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Minireview

How does NO activate hemeproteins?

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Abstract

NO was reported to activate guanylate cyclase and, recently, prostaglandin H synthase. NO interaction with the heme component in different hemeproteins is determined by ligand property, electronic configuration of the heme iron and the specific effects contributed by the protein structure. It is found that although NO interaction with the free heme provides some common rules of interaction, the consequences of NO binding to different hemeproteins should be dealt with individually.

Key words: NO; Heme; Hemeprotein; Guanylate cyclase; Prostaglandin H synthase

1. Introduction

Nitric oxide (NO), Science magazine's compound of the year for 1992, has been shown to be involved in many important biological functions. It serves as a neurotransmitter, a vasodilator, a strong inhibitor of platelet aggregation. It is also involved in the bacteria-killing process by macrophages. The issue of whether NO is a 'good' or 'bad' molecule is currently under extensive investigation [1], and guanylate cyclase is generally accepted to be the principal target of NO action [2]. Guanylate cyclase is a hemeprotein and its activity was found to increase by two orders of magnitude in the presence of NO [3]. The concept that NO activates guanylate cyclase via direct interaction with the heme moiety was advanced by two groups [2,4], and has been further extended to other hemeproteins. One interesting example is the proposal that NO activates prostaglandin H synthase [5,6]. This kind of hypothesis requires careful evaluation. Is NO activation of hemeprotein a general phenomenon? This article reviews the basic chemical nature of NO and heme, and considers the interaction between NO and free heme or heme-containing proteins.

2. Electronic configuration and the chemical reactivity of different gas molecules

Table 1 presents several isoelectronic diatomic and triatomic molecules. Among the series of molecules with 10 electrons in the outer shell, CO, but not nitrogen, was found to be a strong field ligand for ferrous heme (Table 2). The molecular orbital theory provides a clear explanation for this difference (Fig. 1). In the nitrogen molecule, the three p orbitals are perfectly aligned for bonding. The bond order is 3, one σ and two π bondings, accommodating all six electrons paired in the fully-occupied bonding orbitals. This electronic configuration makes it stable and very inert. Carbon monoxide also has a bond order of 3, but the difference of electronegativity between the oxygen and carbon atom results in molecular orbitals which are quite different from nitrogen (Fig. 1). The CO orbital with *sp* hybridization has 4 electrons in 2 non-bonding orbitals, with one lone pair producing decent reactivity. Though oxygen, with 12 outer-shell electrons, appears very symmetrical (based on valence bond theory), and is a quite active molecule. Simple analysis by an LCAO (linear combination of atomic orbitals) method reveals that the two additional electrons (compared with nitrogen), are located in two antibonding π orbitals. These two 'high energy' electrons give oxygen its reactive attributes and the potential to be a heme ligand. For similar reasons, we would expect that isoelectronic NO^- (Table 1), which was recently proposed as another physiologically active form of nitric oxide, will be an active molecule [7]. NO, with 11 electrons in the outer shell, is intermediate between oxygen and CO, thus the last electron has to be located in a π^* orbital. The energy difference between this antibonding orbital and the σ nonbonding orbital in NO is not as well separated as those in CO (Fig. 1). This electronic configuration results in some unique properties of NO in terms of its action as a heme ligand.

