Competitive Binding of Bismuth to Transferrin and Albumin in Aqueous Solution and in Blood Plasma*  

Received for publication, June 2, 2000, and in revised form, October 11, 2000  
Published, JBC Papers in Press, December 7, 2000, DOI 10.1074/jbc.M004779200  

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Bismuth compounds have long been associated with medicine for the treatment of a variety of gastrointestinal disorders including diarrhea, constipation, gastritis, and ulcers (1–4). The effectiveness of bismuth has been attributed to its bactericidal action against the Gram-negative bacterium, Helicobacter pylori. There is also a growing interest in using compounds containing radioactive bismuth isotopes as targeted radiotherapeutic agents (5). However, the molecular basis for the mechanism of action of bismuth drugs is not well understood, including bismuth-induced toxicity, especially encephalopathy, which led to the withdrawal of bismuth drugs in France and Australia in the 1970s (1). The diagnosis of encephalopathy is generally defined by the detection of bismuth in blood, plasma or serum, the so-called “Hillemand safety level” (6, 7). Bismuth is primarily present in red blood cells, possibly binding to glutathione, with the remainder in serum or plasma (8–10). The speciation of bismuth in blood plasma, and in particular the nature of interactions of Bi³⁺ with plasma proteins, are in need of investigation.

Recently we have found that the binding of Bi³⁺ to human serum transferrin (hTF) and recombinant N-lobe of transferrin is unexpectedly strong (11, 12). Transferrin is a single-chain glycoprotein (80 kDa) present in blood at a concentration of about 35 μM, and consists of two similar lobes, each of 40 kDa, connected by a short peptide. Its normal function in blood is to carry iron between sites of uptake, utilization, and storage (13–16). It contains two specific iron-binding sites per molecule, one in the N-terminal lobe and one in the C-terminal lobe. Iron binds as Fe³⁺ in a cleft formed by two domains in each lobe. Iron cannot bind strongly without concomitant binding of a synergistic anion. Since transferrin is only about 30% saturated with iron in normal serum (13, 17, 18), there is potential binding capacity for other metal ions that enter the blood. This has led to the idea that transferrin acts as a “delivery system” for therapeutic, diagnostic or toxic ions, including Ga³⁺, Ru³⁺, and Al³⁺ (19–21). Recently we have shown that Bi₂⁺-hTF can block both membrane binding and cellular uptake of ²⁰⁹Fe²⁺-hTF into BeWo placental cancer cells (22). It is therefore now important to establish whether Bi³⁺ binding to transferrin can occur under physiologically relevant conditions, especially in the presence of excess albumin and in blood plasma itself. We have shown previously that the order of lobe loading of hTF with metal ions can readily be determined via two-dimensional ¹H,¹³C NMR studies of recombinant [¹-¹³C]Met-hTF (23). It is known that the strength of binding to the two lobes is slightly different, and that Fe³⁺ is primarily situated in N-lobe in serum (14, 18).

Previous investigations of the interaction of Bi³⁺ with serum albumin has led to the suggestion that albumin may be the major target for Bi³⁺ in plasma (8), especially since albumin has a free thiolate group at Cys³⁴. Human serum albumin, the most abundant protein in blood at a concentration of about 40 mg ml⁻¹ (about 0.63 mM, > 10 times that of transferrin), is a single-chain 66.5-kDa protein, which is largely α-helical, and consists of three structurally homologous domains (24). It is the major transport protein for unesterified fatty acids, drugs, organic compounds, and metal ions, e.g. Ca²⁺, Zn²⁺, Cu²⁺, and Ni²⁺ (25, 26).

In the present work, the binding of a bismuth antiulcer drug  

This work was supported by GlaxoWellcome, the Engineering and Physical Sciences Research Council (EPSRC), Biotechnology and Biological Sciences Research Council (BBSRC), Wolfson Foundation, the University of Hong Kong, and the Committee of Vice-Chancellors and Principals (Overseas Research Student award to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* This abbreviations used are: hTF, human serum transferrin; Bi(h3cit), Bi(III) citrate; HSA, human serum albumin; HSCQ, heteronuclear single-quantum coherence; hTF/2N, recombinant N-lobe of hTF; NTFA, nitritroacetate; pH*, pH meter reading in D₂O; RBC, ranitidine bismuth citrate (an amorphous solid containing ranitidine, bismuth, and citrate in an approximate 1:1:1 molar ratio); ranitidine, N,N-dimethyl-5-(3-nitromethylene-7-thia-2,4-diazaoctyl)furaz-2-methanamine; HRA, recombinant human albumin.

