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CHARACTERIZATION OF INTERACTIONS OF NITRIC OXIDE WITH HUMAN HEMOGLOBIN A BY INFRARED SPECTROSCOPY

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Infrared spectra permit direct measurements of cysteine thiols as well as nitric oxide bound to heme iron in human hemoglobin A nitrosyl. A single symmetric N-O stretch band of nitric oxide bound to Fe^{2+} is detected amid strong water and protein bands in the $Hb^{14}N^{16}O$ minus $Hb^{15}N^{16}O$ difference spectrum. Nitric oxide accepts electron density from metal in bent-end-on Fe^{2+}-^{14}N -^{16}O (ν_{NO}=1616.5 cm^{-1}) and donates electron density to metal in linear Fe^{3+}-^{14}N -^{16}O (ν_{NO}=1925 cm^{-1}). S-H stretch bands reveal that changes in protein conformation occur at α-104, β-93, and β-112 cysteines upon conversion of deoxyHb to HbNO but that no reactions of thiols with NO occur. Furthermore, no infrared band for S-nitrosothiol is detected. Changes in amide I spectra reflect NO binding induced changes in protein secondary structure.

Nitric oxide (NO) was denoted "Molecule of the Year" in 1992 by Science because of its recently recognized roles in areas that include blood pressure regulation, antimicrobial defense, and neuronal communication (1-4). An obviously important but still incompletely understood reaction of NO is its binding to heme iron with activation of cytosolic guanylate cyclase (5), a reaction prevented by NO binding to hemoglobin. The reaction of a protein thiol with NO to form an S-nitrosothiol is also considered important in vivo (6,7). Infrared spectra are potentially useful for the direct observation and characterization of FeNO, -SH, and -SNO groups in proteins (8-12). Here we report an infrared study of interactions of NO with human hemoglobin A.

Materials and Methods

Oxyhemoglobin A from normal adult human blood was purified to remove other proteins as well as organic phosphates and other small molecules (13). Prior to use 5% $^{14}N^{16}O/95\%N_2$ (technical grade, Air Products and Chemicals, Inc.) and $^{15}N^{16}O$ (99%, Cambridge Isotope Laboratories) were treated with 1 M aqueous KOH to remove NO_2 (14).

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A solution of HbO_2 9 mM in heme was prepared in H_2O-based 200 mM sodium phosphate buffer pH 7.2. To obtain a deoxyHb solution, the HbO_2 solution was degassed thoroughly with N_2 followed by addition of sodium dithionite to a concentration of 18 mM. Exposure of the deoxyHb solution to excess $^{14}N^{16}O$ or $^{15}N^{16}O$ yielded the nitrosyl complex as shown by visible/Soret spectra (maxima at 573.5, 545 and 418 nm). MetHbNO was obtained by introduction of a small amount of O_2 into the $Hb^{14}N^{16}O$ solution with excess NO present; the $Fe^{3+}NO$ infrared band exhibits $\nu_{NO}=1925$ cm^{-1} and $\Delta\nu_{1/2}= 9$ cm^{-1}. Similar exposure to CO (99.5%, General Air) yielded HbCO.

Infrared spectra of the Hb solutions were measured at 20° C in Beckman FH-01 cells with CaF_2 windows using a Perkin Elmer Model 1800 Fourier transform spectrophotometer with a Hg/Cd/Te detector and 7700 computer. Pathlengths were 6 μm for measurements in the 1700 to 1400 cm^{-1} region and 100 μm for the 2500 to 2620 cm^{-1} region. 1000 scans at a resolution of 2 cm^{-1} were averaged for each spectrum (10). Deconvolution of infrared bands were carried out using the CURVEFIT function of Spectra Calc Software (Galactic Industries Corp.).

