Nitric Oxide in Biology: Its Role as a Ligand

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1 Introduction

Interest in nitric oxide as a chemical affecting human life has increased dramatically over the last twenty years,[1-3] at first through its damaging effects. This simple molecule was recognised in the air many years ago arising as a component of the exhaust fumes of petrol-driven vehicles and is now treated as generally injurious to health. One glance at its electronic structure as a free radical raises some alarm but it is also a powerful oxidising agent and a powerful reducing agent. Its final oxidation product as nitrate, which is carried in drinking water, is also a known risk especially to the health of the foetus and the very young. Much of the nitrate in drinking water comes in fact from agricultural fertilisers and it can be reduced back to NO•. Nitrate acts as a nutrient for nitro-bacteria. However the recent more dramatic increase in interest has arisen not from pollution but from the finding that nitric oxide is a natural, endogenous, chemical messenger between cells in higher animals including humans. The full role of its action is not known but the simplest is as a relaxant of smooth muscles. In essence nitric oxide is released by endothelial (surface) cells to adjacent smooth or posture muscle cells where it causes their relaxation. These findings have revealed the value of nitrates and other organic nitro-compounds, especially nitroglycerines, long used as medicines for heart patients suffering from angina. In essence the action of the NO• formed on reduction of the nitrate or nitro-compound is the relaxation of the arterial wall allowing a faster through-put of blood to the suffering muscle. As Nobel (of the Nobel Prize) remarked it is remarkable that the material, nitro-glycerine, from the properties of which he became rich and famous as the developer of explosives, should be taken by him to alleviate angina. With this short introduction to the biological interest of NO• I shall now go back over its chemistry and then return to its function in living organisms.

Professor Williams is now Emeritus Fellow at Wadham College and Emeritus Professor, Oxford University. He was born in 1926, and educated at Wallasey Grammar School. He studied chemistry at Merton College, Oxford, graduating in 1948. He took his doctor's degree at Oxford in 1951 working with Professor H. M. N. Irving. With Professor A. Tiselius (Uppsala Sweden) 1951–1952 he developed certain analytical chromatographic methods. He became lecturer and tutor in Chemistry at Wadham College and at the University of Oxford 1955–1965. After a year at Harvard University 1965–1966 he changed direction to teach biochemistry until 1974. He became a Reader in 1972 and Napier Royal Society Research Professor at Oxford 1974–1991 and was elected Fellow of The Royal Society in 1972, and is also a foreign member of the Swedish, Portuguese, Czechoslovakian and Belgian Science Academies. He has given named lecture series in several European and American Universities and plenary international lectures at many Chemistry, Biochemistry and Biology Conferences. He is a medallist of The Biochemical Society (twice), The Royal Society (twice), The Royal Society of Chemistry (twice), The European Biochemical Societies (twice) and the International Union of Biochemistry. He was a founder member of The Oxford Enzyme Group which has devised many new methods for the study of in vitro *and* in vivo *biological systems.*

His major research contributions concern metal ions and their function in biological systems. The emphasis in this work is that all biological systems are derived from a combination of inorganic and organic compounds. There is no life without minerals and during the course of evolution there has been considerable changes in mineral chemistry due to life. The work is summarized in two recent books – The Biological Chemistry of The Elements (1991) and The Natural Selection of The Chemical Elements (February 1996).

2 Physical Properties

Nitric oxide has but a small dipole moment and is gaseous down to −152 °C. It is slightly and almost equally soluble in many solvents. At a vapour pressure of 1 atm it has a partition coefficient between solvents and the gaseous phase in the following order: methanol 0.36, benzene 0.30, pentane 0.25 and water 0.07. Thus it is expected that like O_2 it can diffuse relatively easily from water to organic solvents such as biological membranes and the hydrophobic interior of proteins. However it cannot be generated in high concentrations in water since its solubility is quite low and almost the same as that of dioxygen and carbon monoxide.

3 Chemical Properties

3.1 Oxidation State Diagram for Nitrogen

The oxidation state diagram for nitrogen is given in Figure 1, together with that for some other non-metals. Thermodynamically

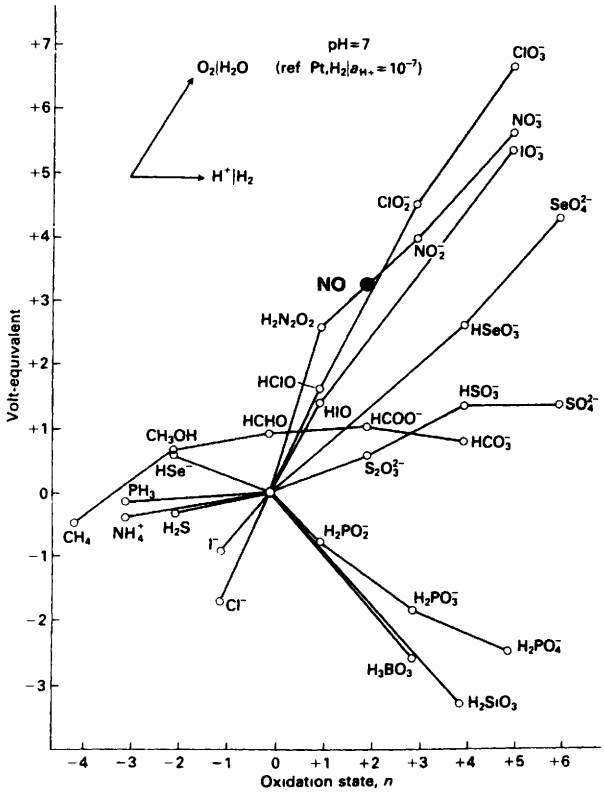

Figure 1 The oxidation state diagram for non-metals at pH = 7.0. Note that dioxygen itself cannot react with N_2 to give NO• but can give NO_3^- at thermodynamic equilibrium. NO• is formed in car engines by less stable oxidation states of O_2 reacting with N_2.

speaking not only the oxidation of NO• by O_2 is readily brought about but so is reduction even to NH_3 by many hydrogen containing molecules. The kinetic control of its reactions is then critical.