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to human serum transferrin in aqueous solution in the presence of a large excess of albumin and to recombinant transferrin (N-lobe of transferrin and the mutant I132M of N-lobe of transferrin labeled with [13C]Met) in intact blood plasma has been monitored directly under physiologically relevant conditions using 1H,13C NMR spectroscopy. The introduction of Met^132 into the N-lobe provides a convenient monitor for metal binding since this residue occupies a similar site within helix 5 of the N-lobe and forms part of the hydrophobic patch around Trp^128 (Leu^122-Trp^128 Ile^132) as Met^464 in the C-lobe (Val^654, Trp^460-Met^464). The interaction of bismuth with human albumin was also studied. Surprisingly, we found that Bi^3+ still binds to the iron-binding sites of transferrin even in the presence of a large excess of albumin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant N-lobe hTF/2N (residues 1–337) was expressed in baby hamster kidney cells using a pNUT plasmid with 1-[13C]methionine in the growth medium, and purified as described previously (27, 28). A gene for the mutant I132M protein was created by site-directed mutagenesis using previously published methods (27, 29). Iron was removed from proteins by treatment with a metal-removal buffer containing 1 mM NTA, 1 mM EDTA, and 0.5 mM sodium acetate, pH 4.9, using ultrafiltration Centricron 10 ultrafilters (Amicon). Human serum albumin (HSA) was purchased from Sigma as essentially globulin-free and fatty acid-free and was purified via ultrafiltration (Centricron 10) using 0.1 M chloride washing 3 times (each 1 h). It was then lyophilized. Recombinant human albumin (rHA) was supplied by Delta Biotechnology Ltd. (batch GS 950292 and R970103). Samples of rHA were dialyzed against 100 mM ammonium bicarbonate, pH 7.9, and freeze-dried. Ranitidine bismuth citrate (RBC) and bismuth citrate ([Bi(Hcit)] were provided by GlaxoWellcome plc. NaHCO3, KCl, 5,5′-dithiobis(2-nitrobenzoic acid), and other chemicals were purchased either from Aldrich or Sigma with the highest quality and used as purchased without further purification. Crystallographic (BiNTA) was synthesized according to a literature procedure (30), and had a satisfactory elemental analysis.

A 50 mM stock solution of [Bi(cit)] was prepared by adding the minimum amount of ammonia solution to a suspension of [Bi(cit)] until the solution became clear. The final pH of this solution was about 7, and it was then diluted before use. A solution of Fe(NTA) was prepared from an iron atomic absorption standard (1000 ppm in 1% HNO3, 454, Trp^460-Met^464) and had a satisfactory elemental analysis. One-dimensional 1H NMR—Preparation of NMR Samples—1H NMR spectroscopy was used to investigate the interaction of bismuth with human serum transferrin and albumin.

**RESULTS**

**NMR Studies of Bi^3+ Binding to I132M hTF/2N and Comparison with Fe^3+**

One-dimensional 1H NMR—It has been shown previously that 1H NMR spectra of human serum transferrin are complicated by the overlap of the very large number of resonances present and by their broadening due to the slow tumbling of this 80-kDa protein (36). However, the high-field region of the spectrum of the N-lobe is relatively well resolved. High-field-shifted resonances have been assigned to protons from residues around Trp^128, i.e., Leu^122, and Ile^132 (37, 38). The mutation of Ile^122 to Met should lead to the disappearance of the resonance at 0.603 ppm, which has previously been assigned as γCH3 of Ile^122. Indeed, this was found to be the case (Fig. 1). Other changes were also observed in the spectrum of the mutant in comparison to that of wild-type hTF/2N. For example, the resonance at 0.170 ppm from hTF/2N disappeared, and the peak for δCH3 of Leu^122 (0.339 ppm in hTF/2N) shifted to 0.324 ppm. Addition of 0.5 mol of Fe^3+ (as [Fe(NTA)]) caused new peaks to appear at 0.016 ppm (C′), 0.025 ppm (B′), and further addition of Fe^3+ (total 1 mol eq) increased the intensity of both C′ and B′ but decreased that of peak B significantly (Fig. 1A).

