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Binding of O₂ and NO to Heme in *Heme-Nitric Oxide/Oxygen-Binding (H-NOX) Proteins. A Theoretical Study*

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Abstract

The binding of O₂ and NO to heme in *Heme-Nitric Oxide/Oxygen-binding (H-NOX)* proteins has been investigated with DFT as well as dispersion-corrected DFT methods. The local protein environment was accounted for by including the six nearest surrounding residues in the studied systems. Attention was also paid to the effects of the protein environment, particularly the distal Tyr140, on the proximal iron-histidine (Fe-His) binding. The Heme-AB (AB = O₂, NO) and Fe-His binding energies in iron porphyrin FeP(His)(AB), myoglobin Mb(AB), H-NOX(AB), and Tyr140 Phe mutated H-NOX: Y140F(AB) were determined for comparison. The calculated stabilization of bound O₂ is even higher in H-NOX than in a myoglobin (Mb), consistent with the observation that the H-NOX domain of *T. tengcongensis* has a very high affinity for its oxygen molecule. Among the two different X-ray crystal structures for the *Tt* H-NOX protein, the calculated results for both AB = O₂ and NO appear to support the crystal structure with the PDB code 1XBN, where the Trp9 and Asn74 residues do not form a hydrogen bonding network with Tyr140. A hydrogen bond interaction from the polar residue does not have obvious effects on the Fe-His binding strength, but a dispersion contribution to E_{bind}(Fe-His) may be significant, depending on the crystal structure used. We speculate that the Fe-His binding strength in the *deoxy* form of a native protein could be an important factor in determining whether the bond of His to Fe is broken or maintained upon binding of NO to Fe.

Keywords

Binding energies; oxygen; nitric oxide; heme; H-NOX proteins; DFT calculations; Dispersion correction

1. Introduction

Hemoproteins are one of the most important classes of biomolecules.¹ One important function of hemoproteins is to sense gaseous diatomic molecules.²⁻⁴ Soluble guanylate cyclase (sGC) is a nitric oxide- (NO-) sensing hemoprotein, catalyzing the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) and thereby playing an important role in cGMP-mediated signaling pathways.⁵ A most remarkable feature of sGC is that it selectively binds NO (and CO) but not O₂, in contrast to myoglobin (Mb) and hemoglobin (Hb), the O₂ storage and transport hemoproteins. Table 1 reports

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Supporting Information

Comparison between the calculated results for FeP(L)(AB) and FePPIX(L)(AB) (Table S1). Calculated binding energies of AB to porphyrin in terms of electronic energy, enthalpy, and Gibbs free energy (Table S2). BP and B3LYP calculated FeP(Im)-AB binding energies from the literature and in this work (AB = CO, O₂, NO) (Table S5). Comparison of the calculated binding energies with DFT and two DFT + E_{disp} methods (-D1, -D3) (Tables S7 – S9). Optimized structures of the H-NOX(O₂), Mb(O₂), and Mb(NO) model systems (Figure S1). This information is available free of charge via the Internet at <http://pubs.acs.org>.

experimental dissociation rate constants for O₂ and NO from different proteins. This ligand selectivity is very important for NO-specific signaling as sGC functions. Since the heme in sGC is identical to that in Mb/Hb, the reason why sGC achieves this “reverse” discrimination among the diatomic ligands, is elusive. So far, there has been no structure determination for sGC.

Recently, a number of bacterial proteins (named H-NOX for *H*eme-*N*itric oxide/*O*Xygen-binding) with sequence homology to sGC have been identified and characterized.^{6–12} Some of these bacterial proteins from eukaryotes and facultative aerobic prokaryotes also exclude O₂ while binding NO selectively, the same characteristic as sGC. These proteins may be called NO-selective H-NOXs. On the other hand, certain H-NOX proteins found in obligate anaerobic bacteria (prokaryotes) reversibly bind O₂ as well as NO and CO (similar to Mb/Hb). The latter proteins may be called O₂/NO binding H-NOXs. Marletta and co-workers⁸ have recently reported the crystal structure of an O₂/NO binding H-NOX from the anaerobe *T. tengcongensis* (*Tt* H-NOX). A similar protein with structure determination was reported by Raman and co-workers.⁷ One of the significant features of the O₂-bound structure of *Tt* H-NOX is the presence of a distal pocket hydrogen-bonding network, which includes Tyr140, Asn74, and Trp9. But these residues are absent in all of the domains of the studied NO-selective H-NOX. Since Tyr140 is involved in a short (2.74 Å) hydrogen bond to the bound O₂, this residue was suggested to be the key molecular factor for ligand discrimination in the H-NOX family.¹⁰

There is another interesting finding on the coordination behavior of the H-NOX family: NO-selective H-NOXs (e.g. VCA0720⁴ and *L1* H-NOX¹²) bind NO in a 5-coordinate Fe^{II}-NO complex but their CO complexes are 6-coordinate; however, the O₂/NO binding H-NOXs (e.g. *Tt* Tar4H⁴) form 6-coordinate Fe^{II}-NO complexes. Figure 1 illustrates the coordination behaviors for a NO-selective H-NOX and an O₂/NO binding H-NOX.

Therefore, the H-NOX proteins are divided into two groups: one group is similar to soluble guanylate cyclase (sGC), forming 5-coordinate NO complexes and rigorously excluding O₂ as a ligand. This is in contrast to Mb and Hb. The other group is similar to the globins, forming a stable oxygen complex and a 6-coordinate NO complex. The behavior of H-NOX is rather special. After a great deal of experimental work,^{4–12} the selection mechanism for the discrimination between NO and O₂ by H-NOX appears to be clear; that is, a tyrosine in the distal heme pocket of the H-NOX heme fold is necessary for stabilization of a bound O₂. However, the coordination behavior (Figure 1) mentioned above for the H-NOX family is not understood yet.

Theoretical studies of H-NOX are very rare. Tangen et al.¹³ performed DFT calculations on two simple models of heme with one distal residue, namely Heme(NO) Tyrosine and Heme(NO) Histidine. For these simple models, the Heme–NO binding energy (E_{bind}) was calculated to increase by 3 – 4 kcal/mol (0.13 – 0.17 eV) due to the presence of histidine; this increase in E_{bind} is significant. According to experiments on nitrosyl porphyrin and Mb (the latter contains a distal histidine), the whole protein environment in Mb has little effect on the Heme–NO binding strength. Considering an inhibitory effect due to the presence of a water molecule residing in the active site of deoxyMb, the stabilization energy of 3 – 4 kcal/mol calculated in ref. 13 may still be too large (see Section 3.2). Hence, calculations on the simple model systems mentioned above may not provide results that are representative of the full system.

Using density functional theory (DFT) methods, we have here investigated binding of O₂ and NO to the heme in the H-NOX proteins; a number of nearest surrounding residues were included in the calculated systems. We were concerned mainly with the effects of the protein

environment on the binding energies of O₂/NO to the heme in the *Tt*H-NOX proteins, for which X-ray crystal structures are available. The interaction of O₂/NO with iron porphyrins FeP and FeP(Im) (P = porphine, Im = imidazole) has been studied in detail in the literature; several recent theoretical studies^{14–23} have provided insight into the geometries, electronic structures, and binding energies for the Heme(O₂/NO) model complexes. For comparison, the Mb(O₂) and Mb(NO) systems were included in the calculations. The binding of O₂ to Mb has been studied extensively in the literature (see ref. 24 for a review about this topic), and considerable experimental information is available for nitrosyl Mb. The main aim of our work is two-fold:

- i. To examine the specific role of the distal tyrosine residue (Tyr140) in the O₂/NO binding to the heme in H-NOX, thereby shedding further light on this ligand discrimination by H-NOX.
- ii. To investigate the effects of the protein environment, particularly Tyr140, on the axial, proximal Fe–His binding. In sGC, the formation of a 5-coordinate NO complex and the breaking of the Fe–His bond are thought to play a central role in activation of this enzyme.²⁵

Proteins are challenging systems for quantum chemical studies because an accurate description of weak intermolecular interactions is necessary. Substantial dispersion contributions can be expected whenever nonpolar, especially aromatic, residues are present. Although DFT has proven to be efficient in calculations on Heme-AB complexes (AB = O₂, CO, NO), it is argued^{26–29} that the DFT methods in common use today do not properly describe the long-range dispersion interactions. In order to correct for this deficiency, one practical method is to add a (semi-) empirical correction of the form C_6R^{-6} to a density functional scheme to yield a DFT + E_{disp} model (it is denoted as DFT-D by some other authors), in which the dispersion energy is calculated separately from the DFT calculation and simply added to the DFT energy, namely $E_{\text{total}} = E_{\text{DFT}} + E_{\text{disp}}$ (see next Section). There have been a large number of DFT + E_{disp} studies on the effect of dispersion on small- to medium-size complexes and satisfactory results have been obtained (e.g. refs. 29–36). This correction method was claimed to be an accurate and efficient scheme for calculating a wide range of intermolecular interactions in which dispersion contributions are important.³⁷ Recently, the DFT + E_{disp} method has been applied by Siegbahn et al.²³ to calculate the FeP(Im)–AB binding energies (see Section 3.1). For the above reasons, both DFT and dispersion-corrected DFT methods were used here; the latter are expected to yield improved quantitative results.