3.2 The Chemistry of Nitric Oxide with Oxygen

Every school child knows (or did know) how to make NO• from nitric acid and copper turnings and such a child also knows that NO• does not exist for long in air due to the reaction with O_2 to give NO_2, a brown gas. However this reaction (1) is termolecular:

$$2NO^{\bullet}+O_2 \rightleftarrows 2NO_2 \quad (1)$$

and hence, although the free energy change is strongly in favour of NO_2, the reaction rate is only fast at high concentrations so that in fact NO• persists in the gas or in solution phases at 10^{-6} mol dm^{-3} or below for at least a minute. Immediately this warns us that to understand the action of NO• we must look not just at the thermodynamics of its reactions but at the kinetics of its release and binding. NO_2 with water goes on to give both nitrite and nitrate as mentioned above while reduction of nitrate *via* NO• is used by both man (in fertilisers) and bacteria as a source of ammonia.

NO• reacts directly with superoxide to give peroxynitrite, an important fast reaction in biological systems:

$$H^+ + O_2^{\bullet -} + NO^{\bullet} \rightarrow OH^{\bullet} + NO_2^{\bullet} \quad (2)$$

The reaction may be involved in some biological defence mechanisms where it is said that OH• is deliberately generated but it exposes organisms which produce it to risk since the hydroxyl radical is an extremely potent reagent.

Reduction of NO• by hydrides requires catalysts usually based on metals showing again that NO• itself is not very reactive. The catalysts used by organisms will be described later but almost any metal surface is catalytic.

From the above redox reactions we can deduce that the standing concentration of NO• in early periods of Earth's history was extremely low since O_2 had a pressure of only some 10^{-12} atm. Today the O_2 pressure is 0.2 atm and N_2 gas is even more available. The equilibrium for reaction of O_2 and N_2 is just in favour of nitrate, Figure 1, and though reaction to NO• is unfavourable:

$$O_2 + N_2 \rightleftarrows 2NO^{\bullet}$$

$$\Delta G^{\circ} = 12.8 \text{ kJ mol}^{-1} \quad (3)$$

we must always always remember that NO• itself will disproportionate to higher and lower oxidation states. There is always a low steady state concentration of NO• in the air.

Why is the present atmosphere as it is? Obviously its gases only exist in their present concentrations in the absence of catalysts.

3.3 Reactivity of NO• with Other Non-metals

Despite its considerable potential in both oxidation and reduction reactions NO• does not attack many non-metal centres rapidly in the absence of catalysts.[4] In this it resembles dioxygen. NO^+ is of course a different and very powerful electrophile in concentrated nitric–sulfuric acid but is not easily obtainable from NO• at neutral pH. Just as proteins and nucleic acids are relatively safe from attack by O_2 so they are relatively kinetically stable in the presence of NO•. NO• does react with thiolates and even with sulfite but these reactions are not known to be of great importance in biological system except possibly in defence mechanisms (see below) and possibly in the clearance of NO•.

Before we consider reactions with metal ions, free or in complexed states, we note that we can conclude that the diffusion of NO• at low concentrations (10^{-6}M) through water or organic solvents is not greatly impeded by either physical or chemical barriers due to non-metals, and it is likely to come to rest on metal ions.

3.4 Coordination Chemistry of NO•

NO• is an extremely powerful ligand giving binding constants to metal ions free or in complex form often greatly in excess of those of CO and almost always much higher than those of O_2. The range of NO• compounds known are illustrated in Table 1.[5] One feature is

Table 1 Some nitroso complexes[5]

Complex	Comment
$[Fe(CN)_5(NO)]^{2-}$	Nitroprusside used as medicine
$[Fe(CN)_4(NO)]^{2-}$	Reduced nitroprusside
$[Ru(NH_3)_5(NO)]^{3-}$	
$[Cr(NO)_4]$	Compare $Cr(CO)_6$
$[IrCl(CO)(PPh_3)_2(NO)]$	Ir–N–O bond angle 124°

the variable nature of the bound NO•. Low oxidation states of metals give M·NO^- while high oxidation states give M·NO^+ with increase or reduction of the effective metal oxidation state on binding. The nature of NO• in a complex, which can be studied by its IR spectrum, has a decided effect on the reactivity of the group in different low or high oxidation state complexes. One example is nitroprusside which is a complex formed from $[Fe(CN)_5(H_2O)]^{2-}$, *i.e.* an Fe^{III} complex, and can be written $[Fe^{2+}(CN)^5{-}NO^+]^{2-}$. The bound NO^+ reacts with electrophilic reagents such as OH^- to give a nitrito complex, and with amines to give a variety of products which have been used to diagnose primary and secondary amino-compounds. Thus:

$$RNH_2 \rightarrow ROH + N_2 \quad (4)$$

$$R_2NH \rightarrow R_2N_2O \quad (5)$$

In the presence of excess of amine the nitroprusside gives finally an amino-complex of $[Fe(CN)_5(H_2O)]^{2-}$, the amine replacing H_2O.

