Contribution of the γ-Carboxyl Group of Glu-43(β) to the Alkaline Bohr Effect of Hemoglobin A†

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ABSTRACT: Glu-43(β) of hemoglobin A exhibits a high degree of chemical reactivity around neutral pH for amidation with nucleophiles in the presence of carbodiimide. Such a reactivity is unusual for the side-chain carboxyl groups of proteins. In addition, the reactivity of Glu-43(β) is also sensitive to the ligation state of the protein [Rao, M. J., & Acharya, A. S. (1991) *J. Protein Chem. 10*, 129–138]. The influence of deoxygenation of hemoglobin A on the chemical reactivity of the γ-carboxyl group of Glu-43(β) has now been investigated as a function of pH (from 5.5 to 7.5). The chemical reactivity of Glu-43(β) for amidation increases upon deoxygenation only when the modification reaction is carried out above pH 6.0. The pH–chemical reactivity profile of the amidation of hemoglobin A in the deoxy conformation reflects an apparent pK_a of 7.0 for the γ-carboxyl group of Glu-43(β). This pK_a is considerably higher than the pK_a of 6.35 for the oxy conformation. The deoxy conformational transition mediated increase in the pK_a of the γ-carboxyl group of Glu-43(β) implicates this carboxyl group as an alkaline Bohr group. The amidated derivative of hemoglobin A with 2 mol of glycine ethyl ester covalently bound to the protein was isolated by CM-cellulose chromatography. The chemical characterization of this derivative of hemoglobin A, involving the separation of the modified β-globin by RPHPLC, isolation of the modified tryptic peptide by RPHPLC, and amino acid sequencing of the modified tryptic peptide, has established that this is a homogeneous derivative of hemoglobin in which both γ-carboxyl groups of Glu-43(β) have been amidated. The amidation of Glu-43(β) increases the O_2 affinity slightly without any influence on the Hill coefficient. A comparison of the Bohr proton titration of hemoglobin A with that of the disubstituted derivative as a function of pH established the contribution of the γ-carboxyl group of Glu-43(β) to the alkaline Bohr effect of the protein. Glu-43(β) is located at the $\alpha_1\beta_2$ interface of hemoglobin A, and the increase in the pK_a of the γ-carboxyl group of Glu-43(β) upon deoxygenation of the protein apparently reflects an increase in the hydrophobicity of the $\alpha_1\beta_2$ interface of the molecule.

Crystallographic analysis of deoxy-HbS[1] (Wishner et al., 1975; Padlan & Love, 1985) has implicated a number of side-chain carboxyl groups of Asp and Glu residues as part of the intermolecular contact region of polymerized deoxy-HbS. In an attempt to establish whether any of these carboxyl groups exhibit an unusual chemical reactivity around neutral pH, the accessibility of the side-chain carboxyl groups of HbS for amidation was investigated (Seetharam et al., 1983; Acharya & Seetharam, 1985). During these investigations, it became clear that Glu-43(β) exhibits an unusual high chemical reactivity around pH 6.0. In addition, the amidation of this residue results in an increase in the solubility of the deoxy-HbS.

In an attempt to establish a direct correlation between the chemical reactivity of the γ-carboxyl group of Glu-43(β) and its protonation state, sulfo-NHS was incorporated into the amidation system as the "rescuer" of the activated carboxyl group (Staros et al., 1986; Rao & Acharya, 1991). In the presence of sulfo-NHS, the carbodiimide activated carboxyl group (*O*-acylurea adduct) is readily trapped as the sulfo-NHS ester (Figure 1). Thus the rearrangement of the *O*-acylurea adduct to the *N*-acylurea (a known side reaction during the carbodiimide mediated amidation of the carboxyl group) is essentially inhibited. Therefore, in the presence of sulfo-NHS, the carbodiimide activated carboxyl group is channeled through nucleophilic attack, i.e., amidation. This improvement in the modification procedure provides a high specificity for the amidation reaction. Therefore, the extent of amidation of HbA under a given set of conditions reflects directly the propensity of the side-chain carboxyl group of Glu-43(β) to be present in its protonated state.

A kinetic study of the amidation of HbS as a function of pH has established that the pK_a of the γ-carboxyl group of Glu-43(β) in the oxy conformation of the protein is around 6.35 (Rao & Acharya, 1991). The normal pK_a for the γ-carboxyl group of Glu residues of proteins is around 4.8. The unusually high pK_a of 6.35 for the γ-carboxyl group of Glu-43(β) of HbS has prompted us to investigate the influence of deoxygenation of HbS on the chemical reactivity of its Glu-43(β). It is conceivable that the pK_a of the γ-carboxyl group of Glu-43(β) could be normalized (decreased) upon deoxygenation. Deoxygenation of the protein increased the chemical reactivity of Glu-43(β) (Rao & Acharya, 1991). This increase in the chemical reactivity of the carboxyl group could be a consequence of an increase in the pK_a, since the protonated carboxyl group is the active species that participates in the formation of the *O*-acylurea intermediate (Figure 1). Alternatively, the increased reactivity may simply reflect the

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[1] HbA, normal human hemoglobin; HbS, sickle cell hemoglobin; EDC, 1-ethyl-3-[3′-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; sulfo-NHS, *N*-hydroxysulfosuccinimide; GA, galactosamine; MES, 4-morpholineethanesulfonic acid; Di-GEE-HbA, disubstituted derivative (glycine ethyl ester as nucleophile) of HbA; Tetra-GEE-HbA, tetra-substituted derivative (glycine ethyl ester as nucleophile) of HbA; RPHPLC, reverse-phase high-performance liquid chromatography; TMV, tobacco mosaic virus.

