S-Nitrosylation-induced Conformational Change in Blackfin Tuna Myoglobin*

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S-Nitrosylation is a post-translational protein modification that can alter the function of a variety of proteins. Despite the growing wealth of information that this modification may have important functional consequences, little is known about the structure of the moiety or its effect on protein tertiary structure. Here we report high-resolution x-ray crystal structures of S-nitrosylated and unmodified blackfin tuna myoglobin, which demonstrate that in vitro S-nitrosylation of this protein at the surface-exposed Cys-10 directly causes a reversible conformational change by “wedging” apart a helix and loop. Furthermore, we have demonstrated in solution and in a single crystal that reduction of the S-nitrosylated myoglobin with dithionite results in NO cleavage from the sulfur of Cys-10 and rebinding to the reduced heme iron, showing the reversibility of both the modification and the conformational changes. Finally, we report the 0.95-Å structure of ferrous nitrosyl myoglobin, which provides an accurate structural view of the NO coordination geometry in the context of a globin heme pocket.

Protein S-nitrosylation, or the formation of an S–NO bond involving the sulfur of a cysteine residue, is an important post-translational modification. Numerous proteins whose functions are altered by S-nitrosylation have been identified, including enzymes, transcription factors, receptors, channels, and structural proteins (reviewed in Ref. 1). Parallels have been drawn between S-nitrosylation and O-phosphorylation, a ubiquitous biological signal (2, 3). Regulation of the function of many proteins by O-phosphorylation is a consequence of structural changes that take place in the protein following phosphorylation. The molecular consequences of protein S-nitrosylation are less well characterized. Enzymes such as caspases that require a cysteine thiol in the active site can be modulated in their activity by S-nitrosylation, which alters the reactive properties of the cysteine (4). S-Nitrosylation can also influence protein-protein interactions, as recently demonstrated with yeast two-hybrid screening (5). Finally, S-nitrosylation may have the ability to modulate the function of a protein via allosteric changes in its structure. Although this mechanism has been hypothesized for several systems, there has thus far been little structural evidence published (6, 7).

Another critical role for NO in cell physiology involves its interaction with heme proteins. The first well defined mechanism for a NO-dependent physiological effect was the relaxation of vascular smooth muscle following the binding of NO to the heme iron of guanylate cyclase, which activates this enzyme to convert guanosine triphosphate to the second messenger cGMP (8). The reaction of the NO group (NO, NO−, NO−) with heme and thiol is complex (9). However, under various in vitro and in vivo conditions, the NO group is capable of binding at one or the other or exchanging between the two. It has been shown that the bioavailability of NO when bound to Cysβ93 of hemoglobin is dependent upon blood O2 concentration as well as the allosteric and redox state and that it acts as an important regulator of vessel tone and blood flow (10–13).

Sperm whale myoglobin (Mb), the first myoglobin whose x-ray structure was determined, lacks a cysteinyl residue. Human myoglobin has a single reactive cysteine at position 110 and is known to form an S-nitrosylated species in vitro and in vascular smooth muscle cell culture when exposed to physiological concentrations of a nitric oxide donor (14, 15). Myoglobins with reactive cysteines are seen scattered throughout the vertebrate species, but there seems to be little to suggest a functional pattern. In fishes, the group that includes tunas frequently possesses a single cysteine within the N-terminal helix. It has been suggested that this reactive -SH plays a role in S-nitrosylation in vivo (16), although there are no hard data yet to support this hypothesis.

With the recent advancement in methodologies for the detection and identification of S-nitrosylated proteins in cell culture and tissue samples, such as the S-nitrosylation site identification (17, 18) extension of the original biotin switch method (19), an extensive data base of S-nitrosylation sites within proteins is being accumulated. In light of these advancements, a more detailed knowledge of the structural consequences of this modification will be critical for understanding the molecular mechanisms by which S-nitrosylation alters protein function.

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The atomic coordinates and structure factors (code 2NRL, 2NRM, and 2NX0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviation used is: Mb, myoglobin.
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In this work, we have demonstrated with atomic resolution the effects of S-nitrosylation on the x-ray crystal structure of blackfin tuna myoglobin. In addition, we have identified conformational changes in this protein that are the direct result of the formation of the S-nitrosothiol group. We also demonstrate that, upon reduction with dithionite, the NO group is transferred from the surface-exposed Cys-10 to the reduced heme iron, showing the reversibility of the S-NO modification and the conformational changes. Finally, we have provided the first atomic resolution structure of nitric oxide bound to the heme iron of a globin protein, which allows the calculation of accurate geometric parameters for this important coordination complex.

