STABILITY OF THE NITROSYL–SIROHAEM COMPLEX OF PLANT NITRITE REDUCTASE, INVESTIGATED BY EPR SPECTROSCOPY

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1. Introduction

Nitrite reductase (nitrite:ferredoxin oxidoreductase EC 1.7.7.1) is an enzyme that contains one sirohaem group [1–3] and one iron–sulphur cluster [2,7] per molecule. The substrate nitrite and other nitrogenous intermediates of the reaction are presumed to bind to the sirohaem. Electron paramagnetic resonance (EPR) spectroscopy showed that in the isolated enzyme, the sirohaem was in the high-spin state. On treatment with dithionite and nitrite, a new signal appeared at $g = 2.007, 2.058$ [2]. This was presumed to arise from an intermediate in the enzyme reaction as it was observed in samples frozen under turnover conditions [4]. The signal was assigned to an Fe(II)–NO complex of sirohaem which would represent the first stage in the reduction of nitrite to ammonia. In the earlier observations, ^{14}N hyperfine structure which would have corroborated this interpretation was not observed. Certain nitrosyl complexes of haem proteins are known not to give resolved ^{14}N hyperfine structure in their powder spectra unless modified in some way. For example, in haemoglobin nitrosyl the splitting is not observed unless the protein structure is perturbed by sodium dodecyl sulphate [5].

Closer examination of the EPR spectrum of nitrosyl–sirohaem nitrite reductase has revealed hyperfine structure. We present here details of the spectra derived from $^{14}NO_2^-$ and $^{15}NO_2^-$. The different hyperfine patterns in the spectra of these two complexes made it possible to investigate the possibility of exchange of bound NO. It was concluded that the complex is very stable and exchange was not detectable unless the enzyme underwent turnover.

2. Experimental

Nitrite reductase was isolated from vegetable marrow (*Cucurbita pepo*) leaves as in [3]. Sodium [^{15}N]nitrite (99.7 atom%) was obtained from BOC Ltd, London, SW19 3UF. ^{14}NO gas was obtained from Cambrian Gases, Croydon, Surrey, 4XB.

N_2 gas ('oxygen-free' grade) was passed through alkaline dithionite in a Nilox apparatus [6] to remove traces of oxygen. ^{14}NO gas was passed through a 1% sodium hydroxide solution to remove higher oxides of nitrogen.

Samples of enzyme were degassed under vacuum and flushed with N_2 (5 cycles) in EPR tubes. Additions were made through a rubber septum by μl syringes fitted with long needles.

EPR spectra were recorded in a Varian E4 spectrometer with an Oxford Instruments liquid helium transfer system. Additions and subtractions spectra were performed on a Datalab DL 4000 waveform calculator, Data Labs., Mitcham, Surrey.

3. Results

Treatment of nitrite reductase with excess nitrite and limiting dithionite resulted in an EPR spectrum with axial symmetry at $g_\perp = 2.058$ and $g_\parallel = 2.007$. By using $^{14}NO_2^-$ and $^{15}NO_2^-$ it was possible to observe clearly differentiated hyperfine splittings at $g = 2.007$ (fig.1). The $g_\perp$ splittings at $g = 2.058$ are masked by the slope of the first derivative spectrum, but are clearly visible on the ^{14}N–^{15}N difference spectrum (1c). The splittings obtained were 0.88 mT for ^{14}N and 1.24 mT for ^{15}N in both $g_\parallel$ and $g_\perp$.