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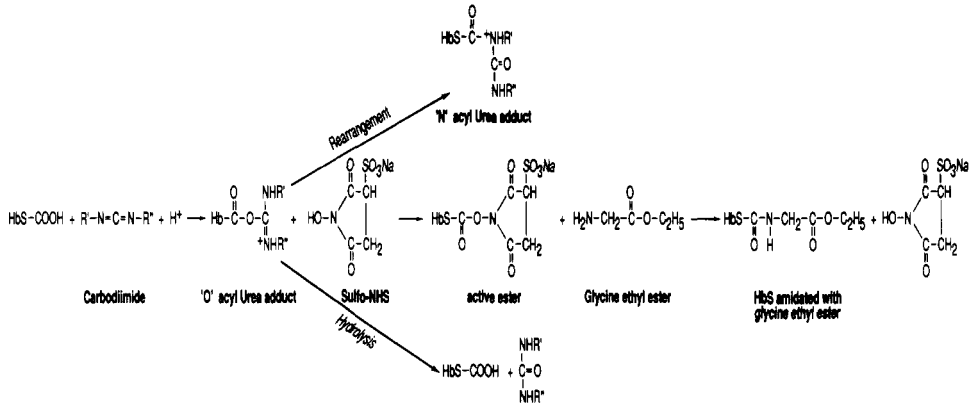

FIGURE 1: Chemistry of the amidation of HbA in the presence of sulfo-NHS. The reaction of the protein carboxyl group in the presence of EDC and GEE, as well as the role of sulfo-NHS in rescuing the activated carboxyl group (*O*-acylurea), is schematically represented. The trapping of the activated carboxyl group as the sulfosuccinimidyl ester inhibits the formation of the *N*-acylurea adduct. The latter has been a side reaction in the carbodimide-activated coupling of carboxyl groups of proteins with the nucleophiles. The sulfosuccinimidyl ester has a higher stability compared to that of the *O*-acylurea adduct and is either channeled through the amidation pathway or hydrolyzed back to the free carboxyl group.

deoxygenation-mediated changes of the microenvironment of Glu-43(β) which lead to an increased accessibility of the carboxyl group to the reagents. If the observed increase in the chemical reactivity of the carboxyl group is indeed a consequence of an increased pK_a, this would qualify Glu-43(β) as an alkaline Bohr group. Thus, it became important to resolve the differences between these two molecular concepts. The results of the detailed investigations that demonstrate Glu-43(β) is an alkaline Bohr group of HbA are presented in this paper.

MATERIAL AND METHODS

HbA and HbS were prepared by chromatography of erythrocyte lysates on a column (2 × 30 cm) of DE-52 cellulose equilibrated with 0.05 M Tris–acetate buffer, pH 8.5. A linear gradient generated by using 1 L each of 0.05 M Tris–acetate buffer, pH 8.3, and 0.05 M Tris–acetate buffer, pH 7.0, was used to elute the protein from the DE-52 column. Stripped hemoglobins were prepared as described previously (Rao & Acharya, 1991).

Amidation of HbS and HbA. Amidation of Glu-43(β) of HbA and HbS was carried out by the sulfo-NHS–EDC coupling procedure, as described previously (Rao & Acharya, 1991). Kinetics of amidation reactions of HbA/HbS (0.5 mM) (oxy and deoxy) with different nucleophiles (100 mM) in the presence and absence of chloride (100 mM), EDC (10 mM), and sulfo-NHS (2 mM) at different pH's were carried out in 20 mM MES buffer for the required length of time. Proteins were extensively dialyzed with MES buffer of the required pH before use. Kinetics of amidation reactions in the deoxy conformation of protein were carried out as described previously (Rao & Acharya, 1991).

Purification of Amidated HbA. The amidated HbA was chromatographed on a (carboxymethyl)cellulose (CM-52) column (2 × 30 cm) equilibrated to pH 6.0 with 10 mM potassium phosphate buffer containing 1 mM EDTA. The protein was eluted using a linear gradient generated by using 1 L each of 10 mM potassium phosphate buffer, pH 6.0, and 50 mM potassium phosphate buffer, pH 8.5, as the initial and final buffers, respectively. Both buffers contained 1 mM EDTA. Amidated samples of HbA, eluting after HbA from the CM-cellulose column, were isolated. The major amidated HbA was further purified by rechromatography on a (0.9 × 30 cm) CM-cellulose column using a similar gradient, except that the gradient was generated using 250 mL each of initial and final buffers.