EXPERIMENTAL PROCEDURES

Purification of Myoglobin from Blackfin Tuna—Myoglobin was purified from the red muscle of blackfin tuna (*Thunnus atlanticus*) caught near Rincon, Puerto Rico. The red muscle was dissected, homogenized, and centrifuged, and myoglobin was precipitated from the supernatant between 65 and 95% ammonium sulfate. Following resuspension of the precipitated myoglobin and dialysis against phosphate-buffered saline, the protein was separated by size exclusion chromatography on a Sephadex G-75 column. Myoglobin-containing fractions were concentrated to ~1.2 mM and stored at ~80°C. Myoglobin isolated by this method was nearly 100% ferric aquo (met)myoglobin, as judged by UV-visible spectroscopy.

Trans-S-nitrosylation of Myoglobin—Purified myoglobin was S-nitrosylated by reaction of 300 μM myoglobin with 3 mM S-nitrosothiocyante in borax buffer containing 500 μM diethylenetriamine pentaacetate at pH 9. S-Nitrosothiocyante was removed by passing the sample through two sequential Micro Bio-Spin 6 size exclusion columns (Bio-Rad). The amount of S-nitrosylaion in purified myoglobin samples was quantitated using the Saville (20) and Griess (21) reactions in acidified aqueous solution as described previously (22–24), which results in the production of an azo dye product that can be quantitated spectrophotometrically using an extinction coefficient of 50,000 M⁻¹ cm⁻¹ at 545 nm. A calibration curve was generated for this assay using S-nitrosoglutathione, which was quantitated using an extinction coefficient of 767 M⁻¹ cm⁻¹ at 337 nm (25). A chemiluminescence nitric oxide analyzer (Sievers) was also used to verify production of S-nitrosyl myoglobin, again using S-nitrosoglutathione or S-nitrosothiocyante for calibration.

Crystallization and Data Collection—Purified myoglobin, at a concentration of 1.2 mM in phosphate-buffered saline, was crystallized by hanging-drop vapor diffusion at room temperature by mixing 2 μL of protein solution with 2 μL of a precipitant solution containing 0.2 M potassium/sodium tartrate, 0.1 M sodium citrate (pH 5.6), and 2.0 M ammonium sulfate. Crystals grew as clusters of large plates in 2–7 days. Crystals were cryo-protected by soaking for 30 s in the precipitant solution containing either 30% ethylene glycol or 30% glycerol and rapidly cooled to 100 K in a gaseous nitrogen stream for data collection. Crystals of Mb–SNO were grown and cryo-protected in the same way as unmodified myoglobin.

Crystals of ferrous nitrosyl myoglobin were prepared by soaking of metmyoglobin crystals in deoxygenated precipitant solution containing 2.2 M ammonium sulfate, 0.2 M potassium/sodium tartrate, 0.1 M sodium citrate, pH 5.6, and 20 mM sodium dithionite for 20 min followed by anaerobic transfer to precipitant solution supplemented with 3 mM nitric oxide donor 2-(N,N-diethylamino)-diazenolate-2-oxide (kindly supplied by Dr. Katrina Miranda). After 10 min, the crystals were quickly transferred to 3.5 M ammonium sulfate, 0.16 M potassium/sodium tartrate, 0.1 M sodium citrate, pH 5.6, and flash-frozen in liquid N₂.

Data were collected either on a rotating copper anode home x-ray source equipped with a Saturn 92 CCD detector (Rigaku/Molecular Structure Corporation) or at the Advanced Photon Source synchrotron beamlines indicated in Table 1. Diffraction data were integrated and scaled using either d*TREK (26) from within the CrystalClear software suite (Rigaku/Molecular Structure Corporation) or HKL2000 (27). Data collection statistics are provided in Table 1.

