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Affinity Labels for the Anion-Binding Site in Ovotransferrin†

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ABSTRACT: Bromopyruvate, a known alkylating agent, has previously been reported to function as an affinity label for the anion-binding site in the iron-binding protein ovotransferrin [Patch, M. G., & Carrano, C. J. (1982) *Biochim. Biophys. Acta 700*, 217-220]. However, the present results indicate that hydroxypyruvate also functions in an almost identical manner, which implies that alkylation of a susceptible nucleophile cannot be the mechanism responsible for the covalent attachment of the anion. Model complexes and amino acid analysis of labeled ovotransferrin suggest that initial Schiff base formation, followed by reduction of the imine bond between the affinity anion and a lysine within the locus of the anion-binding site, accounts for the irreversible labeling. As expected, the covalently attached anions render the iron in the ovotransferrin–iron–anion ternary complex much more resistant to loss at low pH. It is proposed that the covalently labeled protein be used to test the hypothesis that iron removal from transferrin occurs by protonation and loss of the anion in low-pH lysosomal vesicles.

The term "transferrin" is applied to a whole class of iron-binding glycoproteins which includes the ovotransferrins, the lactoferrins, and the serum transferrins. The various transferrins are structurally and chemically quite similar, with the ability to tightly, but reversibly, bind 2 mol of ferric ion (Aasa et al., 1963). All have molecular masses of approximately 80 000 daltons and consist of single polypeptide chains of approximately 650 amino acids (Jeltsch & Chambon, 1982; MacGillivary et al.; Williams et al., 1982) folded into two compact regions, each of which can bind a single iron. An X-ray crystal structure of human lactoferrin has recently been published (Anderson et al., 1987). A number of excellent reviews on these important proteins are available (Aisen & Listowski, 1980; Chasteen, 1977, 1983a).

One of the unique features of the transferrins is that, in order for iron to bind, a suitable anion must also be present (Bates & Wernicke, 1971; Bates & Schlabach, 1973; VanSnick et al., 1973; Zweier et al., 1981). In physiological media, this necessary anion is carbonate (or possibly bicarbonate). In the absence of carbonate, numerous other organic anions can promote binding (Bates & Schlabach, 1973; Schlabach & Bates, 1975), although most simple inorganic anions are ineffective. The function of these synergistic anions may be to "lock in" the bound metal ion, thereby protecting it from

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competing reactions (Kojima & Bates, 1979; Williams & Woodworth, 1973). Schlabach and Bates (1975) determined that the anion-binding site is at least 3 Å deep, 6 Å wide, and 6 Å or more in length. The site appears to be asymmetric and located near the surface of the protein. In addition to a carboxylic acid moiety, all of the known synergistic anions for transferrin possess a Lewis base group within 6.3 Å of the acid. An "interlocking-sites" model (Bates & Schlabach, 1973) has been proposed for the interaction of the anion with the metal and protein.

The purpose of the present work was to develop an affinity label analogue to the synergistic anion bound to ovotransferrin to be used as a probe for elucidation of the structural details of the active site and in the examination of the mechanism of iron binding and release. In work previously performed in this laboratory (Patch & Carrano, 1982), it was reported that bromopyruvate behaved as a synergistic anion and affinity label for the anion-binding site of ovotransferrin. However, it has been subsequently determined that, due to the high pH at which these experiments were performed, bromopyruvate may not have been the actual species involved in these reactions. We report here the effects of bromopyruvate and its hydrolysis product, hydroxypyruvate, as affinity anions for ovotransferrin at near neutral pH.

Materials and Methods

Initial preparations of ovotransferrin, the generous gift of Dr. R. C. Woodworth of the University of Vermont, were prepared by published methods (Woodworth & Schade, 1959) and highly purified. Later, the protein was purchased and used as received from Sigma. No differences between the two preparations were noted in regard to this study. Bromopyruvate, hydroxypyruvate, fluoropyruvate, pyruvic acid, and N^{α}-acetyl-L-lysine were all purchased from Sigma and used as received. The synthesis of radiolabeled bromopyruvate was based on a previously described method (Meloche, 1970). The purity of the radiolabeled complex was checked by autoradiography of TLC plates after spraying with EN^{3}HANCE from New England Nuclear.

