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Resonance Raman Investigation of Nitric Oxide Bonding in Nitrosylhemoglobin A and -myoglobin: Detection of Bound N–O Stretching and Fe–NO Stretching Vibrations from the Hexacoordinated NO–Heme Complex†

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ABSTRACT: With excitation at 406.7 nm, we have observed the resonance Raman enhancement of the bound ν(N–O) stretch at $\sim$1623 cm^{-1} in nitrosylhemoglobin A and nitrosylmyoglobin, indicating the existence of a charge-transfer transition underlying the strong Soret band. The ν(Fe–NO) stretch at 551 cm^{-1} has also been detected in the Soret as well as in the Q-band region, a phenomenon similar to the ν(Fe–O_2) and ν(Fe–CO) stretches in oxy and carbon monoxy hemoproteins. It appears that these iron–ligand vibrations may be resonance enhanced via porphyrin $\pi \rightarrow \pi^*$ transitions. Upon addition of inositol hexaphosphate at pH 6.0, the ν(Fe–NO) stretch at 551 cm^{-1} and a low-frequency mode at 301 cm^{-1} exhibit an intensity decrease by approximately one-half. Contrary to the work of Stong et al. [Stong, J. D., Burke, J. M., Daly, P., Wright, P., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 5815], who employed an excitation wavelength at 454.5 nm, we observed no intensity increase at 592 cm^{-1} attributable to the ν(Fe–NO) stretch from the pentacoordinated NO–heme complex in the α subunits.

Nitric oxide (NO) has been employed as a probe to detect the conformational change of the heme moiety in hemoglobin (Hb) when the quaternary structure is switched from the R to the T form (Rein et al., 1972; Cassoly, 1974; Taketa et al., 1975; Maxwell & Caughey, 1976; Perutz et al., 1976). Electron paramagnetic resonance (EPR) studies revealed that human nitrosylhemoglobin A (nitrosyl-HbA) in the R structure has four hexacoordinated hemes, whereas in the T structure, as induced by inositol hexaphosphate (IHP), it is a hybrid of penta- and hexacoordinated NO–heme complexes (Maxwell & Caughey, 1976; Perutz et al., 1976). Supporting evidence has come from both infrared (IR) and resonance Raman spectroscopic studies. Bound NO in the R structure of nitrosyl-HbA gives rise to a single IR absorption band at 1615 cm^{-1}, characteristic of the ν(N–O) stretch in a hexacoordinated NO–heme, and addition of IHP causes the appearance of a second IR band at 1668 cm^{-1}, characteristic of the ν(N–O) stretch in a pentacoordinated NO–heme (Maxwell & Caughey, 1976). On the other hand, the resonance Raman spectrum of nitrosyl-HbA displays a depolarized porphyrin ring mode at 1633 cm^{-1}; half of its intensity is shifted to 1644

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cm^{-1} upon the addition of IHP (Szabo & Barron, 1975). Indeed, studies of model compounds indicated that the hexacoordinated NO–heme exhibits a depolarized ring mode at $\sim$1637 cm^{-1}, whereas the pentacoordinated NO–heme has the corresponding line at $\sim$1644–1648 cm^{-1} (Spaulding et al., 1975).

Perutz et al. (1976) and Nagai et al. (1978) suggested that the unusual pentacoordinated NO–heme in the T structure occurs mainly in the α subunits. This has been confirmed by Nagai et al. (1980) in their elegant resonance Raman studies of reconstituted nitrosyl-HbA which was labeled in either α or β subunits with the mesodeuterated heme.