For comparison, titrations of Fe^3+ (as Fe(NTA)) with the mutant protein were also performed under similar conditions (pH 7.8, 310 K and 10 mM bicarbonate) and the results are shown in Fig. 1B. Upon addition of 0.5 mol of Eq Fe^3+, the resonance at 0.324 ppm decreased in intensity, and almost completely disappeared with 0.9 mol of Eq Fe^3+ present. Broad new peaks at −0.004 ppm −0.392 ppm appeared and increased in intensity. It is reasonable to assume from these titration studies that the resonance at −0.324 ppm consists of two overlapped peaks, one of which (peak B) can be assigned to the...
Fig. 1. The high-field region of the 1H NMR spectrum of apo-I132M hTF/2N and its Bi3+ and Fe3+ complexes in the presence of 10 mM bicarbonate 0.1 M KCl, pH 7.8. A, apo-I132M hTF/2N (bottom), and after addition of 0.5 (middle), and 1.0 mol eq of [Bi(NTA)]2−; B, apo-I132M hTF/2N (bottom), and after addition of 0.5 (middle) and 0.9 mol eq of [Fe(NTA)]2−; C, stack plot showing the changes in shifts of the methyl protons of Leu122 for wild-type hTF/2N and I132M mutant after metal binding.

δCH3 of Leu122 as judged from the change in pattern of this peak on titration of both wild-type and mutant proteins with metal ions. The other peak (C) cannot be assigned. Two-dimensional total correlation spectroscopy and NOESY experiments support these assignments (data not shown). Peak B has identical associated NOESY cross-peaks for both wild-type and mutant hTF/2N, which suggests that peak B belongs to the δCH3 of Leu122. Similar cross-peak patterns were observed for B and C in the NOESY spectrum, which indicates that peak B for the metal-bound protein is the analogue of peak B. A comparison of the changes in the chemical shifts of the δCH3 peak of Leu122 after binding of hTF/2N and I132M-hTF/2N to Bi3+ and Fe3+ is shown in Fig. 1C.

Table I

| Residue   | Apo-form, δ ppm, (1H/13C) | Bi-bound, δ ppm, (1H/13C) |
|-----------|---------------------------|---------------------------|
| Met109    | 1.97/14.65                | 1.97/14.60                |
| Met109    | 1.94/16.15                | 1.97/16.22                |
| Met132    | 1.51/17.98                | 1.45/17.93                |
| Met132    | 2.13/15.54                | 2.13/15.54                |
| Met132    | 2.15/16.16                | 2.14/16.10                |
| Met132    | 2.21/15.76                | 2.20/15.75                |

Addition of 0.5 mol eq of Fe3+ (added as [Fe(NTA)]2−) to I132M hTF/2N caused similar changes in the two-dimensional HSQC spectrum as for 0.5 mol eq Bi3+, except that the peak for Met132 for the bound form was significantly broadened. The resonance for Met132 in the apo-protein disappeared after addition of 1.0 mol eq of Fe3+. The changes to the shifts of the other Met resonances were identical to those observed on addition of 1.0 mol eq of Bi3+.

Binding of Bi3+ to I132M hTF/2N in the Presence of Excess of Albumin—The 1H and 13C NMR chemical shift changes induced by metal ions (e.g., Fe3+ and Bi3+) provide convenient probes for investigation of Bi3+ translocation between transferrin and proteins such as albumin. These experiments were performed using low concentrations of I132M hTF/2N (150 μM) in the presence of 12 mol eq of HSA or rHA (1.8 mM), pH 7.8, 10 mM bicarbonate, 310 K. We choose an [albumin]/transferrin ratio of 12:1 to mimic biological conditions. The concentration of albumin in blood plasma (about 0.63 mM) is about 18 times higher than that of transferrin (about 35 μM), but hTF is only about 30% saturated with Fe3+. The 1H,13C two-dimensional HSQC NMR spectrum of this protein mixture shows sharp resonances from the six labeled Met residues of transferrin and broadened (natural abundance) resonances from albumin. The Met132 peak was overlapped with peaks from albumin (data not shown). Since I132M hTF/2N is present at low concentration, one-dimensional 13C-edited 1H NMR spectra were recorded over a period of 30 min each. Fig. 3 shows the...
The change in the peak for Met 309 (2.15 ppm) was simultaneously decreased in intensity and finally disappeared. The single H NMR peak for Met 132 in [13C]citrate on the one-dimensional 13C-edited 1H NMR spectrum of apo-I132M hTF/2N increased in intensity, reaching a maximum after 1 mol eq of Bi3+ added in 0.25 mol eq steps to I132M hTF/2N in the presence of 12 mol eq of rHA, 10 mM bicarbonate. With increase in Bi3+ concentration, a new peak at 1.45 ppm appeared. This can be assigned to Met 132 in Bi-hTF/2N, and it gradually in -

\[ \text{Bi}^{3+} + \text{metalloprotein} \rightarrow \text{Bi-protein complex} \]

