HAEM OF SPINACH NITRATE REDUCTASE: LOW TEMPERATURE SPECTRUM AND MID-POINT POTENTIAL

R. J. FIDO, E. J. HEWITT, B. A. NOTTON, O. T. G. JONES+ and A. NASRULHAQ-BOYCE+

Long Ashton Research Station, University of Bristol, Long Ashton, Bristol, BS18 9AF and *+Department of Biochemistry, University of Bristol, Bristol, BS8 1TD, England*

Received 17 December 1978

1. Introduction

Nitrate reductase purified from a number of eukaryotic sources, including *Neurospora crassa* [1,2], *Chlorella vulgaris* [3,4], *Chlorella fusca* [5], *Thalassiosira pseudonana* [6], *Ankistrodesmus braunii* [7] and spinach (*Spinacea oleracea*) [8,9] displayed light absorption spectra at room temperature, and in one case [2] at liquid nitrogen temperature, typical of a *b*-type cytochrome. This cytochrome was shown, for the enzyme from *N. crassa* [2] and *C. vulgaris* [3], to have a protohaem IX prosthetic group from the spectra of its pyridine hemochromogen derivative. Haem involvement in the overall electron-transfer pathway was implicated by the reoxidation of NADH-reduced haem by nitrate [1,3,8], additionally the NADH-reduced haem was reoxidised by the dehydrogenase electron acceptor dichlorophenolindophenol (DCPIP) [8] although the enzymic involvement of the haem in this partial function of the enzyme was not proved [8,10].

We now report further characterisation of the haem prosthetic group of spinach nitrate reductase based on its visible absorption spectrum at liquid nitrogen temperature and the spectrum of its pyridine hemochromogenic derivative. We also report the mid-point potential of the haem and discuss its involvement with the dehydrogenase function of the enzyme.

2. Materials and methods

2.1. *Purification of nitrate reductase*

Spinach (*Spinacea oleracea* L.cv Noorman) plants were grown for 6–8 weeks with complete nitrate nutrient [11] using a nutrient film technique. Leaves were sampled; the enzyme was extracted and purified by a modification of the procedure as in [8]. Gel filtration was performed through Biogel A 1.5m (Bio-Rad Labs.) and the affinity chromatography utilised Blue–Sepharose (Pharmacia). The enzyme-loaded column was washed with 0.08 M phosphate buffer (pH 7.5), 1 mM EDTA and then with 0.4 M phosphate buffer (pH 7.5), 1 mM EDTA until the eluates were free of 280 nm absorbing material before elution of the enzyme with 0.08 M phosphate buffer (pH 7.5), 1 mM EDTA containing 1 M KCl. All operations were carried out at 0–4°C.

2.2. *Pyridine hemochromogen derivative*

Haem was extracted from the purified enzyme and the pyridine hemochromogen derivative formed using the acid–acetone method, without dialysis, as in [3]. Spectra were measured at 20°C using a Pye-Unicam SP 1700 recording spectrophotometer.

2.3. *Low temperature spectrum*

Spectra at liquid nitrogen temperature (77 K) were measured using a spectrophotometer constructed in the Medical School workshops, University of Bristol, according to the principles outlined in [12] and detailed in [13]. Cuvettes had a 0.2 cm light path and were pre-cooled before measuring spectra of the enzyme in 50 mM MOPS (pH 7.0) containing 50% sucrose.

2.4. *Mid-point potential*

Potentiometric titrations were carried out at 22°C

Elsevier/North-Holland Biomedical Press

under anaerobic conditions in a dual wavelength spectrophotometer using the method in [14]. The spectrophotometer was constructed in the Medical School workshops, University of Bristol, by modification of a basic design [15] as detailed in [13]. The modified glass cuvette of 1 cm light path was constructed in the same workshops and is described in [16]. The enzyme, dissolved in 50 mM MOPS, 50 mM KCl (pH 7.0) was deaerated by bubbling with argon scrubbed with Fiesers reagent [17] for 30 min before starting the titrations and the argon was blown onto the surface of the mixture during the titrations. Measuring and isobestic wavelengths used were 557 nm and 540 nm, respectively. Final concentrations of mediators and their mid-point potentials at pH 7 (E_{m7}) as given in [18] were: 50 μM diaminodural (E_{m7} = +220 mV); 25 μM phenazine methosulphate (E_{m7} = +80 mV); 25 μM phenazine ethosulphate (E_{m7} = +55 mV); 6 μM pyocyanine (E_{m7} = −34 mV) and 20 μM 2-(OH),1,4,-naphthoquinone (E_{m7} = −145 mV). Ferricyanide (100 mM) was used to oxidise the enzyme and reductive titration used 1–2 μl additions of 7 mM NADH. Changes in absorbance and the redox potential of the mixture were monitored simultaneously and continuously. A plot of the redox potential in the system versus the logarithm of the ratio of oxidised to the NADH-reduced haem was used to estimate the mid-point potential.

