Oxidation–reduction midpoint potentials of the flavin, haem and Mo-pterin centres in spinach (*Spinacia oleracea* L.) nitrate reductase

Christopher J. KAY,* Michael J. BARBER,*† Brian A. NOTTON† and Larry P. SOLOMONSON*

*Department of Biochemistry and Molecular Biology, University of South Florida, College of Medicine, Tampa, FL 33612, U.S.A., and †University of Bristol Department of Agricultural Sciences, Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, U.K.

Oxidation–reduction midpoint potentials have been determined for the flavin, cytochrome b_{557} and Mo-pterin prosthetic groups of spinach (*Spinacia oleracea* L.) assimilatory nitrate reductase using visible, c.d. and room-temperature e.p.r. potentiometric titrations. At pH 7 and 25 °C, the midpoint potential for the FAD/FADH₂ couple was determined by c.d. potentiometry to be -280 ± 10 mV ($n = 2$). The redox potential for reduction of the haem was determined by visible potentiometry to be -123 ± 10 mV ($n = 1$), significantly lower than the previously published value of -60 mV [Fido, Hewitt, Notton, Jones & Nasrulhaq-Boyce (1979) FEBS Lett. **99**, 180–182]. Potentials for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) redox couples, determined by room-temperature e.p.r. potentiometry, were found to be $+2 \pm 20$ and -6 ± 20 mV respectively. These values are very similar to the values previously determined for the FAD, haem and Mo-pterin centres in assimilatory nitrate reductase isolated from the unicellular green alga *Chlorella vulgaris* and indicate a close thermodynamic similarity between the two enzymes.

INTRODUCTION

Assimilatory nitrate reductases (NRs; EC 1.6.6.1–3) catalyse the rate-limiting step in inorganic-nitrogen metabolism (Guerrero *et al.*, 1981), the reduction of NO₃[−] to NO₂[−], in algae, fungi and plants. The enzyme has been isolated from a variety of sources and shown to be a multimeric complex metalloflavoprotein containing FAD, cytochrome b_{557} and Mo-pterin prosthetic groups in a 1:1:1 stoichiometry per subunit (M_r 100 000) (Hewitt & Notton, 1980). Reducing equivalents, from the physiological electron donor NAD(P)H, are added to the enzyme at the flavin, whereas electron egress to NO₃[−] occurs via the Mo-pterin prosthetic group.

Visible and e.p.r. spectroscopic studies have indicated that the flavin, haem and Mo-pterin prosthetic groups of both *Chlorella vulgaris* (a unicellular green alga) and spinach (*Spinacia oleracea* L.) NR exhibit very similar spectral properties, suggesting that the environments of the individual prosthetic groups are closely related in both enzymes. Partially reduced NRs exhibit flavin semiquinone (FAD^{•−}) and Mo(V) e.p.r. spectra, the latter existing in multiple forms that are related by the ionization of a single exchangeable weakly coupled proton (Gutteridge *et al.*, 1983; Kay & Barber, 1989).

Oxidation–reduction midpoint potentials have been obtained for the FAD, haem and Mo-pterin centres of the *Chlorella* enzyme by using a variety of techniques including microcoulometry (Spence *et al.*, 1988), visible, c.d. and e.p.r. potentiometry (Kay *et al.*, 1988). In addition, comparison of the results obtained for the Mo centre using room-temperature and low-temperature e.p.r. analysis has indicated that potentials obtained using frozen samples were 20–30 mV lower than the corresponding values obtained at room temperature.

For the spinach enzyme, values have been previously obtained for the b -type cytochrome [E_0' (standard electrode potential) = -60 mV] using visible potentiometry (Fido *et al.*, 1979) and the Mo couples [Mo(VI)/Mo(V) = -8 mV, Mo(V)/Mo(IV) = -42 mV] using low-temperature e.p.r. spectroscopy (Barber *et al.*, 1987).

