Isolation of Two Glycolipid Transfer Proteins from Bovine Brain: Reactivity toward Gangliosides and Neutral Glycosphingolipids†

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ABSTRACT: Two glycolipid transfer proteins that catalyze the transfer of gangliosides and neutral glycosphingolipids from phosphatidylcholine vesicles to erythrocyte ghosts have been isolated from calf brain. Purification procedures included differential centrifugation, precipitation at pH 5.1, ammonium sulfate precipitation, and gel filtration on Sephadex G-50 and G-75. The final stage employed fast protein liquid chromatography (Mono S), producing two peaks of activity. Apparent purity of the major peak (TP I) was approximately 85–90%, as judged by sodium dodecyl sulfate/urea–polyacrylamide gel electrophoresis. That of the minor fraction (TP II) was less. The major band of both fractions had a molecular mass of approximately 20000 daltons. Both proteins catalyzed the transfer of ganglioside GM1 as well as asialo-GM1, but transfer protein I was more effective with di- and trisialogangliosides. Transfer protein II appeared to be somewhat more specific for neutral glycolipids in that GA1 was transferred more rapidly than any of the gangliosides; however, lactosylceramide transfer was relatively slow. Neither protein catalyzed transfer of phosphatidylcholine.

Proteins that catalyze the transfer of glycolipids between membranes have been described in several recent reports. These are analogous to the widely distributed and well-characterized phospholipid transfer proteins [for reviews, see Zilversmit and Hughes (1976), Wirtz and van Deenen (1977), and Helmkamp (1986)]. Glycolipid exchange activity for neutral glycolipids has been observed in spleen (Metz & Radin, 1982), liver (Bloj & Zilversmit, 1981; Yamada & Sasaki, 1982a), and brain (Yamada & Sasaki, 1982b; Abe et al., 1984; Sasaki et al., 1984; Sasaki & Demel, 1985; Wong et al., 1984; Yamada et al., 1985; Brown et al., 1985). While these studies have dealt for the most part with relatively simple glycolipids, a few have also described activity toward gangliosides GM1[1] (Bloj & Zilversmit, 1981; Brown et al., 1985) and GM3 (Yamada et al., 1985). We recently presented evidence for ganglioside transfer activity in bovine and rat brain that catalyzes transfer of GM1 from sonicated vesicles to erythrocyte ghosts and neuronal membranes (Gammon & Ledeen, 1985). Since gangliosides are major glycolipids of both neurons and glia (Yu & Iqbal, 1979; Ledeen, 1983, 1985), it was of interest to determine whether a transfer protein(s) exist(s) in brain that act(s) on the bulk of these substances. We describe here isolation from calf brain of a protein that catalyzes transfer of the four major gangliosides of this tissue, and certain neutral glycolipids as well. We also obtained evidence for a second transfer protein with somewhat less activity toward gangliosides and relatively more toward neutral glycolipid(s).

Materials and Methods

Materials. Radiolabeled [^{3}H]GM1 (2 Ci/mmol), NaB^3H_4 (7.9 Ci/mmol); [^{3}H]triolein (112 mCi/mmol), [^{14}C]triolein (109 mCi/mmol), and [^{14}C]phosphatidylcholine (112 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled gangliosides were gifts of Fidia Research Laboratories (Abano Terme, Italy), and asialo-GM1 (GA1) was a gift of Dr. Robert Yu. The following substances were purchased as indicated: egg yolk phosphatidylcholine (type V-EA), phenylmethanesulfonyl fluoride (PMSF) and other protease inhibitors, fatty acid free bovine serum albumin, and palladium on barium sulfate from Sigma Chemical Co. (St. Louis, MO); electrophoresis reagents and Bradford protein assay kit from Bio-Rad Laboratories (Rockville, NY); electrophoretic-grade urea from Schwarz/Mann (Cambridge, MA); Sephadex G-50 and G-75 and FPLC Mono-S column (analytical) from Pharmacia Fine Chemicals (Piscataway, NJ). Outdated human blood samples were obtained from a blood bank for preparation of erythrocyte ghosts.