Structure Solution, Modeling, and Refinement—The blackfin tuna myoglobin structure was solved by molecular replacement using the program Phaser (28) with yellowfin tuna myoglobin as a search model (Protein Data Bank (PDB) code 1MYT) (29). The structure was rebuilt and refined using Coot (30) and Refmac5 programs within the CCP4 suite (31), respectively. Although the amino acid sequence of blackfin tuna myoglobin is not available in any data bases, the sequence was tentatively assigned based on the excellent quality electron density maps and appears to be identical to bluefin and bigeye tuna myoglobins, except for a His → Gln substitution at position 112, which is also present in other tuna species. Positive difference electron density at the N terminus was modeled as an N-acetyl group, given that this modification is known to occur in myoglobins from other tuna species (32). Electron density at position 10 in crystals of myoglobin trans-S-nitrosylated with S-nitrosothiocyante was interpreted as two conformations of Cys S–NO (Fig. 1A and B) and one conformation of unmodified Cys-10 (Fig. 1C). Geometric restraints for the refinement of the Cys-10 S–NO group were generated based on a small molecule crystal structure of S-nitroso-L-cysteine ethyl ester (33). For the ferrous nitrosyl myoglobin structure, no restraints were placed on the Fe–NO bond lengths or angles during the final stages of refinement. During the later stages of refinement of the atomic resolution Mb structures, anisotropic B-factors were refined for all atoms, and hydrogens were added in their riding positions. Refinement statistics are provided in Table 1.

RESULTS

Structure of Blackfin Tuna Myoglobin—The structure of myoglobin isolated from blackfin tuna was determined to 0.91 Å resolution by molecular replacement. The overall structure is very similar to that of myoglobins from other species, especially yellowfin tuna (29), and will not be discussed further here. Detailed descriptions of various heme oxidation and ligation states of blackfin tuna myoglobin from high-resolution crystal structures will be published separately.

Generation and Stability of S-Nitrosomyoglobin—Blackfin tuna myoglobin was successfully S-nitrosylated at Cys-10 via
reaction with excess S-nitrosocysteine or S-nitrosoglutathione. Although the characteristic absorption band indicative of S-nitrosothiols at ~330 nm (extinction coefficient ~600–800 M⁻¹ cm⁻¹) was masked by the much stronger heme absorption at that wavelength (extinction coefficient ~20,000 M⁻¹ cm⁻¹), the presence of S-nitrosomyoglobin was confirmed using the Saville-Griess assay as well as chemiluminescence after extensive desalting of the sample to remove S-nitrosocysteine and free cysteine. Not all of the myoglobin Cys-10 was S-nitrosylated under these reaction conditions, with an average yield of 50–70% Mb S–NO. The amount of S-nitrosothiol in the S-nitrosomyoglobin sample did not decrease significantly over the course of a month at room temperature when stored in the dark in a metal ion-free buffer.

Structure of S-Nitrosomyoglobin—Myoglobin trans-S-nitrosylated by reaction with excess S-nitrosocysteine crystallized in the same form as unmodified myoglobin. Data were collected to 1.09 Å resolution using a rotating copper anode x-ray generator. Attempts to collect data to higher resolution using a synchrotron source led to complete removal of the S–NO group. The x-ray sensitivity of protein S–NO groups has been observed in other systems as well (34). Slight loss of the S–NO group was also observed using a rotating anode source when long exposure times were used; therefore, exposure times were minimized to prevent degradation of the S–NO. The electron density map around position 10 clearly indicated a chemical modification of the sulfur of Cys-10 as well as multiple conformations of the Cys-10 side chain (Fig. 1). The final model included three conformations of Cys-10, two that had been S-nitrosylated, whereas conformation C is not.

TABLE 1
Data collection and refinement statistics

| Data collection | Crystal | Mb–SNO | Mb–SNO 2 | Mb–SNO 2 | Mb-unmodified | Mb–Fe(NO) |
|-----------------|--------|--------|---------|---------|-------------|----------|
| Mb–SNO          | P2_1   | 51,93  | 51,95   | 51,95   | 51,98       | 51,98    |
| Mb–SNO 2       | P2_1   | 34,00  | 38,87   | 34,34   | 38,53       | 38,78    |
| Mb–SNO 2       | P2_1   | 34,34  | 38,53   | 34,51   | 38,87       | 38,78    |
| Mb–unmodified   | P2_1   | 34,34  | 38,53   | 34,51   | 38,87       | 38,78    |
| Mb–Fe(NO)      | P2_1   | 34,34  | 38,53   | 34,51   | 38,87       | 38,78    |