Other examples of the binding of NO• in different electronic states are given in Table 1.

3.5 The *trans*-Effect

NO• is such a powerful ligand that it can dominate the coordination sphere of a metal. Thus reduction of $[Fe(CN)_5(NO)]^{2-}$ gives $[Fe(CN)_5(NO)]^{3-}$ which releases CN^- to give five-coordinate $[Fe(CN)_4(NO)]^{2-}$. Here the paramagnetic electron appears to reside somewhat equally on the Fe and the NO•. In any event the structure shows the Fe out of the plane of the four cyanides and the Fe–NO bond distance is very short, 1.5 Å, Figure 2.[6] The domination of NO• as a ligand here shows that it has such a strong *trans*-influence as to

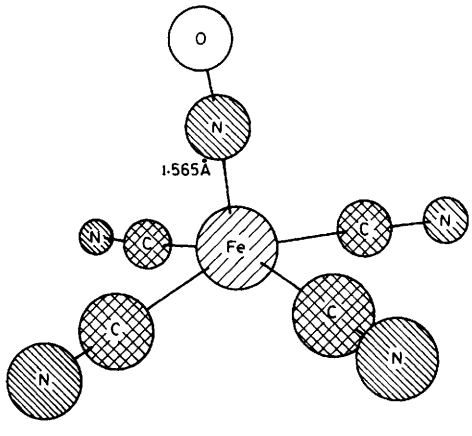

Figure 2 The structure of $[Fe(CN)_4(NO)]^{2-}$. Fe·NO is almost linear. Note bond lengths.

remove the sixth ligand. This situation is common to iron porphyrin complexes, and probably to haem-proteins, but there is a curious parallel in vitamin B_{12} chemistry where strong σ-donor ligands so reduce the *trans*-bonding strength of Co^{III}, also of $3d^6$ configuration, as to give a five-coordinate complex.[7] The over-riding of the 18-electron rule is reminiscent of the fact that this rule often fails in complexes later in transition metal series, *e.g.* $[Ni(CN)_4]^{2-}$, which have a larger number of *n*d electrons.

4 Detection of NO•

The difficulty of detection of NO• especially in living organisms lies in the low range of concentration of this chemical, *i.e.* 10^{-9}–10^{-6}M. One way of detecting NO• uses spin-traps together with EPR measurements.[8] One reagent for following these levels of NO• is the *N*-methyl-D-glucamine dithiocarbamate (mgd) Fe^{2+} complex. The Fe^{2+} forms an NO• complex, $[Fe(mgd)_2(NO)]$, with a simple three-line EPR spectrum. The method can be used on urine samples to estimate the *in vivo* level of NO•. An alternative detection method is to use the NO• scavenger, 1,2-diisopropylidene cyclohexa-3,5-diene, as a spin trap.

NO• can also be detected by electrochemical oxidation using an NO• selective microprobe electrode.[9]

O_2, CO and NO•: Comparative Ligand Strength

Uppermost in a chemist's mind when considering the value of NO in biological organisms is the relative properties of this molecule and those of O_2 and CO. Obviously O_2 is overwhelmingly the most concentrated in ambient conditions since its atmospheric partial pressure is so much greater. However its binding to metal ions is intrinsically weaker for the obvious reason that it is neither a really good donor nor a good acceptor. Thus while some iron complexes bind O_2 quite well, and bind CO and NO• more strongly, others bind NO• or CO but do not seem to bind O_2 at all at ambient pressure. The factors affecting binding, apart from the metal ion itself and its other coordination partners, are the steric demands of the neighbouring groups to the coordinated complexes and the effect of their electronic structure and that of bound O_2, NO or CO. While dioxygen binds to reduced metal ions giving a state close to $M^+O_2^-$, CO remains neutral and NO• may be NO^- but NO^+ is most likely. Clearly O_2 as O_2^- and in some cases NO as NO^- bindings, which take on negative electrostatic charge, are favoured by nearby (distal) positive charge and/or H-bonding capability while NO^+ binding is favoured by nearby negative charge. The conformations preferred by the small molecules themselves are M–O–O: bent, M–C–O: linear, and M–N–O: linear as NO^+ (iso-electronic with CO) or bent NO^- (iso-electronic with O_2). Thus, for example, steric inhibition around the binding metal-ion to the linear forms can favour bent O_2-binding over linear CO or NO^+ binding. This appears to be important in the enhancement of dioxygen binding to haemoglobin relative to that of CO; see below.

We turn next to the thermodynamic binding strength of the three small molecules to specific free-iron porphyrin complexes and then to haem-proteins.

5 Haem Complexes

An understanding of the reactions of haem with NO• is a pre-requisite to the understanding of the function of NO• in organisms since the receptor for NO• is a heam protein. The parallel with receptors and carriers of dioxygen[10] and probably carbon monoxide is very close. The usefulness of the parallel is increased by the fact that the synthase for NO• is also a haem protein and its activity is due to dioxygen and not nitric oxide interaction with the haem. All of the O_2 or NO• systems are poisoned by CO to some degree. Thus we need to understand the relative binding strengths of NO•, CO and O_2 to haem. Fortunately there are structures and much detailed study on the equilibrium binding constants, spectra and magnetism of many model haem compounds of a variety of degrees of sophistication.

