Structural Evidence for an Anion-Directing Track in the Hen Ovotransferrin N-Lobe: Implications for Transferrin Synergistic Anion Binding†

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ABSTRACT: The transferrins are a class of iron-binding proteins that require the presence of a synergistic anion for conformation-dependent binding of ferric ions. Bromopyruvate, a known synergistic anion and affinity label of ovotransferrin (oTF) [Bailey, C. T., Patch, M. G., & Carrano, C. J. (1988) *Biochemistry 27*, 6276−6282], was used to probe the structure of the metal- and anion-binding sites of the functional N- and C-terminal proteolytic halves (oTF/2N and oTF/2C, respectively) of ovotransferrin. Incubation of oTF/2N with [2-^{14}C]bromopyruvate in the presence of Fe^{3+} ions resulted in the incorporation of 0.70 mol of ^{14}C label/mol of oTF/2N; ^{14}C-labeled oTF/2N was then purified and digested sequentially with trypsin and V8 protease to determine the sites of modification. Quantification of ^{14}C radioactivity, analysis of purified ^{14}C-labeled peptides by gas-phase sequencing and mass spectrometry demonstrated that chemical modification was restricted to nucleophilic residues contained in a fragment corresponding to residues 189−204 of oTF/2N, including Lys 199, Lys 202, and His 196. Lysine 199 was also protected from modification with [^{3}H]CH_2O in iron-saturated oTF/2N, suggesting the involvement of this residue in anion binding by the apo conformation [Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V., & Baker, E. N. (1990) *Nature 344*, 784−787]. Lysine 199 is conserved as a basic residue in the N-terminal metal-binding domains of the transferrins but not in the homologous C-terminal metal-binding domains. Identical trials with oTF/2C showed binding, but not modification, with bromopyruvate. These data suggest that Lys 199, Lys 202 and His 196, which are located on an α-helix (8) that terminates at the anion-binding site [Dewan, J. C., Mikame, B. Hirose, M., & Sacchettini (1993) *Biochemistry 32*, 11963−11968], attract and channel the synergistic anion to the anion-binding site. The presence or absence of basic residues in the metal-binding lobes of transferrins may account for the different anion- and metal-binding characteristics observed for the iron-binding sites of these proteins.

The transferrins comprise a class of 80-kDa, non-heme, iron-bonding glycoproteins, which include ovotransferrin (oTF)[1] from egg white, serum transferrin from blood plasma, lactoferrin from mammalian milk, and melanotransferrin from melanocytes (Aisen & Listowski, 1980; Brock, 1985). These proteins are characterized by a single polypeptide chain which is folded into two structurally similar lobes, each capable of binding a high-spin ferric ion in a conformationally dependent manner (Anderson et al., 1989, 1990; Grossman et al., 1992). Each lobe is connected by a small peptide linker region, which can be cleaved proteolytically to yield functional N- and C-terminal half-molecules (oTF/2N and oTF/2C, respectively). X-ray crystallographic studies of human lactoferrin, rabbit serum transferrin, and oTF/2N have demonstrated that each iron-binding lobe contains two domains that define an interdomain cleft containing the iron-binding site (Anderson et al., 1989; Bailey et al., 1988b; Dewan et al., 1993). Each site possesses the same protein ligands for iron binding: the phenolic oxygens of two tyrosines, a carboxylate oxygen of aspartate, and an imidazole nitrogen of histidine. Conservation of these residues in regard to iron binding is thus far supported by physical studies and the available sequences of other transferrins (Aisen, 1989).

The transferrins require the presence of a synergistic anion, carbonate, to bind iron (Harris & Aisen, 1989). The anion occupies two cis-octahedral sites in the inner coordination sphere of iron in the iron-binding sites of lactoferrin (Anderson et al., 1989; Smith et al., 1991), serum transferrin (Wang et al., 1993), and oTF/2N (Dewan et al., 1993) and is bound directly between the metal and a region of positive charge density in the interdomain cleft, comprised of a conserved arginine and the N-terminus of an α-helix (Baker & Lindley, 1992). A series of compounds each containing a Lewis base proximal to a terminal carboxylate group also serve as synergistic anions (Schlabach & Bates, 1975; Woodworth et al., 1975), including bromopyruvate and hydroxypyruvate (Carrano & Patch, 1982; Bailey et al., 1988a). Carrano and co-workers have demonstrated that both anions also function as affinity labels of oTF, resulting in the preferential modification of lysine residues on the protein (Carrano & Patch, 1982; Bailey et al., 1988a).

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[1] Abbreviations: DTT, dithiothreitol; LSIMS, liquid secondary ion mass spectrometry; *m/z*, mass to charge ratio; OPA, *o*-phthalaldehyde; oTF, ovotransferrin; oTF/2C, C-terminal half-molecule of ovotransferrin; oTF/2N, N-terminal half-molecule of ovotransferrin; oTF/2N·-$(CH_3)_n$ and oTF/2C·$(CH_3)_n$, methylated ovotransferrin half-molecules; ppm, chemical shift in parts per million; TFA, trifluoroacetic acid; TMS, tetramethylsilane.

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Several lines of evidence suggest that the synergistic anion binds first in a series of events that culminate in formation of the iron−transferrin−anion ternary complex (Kojima & Bates, 1981; Cowart et al., 1982; Anderson et al., 1990, Lindley et al., 1993). On the basis of the X-ray crystallographic structure for apolactoferrin (Anderson et al., 1990), it has been suggested that the negatively charged anion is attracted by conserved positively charged residues that are exposed in the open conformation. The anion presumably reduces the positive charge in the immediate vicinity of the binding site and thereby facilitates entry of the metal cation into the iron-binding site and subsequent conformational charge, resulting in closure of the interdomain cleft (Kilar & Simon, 1985; Baker & Lindley, 1992; Grossman et al., 1992). To test this notion, we have carried out conformation-specific labeling of the apo and iron-bound forms of oTF/2N with [^{3}H]CH_2O and the synergistic anions bromo-2-[^{13}C]pyruvate and bromo-2-[^{14}C]pyruvate. Several basic residues were modified by bromopyruvate, including His 196 and Lys 199, which was also protected from modification by [^{3}H]CH_2O in the iron-bound conformer of oTF/2N. Both Lys 199 and His 196 are conserved as basic amino acids in the homologous N-terminal metal-binding domains of the transferrins and are located on α-helix 8 (Dewan et al., 1993), which terminates at the anion-binding site (Baker & Lindley, 1992). The results suggest that these residues may attract and channel the synergistic anion to the anion-binding site, which is consistent with the mechanism for metal binding described above.

Although the N- and C-terminal lobes of the transferrins contain equivalent ligands for anion and metal binding (Baker & Lindley, 1992), a large body of evidence indicates that there are differences in metal and anion binding between the two sites (Princiotto & Zapolski, 1975; Folajtar & Chasteen, 1982; Williams et al., 1982a; Harris, 1989; Sola, 1990; Dewan et al., 1993). This suggests that additional amino acid side chains on the transferrins and/or secondary effectors (nonsynergistic anions) may mediate iron binding and release (Folajtar & Chasteen, 1982; Kretchmar & Raymond, 1988; Marques et al., 1991). Several corroborative reports have demonstrated the presence of two distinct anion-binding sites, which can be attributed to the individual lobes of serum transferrin and oTF (Oe et al., 1989; Harris, 1989; Harris et al., 1990). To further understand differences in anion binding by these sites, oTF/2C was treated with CH_2O and bromopyruvate in trials identical to those described above for oTF/2N. Positively charged residues were protected by modification from [^{3}H]CH_2O in the iron-bound conformer; however, in contrast to oTF/2N, covalent modification of apo-oTF/2C by bromopyruvate was not detected. The results suggest that the binding of synergistic anion by each lobe occurs by a different mechanism.