The number in parentheses is for the highest resolution shell.

| Refinement | Resolution Range (Å) | 30–1.09 | 30–1.50 | 40–1.35 | 30–0.91 | 22–0.95 |
|------------|----------------------|---------|---------|---------|---------|---------|
| No. of reflections | 51,131 | 13,018 | 16,020 | 14,914 | 15,617 |
| Rwork/Rfree (%) | 13.5/17.1 | 13.5/17.1 | 13.3/17.1 | 15.1/17.1 | 15.1/17.1 |
| No. of atoms | 1601 | 1596 | 1341 | 1484 | 1539 |
| Average B-factor (Å²) | 8.5 | 8.8 | 9.4 | 11.2 | 9.0 |
| Root mean square deviations | Bond lengths (Å) | 0.014 | 0.018 | 0.018 | 0.014 | 0.017 |
| Bond angles (°) | 1.54 | 1.54 | 1.54 | 1.54 | 1.54 |
| Ramachandran (%) | Most favored | 93.7 | 92.9 | 92.6 | 92.0 | 91.3 |
| Additionally allowed | 6.3 | 7.1 | 7.4 | 8.0 | 8.7 |
| Generously allowed/disallowed | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

* The number in parentheses is for the highest resolution shell.

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| X-ray source | Rotating Cu anode | Rotating Cu anode | Rotating Cu anode | APS 31-ID | APS 14BM-C |
|--------------|------------------|------------------|------------------|---------|-----------|
| Space group  | P2_1             | P2_1             | P2_1             | P2_1    | P2_1      |
| a, b, c (Å) | 33.94, 51.93, 38.9 | 34.00, 51.95, 38.9 | 34.34, 51.78, 38.53 | 34.34, 51.98, 38.78 | 34.34, 51.98, 38.78 |
| α, β, γ (°) | 90, 103.75, 90   | 90, 103.64, 90   | 90.053, 90.053   | 90.053, 90.053   | 90.053, 90.053   |
| Wavelength (Å) | 1.5418 | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Resolution Range (Å) | 30–1.09 (1.13–1.09) | 30–1.50 (1.55–1.50) | 40–1.35 (1.40–1.35) | 30–0.91 (0.94–0.91) | 22–0.95 (0.98–0.95) |
| Rwork (%), | 6.1 (36.5) | 3.5 (17.0) | 6.4 (40.3) | 5.5 (38.6) | 8.9 (47.0) |
| Rfree (%) | 18.1 (3.3) | 18.8 (3.3) | 24.4 (2.2) | 16.0 (3.3) | 12.0 (3.5) |
| Completeness (%) | 98.4 (93.2) | 96.4 (74.5) | 98.5 (91.2) | 98.2 (82.5) | 98.7 (99.0) |
| Redundance | 6.1 (3.5) | 2.0 (1.2) | 7.2 (1.6) | 7.2 (4.0) | 9.9 (10.1) |

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**FIGURE 1. Electron density for S-nitrosylated Cys-10 of blackfin tuna myoglobin.** Stereo view of the 2Fo–F electron density omit map for Cys-10 in S-nitrosylated myoglobin. The map is contoured at 1.0 σ and shown in blue mesh. The final refined model is shown as sticks colored by atom type. The three modeled conformations of Cys-10 are shown: conformations A and B are S-nitrosylated, whereas conformation C is not. All figures were produced using PyMOL.
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A) Conformational change in myoglobin upon S-nitrosylation. A, stereo view of the superimposed structures of unmodified myoglobin (yellow) and also following trans-S-nitrosylation (magenta). Black arrows depict the direction of movement of various portions of the structure upon S-nitrosylation at Cys-10. Helices and loops discussed in the text are labeled. The two modeled conformations of loop EF are shown for each structure. B, stereo view a close-up of structural elements surrounding the S-nitrosocysteine that undergoes a conformational change. The S-nitrosocysteine at Cys-10, the side chain of Leu-117, and the backbone carbonyl of Ala-6 are shown as sticks with the same color scheme as A. Semitransparent van der Waals spheres demonstrate the steric clash that would occur between the S-nitrosocysteine group and Leu-117 and Ala-6 if no conformational change occurred. These clashes are also represented as dashed red lines. In each panel, only the major conformation of each residue is shown for clarity.