2. Computational Details

2.1. Models

For H-NOX, only the crystal structures of the O₂-binding H-NOX domain from the anaerobe *T. tengcongensis* (*Tt*H-NOX) are available. They were determined by two research groups,^{7,8} but show some differences. Figures 2a and b illustrate the distal heme pockets of H-NOX determined by the two different research groups. The six (6) closest distal residues surrounding the bound O₂, namely isoleucine 5 (Ile5), tryptophan 9 (Trp9), Tyrosine 140 (Tyr140), asparagine 74 (Asn74), leucine 144 (Leu144), and phenylalanine 78 (Phe78), have atoms falling within a ca. 8.0-Å radius of the Fe atom. It is seen that the side chain of Tyr140 forms a hydrogen bond with the bound O₂ ($R_{\text{O}_2 \cdots \text{H}} \approx 1.6 - 1.8 \text{ Å}$). In the structure determined by Marletta and co-workers⁸ (Figure 2a, PDB code 1U4H), the orientation of Tyr140 is fixed by a hydrogen bonding network involving Trp9 and Asn74, which may contribute to a high affinity of the protein for oxygen. In the structure reported by Raman and co-workers⁷ (Figure 2b, PDB code 1XBN), the indole -NH group of Trp9 and the ammonium group -NH₂ of Asn74 swing out away from the bound O₂. Therefore, the

distal environment in the structure with PDB code 1U4H is different from that with PDB code 1XBN.

No structural data are available for an NO complexed to any of the H-NOX proteins. To model H-NOX(NO), the crystal structure of H-NOX(O₂) was used, where O₂ is just replaced with NO.

For comparison, the different crystal structures were considered in our investigations of the O₂/NO binding to H-NOX, and Figures 2a and b are the model systems we used. In our actual calculations, the systems were simplified somewhat. For example, iron protoporphyrin IX (FePPIX) of the heme group was modeled as a porphine (P) without substituents, as shown in details in Figure 3. The proximal His102 was modeled as a 4-ethylimidazole (4-EtIm). Such simplifications have been justified in previous studies.^{38,39} In Supporting Information (Table S1), a comparison is given between the calculated results for FeP(L)(AB) and FePPIX(L)(AB). The calculated FeP(L)–AB binding energy differs from the FePPIX(L)–AB value by at most 0.03 eV; the optimized structural parameters of FeP(L)(AB) are also very similar to those of FePPIX(L)(AB). Considering the fact that the residues around the heme are anchored to the polypeptide in the protein matrix, constraints are imposed on the residues in the geometry optimizations; i.e. the terminal amino nitrogen atoms are fixed according to the crystal structure.

To examine the effect of electrostatic interactions on the Fe–O₂/NO and Fe–His binding, the distal Tyr140 was replaced by a Phe in H-NOX, yielding an Y140F mutant.

As mentioned in the Introduction, Mb(O₂) and Mb(NO) were also included in our investigations for comparison. The model systems used are illustrated in Figures 2c and d; they are based on crystal structures of Mb(O₂) (PDB code 1A6M)⁴⁰ and Mb(NO) (PDB code 1HJT),⁴¹ respectively. Here the given closest residues surrounding the bound O₂/NO are isoleucine 107 (Ile107), valine 68 (Val68), leucine 29 (Leu29), histidine 64 (His64), and phenylalanine 43 (Phe43). The systems/proteins used in this work are as follows.

Por: pure porphyrin, i.e. FeP(4-EtIm): the model system of the heme.

H-NOX^{1U4H}: H-NOX based on the crystal structure with the PDB code 1U4H.

H-NOX^{1XBN}: H-NOX based on the crystal structure with the PDB code 1XBN.

Y140F: (Tyr140 → Phe) mutated H-NOX.

Mb: myoglobin.

2.2. DFT and Dispersion-Corrected DFT methods

The calculations were carried out using the Amsterdam Density Functional (ADF) program package – ADF2012.01.^{42,43} Three density functionals, namely BP (Becke's 1988 gradient correction for exchange⁴⁴ plus Perdew's 1986 gradient correction for correlation⁴⁵), revPBE (revised Perdew-Burke-Ernzerhof functional proposed in 1998 by Zhang and Yang⁴⁶), and B3LYP were employed. BP has been shown to give an excellent description of molecular structure in previous studies^{18,19} as well as in our recent calculations.³⁹ revPBE provides an excellent binding energy for O₂ to Heme.⁴⁷ B3LYP is the most popular hybrid density functional due to its overall high accuracy for main-group systems⁴⁸ and good performance for many bioinorganic systems.^{49,50} This functional gives the correct ground state for deoxyHeme.⁵¹ The STO (Slater-type orbital) basis set used is the standard ADF-TZP, which is triple- plus one polarization function.

As mentioned above, the current DFT functionals fail to describe dispersion properly. In order to correct for this deficiency, one practical method is to add a (semi-)empirical

correction of the form C_6R^{-6} & C_8R^{-8} to a density functional scheme to yield a DFT + E_{disp} model, in which the dispersion energy is calculated separately from the DFT calculation and simply added to the DFT energy, namely

$$E_{\text{total}} = E_{\text{DFT}} + E_{\text{disp}}$$

Three versions of Grimme's dispersion correction methods (DFT + E_{disp}) have been implemented in the program package used here; they are labeled⁵² as DFT-D1, -D2, and -D3, respectively. The first version of the dispersion correction -D1²⁹ was designed mainly for noncovalent interactions between molecular fragments, assuming that within a covalent molecule, the effects of correlation are covered well by the particular exchange-correlation (XC) functional; E_{disp} within a covalent molecule is not calculated. This version has been shown to give good results.⁵³ The -D3 version was developed very recently⁵² and contains many modifications and new features. It is claimed to have the advantages of higher accuracy, broader range of applicability, and less empiricism.⁵² In a recent work,⁴⁷ we investigated the application of the DFT + E_{disp} approaches to several genuine, large biological systems. It was shown that DFT-D1 performed very well, ensuring structural and energetic features in close agreement with experiment. Both the DFT-D2 and DFT-D3 approaches gave too short distances between the residues and the heme moiety in the proteins, but DFT-D3 is a great improvement over DFT-D2 for the energetic results.

For the dispersion correction made here, the -D1 version was adopted. Calculations with the DFT-D3 method were performed as well; its results are reported in Supporting Information, together with the DFT-D1 calculated results for comparison. In view of the fact that the DFT-D1 method is able to give a good description of the molecular structures for proteins,⁴⁷ we have performed the DFT-D3 calculations at the DFT-D1 optimized structures. The density functional used in our geometry optimizations is BP. It is shown that for the FeP(L) (AB) systems, the changes in the calculated structures by using different functionals employed here are insignificant;³⁹ in particular these changes do not lead to notable errors in the calculated energies.³⁹ In other cases, significant discrepancies between different functionals were found for structure predictions.¹⁷

3. Results and Discussion

We first calculated the Por-AB (AB = O₂, NO) binding energies E_{bind} , as they are the reference points for discussing the effects of the local protein environment on the Heme-AB binding. E_{bind} is defined as

$$-E_{\text{bind}} = E[\text{Por}(\text{AB})] - \{E(\text{Por}) + E(\text{AB})\} \quad (1)$$

In the case of a protein, e.g. H-NOX(AB), E_{bind} is defined as

$$-E_{\text{bind}} = E[H - \text{NOX}(\text{AB})] - \{E(\text{deoxyH-NOX}) + E(\text{AB})\} \quad (1a)$$

Here $E[\text{Por}(\text{AB})]$, $E(\text{Por})$, $E[\text{H-NOX}(\text{AB})]$, $E(\text{deoxyH-NOX})$, and $E(\text{AB})$ are the total energies of the indicated species, which are optimized independently. The ground states of Por [i.e. FeP(4-EtIm)] and deoxyHeme can be different when different functionals are used;⁵¹ BP predicts a singlet ($S = 0$) ground state, while a triplet ($S = 1$) ground state is predicted by revPBE. Finally B3LYP gives the correct (experimental) ground state multiplicity ($S = 2$) for these systems. The calculated E_{bind} refers to the species at the calculated ground state; i.e. each functional is used for its predicted ground state. Some

previous calculations of the Por–AB binding energies (e.g. ref. 17) may refer to Por at the experimental ground state ($S = 2$) for each functional used.

