Chemical Modification of the Arginines in Transferrins†

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ABSTRACT: Phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione were used to modify the arginines in ovotransferrin and human serum transferrin. As much as 88% of the arginines of iron-free ovotransferrin was modified with a 7.5-fold excess of 2,3-butanedione in 200 mM borate. The iron-binding activity decreased to 50% in this reaction. The rate and extent of arginine modification and inactivation of the protein were directly related to the concentration of borate in solution. When borate was removed from the modified protein solution, the iron-binding activity increased from 50 to 80% and some of the arginine was regenerated. Cyclohexanedione also inactivated the protein in borate buffer but was not used further due to apparent side reactions. When iron-free ovotransferrin or human serum transferrin was modified with a 7.5-fold molar excess of phenylglyoxal, as compared to arginines, in bicarbonate buffer, a pseudo-first-order loss in arginine and activity was observed. There was no significant modification of arginines or inactivation of the protein when the phenylglyoxal reaction was done in borate buffer. Analysis of the reaction data by a kinetic method or a statistical analysis suggests that there is one critical arginine involved in the binding of each iron atom to the protein. Arginine may function as an essential residue in the anion-binding site of transferrin, i.e., the bicarbonate-binding site.

The transferrins are a group of metal-binding proteins found in a wide variety of vertebrate fluids (Brown et al., 1977). All known transferrins bind two atoms of iron per molecule at two nearly identical sites, forming a salmon-pink colored complex with an absorption maximum near 470 nm. There are no prosthetic groups, such as heme, and the metal-binding site is dependent upon the conformation of the protein with the ligands contributed by the side chains of properly positioned constituent amino acids (Fraenkel-Conrat and Feeney, 1950). There are most likely two histidines and two or three tyrosines involved in the binding of each iron (Feeney and Komatsu, 1966; Komatsu and Feeney, 1967; Rogers et al., 1977b; Tomimatsu et al., 1976). Amino and indole side chains do not appear to be directly involved (Buttkus et al., 1965; Ford-Hutchinson and Perkins, 1972).

In order to form a stable metal–protein complex, it is necessary for the transferrin molecule to bind an anion, normally carbonate or bicarbonate. It seems most likely that the anion interacts directly with the Fe^{3+} while binding to the protein in a specific anion-binding site (Aisen et al., 1973; Harris et al., 1974; Schlabach and Bates, 1975; Campbell and Chasteen, 1977). Although the metal-binding site has been extensively studied, little is known about the amino acids involved in the anion-binding site. It has been suggested that the anion might interact directly with a protonated guanidinium group of an arginine (Bates and Schlabach, 1973; Schlabach and Bates, 1975). A recent report on the sequences of two homologous peptide fragments of human serum transferrin indicates that two tyrosines and an arginine are conserved (MacGillivray and Brew, 1975).

Reactions of 2,3-butanedione or 1,2-cyclohexanedione with proteins in borate have been shown to have specificity for arginine residues under mild conditions (Patthy and Smith, 1975a; Riordan, 1973). There are numerous reports in the literature on the functional role of arginines in enzymes using these improved chemical techniques (Demaille et al., 1977; Frigeri et al., 1977; Kantrowitz and Lipscomb, 1976, 1977; Marcus, 1976; Powers and Riordan, 1975). A conclusion derived from the many recent studies is that arginine may function in the binding site of anionic substrates and cofactors (Riordan et al., 1977).

We have investigated the possible essentiality of arginines in transferrins. Phenylglyoxal and 2,3-butanedione were used to modify both human serum transferrin and chicken ovotransferrin.

Experimental Procedure

Materials

Chicken egg white ovotransferrin was isolated as previously described (Rogers et al., 1977b). Human serum transferrin was purchased from Behring Diagnostics. 2,3-Butanedione was purchased from Eastman Chemical Co. and was distilled before use. Phenylglyoxal monohydrate was obtained from K & K Laboratories and was recrystallized from CCl_4–acetone (90:10, v/v) prior to use in reactions with the proteins. [^{14}C]Acetophenone was purchased from New England Nuclear. The [^{14}C]acetophenone was used to prepare [^{14}C]-phenylglyoxal, with a specific activity of 1.55×10^9 cpm/mol according to the method of Riley and Gray (1947). All other materials used were purchased as reagent grade.

