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An abundant haem protein in *Riftia pachyptila* symbionts is a nitrite reductase

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Introduction

A large proportion of the biomass at hydrothermal vents is made up of the tube-worm species *Riftia pachyptila* Jones, 1981. *R. pachyptila* relies exclusively on its chemosynthetic bacterial symbionts for its source of organic carbon. The symbionts live in an organ, inside the worm, connected to the surrounding seawater only via the worm's closed circulatory system. This symbiont association takes place, of necessity, in a zone exposed alternately to hypoxic, sulphide-rich waters and oxic, sulphide-poor, nitrate-rich waters (see reviews by Nelson & Fisher (1995) and Childress & Fisher (1992) for discussions of the biological aspects of hydrothermal vents and *Riftia pachyptila*).

It is not fully understood how *R. pachyptila* and it symbiont survive the frequent periods of anoxia they must encounter. *Riftia pachyptila* symbionts have been shown to respire both oxygen and nitrate. *R. pachyptila* itself contains widely variable levels of nitrite, potentially a product of nitrate respiration, in its blood stream. Thus, it is possible that *R. pachyptila*'s symbiont may utilize nitrite respiration as well as nitrate respiration in order to survive extended periods of anoxia. In the following paper we present a description of the purification of a nitrite reductase (NR) from *R. pachyptila* (RpNR). It is not known whether or not this RpNR is assimilatory or dissimilatory. We hope that future experiments by others will determine the role of this abundant protein in the metabolism of *R. pachyptila*.

Purification of RpNR

All steps should be done on ice or in a 4°C cold room. First, choose a live worm, with a healthy looking trophosome and dissect the bacteria containing tissue away from the connective tissue. Rinse in iced *Riftia* saline (0.4 M NaCl, 2.6 mM KCl, 30 mM $MgSO_4$ $7H_2O$, 10 mM $CaCl_2$ $2H_2O$, 30 mM HEPES, pH 7.5) until no more blood is seen in the rinse. Bring 2-5 ml settled wet volume of trophosome tissue to a total volume of 9 ml in *Riftia* saline (RS) and homogenize in a 15 ml glass dounce with a loose fitting pestle (approx. 100 µm clearance) using 2-3 gentle strokes. Spin 20 min in a clinical centrifuge on the #5 setting. Remove the supernatant (host fraction) and gently resuspend the bacterial pellet in 3 ml RS. Overlay onto 10 ml 90% percoll made with *R*S and spin 5 min in a clinical centrifuge on setting #5. Remove supernatant (SN) and resuspend pellet in 10 ml RS then spin on setting #3 for 3 min. Repeat this rinse step once more then resuspend pellet in 3 ml RS (15% glycerol may be added) and freeze in liquid nitrogen.

It is best to begin with the above purified bacteria but is possible to begin with whole frozen trophosome. Next, lyse the bacteria either by sonication or by French press. Dilute the purified bacterial stock with RS by 2-3 fold, add PMSF (Phenyl methyl sulphonyl fluoride) to a final concentration of 1 mM and run twice through a French press at 20,000 psi. Spin lysate at 14,000 rpm for 25 min at 4°C and save SN. Fractionate this SN by ammonium sulphate precipitation on ice (maintain pH 7.0) allowing at least 20 min equilibration after addition of the salt and then spinning at 15,000 rpm at 4°C for 15 min to pellet. Serially collect the following fractions: 0-35%, 35-50%, 50-70%, 70-90% and 90-100%. Most of the RpNR is in the 70-90% pellet. Resuspend the green 70-90% pellet in RS diluted by half for sucrose gradient purification or in phosphate-buffered saline solution (PBS) (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 $7H_2O$, 2mM KH_2PO_4, pH 7.5 at 4°C) for gel filtration.