MATERIALS AND METHODS

Materials. Ovotransferrin from hen egg white and its half-molecules, oTF/2C and oTF/2N, and the corresponding iron-saturated forms, Fe-oTF/2N and Fe-oTF/2C, were prepared as previously described (Brown-Mason & Woodworth, 1984). Protein concentrations were determined from millimolar absorptivities at 280 nm obtained from dry-weight measurements of the apo and iron forms of ovotransferrin and the corresponding half-molecules (Brown-Mason & Woodworth, 1987). The synthesis of 3-bromo-2-[^{14}C]-pyruvate from [2-^{14}C]pyruvate (5−20 mCi/mmol, DuPont) was based on previously described methodologies with [1-^{14}C]pyruvate (Meloche, 1970; Bailey et al., 1988a). The specific activity of the bromopyruvate solutions was determined by published methods (Meloche, 1970). Bromo-2-[^{13}C]pyruvate was synthesized with modifications of the method of Dickens (1962) with 2-[^{13}C]pyruvate (Merck Stable Isotopes). The purity of the labeled complexes was assessed as previously described (Bailey et al., 1988a). Tritiated formaldehyde solutions (40−60 mCi/mmol, DuPont) were purified and assayed for specific activity by published procedures (Jentoft & Dearborn, 1983). All other chemicals used were reagent grade unless otherwise stated.

Preparation of Proteins and Reagents. Proteins were prepared for titration under CO_2-free conditions with modifications of previously described methods (Bailey et al., 1988a). All titrations and modifications were carried out using Schlenk inert atmosphere techniques. CO_2-free solutions were prepared by cannula transfer of appropriate volumes of N_2-saturated H_2O to preweighed reagents exposed to six N_2-atmosphere vacuum−purge cycles. Iron solutions were prepared in the same manner by addition of 1 drop of H_2SO_4 to a 40 mM solution of $Fe(SO_4)\cdot 7H_2O$ to prevent formation of insoluble polyhydroxides (Harris, 1975). N_2-saturated water was prepared by boiling H_2O under a N_2 atmosphere and passing N_2 through a submerged sintered glass tube until the water reached room temperature for three repetitions. N_2 was freed of CO_2 and O_2 by passing the gas through an R-311 catalyst column and a 35% (w/w) NaOH solution. CO_2-free NH_3 was obtained from an ammonia generator containing a 1:1 NH_4OH:25% (w/w) NaOH solution over a $CaCl_2$ bed with purified N_2 as the carrier gas. Lyophilized proteins and reagents were subjected to six N_2-atmosphere vacuum-purge cycles prior to anion-binding studies. The removal of iron from the half-molecules was performed by previously described methods (Mason et al., 1987).

Chemical Modification of oTF/2N and oTF/2C. Reductive methylation of amine side chains on the oTF half-molecules with [^{3}H]formaldehyde was carried out as previously described (Jentoft & Dearborn, 1983). Nonspecifically bound anion was removed by passing the protein successively through two PDG-6 desalting spin columns (5 mL gel volume), and the labeled proteins were then purified on a G-75 Sephadex (Pharmacia) column (5 × 75 cm). To determine the extent of labeling, aliquots of known amounts of the purified protein (10 μL) were transferred into vials containing 5 mL of Hydrofluor (National Diagnostics), and ^{3}H activity of the samples was determined.

Affinity labeling of the half-molecules with bromopyruvate and [2-^{14}C]bromopyruvate was carried out as previously described (Bailey et al., 1988a), except that $NaCNBH_3$ was used to reduce the carbonyl addition products of bromopyruvate and target amine side chains on the protein. Formation of the Fe−protein−bromopyruvate ternary complexes was monitored by visible spectroscopy (Bailey et al., 1988a). The extent of labeling was determined by SDS−PAGE (Laemmli, 1970). Known amounts of protein modified with 3-bromo-2-[^{14}C]pyruvate were purified by electrophoresis on 5−12% gradient polyacrylamide gels; protein bands were sectioned from the gel, and each section was incubated with 600 μL of Protosol (NEN DuPont) at 40 °C for 20 h in 7-mL polypropylene tubes. The Protosol solutions were diluted

to 5.6 mL with Hydrofluor and assayed for ^{14}C activity.

Proteolytic Cleavage of ^{14}C-oTF/2N. OFT/2N was prepared for proteolytic cleavage by published procedures (Butkowski et al., 1977). Tryptic proteolysis of ^{14}C-labeled oTF/2N was performed by previously described procedures (Williams et al., 1982b). Purified tryptic preparations (below) were lyophilized and treated with V8 protease from *Staphylococcus aureus* (Sigma) (Drapeau et al., 1972).

FPLC and HPLC of ^{14}C-oTF/2N Peptides. Lyophilized proteolysates were dissolved in 1.1 mL of 0.1% (v/v) TFA. Aliquots (100 μL) were injected onto a Pep RPC-18 column (Pharmacia) and were chromatographed in the reverse phase by FPLC. The effluent was monitored at 214 nm, and the radioactivity of each fraction was determined as previously described. The percent yields and concentrations of the labeled peptides were determined from the initial amount of soluble radioactivity, the specific activity of 3-bromo-2-[^{14}C]-pyruvate (1.67 $\times$ 106 dpm/μmol), and the amino acid content. Peptide fractions with detectable activity and identical retention times were pooled and lyophilized for HPLC.

High-performance liquid chromatography was performed on a Varian Model 5000 HPLC system equipped with a V4 variable-wavelength detector. High molecular weight peptides (>1400 Da) were chromatographed in the reverse phase on a LiChrospher 100 RP-18 column (5-μm particle diameter) (Merck). Low molecular weight hydrophilic peptides (<1400 Da) were initially separated in the reverse phase on a LiChrospher RP-18 column, and subsequently with a LiChrospher 100 RP-8 column (5-μm particle diameter).

Amino Acid Analysis. Peptides were hydrolyzed in 500 μL of 6 N constant-boiling HCl and 150 μL of thioglycolic acid (Sigma) under reduced N_2 atmosphere for 24 h. The acid hydrolysate was dried in vacuo, and amino acid analysis was performed by ion exchange with postcolumn OPA derivatization. The effluent of hydrolysates from ^{14}C-labeled peptides was fractionated, and each sample was analyzed by scintillation spectrophotometry to correlate retention times of modified amino acids (model compounds) to ^{14}C-labeled residues on the peptide. Dityrosine was assayed as previously described (Fry, 1984).

Gas-Phase Sequence Analysis. Peptides were prepared for sequence analysis by HPLC with acetonitrile−water gradients (0.1% in TFA) to remove nonvolatile salts. Appropriate fractions were lyophilized and diluted to 30−50 μL with 60% acetonitrile. Sequence analysis was performed on a Model 470A gas-phase protein sequencer (Applied Biosystems) by automatic Edman degradation (Edman & Begg, 1967). Identification of phenylthiohydantoins was by HPLC.

Synthesis of Model Complexes. To determine specific structural modifications of amino acids resulting from derivatization of the protein, the following model compounds were synthesized for comparative analysis. The synthesis and purification of dityrosine and isodityrosine (oxidative coupling products of tyrosine) was carried out as previously described (Fry, 1984). *N,N*-Dimethyl lysine was synthesized from *N*-acetyllysine by the procedures described above (Means, 1977). The reductive amination product of bromopyruvate and *N*-acetyllysine was synthesized and purified by previously described procedures (Bailey et al., 1988a).