Resonance Raman spectroscopy is an ideal tool for locating charge-transfer transitions (Asher et al., 1977; Yu & Tsubaki, 1980; Tsubaki & Yu, 1981; Wright et al., 1979). In resonance with the $\pi \rightarrow \pi^*$ transitions such as the Q and Soret bands, Raman scattering intensities are mainly derived from in-plane porphyrin ring vibrations. However, axial ligand-related vibrations can be enhanced *via* the Franck-Condon and/or Herzberg-Teller vibronic coupling mechanism (Felton & Yu, 1978) by tuning the laser-exciting wavelength into a suitable charge-transfer transition. In this work, we demonstrate that both ν(N–O) and ν(Fe–NO) in the hexacoordinated NO–heme (but not in the pentacoordinated NO–heme) can be resonance enhanced with excitation at 406.7 nm, indicating the existence of a charge-transfer transition involving the π electron on bound NO. The mechanisms for the enhancement of these two modes are discussed in terms of d_π(Fe)–π^*(porphyrin) and d_π(Fe)–π^*(axial ligand) interactions.

Materials and Methods

Preparation of Nitrosylhemoglobin and -myoglobin. Sperm whale myoglobin (Mb) (Sigma Chemical Co.) was purified in the carbon monoxy form as described previously (Tsubaki et al., 1981a). Human hemoglobin A (HbA) was prepared in the oxy form by the usual procedure from whole blood and then was converted to the carbon monoxy form. HbA solution was gel filtered against 1 mM Na_2HPO_4 and deionized by passage through a Dintzis column (Nozaki & Tanford, 1967). Mb and HbA were diluted with the appropriate buffer and then transferred into a rotating quartz Raman cell fitted with a rubber septum. The sample solution was completely carbon monoxygenated and deoxygenated by repeated evacuation and flushing with pure nitrogen gas (Matheson, CP grade). After the final evacuation, nitric oxide (NO) (Matheson) treated with 0.1 N NaOH solution was introduced. The pressure of the NO gas was maintained slightly higher than 1 atm. By this procedure, Mb and HbA can be converted to the nitrosyl form without using sodium dithionite.

Preparation of the Pentacoordinated NO–Heme Model Complex. Hemin (Sigma Chemical Co., bovine type I) was dissolved in a 3% sodium dodecyl sulfate ($NaDodSO_4$) aqueous solution at alkaline pH. The hemin solution was deoxygenated by repeated evacuation and flushing of pure nitrogen gas and then equilibrated with NO gas which had been shaken with 0.1 N NaOH solution. The hemin solution was gradually reduced to produce pentacoordinated (PP)Fe^{II}(NO) model complex (PP = protoporphyrin IX). ^{15}NO gas (Prochem, 95.0% enrichment) was used for isotope substitution experiments for both proteins and NO–heme model complexes.

Measurements of Raman Spectra. The exciting laser line at 406.7 nm was provided by a Spectra-Physics Model 171–01 krypton ion laser, and a 90° scattering geometry was used. The sample in the rotating Raman cell was kept spinning during the measurements to reduce local heating and photodissociation of bound NO. The Raman spectra were obtained by using a highly sensitive multichannel Raman system, which has been described in detail previously (Yu & Srivastava, 1980).

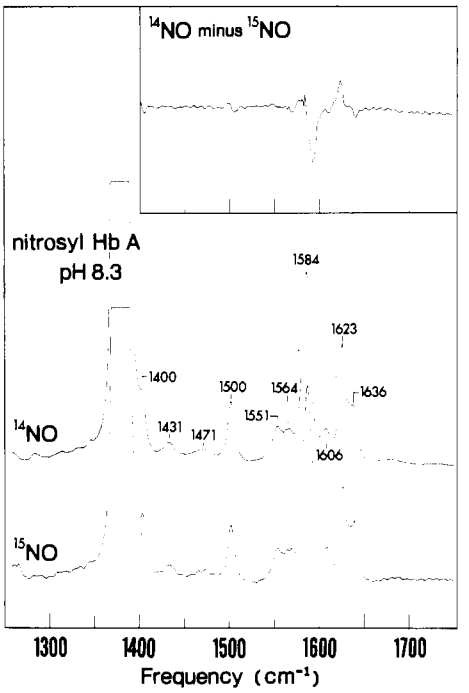

FIGURE 1: Higher frequency region (1250–1750 cm^{-1}) spectra of nitrosyl-HbA ($\sim$60 μM, heme basis) in 0.05 M Tris-HCl, pH 8.3, buffer. Raman spectra of Hb$A^{14}NO$ (upper spectrum) and Hb$A^{15}NO$ (lower spectrum) are shown, and their computer-subtracted difference spectrum is presented in the inset on the same scale. Conditions were the following: excitation wavelength, 406.7 nm; excitation laser power, 20 mW; slit width, 100 μm; slit height, 0.2 cm; delay, 10000 (303 s).