If we start our discussion from free iron porphyrins in water or organic solvents then in both the ferrous, Fe^{II}, and ferric, Fe^{III}, states they are high-spin. The Fe^{II} ion and the Fe^{III}, to a lesser degree, are bound out-of-plane. Now on binding alternative donors to the z-axis coordination sites, Figure 3, in either one or two steps, the iron in both oxidation states may switch to the low-spin state and move in-plane. A weak donor such as fluoride is not effective in this regard and we can write a series of binding strengths (assisted by π-bonding) which is: neutral oxygen (H_2O)<charged oxygen (OH^-)<amines (NH_3, imidazole or pyridines)<thiolate<CO (CN^-) NO^+, *i.e.* the strength with which the iron is driven to become low-spin. The exact order is somewhat erratic where combinations

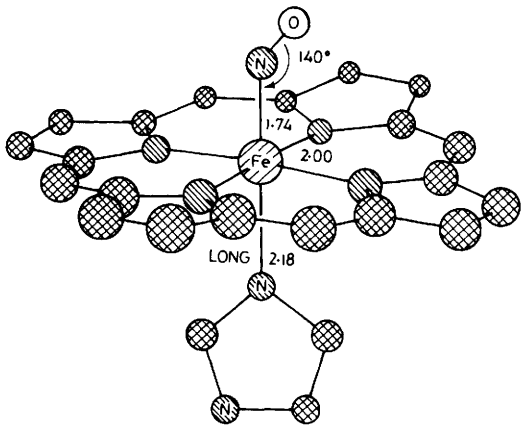

Figure 3 A typical six-coordinate haem complex here showing NO• bound opposite imidazole. Fe·NO is bent. Note bond lengths.

of two donor types are used to drive the metal ions to low-spin but they do so in roughly the expected way. Thus one nitrogen base and one water in the fifth and sixth coordination positions respectively give high-spin complexes but replacement of the water by many other donors (not oxygen donors) drives the system to low-spin.[11] NO is so strong a ligand that in the Fe^{II} condition the iron goes low-spin even in the absence of a sixth ligand.

The stereochemical adjustments on binding of a molecule to the sixth position are considerable. If the Fe^{II} ion moves in-plane then the fifth ligand bound at right angles to the haem moves with it and adjusts its bond length, Figure 4. On the binding of simple ligands such as ammonia, or even O_2, CO or CN^-, the movement is always to give shorter bond lengths, Figure 4. For NO• binding to a haem, replacing say water, but with iron bound initially to a nitrogen-base with iron out-of-plane on the nitrogen-base side, the change could be dramatically different in that the NO• displacement of the water could cause a complete loss of the nitrogen-base and the creation of a low-spin five-coordinate nitroso complex with the iron moved out-of-plane on the opposite side of the porphyrin from the nitrogen base which then moves away from the iron. We shall see the value of these adjustments later, when we consider haem-proteins.[12]

6 Reactions of NO• in Metal Complexes

6.1 Reversible Addition of NO• to Non Metals in Complexes

The classical test for thiols is the nitroprusside reaction:

$$[Fe(CN)_5(NO)]^{2-} + RS^- \rightleftharpoons \left[Fe(CN)_5N\begin{matrix} O \\ \diagup \\ \diagdown \\ SR \end{matrix} \right]^{3-}$$

which is reversible. This somewhat curious addition occurs with other sulfur compounds, for example many thiols, and with the Fe_n/S_n centres known in many electron transfer proteins (ferredoxins):

$$RS^- + NO^\bullet \rightleftarrows RSNO^- \quad (6)$$

$$(RS^-)_4Fe_4S_4 + NO^\bullet \rightleftarrows [(RS^-)_4Fe_4S_4NO] \quad (7)$$

NO also reacts with sulfite to give a variety of intermediates which decompose to give sulfate and reduced NO compounds. The importance of these reactions in living systems is not known at present.

7 Biological Uses of Nitric Oxide

The full involvement of nitric oxide in biological systems is not yet known.[1–3] As stated already we need to have in mind the involvement of nitric oxide in bacterial metabolism as well as its function as a cell-to-cell messenger and detoxifying agent in higher animals,

