PURIFICATION AND SOME PROPERTIES OF THE MEMBRANE-BOUND RESPIRATORY NITRATE REDUCTASE OF *AEROBACTER AEROGENES*

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1. Introduction

Respiratory nitrate reductases of various bacteria are membrane-bound proteins [1–3], and mediate the transfer of electrons from cytochromes to nitrate, which is used as the terminal electron acceptor under anaerobic conditions [1,4,5]. To the best of our knowledge, only two respiratory nitrate reductases have been isolated [3,6], that were devoid of cytochromes and appeared to be homogenous as judged from the electrophoretic and the sedimention [6] analysis of the purified enzyme. The purified respiratory nitrate reductase from *Escherichia coli* (Yamagutchi strain) was found to have a sedimentation value of 25 S [6], whereas the respiratory nitrate reductase from *E. coli* K-12, after solubilization from the membranes, was reported to sediment at about 11 S [2].

This paper reports on the purification of the respiratory nitrate reductase of *Aerobacter aerogenes*. It is shown that the enzyme can be completely solubilized from the membrane fraction with the aid of deoxycholate (DOC). The presence of DOC during the first stages of the purification after solubilization proves to be necessary in order to avoid reaggregation of the enzyme with other membrane components still present. The purified enzyme does not contain cytochrome material, appears to be homogenous, and can be present in a 8.5 S monomeric state or in a 13.9 S dimeric state.

2. Materials and methods

A. aerogenes, strain S 45, was grown in a minimal medium under anaerobic conditions as described previously [7]. The bacteria were collected in the mid-log phase, where they have a high level of nitrate reductase activity [7]. Washing and storage of the bacteria, and the preparation of the cell-free extracts were essentially the same as described previously [7].

The membrane fraction was obtained by centrifuging the cell-free extract at 122,000 × *g* for 1 hr in a Spinco preparative ultracentrifuge. The pellet was washed once with 0.07 M phosphate buffer, pH 7.0, and resuspended (3 mg protein/ml) in 0.05 M imidazole buffer, pH 7.8, with the aid of a Potter-Elvehjem homogenizer. Deoxycholate (DOC) was added at a final concentration of 1.5% and, after stirring for 15 hr at 4°C, the suspension was centrifuged again at 122,000 × *g* for 1 hr. The supernatant, referred to as the DOC-solubilized supernatant, was carefully separated.

Nitrate reductase activity with reduced benzylviologen as the electron donor, was assayed and defined as before [7]. Catalase activity was measured according to Bergmeyer [8], and protein acoording to Lowry et al. [9].

3. Results and discussion

3.1. *Solubilization of the nitrate reductase*

The best solubilization result was obtained by treating the membrane fraction with DOC at a final concentration of 1.5%. In this way 85% of the nitrate reductase activity and only 30% of the total membrane protein became soluble. A good recovery (90%) of the enzyme was also obtained with the detergent Tri-

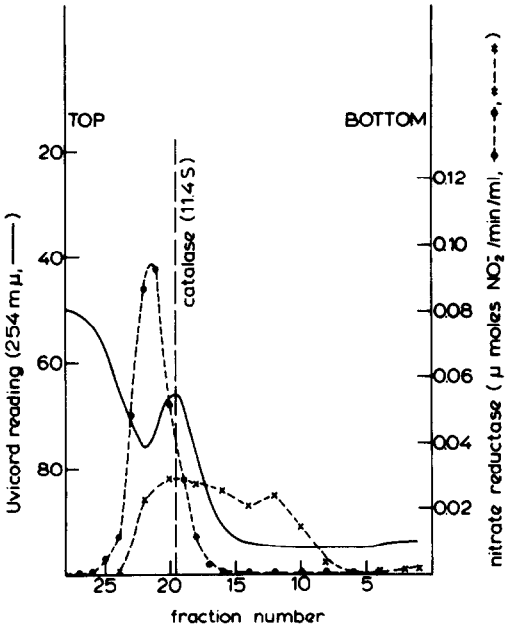

Fig. 1. Sedimentation analysis of the solubilized nitrate reductase. Samples (0.5 ml) of the DOC-solunilized supernatant (see Methods) and 0.15 ml of a diluted catalase solution (15 000 U./ml, Sigma) were layered over 32 ml sucrose gradients (5-25%, w/v) with (0.2%, •- - - •) or without (x- - -x) DOC. The gradients were centrifuged in a S.W.25-1 rotor of a Spinco ultracentrifuge at 22 500 rpm for 18 hr at 4°C. Fractions (1 ml) were assayed for nitrate reductase activity [14] and catalase activity [16]. The transmission at 254 mμ was continuously monitored with a Uvicord I (LKB).