Mass Spectrometric Analysis. Mass spectrometry was carried out at the Mass Spectrometry Facility, University of California, San Francisco. Peptide molecular weights were measured by means of liquid secondary ion mass spectrometry (LSIMS). Mass measurements were carried out on a Kratos Concept IH double-focusing mass spectrometer (Kratos Analytical Instruments, Manchester, U.K.) equipped with an LSIMS source (Falick et al., 1986a) and a cooled sample introduction probe (Falick et al., 1986b). Peptide lyophilysates were dissolved in 0.1% aqueous TFA, and aliquots of the solution were added to a 1:1 glycerol: thioglycerol/0.1 M HCl matrix on the probe tip.

^{13}C NMR Spectrometry of Model Compounds and Reagents. Carbon-13 nuclear magnetic resonance spectra were recorded on the 5.87-T Bruker NMR spectrometer. Spectra were acquired in the Fourier transform mode with a sweep width of 25 000 Hz and a relaxation delay of 1.0 s. All samples were run at 2.5 mM in 98% D_2O at pH* 7.0 (pD). ^{13}C chemical shifts were related to a zero assignment for the methyl carbons of TMS.

^{13}C NMR of Proteins. Proteins were prepared for NMR by lyophilizing samples and dissolving them in 98% D_2O at 100 mg/mL (2.6 mM) and pH* 8.5. All samples were prepared with bromo-2-[^{13}C]pyruvate as the anion and Fe(III) as the bound metal ion. The labeled protein was purified by eluting it on two successive PDG-6 spin columns (Bio-Rad) and a G-75 Sephadex column (see above) before spectral acquisition. A reference capillary filled with 100 μL of TMS was inserted into a 5-mm NMR tube containing 500 μL of the protein solution. Spectra (25 000 Hz sweep width) were obtained at a probe temperature of 304 K without spinning. Between 30 000 and 80 000 transients were collected with a relaxation delay of 1.0 s and a pulse width of 10 μs.

^{13}C NMR studies of denatured proteins were carried out by dissolving lyophilized samples in 6 M guanidine hydrochloride in D_2O, pH* 8.0, and immersing the samples in boiling H_2O for 5 min. The proteins were exchanged with several 2-mL volumes of the same buffer by ultrafiltration to remove nonspecifically-bound ^{13}C label. Spectral acquisition was carried out in the denaturing buffer. The major chemical shifts observed for denatured ^{13}C-labeled oTF/2N•-$(CH_3)_n$ were identical to those observed for the purified nondenatured protein (above).

^{13}C Resonance Shift Assignments. The ^{13}C chemical shifts for the amino acids and modifications thereof have been determined (Breitmaier & Voelter, 1978; Horsley, 1970; Horsley & Sternlicht, 1968; Del Re et al., 1963). ^{13}C chemical shifts for the modified protein were assigned to structures on the basis of the previously mentioned empirical models and the additivity rule for the ^{13}C chemical shifts of alkanes (Table 1) (Grant & Paul, 1964). The predicted structures were based on chemical shift analysis, mass spectral analysis, and the chemistries observed for reagents and specific functional groups under similar conditions.

RESULTS

Conformation-Dependent Covalent Modification of oTF/2N and oTF/2C. Several conserved lysine residues, exposed only in the open apo conformation of the transferrins, are thought to function in the preliminary steps of metal binding by attracting the synergistic anion (Anderson et al., 1990). To test this, exhaustive reductive methylation of the iron-saturated forms of oTF/2N and oTF/2C was carried out with CH_2O and $NaCNBH_3$ to selectively modify solvent-acces-

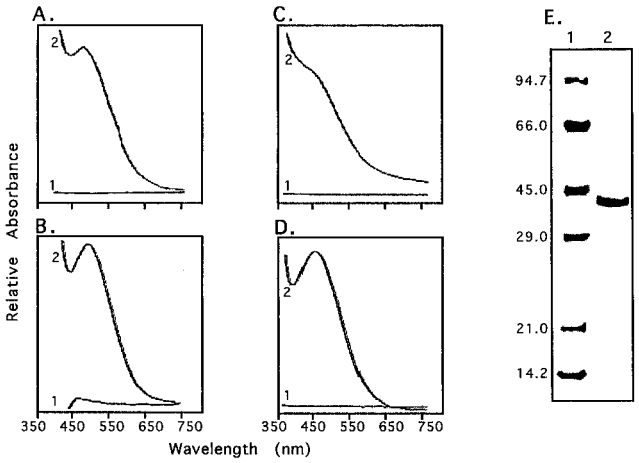

FIGURE 1: Difference visible spectra of iron-binding by oTF/2C·-$(CH_3)_n$ (panels A and B) and oTF/2N·$(CH_3)_n$ (panels C and D) in the presence of the synergistic anions carbonate (panels A & C) and bromopyruvate (panels B and D). The spectra were generated by scanning from 750 to 350 nm for the apo sample (trace 1) and 1 h after addition of the synergistic anion (trace 2). The spectrum of the apoprotein was recorded as the baseline, and further difference spectra were plotted by subtracting the baseline from each scan. Absorption increases (trace 2) result from Fe^{3+}-tyrosine charge transfer upon formation of the ternary complex of each protein; the absorbance maxima recorded for the bromopyruvate complexes of oTF/2C·$(CH_3)_n$ (470 nm) and oTF/2N·$(CH_3)_n$ (460 nm) were consistent with the λ_{max} observed for the corresponding complex of holoovotransferrin (470 nm) (Bailey et al., 1988). Final concentrations were protein (0.21 mM), $FeSO_4 \cdot 7H_2O$ (0.21 mM), KCl (0.01 mM), bromopyruvate (0.84 mM), carbonate (0.84 mM), and Hepes (50 mM, pH 7.0). SDS−PAGE (E) of proteins was carried out as described under Materials and Methods. Lanes 1 and 2 contain Coomassie-stained molecular weight markers (indicated by mass in kilodaltons) and oTF/2N·$(CH_3)_n$ (39 kDa) labeled with 3-bromo-2-[^{14}C]pyruvate, respectively.

sible lysines in the closed metal-binding conformation (Grossman et al., 1992). The reaction is lysine-specific (Jentoft and Dearborn, 1979), resulting in bis-methylation of the ϵ-amino group (Jentoft & Dearborn, 1983). After removal of iron (see Materials and Methods), the iron-free, methylated half-molecules, oTF/2N·$(CH_3)_n$ and oTF/2C·-$(CH_3)_n$, were subjected to a second round of reductive methylation using [^{3}H]CH_2O to determine the percentage of lysine residues that were protected by the metal-binding conformation. Incorporation of ^{3}H label into oTF/2N·$(CH_3)_n$ and oTF/2C·$(CH_3)_n$ corresponded to 18.0% and 4.20% of the total number of lysines per molecule, respectively. As a control, the apo forms of each half-molecule were also treated successively with CH_2O and [^{3}H]CH_2O; less than 0.1% of the lysines on each half-molecule incorporated label. Thus, the additional modification of oTF/2N·$(CH_3)_n$ and oTF/2C·$(CH_3)_n$ resulted directly from the effects of iron binding. Protein degradation did not contribute significantly to the observed protective effects, based on the iron-binding capabilities of the methylated half-molecules. Both oTF/2N·$(CH_3)_n$ and oTF/2C·$(CH_3)_n$ (Figure 1A,B) and their corresponding nonmodified half-molecules specifically bound iron to the same extent in the presence of carbonate (data not shown). Both of the methylated half-molecules also readily bound iron in the presence of bromopyruvate (Figure 1C,D). Furthermore, the extent of iron bound by each half-molecule was consistent with the extent of iron binding and the maximum absorbances (470 nm) observed for the holoprotein in the presence of bromopyruvate (Bailey et al., 1988). The results indicate that several lysine residues are protected by the iron-binding conformations of each half-molecule.