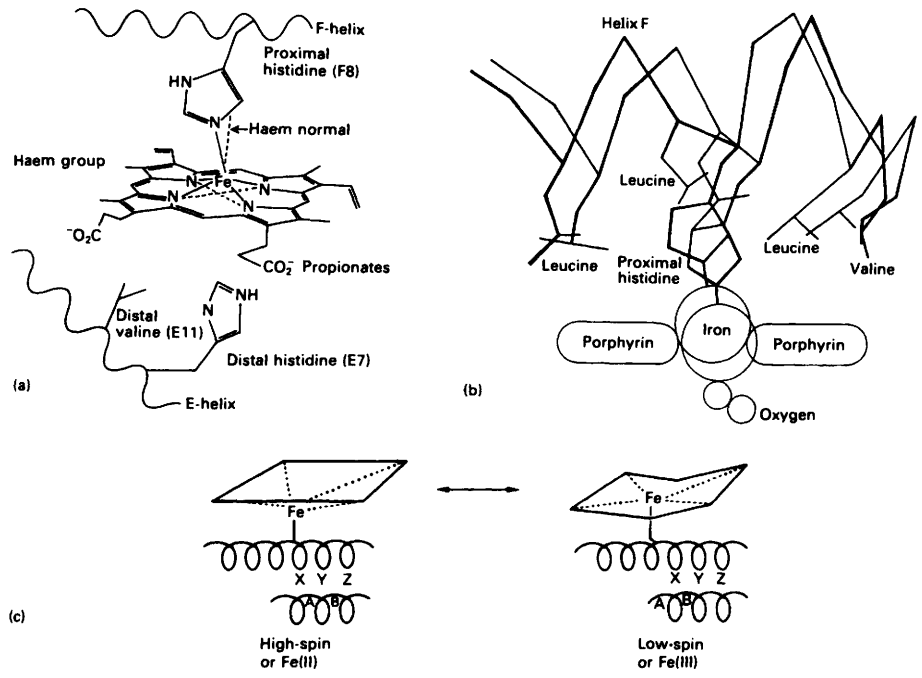

Figure 4 (*a*) The binding site of O_2 to iron of haemoglobin; the Fe^{II} is high-spin and five-coordinate. (*b*) The change in the structure of haemoglobin after O_2 binding. (*c*) The intricate rearrangement of more distant parts of haemoglobin on binding ligands such as O_2, CO or NO•. The figure is taken from ref. (11) but it is based on the work of M. F. Perutz.

Table 2 Functions of Nitric Oxide Synthase[1-3]

1 Relaxation of smooth muscle by endothelial cells: regulation of blood flow (NO• as messenger)
2 Central nervous system responses: memory? (NO• as messenger)
3 Resistance to bacteria and lower organisms (NO• with $O_2^{\bullet-}$ as poison). The enzyme is in macrophages

N.B. Three different but very similar enzymes are involved in the different tissues.

Table 2. As discussed earlier nitric oxide entered the environment as dioxygen pressure rose and it could well be that there are signalling proteins or even buffering proteins against the presence of NO• even in some early prokaryote (bacterial) cells. Thus NO• was probably treated at first by cells as a poison. The uses of NO• today in higher animals relate mainly to signalling between cells. Some of the systems are listed in Table 2. However a second use may well be deployment as a protective poison to kill certain bacteria and yet a third function is in certain activities of the brain. The use of NO• as a poison or a messenger needs a generating system but of course the generator and the receptor have to be in appropriate places and reasonably close proximity, since NO• has not a long life if O_2 or $O_2^{\bullet-}$ is available in the presence of metal ions. Only certain cells are included in a particular biological message system. It is important to realise that a message- or poison-generating system requires *timed activity* so that it is not always switched to the on-situation. The switches will be described below.

8 NO binding to Proteins

Turning to haem proteins, from the functional point of view the two factors of importance within the proteins are the relative affinity for O_2, CO and NO• and the stereochemical adjustments forced upon the protein by binding the diatomic molecules. The affinity is described in Table 3. It shows that many haem sites are likely to be partially saturated with O_2 under normal aerated conditions. To avoid this binding of O_2 the protein must be designed to reduce log$K$ to below 3 *i.e.* binding is made slight in the atmosphere [p−(O_2)=0.2 Torr]. CO is not a competitor except by accident (CO-poisoning) since CO is at very low concentration in most environments but not all. To be

Table 3 Binding constants for NO, CO and O_2 to haem and haemproteins in water[a] (37 °C): units log K/moles l^{-1}[b]

Compound	NO	CO	O_2
Model haem compounds	15.0	9.0	5.0
Haemoglobin	12.0	7.0	5.0
Haemocyanin			4.0
Hemerythrin			4.0

[a] Note the saturation concentration of all three gases in water is around 10^{-3} M. While O_2 may be close to this level CO is far from it ($<10^{-8}$ M) while NO is produced locally and effective at $<10^{-9}$ M in living systems. [b] Changes in the fifth ligand or the protein can change all the values by at least ±2, so that rounded values only are given.

effectively bound NO• need not be present at much greater than nanomolar levels due to the great strength of NO• binding. Thus any local production of NO• almost inevitably leads to binding by any haem which is nearby and open-sided or bound by water in the sixth position.

To understand the detailed functioning of NO• we need next to analyse the nature of haem protein structures.

8.1 The Nature of Helical Proteins[13]

A major feature of haem proteins is that they are usually helical in construction, though one or two exceptions such as cytochrome f are known. The calcium-binding protein, calmodulin, which will interest us too in this article, is also helical. In fact calmodulin is quite like cytochrome c′ (see below) in that both are based on four-helical bundles, Figure 5, the one binding Ca^{2+} and the other haem and NO•. In both cases the protein structure is adjusted by the binding of the small group. This is a common property of many helical proteins which means that they are extremely useful as mechanical signalling devices on receipt of a message from a small molecule. If the proteins are made of subunits each of which can bind Ca^{2+} or NO• (or for that matter O_2) then the binding of the several units can become cooperative. This is seen in haemoglobin ($4O_2$), Figure 4(*c*), in calmodulin ($4Ca^{2+}$) and in some cytochromes c′ (NO). However a helical segment of a protein can also be part of an enzyme so that the enzyme conformation and activity are adjusted by changes in its helices. This is a common