Separation of α- and β-Globins of Di-GEE-HbA and HPLC Mapping of Tryptic Peptides. α- and β-globins of HbA and Di-GEE-HbA were separated by RPHPLC on a semipreparative Vydac C4 column (10 mm × 250 mm). The original procedure (Shelton et al., 1984) was modified slightly to get a better resolution of α- and β-globins. Globins were eluted from the RPHPLC column using a linear gradient (over 100 min) of 35–50% acetonitrile, each containing 0.1% trifluoroacetic acid at a flow rate of 120 mL/h. Purified α- and β-globins were digested with TPCK trypsin in 50 mM ammonium bicarbonate, pH 8.0 and 37 °C. An enzyme to protein ratio of 1:100 was used in all the digestions, and a period of 6 h was generally employed. The tryptic digests were analyzed by RPHPLC on an analytical Aquapore RP-300 (4.6 × 250 mm) column using a linear gradient (over 160 min) of 5–50% acetonitrile, each containing 0.1% trifluoroacetic acid. Fractions containing the modified peptide i.e., radioactive material, were pooled and isolated by lyophilization. Further characterization of the derivatized peptide involved the second RPHPLC at pH 6.0 (ammonium acetate–acetonitrile system); amino acid analysis and sequence analysis of the peptide was carried as described previously (Rao & Acharya, 1991).

Proton Titrations of the Oxy–Deoxy Transition of HbA and Di-GEE-HbA. The uptake or the release of proton as a consequence of conformational isomerism of HbA or amidated HbA was estimated using a Radiometer pH Stat at room temperature (23 ± 2 °C). Purified oxy-HbA and oxy-Di-GEE-HbA were dialyzed with 0.1 M KCl before the proton titration studies. The protein concentration was 0.1 mM. The total change in protein concentration during these sets of measurements was less than 1% of the original protein. The total region of pH titration (from pH 5 to 8) was divided into four intervals, and each interval was covered at least twice (Antonini et al., 1963). The forward and backward titrations agreed within a 5% difference in terms of the amount of acid or alkali consumed.

Oxygen Equilibrium Measurements. Oxygen equilibrium measurements of HbA and Di-GEE-HbA were recorded using an Amicon Hem-O-Scan, at pH 7.0 and 37 °C in 50 mM Bis-Tris–acetate buffer as described by Benesch et al. (1978).

RESULTS

Reactivity of Glu-43(β) of HbA in the Oxy and Deoxy Conformations. Glu-43(β) of HbS demonstrates a high

Table I: Influence of Chloride and β_6 Mutation on the Reactivity of Glu-43(β) of HbA

protein	rate of amidation[a] ($\times 10^{-2}$ min^{-1}) oxy	deoxy	relative increase in chemical reactivity, deoxy/oxy
HbA (–KCl)	1.12	2.55	2.27
HbA (+KCl)[b]	1.20	2.94	2.45
HbS (–KCl)	1.20	2.37	1.97

[a] Amidation of HbA and HbS (0.5 mM) was carried out in 20 mM MES buffer, pH 7.0, at room temperature using 10 mM EDC and 100 mM GEE in the presence of 2 mM sulfo-NHS. The pseudo-first-order rate constants are presented. [b] Amidation of HbA was carried out in the presence of 100 mM KCl in addition to the reagents and buffer discussed above.

Table II: Influence of the Nucleophile on the Amidation[a] of Glu-43(β) of HbS

nucleophile	rate of amidation ($\times 10^{-2}$ min^{-1}) oxy	deoxy	relative increase in chemical reactivity, deoxy/oxy
glycine ethyl ester	1.20	2.37	1.97
galactosamine	0.82	1.60	1.95

[a] Amidation of HbS (0.5 mM) with GEE or GA was carried out at pH 7.0 (20 mM MES buffer, 23 °C) using 2 mM sulfo-NHS, 10 mM EDC, and 100 mM nucleophile. The pseudo-first-order rate constants for the amidation of oxy- and deoxy-HbS with two nucleophiles (GEE or GA) are given.

selectivity for amidation. The chemical reactivity of HbS increases by nearly 2-fold upon deoxygenation (Rao & Acharya, 1991). The amidation of HbA has been now carried out at pH 7.0 in the oxy and deoxy conformations of the protein to establish whether the conformational transition mediated increase in the chemical reactivity of Glu-43(β) is unique to HbS or whether it is a general property of hemoglobins. The kinetics of amidation of oxy- and deoxy-HbA (5×10^{-4} M) at pH 7.0 are comparable to that of amidation of oxy- and deoxy-HbS, respectively. Therefore, it is clear that the deoxygenation of Hb induces a higher chemical reactivity of the γ-carboxyl group of Glu-43(β) (Table I). Thus, the increase in the reactivity of Glu-43(β) in the deoxy conformation of hemoglobin does not appear to be a consequence of the mutation at the β^6 site, i.e., mutation of Glu-6(β) to Val; it appears to be a general property of the basic structural organization of the hemoglobin molecule. Nonetheless, it should be noted here that the relative increase in the chemical reactivity of Glu-43(β) of HbA on deoxygenation is somewhat higher than that of HbS. Further studies will be needed to establish whether this reflects a long-range influence of mutation at the β^6 site on the conformational aspects of the $\alpha_1\beta_2$ interface in the deoxy conformation.

