Exogenous ferrous iron is required for the nitric oxide-catalysed destruction of the iron–sulphur centre in adrenodoxin

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No effects of gaseous NO added at a pressure of 19.95 kPa on the stability of the binuclear iron–sulphur centre (ISC) of reduced iron–sulphur protein adrenodoxin (0.2 mM) have been observed using the EPR method. However, the incubation of the protein with NO in the presence of ferrous iron (1.8 mM) led to complete ISC degradation, accompanied by the formation of protein-bound dinitrosyl iron complexes (DNICs; 0.3 ± 0.1 mM). Similar results were obtained when low-molecular-mass DNIC with phosphate or cysteine (1.8 mM) were added to solutions of pre-reduced adrenodoxin. The degradation of the ISC was

suggested to be due to the attack of the $\text{Fe}^+(\text{NO}^+)_2$ group from low-molecular-mass DNICs added or formed during the interaction between NO and ferrous ions on the thiol groups in active centres of adrenodoxin. This attack leads to a release of endogenous iron from the centres, which is capable of forming both low-molecular-mass and protein-bound DNIC, thereby ensuring further ISC degradation.

Key words: dinitrosyl iron complex, iron–sulphur protein, nitric oxide (NO).

INTRODUCTION

The interaction between NO and the active centres of iron–sulphur proteins (ISPs) is a way of NO signalling in living systems that ensures both regulatory and cytotoxic NO effects on diverse biochemical and physiological processes [1–6]. As a rule the interaction leads to the degradation of the iron–sulphur centre (ISC). This is accompanied by the formation of EPR-detectable dinitrosyl iron complexes (DNICs) incorporating protein thiol groups [with the formula $(\text{RS}^-)_2\text{Fe}^+(\text{NO}^+)_2$] [3–13]. The complexes give a characteristic EPR signal with $g_{\perp} = 2.04$ and $g_{\parallel} = 2.014$ (d^7 electron configuration) [14–16]. It was proposed that the mechanism of this process was via replacement of inorganic sulphur atoms (*S) in the ISC with NO molecules, resulting in the contemporaneous formation of DNIC [6,7]. The simplest ISC occurring in an ISP is binuclear. The proposed mechanism suggested to operate in this case is illustrated by Scheme 1. However, it is known that paramagnetic DNICs are formed only during the interaction between NO and ferrous iron (Fe^{2+}) [14–16]. Therefore, DNIC should only be able to appear following the reaction of NO molecules with reduced ISC.

Adrenodoxin is a simple ISP, containing a binuclear ISC. Our previous experiments on oxidized adrenodoxin supported the idea that NO does not affect the oxidized centres [17]. However, incubation of the protein with NO in the presence of ferrous iron led to significant DNIC formation, accompanied by complete

ISC degradation. Similar results were obtained when the protein was incubated with low-molecular-mass DNICs containing thiol or non-thiol ligands [17]. The effect was suggested to be due to the interaction of exogenous iron in the DNIC with protein thiol groups, leading to replacement of the endogenous iron atom in the ISC (Scheme 2).

The results of similar experiments on reduced adrenodoxin proved to be rather equivocal [17]. As early as 1–2 min after gaseous NO addition to the solution of adrenodoxin pre-reduced with dithionite the EPR signal at $g = 1.94$ characteristic of native ISC disappeared, consistent with the mechanism of ISC degradation presented in Scheme 1. However, the amount of DNIC that appeared in the solution was negligible, compared with the total iron content of the protein. Moreover, the addition of dithionite to the solution of adrenodoxin treated with NO resulted in notable recovery in the intensity of the EPR signal at $g = 1.94$. However, it could not be excluded that NO_2 was present as a contaminant in the gaseous NO used (formed by the process of dismutation of NO to form NO_2 and N_2O [18]). As it was therefore possible that NO_2 had oxidized the reduced ISC in adrenodoxin, it could not be excluded that the small non-quantitative loss of the $g = 1.94$ signal resulted from an interaction with the oxidized, not the reduced, protein. In the

Scheme 1 The mechanism of NO-catalysed destruction of binuclear iron–sulphur centres

Scheme 2 The mechanism of binuclear iron–sulphur centre degradation by dinitrosyl iron complexes

L, non-thiol ligand.

