Electrostatic Modification of Protein Surfaces: Effect on Hemoglobin Ligation and Solubility†

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ABSTRACT: Protein amino groups can be carboxymethylated under mild conditions by the combined use of glyoxylate ion and cyanoborohydride. The amino group is converted to a zwitterionic residue where the pK of the secondary amine is only slightly altered and a carboxyl group has been added some 3 Å from the nitrogen atom in the amine. Modification of hemoglobin to low levels of carboxymethylation yields derivatives specifically modified at the terminal α-amino groups. These modified hemoglobins are models for the interactions between the protein and small anions. When the extent of modification is increased by treating the protein with a higher concentration of the modifying agents, lysine residues become converted to N^{ϵ}-(carboxymethyl)lysine. In excess of 90% of lysine residues in hemoglobin and myoglobin can be modified by this technique. The increased negative charge can be adjusted to any intermediate level of modification. The change in electrostatic free energy that results from the altered distribution of charge on the protein surface can be correlated with functional properties. Thus, the increased repulsion between the hemoglobin dimers leads to dimerlike oxygen binding properties at a high degree of modification. Similarly, changes in protein solubility secondary to modification reflect altered tetramer–tetramer interactions in the solid state. This method for achieving an altered distribution of charge on the protein surface, a method which can be carried out in a specific or nonspecific fashion to achieve varying degrees of modification, represents a powerful tool for the study of electrostatic interactions in protein chemistry.

Electrostatic forces between proteins and protein subunits and the interactions between charged residues and solvent have been intensively studied by a variety of researchers interested in protein solution properties (Linderstrøm-Lang, 1924; Tanford & Kirkwood, 1957; Lee & Richards, 1971). Major contributions pertaining to analytical estimates of stabilizing free energy in heme proteins have been developed by Gurd and associates (Shire et al., 1974; Matthew et al., 1979a,b, 1981). The interactions between a number of charged amino acids in hemoglobin (Hb) and anions have been identified in terms of functional aspects of ligand binding by means of X-ray crystallography (O'Donnell et al., 1979). Calculations of summed electrostatic forces in determining the stability of the hemoglobin tetramer have been carried out for both oxy- and deoxy-Hb (Flanagan et al., 1981).

The importance of electrostatic forces as determinants of protein solubility was realized early. The pH dependence of protein solubility normally exhibits a minimum at or near the isoionic point which can be related to the appearance of increasing repulsive forces between protein molecules at both sides of the isoionic point (Green, 1931). Systematic electrostatic modification of hemoglobin using the technique described in this paper may provide a useful tool for further evaluating the thermodynamics of protein solubility.

The modification of proteins using reduction of the unstable Schiff base that is formed between amino groups and aldehydes is a well-established biochemical tool for modification of protein amino groups. The reaction is utilized for reductive methylation using formaldehyde and borohydride (Means & Feeney, 1968). Similarly, pyridoxal phosphate can be covalently linked to protein amino groups (Fisher et al., 1958). This compound and a number of analogues have also been used for affinity labeling of the binding site for 2,3-diphosphoglycerate to hemoglobin (Benesch et al., 1972). Labeling of amino groups with glyoxylic acid differs from the other methods by virtue of the rather unique properties of the aldehyde itself. It is a relatively small anion, and when added to proteins in the absence of reducing agents, it behaves very much like a simple anion, e.g., acetate ion. When the reducing agent is added, we "freeze" certain aspects of the behavior of this ion into the structure of the protein, thus acting as covalently bound counterions. The protein has been equipped with a new amino acid, N^{ϵ}-(carboxymethyl)lysine, which can replace lysine quite effectively. The influence of the zwitterionic side chain of this amino acid, in terms of its effect on a variety of aspects of protein chemistry in general, and hemoglobin chemistry in particular, is the basis for this paper.

Preliminary aspects of this work have been presented in abstract form (Saunders & Hedlund, 1983). Similarly, Acharya et al. (1982) have presented certain aspects of the reaction between glyoxylic acid and hemoglobin. The earliest study we have found in which a protein was covalently modified by glyoxylic acid was a report by King et al. (1977).

Materials and Methods

Human hemoglobin was purified by chromatography on DEAE-Sepharose. The hemoglobin solution was dialyzed against distilled water and then deionized by passage through a mixed-bed ion-exchange resin. Hemoglobin concentrations were measured as the cyanomethemoglobin derivative by using an extinction coefficient of 44.0 at 540 nm for a millimolar solution of hemoglobin tetramer.