The DFT and DFT + E_{disp} (i.e. DFT-D1) calculated E_{bind} results are collected in Table 2, together with available, estimated experimental data.^{17,54–57} As is shown in other studies in the literature,^{17,21,58} the calculated E_{bind} is rather sensitive to the choice of functional. We have thus presented the relative binding energies between protein–AB and Por–AB,

$E_{\text{bind}}(\text{protein-AB/Por-AB})$. These are given in Table 3. Owing to error cancellation, E_{bind} is much less dependent on a specific functional and should be more useful to examine the effect of the local protein environment on the Heme–AB binding.

Some comments have to be made on the experimental data for the AB binding energies to porphyrins and proteins, as they are in fact the dissociation barriers estimated from the measured dissociation rate constants, using transition state theory. Blomberg et al.⁵⁴ gave a rough estimate of the experimental Heme–AB binding energies in terms of the Gibbs free energy G (called absolute binding energy), where several assumptions were made: (1) The barriers for the reverse association of the diatomic molecules to Heme are similar for all ligands; (2) there are little changes of entropy between the bound complex and the transition state for Heme–AB dissociation; (3) the reverse association barrier roughly corresponds to the loss of entropy when binding AB to Heme. The loss of entropy ($T \Delta S$) is estimated to be 10 kcal/mol in ref. 54, and the experimental values for the Gibbs free energy of binding were then obtained by subtracting 10 kcal/mol from the dissociation barriers. Meanwhile,⁵⁴ the same entropy correction (10 kcal/mol) was used for correcting the calculated binding energies. In this case, the dissociation barriers actually correspond to the binding energies in enthalpy (H).

Based on an average of available theoretical and experimental results, Rado and Pierloot¹⁷ made an estimate of the protein effect on the AB binding energy to heme. A value of ~6 kcal (0.26 eV) was assumed for $\text{AB} = \text{O}_2$ and ~0 kcal/mol for $\text{AB} = \text{NO}$. Table 2 gives two sets of experimental binding energies for O_2 to porphyrin. One set refers to the measured dissociation barriers for chelated protoheme [mono-3-(1-imidazolyl)-propylamide monomethyl ester] dissolved in benzene⁵⁶ and it is 0.53 eV (1 eV = 23.06 kcal/mol = 96.5 kJ/mol). The other set (0.44 eV) refers to the dissociation barriers for Mb, corrected for the absence of the protein environment.¹⁷ The values for Por in benzene may not be best suited for comparison with computational results because they are not measured in the gas phase. It is known that the binding properties of AB to chelated protoheme can be different when the porphyrin is dissolved in different solvents.⁵⁹

To examine thermal corrections as well as entropy effects on the Heme–AB binding, we have calculated binding energies of AB to porphyrin in terms of electronic energy ($E = E_{\text{bind}}$ defined in Eq. 1, without zero-point vibrational energy correction), enthalpy [$H = E + E(\text{translation}) + E(\text{rotation}) + E(\text{vibration}) + RT$], and Gibbs free energy ($G = H - T \Delta S$) and given them in Supporting Information (Table S2), where the relevant calculated zero-point vibrational energies (ZPVEs), thermal corrections (which contain translational, rotational, and vibrational contributions) to the electronic energy (E), to the enthalpy (H), and to the Gibbs free energy (G) for various systems are reported in Table S3. The thermal corrections are calculated according to the rigid rotor/harmonic oscillator/ideal gas approximation at 298.15 K. From Table S2, we see that the difference between E and H is only 0.03 eV. (The correction for ZPVE is ca. 0.1 eV.) Thus, it is adequate to compare the calculated E_{bind} ($= E$) with the experimental dissociation barrier, as is done and discussed in other studies.¹⁷ Our calculated $T \Delta S$ values are 0.50 eV (11.5 kcal/mol) for both $\text{AB} = \text{O}_2$ and NO, quite comparable to the estimate of 10 kcal/mol in ref. 54. We use here dissociation

barriers instead of the Gibbs free energies of dissociation because frequency calculations are impractical for a number of large protein model systems considered here.

Kepp⁵⁸ has recently investigated the thermal correction and entropy effect on the spin-state energetics for Fe^{III} porphines and shown that neglect of these effects may lead to incorrect conclusions about chemical energies and the adequacy of functionals. We have calculated relative energies for selected states of FeP(4-EtIm) in terms of electronic energy (E), enthalpy (H), and Gibbs free energy (G) and given them in Supporting Information (Table S4). If the energy of the triplet state ($S = 1$) is set to zero, the changes of the relative energy, from E to H, are 0.01 eV for the $S = 0$ state and -0.04 eV for the $S = 2$ state. From E to G, the changes are 0.06 and -0.07 eV for the $S = 0$ and $S = 2$ states, respectively. The thermal correction and entropy favor the high-spin state, as is shown by Kepp.⁵⁸ Nevertheless, these effects are not large enough for the BP and revPBE functionals to change the ground state of FeP(4-EtIm) from low spin to high spin.

Table 4 presents the calculated binding energies of axial histidine (His) to the porphyrins [FeP, FeP(AB)] and to the various proteins. It is shown that the calculated FeP(AB)–His binding energies are less sensitive to the choice of functional than the FeP(His)–AB ones; the same is true for the proteins.

3.1. Por–AB Binding Energies (AB = O₂, NO)

Accurate prediction of binding energies for the diatomic molecules to heme proves to a difficult issue in theoretical computations.⁵¹ Rovira et al.¹⁴ were probably the first to provide a detailed set of DFT data for binding energies and electronic properties for FeP(Im)(AB). Their calculations used the BP functional and yielded binding energies of 15 and 36 kcal/mol for AB = O₂ and NO, respectively. Blomberg et al.⁵⁴ studied binding of AB to FeP(NH₃) with the B3LYP functional; they reported binding energies of 7.6 and 12.5 kcal/mol for AB = O₂ and NO, respectively. Comparative theoretical studies of FeP(Im)(AB) with *ab initio* and various DFT methods were performed by Strickland and Harvey²¹ and by Radon and Pierloot.¹⁷ More recently, Siegbahn et al.²³ carried out a dispersion-corrected DFT study for FeP(Im)(AB); their B3LYP estimates of dispersion were 7.7 kcal/mol for AB = O₂ and 9.3 kcal/mol for AB = NO.

Our $E_{\text{bind}}(\text{Por-AB})$ values calculated with BP are 0.71 and 1.70 eV for AB = O₂ and NO, respectively. They are larger than the experimental data (O₂: 0.44 eV, NO: 0.99 eV) by 0.27 and 0.71 eV, respectively for the two ligands. The revPBE functional performs much better than BP and gives $E_{\text{bind}}(\text{Por-O}_2)$ which is excellent agreement with experiment. Nevertheless, this functional still overestimates $E_{\text{bind}}(\text{Por-NO})$ by 0.48 eV. For B3LYP, the $E_{\text{bind}}(\text{Por-O}_2)$ is too small by 0.40 eV, but this functional also overbinds considerably for Por–NO. When the dispersion correction is made through the -D3 version, the Por–AB binding energies of B3LYP increase by 0.26 – 0.28 eV (Supporting Information); the dispersion term E_{disp} improves greatly the B3LYP result on Por–O₂, but shows deficiency when this functional is applied to Por–NO. The addition of E_{disp} obtained with the -D3 version makes BP and revPBE worse for the Por–AB binding energies. Note that E_{disp} is not calculated within the covalent Por(AB) molecule by the DFT-D1 method.