Results and Discussion

Higher Frequency Region and Bound ν(N–O) Stretch. Resonance Raman spectra (1250–1750 cm^{-1}) of nitrosyl-HbA (^{14}NO and ^{15}NO) are presented in Figure 1 (without IHP) and Figure 2 (with IHP). The insets show the difference spectra (^{14}NO minus ^{15}NO) in the 1400–1750-cm^{-1} region.

The spectral features here are considerably different from those reported previously (Szabo & Barron, 1975; Scholler et al., 1979; Stong et al., 1980; Nagai et al., 1980) by using excitation wavelengths between the Soret and the β band. With Soret excitation at 406.7 nm, polarized porphyrin ring modes dominate the spectra as in the cases of oxidized hemoglobin and myoglobin derivatives (Tsubaki et al., 1981a). Upon addition of IHP, both Hb$A^{14}NO$ and Hb$A^{15}NO$ spectra (Figure 2) exhibit a weak line at 1647 cm^{-1}, which presumably corresponds to the depolarized ring mode from pentacoordinated NO–heme (Szabo & Barron, 1975). In addition, we detected a slight broadening of the 1500-cm^{-1} line on the high-energy side (Figure 2) upon conformational change from R to T, consistent with that reported by Nagai et al. (1980). However, these spectral changes upon the addition of IHP are less pronounced than those reported previously, indicating that with excitation at 406.7 nm the intensity of resonance Raman scattering from the pentacoordinated NO–heme is weaker than that from the hexacoordinated NO–heme.

The absorption spectral change induced by IHP is far greater for nitrosyl-HbA than for any other hemoglobin derivatives reported so far. The Soret band reduces its intensity by 23% and is slightly blue shifted by 1.5 nm (Pertuz et al., 1976). Studies of hybrid nitrosyl-HbA containing mesoheme

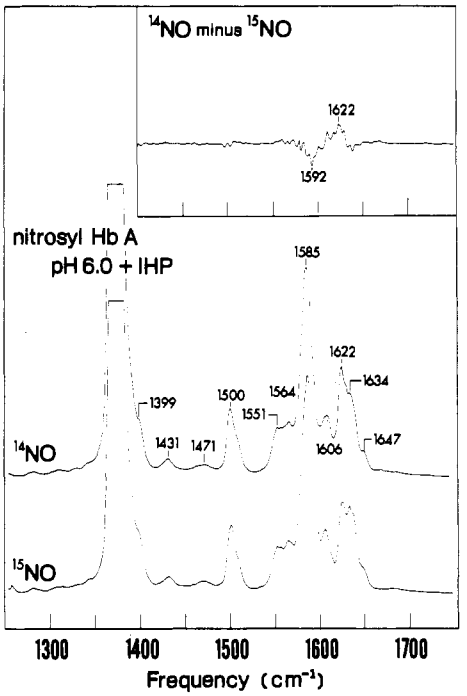

FIGURE 2: Higher frequency region (1250–1750 cm^{-1}) spectra of nitrosyl-HbA (~60 μM, heme basis) in 0.05 M citrate–phosphate, pH 6.0, buffer in the presence of IHP (3.8 mM). Raman spectra of HbA^{15}NO + IHP (upper spectrum) and HbA^{15}NO + IHP (lower spectrum) are shown, and their computer-subtracted difference spectrum is presented in the inset on the same scale. Other conditions are the same as in Figure 1.

