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Review

Nitric oxide and iron proteins

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Abstract

Nitric oxide interactions with iron are the most important biological reactions in which NO participates. Reversible binding to ferrous haem iron is responsible for the observed activation of guanylate cyclase and inhibition of cytochrome oxidase. Unlike carbon monoxide or oxygen, NO can also bind reversibly to ferric iron. The latter reaction is responsible for the inhibition of catalase by NO. NO reacts with the oxygen adduct of ferrous haem proteins (e.g. oxyhaemoglobin) to generate nitrate and ferric haem; this reaction is responsible for the majority of NO metabolism in the vasculature. NO can also interact with iron–sulphur enzymes (e.g. aconitase, NADH dehydrogenase). This review describes the underlying kinetics, thermodynamics, mechanisms and biological role of the interactions of NO with iron species (protein and non-protein bound). The possible significance of iron reactions with reactive NO metabolites, in particular peroxynitrite and nitroxyl anion, is also discussed.

Keywords: Nitric oxide; Iron; Peroxynitrite; Nitroxyl anion; Kinetics; Mechanism

Contents

Abbreviations: Hb, haemoglobin; EDTA, ethylenediaminetetraacetic acid; DNIC, dinitrosylirondithiol complex; IRE, iron-responsive element; IRP, iron regulatory protein; mitochondrial complex I, mitochondrial NADH dehydrogenase; mitochondrial complex II, succinate dehydrogenase; mitochondrial complex III, cytochrome bc_1 complex

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1. Introduction

The interaction of nitric oxide with metals has been studied since the early years of this century [1]. It is unlikely that the chemists of that time would have imagined that as the millennium approached there would still be unsolved questions about metal: NO interactions, nor that these would be addressed primarily by life scientists, not inorganic chemists.

Nitric oxide can bind to most transition metals [2]. However, its interactions with iron are by far the most studied transition metal:NO reactions (Fig. 1). The main NO signal transduction pathway so far elucidated, the activation of guanylate cyclase, proceeds via direct binding of NO to haem iron [3]. Furthermore, it has been suggested that NO binding to ferrous haem in cytochrome oxidase [4–7], ferric haem in catalase [8] and iron or sulphur atoms in iron–sulphur proteins [9,10] can all occur under physiological or pathophysiological conditions.

This review will be an overview of the chemical interaction of nitric oxide with iron in biological systems, with particular emphasis on the benefits and disadvantages of the selection of iron proteins as evolutionary targets for NO action. By the very nature of the field, the papers cited will be a selection of those available, chosen to highlight the chemical points made ('nitric oxide+iron' yielding 129 hits in a search of the Science Citation Index for papers published in 1997 alone). The reader is directed to other reviews in this area for more detailed discussion of the chemistry [2,11–15] and spectroscopy [16,17] of iron reactions with NO. In particular, there are many relevant chapters, especially with regard to spectroscopic techniques, in several recent books describing methodology in nitric oxide research [18–21].

The reactions of NO with iron can be divided depending upon whether they involve mere binding or binding+metabolism. The redox state of the iron (ferrous, ferric or ferryl) also dramatically modulates whether binding is favoured over metabolism, and in the former case the strength of the iron–NO bond.

2. Binding reactions of NO and iron

2.1. Binding of NO to ferrous iron

$$NO + Fe^{2+} \leftrightarrow Fe^{2+} - NO \quad (1)$$

NO binds reversibly to ferrous iron. Table 1 illustrates the relative on rates, off rates, and dissociation constants for this reaction in a selection of biological and chemical systems. It is instructive to compare NO binding to that of CO and O_2 for a number of reasons: (1) their size, charge (neutral) and hydrophobicity are similar suggesting similar access to cellular iron pools, whether the latter are low-molecular-weight or protein-bound; (2) O_2 (and to a lesser extent CO) is likely to be present in higher concentrations in most in vivo situations than NO and therefore could theoretically compete for NO binding sites on ferrous iron; and (3) once bound, the paramagnetic properties of NO and O_2 result in the addition of unpaired electrons to the system with the

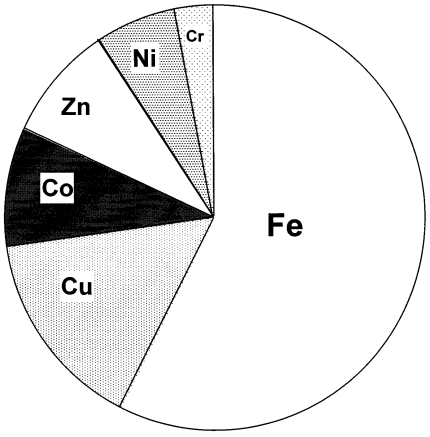

Fig. 1. Publications in 1997 describing nitric oxide interactions with transition metals. An initial search of titles in the Science Citation Index for 1997 was made. Articles were selected on the basis that they contained both the words 'nitric oxide' and a transition metal. The abstracts of the articles selected were then read and only articles describing direct NO/metal interactions were selected. The total number of such articles was 33. The distribution between different transition metals is illustrated in the pie chart above. In fact, there was little change if the abstract checking procedure was ignored. A similar direct search using the titles, keywords, and abstract fields also revealed the preponderance of references on iron (129 out of the 260 articles selected).

result that, unlike CO, simple reversible binding is not always the only interaction observed.

CO, NO and O_2 can all bind reversibly to ferrous iron. In most proteins, oxygen metabolism by ferrous iron is faster than its dissociation and therefore the oxygen adduct is not stable. The exception, of course, is in the oxygen-transport proteins, such as haemoglobin and myoglobin, which make stable O_2 adducts with measurable on and off rates. A general comparison for the on rates reveals that O_2 and NO have similar rates (in the order 10^7–10^8 M^{-1} s^{-1}). CO on rates, however, are considerable lower (always $< 10^7$ M^{-1} s^{-1}). However, the most interesting comparison is in the off rates. The greater affinity of NO over CO for ferrous haem is reflected in both a faster on rate *and* a slower off rate; in some cases the dissociation rate is so slow ($< 10^{-5}$ s^{-1}) as to be technically very difficult to measure. This creates clear problems for the role of NO as a signalling molecule. For example, the half-time of guanylate cyclase deactivation when NO is removed is less than a minute, which is clearly impossible if NO dissociates from this enzyme as slowly as from haemoglobin or myoglobin. A similar problem exists for mitochondrial respiration where there is clear evidence that low levels of NO generate the ferrous cytochrome oxidase–NO complex, yet respiration returns to normal within seconds once NO is removed from the solution.

As NO binds so tightly to ferrous haem, it has been suggested that it might be easier to remove the haem–NO complex from the protein rather than simply dissociate the NO from the haem. NO can dissociate the *trans* ligand from haem iron and, therefore, has the potential to destabilise haem binding to the protein. Indeed guanylate cyclase is frequently purified as an apoprotein (without the haem cofactor). It has therefore been suggested that NO-induced haem dissociation may be responsible for changes in enzyme activity in vivo [11,13]. Although this seems an intrinsically inefficient mechanism for short-term regulation of the NO signal, haem dissociation may be important [11] in limiting cGMP synthesis following prolonged exposure to high NO concentrations (and may therefore be a contributing factor in the development of nitroglycerin tolerance). In fact, recent data demonstrates that haem dissociation from proteins is unnecessary for fast NO dissociation and that proteins are able to modify the microenvironment of the iron–nitrosyl bond to have substantially higher than expected dissociation rates for NO: 0.05 s^{-1} for guanylate cyclase [30] and 0.13 s^{-1} for cytochrome oxidase [29]. Thus, increased NO dissociation rates within proteins allow NO to have a *short-term* role in increasing cyclic GMP levels or in modulating mitochondrial respiration. The on rates for NO are fixed at about 10^8 M^{-1} s^{-1} for most ferrous haem proteins; this suggests, paradoxically, that NO binds *weaker* to ferrous iron in its target molecules than to other haem proteins, e.g. myoglobin or haemoglobin.

