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

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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 protein-bound dinitrosyl iron complexes (DNICs) [3–13]. The complexes give a characteristic EPR signal with \( g \approx 2.04 \) and \( g_s = 2.014 \) (d 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(II)) [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 NOX was present as a contaminant in the gaseous NO used (formed by the process of dismutation of NO to form NOX and N2O [18]). As it was therefore possible that NOX 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

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 \( Fe(\text{NO})_x^+ \) group from low-molecular-mass DNICs incubated with NO in the presence of ferrous iron (1.8 mM) led to complete ISC degradation. 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).

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 NO\textsubscript{2} 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 Fe\textsuperscript{2+} 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 FeSO\textsubscript{4}\cdot7H\textsubscript{2}O (Fluka, Buchs, Switzerland) were used in the experiments. Gaseous NO was synthesized in the reaction of FeSO\textsubscript{4} with NaNO\textsubscript{3} in 0.1 M HCl with subsequent low-temperature sublimation in an evacuated glass system. \textsuperscript{57}Fe\textsuperscript{2+} ion solutions were obtained from a powder of \textsuperscript{57}Fe\textsubscript{2}O\textsubscript{3} 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 [20] was evaluated using the ratio of the absorption band at 414 nm

\[ \text{ratio} = \frac{\text{absorption at 414 nm}}{\text{absorption at 280 nm}} \]

The concentration of isolated adrenodoxin was measured spectrophotometrically as described elsewhere [16]. The concentration of isolated adrenodoxin was estimated by the method of double integration of EPR signals using a known concentration of DNIC (prepared with cysteine) as a standard sample.

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 \( \mu \)M (i.e. < 5 % of the adrenodoxin). A small signal at \( g = 2.04 \) was also observed, due to the formation of 2 \( \mu \)M 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 Fe\textsuperscript{2+}–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

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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 Fe(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 Fe$^{2+}$ ions (Scheme 2), the process must lead to a release of Fe$^+$S$_2^-$ groups from the centres. It is reasonable to suggest that when reduced to
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 $^{54}$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 $^{54}$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-$^{54}$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 $^{54}$Fe signal.

This suggests that some of the iron in the DNIC has arisen from the ISC iron (which is predominantly $^{54}$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 $^{54}$Fe/$^{57}$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 $^{54}$Fe-citrate and NO to reduced adrenodoxin containing natural levels, predominantly of $^{54}$Fe, in its ISC. The subsequent thiosulphate-ligated DNIC shows clear evidence of $^{54}$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 $g_z$ component...
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 4Fe–4S 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 (Fe6S6)6 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 ISP 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 µM NO and 200 µM 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 4Fe–4S 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

![Image](66x438 to 284x735)

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

(a) EPR spectra from 0.2 mM reduced adrenodoxin. (b) Addition of 57Fe2+ (0.3 mM) + NO to spectrum (a). (c) Addition of dithionite and NO to spectrum (b). Recording condition 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 57Fe resulted in a significant broadening of the protein-bound DNIC formed following NO addition to reduced adrenodoxin (Figure 5). The width of the g⊥ 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 55Fe (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⊥ component (Figure 5c). Thus the initial event appears to be eligation 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 [(L)Fe(NO)3], 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 *Fe4*S2* 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
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 μM 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, surprisingly, iron complexes in tumor target cells cocultivated with activated macrophages. Exp. Cell Res. 214, 415–424


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