Effect of Amidination of Lysyl Residues on the Oxygen Affinity of Human Hemoglobin

SPECIFICITY OF METHYL ACETIMIDATE FOR LYSINE C5(40)α*

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SUMMARY

Treatment of human oxyhemoglobin with methylacetimidate results in selective amidination of the ε-amino group of lysine C5(40)α. The modified hemoglobin exhibits increased oxygen affinity, high cooperativity, and normal Bohr effect. Hybrid molecules containing amidinated β chains and normal α chains have normal ligand-binding properties, whereas hybrid molecules containing amidinated α chains have ligand-binding properties identical with fully amidinated hemoglobin. Amidination of deoxyhemoglobin produces only minimal changes in ligand-binding properties. We propose that amidination of lysine C5(40)α prevents its participation in the salt bond with histidine HC3(146)β in deoxyhemoglobin, thus shifting the allosteric equilibrium in favor of the high affinity oxy conformation.

The cooperative binding of oxygen by hemoglobin is proposed to be a result of the existence of two different conformational states of the tetrameric molecule (1). These two states represent the two distinct quaternary structures of crystalline deoxyhemoglobin and oxyhemoglobin, as determined by Perutz (2). The low affinity deoxy (T) form and the high affinity oxy (R) form are in equilibrium in solution, the position of equilibrium being determined by the degree of ligation and by other factors. The deoxy (T) configuration is proposed to be stabilized by a number of salt bridges which are not present in the oxy (R) configuration. These salt bridges exist between the carboxyl group of histidine HC3(146)β and the ε-amino group of lysine C5(40)α; between the imidazole group of histidine HC3(146)β and the β-carboxyl group of aspartate FG1(94)β of the same chain; between the carboxyl group of arginine HC3(141)α and the β-carboxyl group of aspartate H9(126)α of the opposite chain (2). A modification of the hemoglobin structure which specifically interferes with the formation of these salt bonds is expected to result in a lower stability of the deoxy (T) conformation, and a shift in the position of the equilibrium toward the oxy (R) conformation. This shift could be observed as an increase in the oxygen affinity of the modified hemoglobin.

In this investigation we have utilized chemical modification to elucidate the role of the amino groups of human hemoglobin in the binding of oxygen. We have selected methyl acetimidate as the reagent of choice for two reasons. The conditions for extensive modification are rather gentle, pH 9 at room temperature for 15 to 60 min; and the modification does not alter the net charge of the protein at neutral pH, the acetamidino group having a pK above 12 (3). In addition, a number of proteins have been subjected to extensive amidination with few if any accompanying changes in physical or biological properties (4–9).

A preliminary account of this work has been presented (10).

METHODS

Synthesis of Methyl [8-14C]Acetimidate—Methyl acetimidate was synthesized from [2-14C]acetoniitrile (ICN) and methanolic HCl by the procedure of Hunter and Ludwig (3). The specific radioactivity of the product was determined directly by measurement of the radioactivity per unit dry weight, and indirectly by formation of [14C]acetamidinovaline.

Preparation of Hemoglobin—Erythrocytes were collected from the junior author in lithium oxalate-containing tubes, immediately centrifuged, and washed three times with 0.14 M NaCl + 0.02 M sodium phosphate, pH 7.4. Hemolysis was achieved by addition of 10 volumes of distilled water, the hemolysate was stirred for 30 min at 0°C, and the red cell stromata were removed by centrifugation. The hemolysate was concentrated 4-fold by ultrafiltration and dialyzed against 0.01 M sodium phosphate, pH 6.8. Hemoglobin A was purified by chromatography on CM-Sephadex C-50 (11), and was concentrated to 60 mg/ml by ultrafiltration. Hemoglobin concentration and ferrihemoglobin content were determined spectrophotometrically (12). Preparation of globin was achieved by the acid-acetone precipitation method of Winterhalter and Huchu (11). Native and amidinated hemoglobin were dissociated into α and β chains by treatment with excess p-hydroxymercuribenzoate (13) followed by chromatography on DEAE-Sephadex A-50 (14). Purity of the separated chains was ascertained by disc gel electrophoresis at pH 8.9 and by amino acid analysis. Sulphydryl groups of the α chains were regenerated on a column of CM-Sephadex.