Preparation of Radiolabeled Glycolipids. [^{3}H]GM1 and [^{3}H]lactosylceramide ([^{3}H]Lac-Cer) were prepared by the method of Leskawa et al. (1984). Individual gangliosides and GA1 were labeled with ^{3}H according to a modification of the Schwartzmann (1978) method; the catalyst employed was palladium on barium sulfate, and the solvents were water (for gangliosides) and tetrahydrofuran/water (4/1, for GA1). Some [^{3}H]GM1 was prepared by this method, and the rest was purchased (see above). Radiolabeled lipids were purified by preparative TLC or the DEAE-Sephadex method (Ledeen & Yu, 1982). Radiopurity, determined by TLC, was >90% for each glycolipid used.

Preparation of Donor/Acceptor Membranes and Assay of Transfer Activity. Small sonicated vesicles and erythrocyte ghosts were prepared as described previously (Gammon & Ledeen, 1985) with slight modifications. Phosphatidylcholine vesicles containing 5–10 mol % GA1, Lac-Cer, or an individual ganglioside were prepared by sonication of a suspension of the phospholipid, ^{3}H-labeled glycolipid [(5–20) × 10^3 dpm], and

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Lac-Cer, lactosylceramide; GA1, asialo-GM1 (gangliotetraosylceramide); TLC, thin-layer chromatography; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid. Ganglioside nomenclature is that of Svennerholm (1963).

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enough unlabeled glycolipid to give the desired concentration. [^{14}C]Triolein was included as a nontransferable vesicle marker. Phosphatidylcholine transfer was determined with vesicles containing trace amounts of [^{14}C]phosphatidylcholine and [^{3}H]triolein (no glycolipid). Transfer activity was measured as described (Gammon & Ledeen, 1985). Correction was made for cosedimentation of vesicles by quantitation of the [^{14}C]- or [^{3}H]triolein. Correction was also made for uncatalyzed transfer by conducting parallel incubations without transfer protein.

Purification of Glycolipid Transfer Proteins. Extracts enriched in transfer activity were routinely prepared from approximately 600 g of whole calf brain by an extension of the method previously described (Gammon & Ledeen, 1985). A 35% homogenate of brain was prepared in SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4) containing 2 mM PMSF with a Waring blender and Polytron tissue processor. Following centrifugation at 13000*g* for 30 min, the pH of the supernatant was adjusted to pH 5.1 and the resulting precipitate sedimented by centrifugation. The pH of the supernatant was readjusted to 7.4 and ammonium sulfate added. Protein precipitating between 40 and 90% ammonium sulfate was sedimented, resuspended in 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol (DTT) and 0.02% sodium azide, and dialyzed against the same buffer. The dialyzate was concentrated to approximately 60 mL (concentration of 40 mg/mL) by ultrafiltration with an Amicon YM10 membrane of 43-mm diameter. It was centrifuged at 100000*g* for 60 min and the supernatant divided into 10–12-mL portions for application to a Sephadex G-75 column (5 × 50 cm) equilibrated in the same buffer. The column was eluted by gravity flow, and the included fractions 7–24 (340–360 mL; cf. Figure 1) were pooled and concentrated (Amicon YM10 membrane, 25-mm diameter) to approximately 20 mg/mL protein (2.5-mL total volume). To this was added bovine serum albumin (BSA), 50 μg/mL, 1 μg/mL leupeptin, and 5 mg/mL aprotinin as protease inhibitors (later on these inhibitors were added at an earlier stage). The concentrate was then applied to a Sephadex G-50 column (1.5 × 86 cm) to remove more of the remaining high molecular weight proteins. Elution was carried out with the above phosphate buffer (pH 7.4) containing leupeptin and aprotinin, at a flow rate of 24 mL/h; a peristaltic pump was used to adjust the flow rate. Fractions of 3 mL were collected.