Comparisons of NO binding between haem proteins and low molecular weight non-haem iron complexes are not straightforward as many of the studies on the latter are carried out at rather acid pH values (given the problems of maintaining the ferrous iron in solution at neutral pH). However, Table 1 reveals that most of these complexes (including some measured at close to physiological pH) have significantly less affinity for NO than haem proteins. In particular the fast off rates suggest that in vivo NO will not get

Table 1
A comparison of the kinetic and thermodynamic constants for nitric oxide, carbon monoxide and oxygen binding to ferrous iron in different proteins and model systems

Compound	O_2 on rate (M^{-1} s^{-1})	O_2 off rate (s^{-1})	O_2 dissociation constant (M)	NO on rate (M^{-1} s^{-1})	NO off rate (s^{-1})	NO dissociation constant (M)	CO on rate (M^{-1} s^{-1})	CO off rate (M^{-1} s^{-1})	CO dissociation constant (M)	References
Haemoglobin (R-state)	5×10^{7}	15	3×10^{-7}	2×10^{7}	1.8×10^{-5}	0.9×10^{-12}	6.0×10^{6}	0.01	1.7×10^{-9}	[22]
Haemoglobin (T-state)	4.5×10^{6}	1900	4.2×10^{-4}	2×10^{7}	3×10^{-3}	1.5×10^{-10}	8.3×10^{4}	0.09	1.1×10^{-6}	[22,23]
Myoglobin	1.4×10^{7}	10	7.1×10^{-7}	1.7×10^{7}	1.2×10^{-4}	0.7×10^{-11}	5.0×10^{5}	0.17	3.4×10^{-7}	[24,25]
Cytochrome *c*	–	–	–	8.3×10^{0}	2.9×10^{-5}	3.5×10^{-6}	–	–	–	[26,27]
Cytochrome oxidase	1×10^{8}	–	–	1×10^{8}	0.13	1.3×10^{-9}	7×10^{4}	0.023	3×10^{-7}	[28,29]
Guanylate cyclase	–	–	–	7×10^{8}	7×10^{-4}	1×10^{-12}	–	–		[30,31]
Guanylate cyclase+GTP and Mg^{2+}	–	–	–	7×10^{8}	0.05	0.7×10^{-10}	–	–		[30,31]
Fe^{2+} $(acac)_2$, pH 6.6	–	–	–	4×10^{2}	24	5.9×10^{-2}	–	–	–	[32]
Fe^{2+} citrate, pH 5.1	–	–	–	4.4×10^{5}	6.6×10^{2}	1.5×10^{-3}	–	–	–	[32]
Fe^{2+} EDTA, pH 5.1	–	–	–	$>6\times10^{7}$	>60	1×10^{-6}	–	–	–	[32–34]
$Fe(H_2O)_6$ $^{2+}$ (in sulphuric acid)	–	–	–	6.2×10^{5}	1.4×10^{3}	2×10^{-3}	–	–	–	[35]

The references in the table refer to the nitric oxide data. Values for CO and O_2 binding taken from references [36–38]. –, not determined or not measurable. The pH for the protein studies was in the physiological range (7–7.4). The pH for the inorganic complexes is indicated in the table. Apart from the case of Fe^{2+} EDTA, the dissociation constant has been determined as the ratio of the off and on rates, rather than being measured directly.

'trapped' in non-protein iron pools. Interestingly, model haem iron complexes can have very high affinity for NO [25,27] and this demonstrates that the polypeptide chain is not required to facilitate NO binding to haem proteins. Instead, the role of the protein microenvironment is likely to be to regulate the strength of binding (and subsequent protein conformation and activity changes) to the physiological requirements.

There is one further important point to make about NO binding to haem proteins. In stark contrast to CO and O_2, NO in general binds more tightly to ferrous iron in the absence of a *trans* ligand [13]. In practice, this means that NO binding will frequently dissociate the *trans* ligand; alternatively, the binding of a *trans* ligand can force NO dissociation. The former effect has been demonstrated in a number of proteins by EPR spectroscopy as the 5-coordinate and 6-coordinate complexes have distinct spectra [39]. In guanylate cyclase, for example, binding of NO displaces the proximal histidine ligand and this may induce conformational changes in the protein that increase the cyclase activity [3] (as well as increasing the tendency for haem dissociation described above).

Conversely, changing the protein's structure to force a ligand to bind *trans* to the NO (and hence weaken the Fe^{2+}–NO bond) is one possible answer to the biological necessity of removing NO from ferrous haem proteins. NO dissociation from 6-coordinate model haems occurs at least 1000 times faster than from otherwise similar 5-coordinate haems [25]. The dissociation rate of NO from guanylate cyclase is increased 100-fold (Table 1) in the presence of