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C-50 by treatment with 0.3 mM 2-mercaptoethanol in 0.01 M sodium phosphate, pH 6.7 (14). Sulphydryl groups of the globin chains were regenerated on a column of DEAE-Sephadex A-50 by treatment with 0.03 M 2-mercaptoethanol in 0.1 M Tri-HCl, pH 8. Hybrid hemoglobins were formed by mixing stoichiometric amounts of the appropriate α and β chains at 0°C for 1 hour. The samples were then dialyzed against 0.01 M sodium phosphate, pH 6.8, and purified by chromatography on CM-Sephadex (11).

Amidination of Hemoglobins—A stock solution of methyl [2-14C]-acetimidate (0.027 mCi/mmol) in 0.1 M Tri-HCl, pH 9, was prepared and neutralized with 1 eq of NaOH immediately before use. This solution was diluted to the desired concentration with 0.04 M NaCl in 0.1 M Tri-HCl, pH 9, and was added to purified hemoglobin which had been dialyzed against 0.01 M Tri-HCl, pH 9. The hemoglobin concentration in the reaction mixture was 0.07 m. After 15 min at room temperature the reaction was terminated by the addition of 0.2 mmol of NaHPO₄ per ml of sample. The amidinated sample was dialyzed against 0.01 M sodium phosphate, pH 6.0, containing 0.1 M NaCl and 1 m EDTA.

Determination of Number of Residues Amidinated—The radioactivity of samples of amidinated hemoglobin or methyl hemoglobin peptides was measured in a Beckman LS200B liquid scintillation spectrometer using a scintillation fluid of 0.45% 2,5-diphenyloxazole in toluene-Triton X-100 (2:1). The aqueous sample plus water made up 10% of the total volume. Counting efficiency was determined using an internal standard of [14C]toluene. The number of residues amidinated was calculated by dividing the radioactivity incorporated per mmol of protein or peptide by the specific radioactivity of the methyl [14C]acetimidate. The number of lysyl ε-amino residues amidinated was calculated by subtracting the number of amidinated NH₂-terminal residues from the total number of amidinated residues.

The number of NH₂-terminal valyl ε-amino groups amidinated was determined indirectly by quantitating the nonamidinated amino groups of the intact heme protein with KNa[14CO₃] (15). Amidinated α- or β-globin chains were dissolved in 8 M urea containing 20% N-ethylmorpholine, pH 8, and 0.6 M KNa[14CO₃] (7.3 mCi/mmol). The protein concentration was 10 mg/ml. After 24 hours at 40°C unreacted cyanate was removed by exhaustive dialysis against 10⁻⁴ M HCl. The sample was lyophilized and dissolved in 6 ml HCl and a portion was hydrolyzed at 145°C for 4 hours (16) and the protein concentration was determined by amino acid analysis. The remainder of the sample was heated at 100°C for 1 hour to cyclize the carbamylated residues. The resulting hydantoins were eluted from Dowex 50 H⁺ with formic acid, and chromatographed on Sephadex G-50. Peptides were detected by their absorbance at 280 nm and by their radioactivity. The peptide fraction was identified as valine hydantoin by chromatography on CM-Sephadex (11). The number of amidinated NH₂-terminal residues from the total number of amidinated residues.

Cyanogen Bromide Cleavage of α-Globin Chains—The α chain was cleaved into three fragments by treatment with cyanogen bromide (18), after reduction of methionine sulfoxide residues with 25% 2-mercaptoethanol (19). After 20 hours of incubation with CNBr the protein solution was evaporated to dryness, redissolved in 75 m M formic acid, and chromatographed on Sephadex G-50. Peptides were detected by their absorbance at 280 nm and by their radioactivity. Fragments corresponding to residues 77 to 141 were detected by their absorbance at 280 nm and by their radioactivity. The α chain was separated into two fragments and the extent of amidination of the two fragments was determined by amino acid analysis. The fragment corresponding to residues 77 to 141 was purified by chromatography on CM-Sephadex (11).