Fractions 21–28 (cf. Figure 2) were pooled, the pH was adjusted to 6.1, and portions (6 mL = 3 mg of protein) were applied directly to an FPLC Mono S cation-exchange column adapted to a Beckman Model 420 HPLC. FPLC applications were always filtered thru a 0.2-μm Millipore filter. Prior to application, the Mono S column was equilibrated in 5 mM sodium phosphate (pH 6.1) containing 1 mM DTT (buffer A). After being loaded, the column was eluted (1 mL/min) with 10 bed volumes (10 × 1 mL) of buffer A. The column was then developed at the same flow rate with an increasing gradient of buffer B (buffer A containing 1 M NaCl) from 0–265 mM NaCl over 35 min. Aliquots from collected fractions (2 mL each) were assayed for protein [200–400 μL—Bradford (1976)] and glycolipid transfer activity (100 μL). Those fractions enriched in activity were pooled, diluted 5 times with buffer A and reapplied to the same Mono S column, which had been regenerated with 1 M NaCl and buffer A in sequence. Fractions containing transfer activity were pooled and lyophilized for gel electrophoresis. Alternatively, the transfer protein was stored for 4–6 weeks at −20 °C without appreciable loss of activity; for this purpose we added BSA (3 mg/mL), DTT (1 mM), and sodium azide (0.02%).

Table I: Summary of Isolation of Transfer Protein from Calf Brain[a]

steps	total protein (mg)	protein recovery (%)	transfer activity (pmol min^{-1} mg^{-1})	purification (*x*-fold)
(1) first 13000*g* supernatant	9310	100	NM	1
(2) pH 5.1, 13000*g* supernatant	4630	50	NM	2
(3) 90% $(NH_4)_2SO_4$	2320	25	0.74	4
(4) G-75 column, fractions 7–24	798	8.60	63.5	343
(5) G-50 column, fractions 21–28	54	0.60	74.3	401
(6) Mono S column	4.0	0.04	261	1411

[a] Calf brain, 600 g wet weight, was homogenized as described and centrifuged at 13000*g* for 30 min to give supernatant in step 1. Purification for the first three steps was estimated from percent recovery, since these fractions could not be assayed with accuracy. The run shown here was typical of results in our standardized procedure. Transfer activity was assayed with GM1. NM, not measured.

Protein Analysis. Protein was assayed by the method of Lowry (1951) or Bradford (1976). The latter was employed when protein concentration was low or DTT concentration was sufficient to cause interference by the Lowry method. Lyophilized proteins were subjected to polyacrylamide gel electrophoresis (PAGE) on 15% acrylamide gels (Weber & Osborn, 1969). We also employed the method of Swank and Munkres (1971) with the modification that electrophoresis was on 15% polyacrylamide/6 M urea gels. Electrophoresed proteins were visualized by staining with Coomassie brilliant blue overnight. Gel patterns were scanned with a Gilford 2400 spectrophotometer.

Results and Discussion

Most of the procedures employed here for purification of the glycolipid transfer proteins were similar to those previously employed for related proteins from bovine (Wong et al., 1984) and pig brain (Abe et al., 1984; Sasaki et al., 1984) and the nonspecific lipid transfer protein of bovine liver (Crain & Zilversmit, 1980), which showed activity toward globoside and GM1 (Bloj & Zilversmit, 1981). In addition, we have employed an FPLC Mono S column, which provided effective resolving power at the final stage of purification and facilitated isolation of two glycolipid transfer proteins from calf brain.