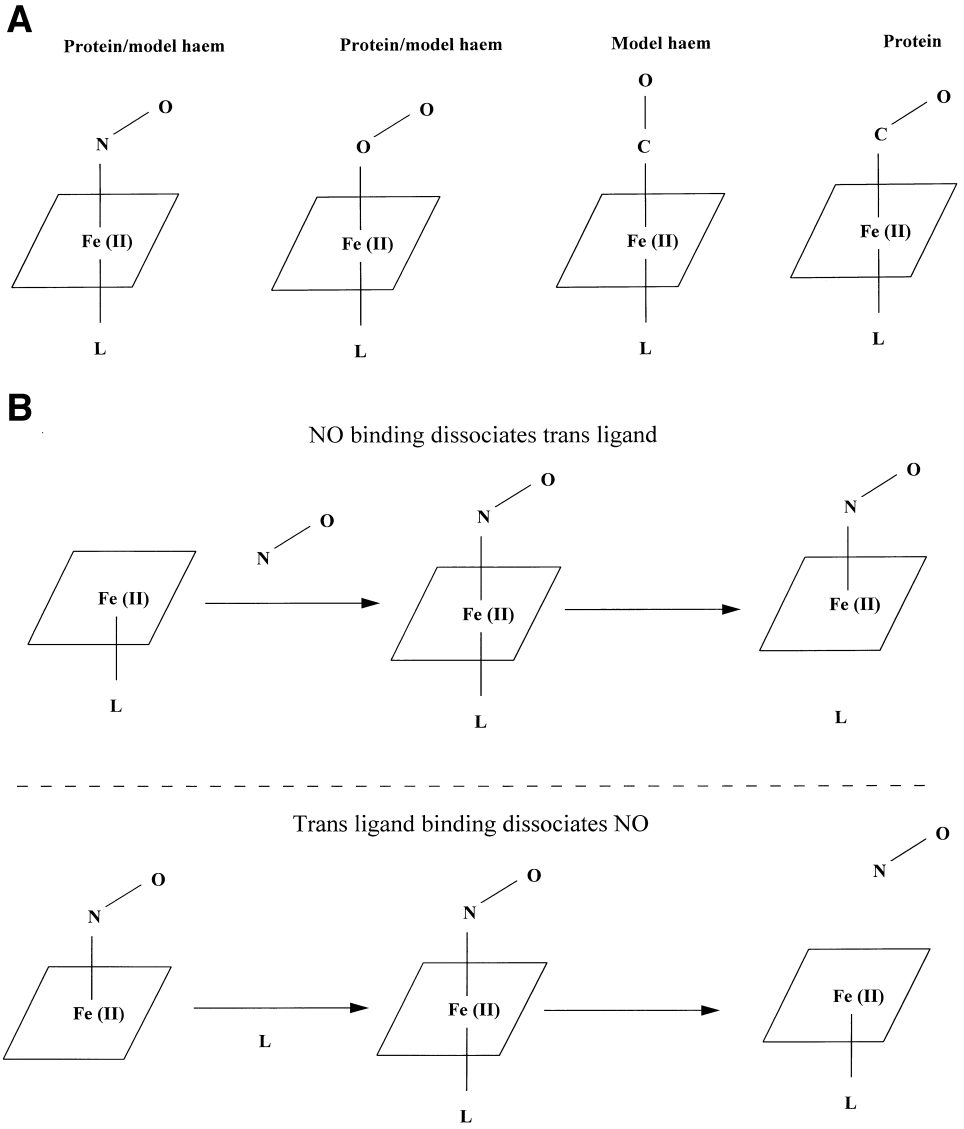

Fig. 2. (A) Geometry of binding of nitric oxide, oxygen and carbon monoxide to ferrous haem iron. NO and O_2 bind in a 130–150° angle. CO prefers a linear geometry, but due to the presence of steric hindrance in the distal side of the haem pocket is frequently forced to bind with a non-preferred bent angle. Thus, as well as the intrinsic weakness of the Fe(II)–CO bond (compared to that of Fe(II)–NO), steric factors can further contribute to the increased affinity of haem proteins for NO, rather than CO. (B) Possible effects of NO on *trans* ligand binding (and vice versa) in ferrous haem nitrosyl complexes. Unlike CO and O_2, NO binding decreases the affinity of a *trans* ligand and can thus dissociate it, forming a 5-coordinate complex. The displaced ligand (L) can either directly perform enzyme catalysis (e.g. acid/base chemistry if it is a histidine) or induce protein conformational changes that affect catalysis at sites distant to the haem. Similarly, forcing a *trans* ligand to bond to a 5-coordinate nitrosyl complex weakens the Fe–NO bond and may induce NO dissociation.

Mg^{2+} and GTP. GTP has been shown to perturb the Raman spectrum of the Fe^{2+}–NO bond [40] and it may therefore increase NO dissociation by forcing the proximal histidine to re-bind to the iron [30].

These different roles of NO, CO and O_2 as ferrous haem ligands are illustrated in Fig. 2.

2.2. Binding of NO to ferric iron

$$NO + Fe^{3+} \leftrightarrow Fe^{3+} - NO\ (Fe^{2+} - NO^{+}) \quad (2)$$

Another distinction between NO binding to iron and that of CO and O_2, is that NO can also bind reversibly to ferric iron. In contrast to the case with

Table 2
Kinetic and thermodynamic constants for nitric oxide binding to different forms of ferric iron

Compound	On rate ($M^{-1}\,s^{-1}$)	Off rate (s^{-1})	Dissociation constant (M)	Reference
Haemoglobin	4×10^{3}	1	2.5×10^{-4}	[41]
Haemoglobin α-chains	3.3×10^{3}	2.1	1.4×10^{-4}	[41]
Haemoglobin β-chains	1.3×10^{4}	3	2.3×10^{-4}	[41]
Myoglobin (sperm whale)	5.3×10^{4}	14	2.6×10^{-4}	[27,42,43]
Myoglobin (elephant)	2.2×10^{7}	40	1.8×10^{-6}	[41]
Microperoxidase	1.1×10^{6}	3.4	3.1×10^{-6}	[42]
Cytochrome *c*	1×10^{3}	0.03	3×10^{-5}	[27,43,44]
Catalase	2×10^{6}	1	5×10^{-7}	This paper (see text)
	3×10^{7}	170	6×10^{-6}	[27]

For haemoglobin, myoglobin and microperoxidase, the dissociation constant has been determined as the ratio of the off and on rates, rather than being measured directly. For catalase and cytochrome *c*, direct measurements of the dissociation constant gave very close agreement to that determined from the kinetic data.

Fe^{2+}, a linear geometry of the Fe^{3+}–NO bond is favoured [45]. Optical and EPR spectroscopy [45] suggest that the unpaired electron originating from NO transfers to the iron atom, making the spin-coupled ferrous–nitrosonium formulation more accurate in this case (Fe^{2+}–NO^{+}). This is consistent with the linear Fe–N–O bond as this is the favoured geometry for NO^{+} ligation [12]. Table 2 illustrates the kinetic and thermodynamic parameters for this reaction. It can clearly be seen that NO binds less tightly to ferric, as opposed to ferrous, iron. The NO on-rate can approach 10^{7} M^{-1} s^{-1} when the distal side of the haem is free as in catalase, elephant myoglobin (which lacks a distal histidine ligand) or the 5-coordinate artificial undecapeptide microperoxidase; however, for all proteins (Table 2) and model haems [27] the off rate for the ferric–NO complex is much faster (by between 10^{4} and 10^{8} times) than for the respective ferrous–NO complex.

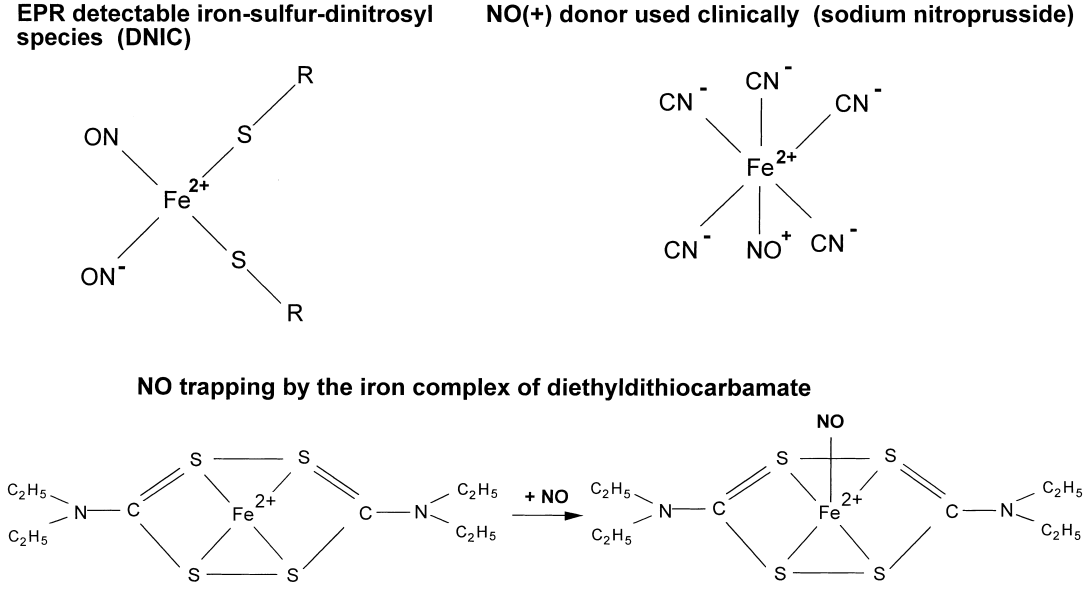

Fig. 3. Structures of some important non-haem iron nitrosyl complexes. Iron–sulphur–dinitrosyl complexes are detectable in most physiological circumstances where NO production is enhanced. Sodium nitroprusside is a NO donor used to induce hypotension clinically. Diethyldithiocarbamate makes an iron complex that readily binds NO and has been used to trap NO in a number of animal models of human diseases where NO overproduction is implicated (e.g. sepsis, hypoxia-ischaemia, transplant rejection).

NO binding to ferric haem is also less reversible than to ferrous haem and, in many cases, reductive nitrosylation converts the ferric–NO complex to the ferrous iron (cf. later). Even this reaction, however, occurs slowly on the biological time scale (with a half-life of 10 min at neutral pH for haemoglobin, and longer for other proteins [43,45].