In Supporting Information (Table S5), a comparison is given of the calculated Por–AB binding energies (AB = CO, O₂, NO) from the literature and in this work. We see that there is some difference of the E_{bind} results obtained with the same functional in two different references. The origin of this difference has been discussed in a previous paper;⁵¹ the basis sets employed could be a major factor. However, we notice that in the other studies^{17,21,23,54} that used GTO (Gaussian-type orbital) basis sets, the B3LYP functional was shown to greatly underestimate the binding energy of NO to Por, in strong contrast to the present

work that uses STO basis sets. No clear or satisfactory explanation could be given here for such a difference, which is as large as 1 eV. From Table S2 (Supporting Information), our B3LYP calculated Por–NO binding energy in terms of the Gibbs free energy, $-G$, is 0.88 eV, which is ca. 0.3 eV larger than the estimated experimental $-G$ value (0.56 eV); taking into account thermal correction and entropy reduces the discrepancy between the calculated and experimental data. Our B3LYP $E_{\text{bind}}(\text{Por-NO})$ is larger than $E_{\text{bind}}(\text{Por-CO})$, in agreement with the trends in the experimental data and in the high-level *ab initio* CASPT2 calculated values.¹⁷ This is in contrast to the B3LYP results from the other authors, which show the opposite trend.

3.2. Mb–AB Binding Energies

Among the different hemes investigated, the AB adducts of myoglobin are probably some of the most-studied systems in biology.⁶⁰ We therefore calculated Mb(AB) here for comparison. There is experimental information available for the Mb–AB binding energies,^{54,57} which can be used to test the computational methods adopted here.

First looking at the results obtained with pure DFT, the calculated Mb–O₂ binding energies are 0.91, 0.61, and 0.28 eV for the BP, revPBE, and B3LYP functionals, respectively; they are 0.17 – 0.24 eV larger than those for Por–O₂, the $E_{\text{bind}}(\text{Mb-O}_2/\text{Por-O}_2)$ values being quite close to the experimental one (0.26 eV). A hydrogen bond (H-bond) interaction between His64 and the terminal oxygen atom is responsible for the extra stabilization of the bound O₂ in Mb (see Figure 2c). To show this point more clearly, additional calculations were performed on a model system Mb^{no His64}(O₂) that excludes His64 from the residues; this leads to a decrease of ~0.15 eV in E_{bind} and the calculated Mb^{no His64}–O₂ binding energies are only slightly larger than the Por–O₂ ones. When a dispersion correction is made, there are little changes in $E_{\text{bind}}(\text{Mb-O}_2)$. With the BP functional, the DFT + E_{disp} calculated $E_{\text{bind}}(\text{Mb-O}_2)$ is even slightly smaller than that calculated with pure DFT. We should point out that the dispersion energy between bound AB and the surrounding residues is always attractive. But according to the above definition for E_{bind} , the calculated Mb–AB binding energy is also related to the energy of *optimized* deoxyMb. A negative dispersion contribution to $E_{\text{bind}}(\text{Mb-AB})$ means that the non-covalent (dispersion) interaction between the protein environment and the heme is enhanced when AB departs.

In the case of Mb(NO), the calculated $E_{\text{bind}}(\text{Mb-NO}/\text{Por-NO})$ values with pure DFT indicate that a H-bond with bound NO provides no or much less stabilization than that with bound O₂, owing to the much less polar nature of the Fe–NO complex. When a dispersion correction is made, the calculated E_{bind} increases by 0.02 – 0.06 eV from Por–NO to Mb–NO, which is slightly different from the experimental $E_{\text{bind}}(\text{Mb-NO}/\text{Por-NO})$ value (0.00). The analysis of binding and kinetic data for Mb(NO) and its mutants suggest that bound NO is stabilized ~ tenfold (0.05 eV) by the protein environment,⁵⁷ in good agreement with the calculation. It is argued⁵⁷ that this favorable interaction with bound NO compensates for the (tenfold) inhibition due to the presence of distal pocket water, and the net result is little or no change in experimental E_{bind} from Por–NO to Mb–NO.

3.3. H-NOX–AB Binding Energies

We first examine the results calculated in the 1U4H crystal structure (i.e. based on the crystal structure with PDB code 1U4H). The calculations with pure DFT give an H-NOX–O₂ binding energy which is comparable to the Mb–O₂ one. But there is a large increase of 0.22 – 0.25 eV in $E_{\text{bind}}(\text{H-NOX-O}_2)$ when E_{disp} is included, in contrast to Mb–O₂, where the DFT and DFT + E_{disp} calculated E_{bind} values are close to each other. Table 2 also provides measured dissociation rate constants [k_{AB} , i.e. $k_{\text{off}}(\text{AB})$] of the diatomic AB molecule from the heme of the various proteins. There is a relationship between the change

() in the ligand binding energy $E_{\text{bind}}(\text{AB})$ and the change in k_{AB} : $E_{\text{AB}} = kT \ln k_{\text{AB}}$.⁵⁵ According to this formula and the measured k_{AB} , E_{bind} for H-NOX- O_2 is estimated to be 0.76 eV, which is 0.06 eV higher than that for Mb- O_2 . The calculation with DFT + E_{disp} yields $E_{\text{bind}}(\text{H-NOX-}\text{O}_2)$ which is 0.15 – 0.30 eV larger than $E_{\text{bind}}(\text{Mb-}\text{O}_2)$, in great contrast to 'experiment'. In the case of AB = NO, the calculated E_{bind} with pure DFT is 0.02 – 0.07 eV larger than that in Mb; $E_{\text{bind}}(\text{H-NOX-NO/Mb-NO})$ is enlarged to 0.11 – 0.18 eV when the dispersion correction is made. The measured k_{NO} values indicate that E_{bind} decreases by 0.04 eV from Mb-NO to H-NOX-NO; the trend in the calculated $E_{\text{bind}}(\text{NO})$ values is opposite to the experimental one.

When Heme(AB) is put in the 1XBN crystal structure, Tyr140 is the only H-bond donor for Heme(AB). As a result, the calculated H-NOX^{1XBN}-AB binding energies are all significantly smaller than the H-NOX^{1U4H}-AB ones, and even smaller than the Mb-AB ones without dispersion correction. In this structure, the BP/B3LYP + E_{disp} calculations show $E_{\text{bind}}(\text{H-NOX-}\text{O}_2)$ to be ca. 0.10 eV larger than $E_{\text{bind}}(\text{Mb-}\text{O}_2)$, quite close to the 'experimental' value (0.06 eV) estimated with the measured k_{O_2} data. (It is 0.01 eV for the binding energy difference between H-NOX- O_2 and Mb- O_2 with revPBE + E_{disp}). The dispersion contributes 0.12 – 0.15 eV to $E_{\text{bind}}(\text{H-NOX}^{\text{1XBN}}\text{-}\text{O}_2)$, in contrast to 0.22 – 0.25 eV in the 1U4H structure. In the case of AB = NO, the dispersion contribution is 0.07 – 0.10 eV, while it is 0.13 – 0.16 eV in the 1U4H structure. The BP/revPBE + E_{disp} calculated $E_{\text{bind}}(\text{H-NOX-NO/Mb-NO})$ is now –0.09/–0.07 eV, both the trend and value agreeing well with 'experiment', which is –0.04 eV estimated with the measured k_{NO} data. In the 1U4H structure, a hydrogen bonding network involving Trp9 and Asn74 contributes to the high affinity of the protein for both O_2 and NO. However, it appears that the calculated results support the crystal structure with the PDB code 1XBN, where Trp9 and Asn74 do *not* form a hydrogen bonding network with Tyr140.

3.4. Y140F-AB binding energies

To examine the effect of hydrogen bonding in the distal pocket of H-NOX, we focus here mainly on the difference between $E_{\text{bind}}(\text{Y140F-AB})$ and $E_{\text{bind}}(\text{H-NOX-AB})$. When Tyr140 in H-NOX^{1U4H} or H-NOX^{1XBN} is replaced with a non-polar Phe, the DFT calculated binding energy for AB = O_2 decreases by as much as ~0.3 eV, which represents the contribution of an H-bond from Tyr140 to the bound O_2 . From the calculations with DFT + E_{disp} , this decrease in $E_{\text{bind}}(\text{O}_2)$ is 0.3 – 0.4 eV for the 1U4H structure and ca. 0.2 eV for the 1XBN structure. The dispersion contributions to $E_{\text{bind}}(\text{Y140F-}\text{O}_2)$ are 0.14 – 0.18 eV in the 1U4H structure and 0.19 – 0.24 eV in the 1XBN one.