Methods

Preparation of Buffers, Iron-Free Transferrins, and Iron Complexes of Transferrins. All of the buffers that were used in the protein-modification reactions and activity determinations were treated to ensure a minimum of contamination due to iron or other heavy metals as previously described (Rogers et al., 1977a). Iron-free and the diferric transferrins were also prepared as previously described (Rogers et al., 1977a).

Reactions of Transferrins with 2,3-Butanedione. Usually,

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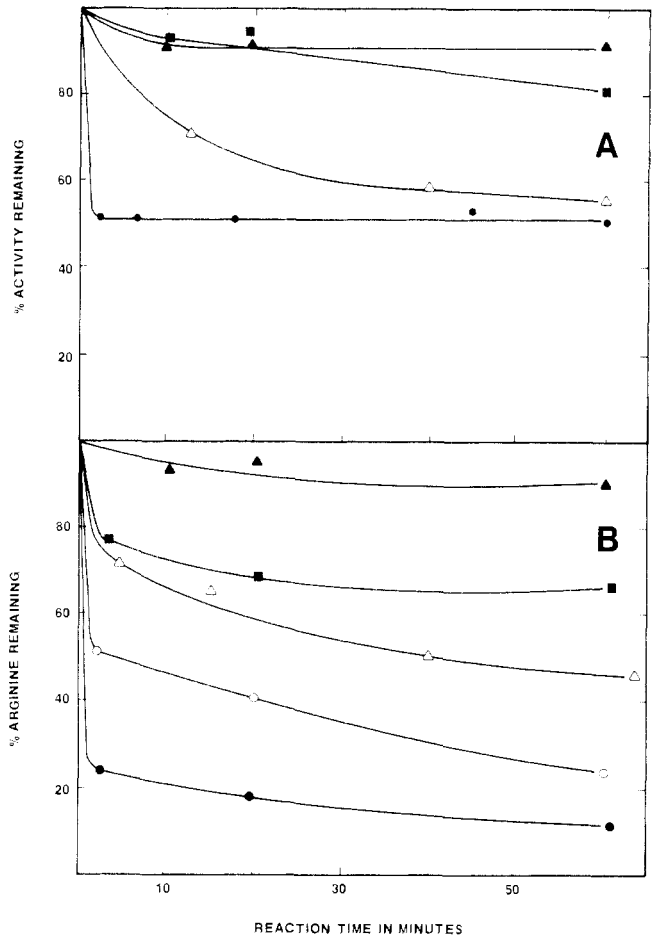

FIGURE 1: The effect of borate on the reaction of 2,3-butanedione (8.6 mM) with ovotransferrin (0.26 mM) at pH 7.8; inactivation of ovotransferrin (panel A) and modification of arginines (panel B). The concentration of borate in the buffers was varied: 100 mM $NaHCO_3$ (0 mM borate) (▲-▲); 20 mM borate (■-■); 50 mM borate (△-△); 200 mM borate (●-●); 200 mM borate with borate removed from solution after reaction (○-○).

54 μL of 2,3-butanedione was added to 3 mL of borate buffer (20–200 mM) at pH 7.8. The pH must be readjusted to 7.8 due to interaction between borate and 2,3-butanedione (Riordan, 1973). A 1.0-mL sample of this solution was added to 1.0 mL of a 0.52 mM solution of iron-free transferrin or ovotransferrin in the same borate buffer, and the pH was readjusted. At intervals, 500-μL aliquots of the reaction solution were passed through a 0.7 × 15 cm column containing Bio-Gel P-4 equilibrated with borate (the same concentration as the reaction buffer) and 100 mM NH_4HCO_3 at pH 7.8. The protein eluates were used directly in a spectrophotometric titration with Fe-NTA[1] to determine the residual iron-binding activity as previously reported (Rogers et al., 1977b). A 200-μL aliquot of this solution was hydrolyzed for amino acid analysis. Riordan (1973) reported that no free arginine was regenerated under these conditions.

