Conformational Restriction of Cysteine-Bound NO in Bovine Serum Albumin Revealed by Circular Dichroism

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Nitric oxide (NO) has recently been shown to play many intriguing roles in bioregulation. It is thought that NO binds to thiols on messengers and acceptors and that protein or peptide nitrosothiols (RS−NOs) serve a pivitol role in regulating NO reactivity and toxicity.[1−6] We present in situ characterization of RS−NO by circular dichroism (CD). We demonstrate that NO bound to the single free thiol of cysteine (Cys), glutathione (GSH), or bovine serum albumin (BSA) is oriented with respect to the asymmetric center of the ligated amino acid. We also show that BSA conformational change is correlated with change in the RS−NO CD signal. We associate this with a change in NO packing between helices as a function of pH. This work provides novel, direct spectroscopic characterization of NO restriction within BSA. The protein studied, BSA, serves as a reservoir for NO control of vasodilation.[3]

Circular dichroism is widely used to characterize the secondary structure of biomolecules, where strong magnetooptical signatures of peptide backbone conformation reveal, for example, the percentage of α-helix, β-sheet, or random coil. However, the spectral overlap of the backbone CD signatures often limits the analytical usefulness of the technique. CD is an ideal tool for studying an active, spectrally isolated chromophore, and we now show that primary nitrosothiols are unusually suitable subjects.

Figure 1a shows the CD spectrum of nitrosylated cysteine (Cys−NO) and glutathione (GS−NO).[7] A strong magnetooptic response is seen at 550 nm. Another band, not shown, exhibits the opposite Cotton effect at 335 nm. The linear absorption spectrum (not shown) indicates the 550 nm band is nearly forbidden while the 335 nm transition is electric dipole allowed (14 and 725 cm^{-1} M^{-1} in nitrosocysteine and 16 and 890 cm^{-1} M^{-1} in nitrosoglutathione, respectively). On the other hand, the 550 nm band in the CD spectrum is stronger than the 335 nm band (0.22 and 0.09 cm^{-1} M^{-1} in nitrosocysteine and 0.24 and 0.16 cm^{-1} M^{-1} in nitrosoglutathione, respectively) and is well separated from other protein CD signals. These transitions are largely n−π* and involve states derived from nonbonding electron atomic orbitals of nitrogen and sulfur, with some σ_{SN} character.[8−10] There is little structure in the 335 nm band, but much more is evident in the 550 nm band. The vibronic bands seen with ca. 1300 cm^{-1} spacing reflect the NO vibrational frequency in the dissociative excited state. Photochemical cleavage of the S−N bond results from photoexcitation into either band, and the subbands seen at 550, 515, and 485 nm are primarily lifetime broadened.[7]

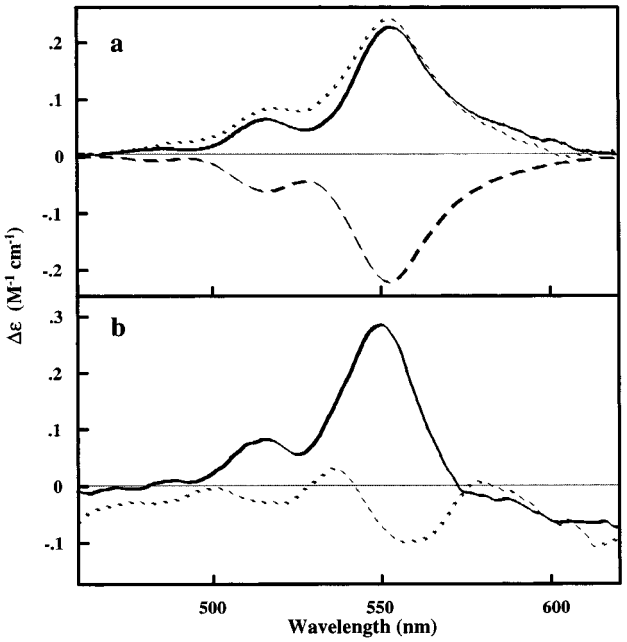

Figure 1. (a) UV−vis circular dichroism spectra of 10 mM nitroso-L-cysteine (solid), nitroso-D-cyteine (dashed), and nitrosoglutathione (dotted) at pH = 2.5. (b) Circular dichroism spectrum of 700 μM BSA−NO at pH = 2.5 (solid) and pH = 7.0 (dotted). The pH-dependent BSA−NO spectrum is collected using the same sample by raising and lowering the pH using 0.1 M HCl and 0.1 M NaOH. The signal reversibly switches between positive and negative by raising and lowering the pH. The BSA−NO signal cross section allows the identification of 5 μM BSA−NO with a signal-to-noise ratio of 20:1 within 2 min.

As neither S nor N itself is a chiral center, we next consider the origin of the magnetooptical signal. Figure 1 shows the CD response of nitroso-D-cysteine, nitroso-L-cysteine, and nitroso-bovine serum albumin. The 550 nm band in the D-enantiomer maintains exactly the same shape and absolute amplitude as the L-enantiomer, but the signal is inverted. This shows that the magnetooptical signal derives from the influence of the nearby chiral center environment. The exact nature of this influence has not yet been quantified, but either one-electron or coupled chromophore effects could be involved. The absence of nearby, strongly dipole-allowed transitions further suggests that the rotational strength does not derive from $\mu\cdot\mu$ terms but rather from $\mu\cdot m$ terms in the rotational strength; the observed transition is much more strongly magnetically allowed than it is electrically dipole allowed.