Abbreviations used: DNIC, dinitrosyl iron complex; HFS, hyperfine structure; ISC, iron–sulphur centre; ISP, iron–sulphur protein.

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present studies we performed experiments using freshly synthesized gaseous NO (to prevent NO₂ accumulation) and determined whether NO alone is capable of inducing the degradation of reduced ISC in adrenodoxin or whether the process can be initiated only in the presence of free Fe²⁺ ions.

EXPERIMENTAL

Materials

L-Cysteine, sodium phosphate, glutathione, methyl viologen, sodium thiosulphate (Sigma, St. Louis, MO, U.S.A.), sodium dithionite (Merck, Darmstadt, Germany) and FeSO₄·7H₂O (Fluka, Buchs, Switzerland) were used in the experiments. Gaseous NO was synthesized in the reaction of FeSO₄ with NaNO₂ in 0.1 M HCl with subsequent low-temperature sublimation in an evacuated glass system. ⁵⁷Fe³⁺ ion solutions were obtained from a powder of ⁵⁷Fe₂O₃ by the method described in [16].

Synthesis of DNICs

DNICs with phosphate, cysteine or thiosulphate were synthesized as described elsewhere [16,19].

Experiments on adrenodoxin

Adrenodoxin was isolated from bovine adrenal glands according to the method described in [20]. The concentrations of isolated protein were evaluated optically, using the intensity of the absorption band at 414 nm [20]. The concentrations estimated were within the range 0.2–0.23 mM. The purity index of the adrenodoxin preparation was evaluated using the ratio of the intensities of absorption bands at 414 and 280 nm; a value of 0.8 indicated high purity of the adrenodoxin preparation [20]. The reduced species of adrenodoxin was obtained by treatment of the initial oxidized preparations with sodium dithionite dry powder (final concentration, 5 mM) and methyl viologen as a redox mediator (final concentration, 0.1 mM). The concentration of reduced adrenodoxin estimated by EPR (see below) was approximately equal to that of oxidized protein determined by the optical method. The treatment of pre-reduced adrenodoxin preparations with gaseous NO was performed in a Thunberg tube apparatus pre-evacuated before the administration of gaseous NO. The latter was added at the pressure of 19.95 kPa for 5 min followed NO evacuation. In some experiments 1.8 or 0.3 mM ^{56/57}Fe²⁺-citrate complex (iron/citrate molar ratio, 1:5) was added to the solutions of pre-reduced adrenodoxin before the NO treatment. DNICs with phosphate or cysteine were added to the solutions of pre-reduced protein in air for 5 min. The decomposition of the active-centre ISC in adrenodoxin molecules induced by these agents was monitored by the decrease in the intensity of the EPR signal at *g* = 1.94, characteristic of the reduced species of adrenodoxin.

EPR measurements

EPR spectra were recorded at 77 K or ambient temperature using an EPR spectrometer, either a modified Radiopan (Radiopan, Warsaw, Poland) or an ESC-106 (Bruker, Karlsruhe, Germany) at X-band, respectively. The concentrations of various paramagnetic centres were estimated by the method of double integration of EPR signals using a known concentration of DNIC (prepared with cysteine) as a standard sample.

Statistical analysis

Results are presented as means ± S.E.M. from at least three experiments.

RESULTS

The addition of freshly prepared NO gas (at a pressure of 19.95 kPa) to pre-reduced adrenodoxin (0.2 mM; 1 mM dithionite) for 5 min at ambient temperature did not result in any significant degradation of the protein ISC: the intensity of the EPR signal at *g* = 1.94 did not decline (Figures 1a and 1b). A broad signal was recorded in the *g*-factor range 2.07–1.98 (Figure 1) with a triplet hyperfine structure (HFS), characteristic of haem-nitrosyl complexes from contaminating haem-containing proteins in the solution. The concentrations of the latter did not exceed 10 μM (i.e. < 5 % of the adrenodoxin). A small signal at *g* = 2.04 was also observed, due to the formation of 2 μM DNIC. The degradation of ISC and the formation of DNICs was much faster (< 5 min) following the addition of NO gas in the presence of 1.8 mM Fe²⁺-citrate complex (1:5 molar ratio; Figure 1c). The DNIC formation in this preparation (0.3 ± 0.1 mM) was accompanied with practically complete ISC degradation in