Reactions of Phenylglyoxal with Transferrins. In a typical experiment, 65 μM transferrin or ovotransferrin was incubated with 17 mM phenylglyoxal in 0.10 M $NaHCO_3$ at pH 7.8. Two-milliliter samples of the reaction solution were removed at intervals and passed through a 1.5 × 30 cm Sephadex G-25 column equilibrated with 50 mM NH_4HCO_3. The protein eluate was lyophilized directly. The residual activity was determined using an A_{470} titration, and losses in arginine were followed by amino acid analysis. Several experiments were done using [^{14}C]phenylglyoxal. The conditions were identical to those above. The extent of modification was determined after gel filtration by scintillation counting using a Beckman LS-250 liquid scintillation counter. In the reactions of phenylglyoxal with ovotransferrin in 200 mM borate, the experimental conditions were like those for the 2,3-butanedione reactions.

Amino Acid Analysis. Possible losses in amino acids after some reactions were determined by amino acid analysis on a Technicon Auto Analyzer as previously described (Rogers et al., 1977b).

Quantitation of Essential Arginines in Transferrins. A statistical method of analysis of the phenylglyoxal modification reactions was used to determine the number of essential arginines in transferrin (Tsou Chen-Lu, 1962). The number of essential groups is determined simply from quantitation of the number of groups modified and the residual activity in samples of partially modified protein. The simplest case is of an enzyme with a total of n residues of type A, all equally reactive toward the reagent, but only i of which are essential for activity. The fraction of each essential group unmodified is equal to the total fraction, χ, of type A residues remaining. The relation between χ and the fraction of the activity remaining, a, is given by $a^{1/i} = \chi$. The value of i is found from a plot of χ vs. $a^{1/i}$ which gives the best straight line. This approach may be extended to more complicated situations. In the reactions of phenylglyoxal with iron-free ovotransferrin the following relation was used:

$$a^{1/i} = \frac{n\chi - (n - p - s)}{p}$$

where s groups react most rapidly, none of which are essential. This is followed by the modification of p groups, i of which are essential. Finally, $(n - p - s)$ groups are unreactive. Again, i can be found from a plot of $a^{1/i}$ vs. χ. This method has been successfully utilized by Paterson and Knowles (1972).

The much more commonly used kinetic method described by Ray and Koshland (1962) was also employed.

Results

Modification of Iron-Free Ovotransferrin with 2,3-Butanedione. Initial experiments were done using 200 mM borate, at pH 7.8, with various excesses of 2,3-butanedione. When a 7.5-fold molar excess of reagent over the amount of arginine present in solution was added, approximately 50% of the iron-binding activity of iron-free ovotransferrin was lost in 2 min and did not decrease further through 60 min (Figure 1). When a 100-fold excess of reagent was used under the same conditions, 60% of the activity was lost over the same time period. Therefore, in most of the following experiments a 7.5-fold excess of reagent was used in order to minimize side reactions which would be more likely to occur at the higher reagent concentrations.

The Effect of Borate on the Modification of Iron-Free Ovotransferrin by 2,3-Butanedione. A number of experiments were done in order to determine the effect of borate on the rate of loss of iron-binding activity and arginines when iron-free ovotransferrin was exposed to 2,3-butanedione at pH 7.8 (Figure 1). Both the rate and extent of the loss in activity and arginine content in ovotransferrin were directly related to the concentration of borate in the buffer. In the absence of borate, only 10% of the arginines were modified after 1 h. In 200 mM borate, 76% were modified after 2 min.

[1] Abbreviations used are: Fe-NTA, iron nitrilotriacetate; EDTA, (ethylenedinitrilo)tetraacetic acid; EPR, electron paramagnetic resonance.