Overlay onto a 5-20% sucrose gradient made with RS at 4°C and spin at 50,000 rpm in a Beckman TLS-55 rotor at 4°C for 6 hours. The RpNR yellow-green band will be approximately 11 mm from the bottom of the tube corresponding to a sedimentation coefficient of 4.38 and an approximate weight of 300 kDa as internally calibrated against the red 400 kDa *R. pachyptila* small blood protein band (*Riftia pachyptila* vascular blood added to overlay for calibration but not for routine purification). Collect band with a syringe and rinse several times with PBS in a Centricon-100 spin column to remove the sucrose and RS and store the purified protein as concentrated as possible at -80°C.

Optimal gel filtration has not been worked out but may be done as follows: prepare Sephadex G-200 in PBS and pack into a 2.5 cm x 37 cm column at a 21 cm pressure drop and rinse at 16 cm pressure drop. Load protein purified from 5g whole trophosome equivalent or 3-4g of purified bacteria equivalent in 1 ml PBS and collect 1 ml fractions at a rate of 730 µl min^{-1}. The RpNR will be at Ve = 54 ml which is around the void volume of this column and corresponds to a theoretical weight of greater than 550 kDa. The lower salt concentration in the gel filtration column as compared to the sucrose gradient may be responsible for the disagreement in size since RpNR may aberrantly associate with other proteins at low salt concentrations leading to a larger apparent size.

Analytical Methods and Results

I. Protein

Riftia pachyptila nitrite reductase was first observed as a ca. 60 kDa band on an unstained SDS-PAGE gel. This band was shown to be protein by its disappearance upon Proteinase K treatment for 2 hrs at 55 °C. The colour of the band varied from orange, when the reducing agent dithiothreitol (DTT) was not added to loading buffer, to yellow, when DTT was added to a final concentration of 100mM. Initially, experiments were performed with whole frozen trophosome. Therefore, it was not known if the 60 kDa protein was contained in the host or the symbiont, or whether or not it was a blood component. To determine the localization of the protein, the purification procedure above was carried out on both the host fraction and the purified bacteria. All of the 60 kDa protein appears to be contained within the purified symbiont bacterial fraction. In addition, overloading a gel with 60 µl undiluted vascular or coelomic blood did not produce a band of this size or colour.

The protein was further characterized by isoelectric focusing and N-terminal sequencing. A Novex isoelectric focusing gel was run according to manufacturers instructions and the pI of the RpNR complex was found to be 5.2. The N-terminal sequence was determined by the protein sequencing facility at the University of California San Diego, following their instructions for blotting a SDS-PAGE gel to PVDF membrane and cutting out the unstained yellow-green band. The sequence is SVKAPPKEMSTETKAAVKKHK and shows no significant identity to proteins in the SwissProt database.

Since RpNR was shown by both sucrose gradient and gel filtration to exist as a large complex of at least 300 kDa, we attempted to determine whether the other proteins we saw in the sucrose purified fraction (Fig. 1) were contaminants or were actually associated with the complex. Native polyacrylamide tube gels were run with purified RpNR and the visible yellow-green band was cut out. This gel slice was

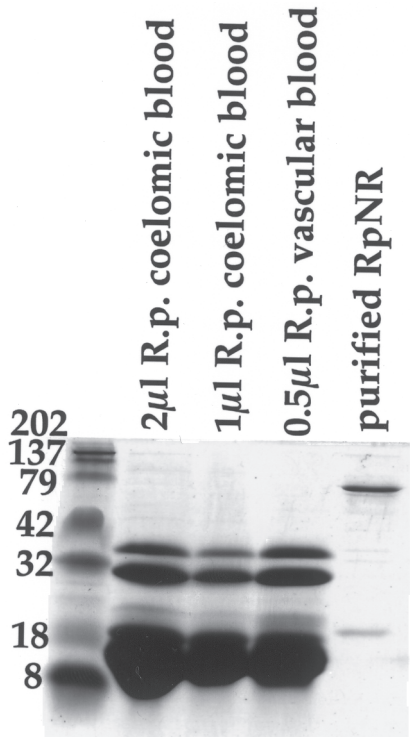

Figure 1. Coomassie stained SDS-PAGE gel, 12% with 4% stack, showing purified RpNR with *R. pachyptila* coelomic and vascular blood for comparison. Weights determined relative to Bio-Rad Kaleidoscope Prestained standards.