ton X-100 (1%), but in this case as much as 70% of the membrane protein was solubilized. Much less successful were treatments with other detergents, such as Tween-40, -60, or -80 and Brij-35 or -58, or extraction with 0.25-1 M NaCl or 0.03 M K_2CO_3, giving either inactivation of the enzyme or poor solubilization or both.

The DOC-solubilized supernatant (see Methods) was analyzed by sucrose gradient centrifugation, both in the absence and in the presence of DOC (0.2%), using catalase as a reference (fig. 1). In the presence of DOC the nitrate reductase moves as a single zone with a sedimentation value of 8.5 S as compared with the known value (11.4 S) of catalase. However, in the absence of DOC the nitrate reductase activity is found scattered all over the gradient, whereas the sedimentation behaviour of catalase remains unaltered. Apparently, in the absence of DOC the nitrate reductase reaggregates, presumably with other membrane components, to particles of a variable size with sedimentation values up to 30 S. This result might provide an explanation for the high sedimentation constant, sometimes found for a partially purified nitrate reductase in the absence of a solubilizing agent (cf. Discussion in ref. 2). This aggregation phenomenon should be taken into account before a variability in the sedimentation behaviour of the enzyme is interpreted as a change in the physical state of the enzyme itself. It also illustrates the necessity of the presence of DOC during the next purification steps.

3.2. *Purification and properties of the nitrate reductase*

The DOC-solubilized supernatant was fractionated by means of gelfiltration as shown in fig. 2. The presence of DOC during the gelfiltration was indeed found to be essential for a sharp elution of the enzyme. As a result of the gelfiltration the enzyme activity was apparently separated from membrane components, since no random aggregation of the enzyme in the absence of DOC was observed after this step.

For a futher purification the pooled enzyme fractions of the gelfiltration were chromatographed on a QAE-Sephadex column at pH 6.2 (fig. 3). In this way a 100-fold purification of the enzyme could be achieved, the recovery of the enzyme being about 20%. The purification factor depends somewhat on the initial specific activity of the cell-free extract. However, in all purifications the final level of specific activity obtained was nearly the same, *viz.* 21 μmole of NO_2^- produced per mg protein per min. The purified enzyme appeared to be homogeneous as judged by sedimentation analysis (fig. 4) and by electrophoresis in 7.5% polyacrylamide gels at pH 9.5 [10]. The purified enzyme contained neither cytochromes, nor any other material absorbing in the 400-500 mμ region (which was found to be present in the purified *E. coli* enzyme [6]).

The sedimentation coefficient ($S_{20,w}$) of the purified enzyme was estimated to be 13.9 S (fig. 4). If, however, DOC or Triton-X-100 was added to the enzyme solution at a final concentration of 0.2% a sharp transition to a form sedimenting at 8.5 S was observed (cf. also fig. 1). Since the preparation appeared to be clearly monodispersive in both cases and since factors

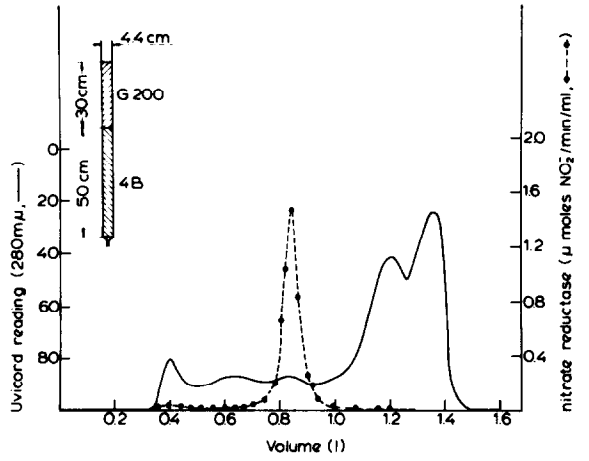

Fig. 2. Gelfiltration of the DOC-solubilized supernatant on a combined Sephadex G 200-Sepharose 4 B column. The column (see inset) was equilibrated with 0.05 M imidazole buffer, pH 7.8, containing 0.2% DOC. After applying 20 ml of the solubilized supernatant, the column was eluted with the same buffer at a flow rate of 60 ml per hr. Fractions of 15 ml were collected and assayed for nitrate reductase activity. The transmission at 280 mμ was continously monitored with a Uvicord II (LKB).