Observation of the Met two-dimensional cross-peaks on titration is shown in Fig. 4. Surprisingly, the peak for Met 132 in the apo-protein almost disappeared and a new peak (bound form) appeared at slightly lower frequency (1.70 ppm) in apo-hTF/2N decreased in intensity and the peak at 1.45/17.93 ppm for Bi3+ was almost identical with and without addition of Bi3+. The shift of the peak for Met309 (2.15/16.16 ppm) shifted to high field, and that for Met309 (2.15/16.16 ppm) shifted to high field.

Uptake of Bi3+ by Transferrin in Plasma

The concentration of transferrin in human plasma is about 35 μM. It is only about 30% saturated with iron (18) and therefore has about 50 μM capacity for binding to other metal ions. To determine if transferrin is a target for bismuth, isotopically labeled [13C]Met I132M hTF/2N (50 μM) was directly added to human plasma. The whole plasma concentration (including the added transferrin) was lyophilized, and the sample was redissolved in half-volume of the original plasma solution. This gave an I132M hTF/2N concentration of 100 μM.

Even with resolution enhancement, the peak for Met309 was still overlapped with other peaks in the 13C-edited 1H NMR spectrum (data not shown). Therefore only the two-dimensional HSQC method was used. The two-dimensional HSQC spectra of this solution containing 100 mM KCl and 20 mM bicarbonate is shown in Fig. 4. Surprisingly, the peak for Met309 became severely broadened but the rest of the Met cross-peaks from transferrin were clearly observed. Many other cross-peaks are present but are difficult to assign, partly due to the limited frequency width used (12 ppm in 13C dimension). The peaks at about 1.46/17.2 ppm, and 1.67/15.8 ppm (folded in 13C dimension) can be assigned to Ala and Lys residues, respectively, of albumin, and the peaks at about 1.24/19.2 ppm to lipids in plasma. After addition of 0.5 mol eq of RBC (relative to the albumin at 1.45/17.9 ppm) in apo-hTF/2N decreased in intensity and the peak at 1.45/17.93 ppm for Bi3+-I132M hTF/2N increased in intensity. Similarly, the peak for Met309 (1.94/16.15 ppm) disappeared and a new peak (bound form) appeared at slightly lower field, and that for Met309 (2.15/16.16 ppm) shifted to high field. The cross-peak for Met309 in the apo-protein almost disappeared and the analogous peak for the bound-form further increased in intensity (Fig. 4). After addition of 1.0 mol eq of RBC, this behavior was similar to that observed for I132M hTF/2N with and without 12 mol eq of serum albumin or recombinant albumin under same conditions. Interestingly, with Bi3+ bound to the protein, the peak for Met309 became sharper and observable.

A second experiment was carried out with blood plasma containing twice the concentration of I132M (200 μM), but the behavior of the Met two-dimensional cross-peaks on titration with RBC was similar. The normal one-dimensional 1H NMR
Interactions of Bismuth Complexes with Albumin

Effect of Bismuth on the Free Thiol Content of Albumin—The free thiol of albumin at Cys\(^{34}\) is a potentially strong binding site for Bi\(^{3+}\). The thiol contents of human serum albumin and recombinant human albumin were determined before and after reaction with bismuth citrate (either RBC or [Bi(cit)]\(^{-}\)) by the 5,5'-dithiobis(2-nitrobenzoic acid) method. The rHA (recombinant) sample contained 0.77 ± 0.01 mol of thiol/mol of protein, while thiol content of (isolated) HSA was significantly lower, only 0.29 ± 0.01 mol/mol HSA. After reaction with various amounts of bismuth citrate in 0.1 M Tris-HCl buffer at pH 7.4 for 12 h, the SH contents decreased by less than 12%, from 0.77 to 0.68 for rHA and from 0.29 to 0.26 for HSA, respectively. This suggests that little Bi\(^{3+}\) binds to Cys\(^{34}\) of albumin.