Deoxygenation-Mediated Increase in the Chemical Reactivity of Glu-43(β) of HbS Independent of the Chemical Nature of the Nucleophile. The increase in the chemical reactivity of Glu-43(β) in the deoxy conformation of HbA and HbS could be simply a reflection of the differential affinity of the reagent GEE to the microenvironment of Glu-43(β) in the two conformational states of the protein. Therefore, the kinetics of amidation reactions of oxy- and deoxy-HbS were carried out with another nucleophile, galactosamine, at pH 7.0 and amidation rates were calculated (Table II). The chemical nature of GEE is distinct as compared to that of GA. Consistent with this, the overall rate of amidation of Glu-43(β) by GA is slower compared to that with GEE as the nucleophile. Nonetheless, the chemical reactivity of Glu-43(β) for amidation with GA increased considerably upon deoxygenation of the protein, in much the same way as seen with GEE. A much more significant observation is that the deoxygenation-mediated relative increase in the chemical reactivity of Glu-43(β) is also nearly the same as that seen with GEE (Table II). This observation suggests that the increase in the chemical reactivity of Glu-43(β) in the deoxy conformation of Hb is a reflection of a change in the pK_a of the γ-carboxyl group of Glu-43(β) rather than the change in the affinity of the microenvironment of this glutamic acid residue toward the nucleophile.

Table III: Influence of pH on the Rate of Amidation of Glu-43(β) of HbS

pH	rate of amidation[a] ($\times 10^{-2}$ min^{-1}) oxy	deoxy	relative increase in chemical reactivity,[b] deoxy/oxy
5.5	6.99	6.99	1.00
6.0	4.48	4.62	1.03
6.5	2.19	3.40	1.55
7.0	1.20	2.37	1.97
7.5	0.63	1.08	1.71

[a] Amidation of HbS (0.5 mM) was carried out at different pH values as described in Table II. The pseudo-first-order rate constants are presented. [b] Represents the increase in the rate of amidation on deoxygenation as compared to that of the oxy form.

Chemical Reactivity of Glu-43(β) of HbA in the Presence of Chloride. The chloride ion binds to deoxy-Hb and modulates its O_2 affinity. Therefore, it is conceivable that the chemical reactivity of Glu-43(β) is influenced by chloride ions. The amidation of HbA was carried out in the presence of 0.1 M KCl and in both conformational states of the protein (Table I). The influence of chloride ion on the chemical reactivity of Glu-43(β) in the oxy conformation of HbA is very limited. The result suggests that the binding of the chloride ion to the deoxy-HbA has very little influence on the conformation of the $\alpha_1\beta_2$ interface as reflected by the chemical reactivity of Glu-43(β).

Chemical Reactivity of Glu-43(β) of Deoxy-HbS as a Function of pH. The increase in the reactivity of Glu-43(β) of HbS upon deoxygenation appears to be independent of the chemical nature of the nucleophile. Therefore, it may be argued that the increased chemical reactivity of Glu-43(β) in the deoxy conformation of the protein is a consequence of the increase in the pK_a of Glu-43(β). If the observed increase in reactivity is indeed a reflection of changes in pK_a, the relative increase in chemical reactivity of Glu-43(β) will vary as a function of pH. Accordingly, the kinetics of amidation reactions of Glu-43(β) of HbS in the deoxy conformation have been studied as a function of pH (Table III). Above pH 6.0, the deoxy protein exhibited a pH-dependent increase in the chemical reactivity, whereas at pH's 6.0 and 5.5, the chemical reactivity of Glu-43(β) of deoxy-HbS was nearly the same as that in the oxy conformation. The relative increase in the chemical reactivity of Glu-43(β) upon deoxygenation of HbS as a function of pH has been calculated and presented in Table III. The maximum increase in the chemical reactivity occurred at pH 7.0. This suggests that the pK_a of the γ-carboxyl group of Glu-43(β) is around 7.0 in the deoxy conformation of Hb. The increase in the apparent pK_a of the γ-carboxyl of Glu-43(β) from 6.35 to 7.0 as a consequence of the R $\rightarrow$ T conformational transition of Hb qualifies Glu-43(β) as an alkaline Bohr group. The independent confirmation of this identification could be made by a study of the oxy–deoxy transition-dependent proton titration of the amidated derivative of HbA with modification on both Glu-43(β) and comparison of it with that of HbA (Kilmartin et al., 1980).