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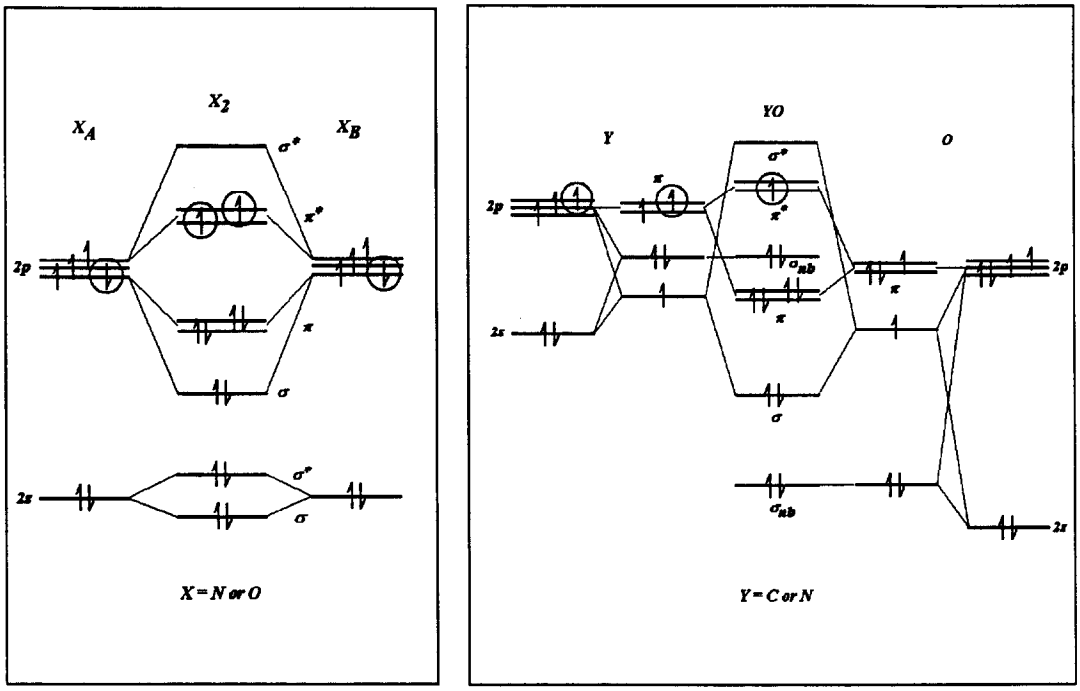

Fig. 1. Energy diagram of diatomic molecules. Panel A shows the molecular orbital energy diagram of homo-diatomic molecules, nitrogen and oxygen. Panel B shows the energy levels of different orbitals in hetero-diatomic molecules, CO and NO. Orbital hybridization and energy promotion of carbon (or nitrogen) atom relative to oxygen atom is shown. The unpaired electron included in the circle indicates electron comes from the atom with higher atomic number. (Adapted from Huheey, [8]).

3. Factors that determine the interaction between free heme and the ligands

A major factor in heme–ligand coordination is the redox state of the heme iron. At physiological pH, ferric heme has a net unitary positive charge (+3 from Fe^{3+} and −2 from the four pyrrole nitrogens) whereas ferrous heme is neutral (+2 from Fe^{2+} and −2 from pyrrole nitrogens). Ferric heme therefore tends to react more strongly with anionic ligands because of electrostatic interaction. The binding strength of the charged ligands follows the so-called spectrochemical series [8], cyanide > nitrite, azide > hydroxide > halides, and can be ranked simply by strength of the Lewis base. In other words, the conjugated base of a strong acid is a weak base, and also a weak ligand. On the other hand, ferrous heme preferentially interacts with neutral gaseous ligands compared to ferric heme, and this is due to the increased orbital interactions. Ferrous heme (d^6) has one additional d-orbital electron compared to ferric iron (d^5). This additional electron doubles the interaction between the iron d_{xz}/d_{yz} orbitals and the antibonding p orbital of either the carbon (in CO) or the nitrogen (in NO) (Fig. 2). Such backbonding appears to play an important role in the interaction between ferrous heme and gaseous ligands. The ligand strength with ferrous heme follows the order: NO > CO > O_2 (Table 2). The fact that CO is a stronger ligand than oxygen can be rationalized by the type of bonding between the ligand and the heme iron. It has been found that the Fe–C–O arrangement is linear with ferrous heme, whereas the Fe–O–O arrangement is bent [9]. It is clear that CO adopts an sp hybridized orbital and O_2 uses an sp^2 hybridized orbital for heme ligation. We thus can expect a stronger bonding and shorter bond length between iron–CO than those between iron–oxygen and this expectation is substantiated by experimental observations (Table 2). Furthermore, the presence of two electrons in the antibonding orbitals of O_2 prohibits the backbonding from the iron as compared to CO or NO. The remaining question is why NO is a stronger ligand than CO?