A summary of the isolation procedure and the purification achieved at each step is given in Table I. Considerable enrichment was effected with Sephadex G-75 (Figure 1) while Sephadex G-50 was less effective (Figure 2). It was concluded that the latter step was only of marginal utility. The final chromatography, with a Mono S column (FPLC), gave considerable enrichment for the first transfer protein (TP I) and an indeterminant amount for the second (TP II). The two peaks of glycolipid transfer activity were well separated (Figure 3). The protein elution profile (not shown) coincided with the major peak of transfer activity, with a small shoulder corresponding to the second peak. Since Mono S is a cation-exchange resin, acidic proteins remaining after Sephadex chromatography were washed through before elution with the salt gradient. The first and major peak of transfer activity (TP I) was rechromatographed on the Mono S column to give a protein that, on the basis of densitometric scans, appeared to be approximately 85–90% homogeneous on SDS/urea–polyacrylamide gel electrophoresis (PAGE). Similar results were obtained with the Weber and Osborn (1969) procedure

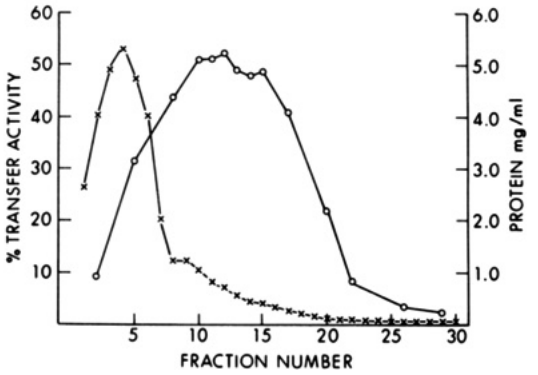

FIGURE 1: Sephadex G-75 chromatography of fraction precipitated by $(NH_4)_2SO_4$. Protein (400–500 mg in 10–12 mL) was applied to a 5 × 50 cm column equilibrated in 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT and 0.02% NaN_3. Fractions of 20 mL were collected, after the first 280 mL of eluate was discarded, and assayed for transfer activity with [^{3}H]GM1. Flow rate, by gravity, was approximately 4 mL/min. Fractions 7–24 were pooled and concentrated to 2.5 mL (50 mg of protein) for application to Sephadex G-50. Protein (×); transfer activity (○).

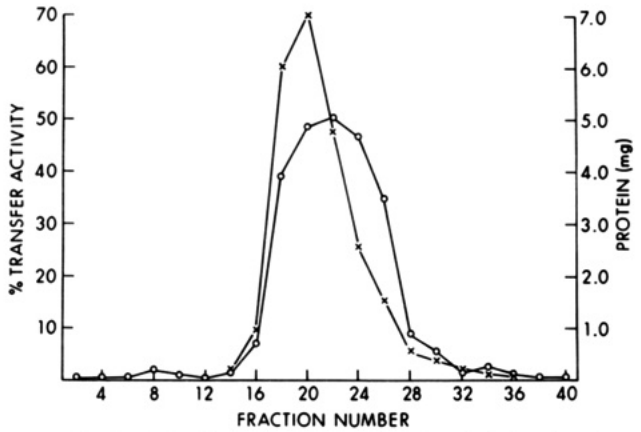

FIGURE 2: Sephadex G-50 chromatography of pooled fractions from Sephadex G-75 column (cf. Figure 1). Protein (50 mg in 2.5 mL) was applied to a 1.5 × 86 cm column equilibrated with the buffered mixture described in Figure 1; also present in some runs were BSA, leupeptin, and aprotinin. Fractions of 3 mL were collected at a flow rate of 24 mL/h (under slight pressure and controlled by peristaltic pump). Transfer activity was assayed with [^{3}H]GM1. Fractions 21–28 were pooled for application to Mono S. Protein (×); transfer activity (○).

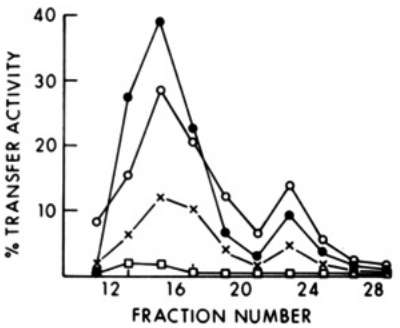

FIGURE 3: FPLC Mono S chromatography of pooled fractions from Sephadex G-50 column. Protein (3 mg in 6 mL) was applied in sodium phosphate buffer (pH 6.1) with 1 mM DTT to a column equilibrated in the same medium. After being loaded, the column was eluted at a rate of 1 mL/min with 10 bed volumes of the above buffer and then with the NaCl gradient as described (Materials and Methods). Fractions of 2 ml were collected and aliquots assayed for protein and transfer activity: (●) GM1; (○) GA1; (×) Lac-Cer; (□) phosphatidylcholine.