Effect of Amidination on Oxygen Affinity—With increasing extent of amidination, the oxygen affinity equilibrium curves are shifted to the left, corresponding to an increase in oxygen affinity (Fig. 1). The p₅₀ values for the native, 29% amidinated, and 99% amidinated samples are 1.75, 1.04, and 0.47 mm Hg, respectively. The oxygen affinities of a number of hemoglobin samples amidinated to varying extents were measured at pH 6.0, 6.8, 7.4, and 9.0, and at pH 7.4 in the presence of 5 mM 2,3-diphosphoglycerate (Table II). An increase in oxygen affinity with increasing extent of amidination is seen at all pH values and in the presence of DPG. The relationship between p₅₀ and the extent of amidination of the ε-amino groups of the globin chains is shown in Fig. 2. An initial sharp decrease in p₅₀ is observed corresponding to amidination of up to 60% of the globin residues. At this point, only 8% of the globin residues have been amidinated. Further amidination of up to 100% of the ε-amino groups of the globin chains does not result in any further increase in oxygen affinity. Thus it is obvious that the effect on oxygen affinity is due to amidination of the ε-amino groups, and not to amidination of the globin chains. Further, since the maximal effect on oxygen affinity is observed after amidination of only 60% of the globin residues, it appears that certain NH₂-terminal residues of the globin chains are more critical for oxygen affinity than others. For example, the NH₂-terminal part of the globin chains might be more accessible to amidination than the more internal residues.

Measurement of Oxygen Affinity—Oxygen equilibrium measurements were made by the method of Imai et al. (23) with minor modifications. Hemoglobin (0.06 mM heme) was dissolved in 0.01 M Tris or bis-tris (2,2-bis(hydroxymethyl)-2,2',2'-nitroethanol) buffer and deoxygenated under a stream of nitrogen. Reoxygenation was measured spectrophotometrically at 576 nm using a Cary 14 spectrophotometer. The partial pressure of oxygen was measured continuously using a YSI 5331 polarographic oxygen sensor with a high sensitivity Teflon membrane. The sensor output was amplified by a YSI model 53 oxygen monitor. The ferrihemoglobin content of the samples did not exceed 5%.

TABLE I

| Sample  | MAP | Tetramer | α Chain* | β Chain* |
|---------|-----|---------|---------|---------|
|         | Total | Valyl | Lysyl | Valyl | Lysyl | Valyl | Lysyl |
| A       | 7.2  | 12    | 13     | 1      | 3     | 14    |
| B       | 6.7  | 6     | 4      | 27     | 5     | 29    |
| C       | 55   | 8     | 59     | 8      | 56    | 8     | 64    |
| D       | 33.3 | 68    | 10     | 73     | 9     | 69    | 10    | 75    |
| E       | 54   | 63    | 16     | 89     | 13    | 90    | 17    | 95    |
| F       | 108  | 05    | 28     | 101    | 26    | 04    | 92    | 203   |

* Hemoglobin was amidinated, then separated into chains.
* MAI, methyl acetimidate.
 Calculated from isolated α and β chains.

RESULTS

Extent of Amidination of Hemoglobin—The extent of amidination of the amino groups in hemoglobin is proportional to the concentration of methyl acetimidate employed. Hemoglobin (0.27 mM) was treated with different concentrations of methyl [14C]acetimidate from 2.7 to 108 mM. The amidinated samples were separated into α and β chains and the extent of amidination of lysyl and valyl residues was determined (Table I). The α and β chains are amidinated to essentially the same extent. The ε-amino groups of the globin residues are considerably more reactive than the NH₂-terminal residues.