Figure 1 EPR spectra following ferrous iron and NO addition to adrenodoxin

EPR spectra from a 0.2 mM solution of adrenodoxin, treated with dithionite (a) followed with NO (b) or with ⁵⁶Fe²⁺ (1.8 mM) + NO (c). (d) Treatment of preparation (c) with dithionite. Measurements were made at 77 K, microwave power 5 mW, and modulation amplitude 0.5 mT. To the right of the spectra, the relative amplification of the signals are indicated (note this means that spectrum c is 20 × larger than indicated when compared with a and b at all magnetic fields or c and d at a higher magnetic field). σ is the width of the signal component at half amplitude.

Figure 2 Effect of thiosulphate on DNIC EPR spectra

EPR spectra from 0.2 mM reduced adrenodoxin treated with ⁵⁶Fe²⁺ (1.8 mM) + NO (a) followed by thiosulphate (b, c). Recordings were made at ambient temperature, microwave power 20 mW and modulation amplitude 0.5 mT (a, b) or 0.01 mT (c). To right of the spectra, relative amplifications of the signals are indicated.

adrenodoxin (Figure 1d). Subsequent treatment of the preparation with dithionite (2 mM) resulted in a decrease of the intensity of the EPR signal from DNIC at $g = 2.04$ and the formation of a transitory EPR signal with $g_{\perp} = 2.01$ and $g_{\parallel} = 1.97$ (Figure 1d). This new signal was the result of the ability of DNIC to accept two electrons, thereby changing the d^7 electron configuration of iron in the complex to d^9 [21]. However, despite this, the EPR signal from reduced adrenodoxin at $g = 1.94$ did not re-appear (Figure 1d). Similar results were obtained when DNIC with phosphate or cysteine (1.8 mM) was added to the solutions of pre-reduced adrenodoxin. However, the DNIC with cysteine was less efficient in ISC degradation (results not shown). The addition of ferrous iron to the solutions alone did not influence the stability of the ISC. It is noteworthy that keeping freshly prepared gaseous NO in a closed glass balloon at a pressure of 19.95 kPa led, within 2–3 weeks, to the gas gaining the capability to oxidize pre-reduced adrenodoxin in the solution. Obviously this time range was long enough for the accumulation of NO₂ via a redox dismutation of the NO molecules [18]. The EPR spectra presented in Figure 1 were recorded at 77 K. As was earlier shown the anisotropic shape of the EPR signal from low-molecular-mass DNIC at this temperature is determined by the g -factor and HFS anisotropy. Low-molecular-mass DNIC solutions change the shape of their EPR spectra when the measurement is made in the liquid or frozen state. However, when increasing the measurement temperature from 77 K to ambient, the shape of the DNIC EPR signal measured in Figure 1(c) remained unchanged (Figure 2a). This indicated unambiguously

Figure 3 Iron isotope effects on thiosulphate DNIC EPR spectra

EPR spectra from the solutions of DNIC with thiosulphate containing ⁵⁷Fe (upper trace), ⁵⁶Fe (middle trace) or ⁵⁷Fe and ⁵⁶Fe in equal amounts (lower trace). Recordings were made at ambient temperature, microwave power 20 mW and modulation amplitude 0.01 mT.

that the complexes remained bound to the protein globula: the low mobility of protein-bound DNIC was not sufficient to average the afore-mentioned anisotropy. However, when 5 mM thiosulphate ions were added to the solution a narrow isotropic signal at $g = 2.03$ appeared, accompanied by a decline in the EPR signal from protein-bound DNIC (Figures 2b and 2c). This suggests that there had been a transfer of Fe²⁺(NO⁺)₂ groups from protein-bound DNIC to thiosulphate ions, resulting in the formation of a DNIC–thiosulphate complex free in the solution. The mobility of these low-molecular-mass complexes at ambient temperature was high enough to average the g -factor and HFS anisotropy.