In the case of AB = NO, there is a decrease of 0.10 – 0.16 eV in both the DFT and DFT + E_{disp} calculated E_{bind} values upon Tyr140 → Phe substitution in H-NOX^{1U4H}. However, when the calculations are performed on the 1XBN structure, this decrease is small (0.02 – 0.04 eV) with pure DFT and even negative when the dispersion correction is made; the BP/revPBE + E_{disp} calculated E_{bind} values of Y140F-NO are 0.06 – 0.07 eV increased as compared to that of H-NOX^{1XBN}-NO (0.01 eV increased from the B3LYP + E_{disp} calculations). The measured k_{NO} results for H-NOX and Y140L (see Table 2) indicate that loss of the distal Tyr140 slows the dissociation rate. This trend is in agreement with the calculated results in the 1XBN structure. The 'experimental' $E_{\text{bind}}(\text{H-NOX-NO/Y140L-NO})$ estimated with measured k_{NO} data is –0.04 eV, also quite close to the calculation on the 1XBN structure. The dispersion contribution to $E_{\text{bind}}(\text{Y140F-NO})$ is 0.1 – 0.2 eV and similar in both 1U4H and 1XBN structures.

3.5. FeP(AB)–His Binding Energies

We now examine the binding of proximal histidine to Fe in porphyrin and the various proteins. The FeP–His binding energies without bound AB were first evaluated as the reference point; they are 0.47, 0.33 and 0.21 eV for the BP, revPBE, and B3LYP functionals, respectively. Upon bonding of O₂ to FeP, this bond is enhanced by more than 0.1 eV according to the calculations with BP and B3LYP. In contrast, NO exerts a repulsive *trans* effect on the proximal ligand; the bonding of NO to FeP(His) results in weakening or rupture of the proximal Fe–ligand bond. The calculations with BP and revPBE show that the Fe–His binding energy in FeP(His)(NO) decreases by 0.26 – 0.30 eV as compared to FeP–His, and the revPBE calculated $E_{\text{bind}}[\text{FeP}(\text{NO})\text{–His}]$ is almost zero (0.03 eV). Thus, NO is distinct from O₂ in its ability to readily form stable five-coordinate heme complexes.^{61,62}

3.6. Mb(AB)–His Binding Energies (Table 4)

The calculations with pure DFT do not give an obvious change of the binding energy from FeP(O₂)–His to Mb(O₂)–His. The dispersion contribution to $E_{\text{bind}}[\text{Mb}(\text{O}_2)\text{–His}]$ is about 0.1 eV. In the case of AB = NO, the DFT calculated $E_{\text{bind}}(\text{Fe–His})$ is ~0.1 eV larger in Mb(NO) than in FeP(His)(NO), and the dispersion contributes an additional 0.1 eV to $E_{\text{bind}}(\text{Fe–His})$ in Mb(NO). Eventually, the DFT + E_{disp} calculated Mb(NO)–His binding energies amount to 0.23 – 0.35 eV, depending on the functional used. Since the Fe–His bond in Mb(NO) is strong enough, Mb would bind NO in a six-coordinate complex. This is in agreement with experimental observation.⁴¹

By excluding His64 from the residues, the DFT calculated Mb^{no His64}(AB)–His binding energies differ little from Mb(AB)–His. In this case, however, the dispersion contributes little to $E_{\text{bind}}(\text{Fe–His})$ as well. Therefore, according to the calculations with DFT + E_{disp} , $E_{\text{bind}}(\text{Fe–His})$ in Mb^{no His64}(NO) is now 0.11 – 0.27 eV, which is about 0.1 eV smaller than that in Mb(NO). On the experimental side,⁶³ Mb(NO) adopts the five-coordinate structure due to cleavage of the Fe–His bond when the pH is lowered to 4, at which the distal His64 becomes protonated (on the N position) and swings out into the solvent away from the bound ligand. This should be similar to the situation of Mb^{no His64}(AB). The calculated results agree qualitatively with the experimental observation.

3.7. H-NOX(AB)–His Binding Energies (Table 4)

The calculated H-NOX^{1U4H}(AB)–His binding energies are all similar to the Mb(AB)–His ones, indicating that the Fe–His bond in the H-NOX^{1U4H} protein is not notably different from that in Mb even after NO binds. The situation is different in the 1XBN structure, where the calculated H-NOX^{1XBN}(AB)–His binding energies are about 0.1 eV smaller compared with H-NOX^{1U4H}(AB)–His. The dispersion contribution to $E_{\text{bind}}[\text{H-NOX}^{\text{1XBN}}(\text{NO})\text{–His}]$ is also small, only 0.02 – 0.04 eV (~0.10 eV in the 1U4H structure). The DFT + E_{disp} calculated NOX^{1XBN}(NO)–His binding energy amounts to 0.06 – 0.23, comparable to or slightly larger than the FeP(NO)–His one (0.03 – 0.21 eV). Experimental studies¹² show that *Tt* H-NOX slowly transitions from a 6-coordinate to a 5-coordinate Fe^{II}–NO complex at 70 °C, indicating that the Fe–His bond in H-NOX is not quite stable. This appears to be in agreement with the calculated results on the 1XBN structure.

3.8. Y140F(AB)–His Binding Energies

Compared to H-NOX^{1U4H}(AB)–His, the Y140F^{1U4H}(AB)–His binding energy decreases by 0.03 – 0.08 eV by the mutation of Tyr140 to Phe in the 1U4H structure. This is true for both AB = O₂ and NO and for both DFT and DFT + E_{disp} calculations. In the 1XBN structure, there is similar change of 0.01 – 0.08 eV in E_{bind} from H-NOX(O₂)–His to Y140F(O₂)–His (i.e. with AB = O₂). For AB = NO, the DFT calculated H-NOX^{1XBN}(NO)–

His and Y140F^{1XBN} (NO)–His binding energies are almost the same. When the dispersion correction is made, a decrease of ca. 0.05 eV is shown from $E_{\text{bind}}[\text{H-NOX}^{\text{1XBN}}(\text{NO})\text{–His}]$ to $E_{\text{bind}}[\text{Y140F}^{\text{1XBN}}(\text{NO})\text{–His}]$; in the latter, the dispersion contribution is –0.03 eV (negative) from the calculations with BP and B3LYP. This means that in the case of Y140F^{1XBN}, the dispersion interaction between the distal residues and the heme(NO) moiety is enhanced when the proximal His departs.

3.9 Optimized Structures

The DFT and DFT + E_{disp} optimized structural parameters of interest for FeP(His), FeP(His)(AB), Mb(AB), and H-NOX(AB) are provided in Tables 5–8, together with available experimental (X-ray) crystal structural data.^{7,8,64–66} Table 6 also provides the experimental structural parameters measured with the multi-scattering XAFS method on Mb(NO) in solution.⁶⁷

In the five-coordinate FeP(His), the proximal histidine pulls Fe out of the porphyrin plane. The three critical structural parameters are $R_{\text{Ct}(4\text{N})\text{–N}(\text{eq})}$ (distance between the center of the ring and the equatorial, porphinato nitrogen), $R_{\text{Ct}(4\text{N})\text{–Fe}}$ (distance between the center of the ring and Fe), and $R_{\text{Fe–N}(\text{ax})}$ (axial Fe–N bond length). $R_{\text{Ct}(4\text{N})\text{–Fe}}$ represents the displacement of Fe out of the 4N-plane toward the axial (proximal) histidine ligand.

Binding AB to the five-coordinate complex moves Fe back into the plane. The structural parameters of interest for FeP(His)(AB) include $R_{\text{Fe–AB}}$ (the axial Fe–AB bond length), $R_{\text{A–B}}$ (the A–B bond length), and $\angle_{\text{Fe–A–B}}$ (the Fe–A–B bond angle). For Mb(AB) and H-NOX(AB), the distances between the bound AB and the H-atom of the –NH or –OH group from His64 (in Mb), Tyr140, Trp9, and Asn74 (in H-NOX) are also given in the tables.

