PERIPLASMIC LOCATION OF THE TERMINAL REDUCTASE IN NITRITE RESPIRATION

Paul M. WOOD

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Received 27 April 1978
Revised version received 23 June 1978

1. Introduction

In respiratory electron transport chains the component which reacts with the inorganic electron acceptor is almost invariably membrane bound. Examples are cytochrome aa_3 in mitochondria, cytochromes aa_3, *o* and *d* in aerobic bacteria, the dissimilatory nitrate reductase in bacteria capable of nitrate respiration, and nitrous oxide reductase in denitrifiers [1]. In many cases it has been established that the protons consumed in the reduction are taken up from the internal phase (the mitochondrial matrix or bacterial cytoplasm), thus contributing to the protonmotive force required by a chemiosmotic mechanism of oxidative phosphorylation [2–4].

By contrast, the dissimilatory nitrite reductase of denitrifying bacteria is a readily solubilised protein which in many cases is released by sonication or French press treatment [5]. In a few bacteria it contains 2 copper atoms/molecule and no iron [6]. The more widespread type, found in *Pseudomonas aeruginosa* and *Paracoccus denitrificans*, is a haem protein with two *c*-type and two d_1-type haems [5] per mol. wt 120 000, and consists of two identical subunits [7,8]. It has been studied mainly as a soluble cytochrome oxidase, and kinetic measurements have been made for reduction by *Pseudomonas* cytochrome *c*-551 and azurin (a copper protein) [9,10]. But in vivo its primary role would seem to be nitrite reduction: its absence has been demonstrated in mutants lacking nitrite respiration [11], it is only synthesised when denitrification is taking place, and denitrification is repressed above low oxygen pressures [1]. Besides, oxygen reduction only proceeds at about 3 s^{-1}, has a high K_m for a terminal oxidase (28 μM), and is inhibited by nitrite [12]. Nitric oxide is the product of nitrite reduction by the purified enzyme [1,13]:

$$NO_2^- + 2\,H^+ + e^- \rightarrow NO + H_2O$$

and the discussion below will mainly be concerned with this reaction. For whole cells there is evidence that free NO is not released [14], but little is known regarding the nature of nitric oxide reductase, or indeed whether it is a separate entity [1,14].

The present study was undertaken to discover whether this nitrite reductase is cytoplasmic or periplasmic. The location is of importance in settling its relationship to the membraneous respiratory chain, and permits experimentally measured stoichiometries for proton pumping and ATP synthesis to be compared with predicted values.

Abbreviation: DCPIP, 2,6-dichlorophenolindophenol

2. Methods

Pseudomonas aeruginosa P6009/1 [15] was obtained from Dr P. E. Reynolds, Department of Biochemistry, Cambridge. It was maintained on nutrient agar slopes and grown in a medium containing 40 mM sodium glutamate, 35 mM KH_2PO_4, 35 mM K_2HPO_4, 0.4 mM $MgSO_4 \cdot 7\,H_2O$, 15 μM $FeSO_4 \cdot 7\,H_2O$, 15 μM $CuSO_4 \cdot 5\,H_2O$, 0.5 μM ammonium molybdate and 40 mM $NaNO_3$ (pH 6.7). After inoculation the flasks were sealed with cellophane and placed on an orbital shaker for 2 days at 32°C. On harvesting, a portion was converted to acetone powder (see below), which was weighed and formed a standard.

Periplasmic proteins were released [16,17] by harvesting the cells at 25°C and resuspending the pellets at 1 g/30 ml in medium at 30°C containing 0.5 M sucrose, 4 mM Na_2EDTA and 40 mM Tris/HCl (pH 8.0), plus 0.25 mg lysozyme/ml. After 2 min, $MgCl_2$ was added to 10 mM. The solution was left at 30°C for 30 min, then centrifuged (15 000 × *g*, 20 min), to give periplasmic proteins (plus some lysozyme) in the supernatant, and a pellet of spheroplasts. Cytoplasmic proteins were released [17] from the spheroplasts by resuspension in 10 mM Tris/HCl plus 2 mM Na_2EDTA (pH 8.0) at the same concentration as before. The release of DNA caused the suspension to become too viscous for centrifugation, so after incubation for 15 min at 20°C, $MgCl_2$ was added to 4 mM, plus a trace of DNase (EC 3.1.4.5). After a few minutes it was centrifuged to yield a supernatant of cytoplasmic proteins and a pellet of lysed membranes. The pellet was resuspended in Tris/HCl (pH 8.0).