TABLE I: Regeneration of Activity and Arginine in Ovotransferrin by Removal of Borate after Reaction with 2,3-Butanedione.

Reaction time (min)	% arginine modified		% activity lost	
	Without borate removal	With borate removal	Without borate removal	With borate removal
2	76	50	47	20
17	82	60	48	19
60	88	76	48	20

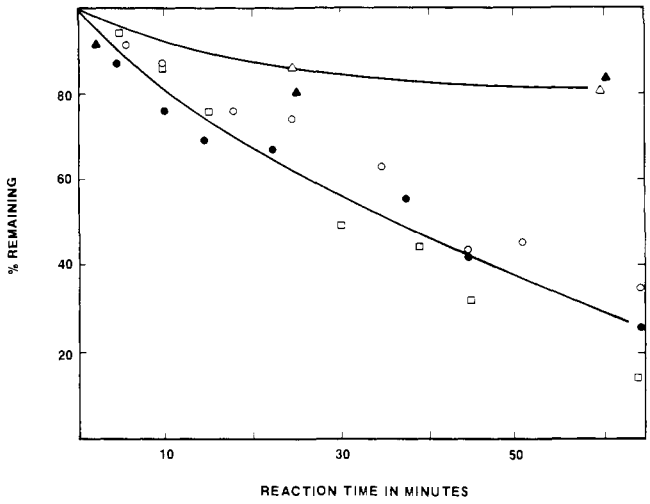

FIGURE 2: The modification of ovotransferrin by phenylglyoxal. The protein (65 μM) was incubated in 100 mM $NaHCO_3$, pH 7.8, with 17 mM phenylglyoxal. The modification of arginine was followed by amino acid analysis (●–●) and incorporation of [^{14}C]phenylglyoxal (○–○). The loss of the iron-binding activity of ovotransferrin was followed by a spectrophotometric titration (□–□). Ovotransferrin (0.26 mM) was also incubated with phenylglyoxal (8.6 mM) in 200 mM borate, pH 7.8, and the losses in arginine (△–△) and iron-binding activity (▲–▲) were determined.

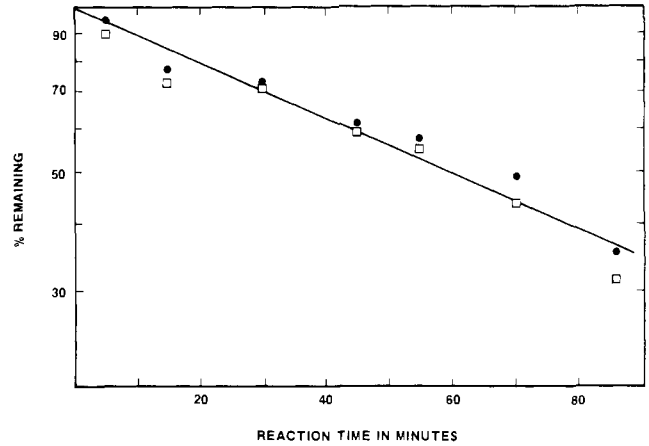

FIGURE 3: A semilogarithmic plot of the loss in iron-binding activity (□) and arginine content (●) for human serum transferrin modified with phenylglyoxal. Human serum transferrin (65 μM) was incubated in the presence of 17 mM phenylglyoxal in 100 mM $NaHCO_3$ at pH 7.8.

Reversal of the 2,3-Butanedione Reaction by Removal of Borate. It has been reported that removal of borate from a solution of modified protein can reverse the modification reaction (Riordan, 1973). When the solution of phenylglyoxal-modified ovotransferrin was passed through a gel-filtration column, equilibrated with 100 mM HCO_3^-, both arginine and activity were regenerated. Correspondingly, some of the iron-binding activity is regenerated when borate is removed as shown in Table I. The regeneration of both arginine and activity was only partial.