We have determined the redox potentials for all three centres in the spinach NR at 25 °C and have compared them with the corresponding values obtained for *C. vulgaris* NR (Kay *et al.*, 1988) to examine the thermodynamic similarities of the prosthetic groups in these enzymes.

MATERIALS AND METHODS

NR was isolated from freshly harvested spinach leaves using immunochromatography as previously described (Fido, 1987). The purified enzyme was stored as a concentrate in 50% (v/v) glycerol and exhibited an NADH:NR activity of approx. 100 units/mg (1 unit corresponds to 1 μ mol of nitrate reduced/min). Enzyme concentrations were determined by using an ϵ_{413} value of 117 mm^{−1}·cm^{−1}. Enzyme samples were freed from glycerol contamination by precipitation with (NH₄)₂SO₄. All experiments were performed using 50 mM-Mops buffer, pH 7 [Ultrol grade (Calbiochem, Los Angeles, CA, U.S.A.) to minimize chloride contamination], which contained 0.1 mM-EDTA and 5 mM-FAD.

Potentiometric titrations using c.d., visible or room-temperature e.p.r. spectroscopy were performed as described by Kay *et al.* (1988) using reduced Methyl Viologen (20 mM) as reductant and K₃Fe(CN)₆ (20 mM) as oxidant. Visible and room-temperature e.p.r. titrations were performed in the presence of the

Abbreviations used: NR, nitrate reductase; E_0' , standard electrode potential.

† To whom correspondence and reprint requests should be sent.

following mediators (10 mM each mediator for visible titrations, 30 mM for e.p.r. titrations) to ensure redox equilibration: Methylene Blue ($E_0' = +10$ mV), 2,5-dihydroxybenzoquinone ($E_0' = -60$ mV), indigodisulphonic acid ($E_0' = -125$ mV), 2-hydroxy-1,4-naphthoquinone ($E_0' = -137$ mV), anthraquinone-2,7-disulphonate ($E_0' = -182$ mV), anthraquinone-2-sulphonate ($E_0' = -225$ mV), phenosafranine ($E_0' = -255$ mV), safranin T ($E_0' = -289$ mV) and Neutral Red ($E_0' = -325$ mV). C.d. titrations were performed in the presence of phenosafranine and Benzyl Viologen ($E_0' = -311$ mV).

Oxidation-reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were obtained from the experimental data points by comparison with theoretical Nernst curves using least-squares analysis (Kay *et al.*, 1988). The uncertainty in measurements of redox potentials, due to electrode errors, curve-fitting etc. for simple redox curves such as the haem, FAD/FADH₂ and Mo(VI)/Mo(IV) couples, is probably of the order of ± 10 mV, though reproducibility from one experiment to another was generally better than this. However, for the FAD/FAD^{•-}, FAD^{•-}/FADH₂, Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples, an additional level of uncertainty was introduced, since the differences in the potentials between the flavin and Mo couples depend upon the integrated intensity of the FAD^{•-} and Mo(V) e.p.r. signals respectively, increasing the errors in these potentials to ± 20 mV (Cammack *et al.*, 1976).

RESULTS AND DISCUSSION

Previous work on the redox potentials of nitrate reductase isolated from *C. vulgaris* (Kay *et al.*, 1988) has demonstrated that the application of a combination of c.d., visible and room-temperature e.p.r. potentiometric titrations enabled determination of the oxidation-reduction midpoint potentials for the five redox couples [FAD/FAD^{•-}, FAD^{•-}/FADH₂, oxidized haem/reduced haem, Mo(VI)/Mo(V) and Mo(V)/Mo(IV)] associated with the three individual prosthetic groups of the enzyme. C.d. spectroscopy yielded values for the flavin potentials, whereas visible and e.p.r. spectroscopy provided values for the haem and Mo centres respectively. Correspondingly we have utilized the same methodologies to determine the redox potentials for the spinach enzyme under equivalent conditions.