Acetone powders were prepared by treating pelleted material with acetone at −5°C [18]. Soluble proteins were released from acetone powders by a double extraction at 45°C with 100 mM ammonium acetate/acetic acid (pH 6.5) [18].

For standardisation of assays, cytochrome *c*-551 and azurin were purified as in [18], and nitrite reductase was separated from other coloured material by concentrating a periplasmic fraction with Aquacide (Calbiochem), and then passing it through a column of Sephadex G-75 [19]. Difference spectra were recorded with a sensitive split-beam spectrophotometer. Solutions containing nitrite reductase were bubbled with N_2 prior to addition of reductant to avoid complications from oxidase activity, with a trace of octan-2-ol to prevent foaming. Pyridine haemochromes were prepared by adding 1 ml pyridine and 0.1 ml 4 M NaOH to 3 ml solution [20]. They were assayed immediately by difference spectra, adding ferricyanide to oxidase d_1-type haem or dithionite to reduce *c*-type.

Nitrite reductase was assayed by $\Delta\epsilon_{620\ nm} - \Delta\epsilon_{680\ nm} = 31.0\ mM^{-1}cm^{-1}$ for difference spectra in alkaline pyridine, untreated minus ferricyanide oxidised. Cytochrome *c*-551 was assayed by difference spectra at ~ pH 7 (ascorbacte + 15 μM DCPIP reduced minus ferricyanide oxidised) by measuring $A_{551\ nm}$ relative to a line drawn from 535–565 nm, subtracting the contribution from nitrite reductase, $\epsilon = 23.3\ mM^{-1}cm^{-1}$, and assuming $\epsilon = 25.2\ mM^{-1}cm^{-1}$ for cytochrome *c*-551. These three extinction coefficients were derived from spectra taken with purified proteins. For cytochrome *c*-551 the basis was $\epsilon_{551\ nm} = 30.0\ mM^{-1}cm^{-1}$ for the reduced cytochrome [18]. For nitrite reductase (see fig.1) it was assumed that in the pyridine haemochrome the two *c*-type haems/molecule each had the same extinction coefficients as mammalian cytochrome *c*: $\epsilon = 21.7\ mM^{-1}cm^{-1}$ for $A_{550\ nm}$ relative to a line drawn from 540–560 nm in dithionite reduced minus untreated difference spectra. (This value was measured with a horse cytochrome *c* solution previously assayed at pH 7 [21].)

For assay of azurin it was necessary to remove nitrite reductase, and advisable to remove cytochrome *c*-551 [18]. The solution was diluted with 4 vol. water and adjusted to pH 3.9 with 1 M acetic acid. After removal of precipitate by centrifugation it was diluted 5-fold again, ferricyanide was added to 10 μM, and it was passed through a small column of

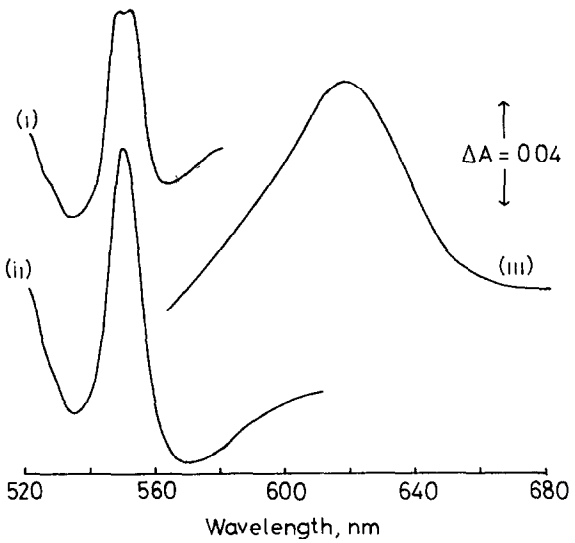

Fig.1. Difference spectra for purified nitrite reductase. (i) Buffer, pH 7, ascorbate + 15 μM DCPIP minus ferricyanide. (ii) Pyridine haemochrome of the same sample, dithionite minus untreated, showing a standard *c*-type haem α-band peak, much higher than the split peak in (i). (iii) As (ii), untreated minus ferricyanide, showing d_1-type haem absorption.

CM-cellulose equilibrated with 25 mM ammonium acetate/acetic acid (pH 3.9). The column was washed with 50 mM ammonium acetate/acetic acid (pH 4.5) to remove brown material, and the blue band (azurin) was eluted with 100 mM potassium phosphate (pH 7). It was assayed in difference spectra, ferricyanide oxidised minus ascorbate reduced, by $\Delta\epsilon_{635\ nm}-\Delta\epsilon_{695\ nm} = 2.7\ mM^{-1}cm^{-1}$, derived from a spectrum for the purified protein assuming $\Delta\epsilon_{625\ nm} = 4.8\ mM^{-1}cm^{-1}$ [22].