Modification with glyoxylic acid was carried out at room temperature in the presence of 0.1 M phosphate buffer, pH 7.0. Glyoxylic acid was obtained as the free acid (Sigma Chemical Co., St. Louis, MO) and titrated to neutrality with sodium hydroxide. Both glyoxylate and cyanoborohydride were added to the hemoglobin solution, which was 0.5 mM tetramer, to various degrees of molar excess. Addition of

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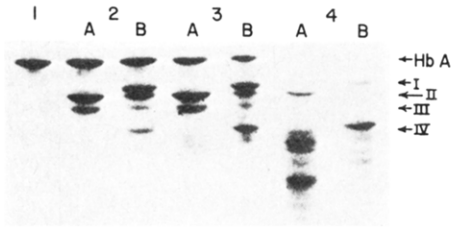

FIGURE 1: Isoelectric focusing patterns of normal and modified human hemoglobin. Lane 1 is control HbA. Lanes 2, 3, and 4 illustrate the degree of modification using 2, 3, and 10 mM glyoxylic acid, respectively. Molar equivalents of cyanoborohydride were used in this instance. Hemoglobin concentration was 0.5 mM tetramer. The lanes labeled A refer to modification carried out on deoxygenated hemoglobin in the presence of 20 mM inositol hexaphosphate. The lanes labeled B refer to modification carried out on fully oxygenated hemoglobin in the absence of inositol hexaphosphate.

merely cyanoborohydride did not alter hemoglobin structure as evidenced by isoelectric focusing and amino acid composition (Borch et al., 1971). Sodium borohydride did not reduce the Schiff base under the conditions used. The reaction appears to be fully complete in 30 min. Additional incubation overnight does not further alter the pattern of modification is evidenced by isoelectric focusing. More complete information pertaining to the rate, the pH dependence, the influence of temperature, and the effect of competing anions will be discussed elsewhere.

The modification of the hemoglobin net charge can be followed by isoelectric focusing, by which a display of clearly identifiable bands is obtained. As the degree of modification is increased, the modified hemoglobin appears as a distribution of bands with an isoelectric point below 6.5. At very high degrees of modification, carboxyl groups become dominating, and the isoelectric point becomes as low as 4.0.

Figure 1 illustrates the modifications obtained with varying but equimolar concentrations of glyoxylic acid and borohydride. Two different incubation conditions were employed. The β-chain terminal amino groups of the hemoglobin were the primary reaction sites when oxyhemoglobin was modified in phosphate buffer (0.1 M, pH 7.0). In order to at least partially block this primary reaction product, every other lane illustrates the altered pattern obtained in a deoxygenated solution in the presence of 20 mM inositol hexaphosphate in the same buffer. Additional details are given in the legend to Figure 1.

In order to structurally characterize the bands illustrated in Figure 1, conditions were chosen so as to provide maximal yield of a given band. A substantial quantity of hemoglobin, i.e., several grams, was reacted and applied to a DEAE-Sepharose column following overnight dialysis against 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.2. The modified hemoglobin was then added to the column (50 × 3 cm), and fractions were collected by using batch elution with decreasing pH to pH 7.0. Fractions were pooled, concentrated by ultrafiltration, and analyzed by isoelectric focusing (LKB Ultrophor, pH gradient 5.5–8.5). Samples that were either pure or contaminated to less than 5% by a neighboring band were used for structural and functional studies. The bands indicated by roman numerals I, II, and IV in Figure 1 were determined to be carboxymethylated on the termini of the β-chain, α-chain, and both α- and β-chains, respectively. The structural assignments were based on canine hemoglobin hybridization studies and isoelectric focusing (Bunn, 1981). Band III has a blocked β-chain terminus and also a modified lysine residue on the α-chain at position α127. The latter assignment was based on an analysis of the tryptic digest of the isolated α-chain. These studies, carried out in Dr. Richard T. Jones' laboratory, indicate that peptides αT12B and αT13 were absent in this hemoglobin fraction. The cleavage point between these peptides is the lysine residue at position 127. Other peptides from both α- and β-chains were normal with the exception of peptides α- and βT1. These peptides lacked a normal valine residue. It is interesting to note that under the conditions used, there is no reaction at the β82 lysine, one of the residues in the binding site for 2,3-diphosphoglycerate. Since peptides βT9 and βT10 from the digested β-chain are observed in normal quantities, the lysine residue between these must be unmodified.