Determination of Amount of Bismuth Bound to Human Serum Albumin—Various mole ratios of ranitidine bismuth citrate were reacted with albumin in 0.1 M Tris-HCl buffer at pH 7.4 and equilibrated overnight at 310 K. Albumin-bound bismuth was then separated from free bismuth by gel filtration chromatography. The Bi\(^{3+}\) content of the albumin fractions was measured by DIN-ICP-MS (data not shown). The amount of Bi\(^{3+}\) bound to albumin increased almost linearly with increase in added RBC and did not reach saturation even with 25 mol eq of RBC present. The gel filtration chromatograms of control albumin and its complex with bismuth were very similar both in terms of peak intensity and retention time (data not shown) suggesting that bismuth does not cause aggregation of the protein. When 40 mol eq of glutathione (relative to the measured Bi\(^{3+}\)) was added to the albumin fraction, a new broad band centred at about 350 nm gradually increased in intensity in a multiphase process, and reached a maximum intensity over a period of 3 h (Fig. 6). This is in contrast to the reaction of bismuth citrate alone with 40 mol eq of glutathione under similar conditions which was complete within minutes. The band at 350 nm is a typical Bi-S absorbance indicating formation of [Bi(SG)]\(^{3-}\).

DISCUSSION

Bismuth compounds are widely used as antiulcer drugs and recently we have shown (22) that bismuth transferrin, Bi\(^{3+}\)-hTF, exhibits marked dose-dependent effects on membrane binding and cell uptake of \(^{59}\)Fe-hTF by placental BeWo cells. This suggested that bismuth transferrin is recognized by the transferrin receptor. The present study was undertaken to determine whether Bi\(^{3+}\) can bind to transferrin under physiological conditions, especially in the presence of excess albumin, and in blood plasma itself. Previously we have shown that NMR can be used to monitor the uptake of metals into the individual lobes of transferrin (23). The \(^{1}H,^{13}C\) NMR cross-peak for Met\(^{464}\) of human transferrin is a sensitive indicator of metal binding to the C-lobe since significant chemical shift changes are induced in both \(^{1}H\) and \(^{13}C\) dimensions. In the N-lobe of intact hTF, however, there is lack of this kind of sensitive indicator. Met\(^{464}\) is situated in the hydrophobic patch (Val\(^{454}\)-Trp\(^{460}\)-Met\(^{464}\)) of helix 5 in the C-lobe (Fig. 7), which backs onto the metal-binding site and H-bonds to the synergistic anion (43, 44). In the N-lobe there is a similar hydrophobic patch in helix 5 near the metal-binding site, consisting of Leu\(^{122}\), Trp\(^{128}\), and Ile\(^{132}\) (Fig. 7) (45). The analogue of Met\(^{464}\) is Ile\(^{132}\) in human serum transferrin, but is Met\(^{132}\) in cow and pig transferrin (46), which suggests that 1132M is a structurally conservative substitution. To provide a possible sensitive indicator for metal ion binding in the N-lobe of human serum transferrin and to investigate the similarity between the two lobes of transferrin, Ile\(^{132}\) was mutated to Met using site-directed mutagenesis. It is easy to produce N-lobe protein in this way in the quantities required for NMR. In contrast, recombinant C-lobe is difficult to prepare, but the N-lobe and C-lobe metal binding constants are usually close (47).

The \(^{1}H\) NMR spectrum of I132M hTF/2N was similar to that of wild-type hTF/2N in the both high-field and His C2H regions, except for the disappearance of the peak for the \(\gamma\)CH\(_{2}\) of...
Ille132 at $-0.603$ ppm. Both Bi$^{3+}$ and Fe$^{3+}$ induce similar chemical shift changes for the high field-shifted peak for $\delta_{CH}$ of Leu122 in the mutant and wild-type hTF/2N (Fig. 1). This suggests that the overall structure of the mutant is similar to that of wild-type hTF/2N. This was also confirmed by molecular modeling, which showed that the protein backbone fold of the mutant is almost identical to that of the wild-type protein. Six of the 10 lowest energy structures placed the side chain of Ile132 above Trp128 (data not shown), a situation which would give rise to a ring current shift for the $\epsilon$-CH of Met132.