The only enzyme where it has been suggested where NO may inhibit the ferric form is catalase [8]. However, the apparent K_i (0.3 μM) measured by enzyme inhibition seems too high for the reported K_d (5 μM) measured spectroscopically [27]. This K_d was measured in an unbuffered solution of ill-defined pH. We have re-measured the K_d at pH 7 (C. Cooper and P. Nicholls unpublished observations) and found a value of 0.5 μM (Table 2) consistent with a role for NO binding to ferric haem in the observed inhibition of catalase at this pH [8].

3. Chemical reactivity of NO with iron

3.1. NO and ferrous iron

Although reversible binding reactions predominate (cf. earlier) some metabolism of Fe^{2+}–NO complexes can occur. This is likely to be important with the very stable haem–NO complexes (e.g. nitrosyl haemoglobin) as otherwise given the very slow off rates, there would be a significant accumulation of these complexes in vivo. For example the dissociation rate of the Hb^{2+}–NO complex is approximately 1×10^{-5} s^{-1}; yet the half-life for the Hb^{2+}–NO complex in vivo following removal of an NO stimulus is 15 min [46], suggesting a much faster dissociation rate. This anomaly can be explained by the reaction of nitrosyl haemoglobin (or myoglobin) with oxygen which generates methaemoglobin and nitrate. Different mechanisms have been proposed for this reaction. However, given that the Fe^{2+}–NO dissociation rate (1×10^{-5} s^{-1}) is slower than observed rates of Fe^{2+}–NO conversion to metmyoglobin and nitrite (which can be as high as 1×10^{-3} s^{-1} at saturating oxygen tension), it seems likely that a direct reaction occurs in the haem pocket between oxygen and the nitrosyl complex, perhaps generating an intermediate haem–peroxynitrite adduct [47].

$$Fe^{2+} - NO + O_2 \rightarrow Fe^{3+} - ONOO^- \rightarrow Fe^{3+} + NO_3^- \quad (3)$$

Clearly, this mechanism opens the possibility of some peroxynitrite formation if dissociation can occur prior to the coordinate sphere rearrangement that forms nitrate.

3.2. NO and ferric iron

Some Fe^{3+}–NO complexes are quite stable (e.g. catalase, horse radish peroxidase, cytochrome *c* peroxidase), whereas others (e.g. metmyoglobin, methaemoglobin, cytochrome *c*) react further [43,45, 48]. In the case of haemoglobin and myoglobin, an autoreduction process in the presence of NO leads to the Fe^{2+}–NO complex. The ferric–NO autoreduction reaction is best characterised by a water (Eq. 5) or hydroxide (Eq. 6) attack on the ferrous nitrosonium ion complex formed following NO addition to the ferric iron (Eq. 4)

$$NO + Fe^{3+} \leftrightarrow Fe^{3+} - NO\ (Fe^{2+} - NO^+) \quad (4)$$

followed by:

$$Fe^{2+} - NO^+ + OH^- \rightarrow Fe^{2+} + NO_2^- + H^+ \quad (5)$$

or:

$$Fe^{2+} - NO^+ + H_2O \rightarrow Fe^{2+} + NO_2^- + 2H^+ \quad (6)$$

In the case of autoreduction of the ferricytochrome *c*–NO complex, ferrocytochrome *c* is the normal product as the ferrocytochrome *c* nitrosyl complex forms so slowly at neutral pH [43,44,49]. However, for myoglobin and haemoglobin the ferrous iron further reacts with the NO in the solution to form the ferrous nitrosyl complex:

$$Fe^{2+} + NO \rightarrow Fe^{2+} - NO \quad (7)$$

An alternative mechanism has been suggested involving binding of a second molecule of NO prior to the hydrolysis reaction [45]. However, at least in the case of cytochrome *c*, this mechanism is not valid

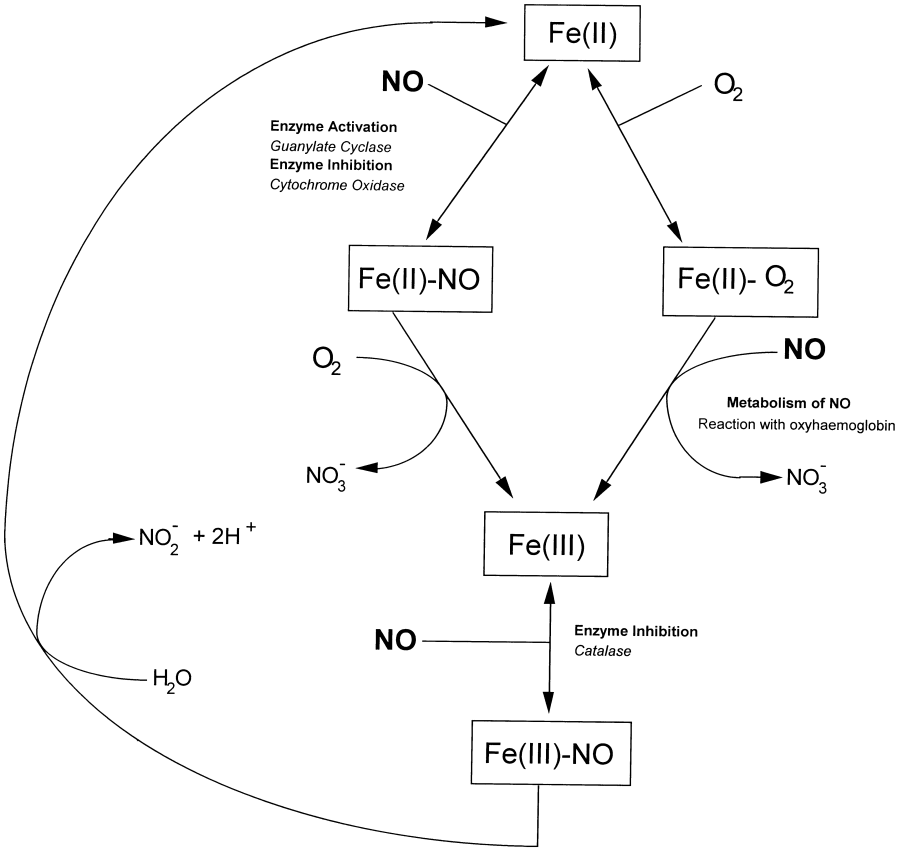

Fig. 4. An overview of haem iron:NO interreactions and their importance. An overview of the interactions of nitric oxide and haem iron described in the text, indicating the most biologically relevant reactions.

because, as described above, ferrocytochrome *c*, not the ferrous nitrosyl complex, is the end-product of the interaction of ferricytochrome *c* with NO [43,44,49].

These reactions are relatively slow [43]. The rate constant for Eq. 5 is 3.2×10^3 M^{-1} s^{-1} for methaemoglobin, 1.5×10^3 M^{-1} s^{-1} for ferricytochrome *c*, and 1.5×10^2 M^{-1} s^{-1} for metmyoglobin. Therefore, although at pH 10 the half-life of a 0.1 μM methaemoglobin–NO solution would be 2 s, at pH 8, it would be 200 s. Eq. 6 appears to occur at a significant rate only in the methaemoglobin–NO case where a rate constant of 1.1×10^{-3} s^{-1} has been measured [43]. Therefore, at pH 7 and below, methaemoglobin–NO still decays with a half-life of approximately 500 s, whilst metmyoglobin–NO and ferricytochrome *c*–NO are significantly more stable.

Although of chemical interest, the biological relevance of these slow reactions is hard to discern. The end-products are non-toxic and the relative slow formation rates and affinities (Table 1) of the ferric nitrosyl complexes will ensure that these pathways are only of minor importance in vivo.