Oxygen binding studies were carried out in dilute solution (25 μM tetramer) by a point by point spectrophotometric technique. Optical densities at 500, 560, and 576 nm were measured at six to eight separate oxygen tensions. The composition of hemoglobin in terms of oxy-, deoxy-, and methemoglobin was obtained by a standard program solving three equations with three unknown parameters. Studies of the effect of chloride were carried out in 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) buffer, pH 7.0, 25 °C. Studies of highly modified Hb were carried out in 0.1 M phosphate buffer at the same pH and temperature.

The amino acid composition of modified hemoglobin samples was carried out following 23-h hydrolysis in constant-boiling HCl at 110 °C. The analysis was carried out on a Dionex D-330 amino acid peptide analyzer using a 0.32 × 14.0 cm column containing Dionex DC-5A resin. Only two modified amino acids were detected, the amino-terminal valine and lysine. The modified lysine residue appears close to methionine by this procedure. Increased time of acid hydrolysis did not alter the composition, suggesting that the derivatized lysine is stable under these conditions. At extremely high levels of modification, a second lysine derivative appears, while the monosubstituted lysine peak is somewhat diminished. This is likely to be due to the appearance of a disubstituted lysine, analogous to dimethylation obtained when carrying out reductive methylation using formaldehyde (Means & Feeney, 1968). This presumed disubstituted lysine residue, being more acidic, appears near cysteic acid. Hemoglobin solubility studies were carried out by the poly(ethylene glycol) solvent exclusion method (Middaugh et al., 1979; Atha & Ingham, 1981). Poly(ethylene glycol) (PEG) of average molecular weight 6000 was used. For these studies, cyanomethemoglobin was employed. The degree of modification and the hemoglobin concentrations were adjusted so that the dimer–tetramer equilibrium would not greatly influence solubilities.

Results and Discussion

Reductive carboxymethylation using glyoxylic acid differs from other means of amino group modification by virtue of the completeness of the reaction and the relatively subtle functional consequences that occur even following extensive lysine modification. A number of other similar protein modifications using related compounds are characterized by a relatively modest degree of modification or profound functional alterations of the modified protein. Similar aldehydes which lead to oxidation and denaturation of hemoglobin at considerably lower degrees of substitution than glyoxylate include acetaldehyde and methylglyoxal. Although reductive carboxymethylation is chemically and conceptually similar to simple reductive alkylation, the glyoxylate procedure appears to differ in two important aspects. First, the glyoxylate anion is a relatively compact anion which will be electrostatically attracted to positive charges on the protein surface in a manner

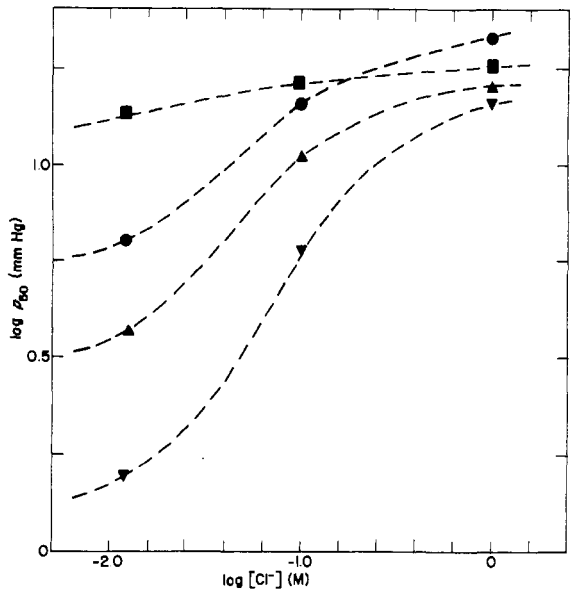

FIGURE 2: Oxygen tension at half-saturation (P_{50}) for three concentrations of chloride in the presence of 50 mM Bistris buffer, pH 7.0, 25 °C. Concentrations of chloride used were 0.013, 0.10, and 1.0 M. Unmodified Hb (▼); α-chains modified (▲); β-chains modified (●); hemoglobin with both α- and β-chain terminal α-amino groups modified (■).