Effect of Amidination on Oxygen Affinity—With increasing extent of amidination, the oxygen affinity equilibrium curves are shifted to the left, corresponding to an increase in oxygen affinity (Fig. 1). The p₅₀ values for the native, 29% amidinated, and 99% amidinated samples are 1.75, 1.04, and 0.47 mm Hg, respectively. The oxygen affinities of a number of hemoglobin samples amidinated to varying extents were measured at pH 6.0, 6.8, 7.4, and 9.0, and at pH 7.4 in the presence of 5 mM 2,3-diphosphoglycerate (Table II). An increase in oxygen affinity with increasing extent of amidination is seen at all pH values and in the presence of DPG. The relationship between p₅₀ and the extent of amidination of the ε-amino groups of the globin chains is shown in Fig. 2. An initial sharp decrease in p₅₀ is observed corresponding to amidination of up to 60% of the globin residues. At this point, only 8% of the globin residues have been amidinated. Further amidination of up to 100% of the ε-amino groups of the globin chains does not result in any further increase in oxygen affinity. Thus it is obvious that the effect on oxygen affinity is due to amidination of the ε-amino groups, and not to amidination of the globin chains. Furthermore, since the maximal effect on oxygen affinity is observed after amidination of only 60% of the globin residues, it appears that certain NH₂-terminal residues of the globin chains are more critical for oxygen affinity than others. For example, the NH₂-terminal part of the globin chains might be more accessible to amidination than the more internal residues.

The abbreviations used are: DPG, 2,3-diphosphoglycerate; Hb, hemoglobin.
if the reagent is somewhat selective toward certain lysyl residues which are functionally involved in oxygen binding.

**Effect of Amidination on Cooperativity**—The cooperativity of oxygen binding, as measured from the slope of the Hill plot at 50% ligand saturation, is only slightly decreased by amidination. The n values for native, 29%, and 99% amidinated hemoglobin (Fig. 1) are 2.7, 2.5, and 2.3, respectively. The n values for the samples presented in Table II are all above 2.0. Since the cooperativity of ligand binding is high, it is unlikely that extensive structural changes occur upon amidination.

**Effect of Amidination on Alkaline Bohr Effect and DPG Effect**—The p50 values of native and amidinated hemoglobin samples decrease as the pH is increased from 6.0 to 9.0 (Table II). The percentage of the Bohr effect which is maintained on amidinated samples is calculated from the following formula (24)

\[
\% \text{ Bohr effect} = \frac{(\log p_{50} \text{ @ pH}_X - \log p_{50} \text{ @ pH}_Y)}{(\log p_{50} \text{ @ pH}_X - \log p_{50} \text{ @ pH}_Y)} \times 100
\]

where \(p_{50}\) and \(p_{50}^*\) represent the oxygen affinities of native and amidinated hemoglobin at the indicated pH. The Bohr effect present in native hemoglobin is defined as 100%. Amidination of hemoglobin to the extent of 95% decreases the Bohr effect by only 25 to 30%. We recognize that there are inherent limitations in these calculations of the relative Bohr effect, but the data (Table III) taken as a whole show that amidination of hemoglobin causes minimal change in the Bohr effect between pH 6.0 and 7.4.

The p50 values of native and amidinated hemoglobin are increased in the presence of DPG (Table II, Fig. 2). For purposes of presentation only, we have expressed the DPG effect in amidinated hemoglobin as a percentage, according to the following formula:

\[
\% \text{ DPG effect} = \frac{(\log p_{50}^* \text{ DPG} - \log p_{50})}{(\log p_{50} \text{ DPG} - \log p_{50}^*)} \times 100
\]

where \(p_{50}\) and \(p_{50}^*\) represent the oxygen affinities of native and

| Sample | Per cent amidination | pH 6.0 | pH 6.8 | pH 7.4 | pH 8.0 |
|--------|----------------------|--------|--------|--------|--------|
| Native |                      | p50    | n      | p50    | n      | p50    | n      | p50    | n      | p50    | n      | p50    | n      |
| A      | 12                   | 2.04   | 2.4    | 1.18   | 2.8    | 1.07   | 2.8    | 0.58   | 2.6    | 9.5    | 3.0    |
| B      | 26                   | 1.62   | 2.7    | 0.80   | 2.6    | 0.79   | 2.9    | 0.56   | 2.7    | 5.7    | 2.8    |
| C      | 55                   | 1.23   | 2.2    | 0.75   | 2.7    | 0.70   | 2.8    | 0.52   | 2.5    | 3.1    | 2.8    |
| D      | 88                   | 0.92   | 2.2    | 0.64   | 2.7    | 0.52   | 2.9    | 0.44   | 2.2    | 1.8    | 2.7    |
| E      | 83                   | 0.83   | 2.3    | 0.55   | 2.5    | 0.46   | 2.6    | 0.44   | 2.1    | 1.0    | 2.8    |
| F      | 95                   | 0.78   | 2.1    | 0.49   | 2.9    | 0.45   | 2.5    | 0.40   | 2.0    | 1.3    | 2.9    |