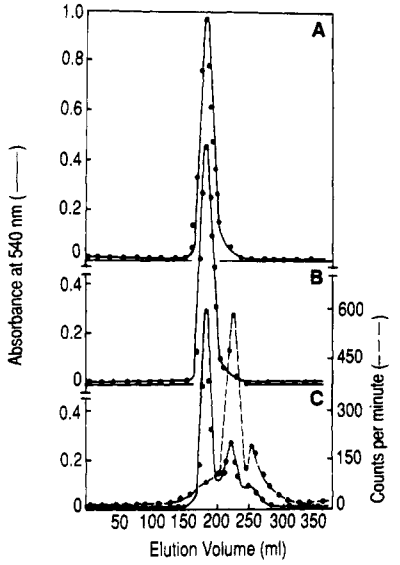

FIGURE 2: Specificity of the amidation of HbA with nucleophile in the presence of sulfo-NHS. The figure illustrates the CM-52 cellulose chromatographic behavior of control HbA (A), HbA treated with EDC and sulfo-NHS (B), and HbA reacted with EDC, sulfo-NHS, and GEE (C). HbA (control sample without any reagents) and HbA treated with EDC (10 mM), sulfo-NHS (2 mM), and GEE (100 mM) incubated at pH 6.0 for 15 min at room temperature were desalted on a Sephadex G-25 column equilibrated and eluted with 10 mM phosphate buffer, pH 6.0, to get rid of the excess reagents. The protein sample from the Sephadex column was dialyzed extensively against 10 mM potassium phosphate buffer. This sample was then chromatographed on a CM-52 column (0.9 × 30 cm) preequilibrated to pH 6.0 with 10 mM phosphate buffer containing 1 mM EDTA. In each case, a 20-mg load of protein was placed onto the column. The chromatogram was developed with a linear gradient of 10 mM phosphate buffer, pH 6.0, to 50 mM phosphate buffer, pH 8.5 (250 mL of each). Both buffers contained 1 mM EDTA. Elution of the protein from the column was followed by measurement of the absorbance of the fractions at 540 nm. A 100-μL aliquot of each fraction was used to measure ^{14}[C]GEE incorporated into each of the component.

Chromatography of Amidated HbA on CM-Cellulose. In order to prepare the homogeneous derivative of HbA with amidation only at Glu-43(β), the chromatographic conditions had to be established. However, this purification is expected to be simple since the amidation of the carboxylate results in the loss of two negative charges of HbA. CM-cellulose chromatography is an appropriate system for the purification of the amidated sample since amidated HbA is more basic than the control HbA and thus is expected to have a stronger interaction with the resin than that of the parent protein. The amidation reaction involves three reagents: EDC, sulfo-NHS, and GEE. Therefore, it is also equally important to establish that one or more combinations of these reagents do not generate modified forms of HbA that could coelute with the desired amidated product. Accordingly, control experiments have been carried out to establish that the reaction is indeed channeled through the amidation pathway as depicted in Figure 1 and leads to the generation of only the expected products. The results of these experiments are presented in Figure 2. This figure compares the chromatographic behavior of a control sample of HbA with those that were incubated either with only sulfo-NHS (2 mM) and carbodiimide (10 mM) or with all three reagents [sulfo-NHS (2 mM), carbodiimide (10 mM), and GEE (100 mM)] at pH 6.0 and 23 °C for 15 min. The sample that contained the two reagents (sulfo-NHS and carbodiimide) without the nucleophile chromatographed exactly the same way as the control sample. Thus, it is clear that no stable covalent adducts are formed in the absence of the nucleophile. This conclusion has been further confirmed by peptide mapping of this sample. On the other hand, the HbA sample that contained the nucleophile, besides sulfo-NHS and carbodiimide, generated two chromatographically distinct components that eluted after HbA (i.e., more basic than HbA). These two new components accounted for nearly 35% of the protein loaded on the column. Both these new components were radioactive. The major radioactive component that eluted just after HbA contained 2 mol of nucleophiles/mol of tetramer and accounted for nearly 28–30% of the protein loaded on the column (Figure 2C). A sample of HbA incubated with GEE alone did not generate any of these components, and the incubated sample is indistinguishable from the control HbA by CM-cellulose chromatography (data not shown). This establishes that the generation of the derivative(s) of Hb is specific to the presence of the nucleophile besides the activating and the rescuing agents.

From the amount of the radioactivity (^{14}C) GEE incorporated into the major radioactive component, it is clear that this is a disubstituted derivative of HbA. Accordingly, this component was designated as Di-GEE-HbA. The minor radioactive component eluting after the disubstituted derivative from the CM-cellulose column has been identified as a tetrasubstituted derivative of HbA, based on the amount of radioactivity incorporated into this sample.

Chemical Characterization of Di-GEE-HbA. The major radioactive component (Di-GEE-HbA) was isolated and subjected to a second purification on CM-cellulose. The repurified Di-GEE-HbA was subjected to detailed chemical characterization to establish the homogeneity of the derivative. The RPHPLC of the acid acetone precipitated globin of Di-GEE-HbA on a Vydac C4 column revealed that all the radioactivity is associated with β-globin. The tryptic peptide mapping of this modified β-globin and isolation of the modified tryptic peptide followed by its amino acid sequencing (data not shown) has established that the amidation of Di-GEE-HbA is on Glu-43(β). Thus, these chromatographic results and the detailed structural analysis have established a simple procedure for the preparation of a homogeneous derivative of HbA in which only the γ-carboxyls of the two Glu-43(β) have been modified.

Accordingly, for large-scale preparation of HbA with amidation of its Glu-43(β), the same reaction conditions and the chromatographic procedures were used for amidation as well as for the purification of the modified product except that cold GEE was used instead of radioactive (^{14}C) GEE. However, as described under Materials and Methods, preparative CM-52 columns were used in view of the larger protein load.