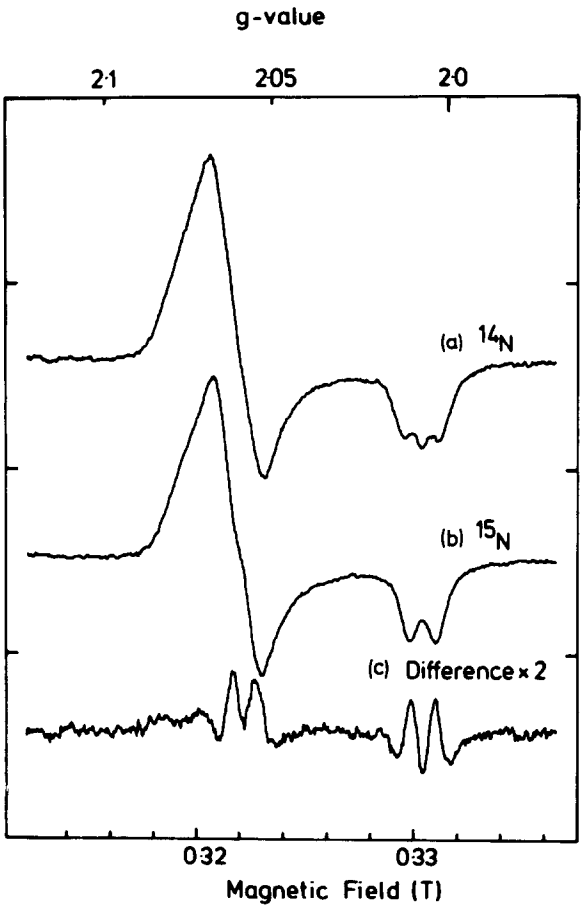

Fig.1. EPR spectra of nitrosyl–sirohaem nitrite reductase. Enzyme (3 mg/ml) treated with 30 mM $NaNO_2$ and 3 mM $Na_2S_2O_4$ at 20°C for 5 min. (a) With [^{14}N]nitrite; (b) with [^{15}N]nitrite; (c) computed difference between (a) and (b). Conditions of measurement: temp. 30 K; microwave power 1 mW; frequency 9.28 GHz; modulation amplitude 0.16 mT; modulation frequency 100 kHz.

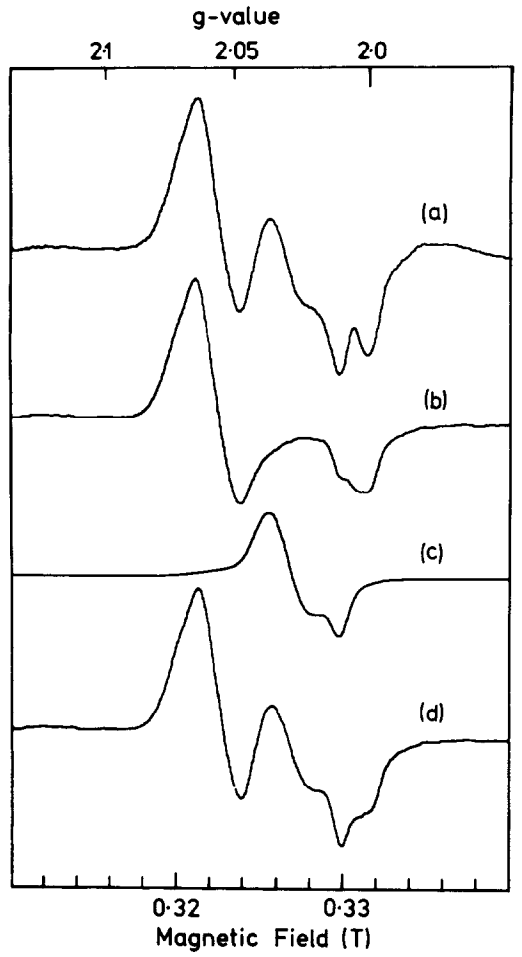

Fig.2. EPR spectra of nitrosyl–sirohaem reductase: (a) enzyme (2 mg/ml) plus 1 mM ascorbate, flushed with NO for 10 min at 20°C; (b) enzyme (2 mg/ml) plus 30 mM nitrite and 1 mM ascorbate; (c) 50 mM cysteine, 5 mM ascorbate, 25 mM nitrite and 12.5 mM ferrous sulphate, incubated at 20°C for 10 min; (d) computed addition of (b) and (c). Conditions of measurement, as in fig.1, except modulation amplitude at 1.0 mT, so that the nitrogen hyperfine splitting is not visible.

The spectrum in [7] of spinach nitrite reductase treated with dithionite and ^{14}NO gas was interpreted as indicating ^{14}N hyperfine splittings of 2.1 mT. Fig.2(a) shows that *C. pepo* nitrite reductase gave a similar spectrum under similar conditions. However, we interpret this spectrum as showing features due to an additional reaction with excess NO. It is known that iron–sulphur proteins treated with NO gas can give an EPR spectrum characteristic of an iron–cysteine–NO complex [8,9]. A spectrum of this complex is shown in fig.2(c). The spectrum of the enzyme treated with excess NO can be simulated (fig.2(d)) by addition of the iron–cysteine–NO spectrum to the spectrum of the enzyme treated with nitrite and ascorbate (fig.2(b)). It appears therefore that nitric oxide can react with at least some of the iron–sulphur clusters in the sample. This effect is probably enhanced by degradation of the enzyme by prolonged exposure to reducing agents.