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(7) Nitrosylated cysteine and glutathione are prepared by combining 100 mM aqueous solutions with an equimolar solution of $NaNO_2$ in 0.5 M HCl (pH = 3.0). The peptides become red/orange immediately upon nitrosylation. Nitrosylated BSA is prepared at 700 μM protein concentration and combined with 1-, 2-, or 8-fold molar excesses of $NaNO_2$ in 0.5 M HCl. The BSA is incubated with the $NaNO_2$ at pH = 3.5−4 for 1 h. The pH is adjusted between pH = 2.5 and 7 using 0.5 M NaOH or 0.5 M HCl. The BSA turns yellow within 1 h of incubation in 2- and 8-fold excesses of $NaNO_2$, indicative of modification of other amino acids, such as tyrosine, tryptophan, and phenylalanine. We see no evidence of irreversible denaturation as Era et al. report (ref 17) for acid-induced changes in BSA. We see no gelation or particulates (evident by light scattering in the UV−vis and CD measurements). Also, the BSA−SNO CD signal reversibly changes between postive and negative values as the pH is raised, lowered, and raised again. The ultraviolet−visible absorption spectra are collected on a Perkin-Elmer Lambda-9 spectropolarimeter. The UV−vis circular dichroism is performed on a nitrogen-purged, Jasco J-710 polarimeter, calibrated with 0.06% (w/v) camphor−sulfonate solution.

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The CD spectra of cysteine NO and glutathione NO show qualitatively similar molar absorbances with relatively little change in bandwidth or peak frequency for the 550 nm progression. Both cysteine and glutathione have a single free thiol. The difference in the rotational strengths (proportional to $\mu \cdot m$) in Cys−NO and GS−NO may be accounted for by the differences in μ contributions which we have determined from the linear absorbance spectra.

The S−NO band at 550 nm for the BSA has a pH dependence not seen in glutathione. The glutathione 550 nm band has similar shape and magnitude in the pH range of 2−7, however, the 550 nm band in BSA partially inverts at neutral pH (see Figure 1b). The CD signal for nitrosylated Cys-34 in BSA is observed when BSA is incubated with 1-, 2-, and 8-fold molar excesses of $NaNO_2$. In the presence of excess $NaNO_2$, other amino acids, such as tyrosine, tryptophan, and phenylalanine, are modified.[15] The 550 nm signal shape and amplitude are unchanged when excess $NaNO_2$ is added, indicating that this S−NO signal is independent of these potential interferences.

Cys-34, the free thiol of BSA, is known to exist in a crevice appoximately 9.5 Å in depth. In the crystal structure of HSA, Cys-34 is partially protected from the solvent, located in a turn between helices h2(I) and h3(I).[11−13] Noel and Hunter have concluded that the thiol group of bovine serum albumin is in a somewhat restricted environment, most likely the crevice that is opened in the course of the N−F transition.[14] BSA undergoes an unfolding transition at pH = 4−4.5 in which the α-helical structure is lost and hydrophobic areas become solvent accessible. Below pH = 3.5, the protein undergoes more expansion in which ca. 25% additional helical structrure is lost.[17] Since Cys-34 resides in a hydrophobic crevice surrounded by helices, we propose that the degree of conformational freedom of the Cys-NO species is dependent on the degree of protein folding.

We now present a model for the pH-dependent changes in the BSA−NO CD spectra. As mentioned above, the sign of the CD signal depends on the relative orientation of the magnetic and electric transition moment vectors, varying as their dot (inner) product. The amplitude of the CD signal for neutral pH BSA−NO is approximately one-third that for pH 2.5 solutions. Linear absorbance spectra indicate that the magnitude of μ is invariant with pH. ^{15}N NMR spectral shifts at neutral pH for BSA−NO are comparable with shifts for other S−NO bonds.[15] Thus, we conclude that the CD signal change derives primarily from the change in the angle between the magnetic and electric moment vectors and not changes in the magnitudes of the transition moments. We note that, as wavelengths of the extreme CD responses in BSA−NO are different, 550 and 558 nm at pH 2.5 and 7, respectively, there may be some variation in $|\mu|$ or $|m|$.

The change in the CD signal for the cysteine SNO electronic transitions (350 nm and 550 nm) may be due to the protein's influence on the CS−NO dihedral angle. Ab initio calculations on isolated Cys−NO predict two energy minima associated with rotation around the CS−NO dihedral angle. The CD signals predicted for the two minimum energy geometries give equal intensity but oppositely signed CD signals.[16] Temperature-dependence studies of Cys−NO show $\Delta\epsilon$ reversibly decreases with temperature over 10−80 °C (experimental data not shown).

In summary, we have presented novel studies revealing how protein conformation and NO binding are coupled. The conformational change associated with acid/base-induced refolding of BSA−NO leads to a significant change in the conformational freedom of NO within the Cys-34-containing protein pocket. We are currently extending this work to time-resolved studies of protein conformational changes coupled to NO photodeligation.

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