analogous to a counterion such as chloride or acetate. Second, the functional consequences that occur appear to be less drastic than those resulting from other types of amino group alterations. The appearance of a secondary amine with a carboxyl group separated by a methylene group seems to only slightly alter the interactions that the lysine ϵ-amino group normally may be involved in, and the negative charge may be considered an equivalent of a covalently attached counterion. As an estimate of the change in pK of the carboxymethylated amino group, it is worth noting that the compound N-tris(hydroxymethyl)methylglycine, $(CH_2OH)_3CNH(CH_2COOH)$, has a pK for its secondary amine only one-tenth of a pH unit below that for the primary amine of its noncarboxymethylated analogue, tris(hydroxymethyl)aminomethane (Good et al., 1966). A protein modified by glyoxylate can be considered to be altered primarily in an electrostatic sense or, in other words, a functionally intact protein surrounded by covalently attached negative charges. The applications of this approach to any problem involving electrostatic interactions of proteins are manifold. In this paper, we describe results pertaining to the effect of electrostatic modification on hemoglobin chemistry, particularly relating to the following three aspects of this protein: the allosteric effect of anion binding, the influence on the tetramer–dimer equilibrium, and the effect of systematic charge alteration on the solubility of the protein.

The α-amino groups of the N-terminal valines of both α- and β-chains have been identified as binding sites of anions to hemoglobin (O'Donnell et al., 1979). Since these derivatives can be purified, the properties of hemoglobins with affinity-labeled anion binding sites can be defined. Studies of the functional properties of these derivatives as well as the differential labeling pattern of oxy- and deoxyhemoglobin provide additional information pertaining to the accessibility of amino groups in this anion binding site. These results are also useful in probing the energetics of anion binding as it relates to allosteric effects in hemoglobin chemistry.

The influence of the covalent modification of the anion binding sites of hemoglobin in terms of the chloride allosteric effect is illustrated in Figure 2. The derivatives modified at the α-chain termini have a significantly decreased chloride effect. Thus, the chloride effect is reduced to about two-thirds of that observed in the control. The behavior of the β-chain-modified derivative shows a more dramatic effect in that the residual chloride effect is only about one-third compared to the binding curve of unmodified hemoglobin. This derivative also exhibits a right-shifted curve in the presence of 1.0 M chloride compared to the unmodified control, suggesting that the covalent addition of negative charge at the β-chain termini allows for more pronounced stabilization of the deoxy form than is obtainable by binding of chloride ion.

The behavior of the derivative modified on all four termini is interesting in that the effects of modification of α- and β-chains appear additive. In this derivative, the chloride effect is all but abolished. The slightly increased oxygen affinity with the β-chain-modified derivative observed in the presence of 1.0 M salt can possibly be accounted for by changes in the alkaline Bohr effect in these derivatives. This problem is presently under study.

The results illustrated in Figure 2 can be discussed along two lines of reasoning. On the one hand, the covalent attachment of negative charges on the hemoglobin molecule can be interpreted as a means of partially satisfying the small anion allosteric effect by means of *differential structural interactions* as opposed to *differential binding*. Alternatively, one might consider the residual anion effect observed in these derivatives as the anion response of a given derivative with some fraction of available anion binding sites blocked. The distinguishing feature between these approaches may be the fact that, from an anion binding point of view, the modifications described here primarily alter the base-line behavior of the response of the protein to anions; i.e., the largest change in oxygen affinity is observed at low chloride concentration. Instead of merely blocking the binding site with a neutral substituent, we have introduced a negative charge, thereby mimicking anion binding. A different response will in all likelihood occur if the substituent is neutral rather than carrying a negative charge. Studies carried out in other laboratories on specifically carbamoylated hemoglobin (Nigen & Manning, 1975; Nigen et al., 1980) represent an interesting comparison in this context. Hemoglobin carbamoylated at the β-chain termini exhibits a slight decrease in oxygen affinity (about 0.1 log unit) over the entire range of chloride concentrations. The α-chain-modified protein, on the other hand, yields an increase in oxygen affinity (about 0.2 log unit in log P_{50}) over the same range of chloride concentrations (O'Donnell et al., 1979; Nigen et al., 1980). The asymmetric effect observed in the present case is therefore more consistent with that expected of a specific anion-like effect, while in the case of carbamoylation the resulting effect represents a relatively subtle alteration in the structure of the binding sites, leading to a symmetric displacement of the curve depicting the chloride response.