Carbonate-free solutions of apoovotransferrin were prepared by dissolving the protein in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (30 mM MES and 0.15 M NaCl, pH 6.1), titrating to pH 4–4.5 with 0.1 M HCl, and allowing ascarite-filtered nitrogen to pass over the solution with gentle stirring for 1 h. Many control experiments, as well as the literature, indicated that this procedure was effective in the removal of all carbonate from the solution (Schlabach & Bates, 1975; Woodworth et al., 1975; Campbell & Chasteen, 1977). After the 1-h degassing procedure, one part iron and an excess of four parts anion (both per binding site) were added to the protein solution. The pH was then raised to approximately 6.5 by addition of aliquots of CO_2-free ammonia gas. Finally, an aliquot of ascarite-filtered air was syringed into the vessel to oxidize the Fe(II) to Fe(III) and initiate the reaction. A flow of nitrogen was maintained to ensure a backpressure which would prohibit the influx of CO_2-contaminated air. Visible spectra were recorded on a Perkin-Elmer 552 UV/vis spectrophotometer. After completion of the reaction, the solution was titrated to pH 8 and an approximately 100-fold molar excess of sodium borohydride added (Okamoto & Morino, 1973). The borohydride served to reduce and hence stabilize the imine bond of the Schiff base formed between the affinity anions and a lysine group of the protein (vide infra).

Reactions involving the protein and radiolabeled affinity anion were treated as above and then immediately purified by precipitation with saturated ammonium sulfate and washing with MES buffer. Equivalent results were obtained when the radiolabeled protein was purified by gel filtration (Patch & Carrano, 1982). Radioactivity was measured on a Beckman LS 250 liquid scintillation counter using Biofluor cocktail from New England Nuclear.

Reconstitution. Following reduction of the imine bond between the protein and the affinity-label anion, the bound iron was removed by dialysis against several changes of 1 mM NTA–1 mM EDTA–0.2 M K_2HPO_4 (pH 6). After further overnight dialysis against MES buffer, the affinity-labeled apoprotein solution was placed in an anaerobic UV/vis cell, the pH was carefully adjusted to 4–4.5, and the cell was degassed to remove any carbonate contamination. After the pH was raised to ~6.5 with gaseous ammonia, iron was back-titrated into the protein in small aliquots (allowing 5 min between aliquots for the system to reach equilibrium) and the optical density at 460 nm recorded after addition of each aliquot.

pH-Dependent Iron Loss from Transferrin. Samples (of similar concentrations) of both modified (1-h reaction with affinity label followed by hydrogenation) and unmodified ferrated ovotransferrin were placed in a 0.15 M MES–0.15 M sodium acetate buffer, titrated to a particular pH value (over a pH range of 7–2) with acetic acid, and allowed to equilibrate for 48 h. No precautions were taken to prevent carbonate interaction *after* hydrogenation of the modified proteins—thus these samples are expected to contain both the affinity label and carbonate in the anion site. The absorbance at 460 nm was converted to percent iron remaining and plotted vs pH.

Model Complex Synthesis. The synthesis of complexes prepared as models for the reaction of the affinity label with the protein was derived from published procedures (Roche et al., 1971) and modified as needed. N^{α}-Acetyl-L-lysine and a slight molar excess of the respective affinity-label anion were dissolved in MES buffer, titrated to pH 6.5, and allowed to stir overnight. With time these solutions turned yellow and then dark brown and were accompanied by a distinct odor. After reaction for 24 h, the solutions were titrated to pH 8 with 0.1 M NaOH, and an excess of $NaBH_4$ was added. The reaction mixture was then titrated back to pH 1.5 (below the pK_a of the carboxylic acids) and lyophilized. Repeated suspensions in MeOH, followed by filtration and rotary evaporation of the filtrate, were performed to remove insoluble salts. Proton and ^{13}C NMR spectra of the products were obtained in either D_2O or MeOH-d_4 on a Bruker FT-250 spectrometer.

Amino Acid Analysis. Modified amino acids were detected after hydrolysis and reaction with the highly fluorescent dansyl chloride (Pierce) by two-dimensional thin-layer chromatography on polyamide sheets (Schleicher & Schuell) as described previously (Woods & Wang, 1967). Spots were detected by fluorescence and/or autoradiography.

Results and Discussion

Bromopyruvate as the Affinity Anion. To monitor the synergistic behavior of bromopyruvate, the reaction of this anion with iron and ovotransferrin was performed in a carbonate-free system and followed by visible spectroscopy. It has been shown previously (Schlabach & Bates, 1975) that iron complexes of ovotransferrin with synergistic anions exhibit visible spectra with λ_{max} values in the 400–500-nm range, whereas use of nonsynergistic anions results in no binding of iron and no distinct peak in this region. When bromopyruvate was employed as the synergistic anion, the visible spectra underwent time-dependent changes as indicated in Figure 1.