When recorded at 77 K the EPR signal of the DNIC with thiosulphate gave an anisotropic EPR signal that practically coincided with that from DNIC with thiol-containing ligands (results not shown). Reducing the value of the high-frequency magnetic-field modulation amplitude from 0.5 to 0.01 mT led to the appearance of a quintet HFS (Figure 2c) in the EPR signal, which was due to hyperfine interaction of unpaired electron from the complex with nitrogen nuclei from two nearby NO⁺ ligands. The parameters of the signal coincided with those described for DNIC with thiosulphate elsewhere [22].

Thus in agreement with the results for oxidized adrenodoxin obtained earlier [17], the present data indicate much more efficient NO-induced ISC degradation in reduced adrenodoxin in the presence of exogenous ferrous iron, as compared with that induced by NO molecules alone. According to the proposed mechanism of ISC degradation induced by NO and Fe²⁺ ions (Scheme 2), the process must lead to a release of *Fe₂*S₂ groups from the centres. It is reasonable to suggest that when reduced to

Figure 4 Effect of varying exogenous iron isotope ratio on DNIC EPR spectra following NO addition to adrenodoxin

Right-hand panel: (a) EPR spectra from 0.2 mM reduced adrenodoxin treated with $^{56}\text{Fe}^{2+}$ (0.2 mM) + $^{57}\text{Fe}^{2+}$ (0.2 mM) + NO followed by subsequent thiosulphate (5 mM) treatment (a). (b) Addition of dithionite and additional NO to sample (a). (c) EPR spectrum from 0.2 mM reduced adrenodoxin treated with $^{57}\text{Fe}^{2+}$ (0.3 mM) followed by subsequent thiosulphate (5 mM) treatment. (d) Addition of dithionite and additional NO to sample (c). (e) EPR spectra from 0.2 mM reduced adrenodoxin treated with $^{56}\text{Fe}^{2+}$ (0.2 mM) + NO followed by subsequent thiosulphate (5 mM) treatment. Left-hand panel: Simulation of EPR spectra from DNIC with thiosulphate containing ^{56}Fe and ^{57}Fe at the ratios indicated in the Figure. Recordings were made at ambient temperature, microwave power 20 mW and modulation amplitude 0.01 mT.

the Fe^{2+} state these iron ions can therefore form DNIC in the presence of NO. To verify the proposition we performed experiments with the addition of isotope ^{57}Fe ions to the adrenodoxin solutions. The ^{57}Fe contains a nuclear spin ($I = 1/2$). The hyperfine interaction of the unpaired electron with this spin leads to an additive doublet HFS in the EPR signal from DNIC with thiol-containing or thiosulphate ligands. Figure 3 illustrates the EPR signals from DNIC with thiosulphate, including isotope ^{57}Fe or ^{56}Fe ions. The difference observed in these signals should allow us to determine whether the iron in the DNIC comes from that added externally, or that already present in the protein. As expected, a complicated HFS pattern is seen in the EPR signal from DNIC with thiosulphate containing equal amounts of ^{57}Fe and ^{56}Fe atoms (Figure 3). A similar complicated HFS pattern was observed for the EPR signal from the solutions of pre-reduced adrenodoxin (0.2 mM) following the addition of NO and 0.2 mM ^{57}Fe – ^{56}Fe –citrate (at equal isotope proportions) for 5 min followed by a subsequent addition of thiosulphate (5 mM; Figure 4a). When compared with a simulation of the spectrum expected if there was an equal amount of the two isotopes

(left-hand panel), there was a slight preponderance of the ^{56}Fe signal.

This suggests that some of the iron in the DNIC has arisen from the ISC iron (which is predominantly ^{56}Fe). The result demonstrates no iron isotope effect on the interaction between iron ions and ISC in the presence of NO. The addition of dithionite and NO to this sample increased the total DNIC and was consistent with a $^{56}\text{Fe}/^{57}\text{Fe}$ ratio of 1.5:1, suggesting that half the DNIC iron now originated from the protein (Figure 4b). The presence of protein iron in the DNIC was confirmed by the addition of pure ^{57}Fe –citrate and NO to reduced adrenodoxin (containing natural levels, predominantly of ^{56}Fe , in its ISC). The subsequent thiosulphate-ligated DNIC shows clear evidence of ^{56}Fe in the DNIC (at an estimated ratio of 0.44:1). Subsequent addition of NO and dithionite to the solution resulted in an increase of this ratio to 0.75:1 (Figure 4d).