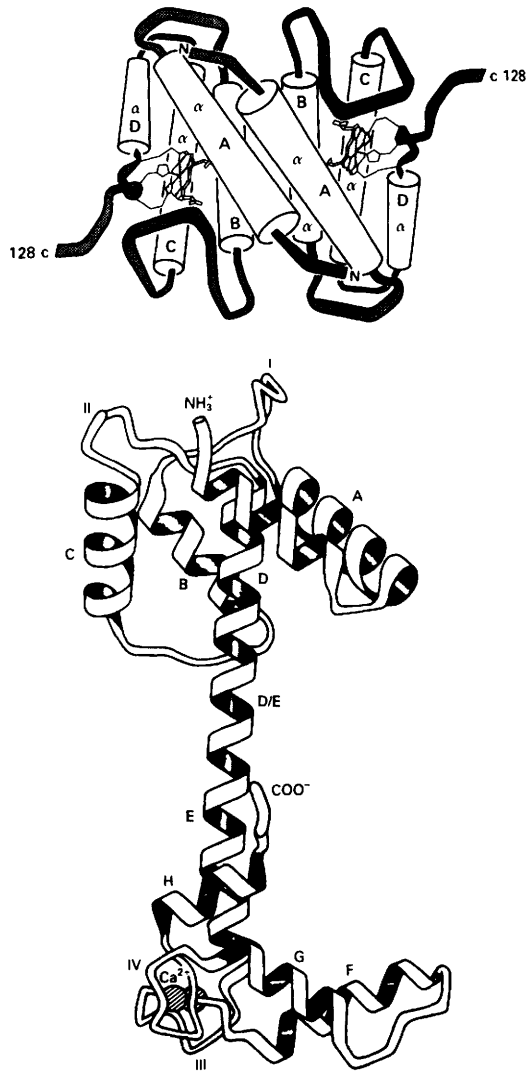

Figure 5 (*a*) The four-helical bundle of cytochrome c′ shown as a side-by-side dimer. (*b*) The four-helical bundle of calmodulin shown as a dumb-bell covalently linked (by a long central helix) dimer.

feature in kinases,[14] which transfer phosphate groups, and is often called a simple helical hinge, but a better description is that of a rising-hinge which requires energy from the binding energy of the substrate molecules for movement, and automatically relaxes when the product molecule leaves.

It takes little imagination to see that a helical signalling protein can be coupled to a helical rising-hinge enzyme so that the signal switches on the enzyme, Figure 6.[14] It has been found that it is a helical calcium-binding protein, calmodulin, which switches on NO•-synthase while it is a helical NO•-binding signal protein, containing haem, which switches on guanyl cyclase so that a message runs through the inorganic components (Scheme 1).

hinge		release	diffuse		hinge	G-protein
Ca^{2+}	→	Fe haem(I)	→ NO• →		Fe haem(II)	→ Phosphate
		(NO• synthesis)			(+NO•)	(Guanyl cyclase)

Scheme 1

In this article we are not concerned with a description of the release of calcium into the cell or with the metabolism of guanyl phosphates but in both these cases too the machinery of protein adjustment to generate gating of ion movement (membrane channels in proteins) and enzyme on/off switches (protein hinges) is based on re-alignment of helices. We turn to the particular cases of the NO•

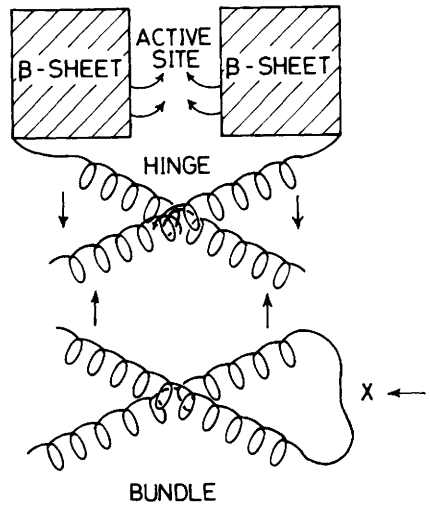

Figure 6 A schematic diagram of the way in which a protein, lower part, which responds to a signal, NO• (or Ca^{2-}), can adjust the activity of an enzyme, upper part, by mechanical changes. The receptor for NO• is shown here as a helical bundle as in haemoglobin which adjust its helices so as to push together the β-sheet binding sites of an enzyme *via* action on a multi-helical hinge for example of a cyclase.

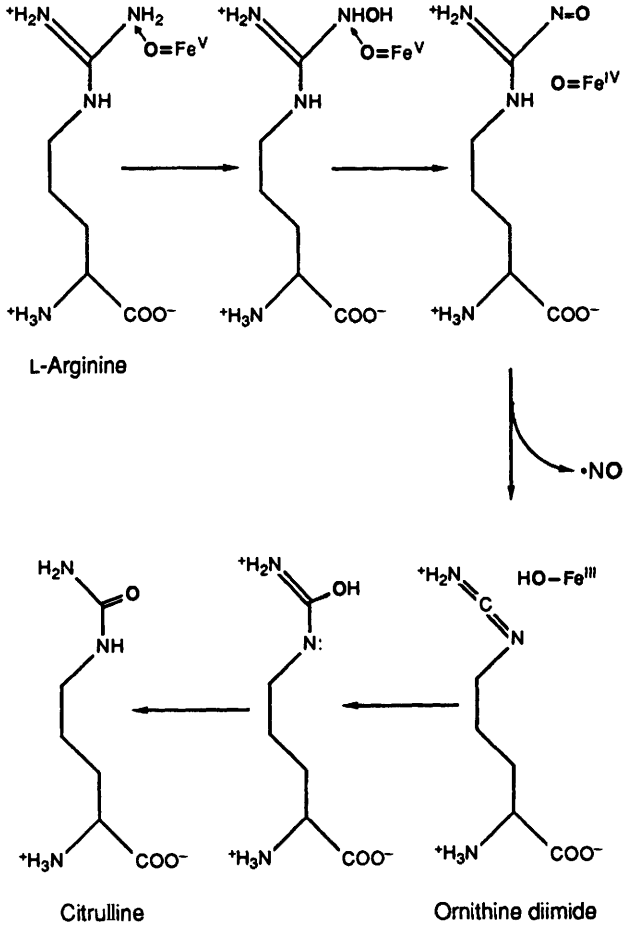

Figure 7 The reaction of arginine to give NO• in the presence of the enzyme NOS. The changes of the iron are also shown.

synthesis by enzymic action, and the NO•-acceptor proteins, and then to the reactions which control them or which they control.