2.5. *Assay methods*

Nitrate reductase activity was determined as in [8] and protein estimated from A_{260} and A_{280} values.

3. Results and discussion

The light absorbance spectrum of NADH-reduced spinach nitrate reductase, measured at 20°C, has been reported and shown to be a *b*-type cytochrome with peaks at 424 nm, 528 nm and 557 nm [8]. Figure 1 shows the absolute absorption spectrum of an NADH-reduced sample of highly purified spinach nitrate reductase at liquid nitrogen temperature (77 K). The sample contained 6.43 mg protein dissolved in 0.7 ml MOPS/sucrose buffer. A shift of the α and β peaks towards shorter wavelengths relative to those obtained at 20°C was observed. The α peak split into two, located at 557 nm and 553 nm; the β peak occurred

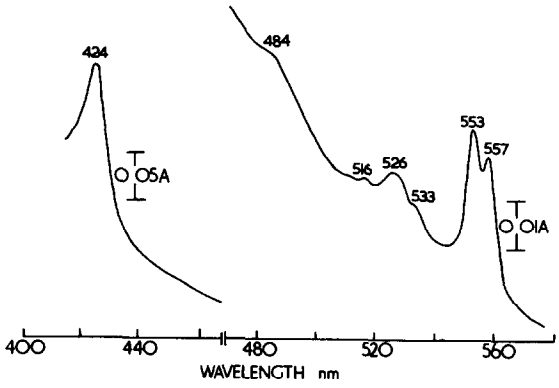

Fig.1. Absolute absorption spectrum of purified, NADH-reduced spinach nitrate reductase measured at 77 K (liquid nitrogen temperature). A 0.7 ml sample of enzyme (6.43 mg protein) in MOPS (pH 7.0) containing 50% sucrose was reduced by adding a few crystals of NADH; the spectrum was determined in a split beam spectrophotometer with MOPS/sucrose in the reference beam using 0.2 cm path length cells.

at 526 nm with shoulders at 533 nm and 516 nm; the Soret band at 424 nm and a broad peak at ~484 nm (not previously observed) were not positionally affected by the decrease in temperature. The shifts and intensification of the α and β peaks as well as the splitting of the α peak are both commonly occurring phenomena for cytochromes at liquid nitrogen temperatures [19].

The only other reported low temperature spectrum of purified nitrate reductase, that of *N. crassa* [2], shows a similar shift of the α and β peaks but the splitting of the α peak was much less extreme being confined to a shoulder at 556 nm on the main peak at 552 nm. A shoulder at 531 nm was reported on a β peak at 524 nm but the shoulder on the shorter wavelength side (seen here) was not reported.

The spectrum of the pyridine haemochromogen derivative prepared from the enzyme was identical in peak position to that obtained from an authentic sample of hemin chloride treated in the same way. Peaks occurred at 555 nm, 524 nm and 418 nm; however the relative peak heights were different suggesting, as with the derivative obtained from the *C. vulgaris* enzyme [3], the presence of other pigments.

The mid-point potential of the haem in spinach nitrate reductase was determined as in section 2 using 14.7 mg purified enzyme dissolved in 4.5 ml MOPS/

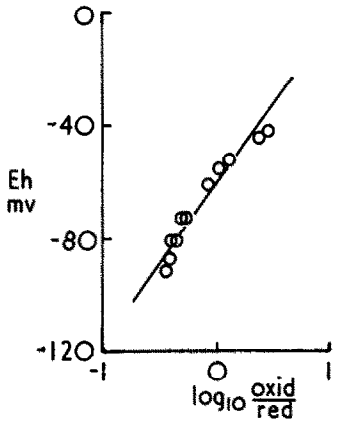

Fig.2. Plot of redox potential of purified spinach nitrate reductase versus $\log_{10}$ ratio of oxidised to reduced haem. A 4.5 ml solution of MOPS/KCl buffer (pH 7.0) containing 14.7 mg protein was deaerated with pure nitrogen, oxidised with ferricyanide and submitted to reductive titration with NADH in the presence of mediators. Potential change and ΔA_{557} compared to A_{540} measured continuously in glass cuvette of 1 cm light path.