then soaked in 20% ß-Mercaptoethanol (BME) for 20 min and incorporated into a second SDS-PAGE gel by pouring the gel around the gel slice. Purified vascular blood was treated in the same way as a control. This procedure revealed four bands for RpNR at 60, 34, 30 and 20 kDa. The bands at 60 and 20 kDa were stronger while the bands at 34 and 30 kDa were weak. The sucrose gradient purified 1.7 MDa vascular blood protein behaved as expected producing two bands around 30 kDa, one at about 40 kDa and several bands below 18 kDa. Thus the RpNR complex has at least two subunits of 20 and 60 kDa and possibly two more subunits of 34 and 30 kDa.

II. Haem

When RpNR was first observed to be a coloured protein an absorption spectrum was made. With a standard spectrophotometer peaks were visible at 348, 372 and 404 nm. Thin layer spectra were made in the laboratory of Dr. David Kraus using partially purified protein which showed direct peaks at 410, 540 and 570 nm. There was little effect when air was replaced with N_2, but upon further reduction with 0.5 torr H2S, maxima in the difference spectra between N_2 and N_2+H_2S appeared at 420, 520 and 551 nm indicating the presence of a cytochrome c551 (Fig. 2). This prompted

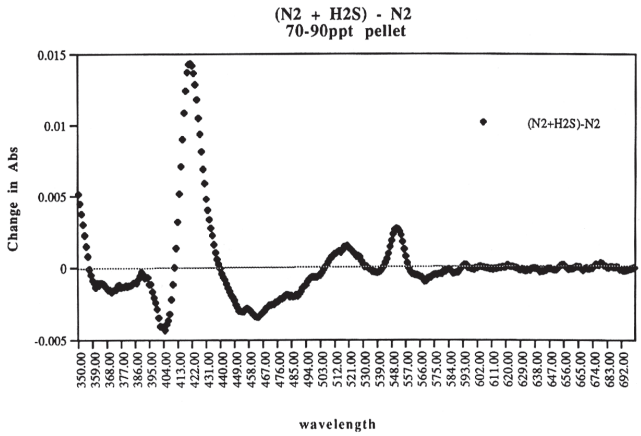

Figure 2. Difference in absorbtion spectrum from a nitrogen reduced state to a 0.5 torr H2S further reduced state of partially purified RpNR. Peaks are at 420, 520 and 551 nm.

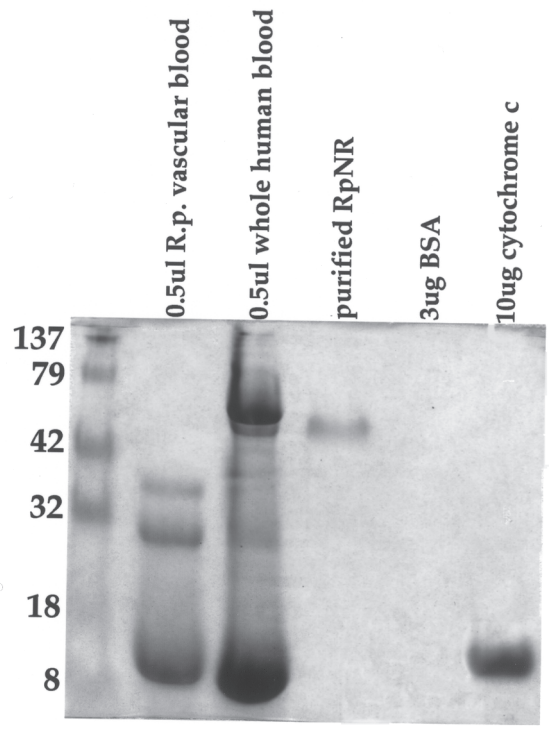

Figure 3. Haem-specific stain of an SDS-PAGE gel (10% with 4% stack). Purified RpNR shown in comparison to whole human blood, R.p. vascular blood, 10 µg cytochrome c from Sigma, and 3 µg BSA (66 kDa) as a negative control.

us to determine whether the purified 60 kDa protein bound haem and if so what type, and also to estimate the number of haems per protein by quantifying the number of iron atoms present per complex.