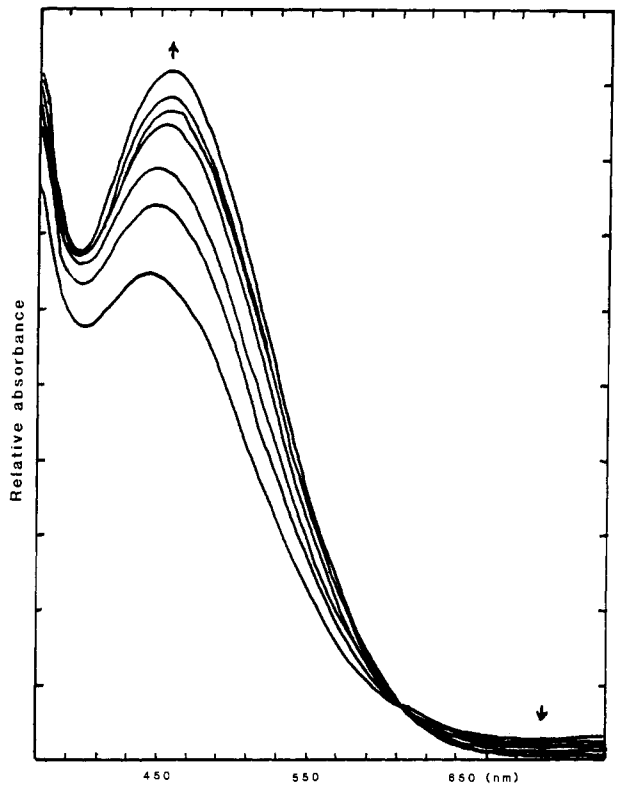


FIGURE 1: Time-dependent spectral changes during the formation of the iron–anion–protein ternary complex in the presence of bromopyruvate at pH 6.5. Spectra recorded at 4 (bottom trace), 8, 11, 30, 45, 65, and 350 (top trace) min.

The initial complex formed exhibited a peak at 440 nm (ϵ = 1400 M^{-1} cm^{-1}/iron) and possessed a distinct yellow color. The λ_{max} for this complex shifted with time (~1 h) from the initial value to 460 nm (ϵ = 2000 M^{-1} cm^{-1}/iron). Spectral changes (as seen by an increase in absorbance) continued for ~6 h with an apparent isosbestic point at 610 nm. A spectrum obtained after sodium borohydride had been added (data not shown) was indistinguishable from the final spectrum obtained above. Thus, on the basis of these spectral data, it appears that bromopyruvate acts as a synergistic anion in the formation of an anion–iron–protein ternary complex.

To determine the extent of any specific labeling (covalent bonding) at the anion-binding site, two parallel experiments were performed. In the first, ovotransferrin and ^{14}C-labeled bromopyruvate were allowed to react as described above. At each indicated time an aliquot was removed, sodium borohydride added in order to stabilize the anion–protein bond (vide infra), and the protein separated from unreacted label by NH_4SO_4 precipitation. In the second, the stable iron–protein–carbonate complex was first prepared, followed by addition of ^{14}C-labeled bromopyruvate. Again sodium borohydride was added and the protein precipitated from each aliquot removed. Assuming that all of the binding sites were protected by the tightly bound iron–carbonate–protein complex, the latter experiment should determine the maximum nonspecific bonding by bromopyruvate away from the anion-binding site. Following precipitation (which preliminary experiments had indicated removes all iron and any noncovalently attached anion) it was determined that after 12 h approximately 2.4 mol of bromopyruvate was bound per mole of protein in the absence of carbonate and that ~0.8 mol of bromopyruvate was nonspecifically bound in the presence of carbonate. Thus, approximately 1.6 of the binding sites had been labeled by the affinity label anion within 12 h (Figure 2). Several 1-h incubations were performed and repeatedly

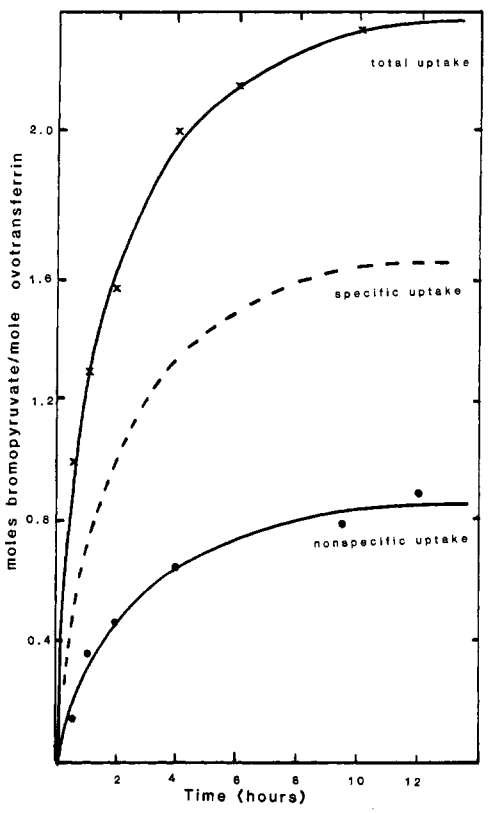


FIGURE 2: Determination of the extent of specific labeling of ovotransferrin by bromopyruvate in the presence of iron. Upper trace is the uptake of ^{14}C-labeled bromopyruvate in the absence of carbonate; lower trace is in the presence of carbonate.