KCl buffer (pH 7.0). Figure 2 shows a least-squares plot of potential versus ratio of oxidised to reduced haem. From this graph the mid-point potential is estimated to be −60 mV. The titration was recycled by reoxidation of the fully reduced haem and a second reductive titration performed with confirmatory results. Complete reduction of the haem by NADH was confirmed by subsequent addition of dilute $Na_2S_2O_4$ to the system.

The question as to whether or not the dehydrogenase function of nitrate reductase is haem dependent, as demonstrated by the reduction of DCPIP, is not resolved by the determination of the mid-point potential of the haem. When NADH-reduced nitrate reductase is titrated with DCPIP to slight excess the haem is found to be reoxidised [8]. The potential of NADH/NAD^+ is −315 mV and therefore addition of DCPIP, which has a potential of +210 mV, causes complete oxidation of the NADH. Oxidation of the haem could therefore occur either because it is on the direct electron pathway from the NADH binding site to the dehydrogenase DCPIP donating site of the enzyme or because the haem is in near equilibrium with the group that donates to the DCPIP and so will itself become oxidised when all the NADH has been oxidised or because reduction of DCPIP occurs also at an alternative site to the dehydrogenase DCPIP donating site (see [10], fig.1).

Acknowledgements

O.T.G.J. thanks the Science Research Council for a Research Grant and A.N.-B. thanks the Government of Malaysia and the University of Malaya for financial support.

References

[1] Garrett, R. H. and Nason, A. (1969) J. Biol. Chem. 244, 2870–2882.
[2] Pan, S. S., Erikson, R. H. and Nason, A. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. 34, no. 3 abstr. 2635.
[3] Solomonson, L. P. and Vennesland, B. (1972) Biochim. Biophys. Acta 267, 544–557.
[4] Solomonson, L. P., Lorimer, G. H., Hall, R. L., Borchers, R. and Bailey, J. L. (1975) J. Biol. Chem. 250, 4120–4127.
[5] Gewitz, H. S., Piefke, J. and Vennesland, B. (1978) Planta 141, 323–328.
[6] Amy, N. K. and Garrett, R. H. (1974) Plant Physiol. 54, 629–637.
[7] Ahmed, J. and Spiller, H. (1976) Plant Cell Physiol. 17, 1–10.
[8] Notton, B. A., Fido, R. J. and Hewitt, E. J. (1977) Plant Sci. Lett. 8, 165–170.
[9] Guerrero, M. G., Jetschmann, K. and Volker, W. (1977) Biochim. Biophys. Acta 482, 19–26.
[10] Maldonado, J. M., Notton, B. A. and Hewitt, E. J. (1978) FEBS Lett. 93, 169–173.
[11] Hewitt, E. J. (1966) Sand and Water Culture Methods used in the Study of Plant Nutrition, Tech. Commun. 22, Commonwealth Bur. Hort., Farnham Royal.
[12] Yang, C. C. and Legallais, V. (1954) Rev. Sci. Instr. 25, 801–807.
[13] Saunders, V. A. (1973) PhD. Thesis, University of Bristol.
[14] Dutton, P. L. and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 165–212.
[15] Chance, B. (1951) Rev. Sci. Instr. 22, 634–638.
[16] Nasrulhaq-Boyce, A. (1978) PhD. Thesis, University of Bristol.
[17] Fieser, L. F. (1924) J. Am. Chem. Soc. 46, 393–407.
[18] Clark, W. M. (1960) in: Oxidation Reduction Potentials of Organic Systems, Ch. 14, Williams and Wilkins, Baltimore.
[19] Keillin, D. (1966) in: The History of Cell Respiration and Cytochrome, Cambridge Univ. Press, London.