Stoichiometry of electron uptake and oxidation–reduction midpoint potentials of NADH:nitrate reductase

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Microcoulometric titrations of NADH:nitrate reductase at 25 °C in Mops buffer, pH 7.0, showed that the native enzyme, containing functional FAD, haem and Mo, required addition of five electrons for complete reduction. Reduction of the native enzyme occurred in three waves corresponding to addition of reducing equivalents to the centres in the order: Mo, haem, FAD. Oxidation–reduction midpoint potentials (E'_0) for the various redox couples were calculated to be as follows: $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$, +16 mV; $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$, –27 mV; $\text{haem}_{\text{oxidized}}/\text{haem}_{\text{reduced}}$, –172 mV; FAD/FADH_2 , –283 mV. The values for the haem and flavin are in excellent agreement with those obtained by visible titrations, namely –164 mV and –288 mV respectively. In contrast, the results for the Mo centre are 28–50 mV more positive than the values previously determined by e.p.r. analysis of frozen enzyme samples poised at defined potentials at 25 °C and suggest different pH-dependencies or entropies of reduction for the Mo couples.

INTRODUCTION

Assimilatory NADH:nitrate reductase catalyses the rate-limiting step in inorganic-nitrogen assimilation, namely the conversion of nitrate into nitrite. The enzyme isolated from the green alga *Chlorella vulgaris* has been shown to be a tetramer of identical subunits (96 kDa), each of which contains one FAD, one *b*-type cytochrome (b_{559}) and one Mo centre (Howard & Solomonson, 1982), the latter associated with a substituted pterin derivative referred to as the 'Mo-cofactor' (Johnson & Rajagopalan, 1982).

Oxidation–reduction measurements of nitrate reductase have been primarily confined to the haem and Mo centres, the former exhibiting a midpoint potential (E'_0) at pH 7 of –164 mV as determined by visible potentiometric titrations in the presence of dye mediators (Kay *et al.*, 1986), whereas the latter exhibited $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ potentials of –34 mV and –54 mV respectively, determined by potentiometric titration followed by e.p.r. analysis of frozen samples (Solomonson *et al.*, 1984). Since oxidation–reduction midpoint potentials are intrinsically temperature-dependent (Palmer & Olson, 1980) and a number of enzymes, including the related Mo-containing protein xanthine oxidase (Spence *et al.*, 1982), have shown electron re-equilibration upon freezing to low temperatures, we have utilized microcoulometry to determine the reduction stoichiometry of nitrate reductase and the oxidation–reduction midpoint potentials for the flavin, haem and Mo centres at 25 °C.

MATERIALS AND METHODS

Assimilatory NADH:nitrate reductase was isolated from *C. vulgaris* as previously described (Howard & Solomonson, 1981) with the addition of a final h.p.l.c. step using a TSK 4000 gel-exclusion column. The enzyme exhibited a specific activity (NADH:nitrate reductase) in

excess of 80 units/mg and an A_{413}/A_{280} ratio greater than 0.7. Enzyme concentration, given in terms of individual subunits, was quantified by using an $\epsilon_{413}^{\text{mm}}$ value of $117 \text{ mM} \cdot \text{cm}^{-1}$.

Microcoulometry was performed as described by Spence *et al.* (1982) in the presence of the following mediators (each present at 75 μM): Toluidine Blue ($E'_0 = 34 \text{ mV}$), pyocyanine ($E'_0 = -60 \text{ mV}$), indigodisulphonate ($E'_0 = -125 \text{ mV}$), anthraquinone-1,5-disulphonate ($E'_0 = -175 \text{ mV}$), anthraquinone-2-sulphonate ($E'_0 = -225 \text{ mV}$), safranine T ($E'_0 = -289 \text{ mV}$), Benzyl Viologen ($E'_0 = -311 \text{ mV}$) and Methyl Viologen ($E'_0 = -440 \text{ mV}$).

Oxidation–reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were obtained from the microcoulometric data by comparison with theoretical $n = 1$ or $n = 2$ Nernst equations added together to represent total electron uptake by the redox centres present in the enzyme by using non-linear least-squares analysis.