Because reductive methylation of iron-saturated oTF/2N and oTF/2C does not significantly affect the ability of either molecule to bind iron or synergistic anion (Figure 1), chemical modification of oTF/2N·$(CH_3)_n$ and oTF/2C·$(CH_3)_n$ with bromopyruvate should allow us to selectively target lysines that are protected in the iron-bound conformation of each half-molecule. Bromopyruvate modifies lysine residues on holoovotransferrin via a Schiff base intermediate (Bailey et al., 1988) and bis-methylation of the ϵ-amino group sterically hinders nucleophilic attack at the 2-carbonyl carbon of bromopyruvate, which is the kinetically preferred reaction between halopyruvates and N-substituted nucleophiles (Fischer et al., 1976). Therefore, only those lysine residues that are protected from methylation in the iron-bound conformation of either half-molecule should be suitable nucleophiles for bromopyruvate.

Covalent Modification of Methylated oTF Half-Molecules with Bromopyruvate. Both oTF/2N·$(CH_3)_n$ and oTF/2C·-$(CH_3)_n$, were treated with 3-bromo-2-[^{14}C]pyruvate in the presence of iron. Formation of the Fe−protein−anion complex (λ_{460} = 2000 M^{-1} cm^{-1}/iron) was monitored by visible spectroscopy (Bailey et al., 1988). When the extent of iron binding was estimated to be greater than 80% (Figure 1C,D), each half-molecule was then covalently modified by reducing the Schiff base intermediates between bromopyruvate and the lysine residues on each protein with $NaCNBH_3$. To determine the extent of covalent modification, known amounts of each protein were resolved by SDS−PAGE, and the bands corresponding to each intact metal-binding lobe were sectioned and measured for ^{14}C radioactivity. Although the protein−metal−anion complexes for each half-molecule were observed (Figures 1A−D), only the N-terminal half-molecule (Figure 1E) was covalently modified by bromopyruvate [0.7 mol of anion/mol of oTF/2N·-$(CH_3)_n$].

Isolation and Characterization of ^{14}C-Labeled Tryptic Peptides. Following purification, oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate was digested with trypsin to determine the sites of covalent modification. The soluble portion of the tryptic proteolysate was found to contain 100% of the ^{14}C activity: 166 nmol of covalently modified peptide. The proteolysate was first analyzed by FPLC in the reverse phase; 87% of the ^{14}C radioactivity (240 802 dpm; 144 nmol) eluted with a major $A_{(214\ nm)}$ peak at 28% acetonitrile (Figure 2A). The ^{14}C-activity-containing fractions bracketing the major $A_{(214\ nm)}$ peak were pooled, lyophilized and further resolved by HPLC. Essentially all the injected ^{14}C radioactivity eluted with one major peak at 27% acetonitrile (trace A, Figure 2B), and over 65% of the peptide was recovered after pooling and lyophilization of the appropriate fractions. Loss of peptide in the following steps was due to surface binding and lyophilization, as judged by quantification of ^{14}C radioactivity in separate trials. Final purification of the tryptic peptide(s) was carried out in the reverse phase at pH 6.5 with TEAP buffer (0.1% (v/v) H_3PO_4 and 0.28% (v/v) triethylamine) (Rivier et al., 1984). One activity-containing peak (Figure 2C) eluted at 22% acetonitrile and was analyzed by gas-phase sequencing and amino acid analysis.

Sequence analysis of the purified peptide(s) through 15 cycles yielded a sequence (Table 1), which corresponds

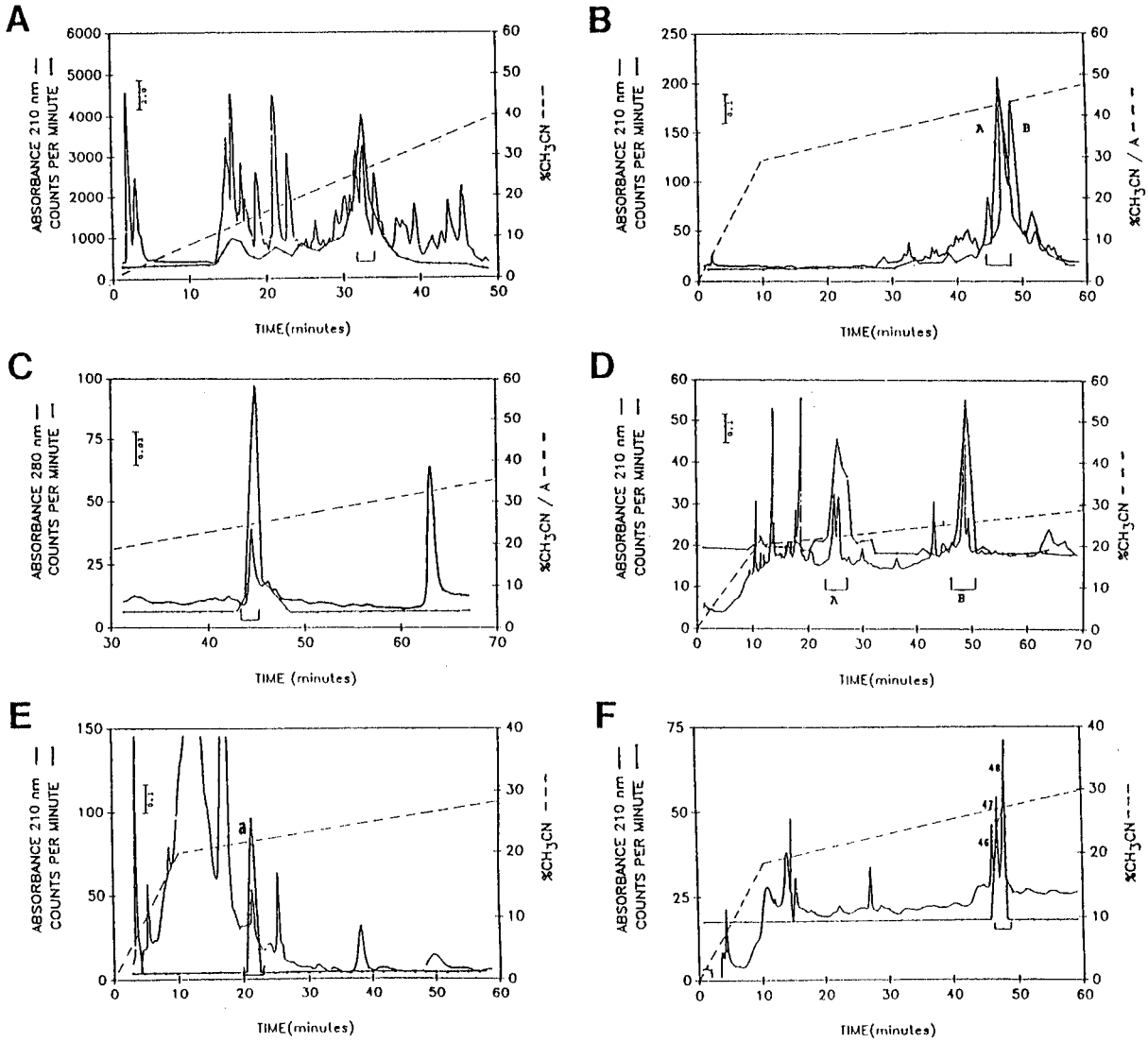

FIGURE 2: FPLC (panel A) and HPLC (panels B−F) of the tryptic and V8 proteolytic digests of oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate. All chromatography was carried out in the reverse phase as described under Materials and Methods. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in CH_3CN, except in panels C and E, were buffer A is TEAP buffer [0.1% phosphoric acid (v/v) and 0.28% triethylamine (v/v), pH 6.5] and buffer B, denoted CH_3CN/A, is 40% buffer A in CH_3CN. The solid and dotted lines shown in panels A−F indicate the respective absorption and ^{14}C activity profiles measured for the peptides generated by tryptic (panels A−C) and V8 (panels D−F) proteolysis of ^{14}C-labeled oTF/2N·$(CH_3)_n$.