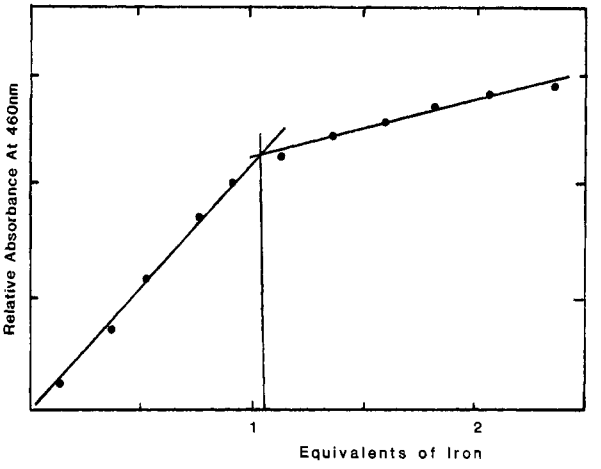


FIGURE 3: Back-titration with iron of 0.13 mM apoovotransferrin previously labeled with bromopyruvate. The absorbance was recorded at 460 nm.

showed approximately half the total number of sites (i.e., one) to be labeled specifically. These 1-h incubations were used for all further studies.

Further support for the existence of a covalent linkage between the protein and the affinity anion comes from reconstitution experiments. After reaction with the affinity anion for 1 h (immediately followed by hydrogenation), the iron was removed from the protein by dialysis and then titrated back in the *absence* of carbonate. Color, which was lost on removal of the iron, was regained upon readdition of just iron. The plots of these back-titrations (Figure 3) have proven reproducible. A break at 1 equiv of iron/protein is consistent with the previous results indicating modification of half of the sites after 1 h of incubation. These results are a strong indication that the anion is irreversibly attached to the protein and has

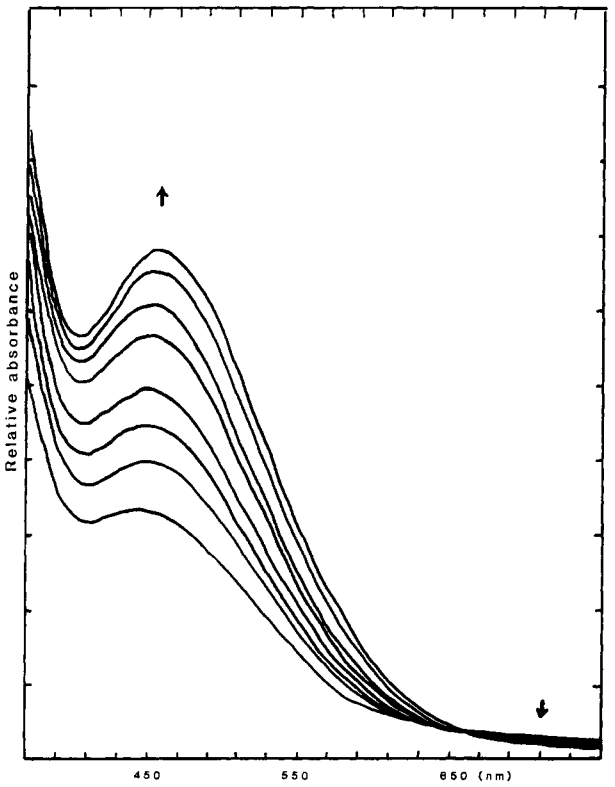


FIGURE 4: Time-dependent spectral changes during the formation of the iron–anion–protein ternary complex in the presence of hydroxypyruvate at pH 6.5. Spectra recorded at 5 (bottom trace), 15, 25, 40, 75, 110, 285, and 420 (top trace) min.