Table 1
Isoelectronic diatomic and triatomic molecules

Molecule	Outershell electrons
CO, N_2, CN^-, NO^+	10
NO	11
O_2, NO^-	12
N_3^-, CO_2, N_2O	16

4. Unique properties of NO

Heme coordination by NO exhibits multiple aspects. Not only does NO interact strongly with ferrous heme, but its odd number of electrons makes NO polar enough to be a decent ligand for ferric heme. NO can interact with heme using either a sp or a sp^2 hybridized orbital,

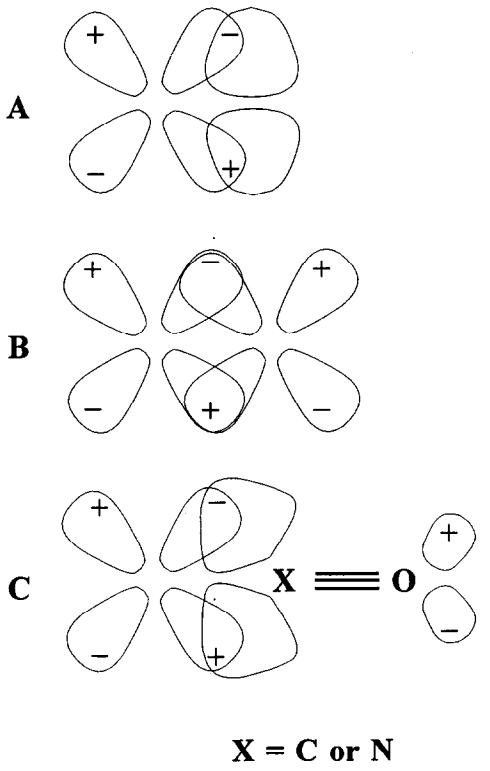

Fig. 2. π bonds between the heme iron *d* orbital(s) and ligand (A) *p* orbital(s), (B) *d* orbital(s), and (C) π anti-bonding orbitals. (Adapted from Huheey, [8]).

thus aligning itself either in a linear or bent arrangement. In fact, complexes containing both linear and bent arrangements of NO ligands are not uncommon [8]. NO can be, as well as a 2-electron donor, a 3-electron donor. This versatility makes it a stronger ligand than CO. The 3rd unpaired electron can also serve as a reducing equivalent to convert the iron to the ferrous state after NO binding to ferric heme. Furthermore, the odd electron of NO makes this molecule paramagnetic. The unpaired electron can interact through the axial ligation with the proximal ligand nucleus, such as histidine nitrogen. This latter property has been extensively exploited in a standard protocol to determine if the proximal heme ligand in a hemeprotein is an imidazole [8]. If the proximal site is unoccupied or not an imidazole, a 3-line hyperfine EPR spectrum is observed as a result of the interaction between the unpaired electron of NO and its nitrogen nucleus (nuclear spin = 1). With a proximal nitrogen ligand, a nine-line hyperfine structure is produced, due to the interaction of the unpaired electron of NO with two nitrogen nuclei [10]. A 3-electron transfer from NO to the heme iron also leads to a repulsive trans effect on any proximal heme ligand. In contrast to CO or O_2 whose binding strength increase with a basic ligand present at the 5th position, NO binds more tightly to heme when the 5th position is empty [4]. This special characteristic has been proposed as a mechanism of guanylate cyclase activation by NO [4], which will be discussed further below.