(Figure 4). Its molecular mass was estimated from these gels to be approximately 20 000 daltons.

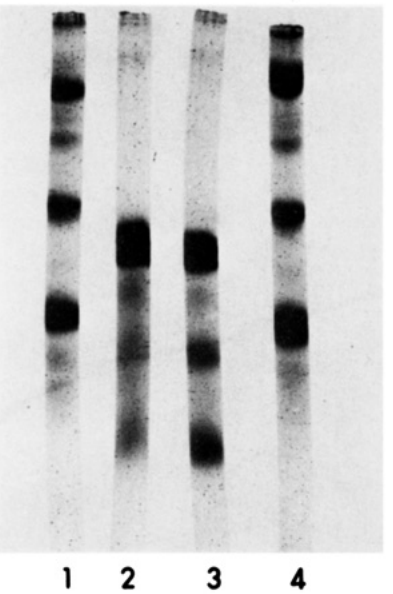

FIGURE 4: Gel electrophoresis of the major purified glycolipid transfer protein (TP I) after second chromatography on FPLC Mono S. Electrophoresis was performed on 12% polyacrylamide–0.1% SDS according to Weber and Osborn (1969), staining with fast green. Lanes 1 and 4 are standards: bovine serum albumin (69 kDa), chymotrypsinogen (23 kDa), and ribonuclease (14 kDa), respectively, from the top. Lane 2 represents the fraction from Mono S containing the peak of transfer activity, pooled fractions 14 and 15 (cf. Figure 3); lane 3 was the following eluent, pooled fractions 16 and 17. Densitometric scanning of lane 2 indicated 85–90% purity.

Table II: Relative Transfer Rates of Different Substrates Catalyzed by Glycolipid Transfer Proteins I and II[a]

substrate	TP I	TP II
GM1	156	83
GD1a	74	54
GD1b	65	59
GT1b	59	55
GA1	100	100
Lac-Cer	27	39

[a] Relative rates of transfer were related to GA1. Fractions of TP I and TP II close to the peaks eluted from Mono S (Figures 3 and 5) were employed. The absolute rate of GA1 for TP I was 199 pmol min^{-1} (mg of protein)$^{-1}$.

The second peak of transfer activity (TP II) off the Mono S column showed a major band on SDS–PAGE that ran parallel to TP I (not shown). Several minor bands were present, and while purity was difficult to estimate, it was judged to approximate 50–60%. In general this fraction had too little protein for effective recycling, and it also tended to lose activity when reapplied to the Mono S column.

Comparison of specific transfer activity toward different glycolipid substrates revealed TP I to be more reactive toward GM1 than GA1, its asialo derivative (Table II, Figure 3). The other gangliosides were only slightly less reactive while Lac-Cer was the least effective substrate. A different order of reactivity was seen for TP II: GA1 was transferred somewhat more efficiently than GM1, and the other gangliosides were transferred at considerably lower rates (Table II, Figure 5). Neither protein catalyzed the transfer of phosphatidylcholine.