Modification of Transferrins with Phenylglyoxal. Ovotransferrin and human serum transferrin were treated with an excess of phenylglyoxal in 100 mM $NaHCO_3$ at pH 7.8. When a 65 μM solution of iron-free ovotransferrin was incubated with a 50-fold excess of reagent over arginines, 50% of the arginines were modified and the iron-binding activity also decreased to about 50% during the first 5 min of the reaction. Also, no significant losses in lysine, histidine, or tyrosine were observed by amino acid analysis.

In subsequent reactions, the relative concentration of phenylglyoxal was decreased so that the effect of partial modifications on the activity could be studied in more detail. When a phenylglyoxal/arginine molar ratio of approximately 8 was used, a gradual loss in arginine was observed by amino acid analysis. This is shown for ovotransferrin in Figure 2. Only 25% of the arginines remained after 65 min. The iron-binding activity of ovotransferrin showed a similar decline over a 65-min reaction period.

Similar experiments were done with [^{14}C]phenylglyoxal, in order to estimate the amount of arginine modification, by an independent method. For these calculations, it was assumed that phenylglyoxal reacts with arginine in a 2 to 1 stoichiometry in bicarbonate buffer (Takahashi, 1968). As shown in Figure 2, the extent of arginine modification determined by either amino acid analysis or by incorporation of [^{14}C]-phenylglyoxal is similar.

When the phenylglyoxal modification reactions were done in 200 mM borate rather than 100 mM $NaHCO_3$, only 15% of the activity was lost in the first 60 min of the reaction (Figure 2). The presence of borate greatly decreased the rate in loss of arginine as well.

When human serum transferrin was exposed to phenylglyoxal in bicarbonate buffer, under the same conditions as for the modification of ovotransferrin, similar rate losses in arginine and iron-binding activity were observed (Figure 3). After 85 min, approximately 35% of the iron-binding activity and arginine remained. These data are plotted as a semilogarithmic relation in order to emphasize that the rates in loss of arginines and activity appear to be pseudo-first-order and nearly identical. Analysis of these data according to the methods of Ray and Koshland (1962) suggests that the inactivation of transferrin is the result of the modification of one arginine essential for the binding of each iron ion.

The statistical analysis of the ovotransferrin–phenylglyoxal reaction data according to the methods of Tsou Chen-Lu (1962) was also used. A plot of $a^{1/i}$ vs. χ is shown in Figure 4. The best straight line is obtained when $i = 1$, implying that one critical arginine residue is involved in the binding of each iron atom. Further information is available from this plot, since the x and y intercepts are $(n - p - s)/n$ and $(p + s)/p$, respectively. From Figure 4, $s = 3$ and $p = 27$, since the total number of arginines, n, is 33. Therefore, under the conditions used, three arginines are rapid reacting, none of which are essential. Then, 27 arginines react, two of which are critical (one for each iron-binding site). Finally, there are three residues which are unreactive, true even when a 50 molar excess of reagent is

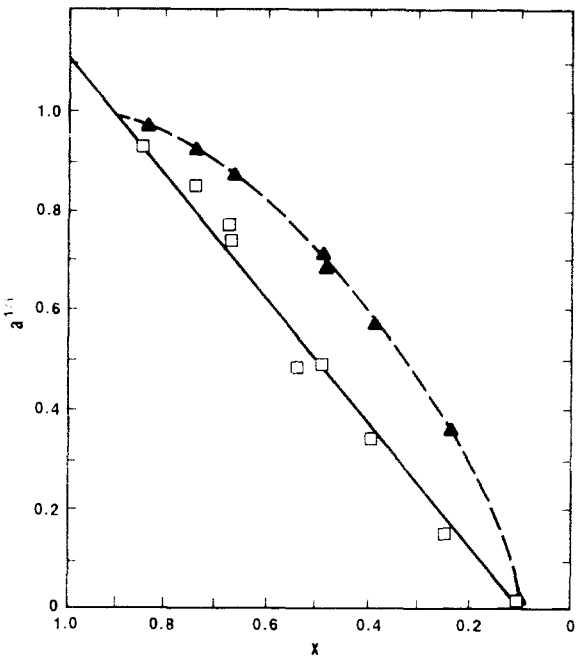

FIGURE 4: The relation between the fraction of activity remaining (a) and the fraction of arginine remaining (χ) for the reaction of phenylglyoxal with ovotransferrin. The data are plotted assuming one essential arginine ($i = 1$) (□–□) and two essential arginines ($i = 2$) (▲- - -▲). The reaction conditions were the same as those described in Figure 2. The plot shows one essential arginine for each iron-binding site.