Once formed, the nitrosyl–sirohaem complex of nitrite reductase appeared to be stable, even when excess nitrite was removed. Fig.3(a,b) show that after gel filtration, the complex could be kept for 4 h at

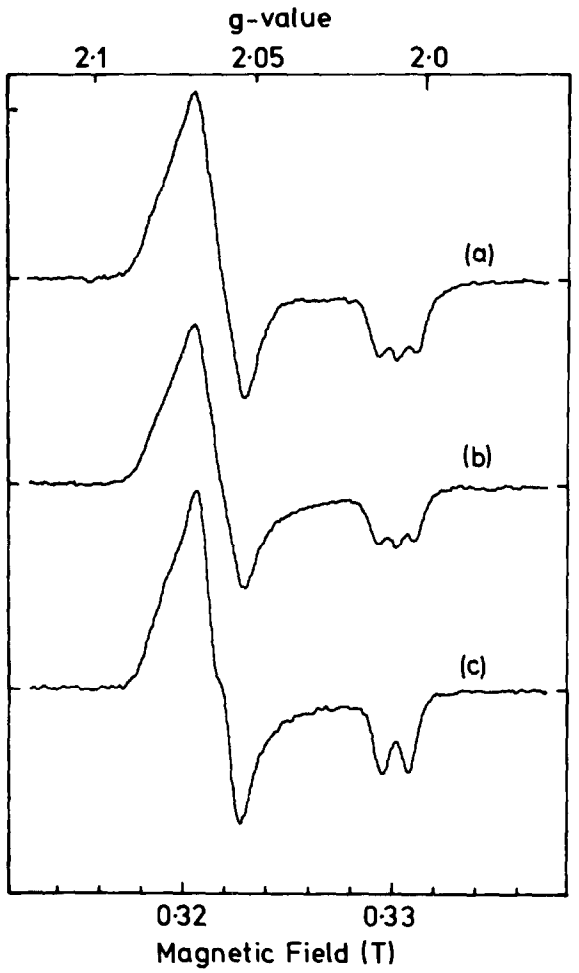

Fig.3. Exchange of ^{14}NO in the ^{14}NO–sirohaem complex with $Na^{15}NO_2$. A sample of enzyme (2 mg/ml) was treated with 30 mM $Na^{14}NO_2$ and 3 mM $Na_2S_2O_4$ at 20°C for 5 min before freezing. Spectrum (a) was recorded under similar conditions to fig.1. The treated enzyme was passed through a 10 × 1.5 cm Sephadex G-25 column and reconcentrated on an amicon concentrator B15 (Amicon Corp. Lexington, MA 02173) at 20°C to ~2 mg/ml, spectrum (b). The sample, in the presence of 30 mM $Na^{15}NO_2$, was then reduced with 3 mM $Na_2S_2O_4$ for 10 min at 20°C before freezing, spectrum (c).

room temperature without loss of signal. The possibility of exchange of the nitrosyl group with added NO_2^- or NO was investigated by using the different hyperfine splittings of the ^{14}N and ^{15}N derivatives in the $g = 2$ region as a 'label' (cf. fig.1(a) and (b)). If $^{15}NO_2^-$ was added to the [^{14}N]nitrosyl complex of the enzyme, the triplet splitting pattern was unchanged. Similarly when [^{15}N]nitrosyl nitrite reductase was incubated with ^{14}NO gas or $^{14}NO_2^-$, no change in the doublet splitting pattern was observed (data not shown). Thus no exchange of NO was detectable, under oxidising conditions. However, under reducing conditions, [^{14}N]nitrosyl nitrite reductase was readily exchanged with $^{15}NO_2^-$ (fig.3(c)). We interpret this as due to turnover of the enzyme, in which the [^{14}N]-nitrosyl derivative is reduced to ammonia, which then dissociates and the $^{15}NO_2^-$ then forms a [^{15}N]nitrosyl derivative with the free enzyme.

4. Discussion

The ^{14}N and ^{15}N hyperfine splittings (fig.1) observed in nitrite reductase–NO are isotropic and small compared with those observed in haemoglobin–NO and some simple Fe–NO complexes (cf. [5]). These narrow splittings suggest that the electron density is localised principally on the Fe atom of the sirohaem, rather than on the nitrogen of the NO ligand.

The results given in fig.3 show that the NO–sirohaem complex is an extremely stable one, since it did not dissociate within 4 h after removal of excess nitrite and dithionite, and did not exchange with added nitrite or nitric oxide.

The ^{14}NO was displaced from the complex by $Na^{15}NO_2$ in the presence of the reducing agent dithionite. This indicates that the enzyme can turn over under these conditions, although the reaction is known to be very slow in the absence of ferredoxin or viologen dye [4]. The results presented here support the assignment of the $g = 2.007, 2.058$ signal as nitrosyl–sirohaem complex, and indicate that it is an intermediate in the enzyme reaction since it undergoes turnover.

Acknowledgements

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