5. Effects of the protein environment on ligand binding by heme

The influences of the protein component on heme–ligand interactions can be classified into three general categories.

a. *Steric effects:* for a given ligand, free heme model compounds usually show faster association and dissociation rates than found in hemeproteins (Table 2). Association of a ligand to five-coordinated heme is usually faster than to six-coordinated heme because breakage of

Table 2
Equilibrium constants and rate constants for NO, CO, O_2 binding to model heme compounds and hemeproteins[a]

Heme	Ligand	K (M^{-1})	k_f ($M^{-1} \cdot s^{-1}$)	k_d (s^{-1})	Reference
Fe(II)PP(1-MeIm)	NO	5.8×10^{11}	1.8×10^{8}	2.9×10^{-4}	17
	CO	7.8×10^{8}	1.8×10^{6}	2.3×10^{-3}	17
Fe(II)TPPS	NO		1.8×10^{9}	~0	18
Fe(III)TPPS	NO	1.1×10^{3}	7.2×10^{5}	6.8×10^{2}	18
Mb(II), whale	NO	3.4×10^{11}	1.7×10^{7}	1.2×10^{-4}	17, 18
	CO	4.5×10^{7}	5.0×10^{5}	2.1×10^{-2}	17, 19
		2.9×10^{7}	5.5×10^{5}	1.9×10^{-2}	20
	O_2	1.9×10^{6}	1.9×10^{7}	10	9
		1.1×10^{6}	1.6×10^{7}	14	20
Mb(II), L29F	CO	3.7×10^{7}	2.2×10^{5}	6.0×10^{-3}	20
	O_2	1.5×10^{7}	2.1×10^{7}	1.4	20
Mb(III), whale	NO	1.4×10^{4}	1.9×10^{5}	13.6	18
			5.3×10^{4}	14	19
Mb(III), elephant	NO		2.2×10^{7}	40	19
Cyt c(II), horse	NO	2.89×10^{5}	8.3	2.87×10^{-5}	18
Cyt c(III), horse	NO	1.6×10^{4}	7.2×10^{2}	4.4×10^{-2}	18
Cat(III), bovine	NO	1.8×10^{5}	3.0×10^{7}	1.7×10^{2}	18

[a]Conditions for the measurements were pH 7.0 and 19–21°C, except pH 9.0 was used for the model compound Fe(II)PP(1-MeIm), and pH 6.5 was used for the experiments performed in [17].

a preexisting bond is required to form the new ligation (compare cytochrome *c* with myoglobin in Table 2). As a consequence of steric hindrance at the distal side of the heme pocket, CO binds to myoglobin heme with a tilted rather than in the linear geometry found in heme model compounds. Steric hindrance which preventing dissociation of bound ligand is well illustrated by the L29F mutant of myoglobin, in which Leu-29 was replaced by the more bulky phenylalanine and resulted in a 10-fold decrease in the dissociation rate constant (Table 2).

b. *Solubility effects:* a hydrophilic heme pocket favors access of ionic ligands to the heme iron, whereas a heme pocket with mainly nonpolar amino acids would facilitate binding of neutral ligands.

c. *Effects of specific amino acids:* a good example is the hydrogen bonding between the distal heme ligand of myoglobin, histidine (E7), which has an adjacent water molecule and has a critical influence on the ligand dissociation rate.

Examples of a combination of several effects are also suggested by the data in Table 2. The rapid association of NO to ferric catalase is probably due to both steric and solubility effects. In elephant myoglobin, the distal histidine (E7) is replaced by a glutamine thereby disrupting the hydrogen-bonding with the specific water molecule. Removal of this steric effect substantially enhances the association of the sixth ligand. However, the unusually large association rate in this myoglobin (Table 2) is likely not to be the consequence of hydrogen bonding disruption alone.

6. Effect of NO on specific hemeproteins

That NO inhibits the oxygen-carrier function of myoglobin (or hemoglobin) is a classical example of specific interaction of NO with hemeprotein. The most recent interesting example of a hemeprotein affected by NO is probably guanylate cyclase, an enzyme that catalyses the conversion of GTP to cyclic GMP and pyrophosphate. This hemeprotein has been purified to homogeneity, and its optical absorption spectrum is indicative of a 5-coordinated ferrous heme [11]. The catalytic activity of guanylate cyclase is increased by 10- to 100-fold in the presence of NO or NO-releasing compounds [3]. Based on the findings that free protoporphyrin IX gave the same activation effect as NO–heme, and that free heme inhibits (not activates) the enzyme [12], Ignarro proposed that a heme structure change is caused by NO binding. He suggested that heme, once bound by NO, elicits an out-of-plane movement of the center iron, to produce a heme core size similar to that of a free porphyrin [2]. This is exactly the opposite of the sequence which occurs in the oxygen–myoglobin interaction.