When the protein modification is carried out with higher ratios of glyoxylic acid to hemoglobin, modification of lysine residues becomes increasingly evident. The isoelectric point of the protein is further decreased, and the distribution of isoelectric points becomes broader. Modification of lysine residues is therefore less well-defined. The modification pattern is not known at the microscopic level; however, the average degree of modification is easily determined by the amino acid composition. It is also possible to determine the width of the distribution by purifying the most and least electrophoretically modified fractions for a given degree of average modification. In Figure 3, we have illustrated the alteration in oxygen binding properties as a function of the degree of modification of normal human hemoglobin. The

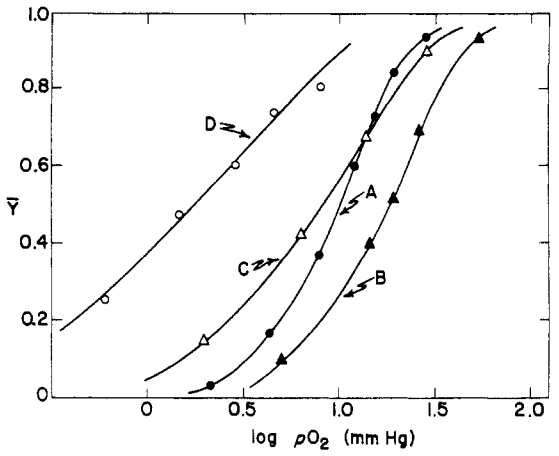

FIGURE 3: Oxygen binding curves in 0.1 M phosphate buffer, pH 7.0, 25 °C. Hemoglobin concentration was 25 μM in all cases. Curve A represents unmodified hemoglobin. The preparation used for obtaining curve B has all four terminal amino groups as well as 12% of the lysine ϵ-amino groups modified. Curve C describes the oxygen binding properties of hemoglobin with 65% of the lysines modified, and curve D refers to a preparation with 85% modification of lysines. Modification was carried out for 30 min at 25 °C in 0.1 M phosphate buffer, pH 7.0. Hemoglobin concentration was 0.5 mM tetramer. 200 mM cyanoborohydride was used for all three preparations. The glyoxylate concentrations in the three samples were (B) 20, (C) 100, and (D) 200 mM.

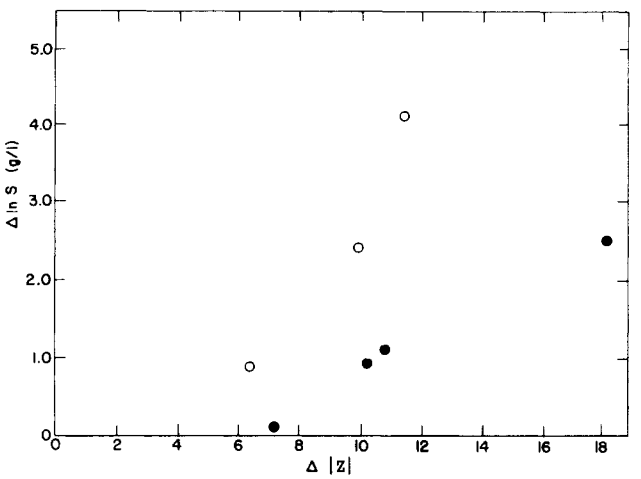

FIGURE 4: Solubility of human cyanomethemoglobin at pH 7.67, 8.20, and 8.72 compared to that obtained at pH 7.0. The open circles illustrate the increase in hemoglobin solubility expressed in a logarithmic scale. A buffer system containing 50 mM each of Bistris and Tris was employed for these measurements. The closed circles refer to measurements of solubility changes observed in four preparations of amino-modified hemoglobin compared to the unmodified control. These measurements were carried out in 0.1 M Bistris, pH 7.0. The actual solubilities refer to the values obtained in 26% poly(ethylene glycol) (M_r 6000), at 22 °C, and were in the range of 2–100 μM hemoglobin tetramer. The abscissa refers to the decreased net charge per hemoglobin tetramer following exposure of the protein to increased pH or modification of amino groups. The former quantity was calculated from the tabulations of Matthew et al. (1979) and includes all histidines that titrate as well as the terminal α-amino groups. The charge change following modification was obtained from the amino acid composition of aliquots of the preparations used for solubility measurements. Under the conditions used, the terminal α-amino groups become fully modified and are included in the total charge change.