3.3. NO and ferryl iron

Ferryl iron $(Fe^{4+}=O^{2-})^{2+}$ is a strong oxidising agent essential for many enzyme-catalysed reactions (e.g. catalases, peroxidases) and may also play a role in pathophysiological processes [50]. There have been no studies demonstrating that NO binds to this species, but reduction of ferryl by NO has been demonstrated in a number of cases [51,52]. The reaction is slow (minutes) compared to more readily available biological antioxidants (e.g. ascorbate), even in the presence of 1.6 mM NO [52]. Although a much faster reduction is seen when NO is added to ferryl cytochrome oxidase [53], we attribute this to side-reactions following rapid NO binding and metabolism at the copper in the enzyme's haem:copper binuclear

centre. The inhibition of catalase discussed previously also seems readily explainable via the reaction of NO with ferric iron, and not the ferryl intermediate. Therefore as the interaction of NO with ferryl intermediates is slow even at unphysiologically high NO concentrations this reaction appears unlikely to be of biological relevance.

3.4. NO and iron–oxy complexes

NO rapidly reacts with the oxy-complex of haem proteins (e.g. oxygenated haemoglobin or myoglobin). The product is nitrate and the ferric (met) protein [54]. The rate has been determined to be 4×10^7 M^{-1} s^{-1} [55], similar to NO binding to ferrous haem enzymes (Table 1). The iron–oxygen interaction in oxyhaemoglobin has both covalent (Hb^{II}–O_2) and ionic characteristics (Hb^{3+}–$O_2^{\bullet-}$). The reaction mechanism with NO is best understood assuming the ionic character of this complex (i.e. ferric:superoxide). As with the reaction of Hb^{2+}–NO with O_2 (cf. earlier) it has been suggested that a transient haem-bound peroxynitrite intermediate is formed [55], in this case, via the interaction of NO with the bound superoxide.

$$Fe^{3+} - O_2^{\bullet-} + NO \rightarrow Fe^{3+} - ONOO^- \rightarrow Fe^{3+} + NO_3^- \quad (8)$$

The speed of this reaction and the large concentration of oxyhaemoglobin in the blood suggests that this is the major reason for the rapid removal of NO in the vasculature.

3.5. NO reactivity with iron proteins is not always due to direct action at the iron site: a cautionary note

One must be careful not to underestimate the protein moiety when studying the reactions of NO with iron proteins. In particular, sulphydryl groups and free radical intermediates are both likely to be targets for NO reactivity and, unless careful spectroscopy is undertaken, reactions outside the iron centre may be falsely attributed to NO binding to iron. Examples of iron proteins where this occurs include haemoglobin, where the formation of cysteine S–NO complexes has been shown to modulate haemoglobin reactivity with oxygen [56]. Recent data suggest that mitochondrial complex I (NADH dehydrogenase) is reversibly inhibited by NO, not at its myriad iron centres, but instead via R–SNO formation at a sulphydryl site [9]. In the case of the non-haem iron enzyme ribonucleotide reductase NO binds both to −SH residues [57] and to a tyrosine radical essential for enzyme activity [14,57–59] and disentangling inhibitory effects at these sites from possible direct effects on the dinuclear iron centre is therefore not trivial.

4. Reactions of NO-related compounds with iron

4.1. Nitroxyl anion

The putative role of nitroxyl anion in biology is controversial [60–62]. However, it has been suggested that it is the initial product of the enzyme nitric oxide synthase [63] and that it can also be formed when NO reacts with ferrocytochrome *c* [44]. The reactions of NO^- with iron are relatively straightforward. It forms a ferrous nitrosyl complex following addition to ferric haem proteins. The rate constant for the reaction of NO^- with ferric haem proteins is unknown, but product analysis shows that it is competitive with the rapid decomposition rate of NO^- to N_2O [64] or its reaction with oxygen to form peroxynitrite [44,65]. Two mechanisms are possible depending on whether outer sphere electron transfer is involved or not:

$$Fe^{3+} + NO^- \rightarrow Fe^{2+} + NO \rightarrow Fe^{2+} - NO \quad (9)$$

or:

$$Fe^{3+} + NO^- \rightarrow Fe^{3+} - NO^- \rightarrow Fe^{2+} - NO \quad (10)$$

In the presence of a large excess of oxygen, the nitroxyl anion is still able to form a myoglobin nitrosyl complex [44]. Given that the rate constants for oxygen and NO binding to Fe^{2+} are similar, and that the oxy-complex would then react with free NO to form ferric(met) myoglobin, these results suggest that free NO in solution would not be able to form the nitrosyl complex under these conditions; this effectively rules out Eq. 9 and favours Eq. 10, [64]. However, in the case of ferricytochrome *c* [54], Eq. 9 must be favoured as the initial product is ferrocytochrome *c* (at pH 7 the formation of the ferrous nitrosyl complex is so slow that the intermediate ferrocytochrome *c* is clearly seen).

Nitroxyl anion also reacts rapidly with oxy- and deoxy-haemoglobin and myoglobin. In the case of the deoxy species, both Fe^{3+} and Fe^{2+}–NO species can be formed, whereas the oxy form results in stoichiometric conversion to the ferric(met) form. The overall stoichiometry is outlined in Eqs. 11 and 12, but the detailed reaction mechanisms are controversial [54,66]; in the case of deoxyhaemoglobin, NO^- has been suggested to be a catalyst for a complicated set of reactions between nitrite and deoxyhaemoglobin [54].

$$2Fe^{2+} - O_2 + NO^- \rightarrow 2Fe^{3+} + NO_3^- + O_2^{2-} \quad (11)$$

$$2Fe^{2+} + H^+ + NO_2^- \xrightarrow{NO^-} Fe^{3+} + Fe^{2+} - NO + OH^- \quad (12)$$

4.2. Peroxynitrite

It has been suggested [11,67] that peroxynitrite can generate a reactive nitronium ion (NO_2^+) intermediate via binding to ferric iron (e.g. in iron–EDTA). This can have pathological implications as it can proceed to oxidise a variety of phenolic compounds, including tyrosine:

$$Fe^{3+} + ONOO^- \rightarrow Fe^{2+} - O^{\delta-} - NO_2^{\delta+} \quad (13)$$

followed by:

$$Fe^{2+} - O^{\delta-} - NO_2^{\delta+} + \text{phenol} \rightarrow NO_2 - \text{phenol} + H^+ + Fe^{2+} - O^- \quad (14)$$

followed by:

$$Fe^{2+} - O^- + 2H^+ \rightarrow Fe^{3+} + H_2O \quad (15)$$

These reactions require that the phenolic compound can react with the nitronium ion intermediate, and may therefore be more likely to occur in low-molecular-weight iron complexes than in metalloproteins (where there may be restricted access for such molecules). Furthermore many ferric haem proteins can stabilise ferryl intermediates and this provides an alternative pathway for the species generated above to dissociate:

$$Fe^{2+} - O^{\delta-} - NO_2^{\delta+} \rightarrow Fe^{4+}{=}O^{2-} + NO_2 \quad (16)$$

Depending on whether NO_2^- or NO_2 is the leaving group, the overall effect is that peroxynitrite can act as a one-electron or two-electron oxidant for ferric haem proteins. It can thus convert ferric iron to the ferryl intermediates with or without formation of a cation radical [68].

$$Fe^{3+} + ONOO^- + R \rightarrow (Fe^{4+}{=}O^{2-})^{2+} + R^{\bullet+} + NO_2^- \quad (17)$$

$$Fe^{3+} + ONOO^- \rightarrow (Fe^{4+}{=}O^{2-})^{2+} + NO_2 \quad (18)$$

One electron oxidation (Eq. 18) seems favoured in the case of lactoperoxidase and myeloperoxidase, whereas two-electron oxidation (Eq. 17) occurs for horseradish peroxidase [68]. Clearly these reactions have possible pathophysiological significance as ferryl iron and the associated free radical species (e.g. ferryl haemoglobin and ferryl myoglobin) are associated with oxidative stress damage to cells [69]. They also shed some doubt on the proposed ferric peroxynitrite intermediate in the reactions of oxyhaemoglobin with NO and nitrosyl haemoglobin with O_2 (Eqs. 3 and 8); according to the above mechanisms, one would expect this intermediate to be converted into ferryl haemoglobin and nitrite, not methaemoglobin and nitrate.