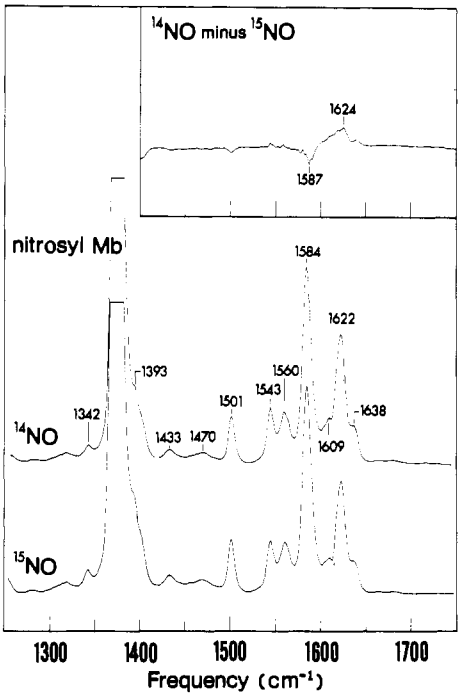

FIGURE 3: Higher frequency region (1250–1750 cm^{-1}) spectra of nitrosyl-Mb (~60 μM, heme basis) in 0.05 M Tris-HCl, pH 8.4, buffer. The computer-subtracted difference spectrum (Mb^{14}NO minus Mb^{15}NO) is presented in the inset on the same scale. Other conditions are the same as in Figure 1.

subunits and protoheme subunits showed that the reduction in intensity of the Soret band is associated almost exclusively with the α(NO) subunits (Nishikura & Sugita, 1976). Comparison of optical absorption spectra between NO-TP-P-Fe^{II} and NO-TPP-Fe^{II}-Im [nitrosyl($\alpha,\beta,\gamma,\delta$-tetraphenylporphinato)(1-methylimidazole)iron(II)] revealed that the Soret band is blue shifted by as much as 10 nm and reduced in intensity by over 40% in the pentacoordinated complex, as compared to the hexacoordinated one (Perutz et al., 1976).

Careful comparison of the spectra between HbA^{14}NO and HbA^{15}NO in Figure 1 (without IHP) and Figure 2 (with IHP) reveals small but definite spectral changes around the 1580–1630-cm^{-1} region, which are manifested as a positive peak at ~1622 cm^{-1} and a negative peak at ~1592 cm^{-1} in the difference spectra (Figures 1 and 2, insets). These two frequencies are appropriate for the assignments of ν(^{14}N–O) and ν(^{15}N–O) stretches, although they are slightly higher (5 cm^{-1} for HbA^{14}NO and 3 cm^{-1} for HbA^{15}NO) than those identified by IR difference spectroscopy (Maxwell & Caughey, 1976). Thus, resonance Raman enhancement of the bound ν(N–O) stretching vibration from hexacoordinated NO–heme can be clearly established. Additional supporting evidence is presented in Figure 3, where the difference spectrum between Mb^{14}NO and Mb^{15}NO also exhibits a positive peak at 1624 cm^{-1} and a negative peak at 1587 cm^{-1}.

However, we could not detect any resonance enhancement of the bound ν(N–O) stretching frequency derived from pentacoordinated NO–heme, which is expected to appear at 1668 cm^{-1} for HbA^{14}NO and at 1615 cm^{-1} for HbA^{15}NO (both with IHP at pH 6.0) according to IR spectroscopy (Maxwell & Caughey, 1976). To confirm this spectroscopic property of pentacoordinated NO–heme, we have carried out resonance Raman studies of the pentacoordinated (PP)Fe^{II}-(^{14}NO) (PP = protoporphyrin IX) model complex in 3% NaDodSO$_4$ aqueous solution at alkaline pH. However, the