9 Nitric Oxide Synthases (NOS)

This enzyme carries out the reaction, Figure 7, of arginine with dioxygen to give NO• and citrulline. It is a multiple redox step

reaction and the enzyme has a complicated requirement for both dioxygen and a source of reducing equivalents as well as for the substrate arginine This multiple requirement is common to a wide range of dioxygen-using enzymes in biological systems, and there is a close similarity between NOS and the cytochromes P-450 Their peculiarity, using reducing power to assist oxidation, is due to the need to activate dioxygen by reduction Thus O_2 and even $O_2^{\bullet -}$ (superoxide) are not very aggressive reactants while peroxide is a fine attacking agent especially in the presence of metal ions Many biological oxidising agents use the pathway

$$O_2 + \text{metal centre (M)} \rightarrow M(O_2) \xrightarrow{2e} MO + H_2O) \quad (8)$$

Attack on the substrate, here arginine, is then through the higher oxene oxidation state of the metal, *e g* $Fe^{V}O$ or $Fe^{IV}O$ Generally

$$MO + RH \rightarrow ROH + M \quad (9)$$

when the enzyme has cycled back to the initial state A large number of such enzymes including nitric oxide synthase use iron as the metal M and as often as not in haem

We can now write a simple scheme of NO• production but this leaves us with some puzzles [15] The foremost is that this haem-protein is not seriously inhibited by NO• itself despite the fact that NO• is produced adjacent to the iron Yet the site reacts with O_2

10 The NO Receptor

This protein is not so well defined It rests in or on a membrane and probably through a conformation change, Figure 4, it activates formation of cyclic guanidine phosphate utilising a G-protein

$$GTP \rightarrow c\ GMP \quad (10)$$

This G-protein product then generates a signal in the cell which activates many synthetic pathways, as well as causing muscle relaxation in smooth-muscle cells If one looks for a simple NO• receptor protein which has the required properties then the obvious choice is cytochrome c′ of bacteria

Cytochrome c′ has already been described in outline in Figure 5 The four-helical bundle re-arranges somewhat on binding NO• (or CO) to the haem which is an effective mechanical signal The mechanism parallels that in haemoglobin (or calmodulin) but whereas haemoglobin is to some degree protected from CO (or NO•) binding, cytochrome c′ is protected from O_2 binding Using this model we can suppose that any haem receptor for NO is protected from O_2, but not CO The haem-to-protein binding alters the haem itself in such a way as to change the protein conformation, Figure 4(*c*) This conformation change alters the binding of the NO receptor to its neighbour guanine cyclase enzyme and switches it to the productive mode, Figure 6

There is then the problem of NO• release from the receptor Some light may be thrown on this question by the enzyme action of nitrite reductase Before turning to this enzyme note in passing that there is a further way in which NO• could generate a message–release of haem – but we do not know if this has any importance

11 Fast and Smooth Muscles: An Aside

It is fascinating to note that the activation of the fastest muscles for animal movement depends on the fastest inorganic messenger which can bind strongly, namely calcium, while the smooth muscles can be activated or deactivated by a kinetically slower inorganic messenger, namely NO• The two cannot be totally dependent in that all contraction depends on the standing calcium concentration Both activities are mechanically driven in very similar ways

12 Release of Nitroso-haem from Proteins

Normally the release of haem from proteins is very slow In the presence of nitric oxide however dissociation is much more rapid [16] As mentioned above the NO• complex of haem iron weakens any *trans* (to NO•) bonding so that the bond may break easily The haem NO complex in such proteins as myoglobin is then free-floating Model studies on the release of haem NO from myoglobin have been summarised by Mitra and co-workers [16] They showed that the rate of release was rapid The physiological importance of the observations is unknown but it is known that removal of haem from proteins such as cytochrome b_5 and myoglobin causes very significant conformational changes in the protein which could be relayed to a partner enzymes such as guanyl cyclase

Table 4 Metals in nitrogen cycle enzymes

Enzymes	Metal
Nitrate reductase	Mo and Fe(haem) or Fe_n/S_n
Nitrite reductase	Fe(haem) or Cu and Fe_n/S_n
Nitric oxide reductase	Fe(haem)
Nitrous oxide reductase	Cu
Nitrogenase	Mo or V or Fe and Fe_n/S_n

13 Denitrification[17]

Bacteria can utilise NO^-_3 as a primary source of oxidising equivalents in the absence of dioxygen The steps of reaction have to include the intermediates

$$NO_3 \rightarrow NO_2 \rightarrow NO^{\bullet} \rightarrow NH_3 \quad (11)$$

$$\text{or } NO_3 \rightarrow NO_2 \rightarrow NO^{\bullet} \rightarrow N_2O \rightarrow N_2 \quad (12)$$

In both cases NO• is produced from nitrite and in many cases it is known that NO• is a free intermediate The presence of NO• in human exhaled breath may well be due to bacterial breakdown of nitrate