Peroxynitrite reactions with ferrous haem proteins are less well-characterised. We have recently demonstrated that mitochondrial cytochrome oxidase catalyses the formation of nitric oxide from peroxynitrite [70]. This leads to *reversible* enzyme inhibition by the nitric oxide produced. However, we [70] and others [71] have demonstrated that at high concentrations of peroxynitrite (>100 μM), cytochrome oxidase is *irreversibly* denatured; NO formation may therefore play a role in protecting cytochrome oxidase and other mitochondrial proteins from peroxynitrite-induced damage. The proposed mechanism is:

$$Fe^{2+} + 2H^+ + ONOO^- \rightarrow Fe^{3+} + NO + H_2O_2 \quad (19)$$

Peroxynitrite can oxidise oxyhaemoglobin to methaemoglobin (forward rate 2.5×10^4 M^{-1} s^{-1}) and reduce ferryl haemoglobin to methaemoglobin, although the reaction mechanisms are not well-characterised [72].

High concentrations of peroxynitrite can also

damage iron prosthetic groups in enzymes e.g. iron–sulphur clusters in aconitase [73,74] and haem groups in cytochrome oxidase [70]. These are not necessarily reactions that occur at the iron atom itself and may result from reactions with sulphydryl groups or porphyrins.

5. NO and iron in biological systems: which chemical reactivity is relevant to which biology?

5.1. Non-protein bound iron

Table 1 shows that non-protein bound iron can react with nitric oxide. However, it is unclear whether these reactions occur in vivo, or if they are, how important they are. Relevant studies are hard to do, given the unclear chemical nature of low-molecular-weight iron pools in cells [75,76]. One reaction that definitely does occur in vivo is the formation of iron–sulphur–nitrosyl complexes. Of these, one particular species, the dinitrosylirondithiol complex (DNIC) [77,78] is readily detectable by EPR spectroscopy (with a g-value in the region of 2.03–2.04) and has therefore been much studied (Fig. 3). This species has been detected in a wide variety of conditions from cancerous tissue [79] to the substantia nigra of Parkinson's disease brain tissue [80]. It is associated with excess NO production in a number of systems including macrophages, tumours, spinal cords, endothelial cells, pancreatic islets and hepatocytes (for extensive reviews of the presence and importance of this signal see [17,21,81,82]). A $g = 2.04$ signal is almost invariably observed following prolonged incubation of any biological material with NO, or NO donors. It has been suggested that DNIC is diagnostic for damage to mitochondrial iron–sulphur enzymes [81]. However, quantitative studies of the amount of DNIC formed compared to the amount of iron–sulphur enzyme damage suggest that in many cases, the majority of the iron and sulphur in DNIC does not come primarily from preformed iron–sulphur centres in proteins [83,84]. DNIC can be readily made in vitro by the addition of ferrous iron, cysteine and NO [85]. It therefore seems likely that the low-molecular-weight iron pool is the main, though not exclusive, source of DNIC iron in vivo. It is not clear whether this reaction perturbs iron metabolism detrimentally; although DNIC formation may have long-term consequences in vivo, short-term function (e.g. of mitochondrial electron transfer) seems unaffected by large increases in DNIC concentration [84].

Whilst the biological relevance of the reactions of non-protein bound iron with NO is unclear, the biomedical and spectroscopic implications of this chemistry have been readily exploited (Fig. 3). For example, sodium nitroprusside is a NO (or more precisely NO^+) donor used to lower blood pressure; NO chelators used to quantify NO in vivo frequently contain iron, the best known of which is the iron complex of diethyldithiocarbamate [86]. Recently NO imaging has been made possible by the use of narrow lines of the NO–Fe complex of *N*-(dithiocarboxy)sarcosine [87].

5.2. Haem proteins

The reactions of NO with haem enzymes are of obvious biological significance. The mammalian NO synthase [88] and the bacterial cd_1 nitrite reductase [89] both synthesise NO and both utilise haem co-factors. The best-characterised mammalian (guanylate cyclase [3]) and bacterial (nitric oxide reductase [90]) target enzymes for NO action, both contain haem iron. The similarity of NO with oxygen suggests that any haem enzyme that reacts with oxygen will be a potential target for NO inhibition (Table 1). Thus, whilst 6-coordinate low spin haem proteins (e.g. the mitochondrial cytochromes *b* and *c*) in general react poorly with oxygen (and therefore CO and NO), any high spin 5-coordinate haem enzymes that can bind and/or activate O_2 will also bind NO. Such enzymes include cytochrome oxidase, the cytochrome P-450 superfamily and haemoglobin. Given the potential strength of NO binding to ferrous haem (and the consequent slow off rates (Table 1)), it is difficult to see how proteins with high spin ferrous haem can avoid being inhibited by NO. As an enzyme that expects to be in the presence of NO rather frequently, nitric oxide synthase, has not been able to completely avoid inhibition by NO [91], it is quite likely that all enzymes with high spin ferrous haem intermediates are inhibited by NO to some extent. It is also likely that this inhibition will be competitive with oxygen and CO as these bind to the same redox

state – the biological question to answer is whether the nM levels of NO usually present are below or above the relevant K_i. In the case of cytochrome oxidase, mechanisms seem to exist to make NO an even stronger inhibitor (probably due to the binuclear haem–copper nature of the oxygen reduction site), and it seems very likely that the inhibition here is significant under pathophysiological, and maybe physiological, conditions [4–6].

However, despite these findings, iron protein enzymology does not permanently grind to a halt for hours following the addition of excess nitric oxide. The solution to the problem of over tight binding of NO to ferrous haem enzymes seems to have been solved by biology via a combination of either preventing bond formation and/or increasing the rate of NO dissociation.

5.2.1. Prevention of Fe^{2+}–NO formation

In the blood cell, NO rapidly reacts with oxyhaemoglobin to form methaemoglobin and nitrate. It is possible that other scavenging enzymes/proteins may prevent NO reaching especially sensitive enzymes in other parts of the body. Some haem enzymes have to react with NO as product or substrate (e.g. bacterial nitrite reductase and nitric oxide reductase). In this case, a complex reaction mechanism has been postulated to prevent the formation of a ferrous–nitrosyl bond during the reaction cycle [90].

5.2.2. Increase in the rate of Fe^{2+}–NO dissociation

The problem of the potential for NO to form strong ferrous nitrosyl bonds in proteins has been overcome in two different ways. Firstly the protein can be modified to increase this dissociation rate as appears to be the case in both guanylate cyclase and cytochrome oxidase; here the faster than expected dissociation rates for NO allowing the enzymes to rapidly become de-inhibited (over seconds, not minutes) when the NO stimulus is removed. Alternatively the Fe^{2+}–NO complex can be 'metabolised'. Nitrosyl haemoglobin is stable for days anaerobically. In the presence of oxygen it is converted to nitrate and methaemoglobin (cf. earlier). The latter is then rapidly reduced to deoxyhaemoglobin by methaemoglobin reductase. Although this whole process still takes many minutes, the NO scavenging by oxyhaemoglobin described above means that the Hb–NO level never reaches a large fraction of the total Hb in the blood cell in the first place. Therefore, this rather leisurely pace of recycling of the active protein is sufficient to ensure that the very low Hb–NO dissociation constant (0.03 nM) does not result in all the blood haemoglobin ending in this inhibitory complex. In fact, even following endotoxaemia in the rat (the biological situation where probably NO production is at its maximum), at most, only 2% of the total haemoglobin concentration is trapped as Hb–NO [92].