Sharma and Traylor [13] combined their study of model heme systems with Ignarro's proposal to derive an interesting interpretation for the enhancement of enzyme activity by NO. Based on the finding that NO binds preferentially to the heme lacking a proximal ligand, Sharma and Traylor proposed that when NO binds heme, there is a tendency to expel the basic ligand on the proximal side of the heme. This mechanism liberates a free base to catalyze the hydrolysis of the phosphate diester bond of GTP. An experiment using NO to release the 1-methylimidazole from a 5-coordinated model heme did in fact promote hydrolysis of *p*-nitrophenolate in the aqueous phase [13]. In contrast to Ignarro's proposal using porphyrin as the active component, Sharma and Trayler suggested that the basic ligand serves as the critical species to cause enzyme activation. However, several important aspects of the proposed mechanism remain to be examined. It is not known whether heme is present in the ferric form in the native enzyme, because reductants such as dithiothreitol are used during enzyme purification [3,11]. Further, NO does bind to ferrous heme with high strength (Table 2) and also promotes dissociation of heme from proteins; both events would tend to severely limit the number of turnovers and thus make for a very inefficient biocatalyst.

The second interesting current example of NO interactions with heme proteins is prostaglandin H synthase (PGHS). Two groups have proposed that NO interacts with the PGHS heme to enhance the cyclooxygenase activity [5,6]. Needleman's group reported that NO or NO-releasing compounds stimulated the formation of prostaglandin by the two isozymes of PGHS by 2- to 8-fold [5]. McCann and his colleagues proposed that NO diffuses into adjacent neurons to activate PGHS and causes the release of PGE_2[6], but no experimental evidence was provided. These proposals of a stimulatory effect of NO on PGHS are contradicted by a report of Kanner et al. [14] who found reversible inhibition of PGHS activity by NO. PGHS has a heme coordination very similar to that in myoglobin, with a histidine as the proximal axial heme ligand [15]. Studies with purified PGHS have indicated that ferric PGHS binds NO very weakly ($K_d \sim$ mM), whereas ferrous PGHS interacts with NO very strongly and leads to release of NO–heme free from the proximal ligand, histidine (Tsai et al., unpublished results). No activation of PGHS activity was observed at NO concentrations ranging from micromolar to mM. It is worth noting that PGHS is a peroxidase and that there is no evidence that ferrous heme is involved in PGHS catalysis. Moreover, it seems quite unlikely that the freely diffusible NO could accumulate to mM levels in any intracellular compartment. Thus, it appears highly improbable that a direct action of NO on PGHS heme could be responsible for the reported increase in prostaglandin synthesis [5]. The possibility of other effects of NO on the PGHS protein remain to be examined.

A recent report of the discovery of reversible heme-

binding proteins in the salivary gland from a blood-sucking insect is very fascinating [16]. Apparently these ferric hemeproteins serve as reservoir for NO in the salivary gland to prevent the blood coagulation to facilitate their blood-feeding routine. It is predicted that these hemeproteins should exhibit NO binding stronger than that of PGHS or metmyoglobin.

In summary, there are general rules to predict the reactivity between NO and free heme. Once the heme is included inside the protein, not only the axial ligands provided by the protein play deciding roles in the NO–heme interaction, many additional factors unique for each protein have to be considered as well. The coordination of NO to the heme moiety of various hemeproteins depends on the redox state of the iron, the presence and the nature of the axial ligand(s), the heme pocket geometry and the specific amino acid residues interacting directly or indirectly with NO. The effect of NO varies from hemeprotein to hemeprotein and can be associated with important changes in catalytic function.

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