Results and Discussion

Measurement of N-O stretch bands. A band for $HbFe^{2+}NO$ near 1615 cm^{-1} could be detected with a dispersive spectrometer by use of D_2O-based solutions (8). The accurate measurement of this band in water is complicated by the strong bands from both water and protein in the same region of the spectrum (10). The much greater sensitivity of the FTIR spectrometer used here permitted measurements in H_2O-based solutions by use of a cell pathlength (6 μm) short enough to let sufficient light through the cell and by careful subtraction of spectra due to protein, liquid water, and gaseous water (15). Success in these subtractions depends critically on the choice of reference spectrum. The most accurate N-O band measurements were obtained from difference spectra of carefully matched solutions of $Hb^{14}N^{16}O$ and $Hb^{15}N^{16}O$ (Fig.1). Use of HbCO as a reference spectrum gave a spurious band at 1608 cm^{-1} (Fig. 1). With deoxyHb as reference, many spurious bands appeared in difference spectra (not shown). These spurious bands arise from differences in protein structure for HbNO, HbCO, and deoxyHb (10). The marked differences in amide I spectra between deoxyHb and HbNO show NO binding alters protein secondary structure whereas the 1608 cm^{-1} band in HbNO minus HbCO spectra is in a region between amide I and amide II bands and is, therefore, likely due to a change in location of an amino acid side chain (10,15). The distinct disadvantage of D_2O-based solutions due to effects of different extents of D/H exchange on protein amide I and II spectra make it important to use H_2O-based solutions (8,15).

Parameters of N-O bands observed in isotope difference spectra are in Table 1. The isotope shift in ν_{NO} is of the magnitude expected whereas the slightly greater width and B value of the $^{15}N^{16}O$ band suggests this band may reflect a small amount of

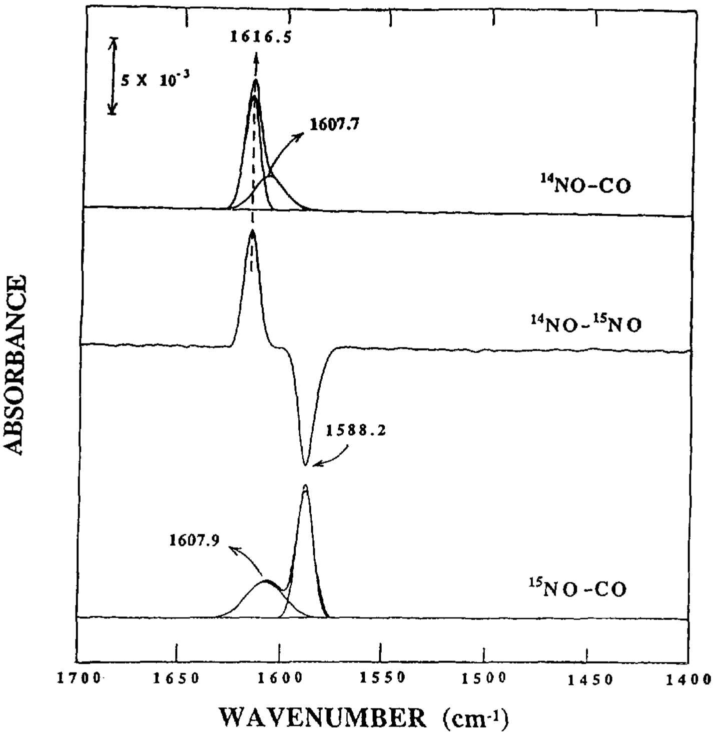

Figure 1. *Difference infrared spectra between solutions of $Hb^{14}N^{16}O$, $Hb^{15}N^{16}O$, and HbCO.* Deconvolution of the $Hb^{14}N^{16}O$ minus HbCO spectrum (top) gives one band with $\nu=1616.5$ cm^{-1} ($\Delta\nu_{1/2}=8.9$ cm^{-1}) and another with $\nu=1607.7$ cm^{-1} ($\Delta\nu_{1/2}=18.0$ cm^{-1}) representing 36% of total band area. The $Hb^{15}N^{16}O$ minus HbCO spectrum (bottom) deconvolutes into a band with $\nu=1588.2$ cm^{-1} ($\Delta\nu_{1/2}=9.9$ cm^{-1}) and a second band with $\nu=1607.9$ cm^{-1} ($\Delta\nu_{1/2}=20.0$ cm^{-1}) representing 38% of the total band area. The major band in each case corresponds closely to the N-O bands of the $Hb^{14}N^{16}O$ minus $Hb^{15}N^{16}O$ spectrum (middle) (Table 1).

vibrational coupling between bound $^{15}N^{16}O$ and adjacent vibrators that does not occur with $^{14}N^{16}O$. Furthermore, the $^{14}N^{16}O$ band shape fits a single band of 100% Gaussian character very closely whereas the $^{15}N^{16}O$ band fits such a shape a little less closely. More extensive vibrational coupling is found in O-O stretch bands of HbO_2 (16-18).