Peroxidases and catalases generally do not form ferrous iron complexes in their reaction cycle; the weaker binding of NO to the ferric (Table 2) and ferryl states therefore makes these enzymes more resistant to NO inhibition (although catalase appears to be an exception here with strong NO binding due to the electron donating properties of the proximal tyrosinate ligand increasing the electronegativity of the ferric state [8]).

A summary of the interactions of haem iron and nitric oxide is illustrated in Fig. 4.

5.3. Non-haem iron proteins

The significance of the reactions of NO with non-haem iron enzymes is less clear. Part of the problem, when comparing haem with non-haem iron enzymes, is that the lack of clear optical spectroscopic signals in the latter case means that far less work has been performed. However, EPR spectroscopy shows that ferrous iron in these enzymes can form well-characterised NO complexes (for a discussion of the iron spin states that result in EPR-detectable signals see [39]).

Again, enzymes that react with oxygen (e.g. monooxygenases and dioxygenases) have the potential to make nitrosyl complexes [16,93–95]. Relevant inhibition studies have only been carried out in a few cases. In the case of lipoxygenase, it has been determined that the levels of NO required for inhibition are higher than those normally found even under pathophysiological conditions [96]. This is in line with the fact that non-haem iron enzymes in general bind ligands at the iron site less tightly than haem proteins. In the case of NO, the ferrous iron frequently remains high spin ($S=2$) resulting in an EPR signal due to an $S=3/2$ spin system (when

spin coupled to the $S = 1/2$ NO molecule). In haem enzymes, the iron is low spin ferrous, $S = 0$, and the resulting EPR signal for the NO complex is, therefore, $S = 1/2$.

As well as iron enzymes, iron storage proteins can react with NO. Ferritin has a complex series of interactions with NO that include signals from $S = 1/2$ and $S = 3/2$ spin states [97,98]. It is possible that these interactions are responsible for the ability of NO to release iron from ferritin stores [99]. Metallothioneins are small, sulphur-rich metal-binding polypeptides produced in response to a variety of physiological and environmental stresses. In the presence of iron and NO, both apo-metallothionein and Zn-metallothionein form EPR spectra similar to DNIC [100]. Again whether this is due to sulphur residues being removed from the metallothionein, or a genuine interaction of iron, cysteine and NO in an undamaged metallothionein molecule is unclear.

The caveats mentioned earlier about demonstrating that NO is definitely acting at the iron site are especially true for some of the non-haem iron enzymes. NO can directly scavenge free radicals. In the case of lipoxygenase, the products of catalysis are free radicals and in ribonucleotide reductase, a protein-bound tyrosine radical is required for enzyme activity. NO interactions and inhibition may, therefore [14], arise from radical coupling reactions (Eq. 20) rather than direct binding to iron and careful spectroscopy is necessary to distinguish between these possibilities.

$$\text{Tyrosine}^{\bullet} + \text{NO}^{\bullet} \leftrightarrow \text{Tyrosine} - \text{NO} \quad (20)$$

5.4. Iron–sulphur proteins

Following the previous suggestions that NO will react at iron sites free to bind oxygen, most iron–sulphur clusters are unlikely targets for NO inhibition/damage. The electron-transferring iron–sulphur centres in the mitochondrial electron transfer chain are likely to be buried in the protein and, therefore, relatively inaccessible to oxygen or NO. Isolated iron–sulphur clusters, free of protein ligation, are inherently sensitive to oxidation by oxygen. This sensitivity is maintained in some protein-bound clusters. For example, if oxygen is present during the purification of iron–sulphur enzymes from anaerobic bacteria, the clusters are generally irreversibly destroyed [101,102]. As these clusters do not need to bind exogenous ligands for their biological function, it is likely that in aerobic organisms, protective mechanisms to prevent oxygen damage will also prevent NO damage. In this context, it is perhaps not surprising that the best characterised direct inhibition of iron–sulphur enzymes by NO is in enzymes from anaerobic bacteria [103–105]. It seems that insensitivity to NO and O_2 damage has co-evolved in iron–sulphur proteins.

However, whilst pre-formed electron transferring iron–sulphur proteins are, in general, insensitive to NO, it is likely that cluster formation requires low-molecular-weight iron and sulphur compounds that will be sensitive to the presence of NO in the cell. Thus, NO could indirectly inhibit electron transfer reactions by preventing the formation of new iron–sulphur clusters with the result that function dropped off with the turnover of old proteins in the cell. It has been suggested that this is the reason for the observed toxicity of nitrite used in food preservation towards Clostridial species [101].

The above arguments do not hold for those relatively recently discovered iron–sulphur enzymes with non-redox roles [106]. Here it is frequently necessary for low-molecular-weight compounds (e.g. citrate) to interact with the cluster and it is harder conceptually to see how access to NO could be prevented. These are generally 4Fe–4S clusters. However, only three of the iron atoms have cysteine coordination; the substrate reacts at the non-cysteine coordinated iron (Fe_a). Most notable of these are mitochondrial and cytoplasmic aconitase [107].

The nature of the NO interaction with aconitase is controversial, with some groups suggesting that peroxynitrite is the reactive species [73,74], whereas others have suggested that NO itself is sufficient [108–110]. It is clear that peroxynitrite can rapidly and irreversibly inhibit both mitochondrial and cytoplasmic aconitase activity [73,74]. Oxidation of the active $[4Fe–4S]^{2+}$ cluster results in loss of the non-cysteine coordinated Fe_a centre, with subsequent formation of the inactive $[3Fe–4S]^{+}$ cluster [107]. Intriguingly, substrate binding seems to prevent this damage [109], suggesting that peroxynitrite cannot access the iron cluster in the presence of citrate in the active site.

High levels (100 μM) of NO can also inhibit aconitase activity [74]. What is less clear is what occurs at physiological (nM) or pathophysiological (low μM) concentrations of NO. A recent study [109] demonstrated specific NO inactivation of aconitase by attack at the non-cysteine coordinated iron site (with eventual formation of protein-bound iron–sulphur dinitrosyl $g = 2.04$ EPR signals). In contrast to the situation with peroxynitrite, substrate binding did not prevent this inhibition. However, this spectroscopic study required high concentrations of the protein (> 50 μM) and consequently high NO concentrations were employed (100 μM 'authentic' NO or the equivalent from slow release NO donors). Even under these conditions, the $t_{1/2}$ for aconitase inhibition was as high as 20 min. However, intriguingly, it has been shown that NO can be a more effective aconitase inhibitor at lower pH or when the enzyme was actively turning over [110]. In agreement with previous studies [73,74], no inhibition of mitochondrial aconitase was observed at pH 7.5 by NO. However, a slow inhibition ($t_{1/2} = 1$ h) was observed at pH 6.5 for the enzyme at rest. This raises the possibility that during hypoxia or ischaemia, when NO levels can rise in tissue [111] and the pH drops, aconitase may become a more sensitive target for NO-mediated damage. Perhaps of greater significance at more physiological pH values inhibition could be observed, but only if the enzyme was actively turning over; this suggests that a turnover intermediate may be susceptible to NO inhibition. The NO concentration was not directly measured in the solution, but assuming perfect equilibration with the gas phase, would have been in the high nM to low μM range and, therefore, of pathophysiological, but not necessarily physiological, relevance.