**TABLE II**

**Effect of amidination on oxygen binding by hemoglobin**

The oxygen equilibrium parameters, oxygen affinity (p50 expressed in mm Hg) and cooperativity (n), were obtained from Hill plots of the reoxygenation of native and amidinated hemoglobins at different pH values and in the presence of 5 mM DPG at pH 7.4. Heme concentration, 0.06 mm; 10 mm bis-tris or Tris-HCl; 22-23°C.

![Fig. 1. Hill plots of native Hb (△—△), Hb amidinated to an extent of 29% (■—■), and Hb amidinated to an extent of 99% (○—○). Heme concentration, 0.06 mm, 10 mm Tris-HCl, pH 7.4, 23°, Po2 in mm Hg.](image)

![Fig. 2. Relationship between extent of amidination of lysyl and NH2-terminal valyl residues and oxygen affinity of stripped Hb (○—○) and of Hb in the presence of 5 mM DPG (△—△). Heme concentration, 0.06 mm, 10 mm Tris-HCl, pH 7.4, 23°.](image)
Effect of amidination on Bohr effect and DPG effect

The Bohr effect and DPG effect of amidinated hemoglobin were compared to those of native hemoglobin. Oxygen affinity data obtained from Table II.

### Table III
Effect of amidination on Bohr effect and DPG effect

| Sample | Per cent amidination | pH 6.0-6.8 | pH 6.8-7.4 | pH 7.4 ± DPG |
|--------|----------------------|------------|------------|-------------|
| Native Hb | 0 | 100 | 100 | 100 |
| A | 12 | 109 | 111 | 85 |
| B | 26 | 88 | 87 | 69 |
| C | 55 | 67 | 88 | 59 |
| D | 68 | 75 | 92 | 55 |
| E | 83 | 94 | 85 | 49 |
| F | 95 | 75 | 70 | 41 |

Oxygen affinity of amidinated hemoglobin is due to selective modification of 1 or more lysyl residues in the α chain. In the presence of DPG the oxygen binding curves of the hybrids containing 90% amidinated chains are no longer superimposable with those of the control or 90% amidinated hemoglobin (Table IV) (see “Discussion”).

### Table IV
Oxygen binding by native, amidinated, and hybrid hemoglobins

Oxygen affinities (p50 expressed in mm Hg) of native Hb, amidinated Hb (Hb*), and hybrids consisting of amidinated (*) and native chains. Heme concentration was 0.00 mm. The measurements were made in the presence and absence of 2 mM DPG in 10 mM Tris-HCl, pH 7.4, at 25°C.

### Table V
Dependence of amidination effect on ligand state of hemoglobin

| Sample | Per cent amidination | p50 (-DPG) | p50 (+DPG) | Per cent amidination | DPG effect |
|--------|----------------------|------------|------------|----------------------|------------|
| Native Hb | 0 | 1.75 | 13.3 | 100 |
| Oxy-Hb* | 26 | 1.05 | 4.5 | 70 |
| Deoxy-Hb* | 28 | 1.45 | 7.3 | 80 |

Oxygen affinities of Hemoglobin Amidinated in Oxy and Deoxy States—Oxyhemoglobin was amidinated using 0.7 mM methyl [3H]acetimidate as described above. Another sample of hemoglobin was deoxygenated under nitrogen, and the amidination was carried out in a glove bag filled with nitrogen. The extent of amidination was approximately the same for the two samples (Table V). However, the oxygen affinity of the sample amidinated in the deoxy state was not altered nearly as much as was that of the sample amidinated in the oxy state. It therefore appears that the key lysyl residue (or residues) in the α chains are less accessible to the reagent when the hemoglobin molecule is in the deoxy conformation. One such lysyl residue which is exposed in the oxy and not in the deoxy conformation is lysine C5(40). This residue is involved in a salt bridge which has been proposed to stabilize the deoxy conformation (2).