Table 1: Sequence Comparison of Purified Peptides Generated from Successive Tryptic and V8 Proteolytic Cleavage of the N-Lobe of Ovotransferrin Labeled with 3-Bromo-2-[^{14}C]pyruvate

source	sequence
N-lobe[a] (189-204)	S G Y S G A F H C L K D G K G D
Tryptic peptides[b]	S G Q S G A F H - L - D G - G
V8 peptides[b]	S G Q S G A F H - L - D G - G D
C-lobe[a] (538-552)	F G Y T G A L R C L V E K G D

[a] Protein sequence from the N- and C-terminal metal-binding lobes of ovotransferrin (Williams, 1982b); alignment of the fragments is based on high sequence (Brock, 1985) and structural (Baker & Lindley, 1992) homology shared between the lobes. [b] The results of gas-phase Edman sequencing of ^{14}C-labeled peptides from oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate. Modification of the residues by bromopyruvate is indicated by dashes. Tyrosine 191 (third cycle) was oxidatively coupled to form dityrosine, resulting in a phenylhydantoin that elutes in the position corresponding to glutamine (see text).

exclusively to a 15-residue fragment from residues Ser 189 to Gly 203 in the sequence of the N-terminal metal-binding lobe of ovotransferrin. Although Gly 203 was the last residue that could be called with high confidence, amino acid analysis was consistent with a 21-mer terminating at Lys 209. The observed cleavage C-terminal to Tyr 188 is likely due to residual chymotropic activity that was observed in the tryptic preparation. From the known sequence, at least three residues on the peptide (Lys 199, Lys 202, and His 196) were possible candidates for amination and alkylation by bromopyruvate (Williams et al., 1982b). Correspondingly, the loss of phenylhydantoins for both Lys 199 and 202 (cycles 11 and 14, Table 1) and a reduced yield for the phenylhydantoin of His 196 (cycle 8, Table 1) was observed. Modification of each lysine was corroborated by protection from hydrolysis C-terminal to either residue by trypsin. Chemical modification of lysine by bromopyruvate (Williams et al., 1982) or reductive methylation (Jentoft & Dearborn, 1979) inhibits proteolysis at lysine by trypsin. An additional site of modification on the peptide was indicated by the loss of phenylhydantoin-tyrosine at the expected position in the sequence for Tyr 191 (cycle 3, Table 1); instead, a phenylhydantoin was observed that coeluted with the derivative

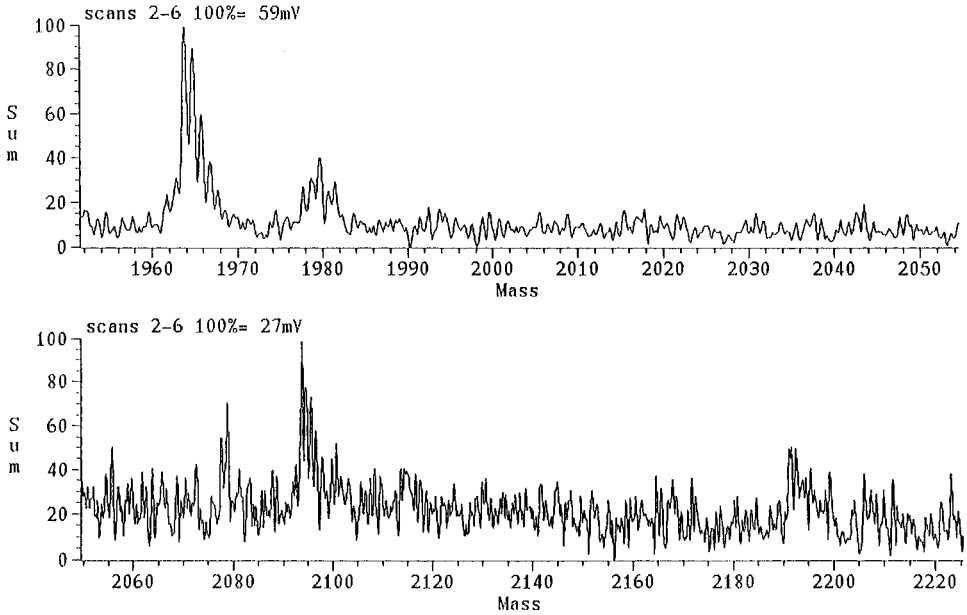

FIGURE 3: Partial liquid secondary ion mass spectra of purified peptides generated from successive tryptic and proteolytic digests of oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate.

expected for glutamine. The concentrations of the peptide(s) determined from the specific activity of the label (150 pmol) and the amino acid content (92 pmol) yielded a ratio of 1.2 mol of bromopyruvate/mol of peptide. The results suggested that the tryptic fraction (Figure 2C) contained peptides with the same sequence, but with different extents of modification. Accordingly, the tryptic peptides were digested with V8 protease to further characterize the sites of covalent modification.

Purification of ^{14}C-Labeled V8 Proteolytic Peptides. The V8 proteolysate of the ^{14}C-labeled tryptic peptide(s) (14 529 dpm; 8.70 nmol) was analyzed by HPLC (Figure 2D). Two major activity-containing bands were eluted at 13% and 17% acetonitrile (R_t = 24 and 48 min), and 70% of the activity (10 170 dpm; 6.09 nmol) was collected. The first activity band (band A, Figure 2D) contained two $A_{(210\ nm)}$ peaks (R_t = 23 and 25 min). The second activity band coeluted with the parent tryptic peptide(s) (band B, Figure 2D) and was the result of incomplete digestion. The fractions from band A (4225 dpm; 2.53 nmol) were resolved by HPLC at pH 6.5 in acetonitrile/TEAP (Figure 2E) (Rivier et al., 1984). One major $A_{(210\ nm)}$ peak (peak a) that eluted at 13.2% acetonitrile (R_t = 22 min) accounted for the total activity (2309 dpm; 1.38 nmol, 54.7% yield); the specific activity of these peptide(s) corresponded to 1.0 mol of ^{14}C label/mol of peptide. The parent peptides (band B, Figure 2D) were further digested for an additional 24 h with V8 protease and rechromatographed under the same conditions (Figure 2F). Two additional $A_{(210\ nm)}$ peaks, (R_t = 46 and 47 min) and the parent peptide (R_t = 48 min) were eluted at ca. 17% acetonitrile. The peptide(s) eluting at 47 min were the only new species to contain activity (specific activity = 1.2 mol of ^{14}C label/mol of peptide). No changes in the specific activity of the peptides were observed after cleavage with V8 protease, indicating that covalent modification of oTF/2N by bromopyruvate was confined to the indicated region on the N-lobe corresponding to residues 189−204.

Characterization of ^{14}C-Labeled V8 Proteolytic Peptides. Gas-phase sequence (Table 1) and mass spectrometric (Figure 3) analysis of the purified V8 proteolytic peptides (Figure 2E,F) demonstrated the presence of several chemically modified peptides derived from cleavage of the parent tryptic peptide(s) C-terminal to Asp 204 (residues 189−204 in oTF/2N). Aspartate 200 was protected from C-terminal proteolysis by V8 protease, even after successive digestions of the tryptic peptides with the protease. Several possibilities could account for the observed cleavage pattern: Asp 200 could be refractory to the protease due to steric constraints imposed either by the secondary structure of the peptides, modification of the adjoining residue (Lys 199), or direct modification by bromopyruvate. The latter possibility is unlikely in terms of the known chemistry for bromopyruvate and the lack of evidence for modification of aspartate by all the methods used. As observed for the tryptic preparation, modification of Tyr 191, His 196, Lys 199, and Lys 202 was indicated by loss of the corresponding phenylhydantoins in the peptide sequence (Table 1) and mass additions to the parent peptide (Figure 3). To further characterize the residues modified on the peptides, the results from mass spectrometric analysis of the ^{14}C-labeled peptides were correlated with chemical models studies and ^{13}C NMR of oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{13}C]pyruvate (Tables 2 and 3).