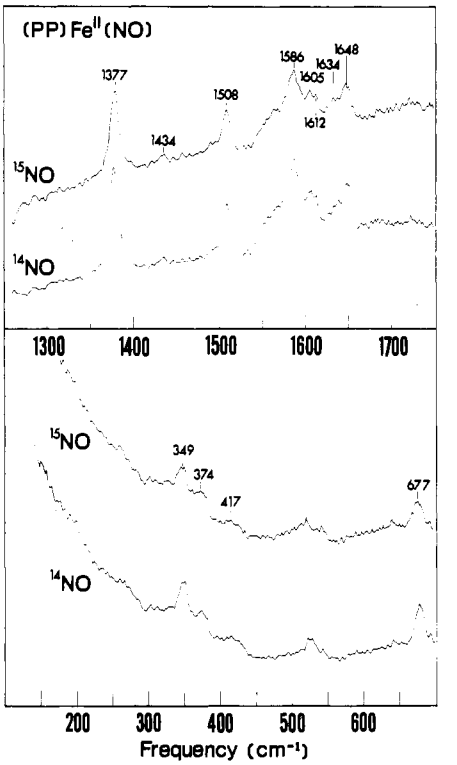

FIGURE 4: (Upper panel) Higher frequency region (1250–1750 cm^{-1}) spectra of pentacoordinated (PP)Fe^{II}(^{14}NO) (lower spectrum) and (PP)Fe^{II}(^{15}NO) (upper spectrum) (PP = protoporphyrin IX) model complexes in 3% NaDodSO$_4$ aqueous solution at alkaline pH. (Lower panel) Lower frequency region (100–700 cm^{-1}) spectra of pentacoordinated (PP)Fe^{II}(^{14}NO) (lower spectrum) and (PP)Fe^{II}(^{15}NO) (upper spectrum) model complexes in 3% NaDodSO$_4$ aqueous solution at alkaline pH. Laser power was 16 mW; other conditions are the same as in Figure 1.

results are inconclusive because of slight background fluorescence. The spectra of both (PP)Fe^{II}(^{14}NO) and (P-

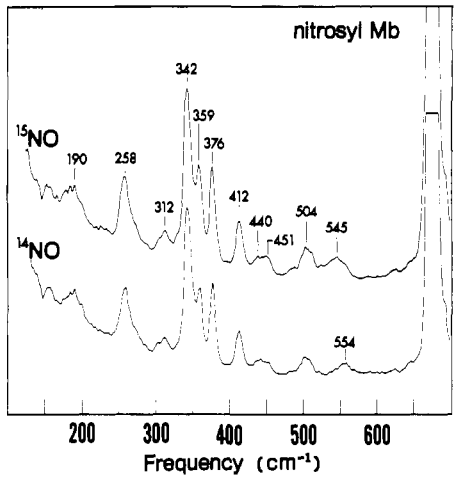

FIGURE 5: Lower frequency region (100–700 cm^{-1}) spectra of nitrosyl-Mb in 0.05 M Tris-HCl, pH 8.4, buffer. Other conditions are the same as in Figure 1.

P)Fe^{II}(^{15}NO) (Figure 4, upper panel) are very similar to those excited at 488.0 nm (Nagai et al., 1980).

Lower Frequency Region and ν(Fe–NO) Stretch. Desbois et al. (1979) reported the lower frequency region spectra of nitrosyl-Mb with excitation at 441.6 nm; however, their spectrum was later found to contain a significant amount of deoxy (or photolyzed) Mb (Tsubaki et al., 1981a). With excitation at 441.6 nm, even a slight degree of photodissociation of nitrosyl-Mb can affect the spectrum considerably because of the closer proximity of the excitation wavelength to the Soret maximum (430 nm) of deoxy-Mb. However, upon excitation at 406.7 nm, such photodissociated Mb does not contribute significantly to the spectrum because the resonance Raman intensity from nitrosyl-Mb is far greater than that from deoxy-Mb. The spectra (100–700 cm^{-1}) of nitrosyl-Mb excited at 406.7 nm, as shown in Figure 5, are quite different from those reported by Desbois et al. (1979) and are similar to those of oxy-Mb and (carbon monoxy)Mb except for several ligand-specific vibrations (Tsubaki et al., 1981b).

Similarities also exist between the spectrum of nitrosyl-HbA (Figure 6) and that of oxy-HbA excited at 413.1 nm (Yu & Srivastava, 1980) except for the ν(Fe–NO) and ν(Fe–O_2) stretches at 551 and 570 cm^{-1}, respectively. Thus, the spectral features related to the porphyrin ring vibrations in "ferric-like" reduced hexacoordinated hemoglobins and myoglobins [such as oxy-Hb, oxy-Mb, (carbon monoxy)Hb, (carbon monoxy)-Mb, nitrosyl-Hb, and nitrosyl-Mb] are very similar to those of the respective low-spin oxidized hemoprotein derivatives.