These thiosulphate studies are informative, but only comment on the nature of the DNIC once it is removed from the protein. Although the HFS due to ^{57}Fe cannot be resolved in protein-bound DNIC, it does lead to a broadening of the $g_{\perp}$ component

Figure 5 Iron isotope effects on protein-bound DNIC in adrenodoxin

(a) EPR spectra from 0.2 mM reduced adrenodoxin. (b) Addition of $^{57}\text{Fe}^{2+}$ (0.3 mM) + NO to spectrum (a). (c) Addition of dithionite and NO to spectrum (b). Recording conditions were as in legend for Figure 1. To the right, relative amplifications of the spectra are shown. σ is the width of the signal component at half amplitude.

of the EPR signal [23]. This allows us to determine whether the iron in protein-bound DNIC comes from exogenous or endogenous sources. Adding exogenous ^{57}Fe resulted in a significant broadening of the protein-bound DNIC formed following NO addition to reduced adrenodoxin (Figure 5). The width of the $g_{\perp}$ component of the DNIC EPR signal at half amplitude (δ) was notably more than that characteristic of the EPR signal of the protein-bound DNIC containing only ^{56}Fe (compare Figure 5b and Figure 1c).

Under the conditions of this experiment 40% of the ISC was still intact. Further addition of dithionite and NO to this sample caused additional cluster decomposition and narrowing of the $g_{\perp}$ component (Figure 5c). Thus the initial event appears to be ligation of exogenous Fe to the cluster resulting in a broad EPR line. Once the cluster is almost completely decomposed, iron from the ISC contributes a larger component of the DNIC.

DISCUSSION

These results illustrate the important role of free ferrous iron in ISC degradation initiated by NO addition. Iron ions sharply accelerate the process, inducing an attack by low-molecular-mass DNICs [$(\text{L})_2\text{Fe}^+(\text{NO})_2$, where L is a non-thiol ligand] on the thiol groups that ligate the iron in the ISC (Scheme 2). This attack results in the formation of protein-bound DNIC and releases $^*\text{Fe}_2^*\text{S}_2$ groups from the ISC. Being reduced, the iron from the latter is capable of forming both low-molecular-mass and, probably, protein-bound DNICs, thereby ensuring further

ISC degradation. The catalytic effects of iron suggest that DNIC formation and ISC degradation become an autocatalytic event as more iron is released from the ISC, although a detailed analysis of the kinetics would be required to verify this proposition. In this context, however, it is interesting to note the data reported by Kennedy et al. [13], who studied tetranuclear $4\text{Fe}-4\text{S}$ ISC degradation in aconitase treated with gaseous NO. The authors observed sigmoidal (auto-catalytic) kinetics for DNIC formation in the solution of aconitase at ambient temperature (see Figure 3 in [13]). DNIC formation was sharply accelerated 40 min after the initiation of the process, resulting in the appearance of 70% of the total DNIC formed in the subsequent 20 min. At that time enzyme activity declined to a negligible level. Kennedy and co-authors [13] did not add any exogenous iron to the aconitase solutions. However, the iron incorporated into $(\text{Fe}^*\text{S})_n$ could appear in the solutions as a result of the slow process of ISC degradation induced by NO molecules alone. The accumulation of the iron released from ISC could provoke the formation of low-molecular-mass DNICs with anionic ligands (L) and lead to the sharp acceleration of ISC degradation and DNIC formation. It is possible that the entire degradation process starts as a result of the low levels of iron contamination present in the solution, followed by this auto-catalytic process.