Oxygen Affinity of Di-GEE-HbA. The oxygen affinity of HbA and Di-GEE-HbA was determined at pH 7.0 and 37 °C, in 50 mM Bis-Tris buffer. The P_{50} of HbA was decreased from 10.3 to 8.3 on amidation of Glu-43(β) (Figure 3). However, this derivatization of HbA has very little influence on cooperativity in the binding of oxygen. The Hill coefficient of Di-GEE-HbA was nearly the same as that of the control HbA (Figure 3 inset). The n value was 2.7 for control HbA and 2.55 for Di-GEE-HbA. Thus, the amidation appears to have very little influence on the overall quaternary structure of HbA.

Proton Titrations of Oxy–Deoxy Transition of HbA and Di-GEE-HbA. The contribution of Bohr protons by a particular ionizable group could be estimated by direct titration of protons released or taken up during the oxy to deoxy

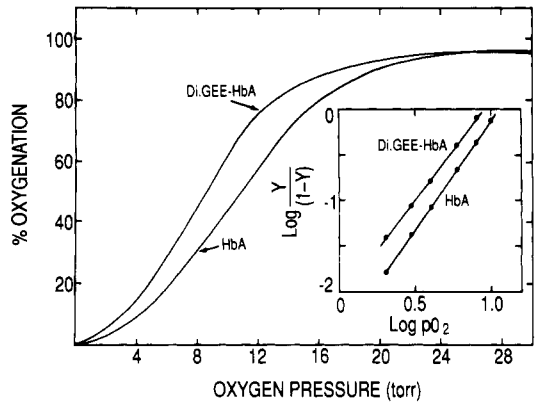

FIGURE 3: Oxygen affinity of HbA and Di-GEE-HbA. The oxygen equilibrium curves were recorded in 50 mM Bis-Tris acetate at pH 7.0 and 37 °C with an Amicon Hem-O-Scan. Inset shows the Hill plots for HbA and Di-GEE-HbA.

conformational transition of the derivative of HbA, in which the ionization of the implicated Bohr group is blocked by the chemical modification or mutation, and comparison of this information with that of the wild type (the parent protein) (Kilmartin et al., 1969, 1973; 1977, 1980; Perutz et al., 1969, 1980, 1984). When such a modified/mutant Hb is available, it is important that it retains the normal quaternary structural aspects of the parent protein. This certainly is the case with Di-GEE-HbA. Proton titration of HbA and Di-GEE-HbA has been carried out to establish the contribution of Glu-43-(β) to the alkaline Bohr effect of HbA. The protons released or taken up as a consequence of the oxy–deoxy transitions of HbA and Di-GEE-HbA have been estimated as a function of pH (Figure 4). It is evident from the titration data that Di-GEE-HbA consumes less acid in the pH region of 6.20–7.80 compared to the parent HbA. However, below pH 6.20, there was no significant difference in consumption of acid or base for the oxy–deoxy transition. The contribution of Glu-43(β) to the alkaline Bohr effect of HbA is maximum at pH 7.0 (Figure 4B) and is about 30%.

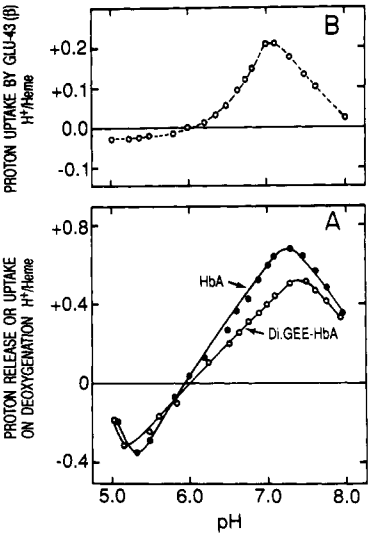

FIGURE 4: Proton titration of the oxy–deoxy conformational transition of HbA and Di-GEE-HbA as a function of pH. Proton release or uptake on deoxygenation is plotted against pH. The uptake or release of protons as a consequence of the conformational transition was estimated by titrating the protein with HCl or KOH using a Radiometer (Copenhagen) pH Stat at room temperature (23 ± 2 °C) containing 0.1 mM protein. Proteins were dialyzed with 100 mM chloride before titration studies. To measure the difference in proton bound between oxy- and deoxyhemoglobin at any given pH, back-titrations were carried out using the pH Stat, which gives the amount of acid or base required to restore the original pH of the deoxygenated protein. (A) Proton uptake or release for HbA and Di-GEE-HbA presented as moles of proton per mole of heme. (B) Proton uptake by Glu-43(β) alone. The data are presented as moles of proton per mole of heme. Proton uptake by Glu-43(β) is calculated from the difference in proton titration of control HbA and Di-GEE-HbA and is plotted against pH.

DISCUSSION

The differential affinity of protons to the two conformational states of HbA is the fundamental molecular basis of the Bohr effect. It has been generally believed that, under physiological conditions, the potential alkaline Bohr groups include the imidazole group of the histidine residues and the α-amino groups of HbA. Consistent with this, His-146(β) and Val-1(α) have been shown to be the major contributors to the alkaline Bohr effect of HbA (Kilmartin et al., 1973, 1980). The results of the present study establish that the apparent pK_a of the γ-carboxyl of Glu-43(β) of HbA is 6.35 in the oxy conformation and it increases to 7.0 in the deoxy conformation. This difference in pK_a qualifies this γ-carboxyl group of Hb to be an alkaline Bohr group. The pH dependence of the deoxygenation-mediated increase in the chemical reactivity Glu-43(β) coupled with the demonstration that the amidation of Glu-43(β) decreases the number of Bohr protons released in the pH region of 6.0–7.5 establishes Glu-43(β) as an alkaline Bohr carboxyl group.