B), and one that remained unmodified (Fig. 1C). This interpretation gave the best fit to the electron density map and was only possible because of the high resolution of the data. The unmodified Cys-10 conformation closely matched that seen in the unmodified myoglobin structure, and was present at ~30% occupancy. The ~70% S-nitrosylation of Cys-10 observed in the crystal matched our synthetic yields of Mb–SNO formation in solution. The major conformation of S-nitrosylated Cys-10 (Fig. 1A) is rotated by only 25° around χ1 (rotation around the Cα–Cβ bond) from the unmodified rotamer (Fig. 1). The second modified conformation (Fig. 1B) has a substantially different χ1 rotamer, rotated by 115°, but the attached NO moiety occupied nearly the same space as that of conformation A. Both Cys-10 SNO conformations are present as the cis dihedral, with C–S–N–O dihedral angles (χ3) close to 0°.

**Structural Changes upon S-Nitrosylation**—Superposition of the structure of S-nitrosylated myoglobin with the unmodified structure reveals that there are significant differences in several portions of the structure. The differences are primarily localized to the structural elements surrounding Cys-10, including helix A, the N terminus of helix H, loop EF, and loop GH (Fig. 2A). Comparison of the Ca traces of S-nitrosylated and unmodified myoglobin yielded a root mean square deviation value of 1.2 Å for portions of the structure surrounding Cys-10 (residues 1–10, 71–83, and 115–127, 34 Ca atoms), whereas the rest of the structure had a root mean square deviation of only 0.2 Å (residues 11–70, 84–114, and 128–146, 109 Ca atoms).

Figure 2: Conformational change in myoglobin upon S-nitrosylation. A, stereo view of the superimposed structures of unmodified myoglobin (yellow) and also following trans-S-nitrosylation (magenta). Black arrows depict the direction of movement of various portions of the structure upon S-nitrosylation at Cys-10. Helices and loops discussed in the text are labeled. The two modeled conformations of loop EF are shown for each structure. B, stereo view a close-up of structural elements surrounding the S-nitrosocysteine that undergoes a conformational change. The S-nitrosocysteine at Cys-10, the side chain of Leu-117, and the backbone carbonyl of Ala-6 are shown as sticks with the same color scheme as A. Semitransparent van der Waals spheres demonstrate the steric clash that would occur between the S-nitrosocysteine group and Leu-117 and Ala-6 if no conformational change occurred. These clashes are also represented as dashed red lines. In each panel, only the major conformation of each residue is shown for clarity.

The NO group of S-nitrosylated Cys-10 packs between the side chain of Leu-117 within loop GH and the backbone carbonyl oxygen of Ala-6 from helix A (Fig. 2B). Superposition of the unmodified myoglobin structure revealed that the S-nitrosothiol group could create steric clashes with Leu-117 and Ala-6 if these portions of the structure did not move. Although two conformations of S-nitrosylated Cys-10 exist in our structure, these observations hold true for both. Therefore, helix A and loop GH were forced in opposite directions, or wedged apart, as a direct consequence of S-nitrosylation at Cys-10. We also observed secondary effects of the movement of helix A. The N terminus of helix H, which was packed against helix A, shifted to occupy the void created by the movement of helix A. Additionally, loop EF was shifted away from helix A. Although loop EF exhibited both static and dynamic disorder in both structures and therefore appeared to be flexible, at least one static conformation of this loop in the unmodified structure was displaced by steric clashes created upon movement of helix A following S-nitrosylation. In contrast to the structural changes surrounding Cys-10 following S-nitrosylation, no significant changes were observed in the vicinity of the heme prosthetic group, the site of oxygen binding in myoglobin.