employed, and no information concerning their essentiality can be ascertained.

Modification of Diferric Transferrin. Diferric human serum transferrin was modified with phenylglyoxal under the same conditions that were used to modify apohuman serum transferrin. In these experiments the iron-binding activity was determined by monitoring the absorbance at 470 nm of the reaction solution. These results indicated that at least 15 of the 25 arginines could be modified without any loss in activity.

When more detailed protection and differential labeling experiments were pursued, phenylglyoxal-modified transferrins were found to be unstable below pH 5.5. Since low pH treatment is usually employed to remove iron from the protein complex, iron could not be conveniently removed.

Modification of Transferrins with 1,2-Cyclohexanedione. The efficacy of utilizing 1,2-cyclohexanedione as a modification reagent in borate buffer was investigated. Initial results indicated that the inactivation of ovotransferrin was similar to that with 2,3-butanedione. However, as the reaction continued, a nondialyzable yellow color was observed. This was probably due to the side reaction of cyclohexanedione with lysine residues previously reported by Liu et al. (1968). Although Buttkus et al. (1965) reported that amino groups are not essential, modification reactions with 1,2-cyclohexanedione were not pursued further because of these interfering side reactions.

Discussion

The arginines in chicken ovotransferrin and human serum transferrin were very reactive toward either 2,3-butanedione or phenylglyoxal as compared to most other proteins. Commonly, larger molar excesses of reagent are employed, often a 200-fold excess, and only a small fraction of the arginines are reactive (Daemen and Riordan, 1974; Kantrowitz and Lipscomb, 1977; Lange et al., 1974; Powers and Riordan, 1975). Since relatively low concentrations of these reagents were needed in our modification reactions, side reactions were less likely, although not ruled out completely, for the 2,3-butanedione reactions.

Phenylglyoxal inactivated iron-free transferrins under mild conditions. For both proteins, the loss in activity was proportional to the loss in arginine, as shown in Figure 3 for human serum transferrin. The losses in arginine were similar when followed by amino acid analysis or by the incorporation of [^{14}C]phenylglyoxal into ovotransferrin, indicating that no significant side reactions were occurring. With diferric transferrin, at least 60% of the arginines could be modified without any loss in the iron-binding activity. This suggests that the inactivation of the apoprotein is due to the modification of a small number of arginine residues.

The inactivation of ovotransferrin by 2,3-butanedione was directly dependent upon the concentration of borate in the reaction solution. This correlation suggests that it is the destruction of arginines which is responsible for the loss in activity (Riordan, 1973). The extents of loss in activity and arginines were approximately proportional in reactions from 0 to 50 mM borate. However, in 200 mM borate not more than 50% of the activity was lost; yet, the arginines were modified up to 85%. The loss in activity in phenylglyoxal reactions showed no similar plateau at 50% and it is not clear why these results are different. Similar effects were seen with alcohol dehydrogenase when modified with these two reagents (Lange et al., 1974).

The reversibility of the 2,3-butanedione inactivation when borate is removed from the reaction solution is consistent with current notions concerning arginine-modification reactions (Riordan, 1973). Some of the arginine was regenerated, but as the reaction continued a decreasing proportion of the modified residues could be regenerated. The results of the reversal experiments suggest that the modification of only a small number of arginines by 2,3-butanedione is responsible for the inactivation. With the most extensively modified product (60 min), approximately only 3.9 of the 29 modified arginines were regenerated while the activity increased from 48 to 80%.