Isocitrate dehydrogenase (EC 1.1.1.42) was assayed at 25°C by monitoring the rate of NADPH formation on a dual-wavelength spectrophotometer. The medium was 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid/KOH, pH 7.4, with 4 mM $MnSO_4$, 4 mM sodium isocitrate and 0.33 mM $NADP^+$ [23].

3. Results

The results of a cell fractionation experiment are presented in table 1. This shows data for a periplasmic fraction prepared by lysozyme treatment in 0.5 M sucrose after a brief exposure to EDTA, a cytoplasmic fraction prepared by osmotic lysis of spheroplasts, and the residual soluble proteins recovered by extraction of an acetone powder prepared from the lysed membranes. Table 1 shows the distribution of NADP-linked isocitrate dehydrogenase (a marker for the cytoplasm), nitrite reductase, and also cytochrome *c*-551 and azurin. Except for azurin, all assays were performed without purification. Interference from the other soluble *c*-type cytochromes reported for *Ps. aeruginosa* is unlikely: the combined levels of cytochromes *c*-550 and *c*-555 [24] (originally lumped together as *c*-554 [18]) proved scarcely detectable on attempted purification (as found in [25]) and were estimated as <2% of the *c*-551 level, while cytochrome *c*-557(551), a peroxidase with *c*-type haem, is only reduced by dithionite and would not contribute to ascorbate versus ferricyanide spectra [25,26]. (Preliminary results with cells grown semi-aerobically indicated a cytoplasmic location.)

Table 1 includes a nitrite reductase assay for the lysed membranes. This was conducted in the usual way, so the limit includes any cytochrome *d*, in practice only significant in aerated cultures. The lysed membranes contained high levels of ascorbate-reducible cytochrome *c* (30 μmol from cells equivalent to 100 g acetone powder), most of which was membrane bound and could not be extracted. The difference spectra in fig.2 show the presence of ascorbate reducible cytochrome *c* with a peak at 552 nm (along with *b*-type cytochromes), and also of a *b*-type cytochrome kept at least partly oxidised by nitrate in the presence of dithionite.

4. Discussion

The results in table 1 show that nearly all of the nitrite reductase and azurin, and most of the cytochrome *c*-551, were recovered in a periplasmic fraction which only accounted for 1.2% of the isocitrate dehydrogenase. This common location supports the general assumption in kinetic studies that cytochrome *c*-551 and azurin are the physiological donors to the reductase; preliminary results suggest that they form an interchangeable pair, with

Table 1
Location of nitrite reductase and other soluble redox proteins in *Ps. Aeruginosa*

	Isocitrate dehydrogenase (% total recovery)	Nitrite reductase (μmol from cells equivalent to 100 g acetone powder)	Cytochrome *c*-551	Azurin
Periplasmic fraction	1.2	12.2	29.3	13.0
Cytoplasmic fraction	98.1	<0.3	6.3	1.5
Acetone powder extract from lysed membranes	0.7	<0.5[a]	2.0	<0.2

[a] Assayed in the lysed membranes, since acetone powder extracts of whole cells gave poor yields, probably because of loss of d_1-type haem

For methods and other details, see text

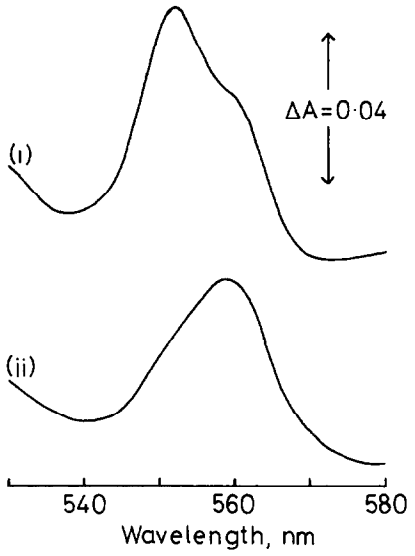

Fig.2. Difference spectra for resuspended lysed membranes (pH 8). (i) Ascorbate + 15 μM DCPIP minus ferricyanide. (ii) Membranes at 6-times the concentration of (i), dithionite minus dithionite + 10 mM $NaNO_3$.