**NO Cleavage from Cys-10 and Rebinding to Reduced Heme**—Following trans-S-nitrosylation with S-nitrosocysteine and desalting, a UV-visible absorption spectrum showed that the heme group of isolated blackfin tuna myoglobin remains in the ferric aquo (met) state (Fig. 3A, black spectrum). The addition
of the reducing agent sodium dithionite to this sample resulted in a shift of the Soret and Q bands of the heme absorption spectrum to wavelengths indicative of the ferrous nitrosyl (FeII–NO) derivative (Fig. 3A, red spectrum). Spectral deconvolution indicated that 40% of the sample was converted to the ferrous nitrosyl form, 37% became unliganded ferrous deoxy, and 23% remained ferric met. Furthermore, the addition of dithionite to solutions of either S-nitro-glutathione or S-nitro-cysteine resulted in the release of nitric oxide that could be detected by a chemiluminescence-based nitric oxide monitor.

This leads us to conclude that, upon reaction of Mb–SNO with dithionite, the NO group is cleaved from the Cys-10 sulfur and can subsequently bind to the reduced heme iron. As expected, treatment of unmodified metmyoglobin with dithionite resulted in primarily the unliganded ferrous deoxy form of the heme (Fig. 3B). Spectral deconvolution showed 0% ferrous nitrosyl, 77% ferrous deoxy, and 23% ferric met.

We were also able to observe this NO group transfer in two structures derived from a single crystal. Rapid data collection to minimize the x-ray dose on a crystal of S-nitrosomyoglobin produced a structure identical to that described earlier (Figs. 1 and 2), with Cys-10 modified by NO (Fig. 3C) and the heme in the ferric aquo state (Fig. 3D). Subsequent soaking of the same crystal in a solution containing 200 mM dithionite and the collection of another dataset showed that the NO group is lost from Cys-10 following treatment with dithionite. D and F illustrate the conversion of the heme group from ferric aquo to primarily ferrous nitrosyl after soaking the crystal in dithionite. Note that the conformational change in myoglobin resulting from S-nitrosylation of Cys-10, which is illustrated in Fig. 2, is observed in these two structures obtained from the same myoglobin crystal.
this conformational change is accommodated within the context of a single crystal. We wish to make clear that the reactions of Mb–SNO with dithionite do not necessarily shed light on the chemistry that may take place in vivo. Dithionite was used in these studies as a means to demonstrate the reversibility of the S-nitrosylation of Mb at Cys-10 and was convenient for the rapid soaking of crystalline samples. Nonetheless, it is interesting to note that if a suitable reductant were present in vivo, the myoglobin heme would efficiently capture the released NO. 

Ultrahigh Resolution Structure of FeII–NO Myoglobin—Crystals of the FeII–NO form of blackfin tuna myoglobin were prepared by anaerobic reduction and treatment with the nitric oxide donor 2-(N,N-diethylamino)-diazenolate-2-oxide, and a dataset was collected to 0.95 Å resolution using synchrotron radiation. At this resolution, individual atoms of the heme-NO complex were resolved in the electron density map (Fig. 4), allowing highly accurate bond lengths and angles to be calculated. Geometrical parameters of the heme Fe–NO ligation from this structure are summarized in Table 2. The heme group was nearly planar, as demonstrated by the small distortion parameters in Table 2, calculated using normal coordinate structural decomposition calculations as described previously (35).

DISCUSSION

Production and Stability of S-Nitrosylated Blackfin Tuna Myoglobin—We were able to S-nitrosylate myoglobin isolated from the red muscle of blackfin tuna by in vitro trans-S-nitrosylation using either S-nitrosocysteine or S-nitrosoglutathione. S-Nitrosylation of the myoglobin was confirmed using chemiluminescence, the Saville-Griess colorimetric assay, and by release of the NO group from Cys-10 and recapture at the heme iron following dithionite reduction monitored by UV-visible spectroscopy and x-ray crystallography (Fig. 3). The SNO group at Cys-10 was quite stable in the dark and in the absence of metal ions and produced highly ordered crystals quite readily, providing an ideal system for the study of the effects of S-nitrosylation on protein structure. Factors previously proposed to affect the formation or stability of S-nitrosothiols include the steric bulk surrounding the thiol (25), redox environment (36, 37), reactivity of the thiol, and pH. The sulfur atom of Cys-10 in blackfin tuna myoglobin is partially solvent-exposed and is located between the side chains of an aspartate (Asp-118) and a
lysine (Lys-9) at the surface of the protein. It has been proposed that similar “acid-base” motifs could be an important determinant of cysteine S-nitrosylation (1). The charged functional groups of the Asp-118 and Lys-9 side chains are located 7–9 Å from the Cys-10 sulfur, however, and therefore may not have a strong influence over its reactivity. In the S-nitrosylated structure, the sulfur-bound NO group is also partially surface-exposed and is surrounded primarily by hydrophobic amino acid side chains from the protein core and backbone carbonyl oxygens. No hydrogen bonds were observed to the NO group. Hydrophobic regions of proteins were also proposed to enhance S-nitrosylation by increasing the local formation of N₂O₃, a potent nitrosylating agent (38). This may play a role in the formation of the Mb–SNO, given that S-nitrosylated blackfin tuna myoglobin, we observed two conformations of the modified cysteine side chain. Both were present in myoglobin closely matched the predicted and observed geometric parameters for S-nitrosylation. Both conformations of Cys-10–SNO that we observed in myoglobin, given that S-nitrosylated Cys-10 orients toward the hydrophobic protein interior in our structure.