Implications of N-O stretch bands re protein structure. The NO bands provide information on the nature of the bonding between iron and NO ligand, on the immediate environment of NO, and on effects of NO bonding to heme iron on protein conformation. The single symmetric band character of the $^{14}N^{16}O$ band indicates little, if any, differences in bonding to α and β subunits occur and gives no evidence for multiple protein conformers as found for HbCO (19). The narrow NO band width ($\Delta\nu_{1/2}$) indicates the immediate environment about the N-O vibrator is consistent with similar immobile environments at each of the four NO binding sites (8,19,20). Band frequencies (ν_{NO}) indicate a marked change in N-O bond order occurs upon bonding NO to Fe. The ν_{NO}

Table 1. *N-O Stretch Band Parameters for $^{14}N^{16}O$ and $^{15}N^{16}O$ Hemoglobin A Nitrosyls*[a]

Nitrosyl isotope	ν_{NO}[b] (cm^{-1})	$\Delta\nu_{1/2}$[c] (cm^{-1})	ϵ[d] ($mM^{-1}cm^{-1}$)	B[e] ($mM^{-1}cm^{-2}$)
$^{14}N^{16}O$	1616.5 ± 0.1	8.9 ± 0.2	1.5 ± 0.1	13.8 ± 0.1
$^{15}N^{16}O$	1588.1 ± 0.1	9.9 ± 0.2	1.5 ± 0.1	16.2 ± 0.1

(*a*) Parameter values are averages from $Hb^{14}N^{16}O$ minus $Hb^{15}N^{16}O$ spectra from four independent experiments. (*b*) Wavenumber at band maximum. (*c*) Bandwidth at one-half intensity. (*d*) Extinction coefficient at ν_{NO}. (*e*) Integrated band area.

for $^{14}N^{16}O$ as a gas is 1876 cm^{-1} compared with 1616.5 cm^{-1} for HbNO. This decrease of 260 cm^{-1} denotes a marked reduction in N-O bond order and the transfer of electron density from Fe^{2+} to NO to give a bent-end-on stereochemistry (8,9). In contrast, when $^{14}N^{16}O$ binds to the Fe^{3+} of metHb, ν_{NO} becomes 1925 cm^{-1} due to electron density transfer in the opposite direction, i.e., from NO to Fe^{3+}, to give linear Fe-N-O bonding. Thus, the optimum ligand site pocket provided by protein is different for HbNO and metHbNO.

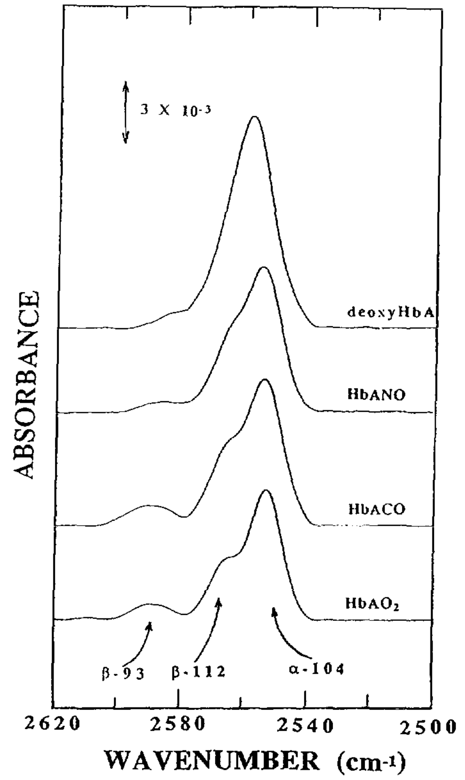

Figure 2. *Infrared spectra of cysteine thiol groups in deoxy, nitrosyl, carbonyl, and oxy species of human hemoglobin A* .

Table 2. *Deconvoluted S-H infrared stretch band parameters of deoxy, nitrosyl, carbonyl, and oxy species of hemoglobin A.* Parameter values represent averages from three independent experiments.