RESULTS AND DISCUSSION

The results of microcoulometric titrations of native nitrate reductase are shown in Fig. 1. Titrations, performed in a reductive phase, showed no electron uptake at potentials more positive than +100 mV. However, as the potential was decreased, reducing equivalents were taken up by the enzyme over the potential range +60 mV to –400 mV. The microcoulometric current/potential wave suggested the presence of three separate regions of electron addition. The first, corresponding to the uptake of two reducing equivalents per haem, occurred with a midpoint potential of approx. –2 mV, whereas the second phase accounted for the addition of a single reducing equivalent with a midpoint potential of approx. –177 mV. The final phase, again accounting for addition of two reducing

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Fig. 1. Microcoulometric titration of NADH:nitrate reductase

Titration were performed in 50 mM-Mops buffer, containing 1 mM-EDTA and 100 mM-KCl, pH 7.0, in the presence of the dye mediators described in the Materials and methods section. Each experimental point represents the electron uptake after addition of 1.2–1.6 nmol of enzyme subunits added to the titration vessel. Error bars indicate the estimated accuracy in each measurement equivalent to $\pm 2 \times 10^{-10}$ coulombs (0.13 electron/FAD) and ± 10 mV. The continuous curve represents the 'best fit' to the data points (+) using the potentials given in Table 1. The broken curve utilizes the previously published potentials for the redox centres in nitrate reductase obtained by using visible spectroscopy and low-temperature e.p.r. potentiometric titrations.

equivalents, exhibited a midpoint potential of approx. -264 mV. Thus the data indicated that each nitrate reductase subunit required the addition of five reducing equivalents for complete reduction. This reduction stoichiometry could be accounted for by addition of two electrons to each flavin and molybdenum, and addition of a single electron to the haem, suggesting that other functional groups, such as thiol groups, do not participate directly in electron-transfer reactions. This finding is consistent with previous studies that have identified the

presence, in the oxidized enzyme, of an essential thiol group involved in NADH binding (Barber & Solomonson, 1986). In addition, the finding that total reduction could be accounted for by addition of two electrons to the Mo centre would suggest that redox states lower than Mo^{IV} , such as Mo^{III} (Jacobs & Orme-Johnson, 1980), need not be considered in the mechanism for this enzyme.

Previous potentiometric studies of nitrate reductase using e.p.r. analysis of frozen samples have shown that the Mo centre accepts two electrons with midpoint potentials of -34 mV and -54 mV (Solomonson *et al.*, 1984) respectively, whereas visible potentiometric titrations of the haem centre have demonstrated an $n = 1$ redox process with a midpoint potential of -164 mV (Kay *et al.*, 1986). Visible titrations of the FAD prosthetic group have also been performed, using the small FAD-containing fragment obtained after limited proteolysis of the enzyme with corn (*Zea mays*) inactivating proteinase (Solomonson *et al.*, 1986), yielding a value of -288 mV for the FAD/FADH₂ couple (Solomonson & Barber, 1987). Since microcoulometry only yields information on the number of reducing equivalents accepted by an enzyme as a function of applied potential and provides no information on the identity of the individual electron acceptors, we utilized the previous potentials established for the Mo, haem and flavin centres as the foundation for the analysis of the microcoulometric data. On the basis of these results, we assigned the high-potential two-electron wave to reduction of Mo and the intermediate one-electron wave to reduction of the haem. We assigned the third low-potential phase of electron uptake to reduction of the flavin, the prosthetic group of lowest potential.

Computer-simulated electron-uptake curves were generated by using the equation:

$$n = \frac{1}{[1 + 10^{(E - E_{\text{Mo},1})/59} + 10^{(E_{\text{Mo},2} - E)/59}] + \frac{2}{[1 + 10^{(E - E_{\text{Mo},2})/59} + 10^{(2E - E_{\text{Mo},1} - E_{\text{Mo},2})/59}] + \frac{1}{[1 + 10^{(E - E_{\text{haem}})/59}] + \frac{1}{[1 + 10^{(E - E_{\text{FAD},1})/59} + 10^{(E_{\text{FAD},2} - E)/59}] + \frac{2}{[1 + 10^{(E - E_{\text{FAD},2})/59} + 10^{(2E - E_{\text{FAD},1} - E_{\text{FAD},2})/59}]}$$

where n represents the number of electrons taken up per nitrate reductase subunit, E is the applied potential, $E_{\text{Mo},1}$ and $E_{\text{Mo},2}$ correspond to the midpoint potentials for the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couples, E_{haem} represents the midpoint potential for the haem centre, and $E_{\text{FAD},1}$ and $E_{\text{FAD},2}$ correspond to the potentials for the FAD/FAD^{••} and FAD^{••}/FADH₂ couples respectively.