Resonance enhancement of the ν(Fe–NO) stretch was first reported by Chottard & Mansuy (1977), who observed a weak line in the Raman spectrum of nitrosyl-HbA at 549 cm^{-1}, which shifted to 539 cm^{-1} upon ^{14}NO → ^{15}NO substitution. This was confirmed by Stong et al. (1980), although their frequencies at 553 (HbA^{14}NO) and 546 cm^{-1} (HbA^{15}NO) were somewhat higher. As shown in Figure 6, we detected the ν(Fe–NO) stretch at 551 cm^{-1}, which shifts to 545 cm^{-1} by ^{15}NO substitution. In the case of nitrosyl-Mb (Figure 5), the corresponding lines appear at 554 (Mb^{14}NO) and 545 cm^{-1} (Mb^{15}NO).

Although Stong et al. (1980) reported the observation of an additional ν(Fe–NO) stretch at 592 (HbA^{14}NO with IHP) or 589 cm^{-1} (HbA^{15}NO with IHP) (both excited at 454.5 nm), we could not detect any corresponding Raman line except the one at 589 cm^{-1}, which is apparently a porphyrin ring mode (Figure 6). Indeed, this 589-cm^{-1} line can be observed in the spectra of other ligated HbA derivatives in both reduced and

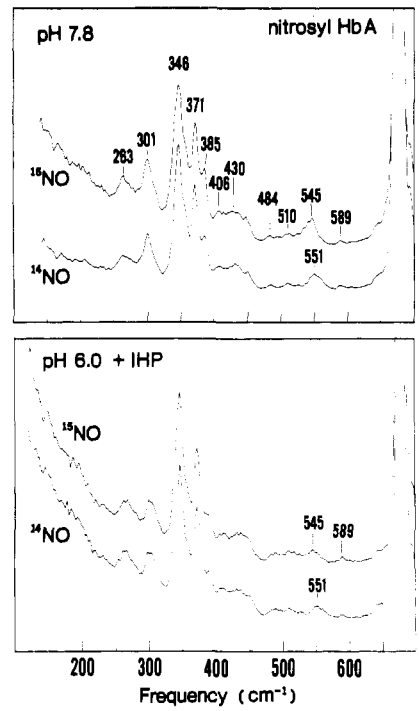

FIGURE 6: Lower frequency region (100–700 cm^{-1}) spectra of nitrosyl-HbA in 0.05 M Tris-HCl, pH 7.8, buffer (upper panel) and in 0.05 M citrate–phosphate, pH 6.0, buffer in the presence of IHP (3.8 mM) (lower panel). Other conditions are the same as in Figure 1.

oxidized states upon Soret excitation (Yu & Srivastava, 1980; Tsubaki et al., 1981b). Close examination of lower frequency region spectra reveals the intensity reduction of the ν(Fe–NO) stretch at 551 cm^{-1} and a low-frequency mode at 301 cm^{-1} upon addition of IHP (Figure 6). This is consistent with the results from the higher frequency region spectra; i.e., only hexacoordinated NO–heme gives rise to a resonance-enhanced ν(N–O) stretch at ~1622 cm^{-1} upon Soret excitation. Likewise, the ν(Fe–NO) stretching vibration from pentacoordinated NO–heme in half of the subunits of nitrosyl-HbA (with IHP) is no longer enhanced with Soret excitation at 406.7 nm. The frequency at 301 cm^{-1} appears too high for the Fe–N_ϵ(His) stretch (Scheidt et al., 1977).

The lower frequency region spectra of pentacoordinated (PP)Fe^{II}(^{14}NO) and (PP)Fe^{II}(^{15}NO) as shown in Figure 4 (lower panel) exhibit no isotope-sensitive line near 590 or 480 cm^{-1}; the latter frequency was identified as the ν(Fe–^{14}NO) stretch in pentacoordinated nitrosyl protoheme IX dimethyl ester in the solid state by IR spectroscopy (Maxwell & Caughey, 1976).