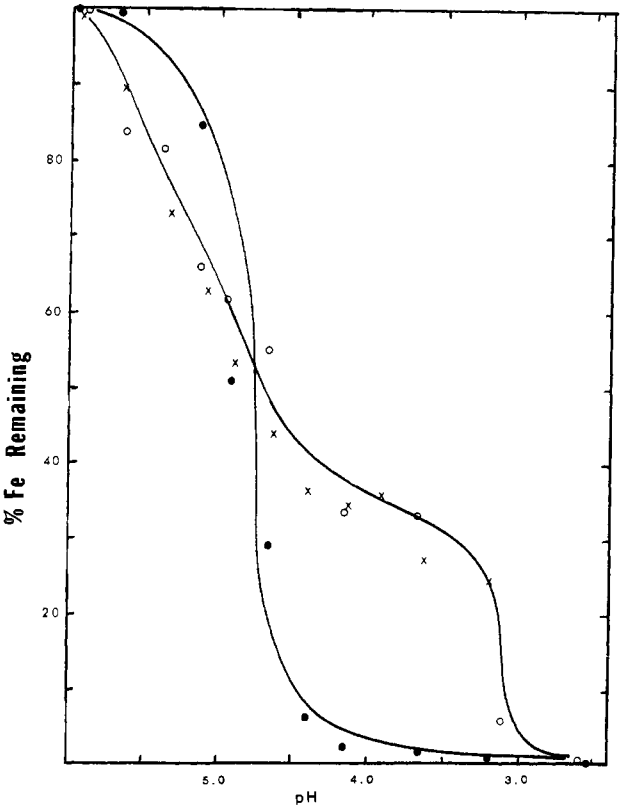


FIGURE 5: Plot of percent iron remaining in ovotransferrin as a function of pH for a series of synergistic anions: (●) bicarbonate; (○) bromopyruvate; (×) hydroxypyruvate.

locked the protein in a position to accept the iron.

Hydroxypyruvate as the Affinity Label. Because reinterpretation of previous work (Patch & Carrano, 1982) had indicated that hydroxypyruvate may have been the reactive species when bromopyruvate was used as a synergistic anion at high pH, experiments were also performed with authentic samples of this anion.

When hydroxypyruvate was utilized as the synergistic anion, spectral features (Figure 4) similar to those found for bromopyruvate were obtained. Here, a shoulder was initially formed at 440 nm and, over a longer period of time (~7 h), shifted to a distinct peak at 460 nm (ϵ = ~1700 M^{-1} cm^{-1}/iron), with an apparent isosbestic point at 660 nm. Thus, hydroxypyruvate also behaves as a synergistic anion in the formation of an anion–iron–protein ternary complex.

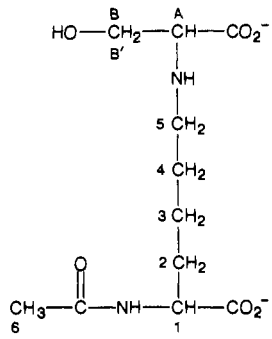
Experiments similar to those performed with bromopyruvate were conducted with hydroxypyruvate to determine the extent of any specific labeling by this anion. It was found that after ~20-h incubation approximately 2.5 mol of hydroxypyruvate was bound per mole of protein in the absence of carbonate and that ~0.8 mol was nonspecifically bound in the presence of carbonate. Thus, approximately 1.7 of the two binding sites were being labeled within 20 h. One-hour incubations repeatedly resulted in the labeling of one site.

A plot of the back-titration of iron to affinity-labeled apoovotransferrin (1-h reaction with hydroxypyruvate, followed by hydrogenation and dialysis) in the absence of any other anion exhibited a break at approximately 1 equiv of iron, similar to that seen with bromopyruvate. This confirms the modification of half of the sites after 1 h of incubation.

Effect of Affinity Labeling on Iron Binding and Release. The formation of the iron–affinity anion–protein ternary complex has been monitored by two methods—optical absorbance and radioactivity. It was determined that when either bromopyruvate or hydroxypyruvate was used as the synergistic anion, the specific irreversible bonding of the ^{14}C label followed virtually the same time course as the spectral changes for the iron. At pH 6.5, pseudo-first-order rate constants obtained from linear regression analysis of plots of ln $[A_\infty/(A_\infty - A_T)]$ vs time are k_{obsd} = 6.7 × 10^{-3} and 6.9 × 10^{-3} min^{-1} for bromopyruvate and k_{obsd} = 5.4 × 10^{-3} and 5.8 × 10^{-3} min^{-1} for hydroxypyruvate from the spectral and radioactive data (corrected for nonspecific binding), respectively. Thus, the rate of the increase in the absorbance (optical spectra) correlates well with the rate of the specific attachment of the anion into the anion-binding site (as seen by the radioactivity data). These results are consistent with an initial rapid (within minutes) formation of a "loosely" bound, noncovalent, ternary complex between anion, protein, and iron, which is followed by a slow (hours) reaction between the anion and the protein, causing a shift in the spectrum to that typical of the more tightly held anions.