Localisation of Amidinated Residues in α Chain. CNBr Fragmentation—Hemoglobin was treated with methyl [3H]acetimidate (3.2 x 10⁶ dpm/μmol) and was separated into α and β chains as described above. The hemoglobin was removed from the α chain and the specific radioactivity of the α-globin was determined to be 3.8 x 10⁴ dpm/μmol. Since the α chain contains 11 lysyl residues, the average specific activity of the lysyl residues is 3.4 x 10⁴ dpm/µmol lysine.

The α-globin chain was treated with CNBr which should result in cleavage at methionines 32 and 76. The resulting mixture was separated on Sephadex G-50 (Fig. 3). Five peaks were observed from measurements of radioactivity and absorbance at 280 nm. The order of elution is intact α-globin (residues 1 to 141), peptide BC (residues 33 to 141), peptide AB (residues 1 to 76), and the three desired CNBr fragments, C (residues 77 to 141), B (residues 33 to 76), and A (residues 1 to 32). Fragments A, B, and C were separated from each other by chromatography on phosphocellulose (20). Peptides A, B, and C were identified and quantitated by amino acid analysis (Table VI). The specific radioactivities of peptides A, B, and C are 4.2 x 10⁴, 4.6 x 10⁴, and 1.8 x 10⁴ dpm/μmol of lysine. These specific activities are more conveniently expressed relative to the specific activity of the average lysine in the α chain, taken as 1.0. Thus the relative specific activities of peptides A, B, and C are 1.2, 1.4, and 0.5, respectively.

Separation and Identification of Chymotryptic Peptides from Fragment A—A chymotryptic digest of the CNBr Fragment A was chromatographed on a column (0.9 x 13 cm) of phosphocellulose previously equilibrated with 0.025 N H₃PO₄ (20). The
Amino acid composition of CNBr fragments of amidinated \( \alpha \)-globin

Fragments were separated by chromatography on Sephadex G-50 and were purified by phosphocellulose chromatography or gel filtration. The fragments are designated by letters which correspond to those shown in Table VI.

**Table VI**

| Amino Acid | \( \alpha_{14-12} \) | \( \alpha_{12-11} \) | \( \alpha_{11-10} \) |
|------------|----------------|----------------|----------------|
| Asp        | 2.0            | 5.0            | 5.0            |
| Thr        | 0.9            | 3.9            | 4.9            |
| Ser        | 0.8            | 2.8            | 3.4            |
| Glu        | 2.8            | 1.4            | 1.6            |
| Pro        | 0.9            | 2.4            | 4.0            |
| Gin        | 3.7            | 3.3            | 0.3            |
| Ala        | 6.2            | 5.7            | 8.9            |
| Cys        | 0.0            | 0.0            | 0.1            |
| Val        | 2.8            | 4.6            | 6.5            |
| Met        | 0.0            | 0.1            | 0.0            |
| Ile        | 0.0            | 0.0            | 0.1            |
| Leu        | 1.9            | 3.6            | 10.5           |
| Tyr        | 0.9            | 1.0            | 0.9            |
| Phe        | 0.0            | 4.3            | 4.3            |
| Trp        | 0.0            | 0.0            | 0.0            |
| Lys        | 2.8            | 4.3            | 4.7            |
| His        | 1.1            | 4.5            | 5.2            |
| Arg        | 0.9            | 0.2            | 2.0            |

(a) Residue number.
(b) Molar ratio of amino acids is calculated relative to aspartic acid.
(c) Theoretical values obtained from amino acid sequences.
(d) Not determined.

Fig. 3. Separation of cyanogen bromide fragments of \( \alpha \)-globin on Sephadex G-50. Six-milliliter fractions were collected.