The Val-1(α) and His-146(β) are considered as the major contributors to the alkaline Bohr effect of Hb in the presence of chloride ion. These two residues of HbA account for nearly 70% of the alkaline Bohr protons (Kilmartin, 1977). Thus, about 30% of the alkaline Bohr groups are still to be identified. It has been suggested that there may be no specific group(s) responsible for the unexplained 30% portion of the alkaline Bohr effect (Kilmartin, 1977). Perutz et al. (1980) have reported that, in addition to Val-1(α) [NA1], Lys-82(β) [EF6] is also responsible for the chloride-induced Bohr effect in the absence of organic phosphates, and its contribution corresponds to the part of the Bohr effect that has not been accounted for previously. The contribution of Lys-82(β) to the alkaline Bohr effect is supported by the observation that all abnormal hemoglobins in which this Lys is replaced by a neutral or anionic residue exhibit a reduced Bohr effect in the presence of 0.1 M KCl. However, it is conceivable that part or all of this contribution of Bohr protons may have come from changes in the ionic interactions at the DPG binding pocket. In recent years it has become clear that the contribution of protons by a given Bohr group is further modulated by the solvent conditions and the heterotrophic effectors of HbA (van Beek et al., 1979; Shih & Perutz, 1987; Ho & Russu, 1987; Busch & Ho, 1990; Russu et al., 1990; Busch et al., 1991). Reviewing all the earlier studies, Imai (1982) suggested that 25% of the Bohr proton contribution in the physiological pH region still remains unexplained. Recently, Busch et al. (1991) suggested that additional sites other than His-146(β) participate in the Bohr effect even in the absence of chloride. The identification of Glu-43(β) as the alkaline Bohr group is in line with such a suggestion. The demonstration that the contribution of Glu-43(β) to the Bohr effect at pH 7.0 accounts for nearly 30% is consistent with the amount of the unexplained portion (25%) of the alkaline Bohr effect (Imai, 1982).

The molecular explanations for the quantification of a particular ionizable group as a Bohr group have been the subject of intensive investigations, as well as of considerable controversy. The formation/breakage of the salt bridges as a consequence of the oxy-deoxy transition of HbA, thereby influencing the ionization behavior of the implicated Bohr

group, has been advanced as an explanation (Perutz, 1970a,b, 1990). The contribution of a Bohr group to the observable macroscopic Bohr effect appears to be influenced by the interactions of heterotrophic effectors with HbA. This concept appears to be now generally accepted (Shih & Perutz, 1987; Ho & Russu, 1987; Busch & Ho, 1990; Russu et al., 1990; Busch et al., 1991). However, the salt bridge interaction of Glu-43(β) does not appear to be the molecular explanation for its contribution as an alkaline Bohr group of Hb. The participation of the carboxyl group in a salt bridge is expected to decrease its pK_a. Glu-43(β) is the most basic carboxyl group of HbA. The accessibility of this residue at neutral pH for derivatization by a variety of nucleophiles is not consistent with a salt bridge concept. The deoxygenation of HbA increases its pK_a. The generation of the salt bridge upon deoxygenation is not consistent with this observation since the salt bridge formation is expected to decrease the chemical reactivity. The pK_a of the carboxyl group is generally increased when it is present in a hydrophobic environment. Therefore, the increase in the pK_a of Glu-43(β) upon deoxygenation of Hb apparently reflects an increase in the hydrophobicity of its microenvironment.

Dependence of the pK_a of the side-chain carboxyl group of Glu and/or Asp residues of proteins on the hydrophobicity of their microenvironment has been invoked previously (Belasco et al., 1978; Lolis & Petsko, 1990). An increase in the pK_a of the carboxyl groups of Glu-95 and Glu-106 has been suggested to occur upon the polymerization of TMV protein (Casper, 1963; Shalaby et al., 1977; Namba & Stubbs, 1986). An elevated pK_a for the β-carboxyl of Asp of bacteriorhodopsin has been demonstrated (Subramaniam et al., 1990; Otto et al., 1990). High pK_a's for side-chain carboxyl groups have been implicated for lysozyme (Kenneth, 1991; Parsons & Raffery, 1972) and triosephosphate isomerase (Belasco et al., 1978). A desolvation of the microenvironment of these carboxyl groups has been hypothesized for this increase in the pK_a of these carboxylates. Tanford et al. (1959) suggested that β-lactoglobulin contains two carboxyl groups with a pK_a of about 7.5, and this observation was confirmed by Susi et al. (1959) using infrared spectroscopy.