For both FeP(His) and FeP(His)(AB), there is good quantitative agreement between the optimized and experimental structures. No obvious changes in the calculated R and $\angle$ values are found from FeP(His)(AB) to Mb(AB) or H-NOX(AB) within the internal structure of the heme. Relatively large discrepancies are shown between the optimized and experimental structural parameters in Mb(NO). There is difficulty in accurately determining bond lengths and bond angles in protein molecules, due to the much lower resolution available in proteins versus small molecule (heme model) structures. We suspect that the Fe–N–O angle of the Mb(NO) crystal structure (ca. 112°) is largely underestimated (~140° according to the calculations, 140° in the crystal structure of FeTPP(1-MeIm)(NO)⁶⁶, 150° from a measurement with the multiple-scattering XAFS method on Mb(NO) in solution⁶⁷). Compared to $R_{\text{Fe–His}}$ in FeP(His), the additions of O₂ and NO to FeP(His) result in a shorter and longer Fe–His distance, respectively, consistent with the trends in the calculated Fe–His binding energies. The Fe–His bond in Heme(NO) is 0.12 – 0.14 Å longer than that in Heme(O₂); histidine is nearly displaced by the binding of NO.

4. Conclusions

The key factor for ligand discrimination in favor of O₂ over NO in the H-NOX proteins has been attributed to an H-bond interaction between the heme-bound O₂ and the distal pocket tyrosine residue (Tyr140).¹¹ According to the calculations, the stabilization of bound O₂, denoted by $E_{\text{bind}}(\text{H-NOX–O}_2/\text{Por–O}_2)$, is 0.35 – 0.50 eV in the 1U4H structure and 0.21 – 0.33 eV in the 1XBN structure; they are even higher than the corresponding value (0.16 – 0.24 eV) calculated in Mb(O₂). This is in agreement with the observation that the H-NOX domain of *T. tengcongensis* has a very high affinity for its oxygen molecule.⁸ Upon mutation of Tyr140 in H-NOX to a non-polar Phe, the calculated $E_{\text{bind}}(\text{Y140F–O}_2)$ decreases by 0.3 – 0.4 eV for the 1U4H structure and ca. 0.2 eV for the 1XBN structure, further supporting the argument that Tyr140 is a critical residue involved in binding O₂. The

difference between $E_{\text{bind}}(\text{H-NOX-O}_2)$ and $E_{\text{bind}}(\text{Mb-O}_2)$ estimated with measured $k_{\text{off}}(\text{O}_2)$ is about 0.06 eV, agreeing well with the calculated results (~ 0.10 eV) in the 1XBN structure. There is also a large effect of the H-bond on the binding of O_2 when the calculations are based on the crystal structure with the PDB code 1U4H.

Polar interactions with distal residues carry much less energy for NO than for O_2 . The calculated stabilization of the bound NO by the protein environment is 0.02 – 0.06 eV in Mb, 0.16 – 0.23 eV in H-NOX^{1U4H}, and –0.03 to 0.04 eV in H-NOX^{1XBN}. The trends in the calculated E_{bind} from Por–NO to Mb–NO and from Por–NO to H-NOX^{1XBN}–NO are in good agreement with experiment. Again for H-NOX^{1U4H}, the calculated stabilization of the bound NO by the polar residue(s) is overestimated when compared to the experimental effect. The dispersion contribution to E_{bind} is small (< 0.05 eV) for Mb–AB, but relatively large for H-NOX–AB (0.1 – 0.25 eV, depending on the crystal structure and the ligand AB). The dispersion correction is necessary in order to obtain the 'correct' trend in E_{bind} from Mb–AB to H-NOX^{1XBN}–AB.

The effect of the protein environment, particularly, the distal Tyr140, on the proximal Fe–His binding was also the subject of this work. When O_2 binds to the five-coordinate heme, the bond of the *trans* axial ligand is strengthened by more than 0.1 eV. In contrast, the Fe–His bond is weakened by more than 0.2 eV upon the binding of NO to the heme. A hydrogen bond interaction from the polar residue does not have obvious effects on the Fe–His binding strength. This is demonstrated from the DFT calculations by removing His64 from the residues in Mb or by mutating Tyr140 to a non-polar residue Phe in H-NOX. But the effects of the whole local residues and of the dispersion on the Fe–His binding in the proteins may still be significant. Compared to FeP(AB)–His, the corresponding Fe–His binding energy increases by ~ 0.1 eV in Mb(O_2) and H-NOX^{1U4H}(O_2), ~ 0.15 eV in Mb(NO) and H-NOX^{1U4H}(NO), and ~ 0.03 eV in H-NOX^{1XBN}(AB); where the increase of $E_{\text{bind}}(\text{Fe–His})$ arises mainly from a dispersion contribution [~ 0.10 eV in Mb(AB), H-NOX^{1U4H}(AB), and H-NOX^{1XBN}(O_2); ~ 0.03 eV in H-NOX^{1XBN}(NO)]. The mutation of Tyr140 in H-NOX to Phe leads to a decrease of ~ 0.05 eV in $E_{\text{bind}}(\text{Fe–His})$ for both AB = O_2 and NO. This indicates that in the H-NOX protein, interactions (mainly dispersion) from the distal polar residue(s) play only a minor role in the breakage of the axial Fe–His bond upon NO binding. Interestingly, the distal His64 in Mb has a relatively large stabilizing effect (~ 0.1 eV) on the Fe–His bond (this effect arises mainly from a contribution from dispersion, not from hydrogen bonding).

Concerning a NO-selected H-NOX protein (such as sGC), its distal heme pocket does not contain any polar residues that are capable of forming a strong H-bond with a heme-bound ligand. This explains why those proteins do not bind O_2 and are selective for NO. However, the absence of a polar residue in the distal heme pocket is not the reason why the proximal Fe–His bond breaks upon NO binding to Fe. Experimental results have shown that removal of Tyr140 from *Tt*H-NOX has little effect on the coordination number of the NO complex;¹² this observation is consistent with the calculations. Not enough information is provided here to understand the five- versus-six coordination issue.

We speculate that in a native protein, the interactions of the proximal His with the surrounding protein environment would determine whether the bond of His to Fe is broken or maintained upon binding of NO to Fe. The Fe–His bond in the *deoxy* form of these NO-selected H-NOX proteins is relatively weak compared with that in *deoxy* O_2 /NO-binding H-NOX. To show this point, Table 9 gives a comparison of measured Fe^{II} –His stretching frequencies ($\nu_{\text{Fe–His}}$) in different *deoxy* proteins^{68–72} and the coordination numbers (CNs) upon binding of NO to Fe of the heme. A relationship between $\nu_{\text{Fe–His}}$ and CN is quite obvious. A higher $\nu_{\text{Fe–His}}$ value means a stronger Fe–His bond and it corresponds to a CN of

6; the Fe–His will be maintained upon binding of NO to Fe. On the contrary, a lower Fe-His corresponds to a CN of 5; the Fe–His bond tends to break upon binding of NO to Fe. In T-state Hb and sGC, the covalent attachment of the proximal histidine to the polypeptide backbone appears to impose constraints on the proximal histidine. These constraints destabilize the Fe–His bond and thus facilitate the His release upon binding of NO to Fe. In the other proteins such as Mb and *Tt*H-NOX, there are no or less constraints on the proximal histidine and thus His remains coordinated to Fe upon binding of NO.

Phenomena observed in experiments cannot always be explained. For example, when a distal pocket Tyr is introduced with the *L2F142Y* mutation,¹² the equilibrium is shifted primarily to a 6-coordinate Fe^{II} -NO complex, indicating that a Tyr in the distal pocket is necessary for stabilization of the Fe–His bond. However, the opposite result was obtained by mutation in the H-NOX protein from *Clostridium botulinum*,⁷ replacement of Tyr with Phe converted the 5-coordinate NO complex to 6-coordinate NO complex. These differences in a family of sequence-related proteins are not understood yet. Further investigations are needed to reveal factors that control the coordination state of the NO complexes of hemoproteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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For a NO-selective H-NOX (e.g. VCA0720): **For an O₂/NO binding H-NOX (e.g. TtTar4H):**

Figure 1.
Comparison of coordination behaviors between a NO-selective H-NOX and an O₂/NO binding H-NOX

Figure 2.