experimental conditions employed for the modification of the hemoglobins used in Figure 3 are outlined in the legend. A complete analysis of the results illustrated in Figure 3 should include measurements of the tetramer–dimer association constant for both the unliganded and liganded forms, results which are not available at this time. However, the oxygen binding curves in Figure 3 provide clues to the magnitude of repulsive forces that occur between dimers secondary to charge modification. Curve B, which illustrates the oxygen binding properties of a sample with a modest degree of lysine modification, but complete α-amino group modification, suggests that the protein remains in the tetrameric form under the experimental conditions. The right-shifted curve is due to the covalently bound anion effect discussed above. Curve C has a midpoint similar to that for the control but considerably decreased cooperativity. Since the electrostatic component of the total free energy of tetrameric stabilization is relatively larger in the liganded form (Flanagan et al., 1981), it is reasonable to expect that oxyhemoglobin has a stronger tendency to dimerize at a given degree of electrostatic repulsion than the unliganded form. The observed increased oxygen affinity suggests the presence of a considerable amount of dimeric hemoglobin in the fully and partially oxygenated forms. The dramatically left-shifted curve D is the result obtained following modification of approximately 85% of the lysine residues. At a concentration of 25 μM hemoglobin tetramer, the electrostatic repulsion from about 20 additional negative charges per dimer is sufficient to be the dominating factor even in the unliganded form, suggesting an extraordinary degree of destabilization of the hemoglobin tetramer. Curve D, by virtue of the absence of cooperativity and high oxygen affinity, is similar to the extrapolated binding properties obtained for the human hemoglobin dimer (Mills & Ackers, 1981).

The third area to which the systematic electrostatic modification has been applied is that of hemoglobin solubility. The purpose of these studies was to compare the relative effect of histidine titration with those observed secondary to lysine modification. The results illustrated in Figure 4 demonstrate the relative change in solubility of normal hemoglobin as a function of pH vs. those obtained at constant pH for a series of modified hemoglobin preparations where the degree of modification was known from determinations of amino acid composition. The titration status of the hemoglobin histidines was estimated from theoretical pK values (Matthew et al., 1979). Possible slight differences between histidine pK values of oxy- and cyanomethemoglobin were ignored. The average degree of lysine modification was obtained from the amino acid composition. The results indicate that histidine titration, for a given change in charge units, leads to a much larger alteration in solubility than lysine modification. Thus, in terms of interaction in the solid state, the protein solubility is more affected by the titration status of histidines than the appearance of negative charge on lysine residues. The rather dramatic effect of histidine titration suggests that the loss of positive charge with increasing pH leads to local structural changes. Some of the histidines are involved in the alkaline Bohr effect, which may involve significant local rearrangements. Presumably, such local conformational changes have a stronger influence on protein–protein interactions in the solid state than the introduction of negatively charged residues on the ϵ-amino group of lysine residues. Interactions between the lysine amino group and neighboring residues do not seem to be greatly influenced by the negative charge localized further toward the solvent. The fact that water molecules probably can effectively surround such an exposed residue may account for the rather subtle effect on protein solubility. It should also be noted in this context that the lysine modification is not specific. Therefore, a decreased charge by some quantity implies that "on the average" this quantity of lysines has been modified. It is likely that different residues may have been

modified on different molecules, a fact which probably accounts for the relatively subtle change in solubility. Thus, if one could specifically modify lysine residues which are involved in neighbor–neighbor contacts in the solid phase, it is likely that electrostatic repulsion forces could be more dramatically influenced. There are, on the other hand, conceptual advantages of the relative nonspecificity of lysine modification as described here. In terms of theoretical calculations of electrostatic free energy, we can assume, at least as a first approximation, that lysine modification is distributed in a random fashion over the surface of the protein. Thus, in an attempt to evaluate interactions between proteins or between proteins and ions, the situation does resemble an albeit static, macroscopically "smeared", distribution of charge, thereby amenable to theoretical treatment of the type originally outlined by Linderstrøm-Lang (1924). More recent extensions of the Tanford–Kirkwood theory as applied to electrostatic interactions in hemoglobin have utilized crystallographic information to estimate the location of charged residues in heme proteins (Shire et al., 1974). Incorporation of carboxyl groups at specific locations or random incorporation of a certain number of negative charges at the protein–solvent interface can presumably be incorporated into calculations of total electrostatic energy. It would be of particular interest to compare functional properties of the type illustrated in Figure 3 with theoretical estimates of tetramer stabilization in hemoglobin.

The systematic charge modification of hemoglobin as described in this paper is likely to be of considerable use for the study of electrostatic effects in protein chemistry in general. Although we have limited ourselves to the description of some of the changes that occur in hemoglobin pertaining to ligation properties, subunit equilibria, and solubility, the principles and techniques can be applied to any system where protein structure–function relationships are affected by the incorporation of negatively charged groups in a specific or nonspecific fashion.

Acknowledgments

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Registry No. HbA, 9034-51-9; glyoxylic acid, 298-12-4; cyanoborohydride, 33195-00-5; oxygen, 7782-44-7; chloride, 16887-00-6; N^{ϵ}-(carboxymethyl)lysine, 5746-04-3.

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