SNO Geometry—In the atomic resolution structure of S-nitrosylated blackfin tuna myoglobin, we observed two conformations of the modified cysteine side chain. Both were present as the cis conformer (Fig. 1), with C–S–N–O dihedral angles of −90°. Interestingly, two conformations of this group were similarly observed in the small molecule crystal structure of S-nitroso-L-cysteine ethyl ester (33), implying that some degree of static disorder may be an intrinsic feature of this chemical substituent. Quantum mechanical calculations demonstrate that two energy minima exist for the C–S–N–O dihedral angle, at 0° and 180°, due to delocalization of the N = O π electrons over the S–N bond (a phenomenon that may be familiar from the planar peptide bond within the backbone of proteins). This results in a slightly shorter S–N bond and a 12-kcal/mol energy barrier for rotation of the dihedral between 0° and 180° (39). Both conformations of Cys-10–SNO that we observed in myoglobin closely matched the predicted and observed geometric parameters for S-nitrosothiols.

Very recently, structures of trans-S-nitrosylated human thioredoxin (PDB entries 2HXX, 2IFQ, and 2IIY) revealed S-nitrosylation at multiple cysteines, both buried and accessible. The observed modifications conform primarily to the cis dihedral, with partial occupancy of the trans-conformation observed at one site (34). A structure of the nitrophorin protein (PDB entry 1Y21) from Cimex lectularius revealed S-nitrosylation of a proximal heme ligand cysteine, also present as the cis conformer, with a C–S–N–O dihedral close to 0° (40). Only two other PDB entries are currently annotated as containing a protein S-nitrosocysteine. A structure of human hemoglobin treated with NO gas revealed modification of Cys/93 (PDB entry 1BUW) (41) but was later concluded to be the thionitroxide radical C–S–NH–O based on comparison between the observed C–S–N–O dihedral angle (−90°) and quantum mechanical calculations of several possible sulfur-bound nitrogen oxide species (39, 42). Finally, an atomic resolution structure of a mammalian dimethylarginine dimethylaminohydrolase treated with S-nitroso-L-homocysteine (PDB entry 2CII) showed a two-atom modification of the surface-exposed Cys-83. A C–S–N–O dihedral angle of −69.5° was observed, despite the fact that no steric clashes would occur if the group were present with a cis dihedral angle of 0°. Additionally, the S–N and N–O bond lengths deviated substantially from those observed in small molecule crystal structures (33) and predicted from high level calculations (39), suggesting that it may also be a more reduced sulfur-bound nitrogen oxide species.

Future Directions—Using x-ray crystallography, we were able to observe specific changes in the structure of blackfin tuna myoglobin as a direct result of S-nitrosylation at a surface-exposed cysteine residue (Fig. 2). Although there are technical
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challenges to the biophysical characterization of S-nitrosylation-induced conformational changes, such as the instability and x-ray sensitivity of some S-nitrosothiols, we hope this work will serve as a starting point to begin to understand the molecular effects of this modification on protein structure and function. Future experiments with blackfin tuna myoglobin will examine whether S-nitrosylation at Cys-10 has a functional effect on the oxygen-binding properties of this protein. It will also be important to determine whether S-nitrosylation of myoglobin occurs as a normal physiological process in the muscle tissue of the blackfin tuna.

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