The structure and residues surrounding the bound O₂/NO in (a) H-NOX(O₂) with the PDB code 1U4H, (b) H-NOX(O₂) with the PDB code 1XBN, (c) Mb(O₂), and (d) Mb(NO). The relevant H-atoms of interest are shown in the figure; the other atoms are omitted in the figure for clarity. Optimized structures are shown in Supporting Information (Figure S1).

(a) FePPIX

(b) FeP

Figure 3.

(a) Iron protoporphyrin IX (FePPIX) of hemoproteins and (b) iron porphine (FeP)

Table 1Experimental dissociation rate constants (k_{off} , s^{-1}) for O_2 and NO from different proteins

Protein	$k_{\text{off}}(\text{O}_2)$	$k_{\text{off}}(\text{NO})$	ref.
sGC	--- ^a	$(3.6 \pm 0.8) \times 10^{-4}$	12
Mb (Sperm whale)	15	1.23×10^{-4}	57
Hb (R-state)	15 – 35	$\sim 6 \times 10^{-3}$	57
Tt H-NOX	1.22 – 0.09	$(5.6 \pm 0.5) \times 10^{-4}$	11

^a It does not bind O_2 at all ($k_{\text{off}} =$).

Table 2

Calculated binding energies (E_{bind} , eV) of O_2 and NO to Mb and the H-NOX proteins with DFT and DFT + E_{disp} . The results for pure porphyrin (Por) [i.e. FeP(4-EtIm)] are given here for comparison.

		BP		B3LYP	Exptl	
			revPBE		E_{bind}	k_{AB}^c (s^{-1})
Por- O_2^a	DFT	0.71	0.44	0.04	0.44 ^d , 0.53 ^g	4200 ^h
Por-NO ^a	DFT	1.70	1.47	1.41	0.99 ^d	
Mb- O_2	DFT	0.91	0.61	0.28	0.70 ^e	15 ^h
	DFT + E_{disp}	0.87	0.64	0.28		
Mb ^{no His64} - O_2	DFT	0.73	0.49	0.13		
	DFT + E_{disp}	0.75	0.53	0.12		
Mb-NO	DFT	1.73	1.48	1.40	0.99 ^e	1.23×10^{-4} ^h
	DFT + E_{disp}	1.76	1.52	1.43		
Mb ^{no His64} -NO	DFT	1.70	1.49	1.36		
	DFT + E_{disp}	1.66	1.46	1.32		
H-NOX ^{1U4H} - O_2^b	DFT	0.95	0.57	0.30	0.76 ^f	1.22 ⁱ
	DFT + E_{disp}	1.20	0.79	0.54		
H-NOX ^{1U4H} -NO	DFT	1.77	1.50	1.47	0.95 ^f	5.6×10^{-4} ⁱ
	DFT + E_{disp}	1.93	1.63	1.61		
H-NOX ^{1XBN} - O_2^b	DFT	0.85	0.50	0.24		
	DFT + E_{disp}	0.97	0.65	0.37		
H-NOX ^{1XBN} -NO	DFT	1.59	1.38	1.35		
	DFT + E_{disp}	1.67	1.45	1.45		
Y140F ^{1U4H} - O_2	DFT	0.63	0.40	-0.02		
	DFT + E_{disp}	0.81	0.54	0.12		
Y140F ^{1U4H} -NO	DFT	1.64	1.40	1.31	0.99 ^f	1.3×10^{-4} ^j
	DFT + E_{disp}	1.80	1.51	1.50		
Y140F ^{1XBN} - O_2	DFT	0.53	0.24	-0.08		

	BP	revPBE	B3LYP	Exptl	
				E_{bind}	$k_{\text{AB}}^c \text{ (s}^{-1}\text{)}$
Y140F ¹ XBN ₂ NO	DFT + E_{disp}	0.77	0.43	0.13	
	DFT	1.56	1.34	1.33	
	DFT + E_{disp}	1.74	1.51	1.46	

^aNote that the DFT + E_{disp} method used here is not applied to a covalent molecule.

^bThe superscript '1U4H' or '1XBN' means that the calculation is based on the crystal structure with the PDB code 1U4H or 1XBN; the same is true in other tables.

^c k_{AB} is the dissociation rate constant [i.e. $k_{\text{off}}(\text{AB})$] of the diatomic AB molecule ($\text{AB} = \text{O}_2, \text{NO}$) from the heme of the protein.

^dDissociation barrier for Mb, corrected for the absence of the protein environment (ref. 17).

^eDissociation barrier for Mb (ref. 54).

^fEstimated with the formula $E_{\text{AB}} = kT \ln k_{\text{AB}}$ (ref. 55); $E_{\text{AB}} = E_{\text{bind}}(\text{Mb-AB/mutant-AB})$ is the difference of the binding energies between wild-type Mb and the indicated mutant.

^gDissociation barrier for chelated protoheme in benzene (ref. 56).

^hRef. 57.

ⁱRef. 11.

^jExperimental result for Y140L (ref. 11).

Table 3

Calculated relative binding energies (E , eV) between Protein-AB and Por-AB, $E(\text{Protein-AB/Por-AB})^a$ (Protein = Mb, H-NOX, Y140F; AB = O₂, NO).

	BP	revPBE	B3LYP	Exptl ^b
E(Mb-O ₂ /Por-O ₂)	DFT	0.20	0.17	0.24
	DFT + E _{disp}	0.16	0.20	0.24
E(Mb ^{no His64} -O ₂ /Por-O ₂)	DFT	0.02	0.05	0.09
	DFT + E _{disp}	0.04	0.09	0.08
E(Mb-NO/Por-NO)	DFT	0.03	0.01	-0.01
	DFT + E _{disp}	0.06	0.05	0.02
E(Mb ^{no His64} -NO/Por-NO)	DFT	0.00	0.02	-0.05
	DFT + E _{disp}	-0.04	-0.01	-0.09
E(H-NOX ^{1U4H} -O ₂ /Por-O ₂)	DFT	0.24	0.13	0.26
	DFT + E _{disp}	0.49	0.35	0.50
E(H-NOX ^{1U4H} -NO/Por-NO)	DFT	0.07	0.03	0.06
	DFT + E _{disp}	0.23	0.16	0.20
E(H-NOX ^{1XBN} -O ₂ /Por-O ₂)	DFT	0.14	0.06	0.20
	DFT + E _{disp}	0.26	0.21	0.33
E(H-NOX ^{1XBN} -NO/Por-NO)	DFT	-0.11	-0.09	-0.06
	DFT + E _{disp}	-0.03	-0.02	0.04
E(Y140F ^{1U4H} -O ₂ /Por-O ₂)	DFT	-0.08	-0.04	-0.06
	DFT + E _{disp}	0.10	0.10	0.08
E(Y140F ^{1U4H} -NO/Por-NO)	DFT	-0.06	-0.07	-0.10
	DFT + E _{disp}	0.10	0.04	0.09
E(Y140F ^{1XBN} -O ₂ /Por-O ₂)	DFT	-0.18	-0.20	-0.12
	DFT + E _{disp}	0.06	-0.01	0.09
E(Y140F ^{1XBN} -NO/Por-NO)	DFT	-0.14	-0.13	-0.08
	DFT + E _{disp}	0.04	0.04	0.05

^a $E(\text{Protein-AB/Por-AB}) = E_{\text{bind}}(\text{Protein-AB}) - E_{\text{bind}}(\text{Por-AB})$.

^bBased on the estimated experimental data given in Table 2.