Glu-43(β) is located at the $\alpha_1\beta_2$ interface of HbA. Presumably, upon deoxygenation of Hb, a desolvation occurs in the microenvironment of Glu-43(β). This facilitates the protonation of the carboxylate, i.e., an increase in its pK_a. However, this process of desolvation does not appear to "encage" the γ-carboxyl group, since this residue is still accessible for the nucleophile and the carbodiimide. It should also be added here that, in terms of the primary structure, Glu-43(β) is sandwiched between Phe residues that form a part of the heme binding pocket of HbA (Perutz et al., 1968). The oxygenation–deoxygenation of heme apparently influences the noncovalent, intramolecular interactions of the amino acid residues of this region either between themselves, with heme, or with both, and the changes that are the consequence of deoxygenation are reflected as an increase in the pK_a of Glu-43(β).

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REFERENCES

Acharya, A. S., & Seetharam, R. (1985) *Biochemistry 24*, 4885–4890.

Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C., & Rossi-Fanelli, A. (1963) *J. Biol. Chem. 238*, 2950–2957.

Belasco, J. G., Herlihy, J. M., & Knowles, J. R. (1978) *Biochemistry 17*, 2971–2978.

Benesch, R. E., Edalji, R., Kwong, S., & Benesch, R. (1978) *Anal. Biochem. 89*, 162–173.

Busch, M. R., & Ho, C. (1990) *Biophys. Chem. 37*, 313–322.

Busch, M. R., Mace, J. E., Ho, N. T., & Ho, C. (1991) *Biochemistry 30*, 1865–1877.

Casper, D. L. D. (1963) *Adv. Protein Chem. 18*, 37–121.

Ho, C., & Russu, I. M. (1987) *Biochemistry 26*, 6299–6305.

Imai, K. (1982) in *Allosteric Effects in Haemoglobin*, pp 33–36, Cambridge University Press, Cambridge, England.

Kenneth, M. M., Jr. (1991) *J. Am. Chem. Soc. 113*, 3572–3575.

Kilmartin, J. V. (1977) *Trends Biochem. Sci. 2*, 247–249.

Kilmartin, J. V., & Rossi-Bernardi, L. (1969) *Nature 222*, 1243–1246.

Kilmartin, J. V., & Rossi-Bernardi, L. (1973) *Physol. Rev. 53*, 836–890.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249.

Kilmartin, J. V., Fogg, J. H., & Perutz, M. F. (1980) *Biochemistry 19*, 3189–3193.

Lolis, E., & Petsko, A. G. (1990) *Annu. Rev. Biochem. 59*, 616–617.

Namba, K., & Stubbs, G. (1986) *Science 231*, 1401–1406.

Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018–1022.

Padlan, E. A., & Love, W. E. (1985) *J. Biol. Chem. 260*, 8280–8291.

Parsons, S. M., & Raffery, M. A. (1972) *Biochemistry 11*, 1623–1629.

Perutz, M. F. (1970a) *Nature 228*, 726–734.

Perutz, M. F. (1970b) *Nature 228*, 734–739.

Perutz, M. F. (1990) in *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*, pp 7–27, Cambridge University Press, Cambridge, England.

Perutz, M. F., Muirhead, H., Cox, J. M., & Goaman, L. C. G. (1968) *Nature 219*, 131–139.

Perutz, M. F., Muirhead, H., Mazzerella, L., Crowther, R. A., Greer, J., & Kilmartin, J. V. (1969) *Nature 222*, 1240–1243.

Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., & Butler, P. J. G. (1980) *J. Mol. Biol. 138*, 649–670.

Perutz, M. F., Fermi, G., & Shih, T.-b. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4781–4784.

Rao, M. J., & Acharya, A. S. (1991) *J. Protein Chem. 10*, 129–138.

Russu, I. M., Wu, S. S., Bupp, K. A., Ho, N. T., & Ho, C. (1990) *Biochemistry 29*, 3785–3792.

Seetharam, R., Manning, J. M., & Acharya, A. S. (1983) *J. Biol. Chem. 258*, 14810–14815.

Shalaby Ragaa, A. F., & Lauffer, M. A. (1977) *J. Mol. Biol. 116*, 709–725.

Shelton, J. B., Shelton, J. R., & Schroeder, W. A. (1984) *J. Liq. Chromatogr. 7*, 1969–1977.

Shih, D. T.-b., & Perutz, M. F. (1987) *J. Mol. Biol. 195*, 419–422.

Staros, J. V., Wright, R. W., & Swingle, D. M. (1986) *Anal. Biochem. 156*, 220–222.

Subramaniam, S., Marti, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1013–1017.

Susi, H., Zell, T., & Timasheff, S. N. (1959) *Arch. Biochem. Biophys. 85*, 437–443.

Tanford, C., Bunville, L. G., & Nozaki, Y. (1959) *J. Am. Chem. Soc. 81*, 4032–4036.

Van Beek, G. G. M., Zuiderweg, Erik, R. P., & De Bruin, S. H. (1979) *Eur. J. Biochem. 99*, 379–383.

Wishner, B. C., Ward, K. B., Lattman, E. E., & Love, W. E. (1975) *J. Mol. Biol. 98*, 179–194.

Registry No. HbA, 9034-51-9; HbS, 9035-22-7; Cl^-, 16887-00-6; glycine ethyl ester, 459-73-4; galactosamine, 7535-00-4.