Purified protein was loaded onto an SDS-PAGE gel and stained for haem according to Francis & Becker (1984). The 60 kDa RpNR band stained for haem (Fig. 3) and, if the gel was overloaded, the 20 kDa subunit also stained for haem although faintly. The fact that the 60 kDa protein stained for haem, even under harsh non-native conditions, is consistent with the spectral data in suggesting that the haem is a cytochrome type c. To confirm this, reduced pyridine haemochromogen assays were done using pure protein as follows: 299 µl of protein solution with an absorbance of 0.56 at 404 nm, 166 µl of pyridine, and 33 µl of 5M NaOH were mixed and a few crystals of sodium dithionite added. The spectrum was then read from 400 to 600 nm. Peaks at 518 and 549 nm were seen indicating the presence of a cytochrome type c as the prosthetic group on the 60 kDa protein. The amount of iron haems per protein complex was estimated to be 16. Protein samples were burned in a graphite furnace and the amount of iron present determined on a scale of ppm versus a standard curve. The concentration of the protein samples was measured in triplicate by BioRad BCA assay. The weight of the complex used in calculations was 300 kDa. Among the possible configurations of the RpNR complex, one possibility would be four 60 kDa subunits with two to three haems each and four 20 kDa subunits with one haem each. This hypothetical configuration is based on the relative protein amounts of each subunit and the amount of haem staining for each as well as the overall size of the complex.

III. Function

Once it was known that the 60 kDa protein was a cytochrome c551 and that it was localized to the symbiont bacteria, we compared it in size to other known bacterial cytochrome c type proteins and performed a nitrite reduction functional assay. The nitrite reduction assay was modified from Kajie & Anraku (1986) using PBS to make up the solution of 0.3 mM benzyl viologen. The slope of the decline in absorbance at 600 nm over time changed dramatically from the baseline after addition of $NaNO_2$ and remained constant (linear) to near zero. Furthermore, this slope was reduced by half when the amount of protein added was reduced by half indicating that the purified 300 kDa complex is a nitrite reductase.

Discussion

Nitrite reductases have been isolated from many bacteria. Ammonia forming nitrite reductases have been isolated from *Desulfovibrio desulfuricans* (Beijerinck, 1895), *Wolinella succinogens* (Wolin et al., 1961), *Escherichia coli* (Migula, 1895), and *Vibrio fischeri* (Beijerinck, 1889) which contain six c-type haems per protein monomer. The

monomers have molecular weights of 66 kDa, 63 kDa, 56.3 kDa, and 57 kDa respectively. In the case of *D. desulfuricans,* the nitrite reductase exists as a complex of 750 kDa with an additional subunit of 18.8 kDa in weight (Liu et al., 1988). This is very similar in arrangement to RpNR suggesting the possibility that RpNR is also an ammonia forming nitrite reductase though additional experiments must be done. Specifically, the formation of ammonia upon nitrite reduction must be conclusively shown if RpNR is to be classed with the above group of hexahaem c-type cytochromes. Also, the number of haems per monomer must be more precisely determined although, as seen in Darwin et al. (1993), one may find that RpNR is a tetrahaem rather than a hexahaem nitrite reductase.

If in fact, RpNR is a dissimilatory nitrite reductase, then there exists the possibility that this enzyme, like the nitrite reductase of *E. coli,* is also induced primarily in the absence of oxygen and the presence of nitrate and/or nitrite (Darwin et al., 1993). If this is the case, then the expression of this enzyme might be a useful indicator of the environmental conditions experienced by individual *R. pachyptila* over periods of time on the order of hours to days. Since the environment surrounding *R. pachyptila* changes rapidly and is also variable in the vertical dimension as well as the horizontal, an internal biological indicator of anoxia experienced over time would be a valuable tool in learning about *R. pachyptila*'s experience of its environment.

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