Fig. 4. Separation of chymotryptic peptides of CNBr Fragment A on phosphocellulose. Three-milliliter fractions were collected.
The peptides were separated by chromatography on phosphocellulose and purified by gel filtration. The designations of the peptides correspond to those shown in Fig. 4 and Table IX.

TABLE VII

Amino acid composition of radioactive chymotryptic peptides of CNBr Fragment A

The peptides were separated by chromatography on phosphocellulose and purified by gel filtration. The designations of the peptides correspond to those shown in Fig. 4 and Table IX.

| Amino Acid | A_1 (10-14) | A_2 (1-11) | A_2' (1-14) | A_3 (3-11) | A_4 (15-24) |
|------------|-------------|------------|-------------|-------------|-------------|
|            | Calc | Theor | Calc | Theor | Calc | Theor | Calc | Theor | Calc | Theor |
| Asp | 0.1 | 0 | 2.0* | 2 | 2.0* | 2 | 2.0* | 2 | 0.1 | 0 |
| Thr | 0.1 | 0 | 0.9 | 1 | 0.9 | 1 | 0.9 | 1 | 0.0 | 0 |
| Ser | 0.1 | 0 | 0.8 | 1 | 0.8 | 1 | 0.8 | 1 | 0.2 | 0 |
| Glu | 0.2 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 1.0* | 1 |
| Pro | 0.0 | 0 | 1.0 | 1 | 1.0 | 1 | 0.9 | 1 | 0.0 | 0 |
| Ala | 1.9 | 2 | 1.4 | 1 | 3.0 | 3 | 1.2 | 1 | 2.1 | 2 |
| Cys | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 |
| Val | 1.0* | 1 | 2.1 | 2 | 1.7 | 2 | 1.0 | 1 | 1.4 | 1 |
| Met | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 |
| Ile | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 |
| Leu | 0.1 | 0 | 1.0 | 1 | 0.9 | 1 | 0.9 | 1 | 0.0 | 0 |
| Tyr | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 1.2 | 1 |
| Phe | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 |
| Trp | nd | 1 | nd | 0 | nd | 1 | nd | 0 | nd | 0 |
| His | nd | 0 | nd | 0 | nd | 0 | nd | 0 | nd | 1 |
| Lys | nd | 1 | nd | 2 | nd | 2 | nd | 2 | nd | 1 |
| Arg | nd | 0 | nd | 0 | nd | 0 | nd | 0 | nd | 0 |

(a) Residue number
(b) Molar ratio of amino acids is calculated relative to aspartic acid whenever possible and was rounded off to the nearest tenth of a residue. The reference amino acid is denoted by *.
(c) Theoretical values obtained from the amino acid sequence.
(nd) Not determined.

![Fig. 5. Separation of chymotryptic peptides of CNBr Fragment B on phosphocellulose. Three-milliliter fractions were collected.](http://www.jbc.org/)
TABLE VIII
Amino acid composition of radioactive chymotryptic peptides of CNBr Fragment B

The peptides were separated by chromatography on phosphocellulose and purified by gel filtration. The designations of the peptides correspond to those shown in Fig. 8 and Table IX.