It has recently been demonstrated that iron metabolism is regulated by cytoplasmic aconitase. Cytoplasmic aconitase is identical [112–114] to the iron regulatory element protein (IRP). This protein can bind to the iron regulatory element (IRE) sequences in the mRNA for ferritin (iron-storage protein), the transferrin receptor (cellular iron import protein) and erythroid 5-aminolevulinate synthase (an enzyme that catalyses haem biosynthesis) [115]. IRP binding represses the translation of mRNA for ferritin and erythroid 5-aminolevulinate synthase, but protects the mRNA for the transferrin receptor from degradation. When the cells are starved of iron, IRE is activated, binds to the mRNAs described above and the consequent post-transcriptional gene regulation assists the cell in accumulating iron, whilst preventing what little iron is present being diverted into storage or haem biosynthesis.

Intriguingly, the activation of IRP is triggered by events at the iron–sulphur cluster. The 4Fe–4S cluster has aconitase activity, but does not bind to the IRE. The apoprotein has no aconitase activity, but does bind to the IRE. The 3Fe–4S cluster has neither aconitase activity nor does it bind to the IRE [116].

It has recently been suggested that NO may play a role in activating the IRP [117]. What is the evidence for this? It is clear that when high (μM) levels of NO are produced in activated rodent macrophages and cell lines, the $g = 2.04$ DNIC EPR signal can be detected simultaneously with inhibition of cytoplasmic aconitase activity and activation of the IRP [10,118,119]. Addition of 50 μM 'authentic' NO to pure IRP yielded a similar effect i.e. loss of both its aconitase activity and activation of its binding to the IRE [118]. It is not clear whether the cellular effects are due to the direct action of NO on the iron–sulphur cluster, given the relatively high levels of NO required to remove the 4Fe–4S cluster [109] and activate IRE binding in the pure IRP [109]. The situation may be even more problematic in mammalian systems where less NO is generally produced than in the rodent cells and rodent-derived cell lines that have been primarily studied so far. Recent findings have demonstrated that peroxynitrite can activate IRP binding to IRE in vitro [120]. It is therefore possible that in vivo the iron–sulphur cluster oxidation and removal is catalysed by peroxynitrite, rather than NO directly. However, given the finding described above that the enzyme in turnover is more susceptible to NO inhibition [110], it may be that the in vitro studies have not, as yet, been carried out under the correct conditions to measure an effect at lower, more physiological, NO concentrations.

To summarise, when excess NO (μM) is produced there is a slow inhibition of mitochondrial and cytoplasmic aconitase activity. Mitochondrial respiration is, therefore, inhibited and the IRP activated. It is possible, but not yet proven, that this is solely due to NO binding at the Fe_a centre. The sensitive of the iron–sulphur cluster to peroxynitrite oxidation means

that peroxynitrite cannot be ruled out as a mediator of the NO modulation of aconitase activity and IRE binding.

5.5. NO, iron proteins and mitochondrial respiration: what is the target?

There is confusion in the literature about the mechanism by which NO inhibits mitochondrial respiration (see also the review by Brown in this issue [121]). The initial findings suggested that NO, whether added exogenously or via co-culture with cells expressing NO synthase activity, damaged mitochondrial iron–sulphur enzymes and hence inhibited mitochondrial respiration [122–125]. The evidence for this was two-fold: firstly, that the isolated iron–sulphur enzyme activity (especially aconitase) was inhibited; and secondly, that $g = 2.04$ EPR signals from DNIC were formed in the target cells. The latter was assumed to occur from damage to mitochondrial iron–sulphur centres, e.g. aconitase. We, and others, have countered both of these suggestions with the demonstration that the initial target for NO is mitochondrial cytochrome oxidase [5,7,126–128] and that $g = 2.04$ signals alone do not necessarily correlate with decreases in mitochondrial respiration rate and damage to iron–sulphur enzymes [84]. To add to the confusion, there has been a recent report that the mitochondrial bc_1 complex (complex III) is also a target or NO inhibition [129] and that NO can inhibit NADH dehydrogenase (mitochondrial complex I) by reversibly binding to thiols [9]. Inhibition in the latter case is reversed by glutathione, but becomes significant when mitochondrial glutathione levels decline.

As with many studies with NO, there is some truth in all these assertions, depending on the time scale observed and the cellular NO and O_2 concentrations. If NO is added at nanomolar concentrations to aerobic mitochondria or cells it will undoubtedly inhibit mitochondrial cytochrome oxidase first. The clearest evidence is that the inhibition of cellular respiration is rapid (on a seconds time scale), completely reversible and oxygen concentration dependent [126]. All these properties are identical with the interaction of NO with purified cytochrome oxidase [126,130]. Furthermore, optical spectra demonstrate that cytochrome oxidase haem *a* reduction *increases* following NO addition to isolated mitochondria [7] and cells [126] – this is only possible if electron transfer is blocked between haem *a* and oxygen i.e. at the cytochrome oxidase binuclear centre. Inhibition at aconitase or mitochondrial complexes, I, II or III would all result in an *oxidation* of haem *a*.

However, subsequent to the rapid reversible inhibition of cytochrome oxidase, the situation becomes more complex. Long-term damage to mitochondrial respiration may occur via a variety of subsequent pathways. Peroxynitrite levels rise as superoxide levels are increased when the mitochondrial electron transfer chain is inhibited by NO [131]. Peroxynitrite can irreversibly inhibit mitochondrial complexes I [132], II [71,132] III [71,132], cytochrome oxidase [70,71] and aconitase [73,74]. Persistent longer-term (minutes to hours) incubation with NO itself will lead to a gradual inhibition of mitochondrial complex I, when mitochondrial glutathione levels can no longer protect this enzyme [9]. A slow inhibition of aconitase (over minutes to hours) may also occur especially at higher (μM) NO concentrations [108–110]. Over a similar time period, EPR signals will be seen from DNIC at $g = 2.04$ [10,123,125]. Some of these may be due to NO bound to aconitase [109], although it will be difficult to distinguish this fraction from other non-protein-bound low-molecular-weight DNIC [83]. As there are a multitude of irreversible targets for NO and peroxynitrite in mitochondria, disentangling the specific relevant interactions is difficult. Furthermore, peroxynitrite can *stimulate* mitochondrial respiration by increasing the mitochondrial proton leak rate, either directly by damaging lipids [133] or indirectly by opening the mitochondrial permeability transition pore [134]. It is perhaps not surprising that the literature in this area can appear confusing at times!

To summarise, in the presence of nanomolar concentrations of NO, mitochondrial cytochrome oxidase is inhibited reversibly. Due to the low K_i, this effect will predominate whilst NO is present in the solution. Upon the removal of NO, other less reversible effects can manifest themselves. Given that different enzymes exert different degrees of control over the rate of mitochondrial respiration [135,136], in order to determine the relevance of any irreversible damage, it is not sufficient to measure decreases in the isolated enzyme activity alone. Instead, a careful

study varying the substrates for mitochondrial respiration and using specific inhibitors of key steps in both glycolysis, oxidative phosphorylation and ATP utilisation is required to determine the relevant site of inhibition of the cellular respiration rate. It is, of course, possible that inhibition of several pathways may contribute to the overall depression of respiration; the site of the irreversible damage may also vary depending on a number of factors, e.g. cell type, NO/peroxynitrite concentration, length of exposure, mitochondrial glutathione stores. The recent paper by Clementi et al. [9] demonstrating complex I as the site of long-term damage following NO treatment of J774 cells, is a good example of the approach necessary to elucidate which of the many possible targets is relevant.

6. Conclusion

There is little doubt that the interaction of NO with iron proteins is behind many, though by no means all, of the important biological reactions of NO. The range of possible protein targets is due to the varied biological chemistry of NO which can reversibly bind to iron proteins, as well as having the potential to act as an electron donor or an electron acceptor. Many iron proteins have been suggested to have their activity modulated by NO in vivo. With the exception of guanlyate cyclase, the mechanism and physiological relevance of these interactions is still controversial.

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