtures containing methyl viologen was considerably less than in those without. The data of Fig. 3, therefore, clearly shows that both dithionite and methyl viologen are required for the optimal production of sulfide. These observations again indicate that dithionite and more likely the SO_2^- radical,

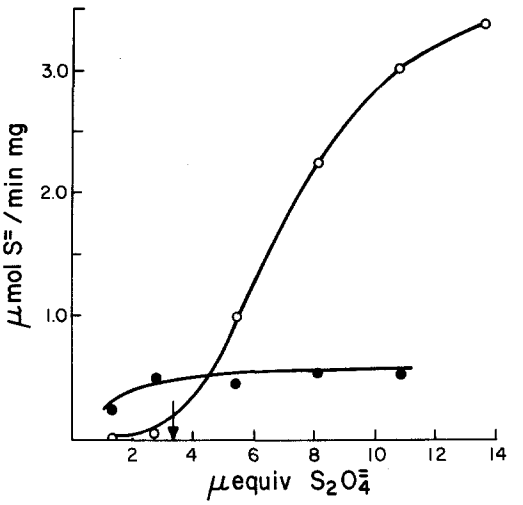

Figure 3: Sulfide formation as a function of dithionite added. To the outer compartment of 10 ml Ehrlenmeyer flasks fitted with center wells was added 2.0 ml 100 mM K-phosphate, pH 7.8 and 42 μg nitrite reductase. Open circles are for determinations in the presence and solid circles for determinations in the absence of 3.36 μmoles methyl viologen in the outer compartment. Center wells contained 0.4 ml 0.5 M NaOH. The assay mixtures were made anaerobic by gassing with argon through serum stoppers and the reaction was started by adding various amounts of dithionite. Reactions proceeded for 10 mins at 37°C and were stopped by addition of 0.4 ml 2 M HCl to the outer well. After a distillation time of 4.5 h the center well was assayed for sulfide. Dithionite was standardized by anaerobic titration against ferricyanide. The arrow indicates the equivalence point between methyl viologen and dithionite.

which should be a substrate analogue of the nitrite anion, functions as a substrate in preference to sulfite in the formation of sulfide.

The presence of dithionite reductase activity in the preparation does not however, account for the failure of reduced methyl viologen to accumulate at the end of a reductive titration involving only this reducing agent. Hydrogenase activity in the preparation could be demonstrated by following hydrogen uptake with benzyl viologen or nitrite as electron acceptors. At 35°C the rate of hydrogen uptake extrapolated to infinite benzyl viologen concentration was about 0.28 μmole H_2 min^{-1} mg^{-1} as opposed to a rate of about 1.5 μmole H_2 min^{-1} mg^{-1} when nitrite was the sole electron acceptor. The fact that nitrite appeared to be a better electron acceptor than benzyl viologen, suggested that the hydrogenase activity could be an integral part of the nitrite reductase. This supposition was reinforced by the finding that the reduction of cytochrome c_3 by the hydrogenase activity with hydrogen as electron donor was too slow to be conveniently measured when an enzyme concentration giving easily measurable reduction of benzyl viologen was used. However, the effect of heat inactivation of the nitrite reductase on hydrogenase activity, shown in Fig. 4, indicated

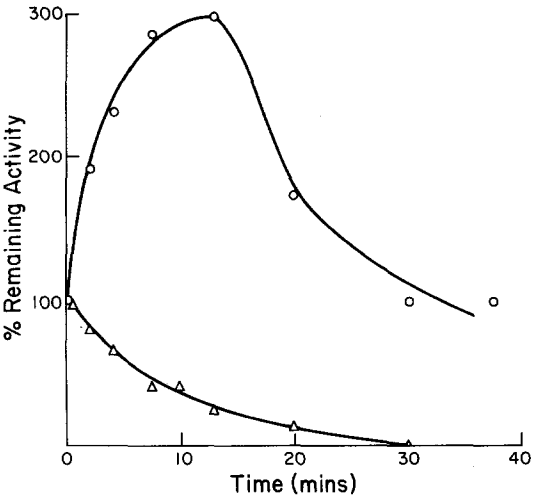

Figure 4: Nitrite reductase at a concentration of 0.677 mg/ml in 50 mM K-phosphate, pH 7.6, was heated at 70°C. Aliquots were withdrawn and pipetted into chilled test tubes at the times indicated. Hydrogenase (O-O) and nitrite reductase (Δ - Δ) activities were measured.

that the hydrogenase activity may be a contaminant. Nitrite reductase from *D. desulfuricans* occurs in solution as a large aggregate of uncertain molecular weight and it is possible that contaminating activities were included during solubilization of the enzyme. Conditions for the dissociation and chromatographic separation of the activities were therefore sought. The results shown in Fig. 5 indicate co-chromatography of nitrite reductase and dithionite re-

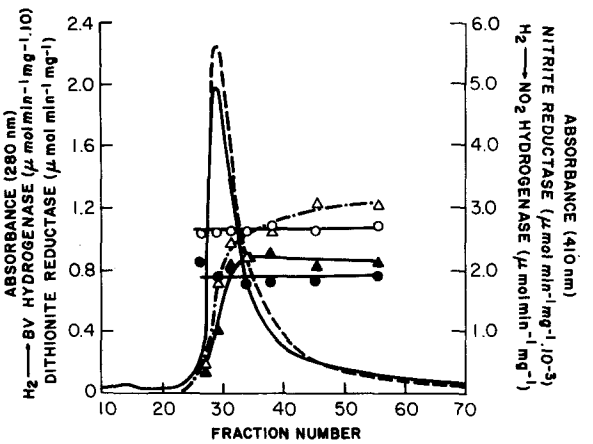

Figure 5: Nitrite reductase in 100 mM K-phosphate, pH 7.6, was 45% saturated by addition of solid $(NH_4)_2SO_4$ at 0°C. The enzyme was applied to a DEAE-cellulose DE-52 column (40 X 2.5 cm) equilibrated with 45% $(NH_4)_2SO_4$ adjusted to pH 8.0 with Tris-base. The column was eluted with a linear gradient using 750 ml of the latter solution and 750 ml 50 mM Tris-HCl, pH 8.0, as limiting buffer. The eluate was analyzed for absorbance at 280 nm (-) and 410 nm (----) and for nitrite reductase (O-O), dithionite reductase (●-●) and hydrogenase with nitrite (▲-▲) or benzyl viologen (Δ - Δ) as electron acceptor. BV: benzyl viologen. Hydrogenase with nitrite as electron acceptor was measured manometrically.

ductase activities, whereas hydrogenase activity does not coincide with the heme Soret band. Also indicated are the low levels of hydrogenase and dithionite reductase activities relative to nitrite reductase.

At present it is uncertain whether the ability of the hydrogenase described here to transfer electrons to the nitrite reductase and also its inability to interact with cytochrome c_3 results merely from tight association with the nitrite reductase *in vitro* or whether it would also manifest itself *in vivo*. Whereas no absolute proof can be presented that the dithionite and nitrite reductase activities are due to the same enzyme, the data does afford an explanation for the unusual stoichiometry observed in the reductive titration with dithionite, and presents evidence for the presence of a dithionite reductase activity as distinct from sulfite reductase.

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