The pH dependence of iron dissociation from protein ternary complexes involving carbonate, bromopyruvate, or hydroxypyruvate as the anion was examined to determine whether the hydrogenated affinity-labeled proteins were more stable to loss of iron by protonation than the carbonate complex. It was found (Figure 5) that both affinity-labeled complexes lost a little more than half of their bound iron over the pH range 6–4.5 (presumably from the non-affinity-labeled sites), where the carbonate complex (pK_a ~ 5) loses virtually all of its bound iron. However, unlike the carbonate complex, the balance of the iron in the affinity-labeled proteins remained bound until about pH 3.1. This biphasic nature, which we attribute to the covalent labeling of only a single site, is consistent with that seen in the reconstitution experiments. The covalently attached anion appears to at least partially stabilize the protein to loss of the iron in the labeled sites by protonation. However, even though the anion is locked in by a covalent bond, at lower pH values there may be protonation of the other

Table I: Chemical Shift Data for the ^1H and ^{13}C NMR Spectra of the Model Complex: N^{α}-Acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine



carbon	chemical shift (ppm) ^1H	^{13}C
1	3.9	53.2
2		30.9
3	1.1–1.7	23.1
4		27.1
5	2.45	39.8
6	1.8	22.2
A	3.85	72.3
B	3.5	64.4
B′	3.6	
$-COO^-$ (2)		174.2 174.7
>C=O		173.7

ligands around the iron, resulting in iron loss. Thus, the pH dependence on iron removal has been changed by locking in the anion but has not been removed entirely.

Nature of the Covalent Linkage. Although bromopyruvate could be expected to alkylate any one of several different amino acid residues, when hydroxypyruvate is employed as the synergistic anion, the only reasonable amino acid residue with which it could form a covalent bond is lysine. Thus, the fact that hydroxypyruvate was seen to form an irreversible attachment to the protein suggests Schiff base formation with a lysine. Indeed, several authors have previously indicated the presence of a lysine residue in the locus of the anion-binding site (Zweier et al., 1981; Chasteen, 1983b; Shewale & Brew, 1982).

When N^{α}-acetyl-L-lysine, a model for the lysine residue, was allowed to react with hydroxypyruvate, followed by reduction of the imine bond by borohydride, the resulting brown complex was confirmed by both ^1H and ^{13}C NMR to be N^{α}-acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine (Table I). The ^1H NMR of this product (pH 6.5) had, as expected, all the resonances assigned to the N^{α}-acetyl-L-lysine moiety in addition to those predicted for the 1-carboxy-2-hydroxyethyl (3-hydroxypropanoic acid) moiety. For the added methylene protons, a chemical shift of ~3.6 was predicted; a new set of resonances at about 3.5 ppm were actually observed. These two protons are chemical shift inequivalent, as they are α to a chiral center. For the methine proton a resonance of ~3.9 ppm was predicted, and a new resonance at 3.85 ppm was observed. The region 3.4–4.0 ppm, which includes these resonances plus the resonance due to the methine proton from the N^{α}-acetyl-L-lysine moiety, was verified by UEANMR (Harris & Woodman, 1966) computer simulation and gave reasonable coupling constants. The ^{13}C NMR also gave the predicted spectrum for this complex with two new resonances observed at 64.4 and 72.3 ppm and assigned to the methylene and methine carbons, respectively. The presence of two carboxylate groups in the model complex was verified by pH titration with standard sodium hydroxide. A plot of pH vs equivalents of base added showed a break at 2 equiv, corresponding to the two carboxylic acid moieties in the compound.

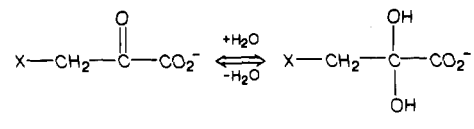
Thus it appears that when hydroxypyruvate reacts with N^{α}-acetyl-L-lysine, N^{α}-acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine is produced, consistent with the initial formation of a Schiff base, the imine bond of which is reduced by borohydride. The ^1H and ^{13}C NMR spectra of the reduced reaction product between N^{α}-acetyl-L-lysine and bromopyruvate were virtually identical with those of the hydroxypyruvate reaction product. This indicates that the bromopyruvate also reacts via a Schiff base mechanism rather than by alkylation.

It was observed, after hydrolysis (6 N HCl, 110 °C, 24 h) and two-dimensional thin-layer chromatography of the dansylated derivatives, that the spots assigned to the lysine–hydroxypyruvate and lysine–bromopyruvate reaction products were all visibly brown. This may be attributed to the similarity between the reaction of lysine with bromopyruvate or hydroxypyruvate and Maillard-type reactions (Ellis, 1959; Rooney et al., 1967), also known as browning reactions. In Maillard reactions, browning products are obtained from the reaction of low molecular weight carbonyl compounds (e.g., glyoxal, dihydroxyacetone, etc.) with amino acids (Hodge, 1953; Kawashima et al., 1977) and are identifiable due to the relatively intense brown color and distinct odors accompanying them. Both of the model complexes produced here were intensely brown and did have quite distinct odors. A very close comparison may be drawn between the reaction of one of the affinity labels, hydroxypyruvate, and a primary amine, e.g., lysine, and the reaction of fructose with a primary amine in a typical Maillard-type reaction.