Cysteine	Ligand	ν_{SH}^{a} (cm^{-1})	$\Delta\nu_{½}$^{b} (cm^{-1})	ε^{c} ($mM^{-1}cm^{-1}$)	B^d ($mM^{-1}cm^{-2}$)
α-104	none	2556.4^e	14.2^e	0.137^e	2.01^e
	NO	2554.6	13.0	0.157	2.11
	CO	2553.8	13.5	0.164	2.30
	O_2	2553.2	12.4	0.148	1.89
β-112	none	2562.8^e	15.6^e	0.091^e	1.46^e
	NO	2566.0	12.1	0.073	0.91
	CO	2566.6	12.3	0.079	1.01
	O_2	2566.3	11.1	0.061	0.71
β-93	none	2577.7^f	17.0^f	0.008^f	0.15^f
	NO	2585.0	17.4	0.011	0.21
	CO	2589.3	16.8	0.025	0.43
	O_2	2588.8	13.8	0.017	0.25

(*a*) Center band frequency. Standard deviations were ca ± 0.1 cm^{-1} except for deoxyHb values. (*b*) Width at one-half band height. Standard deviations were ca ± 0.1 cm^{-1} except for deoxyHb. (*c*) Extinction coefficient. Standard deviations were ca ± 0.001 $mM^{-1}cm^{-1}$ except for deoxyHb. (*d*) Integrated band intensity. Standard deviations were ca ± 0.01 $mM^{-1}cm^{-2}$ except for deoxyHb values. (*e*) Standard deviations can not be reliably estimated (see Methods). (*f*) Standard deviations of ca ± 0.5 cm^{-1} were obtained for ν_{SH} and $\Delta\nu_{½}$, ± 0.005 $mM^{-1}cm^{-1}$ for ε, and ± 0.02 $mM^{-1}cm^{-2}$ for B.

Measurement of S-H stretch bands. The cysteine S-H stretch bands near 2560 cm^{-1} are in a region of the infrared spectrum where neither water nor protein absorbs strongly (Fig. 2) (10,21). Therefore long pathlength cells (100 μm) may be used to measure these weak bands. The NO, CO, and O_2 complexes give spectra with bands for each type of cysteine that can be accurately deconvoluted to give the parameters of Table 2. The deoxyHb spectrum reveals a readily detected β-93 band but the α-104 and β-112 bands overlap too closely to be resolved accurately. However, the parameters given for deoxyHb in Table 2 are reasonable estimates and result in a good fit to the observed spectrum.

Implications of S-H bands re interactions between NO and hemoglobin A. The thiol infrared spectra provide direct evidence of preservation of the -SH groups in the presence of excess NO. Also, there is no evidence in Fig. 1 for bands due to -SNO in the 1400 to 1500 cm^{-1} region where such bands are expected (12). However, it is unlikely for the reaction of -SH + NO to give -SNO to occur in the reducing environment provided by the dithionite in our solutions since the reaction requires an oxidant. However, the -SH spectra show that ligation of NO to the hemes of deoxyHb alters the protein conformation at the thiols of all three cysteines (Fig. 2, Table 2). The binding of either O_2 or CO to deoxyHb also affects the three SH bands.

Conclusions

NO infrared spectra demonstrate binding of NO to both reduced and oxidized heme iron, provide insight into the stereochemistry and bonding in the Fe-N-O linkages, establish that the bonding is very similar at the α and β subunit hemes and that ligand sites in HbANO exist in only one detectable conformer in contrast to the multiple protein conformers of HbACO and $HbAO_2$ (16,19). The high sensitivity of N-O stretch bands in model heme nitrosyls to changes in porphyrin side chains, trans ligand, and medium suggest these spectra can provide direct quantitative and qualitative information on NO binding to metal sites in proteins in general (8,9). S-H stretch bands showed no -SH groups were lost upon exposure of deoxyHbA to NO and no infrared evidence of S-nitrosothiols formation was obtained. However, each type of S-H band shifted position due to changes in protein environment at the thiols, presumably as a result of NO binding to heme iron. These findings show the utility of infrared spectra for monitoring directly the presence and environment of thiols and the formation of S-nitrosothiols in proteins. Finally, the changes in protein amide I infrared bands permit characterization of the NO binding induced changes in protein secondary structure (10,15).

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