Modification of Tyrosine 191. To characterize the modification of Tyr 191, a series of model compounds were chosen, based on the chemistries observed for phenolic groups under similar reaction conditions (Fry, 1984; House, 1972). Of the compounds tested, only the oxidative coupling product of tyrosine (dityrosine) was consistent with the observed modification product of tyrosine 191. Dityrosine was detected in the acid hydrolysates of purified V8 and tryptic peptide preparations of ^{14}C-labeled oTF/2N using TLC (data not shown) (Fry, 1984). The phenylthiohydantoin of dityrosine was prepared and found to coelute with the phenylthiohydantoin of glutamine (data not shown), which was also detected in the sequence position for Tyr 191 in purified ^{14}C-labeled tryptic and V8 preparations (Table 1). The masses observed for all the labeled V8 proteolytic peptides (Figure 3) corresponded to a mass addition (180 Da) to the parent peptide (189−204; oTF/2N) that was also

Table 2: Predicted ^{13}C NMR Chemical Shifts of Reaction Products of Bromopyruvate and Nucleophilic Amino Acid Side Chains

structural modifications			chemical shifts (ppm)		
no.	structure	mass	N_{sub}	S_{sub}	O_{sub}
1	$CH_3C^*(R)(CN)COOH$[b]	98	64.5	48.0	85.0
2	$HOCH_2C^*(R)(CN)COOH$[b]	114	72.5	56.3	93.0
3	$CH_2(R)C^*H(CN)COOH$[b]	98	38.7	42.4	37.7
4	$CH_2(R)C^*OCOOH$[c]	87	190	190	190
5	$HOCH_2C^*H(R)COOH$[e]	89	74.2	58.0	94.7
6	$HOCH_2C^*H(R)CN$[b]	70	52.7	34.5	73.2
7	$CH_2(R)C^*OOH$[d]	59	176.9	176.9	176.9

[a] Chemical shifts were calculated for the ^{13}C-labeled carbon (C*) of the substitution products (1−7) of 3-bromo-2-[^{13}C]pyruvate and nucleophilic amino acid side chains (R), where R is the side chain of lysine (N_{sub}), histidine (N_{sub}), cysteine (S_{sub}), or tyrosine (O_{sub}) and follows the carbon that is modified. [b] The chemical structures are based on known N-, S-, and O-nucleophilic substitution products of bromopyruvate and amino acids in the presence of $NaCNBH_3$ (Lane, 1975), and the products observed when the indicated residues are modified on proteins with bromopyruvate. [c] Modification of cysteine (Fonda, 1976; Yoshida, 1978). [d] Modification of histidine (Crestfield, 1963). [e] Modification of lysine (Bailey, 1988).

consistent with the dityrosine modification (Fry, 1984). On the basis of the work of Hsuan (1987), who demonstrated that both tyrosine iron-binding ligands (Tyr 191 and 92) could be oxidatively coupled to FeoTF/2N by periodate to form dityrosine, we reasoned that Tyr 92 was coupled to Tyr 191. The following amino acid residues are N-terminal (Ser 91) and C-terminal (Tyr 93) to Tyr 92 in the amino acid sequence of oTF (Williams et al., 1982). The proteolytic cleavages that give rise to the observed peptide, those that are N-terminal to Tyr 93 and 92, presumably arose from chymotryptic activity in the tryptic preparation; the cleavages N-terminal to serine are not generally observed with trypsin, but at least one similar case with V8 protease has been reported (Everett et al., 1990).

Modification of Lysine. Bailey et al. (1988a) demonstrated that modification of lysine residues on holo-oTF with bromopyruvate and $NaBH_4$ proceeds via a Schiff base intermediate, using the reductive amination product of *N*-acetyllysine and bromopyruvate [N^{α}-acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine] to model derivatized lysines in the acid hydrolysate of the labeled protein. Correspondingly, we found that the major ^{14}C-labeled amino acid derivative present in the acid hydrolysate of the V8 proteolytic peptides purified from peak a and peak 47 (Figure 2E,F) coeluted with the model compound, which also coelutes with leucine (Figure 4). Evidence for reductive amination of Lys was also obtained by ^{13}C NMR of purified ^{13}C-labeled oTF/2N, previously modified with 3-bromo-2-[^{13}C]pyruvate and $NaCNBH_3$ (Figure 5). In the ^{13}C spectrum of the labeled protein and the model compound (Bailey et al., 1988a), a major chemical shift (72.1 ppm) was observed that was in close agreement with the predicted value of 74 ppm, using empirical additivity rules for N-substitution at the 2-carbonyl position of bromopyruvate by the lysine ϵ-amino group (modification 5, Table 2) (Breitmaier & Voelter, 1978). A mass increase of 89 Da resulting from the indicated modification of lysine was used to calculate the predicted masses of three peptides (II, III, and IV; Table 3) that correspond to peaks at *m*/*z* 1979.6 and 2093.8 in the mass spectrum of the V8 proteolytic digest (Figure 3). Two of the peptides indicated by the former mass peaks resulted from labeling of both Lys 202 and Lys 199. In summation, reductive amination of Lys 199 and/or Lys 202 by bromopyruvate was corroborated by mass spectrometric, ^{13}C NMR, and model compound analyses.

Modification of Histidine 196. A 21% loss in yield of phenylhydantoin-histidine suggested that His 196 was also modified to a limited extent. Using the chemical model technique described above for lysine (data not shown), a ^{14}C-labeled amino acid from the acid hydrolysate of the peptide(s) purified from activity band B (peak 47, Figure 2F) was found to coelute with 3-carboxymethyl-His (modification 7, Table 2), which is formed by oxidative decarboxylation of carboxyketoethyl-His (the alkylation product of bromopyruvate and histidine) under acidic conditions (Crestfield et al., 1963; Heinrikson et al., 1965). Correspondingly, the peak observed at *m*/*z* 1963.8 in the mass spectrum of the V8 peptide digest (Figure 3) was consistent only for a peptide with a mass addition (59 Da) corresponding to (carboxymethyl)histidine (Table 3). The predicted chemical shift (176.9 ppm) for the carboxymethyl substitution was not observed, presumably due to decreased relaxation of the ^{13}C-labeled carboxyl carbon (Levy et al., 1980).

Evidence for additional modification of histidine was supported by mass spectrometric and ^{13}C NMR data: the predicted chemical shift (64.5 ppm) of His modification 1 (Table 2) was in close agreement with the observed chemical shift (62.5 ppm, Figure 5), and the predicted mass of a histidine-modified peptide (Table 3) agreed within 0.3 Da with the largest peak observed (*m*/*z* 2093.5) in Figure 3.

Modification of Cys 197. The ^{13}C NMR spectrum of oTF/2N·$(CH_3)_n$ labeled with 3-bromo-2-[^{13}C]pyruvate showed two upfield absorption bands (58.0 and 42.9 ppm, Table 2) that were best fit by S-substitution of bromo-2-[^{13}C]pyruvate (structures 3 and 5, Table 1). The mass of peptide IV (Table 3) was consistent with structure 3 (Table 1). The remaining peaks were consistent with the presence of (carbamidomethyl)cysteine at position 197, which results from reduction and subsequent alkylation with iodoacetamide prior to proteolysis.