Evidence that Schiff base formation between an ϵ-amino group of lysine and bromopyruvate or hydroxypyruvate accounts for the affinity labeling of ovotransferrin comes from a comparison of the hydrolysis products obtained from model complexes and the protein. When ovotransferrin which had previously been affinity labeled with ^{14}C-labeled bromopyruvate (reaction time = 1 h, followed by reduction with sodium borohydride) was hydrolyzed, dansylated, and subjected to two-dimensional TLC, a spot appeared that was cochromatographic (both by color—it was brown—and by autoradiography) with the reaction product of the model complexes. This strongly supports the notion that it is indeed a lysine residue that has been modified in the anion binding site of the protein.

Interestingly, while other related keto acids including fluoropyruvate and pyruvate itself do function as reversibly bound synergistic anions, they do not function as affinity labels for ovotransferrin. The optical spectra obtained during their interaction with iron and ovotransferrin are time invariant, and no reaction was found with either of these anions and N^{α}-acetyl-L-lysine.

Mechanistic Implications. In order to understand why, among the various substituted pyruvates tested, only the hydroxy and bromo derivatives reacted covalently with either N^{α}-acetyl-L-lysine or ovotransferrin, it is necessary to consider the nature of these anions in aqueous solution. It is well-known that many aldehydes and ketones will undergo reversible hydration in aqueous solution to form *gem*-diols. Indeed, Fischer



X = −OH, −Br, −F, −H

et al. (1982) have reported that the predominant form for bromopyruvate in solution is as its hydrated *gem*-diol form. On the basis of UV spectroscopy, they reported an equilibrium constant of 1.84. This equilibrium can also be observed by ^{1}H NMR. Rather than a single resonance, as would be anticipated for the spectra of bromopyruvate, two resonances are actually observed: a large singlet at 3.4 ppm and a smaller singlet at 4.4 ppm. Because the two methylene protons in bromopyruvate are chemical shift equivalent, these two resonances must derive from different "forms" of bromopyruvate: one being the keto form and the other being the hydrated *gem*-diol form. It was calculated that the methylene protons of the keto form of bromopyruvate should resonate at ~4.3 ppm and those of the *gem*-diol form at ~3.4 ppm. At pH 6.3 the relative intensities of these two peaks were 1:4 in favor of the *gem*-diol form [nucleophilic attack of H_2O or OH^- on the 3-halogen position to form hydroxypyruvate is slow in all cases in comparison with hydration (Fischer et al., 1982)]. Hydroxypyruvate in D_2O also yielded two resonances: singlets at 4.48 and 3.42 ppm. It was predicted that the keto form of hydroxypyruvate would resonate at ~4.5 ppm, while the hydrated *gem*-diol form would resonate at ~3.5 ppm. As opposed to bromopyruvate, the ratio of these two peaks at pH 7 was 1:0.9, in slight favor of the keto form. This difference between bromopyruvate and hydroxypyruvate is not unexpected, however, as the electron-withdrawing halide in bromopyruvate will facilitate nucleophilic attack by water on the keto carbon. The *gem*-diol species was also found to be the predominant form in fluoropyruvate at pH 6.3 (*gem*-diol:keto ratio = 6:1), but not in pyruvate itself (0.1:1). The degree of *gem*-diol formation among these anions can be viewed as a measure of the reactivity of the carbonyl carbon. Because the diol is formed by attack of the weakly nucleophilic water at the carbonyl carbon, it would be expected that the extent of *gem*-diol formation would mirror the electron-withdrawing capabilities of the substituents. This order, F > Br > OH > H, is indeed observed. This ordering of both substituent electronegativities and *gem*-diol formation by the respective anions can also be used to explain why two of these anions react with lysine and behave as affinity labels, while two others do not. The electron-withdrawing groups of the respective anions determine the reactivity of the carbonyl carbon toward nucleophilic attack by both the amine and solvent water. Apparently pyruvate, although existing mainly in the keto form (which is the reactive species in Schiff base formation), does not have a carbonyl carbon that is electropositive enough to react with either amines or water. The carbonyl carbon of fluoropyruvate on the other hand is too reactive; predominant hydration effectively removes most of the reactive keto form from solution. In addition, any imine formed with fluoropyruvate is subject to back-hydrolysis by water since the incipient negative charge on the nitrogen atom in the transition state is stabilized by the electron-withdrawing fluorine. Only in hydroxypyruvate and bromopyruvate is the balance between reactivity of the carbonyl carbon and competitive hydration achieved so that reaction with amine nucleophiles is rapid. Of the two, bromopyruvate reacts faster, consistent with its more electropositive carbonyl carbon.