The differences seen in the relative reactivity of the arginine in transferrins toward phenylglyoxal and 2,3-butanedione are most probably due to the different chemistries involved in each reaction (Riordan, 1973; Takahashi, 1968, 1977). An important additional difference between the reactions of these two reagents was seen in the effect of borate. 2,3-Butanedione inactivation of ovotransferrin was enhanced by borate, whereas the inactivation due to phenylglyoxal was inhibited almost completely by 200 mM borate (Figure 2). A similar effect has been seen with alkaline phosphatase and a number of 1,2-dicarbonyl reagents (Daemen and Riordan, 1974). It appears that the reaction of α-keto aldehydes and arginine is inhibited by borate while the reactivity of 1,2-diketo reagents is greatly increased by borate. The enhanced rate of reaction of the 1,2-diketo compounds is probably the result of stabilization of the products by borate. In contrast, phenylglyoxal appears to form complexes with borate in solution; when phenylglyoxal is added to borate buffers the pH decreases, implying some type of chemical interaction. Complexes between borate and 2,3-butanedione or 1,2-cyclohexanedione have also been reported (Dietl and Tschesche, 1976; Riordan, 1973). High levels of borate may decrease the concentration of the reactive form of this reagent. The results of this work emphasize the importance of using different reagents and procedures for the modification of a particular type of amino acid residue in proteins (e.g., arginines).

The data from the modification reactions with phenylglyoxal were used to quantitate the number of essential arginines in transferrin. It was observed that the pseudo-first-order rates for the inactivation of the transferrin and the modification of

arginines were the same (Figure 3). Analysis of these data according to the method of Ray and Koshland (1962) indicates that one essential arginine is modified for each iron-binding center. Quantitation of the critical arginines was also accomplished by the use of a statistical method of analysis of the reaction data, where no kinetic assumptions are necessary. In agreement with the kinetic approach, analysis of the data by the statistical method suggests that there is one essential arginine for each iron-binding center.

There are limitations to interpretations of changes in protein activities caused by chemical modification. It may be possible that the modification of a nonessential residue near the active site leads to inactivation by steric blocking of the active center by the introduction of a bulky group or that important alterations of charges or conformational changes may occur as a result of the modifications. Confidence in an interpretation that a particular residue is critical, or even essential, for function is increased when it can be proven that a single residue per active center is involved and when different reagents give similar results (Means and Feeney, 1971).

The chemical-modification studies were undertaken for two specific reasons. There is a report that arginines may be important because of some invariance of these residues in the primary sequence of cyanogen bromide cleaved fragments of human serum transferrin (MacGillivray and Brew, 1975), and, secondly, numerous reports have identified at least 25 enzymes that utilize arginyl residues to interact with negatively charged phosphate or carboxylate moieties of substrates or cofactors (Patthy and Smith, 1975b; Powers and Riordan, 1975; Riordan et al., 1977). In fact, it may well be a general functional role of arginines to serve as positively charged recognition sites for negatively charged species that interact specifically with proteins (Riordan et al., 1977). A very likely role for the arginine in transferrin would be to serve as a positively charged critical residue in the anion-binding site. The planar guanidinium group of arginine is well suited to interact with the planar CO_3^{2-} in the Fe-transferrin-CO_3^{2-} complex via two hydrogen bonds in addition to electrostatic attraction.

In such a model, other bifunctional anion substitutes such as pyruvate, glycine, or oxalate would specifically complex with the protein by a carboxylate–arginine interaction in the anion site. The second functional group such as an amino, keto, or another carboxy moiety would form a sixth ligand to the metal center, interlocking the separate binding sites (Schlabach and Bates, 1975; Campbell and Chasteen, 1977). This spatial arrangement of the anion in the metal-binding center is in agreement with a recent report in which EPR spin-labeled anions were studied (Najarian et al., 1978). Such a model differs from the older schematic model of Windle et al. (1963) by including the bonding between the carbonate and the guanidinium group.

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