DISCUSSION

The present study shows that the synergistic anion bromopyruvate undergoes different interactions upon binding by the N- and C-proteolytic halves of ovotransferrin. Although both metal-binding lobes specifically bound bromopyruvate in the presence of Fe^{3+}, only the N-lobe was covalently modified. Lys 199, Lys 202, His 196, and Cys 197 were identified as the primary targets of bromopyruvate by a combination of vapor-phase Edman sequence analysis, mass spectrometry, ^{13}C NMR, and chemical model analysis. The labeled residues are located on peptides corresponding to residues 189−204 in a region of the N-terminal metal-binding domain of ovotransferrin that shares over 50% sequence identity with the known homologous N- and C-lobes of the transferrins (Brock, 1985).

Differential labeling of the N- and C-lobes by bromopyruvate appears to be directly related to the modification of lysine, based on comparison of the primary sequences of the homologous lobes (Table 1) and the work of Bailey et al. (1988a), who demonstrated that lysine residues are the primary targets of bromopyruvate in holoovotransferrin. Cysteine 197 is conserved throughout both lobes of the transferrins (Brock, 1985); however, its modification by

Table 3: Substitution Patterns and Structural Modifications of the Purified, Labeled Peptides Corresponding to Residues 189−204 in the Sequence of oTF/2N[a,b]

no.	amino acids modified	modification[c]	detected in peptide acid hydrolysate	peptide mass (Da)		chemical shifts (ppm)	
				calcd	exptl	calcd	exptl
I	His 196	7	yes	1963.5	1963.8	177	
	Lys 199 and 202	(aminomethyl)-Lys					
II	Lys 199 or 202	5	yes	1979.9	1979.6	74.2	72.1
	Lys 199 or 202	(aminomethyl)-Lys					
III	His 196	1		2093.5	2093.8	64.5	62.5
	Lys 199 and 202	5	yes			74.2	72.1
IV	Cys 197	3		2093.5	2093.8	42.2	42.9
	Lys 199 and 202	5	yes			74.2	72.1

[a] Substitution patterns for peptides modified by bromopyruvate were determined by correlating results from gas-phase sequencing, mass spectrometry, and the patterns of proteolysis for the peptides isolated from oTF/2N•$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate. [b] Parent peptide = ^{189}SGZSGAFHXLKDGKGD, as determined by gas-phase sequencing; Z = dityrosine, X = (carbamidomethyl)-^{197}Cys. (Carbamidomethyl)cysteine is the result of alkylating the protein with iodoacetamide prior to proteolysis; this modification is absent in peptides III and IV. [c] The structural modifications of each peptide, indicated by numbers corresponding to the structures shown in Table 2, were determined on the basis of the mass of each peptide, the chemical shifts observed when oTF/2N was modified with 3-bromo-2-[^{13}C]pyruvate, and coelution of model compounds with ^{14}C-labeled amino acids from acid hydrolysates of peptides labeled with 3-bromo-2-[^{14}C]pyruvate.

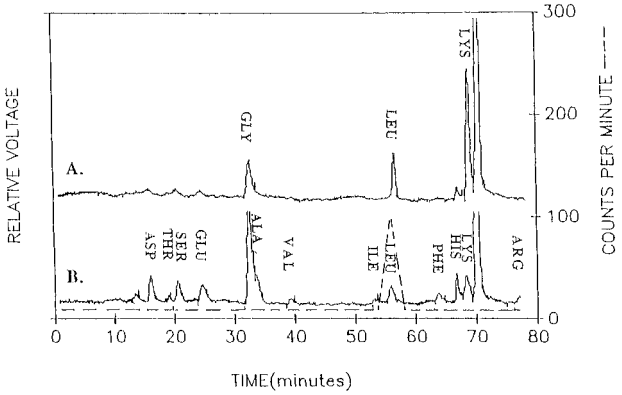

FIGURE 4: Amino acid analysis of the reduced Schiff base product of N^{α}-acetyllysine and bromopyruvate, N^{α}-acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine (A), and the acid hydrosylate (B) of purified peptides generated from successive tryptic and proteolytic digests of oTF/2N•$(CH_3)_n$ labeled with 3-bromo-2-[^{14}C]pyruvate. Amino acid analysis was carried out by postcolumn derivatization as described under Materials and Methods. Additional peaks in trace A result from glycine contamination, ammonia and unreacted, hydrolyzed N^{α}-acetyllysine.

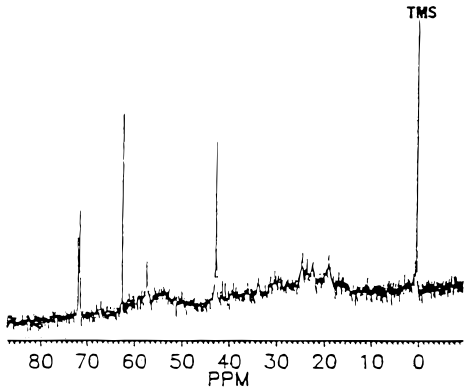

FIGURE 5: ^{13}C NMR of oTF/2N•$(CH_3)_n$ labeled with 3-bromo-2-[^{13}C]pyruvate. Prior to analysis, the protein was purified by size-exclusion chromatography as described under Materials and Methods. The spectrum was acquired over 79 696 transients at a sweep width of 15 151 Hz in D_2O at pD 6.5. All shifts were related to a zero value for TMS. The chemical shifts observed at 72.1, 62.5, 58.0 and 42.9 ppm result from covalent substitution of the ^{13}C-enriched 2-carbon of bromopyruvate by protein side chains.

3-bromo-2-[^{14}C]pyruvate accounted for only a small fraction of the total ^{14}C label in the acid hydrolysate of oTF/2N. Histidine 196, which was also a minor component of the residues labeled by bromopyruvate, is conserved predominantly as a positively charged residue in both lobes of the transferrins. In the C-lobe of ovotransferrin, His 196 is conserved with respect to charge by Arg 545; however, the guanidinium group of arginine does not react with bromopyruvate (Bailey et al., 1988a). Both Lys 199 and 202 were targets for bromopyruvate in the N-lobe. Lysine 202 is conserved in the homologous C-lobe (Table 1); thus modification of Lys 199 presumably reflects differential modification of the lobes by bromopyruvate. Of the possible lysine targets, only lysine 199 is conserved as a cationic residue in the N-lobes of the transferrins (Brock, 1985), which suggests that the presence or absence of positively charged residues corresponding to this residue in the metal-binding domains may account for differences in anion binding observed for each lobe in human serum transferrin (Harris, 1989; Harris et al., 1990) and ovotransferrin (Oe et al., 1989). Several lines of evidence suggest that cationic side chains play important roles in the differences reported for several functions of the individual lobes of transferrin, including anion binding and iron release. For example, previous UV spectrophotometric studies of anion binding to full-length apotransferrin suggest that phosphate anions (synergistic anion mimics) are preferentially bound at the N-terminal site through interactions with protein cationic side chains (Harris, 1989; Harris et al., 1990). Oe et al. (1989) have determined two distinct classes of anion-binding sites for ovotransferrin, similar to those of transferrin, which can also be attributed to the individual lobes. A lysine trigger that is critical for iron release in the N-lobe of ovotransferrin has been implicated as a primary structural difference between the N- and C-lobes (Dewan et al., 1993), possibly relating to differences observed in the pH-dependent release of iron by each lobe of serum transferrin (Princiotto & Zapolski, 1975).