In the two-dimensional $^1$H,$^1$C NMR spectrum of apo-I132M hTF/2N, the $\epsilon$-CH$_3$ resonance of Met132 exhibits a significant $^1$H NMR high-field shift compared with the rest of the Met peaks, as does the analogous cross-peak for Met464 in the C-lobe. Only small changes in shifts of the Met132 resonance ($\Delta \delta = 0.06/-0.05$ ppm for $^1$H and $^1$C, respectively, Table I) occurred when Bi$^{3+}$ or Fe$^{3+}$ binds to the mutant I132M hTF/2N, in contrast to the large shifts for Met464 ($\Delta \delta = -0.20/1.90$ ppm for $^1$H and $^1$C, respectively) suggesting that the structural changes in helix 5 on loading the protein with metal ions are slightly different for the N- and C-lobes. X-ray crystallographic studies have shown that when metal binding domain closure occurs, helix 5 pivots on helix 11 and that a domain movement of about 54$^\circ$ occurs in the N-lobe but only about 15$^\circ$ rotation in the C-lobe (48, 49).

Our studies suggest, for the first time, that transferrin should be considered as a potential mediator for bismuth transport in blood plasma. Previously, it has been assumed (8) that albumin, the most abundant protein in blood serum with a free thiol group at Cys34, is a target site for bismuth drugs, since Bi$^{3+}$ is known to have a high affinity for thiolate sulfur. Glutathione is a thiote sulfur-containing peptide (GSH), for example, can readily displace Bi$^{3+}$ from its complexes with citrate and EDTA at biological pH values (9). Recent reports (8) have shown that only 2% of albumin molecules bind to Bi$^{3+}$ if binding is assumed to occur at the free thiol group of Cys$^{34}$ (pK$\alpha$ about 5 (50)). In this work we have demonstrated that binding of Bi$^{3+}$ to albumin is nonspecific; even 25 mol eq of Bi$^{3+}$ did not saturate albumin, and Cys$^{34}$ is not blocked by Bi$^{3+}$ binding. Previous $^1$H NMR studies of albumin have shown that the imidazole CH resonances of His$^8$ are sensitive to the oxidation of Cys$^{34}$ and to the formation of adducts with gold antiarthritic drugs (41) probably because such reactions lead to movement of the imidazole chain of Cys$^{34}$ which is communicated to His$^8$ via interacting helices. The His regions of $^1$H NMR spectra of albumin in the presence of 1123M hTF/2N or of blood plasma in the presence of intact hTF were found to be almost identical after addition of bismuth compounds (data not shown) which provide further evidence that Cys$^{34}$ is not a major binding site for Bi$^{3+}$.

We have successfully used two-dimensional HSQC NMR spectroscopy to probe changes of Met resonances of transferrin in solution in which the concentration of albumin is 10 times higher. The observation of similar changes in the chemical shifts of the Met residues of I132M hTF/2N on binding Bi$^{3+}$ in the presence or absence of a large excess of albumin, and even in blood plasma, suggests that similar conformational changes are induced by Bi$^{3+}$ under these conditions. Such structural changes could be important for recognition by the transferrin receptor. Bi$^{3+}$ was also observed to bind to intact transferrin in the presence of a large excess (12 mol eq) of serum albumin or recombinant albumin and a similar behavior was observed in blood plasma. Our findings may have implications for the mechanism of neurotoxicity of bismuth drugs (encephalopathy). For a long time it has not been clear how bismuth is transferred to the brain. It is generally accepted that the diagnosis of bismuth encephalopathy can be confirmed by the detection of high Bi$^{3+}$ levels in whole blood, serum, or plasma, the so-called Hillemand safety level (6). It is likely that once bismuth has entered into blood it is transported by transferrin, in a similar manner to Al$^{3+}$. Al$^{3+}$ deposition in the brain is known to cause dialysis encephalopathy and this neurotoxicity is related to transferrin transportation and transferrin receptor recognition in the brain (51).

Selective labeling of the protein in combination with inverse NMR detection is a powerful method for probing the structure and dynamics of high molecular mass proteins, and provides an approach for investigating the translocation of metallo-drugs (and other drugs) between proteins and enzymes at concentrations of biologically relevance without separation, and can also be applied to protein-ligand (in this case for drug screening) (52) and protein-protein interactions.

Acknowledgments—We are grateful to Professor Ross MacGillivray and Bea Tam (University of British Columbia) for providing plasmids. Drs. John A. Parkinson and John Parkinson for assistance with NMR and molecular modeling, and Dr. John Woodrow (Delta Biotechnology) for providing recombinant albumin. We also acknowledge use of the Protein Data Bank (Brookhaven National Laboratory and Structural Bioinformatics).

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