In the above reaction chain there are a variety of enzymes which use different metal ions, Table 4 The structures of the metal sites are of great interest individually but most important for the purposes of this review are those which handle NO• The crystal structures of two nitrite reductases are known but because of its relationship to the NO• receptor we shall concentrate on the haem-containing enzyme[18] leaving the copper enzyme[19] to one side The major interest concerns the way in which NO• leaves the iron since its affinity for haem iron is so high The reaction is the simple one-electron reduction

$$NO_2^- + 2H^+ + e \rightleftarrows NO^{\bullet} + H_2O \quad (13)$$

In the structure in the absence of substrate the haem iron is considered to be in the Fe^{II} state, and open-sided (five-coordinate) Reaction with nitrite leads on to the formation of an Fe^{III}–NO complex and the release of water as in the above equation with the electron coming from Fe^{II} The displacement of NO• is then by a near-neighbour phenolate side chain (of a tyrosine amino acid) The complex which remains is an Fe^{III} phenolate It requires a reductase to release the phenol of the tyrosine and initiate a new cycle of reaction The degree of conformational rearrangement of the enzyme structure during this set of reactions remains to be determined

14 Bacteriocidal Action

Wherever NO• is produced in the same location as superoxide, $O_2^{\bullet -}$, there is an extremely rapid reaction to generate peroxynitrite, ONOO This reaction has been found to occur in certain human white blood cells, macrophages and neutrophils Peroxynitrite is one of the most unstable peroxides and is able to attack a large number of protein side chains such as tyrosine, thiols and imidazole The presence of foreign bacterial cells captured by protective macrophages is known to generate high concentrations of both nitric oxide and superoxide Thus it is highly likely that one method

Table 5 NO-generating drugs

Drug	Formula
Nitroprusside	$Na_2[Fe(CN)_5(NO)]\ 2H_2O$
Glyceryl trinitrate	Propane 1,2,3 triol trinitrate
Amyl (pentyl) nitrate	$(CH_3)_2CHCH_2CH_2NO_2$, fast acting
Sodium nitrite	$NaNO_2$, slow acting
Isosorbide mononitrate[a]	1,4 3,6 Dianhydro D glucitol
Isosorbide dinitrate[a]	1,4 3,6 Dianhydro D glucitol

Fast acting drugs but can be put into tablets (Imdur) so as to be slow acting (over 24 hours)

for the killing of bacteria is a joint action of NO• and $O_2^{\bullet}$ in the form of ONOO

Examination of many primitive organisms such as the horseshoe crab which is at least 500 million years old show that it uses NO• for protection against bacteria Thus maybe NO• has been associated with life for as long as O_2 was available in reasonable quantities some $1-2\times10^9$ years ago

15 Nitric Oxide and the Brain[1,2]

There are a large number of neuro-transmitters in the brain A major transmitter is the amino acid glutamate which was thought to act directly on electrical signals However it now appears that glutamate also acts on a receiving nerve cell by releasing NO• which in turn diffuses back to the donor nerve cell This may be a feed-back regulation of glutamate activity There is the suggestion that this joint action of glutamate and nitric oxide could play a role in potentiating memory

16 Drugs used for NO• Release and Control

Patients with heart disease suffer from angina, nervous pains in the chest, arms or even in the face The pain arises when the arteries or muscles of the heart fail to deliver sufficient oxygen to it to maintain full heart function Major relief arises from dilation of the arteries which is achieved by the release of NO• from orally administered drugs The main drugs used are listed in Table 5 and may be short-acting, say a few hours, or slower acting, say over twelve hours

Unfortunately there are also thought to be diseases where too much NO• is released Here the useful drugs are those which inhibit nitric oxide synthase

In all the applications of these drugs there is the risk of side effects since NO• has so many different roles as a messenger One of increasing interest is the function in the brain

17 The Evolution of Biological Signals[20]

The presence of NO could only arise after the advent of dioxygen $1-2\times10^9$ years ago Essentially, NO and O_2 were initially poisons since life started as a reductive chemical system for CO_2 and N_2 Thus the progression in evolution of oxidative systems had to be

Poison→Protective→Functional Use
Reaction (Signal)

Once a functional use is uncovered for a molecule or ion which has entered the environment then the organism may find a production method It appears that an early use of NO was as a source of nitrogen and energy but before that organisms may well have protected against NO• by evolving a warning signal (cytochrome c′) Once multi-cellular systems arose the signal could be used differently The great advantage of NO• as a transmitter is that it is chemically selective and acts in a very low concentration range The development of NO• in its biological function may be usefully compared with that of Ca^{2+} which is really just a rejected poison by prokaryotes but dominates cell–cell communication

18 Summary

The intention of this article is to reveal the involvement of in particular inorganic elements and one simple compound NO• in living systems and to alert chemists to the ways in which they can help our understanding The inorganic chemist is in danger of letting much of the excitement of this field slip through his fingers In order to pursue the on-going transformation of biological chemistry into a mixture of organic and inorganic chemistry, such as in this case, he or she must study in some depth biological subjects and use the biological literature The revelations concerning NO• outlined here, though yet far from complete, indicate what is unfolding, but nearly all the relevant chemistry is being done outside what are called 'chemistry' departments Much of the biochemistry of NO• is not understood and even less is known of that of CO I hope this article will show that the combination of inorganic and biological chemistries has a very productive future ahead of it NO• is now one of the most important messengers in higher living organisms

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