As mentioned in the preceding paper (Tsubaki et al., 1981b), the Fe–ligand stretching vibrations in oxy-Hb, (carbon monoxy)Hb, and nitrosyl-Hb can be resonance enhanced with the excitation wavelength in both Soret and Q-band regions. Furthermore, the Raman excitation profiles follow the respective absorption spectra from the Soret to the α band. This can be explained by the fact that the electronic structure change in the porphyrin ring upon laser excitation (i.e., $\pi \rightarrow \pi^*$ transition) affects the d_π(Fe)–π^*(ligand) interaction indirectly through the d_π(Fe)–π^*(porphyrin) interaction. The mechanism of resonance Raman enhancement of the Fe–ligand stretching vibration is, therefore, similar for all the ferric-like reduced state hexacoordinated hemoproteins.

When the iron atom moves out of the porphyrin plane upon the breakage or elongation of Fe–N_ϵ(proximal His), the interaction between d_π(Fe) and π^*(porphyrin) becomes so weak due to a large separation in energy between them that the d_π(Fe)–π^*(ligand) interaction is no longer influenced by

electronic promotion ($\pi \rightarrow \pi^*$) within the porphyrin ring. This might be the reason that we have not observed the enhancement of the ν(Fe–NO) stretch in pentacoordinated NO–heme upon Soret excitation. The excitation profile of the 592-cm^{-1} line detected by Stong et al. (1980) in nitrosyl-HbA with IHP should resolve the question of whether it is enhanced *via* a charge-transfer transition.

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Kinetic Isotope Effects in the Oxidation of Isotopically Labeled NAD(P)H by Bacterial Flavoprotein Monooxygenases†

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ABSTRACT: Three bacterial flavoprotein monooxygenases, *p*-hydroxybenzoate hydroxylase, orcinol hydroxylase, and salicylate hydroxylase, have been examined for steady-state kinetic isotope effects with (4*R*)-[4-^{2}H]NAD(P)H and (4*R*)-[4-^{3}H]NAD(P)H. The observed isotope selections are for deuterium, ^DV = 1.8–3.5 and $^D(V/K)$ = 1.7–5.1, and for tritium, $^T(V/K)$ = 5–19. For both orcinol hydroxylase and *p*-hydroxybenzoate hydroxylase, reduction of enzyme-bound FAD by (4*R*)-[4-^{2}H]NAD(P)H in pre-steady-state assays reveals intrinsic deuterium isotope effects of 10 ± 2 on this redox step. These values are at the upper end of substrate deuterium effects seen in enzymatic reactions. Suppression of ~83% of the intrinsic isotope effects in the overall reaction rate (e.g., k_H/k_D = 10 down to ^DV = 2.5) corroborates earlier kinetic data on *p*-hydroxybenzoate hydroxylase [Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254*, 6657] and suggests that these bacterial phenolic monooxygenases balance out internal transition states such that no single barrier is fully rate limiting.

In reactions involving transformations where C–H bonds break at some stage during catalysis, substitution of deuterium or tritium for hydrogen permits mechanistic analysis based on the consequent kinetic isotope effect. Most enzymatic studies using isotopically labeled substrates have focused on steady-state kinetics and measurement of observed isotope effects on overall turnover (on V_{max} or on V_{max}/K_m).[1] The observed isotope effects can vary from full expression of the

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[1] The following definitions shall be used throughout this paper: ^DV indicates a deuterium isotope effect observed on overall turnover in V_{max}; $^D(V/K)$ represents the observed deuterium isotope effect on V_{max}/K_m, determined from comparing overall rates with deuterated and unlabeled substrate; $^T(V/K)$ represents a tritium isotope effect observed on overall turnover in V_{max}/K_m, determined in an internal competition experiment with tritiated substrate; k_H/k_D or ^Dk indicates the intrinsic or primary deuterium isotope effect; similarly, k_H/k_T is the intrinsic or primary tritium isotope effect.

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