Table 4

Calculated binding energies of axial histidine (His) to porphyrins [FeP, FeP(AB)], myoglobin [Mb(AB)], and the H-NOX proteins [H-NOX(AB), Y140F(AB)] (AB = O₂, NO)

		BP	revPBE	B3LYP
FeP-His	DFT	0.47	0.33	0.21
FeP(O ₂)-His	DFT	0.59	0.36	0.39
FeP(NO)-His	DFT	0.21	0.03	0.16
Mb(O ₂)-His	DFT	0.58	0.41	0.33
	DFT + E _{disp}	0.69	0.51	0.46
Mb ^{no His64} (O ₂)-His	DFT	0.59	0.43	0.33
	DFT + E _{disp}	0.54	0.42	0.28
Mb(NO)-His	DFT	0.25	0.11	0.24
	DFT + E _{disp}	0.35	0.23	0.31
Mb ^{no His64} (NO)-His	DFT	0.27	0.10	0.22
	DFT + E _{disp}	0.27	0.11	0.23
H-NOX ^{1U4H} (O ₂)-His	DFT	0.58	0.35	0.42
	DFT + E _{disp}	0.67	0.48	0.54
H-NOX ^{1U4H} (NO)-His	DFT	0.28	0.08	0.19
	DFT + E _{disp}	0.39	0.18	0.29
H-NOX ^{1XBN} (O ₂)-His	DFT	0.49	0.28	0.38
	DFT + E _{disp}	0.60	0.40	0.50
H-NOX ^{1XBN} (NO)-His	DFT	0.21	0.02	0.16
	DFT + E _{disp}	0.23	0.06	0.18
Y140F ^{1U4H} (O ₂)-His	DFT	0.55	0.30	0.34
	DFT + E _{disp}	0.64	0.45	0.46
Y140F ^{1U4H} (NO)-His	DFT	0.24	0.05	0.17
	DFT + E _{disp}	0.32	0.13	0.27
Y140F ^{1XBN} (O ₂)-His	DFT	0.48	0.22	0.30
	DFT + E _{disp}	0.59	0.35	0.42
Y140F ^{1XBN} (NO)-His	DFT	0.20	-0.01	0.16
	DFT + E _{disp}	0.17	0.02	0.13

Table 5

Optimized^a (with DFT and DFT + E_{disp}) and experimental (X-ray) structural parameters (distance R in Å, bond angle in degree) for FeP(His), FeP(His)(O₂), and Mb(O₂)

	FeP(His)		FeP(His)(O ₂)		Mb(O ₂)	
	DFT	Exptl ^b	DFT	Exptl ^c	DFT + E _{disp}	Exptl ^d
R _{Fe-N(eq)}	2.079 ^e	2.057 ^e	2.008	1.98	2.009	2.006
R _{C1(dN)} Fe	0.300	0.291	-0.006 ^f	-0.02	-0.006	0.024
R _{Fe-N(ax)}	2.134	2.149	2.040	2.07	2.049	2.064
R _{Fe-O}			1.844	1.75	1.825	1.806
R _{O-O}			1.288	>1.16	1.309	1.235
Fe-O-O			134.4	<131	130.9	122.5
R _{O1} H (His)					2.611	2.465
R _{O2} H (His)					1.876	1.835
						[2.191] ^g
						[1.939]

^aThe DFT functional used in the geometry optimization is BP, the same is true in the other tables.

^bHigh resolution X-ray crystal structural data on deoxyMb (PDB code: 1BZP) (ref. 64).

^cX-ray structural data on FeTpivPP(1-MeIm)(O₂) [TpivPP = tetra(, , , -orthopivalamide)-phenylporphine] (ref. 65).

^dX-ray structural data on Mb(O₂) (PDB code: 1A6M) (ref. 40).

^eThe distances R_{C1(N4)} N(eq) between the center of the porphyrin ring and the equatorial nitrogen are 2.057 and 2.036 Å for DFT and Exptl, respectively.

^fA negative value denotes an out-of-plane displacement toward the AB ligand.

^gThe values in brackets of [] are the estimated experimental values. The same is true for the other tables.

Table 6

Optimized (with DFT and DFT + E_{disp}) and experimental (X-ray) structural parameters (distance R in Å, bond angle in degree) for Por(NO) and Mb(NO)

	FeP(His)(NO)		Mb(NO)	
	DFT	Exptl ^a	DFT	DFT + E _{disp} Exptl ^b
R _{Fe-N(eq)}	2.017	2.008	2.017	2.018 2.077 (2.00) ^c
R _{Ci(dN) Fe}	-0.071	-0.07	-0.074	-0.071 -0.037
R _{Fe-N(ax)}	2.184	2.180	2.166	2.182 2.179 (2.05)
R _{Fe-N(NO)}	1.734	1.743	1.733	1.728 1.889 (1.75)
R _{N-O}	1.189	1.14	1.197	1.195 1.154 (1.12)
Fe-N-O	141.2	140	139.4	140.9 111.6 (150)
R _{N H (His)}			2.439	2.357 [1.752]
R _{O H (His)}			2.847	3.064 [2.420]

^aX-ray structural data on FeTPP(1-Melm)(NO) (1-Melm = 1-methylimidazole) (ref. 66).

^bX-ray structural data on Mb(NO) (PDB code 1HJT) (ref. 41).

^cThe experimental values in parentheses are those measured by multiple-scattering XAFS method on Mb(NO) in solution (ref. 67).

Table 7

Optimized (with DFT and DFT + E_{disp}) and experimental (X-ray) structural parameters (distance R in Å, bond angle in degree) for H-NOX(O₂)

	H-NOX ^{1U4H} (O ₂)			H-NOX ^{1XBN} (O ₂)		
	DFT	DFT + E _{disp}	Exptl ^a	DFT	DFT + E _{disp}	Exptl ^b
R _{Fe-N(eq)}	2.002	2.001	1.983	2.006	2.008	1.961
R _{Cl(dN)} Fe	-0.035	-0.022	-0.063	-0.016	-0.004	0.104
R _{Fe-N(ax)}	2.044	2.051	2.068	2.066	2.047	1.977
R _{Fe-O}	1.835	1.831	1.814	1.878	1.831	1.821
R _{O-O}	1.321	1.330	1.296	1.331	1.318	1.205
Fe-O-O	128.7	125.7	117.3	113.7	127.5	129.8
R _{O1} H (Tyr140)	2.636	2.448	[2.745]	2.459	2.427	[2.568]
R _{O2} H (Tyr140)	1.741	1.617	[1.829]	1.670	1.676	[1.622]
R _{O1} H (Trp9)	5.333	4.860	[4.675]	7.914	7.819	[8.043]
R _{O2} H (Trp9)	4.301	3.806	[3.782]	7.639	7.606	[8.130]
R _{O1} H (Asn74)	5.604	5.071	[4.219]	10.698	10.203	[7.949]
R _{O2} H (Asn74)	5.286	4.830	[3.825]	10.193	9.662	[7.318]

^aX-ray structural data on H-NOX(O₂) with the PDB code 1U4H (ref. 8).

^bX-ray structural data on H-NOX(O₂) with the PDB code 1XBN (ref. 7).

Table 8

Optimized (with DFT and DFT + E_{disp}) structural parameters (distance R in Å, bond angle in degree) for H-NOX(NO)

	H-NOX^{1U4H}(NO)		H-NOX^{1XBN}(NO)	
	DFT	DFT + E_{disp}	DFT	DFT + E_{disp}
R _{Fe-N(eq)}	2.014	2.012	2.017	2.017
R _{Cl(4N) Fe}	−0.090	−0.078	−0.080	−0.075
R _{Fe-N(ax)}	2.158	2.155	2.166	2.173
R _{Fe-N (NO)}	1.726	1.722	1.727	1.726
R _{N-O}	1.204	1.207	1.201	1.203
Fe-N-O	138.6	136.9	138.0	137.2
R _{N H (Tyr140)}	2.984	2.748	3.085	2.978
R _{O H (Tyr140)}	1.996	1.821	1.972	1.882
R _{N H (Trp9)}	5.954	5.215	9.456	9.422
R _{O H (Trp9)}	4.904	4.124	9.094	9.085
R _{N H (Asn74)}	5.980	5.301	9.518	9.435
R _{O H (Asn74)}	5.437	4.825	8.891	8.830

Table 9

Measured Fe^{II}-His stretching frequencies ($\nu_{\text{Fe-His}}$, cm⁻¹) in different *deoxy* proteins and the coordination numbers (CNs) upon binding of NO to Fe of the heme

	$\nu_{\text{Fe-His}}$ (cm ⁻¹)	CN upon NO binding to Fe	ref.
Hb (R state) () ^a	223	6	68
Hb (T state) ()	207/203	5	68
Hb (R state) () ^b	224	6	68
Hb (T state) ()	220/217	6	68
sGC	203	5	69
FixL	212	5 and 6 mixture	70
Mb	220	6	71
HRP	244	6	72

^a subunit.

^b subunit.