Conclusions

It is now clear that both bromopyruvate and hydroxypyruvate behave as affinity labels for the anion-binding site in ovotransferrin. When these two anions are taken up and form covalent linkages to the protein, a time-dependent optical spectrum is obtained. Both radiolabeling and reconstitution experiments show that the anion is covalently attached. Model complexes, in conjunction with the labeled protein, have helped to identify the labeled amino acid moiety as lysine. This lysine is presumably in the locus of the anion-binding site but not necessarily the residue thought to bind the anion by ionic attraction in the intact protein. Model complexes have also shown that bromopyruvate, although a well-known alkylating agent, here is seen to form a Schiff base in its interaction with lysine. Finally, it was seen that the covalently attached anions rendered the iron in the ovotransferrin–iron–anion ternary complex much more resistant to loss at low pH. If iron removal from transferrin occurs by protonation and loss of the anion in low-pH lysosomal vesicles, the new affinity-labeled proteins should deliver considerably less iron than the intact protein. In vivo studies may be able to confirm this.

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Registry No. Bromopyruvate, 1113-59-3; hydroxypyruvate, 1113-60-6; iron, 7439-89-6; carbonate, 3812-32-6; N^{α}-acetyl-L-lysine, 1946-82-3; N^{α}-acetyl-N^{ϵ}-(1-carboxy-2-hydroxyethyl)-L-lysine, 115290-89-6.

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Isolation of Bovine Angiogenin Using a Placental Ribonuclease Inhibitor Binding Assay†

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ABSTRACT: Angiogenin, which induces the formation of new blood vessels, was isolated previously from two human sources—HT-29 tumor conditioned media and normal plasma. By use of a newly developed binding assay, a similar protein has now been purified from bovine plasma at levels of 30–80 μg/L. This protein has the structural, enzymatic, and biological characteristics expected for an angiogenin molecule. Its amino acid composition is similar to that of the human protein, and 22 of 31 residues in the amino-terminal sequences are identical, including a block of 11 consecutive residues. Like human angiogenin, the bovine protein binds placental ribonuclease inhibitor, is inactive toward conventional RNase A substrates, and displays selective ribonucleolytic activity toward some rRNAs. In addition, the bovine protein induces angiogenesis in vivo in the chick embryo chorioallantoic membrane assay at levels as low as 44 fmol per egg. Thus, angiogenin is present in bovine sera at levels similar to those observed in man, and its enzymatic and biological activities are identical with those of the human protein.

Human angiogenin is a single-chain, basic protein of 14 100 Da (daltons) which induces blood vessel growth in vivo in doses as low as 35 fmol (Fett et al., 1985). It is present in conditioned media from HT-29 human colon adenocarcinoma cells and also in normal human plasma, where it is recovered at levels of 60–150 μg/L (Shapiro et al., 1987a). The primary sequence of angiogenin shows 35% identity with that of human pancreatic RNase A[1] (Strydom et al., 1985; Kurachi et al., 1985). Although inactive toward commonly used substrates for pancreatic RNases, it does exhibit ribonucleolytic activity toward ribosomal and transfer RNAs (Shapiro et al., 1986, 1987a,b; St. Clair et al., 1987). Both the ribonucleolytic and angiogenic activities are completely inhibited by placental ribonuclease inhibitor (PRI) (Shapiro & Vallee, 1987), which binds tightly to the protein with a K_i below 1 pM.[2]

The isolation of angiogenin from human plasma suggested that sera from other species might also serve as sources of the protein. Bovine plasma was chosen initially because of its ready availability and also because of the potential implications that this material might have for cell culture experiments conducted in bovine serum. Preliminary attempts at purification were unsuccessful, however, due to the lack of a convenient assay. This problem was alleviated by the recent development of a binding assay for angiogenin.[3] We now report the use of this procedure to purify to homogeneity a protein from bovine plasma which, on the basis of its physical, enzymatic, and biological properties, is bovine angiogenin.

Experimental Procedures

Materials. Young calf (1–2 weeks old) and mature bovine (at least 1.5 years old) blood from mixed breeds was obtained from local slaughterhouses. Calf serum (sterile, for cell culture) and fetal calf serum (sterile, for hybridomas) were obtained from Whittaker M. A. Bioproducts. CM-cellulose (grade CM-52) ion-exchange resin was a product of Whatman Ltd. Yeast RNA (highly polymerized) and wheat germ RNA

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[1] Abbreviations: RNase(s), ribonuclease(s); RNase A, bovine pancreatic ribonuclease A; PRI, human placental ribonuclease inhibitor; CAM, chorioallantoic membrane; CM 2, salt-eluted fraction from the (carboxymethyl)cellulose column; MS 1, pool of angiogenin-containing fractions from Mono S chromatography in Tris buffer, pH 8.0; MS 2, pool of angiogenin-containing fractions from Mono S chromatography in phosphate buffer, pH 7.0; CM, carboxymethyl; C18, octadecylsilane; HPLC high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HSA, human serum albumin; TFA, trifluoroacetic acid; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); <Glu, pyroglutamic acid.

[2] Frank S. Lee, personal communication.

[3] Manuscript in preparation.

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