the level of azurin depending on the availability of copper to the cells. The location of cytochrome *c*-551 also follows the rule that bacterial cytochromes showing sequence homology with mitochondrial cytochrome *c* are sited in the periplasm (e.g., *Rhodopseudomonas* [27], *Spirillum* [16]). Since *Ps. aeruginosa* also has a membrane-bound cytochrome *c* (fig.2), almost certainly analogous to cytochrome c_1 of mitochondria and *Paracoccus denitrificans*, the entire electron transport chain for nitrite reduction can be predicted as:

. . . (ubiquinone + cytochromes *b*) – cytochrome c_1

– cytochrome *c*-551 (azurin) – nitrite reductase

As far as proton pumping is concerned, the conclusion from a periplasmic location is that the protons taken up in nitrite reduction to NO, 4/2 e^-, cannot contribute to an alkalinisation of the cytoplasm, and are wasted from a chemiosmotic point of view. (NO_2^- is the active species for the reaction, not HNO_2, since the pH optimum is 6.5 [13], whereas protonation has pK 3.4 [28].) This inefficiency is particularly striking since nitrite respiration is unusual in that a net proton consumption occurs [29]. It is only understandable if denitrification is an evolutionary offshoot of aerobic respiration; an explanation can then be found in the redox potential, $E_{m,7}(NO_2^-/NO) = +374$ mV [30]. This is comparable with that for nitrate reduction, $E_{m,7}(NO_3^-/NO_2^-) = +420$ mV, and could not support the same level of proton pumping as oxygen reduction to water, $E_{m,7}(O_2/H_2O) = +810$ mV. Indeed, it was found [31] by measuring molar growth yields for *Pseudomonas denitrificans* that all steps of denitrification had a similar efficiency for oxidative phosphorylation on an electron basis, while all were distinctly less efficient than aerobic growth.

A proton pumping model which agrees with this is shown in fig.3. For nitrite reduction it is reasonable to assume the same stiochiometries of $H^+/2\ e^-$ in sites 1 and 2 as have been measured for aerobic bacterial respiration by pathways which include high potential *c*-type cytochromes, 2 in site 1 and 4 in site 2 [2,32]. For nitrate reduction, the respiratory nitrate reductase has been studied in greatest depth in *E. coli* [3,33], but appears to be very similar in other bacteria [34]. Nitrate reduction is known to bypass *c*-type cytochromes in *Paracoccus denitrificans* [33], and fig.2. implies that this holds for *Ps. aeruginosa*. The standard pattern for nitrate respiration is that the electrons branch off from the aerobic chain in the ubiquinone-cytochrome *b* region, and pass through a specific cytochrome *b*, $b^{NO_3^-}$, to the nitrate reductase complex, which includes nonhaem iron and molybdenum [33,34]. Proton pumping by site 2 only yields

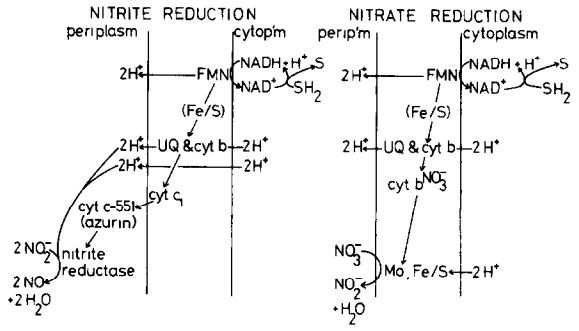

Fig.3. Suggested stoichiometries for proton pumping in the course of nitrite and nitrate reduction by NAD-linked substrates. SH_2, substrate; UQ, ubiquinone.

$H^+/2\ e^-$ of 2, as in aerobic respiration whenever site 2 does not conclude with a *c*-type cytochrome [2,32], but two further protons/$2e^-$ are taken up from the cytoplasm as nitrate is reduced [3,33]. Overall, therefore, the proton pumping efficiency is as predicted for nitrite reduction.

It has frequently been stated that if, or because, nitrite reductase is soluble, nitrite reduction cannot support oxidative phosphorylation [1,31]. However this would only be a valid conclusion if the immediate donor had a negative redox potential, e.g., NAD(P)H or ferredoxin, as for the cytoplasmic nitrate and nitrite ($NO_2^- \rightarrow NH_4^+$) reductases, which normally have an assimilatory role.