Table 1. Comparison of oxidation–reduction midpoint potentials obtained for *Chlorella* NADH:nitrate reductase

Redox couple	E'_0 (mV) calculated by		
	Microcoulometry	Visible spectroscopy	E.p.r. spectroscopy*
$\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$	+16	–	–34
$\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$	–27	–	–54
$\text{Haem}_{\text{oxidized}}/\text{haem}_{\text{reduced}}$	–172	–164†	–
FAD/FADH_2	–283	–288‡	–

* Solomonson *et al.* (1984).

† Kay *et al.* (1986).

‡ Solomonson & Barber (1987).

The best fit to the data was obtained with the potentials listed in Table 1. Previous application of microcoulometry to xanthine oxidase has indicated that the maximum error in determining potentials using this technique is approx. ± 10 mV (Spence *et al.*, 1982). The least-squares analysis indicated that the E'_0 for the $\text{FAD}/\text{FAD}^{\cdot-}$ was significantly lower than that for $\text{FAD}^{\cdot-}/\text{FADH}_2$ (it was found that all fits with $E_{\text{FAD}^{\cdot-}/\text{FADH}_2} - E_{\text{FAD}/\text{FAD}^{\cdot-}} > 70$ mV were essentially identical), so that FAD reduction behaved essentially as a two-electron process in the microcoulometric titrations. This result was consistent with previous work, which showed that the flavin semiquinone ($\text{FAD}^{\cdot-}$) was not detected during e.p.r. potentiometric titrations (Solomonson & Barber, 1984). Thus only the average E'_0 for the FAD/FADH_2 couple could be obtained with any accuracy. The value of -283 mV is in good agreement with the result (-288 mV) obtained from previous visible potentiometric titrations of the flavin-containing small fragment of nitrate reductase (Solomonson & Barber, 1987).

Comparison of the midpoint potentials obtained for the haem by visible potentiometry and microcoulometry at 25°C also showed good agreement. However, in contrast, the values obtained for the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couples at 25°C exhibited a distinct positive shift, corresponding to 28 – 50 mV, when compared with the values obtained by e.p.r. analysis after freezing. Potential shifts of this magnitude may be due to differences in either the pH-dependencies or entropy of reduction ($dE'_0/dT = \Delta S/nF$, where n is the number of electrons, F is the Faraday constant and ΔS is the entropy) of the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couples (Palmer & Olson, 1980). These results are comparable with those obtained for the Mo centre in the related enzyme xanthine oxidase (Spence *et al.*, 1982; Porras & Palmer, 1982) and reinforce the concept that electron distributions between redox centres in multicomponent enzymes determined by using low-temperature e.p.r.

analysis of frozen samples may not accurately reflect those obtained at room temperature.

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REFERENCES

- Barber, M. J. & Solomonson, L. P. (1986) *J. Biol. Chem.* **261**, 4562–4567
- Howard, W. D. & Solomonson, L. P. (1981) *J. Biol. Chem.* **256**, 12725–12730
- Howard, W. D. & Solomonson, L. P. (1982) *J. Biol. Chem.* **257**, 10243–10250
- Jacobs, G. S. & Orme-Johnson, W. H. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 327–344, Pergamon Press, Oxford
- Johnson, J. L. & Rajagopalan, K. V. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6856–6860
- Kay, C. J., Solomonson, L. P. & Barber, M. J. (1986) *J. Biol. Chem.* **261**, 5799–5802
- Palmer, G. & Olson, J. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 187–220, Pergamon, Oxford
- Porras, A. G. & Palmer, G. (1982) *J. Biol. Chem.* **257**, 11617–11626
- Solomonson, L. P. & Barber, M. J. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C. & Mayhew, S. J., eds.), pp. 247–250, Walter de Gruyter and Co., Berlin
- Solomonson, L. P. & Barber, M. J. (1987) in *Inorganic Nitrogen Metabolism* (Ullrich, W. R., Aparicio, P. J., Syrett, P. J. & Castillo, F., eds.), pp. 71–75, Springer-Verlag, Berlin
- Solomonson, L. P., Barber, M. J., Howard, W. D., Johnson, J. L. & Rajagopalan, K. V. (1984) *J. Biol. Chem.* **259**, 849–853
- Solomonson, L. P., Barber, M. J., Robbins, A. & Oaks, A. (1986) *J. Biol. Chem.* **261**, 11290–11294
- Spence, J. T., Barber, M. J. & Siegel, L. M. (1982) *J. Biol. Chem.* **257**, 1656–1661

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