The transfer proteins described by Sasaki and co-workers (Yamada et al., 1985) and Thompson and co-workers (Brown et al., 1985) were also active toward a variety of glycolipids. Comparing relative reactivites, our TP II, with the sequence GA1 > GM1 > Lac-Cer, would appear to resemble the transfer protein described by Brown et al. (1985). Actual numerical values reported here differ considerably from those

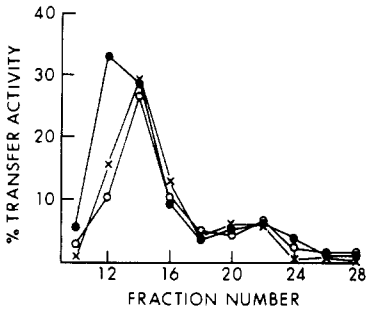

FIGURE 5: FPLC Mono S chromatography of glycolipid transfer proteins employing the major brain gangliosides as substrates. See legend to Figure 3 for details. This represents the first chromatography on Mono S following Sephadex G-50: (●) GD1a; (○) GD1b; (×) GT1b.

of Brown et al. (1985), perhaps owing at least in part to the different assay systems employed. The lower rates we found approximate that reported for GM1 by Bloj and Zilversmit (1981), who also used erythrocyte ghosts as receptor membranes. Sasaki and co-workers concluded from examination of several acidic and neutral glycolipids that an inverse relationship existed between the length of the sugar chain and the transfer rate (Sasaki et al., 1984; Yamada et al., 1985); however, they did not test gangliosides more complex than GM3. Thus, it appears that TP I, with an apparent preference for gangliosides over neutral glycolipids of shorter chain, may be a different protein from that isolated by either Thompson's group from bovine brain or Sasaki's group from pig brain. It may be noted that the assay procedure employed during isolation of our proteins utilized GM1 as substrate and hence may have selected for a ganglioside-reactive species. Additional studies are needed to clarify the relationship between our two glycolipid transfer proteins of brain and those described by the above groups. The fact that neither of our transfer proteins nor those of Thompson or Sasaki catalyzed transfer of phospholipids indicates a basic difference from the transfer protein of bovine liver described by Bloj and Zilversmit (1981).

Conzelmann et al. (1982) have reported that the activator protein which promotes hexosaminidase A catalyzed hydrolysis of GM2 and GA2 can also function as a ganglioside transfer protein in the absence of enzyme. However, the fact that it possessed an acidic isoelectric point (Conzelmann & Sandhoff, 1979) and codistributed with a lysosomal marker (Banerjee et al., 1984) indicates a different protein than those isolated by us and the other groups from brain. The glycolipid transfer protein isolated by Metz and Radin (1982) from bovine spleen bears many similarities to the brain proteins.

The biological role of glycolipid transfer proteins in the nervous system remains to be clarified. The functional location of glycolipids is on the extracellular surface of cells (Steck & Dawson, 1974; Gamberg & Hakomori, 1975; Critchley et al., 1981) while their site of synthesis is in the Golgi apparatus (Morre et al., 1979). In the latter organelles, as well as the vesicles thought to transport them to the cell surface, glycolipids are believed to face the luminal compartment, consistent with the observed resistance of gangliosides in this compartment to neuraminidase (Landa et al., 1981). With this orientation, it is difficult to visualize an intracellular role for glycolipid transfer proteins, unless it is within the lumen of the Golgi. We have speculated on the possibility that ganglioside transfer may be an extracellular process in brain (Byrne et al., 1984), but firm evidence for such a role is not yet available. The presence of a small pool of soluble gangliosides has been reported in brain cytosol (Ledeen et al., 1976; Sonnino et al., 1979), which raises the possibility that at least some glycolipid movement within (or between) cells may proceed by mechanisms other than vesicle transport. Aside from this question of natural biological function, glycolipid transfer proteins can be viewed as promising research tools for manipulation of the glycolipid content of cellular membranes.

ACKNOWLEDGMENTS

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Registry No. GM1, 37758-47-7; GA1, 71012-19-6; Lac-Cer, 4682-48-8; GD1a, 12707-58-3; GD1b, 19553-76-5; GT1b, 59247-13-1.