Sulphate respiration, as in *Desulfovibrio*, provides further examples of soluble terminal reductases [34]. Sulphate enters the cytoplasm and is primed at the expense of two high energy phosphate bonds to form adenylyl sulphate. This is reduced to sulphite by adenylyl sulphate reductase, and further reduction is mediated by bisulphite reductase. Both of these are soluble cytoplasmic proteins [35]. Although there are many unanswered questions concerning the linkage of sulphate respiration to proton gradient formation, it is clear that the protons taken up in the reductions will come from the cytoplasm as normal, unlike the nitrite respiration described here.

Acknowledgements

I should like to thank the Science Research Council for a research grant, Mr D. Willey for expert technical assistance and Dr D. S. Bendall for the use of spectrophotometers.

References

[1] Payne, W. J. (1973) Bacteriol. Rev. 37, 409–452.
[2] Jones, C. W. (1977) Symp. Soc. Gen. Microbiol. 27, 23–59.
[3] Garland, P. B., Downie, A. and Haddock, B. A. (1975) Biochem. J. 152, 547–559.
[4] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[5] Lemberg, R. and Barrett, J. (1973) Cytochromes, Academic Press, London.
[6] Iwasaki, H., Noji, S. and Shidara, S. (1975) J. Biochem. (Tokyo) 78, 355–361.
[7] Gudat, J. C., Singh, J. and Wharton, D. C. (1973) Biochim. Biophys. Acta 292, 376–390.
[8] Kuronen, T., Saraste, M. and Ellfolk, N. (1975) Biochim. Biophys. Acta 393, 48–54.
[9] Parr, S. R., Barber, D., Greenwood, C. and Brunori, M. (1977) Biochem. J. 167, 447–455.
[10] Wharton, D. C. and Gibson, Q. H. (1976) Biochim. Biophys. Acta 430, 445–453.
[11] van Hartingsveldt, J. and Stouthamer, A. H. (1973) J. Gen. Microbiol. 74, 97–106.
[12] Yamanaka, T. and Okunuki, K. (1963) Biochim. Biophys. Acta 67, 379–393.
[13] Yamanaka, T., Ota, A. and Okunuki, K. (1961) Biochim. Biophys. Acta 53, 294–308.
[14] St John, R. T. and Hollocher, T. C. (1977) J. Biol. Chem. 252, 212–218.
[15] Ambler, R. P. and Wynn, M. (1973) Biochem. J. 131, 485–498.
[16] Garrard, W. T. (1971) J. Bacteriol. 105, 93–100.
[17] Wilkinson, S. G. (1975) in: Resistance of *Ps. aeruginosa*, (Brown, M. R. W. ed) pp. 145–188, Wiley, London.
[18] Ambler, R. P. (1963) Biochem. J. 89, 341–349.
[19] Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W. and Melling, J. (1977) Biochem. J. 157, 423–430.
[20] Falk, J. E. (1964) Porphyrins and Metalloporphyrins, p. 181, Elsevier, Amsterdam.
[21] Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570–572.
[22] Ambler, R. P. and Brown, L. H. (1967) Biochem. J. 104, 784–825.
[23] Bernt, E. and Bergmeyer, H. U. (1974) in: Methods of Enzymatic Analysis, 2nd engl. edn (Bergmeyer, H. U. ed) pp. 624–627, Academic Press, New York.
[24] Rönnberg, M. and Ellfolk, N. (1975) Acta Chem. Scand. 29B, 719–727.
[25] Singh, J. and Wharton, D. C. (1973) Biochim. Biophys. Acta 292, 391–401.
[26] Soininen, R. and Ellfolk, N. (1973) Acta Chem. Scand. 27, 35–46.
[27] Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A. and Crofts, A. R. (1975) Biochim. Biophys. Acta 387, 212–227.
[28] Sillén, L. G. and Martell, A. E. eds (1964) Stability Constants of Metal Ion Complexes, Spec. Publ. 17, Chemical Society, London.
[29] Ingledew, W. J., Cox, J. C. and Halling, P. J. (1977) FEMS Microbiol. Lett. 2, 193–197.
[30] Dickerson, R. E. and Timkovich, R. (1975) in: The Enzymes (Boyer, P. D. ed) vol. 11, pp. 397–547, Academic Press, New York.
[31] Koike, I. and Hattori, A. (1975) J. Gen. Microbiol. 88, 11–19.
[32] Jones, C. W., Brice, J. M. and Edwards, C. (1977) Arch. Microbiol. 115, 85–93.
[33] Haddock, B. A. and Jones, C. W. (1977) Bacteriol. Rev. 41, 47–99.
[34] Thauer, R. K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. 41, 100–180.
[35] Bell, G. R., Le Gall, J. and Peck, H. D. (1974) J. Bacteriol. 120, 994–997.