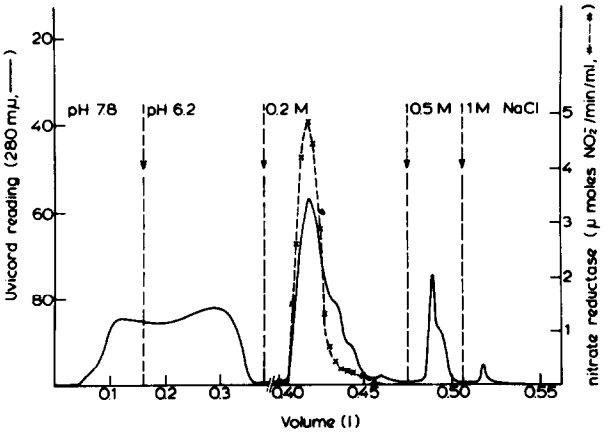

Fig. 3. Ion exchane chromatography of the partially purified nitrate reductase on QAE-Sephadex. The pooled enzyme fractions (75 ml) of the gelfiltration (fig. 2) were absorbed to a column (20 X 2 cm) of QAE-Sephadex (A-25) equilibrated with 0.05 M imidazole buffer, pH 7.8. After the adsorption the pH was lowered to 6.2 (0.05 M imidazole buffer), followed by a stepwise elution with increasing salt concentrations. The flow rate was 6 ml per hr; fractions of 3 ml were collected and assayed for nitrate reductase activity. The transmission at 280 mμ was continuously monitored with a Uvicord II (LKB).

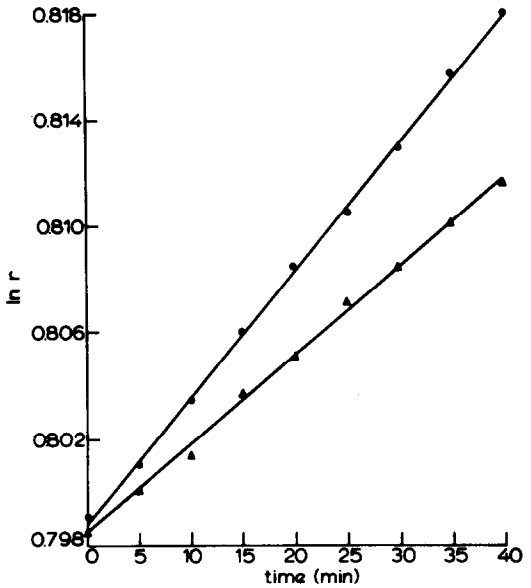

Fig. 4. Sedimentation velocity of the purified nitrate reductase in the absence (•—•; 13.9 S) and in the presence (▲—▲; 8.5 S) of DOC (0.2%). Scanning was started after the An-D rotor of the Spinco E analytical ultracentrifuge attained its required speed (42 040 rpm). The difference in absorbance between enzyme solution and buffer (0.07 M phosphate, pH 7.0) was measured at 264 mμ (double-sector cell). Enzyme concentration: 0.2 mg/ml. Temperature: 6.9°C.

that may aggregate with the enzyme have been removed in the course of the purification, the most likely explanation for the observed sedimentation behaviour is that the enzyme can exist in a 8.5 S monomeric state. This idea is supported by the results of molecular weight determinations by means of sedimentation analysis, using the Archibald method, and by the gelfiltration method. With both methods the estimated molecular weights are about 2×10^5 and 4×10^5 daltons for the 8.5 S and the 13.9 S form, respectivily. Both forms have the same specific activity (the 8.5 S state tested in the presence of 0.2% DOC). The transition of the 13.9 S state to the 8.5 S state can already be brought about by the non-ionic detergent Triton-X-100 in low concentration, without any further change of conditions, suggesting that hydrophobic bonds are involved in the association phenomenon. It is hoped that a further characterization of the nitrate reductase described here will contribute to an adequate understan-

ding of the process of nitrate respiration in *A. aerogenes* [7].

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