The results of a c.d. potentiometric titration of the flavin centre of native spinach NR are shown in Fig. 1(a). C.d. spectra obtained for the spinach enzyme, when compared with the results obtained for the *Chlorella* NR, suggested that changes in the negative c.d. band centred at 460 nm provided a sensitive indicator of the extent of reduction of the flavin chromophore. Spectral changes at 460 nm revealed a single $n = 2$ reduction process corresponding to a midpoint potential of -280 ± 10 mV. Titrations performed in both oxidative and reductive directions were fully reversible and exhibited no hysteresis. The excellent agreement between the experimental data and the theoretical $n = 2$ Nernst curve indicated very little conversion of the flavin into the semiquinone form. Consequently, by analogy with the results obtained for the *Chlorella* enzyme (Kay *et al.*, 1988), which used samples of xanthine oxidase as a standard to estimate the minimal concentration of flavin semiquinone that could be detected by e.p.r. spectroscopy, we estimated that

Fig. 1. Behaviour of the FAD, haem and Mo-pterin centres during potentiometric redox titrations

Spinach NR (2–15 μ M-haem) in 50 mM-Mops buffer, containing 0.1 mM-EDTA and 5 μ M-FAD, pH 7.0, was reduced or oxidized by addition of reduced Methyl Viologen (20 mM) or K₃Fe(CN)₆ (20 mM) respectively in the presence of dye mediators. (a) The degree of reduction of the FAD was monitored at 460 nm; (b) reduction of the *b*-type cytochrome was monitored using $\Delta A_{423-416}$; (c) changes in the concentration of Mo(V) determined by room-temperature e.p.r. spectroscopy. Open and closed symbols represent data points obtained in oxidative and reductive titrations respectively.

approx. 1% or less of the flavin would be expected to be converted into the FAD^{•-} redox state at the midpoint of the titration. This indicated a minimal separation of the FAD/FAD^{•-} and FAD^{•-}/FADH₂ redox couples to be approx. 100 mV. Previous e.p.r. studies of purified spinach enzyme have shown very little conversion of the flavin into the semiquinone form after partial reduction (Gutteridge *et al.*, 1983).

The results of a typical visible potentiometric titration of spinach NR, using the Soret band (423 nm) to monitor the extent of haem reduction, are shown in Fig. 1(b). Similar behaviour was obtained for the changes in the a

band at 557 nm. As for the flavin centre, haem reduction corresponded to a single fully reversible reduction process with a midpoint potential of -123 ± 10 mV ($n = 1$). This value is approx. 60 mV more negative than that previously reported for the midpoint potential of the cytochrome b_{557} prosthetic group of spinach NR (Fido *et al.*, 1979).

The behaviour of the room-temperature Mo(V) e.p.r. signal during potentiometric titrations is shown in Fig. 1(c). At potentials more positive than 130 mV, no paramagnetic species were detected. However, as the applied potential was decreased, a broad Mo(V) signal appeared ($g_{av.} = 1.97$) that reached a maximum signal amplitude at approx. 0 mV and decreased in intensity as the potential became more negative. Reoxidation of the reduced sample resulted in regeneration of the signal, indicating full reversibility. Double integration of the maximum signal amplitude indicated approx. 0.40 spin/Mo. Best-fit potentials obtained for this data, using the Nernst equation modified to represent two coupled one-electron redox processes according to the scheme:

corresponded to potentials of $+2 \pm 20$ mV and -6 ± 20 mV for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples respectively. These values, obtained by room-temperature e.p.r., can be compared with the previously reported values of -8 mV and -42 mV for spinach NR that were obtained using e.p.r. analysis of frozen samples. Thus freezing results in a shift to lower potential of approx. 10–35 mV for both Mo couples. Similar effects of freezing on midpoint potentials have been noted for the *Chlorella* enzyme (Kay *et al.*, 1988) and the related Mo-containing enzyme xanthine oxidase (Spence *et al.*, 1982).