The crystal structure of the iron−carbonate-complexed N-terminal half-molecule of ovotransferrin revealed that His 196, Cys 197, Lys 199, and Lys 202 are located on or at the termini of α-helix 8 (Dewan et al., 1993); modification of these residues by bromopyruvate suggests the presence of an anion-directing track in oTf/2N, based on the following observations. The N-terminus of α-helix 8 terminates at the metal-binding site, where it nearly abuts the terminus of α-helix 5 (Figure 6), which is the known anion-binding site in metal-complexed transferrins (Baker & Lindley, 1992).

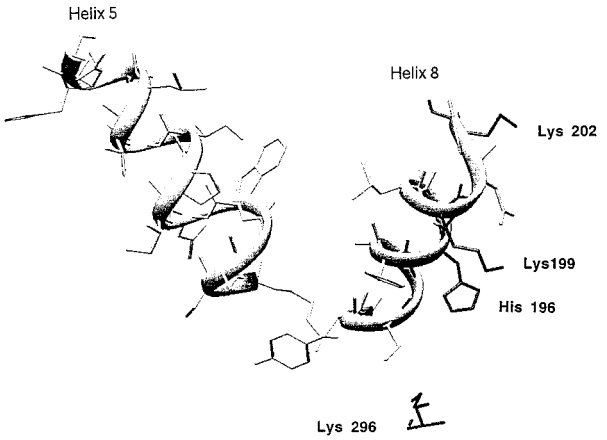

FIGURE 6: Relative positions of α-helices 5 and 8 from the X-ray crystal structure of the hen N-lobe of ovotransferrin (Dewan et al., 1993). The residues labeled by bromopyruvate, in the order Lys 202, Lys 199, and His 196, indicate the predicted path of the synergistic anion along α-helix 8 toward the known anion-binding site at the terminus of α-helix 5 (Baker & Lindley, 1992). Lysine 296 has also been determined to play a role in anion binding (Cheng et al., 1995); the relative position of this residue is shown proximal to the metal-binding site.

The crystal structures of rabbit serum transferrin and human lactoferrin show that the same structural motif is present in these proteins (Bailey et al., 1988; Anderson et al., 1989), where both His 196 and Lys 199 are conserved as positively charged residues (Brock, 1985). Lysine 199 is protected from lysine-specific chemical modification in iron-complexed oTF/2N, which suggests that this residue, and possibly His 196, attract the synergistic anion in the apo form, allowing it to move along α-helix 8 toward the anion-binding site. A recent study of anion binding by wild-type and single-point mutants (K266E, K296E, and K206Q) of the N-lobe of human serum transferrin indicated that two additional lysine residues (Lys 296 and 206) have important roles in anion binding and that Lys 296 may be the temporary binding site for the synergistic anion prior to metal binding (Cheng et al., 1995). Lysine 296 is positioned beyond the C-terminus of α-helix 8 at a distance of 13.9 Å from the carbonyl carbon of the synergistic anion in the metal-binding site (Figure 6). The crystal structure of oTf/2N reveals that only two lysines, Lys 301 (6.3 Å) and Lys 209 (8.0 Å), are in close proximity to the carbonate carbon; however, the distance (2.3 Å) separating the ϵ-nitrogens of these lysates has been attributed to a low-barrier hydrogen-bonding interaction (Dewan et al., 1993), which likely limits targeting of bromopyruvate by these lysines in the native ternary complex. Correspondingly, neither of these residues was labeled in our study of the N-lobe. The next closest possible reactive residues are the imidazole of His 196 at 14.4 Å, which is partially labeled, and the ϵ-amino groups of Lys 296, Lys 19 (15.4 Å), and Lys 291 (16.6 Å), none of which is labeled. All the remaining ϵ-amino groups are distances of 20 Å or more from the carbonate carbon. Thus, preferential labeling of His 196 and Lys 199 by bromopyruvate would suggest that only these residues are accessible to the synergistic anion as it approaches the anion-binding site and that bromopyruvate is no longer susceptible to nucleophilic attack by other side chains when bound in the ternary complex.

Covalent modification of oTf/2N and oTf is rate-limiting with respect to binding of the synergistic anion (Bailey et al., 1988a); thus bromopyruvate likely targets Lys 199 after its binding by each protein. Bailey et al. (1988a) attributed the slow chemical modification step to a "loose" form of anion binding that effectively concentrates targeting lysines in close proximity to the binding site. On the basis of the residues identified in this study and the crystal structure of the N-lobe, however, it is rather unlikely that there are any available lysine targets in close proximity to the binding site (Dewan et al., 1993). Correspondingly, Bailey et al. (1988a) showed that the maximum rates of conversion of lysine to (*N*-hydroxypyruvyl)lysine by bromopyruvate are slow for both free or protein-bound forms of the amino acid; i.e., anion binding does not increase the rate of lysine chemical conversion. Taken together, the data indicate that mechanism(s) other than anion binding must account for the slow chemical modification step. If bound bromopyruvate is the primary source of covalent modification of oTf, as suggested by Bailey et al. (1988a), then the anion is apparently displaced from the binding site (20 Å in the case of Lys 199) by an unknown mechanism. Whether His 196 and Lys 199 are modified by "free" anion during binding, anion displaced from the binding site, or a combination of both forms of the anion, they are preferentially targeted in the protein by the synergistic anion, indicating the presence of an anion-directing track.

Modification of Cys 197 by bromopyruvate further suggests that the synergistic anion moves in a track directed along α-helix 8. Cystein 197 and Cys 115 form a disulfide bond in the native N-lobe of ovotransferrin (Dewan et al., 1993) that is conserved by corresponding cysteines in the N-lobes of rabbit serum transferrin and human lactoferrin (Bailey et al., 1988b; Anderson et al., 1989). Alkylation of Cys 197 by bromopyruvate, therefore, indicates that the disulfide bond is reduced prior to chemical substitution. Although $NaCNBH_3$ reportedly does not reduce disulfide bonds (Lane, 1975), a previous study has shown that a disulfide bridge (Cys 78 and 671) in the C-lobe of oTF is sensitive to reduction by dithiothreitol (Williams et al., 1985). Therefore, the disulfide bond between cysteines 197 and 115 may also have an unusually high reduction potential. Alternatively, reduction of the cysteine bond may be mediated by redox reactions in the metal binding site. The cysteine bond is located in close proximity to the metal-binding site C-terminal to the tyrosine (191) metal-binding ligand, which also undergoes redox-specific modifications (Hsuan, 1987).

The presence of dityrosine in all the isolated peptides likely results from hydrolysis of $NaCNBH_3$. Fry (1984) has shown that tyrosine undergoes oxidative coupling in the presence of ferricyanide. Correspondingly, the formation of $Fe(CN)_n$-transferrin complexes can be observed at high concentrations of CN^- (Harris & Aisen, 1989). Thus, ligation of CN^- by protein-complexed iron or other unknown redox processes could promote oxidative coupling of the tyrosine iron-binding ligands (Tyr 191 and Tyr 92). Precedence for this phenomenon derives from the work of Hsuan (1987), where Tyr 191 and Tyr 92 were oxidatively coupled in ovotransferrin in the presence of periodate, which can replace the synergistic anion in transferrin.

In conclusion, the lack of covalent modification of the C-lobe by bromopyruvate does not necessarily reflect the absence of residues that may direct the synergistic anion to its binding site. From an analysis of the crystal structures of serum transferrin and lactoferrin (Baker & Lindley, 1992),

it seems likely that cationic residues exposed in the apo-C-lobe may attract the synergistic anion; however, they may not be suitable targets for bromopyruvate because of incompatible side-chain chemistries (e.g., arginine guanidinium group), insufficient reactivity, or steric constraints imposed by protein structure. Differential labeling of the lobes of ovotransferrin, however, suggest that the synergistic anion interacts with different residues on the surface of each lobe, which may relate to the differences in anion binding observed for each lobe (Oe et al., 1989).

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