Although the investigators [5–7] suggest that the interaction of NO alone with active centres of ISPs is sufficient for their rapid degradation, the afore-mentioned study of Kennedy and co-authors [13] indicates rather slow kinetics of the process from the physiological standpoint (1 h was required for inactivation at $450\text{ }\mu\text{M}$ NO and $200\text{ }\mu\text{M}$ aconitase, concentrations that are approx. 100–1000-fold greater than those likely to be present *in vivo*). The low efficiency by which NO alone catalyses ISC transformation into DNIC is also seen in the binuclear ISC in the ferredoxin which functions as a SoxR transcription factor in *Escherichia coli* [6]. The concentration of DNIC in the protein never exceeded 18% of the concentration of ISC under the most 'optimal' conditions (equivalent to only 9% of the total concentration of 'iron–sulphur' iron in the ISC). A similar result was obtained when NO was added to the tetranuclear $4\text{Fe}-4\text{S}$ ISC from *Chromatium vinosum* high-potential ISP [5]. DNIC formation even at its maximum was only equivalent to approx. 60% of the protein concentration [5], corresponding to only 15% of this iron content of the ISC. So, on closer inspection, it can be seen that the yield of DNIC in experiments when only NO is added to ISP is rather low.

DNIC formation is clearly observed in cultured cells or isolated tissues following NO addition, either added exogenously or by activating endogenous NO synthase [3,6–10,12,23–28]. However, as we showed previously, the appearance of DNIC in animal tissues or macrophage cells can be explained as being mostly due to loosely bound (so-called free) iron in the samples [24,25]. Taking into account the present data, it is reasonable to suggest that low-molecular-mass DNICs formed from this free iron pool could attack active ISC, resulting in the formation of apo-ISP-bound DNICs. It is tempting to propose that this mechanism operates in the process of formation of DNICs bound with SoxR ferredoxin in *E. coli* cells treated with NO [6], especially as the iron chelator, *o*-phenanthroline, has the ability to block the activation of the *E. coli* SoxRS regulon by NO [28]. However, in contrast with the data obtained in [6], the appearance of DNIC in animal tissues, macrophage cells [24,25] or *Clostridium sporogenes* cells [26] was not accompanied by the degradation of the ISC. Evidently, low-molecular-mass DNICs in these cellular systems could not interact with ISC localized in electron transport chains. We did not perform experiments on pre-reduced adrenodoxin treated with S-nitrosothiols. However, our earlier studies of the

reaction between *S*-nitrosoglutathione and oxidized adrenodoxin demonstrated that this was not an efficient means of degrading the ISC. However, the presence of added ferrous ions later sharply accelerated the *S*-nitrosoglutathione effect [17]. The observed effect of ferrous ions is likely to be due to the formation of low-molecular-mass DNIC, characteristic of the reaction between *S*-nitrosothiols and ferrous iron [19,29,30]. The resulting DNIC would then initiate the rapid degradation of the ISC.

What are the implications for the effects of NO on ISP *in vivo*? Clearly in the absence of free ferrous iron, NO is ineffective in degrading ISP. Once the degradation starts, however, it may become an auto-catalytic event due to iron release from the cluster. Therefore it is clearly important to know the state of the low-molecular-mass non-protein-bound iron in the cell. Unfortunately, while recent studies have clarified the intracellular transporters (chaperones) for redox-active copper in the cell [31], the nature of the free iron pool (if indeed it exists as such) has proved more elusive. We, and others, have identified an intracellular pool of 3–10 μ M iron in cells that is rapidly chelatable by membrane-permeable iron chelators [32–34]. Most candidate ligands for this putative low-molecular-mass iron pool (e.g. citrate, ATP) are EPR-detectable in their ferric state [35,36]. Yet the total pool of all EPR-detectable mononuclear non-haem ferric iron in cells is 3 μ M at most, and probably significantly less [32]. These data are therefore consistent with the possibility that there is a significant population of ferrous iron in cells, as supported by direct Mossbauer spectroscopy studies [37,38]. The relative ease and magnitude of DNIC formation from non-ISP sources in cells following the addition of NO [24,25] or NO⁺ donors [39] is also consistent with a relatively accessible pool of intracellular low-molecular-mass ferrous iron. As reported in this paper, the excess levels of NO produced in disease states is not sufficient, in and of itself, to catalyse ISC degradation. The combination of the ferrous iron pool with NO would, however, be sufficient and could be responsible for the association of diseases linked to NO overproduction and to damage to mitochondrial ISPs, e.g. Parkinson's disease [40]. The assignment of the Mossbauer-detectable ferrous iron in bone marrow to a mitochondrial pool is consistent with this idea [41].

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