These results, which provide the first complete description of the oxidation–reduction midpoint potentials for the flavin, haem and Mo-pterin centres in a higher-plant NR, are compared with those obtained for *C. vulgaris* NR in Table 1. Similar values for the potentials of the flavin, haem and Mo centres were found for both

enzymes, indicating that the environments of the individual chromophores are very similar in the two forms of NR and confirming that electron transfer between prosthetic groups during turnover is a thermodynamically favourable process.

These results also show that the flavin chromophore exhibits the most negative potentials in the enzyme, although still more positive than the physiological electron donor, NADH. The separation between the potentials of the two couples suggests that, in the absence of NADH (or NAD^+), the flavin acts as an obligatory two-electron acceptor, which presumably transfers reducing equivalents in a stepwise manner to haem.

The value obtained during these experiments for the oxidation–reduction midpoint of the cytochrome component is significantly lower than previously reported ($E'_0 = -60$ mV; Fido *et al.*, 1979) for spinach NR. This difference in potential may be due to use of enzyme of higher purity, which enabled improved quantification of the redox state of the haem. In addition, the significantly decreased contamination of the enzyme by proteolysis products of NR and other proteins may have decreased sample heterogeneity, which could result in modified potentials. Further, we utilized reduced Methyl Viologen as an electron donor, in contrast with NADH, and a selection of mediators that more closely matched the redox potential of the haem. These substitutions may have resulted in more efficient sample reduction and improved equilibration. Thus our value for the spinach NR haem potential more closely resembles the values determined for both the *Chlorella* (-164 mV; Kay *et al.*, 1988) and yeast (-174 mV; Notton *et al.*, 1987) enzymes, suggesting the potential of this centre may be very similar in all forms of assimilatory NR.

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REFERENCES

- Barber, M. J., Notton, B. A. & Solomonson, L. P. (1987) *FEBS Lett.* **213**, 372–374
- Cammack, R., Barber, M. J. & Bray, R. C. (1976) *Biochem. J.* **157**, 467–478
- Fido, R. J. (1987) *Plant Sci.* **50**, 111–115
- Fido, R. J., Hewitt, E. J., Notton, B. A., Jones, O. T. G. & Nasrulhaq-Boyce, A. (1979) *FEBS Lett.* **99**, 180–182
- Guerrero, M. G., Vega, J. M. & Losada, M. (1981) *Annu. Rev. Plant Physiol.* **32**, 169–214
- Gutteridge, S., Bray, R. C., Notton, B. A., Fido, R. J. & Hewitt, E. J. (1983) *Biochem. J.* **213**, 137–142
- Hewitt, E. J. & Notton, B. A. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 273–325, Pergamon Press, New York
- Kay, C. J. & Barber, M. J. (1989) *Biochemistry* **28**, 5750–5758
- Kay, C. J., Barber, M. J. & Solomonson, L. P. (1988) *Biochemistry* **27**, 6142–6149
- Notton, B. A., Kay, C. J., Barber, M. J., Solomonson, L. P., Kau, D., Cannons, A. C. & Hipkin, C. R. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 1007
- Spence, J. T., Barber, M. J. & Siegel, L. M. (1982) *Biochemistry* **21**, 1656–1661
- Spence, J. T., Barber, M. J. & Solomonson, L. P. (1988) *Biochem. J.* **250**, 921–923

Table 1. Comparison of the oxidation–reduction midpoint potentials for the FAD, cytochrome b_{557} and Mo-pterin prosthetic groups of spinach and *C. vulgaris* NR

For the spinach enzyme, all values were obtained in 50 mM-Mops buffer, pH 7, containing 0.1 mM-EDTA and 5 μM -FAD in the presence of dye mediators.

Redox couple	E'_0 (mV)	
	Spinach	<i>C. vulgaris</i> *
Mo(VI)/Mo(V)	+2	+15
Mo(V)/Mo(IV)	–6	–25
Mo(VI)/Mo(IV)	–2	–5
Oxidized haem/reduced haem	–123	–164
FAD/FAD ^{•–}	–380	–372
FAD ^{•–} /FADH ₂	–180	–172
FAD/FADH ₂	–280	–272

* Data taken from Kay *et al.* (1988).