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Conformational Sensitivity of β-93 Cysteine SH to Ligation of Hemoglobin Observed by FT-IR Spectroscopy†

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ABSTRACT: The SH vibrational absorption of cysteine F9(β-93) in concentrated aqueous solutions of native liganded hemoglobin (human HbA, horse, and bovine) has been observed by use of Fourier transform infrared spectroscopy. The pattern of β-93 SH absorption intensity is ligand dependent. In bovine hemoglobin derivatives the SH absorption intensity pattern is (carbonmonoxy)hemoglobin (HbCO) > oxyhemoglobin (HbO_2) = cyanomethemoglobin (HbCN) ≫ aquomethemoglobin (metHb) and deoxyhemoglobin (deoxyHb). In horse and human hemoglobin derivatives the pattern is HbCO ≥ HbO_2 > HbCN > metHb. The bovine metHb β-93 SH shows a much lower absorptivity than that of horse or human metHb, and thus it has a different local tertiary equilibrium conformation than does horse or human hemoglobin. X-ray diffraction studies have shown the β-93 SH in carbon monoxide or oxygen bound hemoglobin to be situated within a nonpolar pocket between the F, G, and H helices. The higher than usual SH absorption frequency (2592 cm^{-1}) that we observe implies there is no hydrogen bonding for this thiol group while situated within this nonpolar pocket. A similar β-93 SH absorption has been observed in the β-chain tetramer (thalassemic hemoglobin H in vivo). The β-112 SH stretching band, previously observed in the $\alpha_2\beta_2$ tetramer, was observed for the first time in the β-chain tetramer. A band at 2610 cm^{-1} that is not due to SH was resolved and found to be ligand dependent.

Mammalian hemoglobin is a tetrameric protein consisting of two α and two β subunits that cooperatively bind heme ligands. The oxygen affinity of the isolated subunits is unaltered when they assemble into $\alpha_1\beta_1$ dimers, and ligand binding is noncooperative for both monomers and dimers (Mills & Ackers, 1979). Comparisons of the X-ray crystallographic structures of tetrameric hemoglobin (Baldwin & Chothia, 1979; Fermi et al., 1984) show that ligand binding causes large intersubunit changes about the hemes that extend to the $\alpha^1\beta^2$ contact region with only minor differences at the $\alpha^1\beta^1$ interface. X-ray diffraction does not allow individual bond lengths to be determined with sufficient precision to identify very small changes in large molecules, such as those that might be present at the $\alpha^1\beta^1$ contact. Detection of bond vibrations may enable one to do this, but the problem has always been to isolate and identify the individual vibrating group. We have previously shown that cysteine sulfhydryl vibrations absorb in an isolated spectral region and that FT-IR[1] spectroscopy has the sensitivity to measure the absorption from these groups in concentrated protein solutions (Bare et al., 1975). Cysteine SH groups have proven to be very sensitive molecular probes of protein conformation. The frequency of the SH vibrational absorption is decreased by H-bonding to a nucleophilic group, and the extent of this decrease is determined by the base strength of the associated nucleophile. In similar fashion, the area of the absorption band is proportional to the integrated absorption coefficient (B), which increases with H-bonding due to the greater change in SH

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[1] Abbreviations: FT-IR, Fourier transform infrared; Hb, hemoglobin; HbCO, (carbonmonoxy)hemoglobin; HbO_2, oxyhemoglobin; HbF, fluoromethemoglobin; HbCN, cyanomethemoglobin; HbN_3, azidomethemoglobin; HbNO, nitrosylhemoglobin; metHb and $HbOH_2$, aquomethemoglobin; deoxyHb, deoxyhemoglobin; HbOOCH, formatomethemoglobin; $(\beta CO)_4$, tetramer of carbonmonoxy β-chains; $(\beta O_2)_4$, tetramer of oxy β-chains; deoxyβ_4, tetramer of deoxy β-chains; $\alpha_1\beta_1$, α–β dimer; $\alpha_2\beta_2$, α–β tetramer; PMB, *p*-(chloromercuri)benzoate. Superscripts denote interfacial contacts between α- and β-chains, as in $\alpha^1\beta^1$ or $\alpha^1\beta^2$.

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