| Amino Acid | Calc b | Theor c | Calc b | Theor c | Calc b | Theor c | Calc b | Theor c | Calc b | Theor c |
|------------|--------|---------|--------|---------|--------|---------|--------|---------|--------|---------|
| Asp        | 0.1    | 0       | 1.0*   | 1       | 1.0*   | 1       | 1.0*   | 1       | 1.0*   | 1       |
| Thr        | 2.8    | 3       | 2.5    | 3       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       |
| Ser        | 0.9    | 1       | 0.7    | 1       | 1.3    | 2       | 0.0    | 0       | 0.0    | 0       |
| Glu        | 0.0    | 0       | 0.0    | 0       | 1.0    | 1       | 0.1    | 0       | 0.1    | 0       |
| Pro        | 1.0*   | 1       | 1.8    | 2       | 0.1    | 0       | 0.0    | 0       | 0.0    | 0       |
| Gly        | 0.0    | 0       | 0.1    | 0       | 1.0    | 1       | 0.0    | 0       | 0.0    | 0       |
| Ala        | 0.0    | 0       | 0.1    | 0       | 1.0    | 1       | 2.1    | 2       | 2.0    | 2       |
| Cys        | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       |
| Val        | 0.0    | 0       | 0.1    | 0       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       |
| Met        | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       |
| lle        | 0.0    | 0       | 0.0    | 0       | 0.9    | 1       | 0.9    | 1       | 1.0    | 1       |
| Leu        | 0.9    | 1       | 1.6    | 2       | 0.9    | 1       | 0.9    | 1       | 1.0    | 1       |
| Tyr        | 0.9    | 1       | 1.1    | 1       | 0.0    | 0       | 0.0    | 0       | 0.0    | 0       |
| Phe        | 1.5    | 2       | 2.0    | 3       | 0.1    | 0       | 0.0    | 0       | 0.0    | 0       |
| Trp        | nd     | 0       | nd     | 0       | nd     | 0       | nd     | 0       | nd     | 0       |
| His        | nd     | 0       | nd     | 1       | nd     | 1       | nd     | 1       | nd     | 1       |
| Lys        | nd     | 1       | nd     | 1       | nd     | 1       | nd     | 2       | nd     | 2       |
| Arg        | nd     | 0       | nd     | 0       | nd     | 0       | nd     | 0       | nd     | 0       |

(a) Residue number.
(b) Molar ratio of amino acids is calculated relative to aspartic acid whenever possible and was rounded off to the nearest tenth of a residue. The reference amino acid is denoted by *.
(c) Theoretical values obtained from the amino acid sequence.
(nd) Not determined.

TABLE IX
Extent of amidination of lysine residues of α chain

Specific activities (S.A.), relative specific activities (R.S.A.), and per cent amidination of each radioactive chymotryptic peptide isolated from Fragments A and B.

| Peptide | Residues | Lysine | S.A. a | R.S.A. | Per cent amidination b |
|---------|----------|--------|--------|-------|------------------------|
| A1      | 10-14    | 11     | 4.1    | 1.2   | 13                     |
| A2      | 1-11     | 7, 11  | 8.8    | 1.1   | 12                     |
| A3      | 1-14     | 7, 11  | 4.0    | 1.2   | 12                     |
| A4      | 3-11     | 7, 11  | 3.9    | 1.1   | 12                     |
| A5      | 15-24    | 16     | 5.2    | 1.5   | 16                     |
| B1      | 33-42    | 40     | 10.3   | 4.0   | 42                     |
| B2      | 33-48    | 40     | 12.3   | 3.7   | 38                     |
| B3      | 47-56    | 56     | 0.9    | 0.2   | 3                      |
| B4      | 59-66    | 60, 61 | 3.1    | 0.9   | 10                     |
| B5      | 57-66    | 60, 61 | 2.9    | 0.8   | 9                      |

a S.A. units are disintegrations per min per µmol lysine × 10^-4.
b Based on the specific radioactivity of methyl [14C]acetimidate (3.2 × 10^9 dpm/µmol).

residues 59 to 66 (Table VII). B5 was separated into three fractions of which the second contained all the radioactivity and was identified as residues 57 to 66 (Table VIII).

The distribution of radioactivity within CNBr Fragment C was not investigated, since the relative specific activity was low relative to that of the other two fragments.

Specific Radioactivity of Individual Lysyl Residues—The specific radioactivity of each of the chymotryptic peptides is presented in Table IX. Lysyl residues 11, 16, 40, and 56 are each uniquely present in one or more peptides, and their specific activities are directly obtainable. The specific activity of lysine 7 is calculated from the specific activity of peptide A1, containing lysine 11, and peptides A2, A3, and A3, containing lysines 7 and 11. The average specific activity for lysine 7 in these peptides is 3.0 × 10^9 dpm/µmol of lysine, corresponding to a relative specific activity of 1.1. No unique peptides were isolated for lysines 60 and 61, and an average value for the 2 residues is presented.

Of all of the lysyl residues shown in the table, lysine 40 has by far the highest specific activity, showing a selectivity of the reagent for this residue. This finding is consistent with our other findings that increased oxygen affinity is a result of selective amidination of an α chain lysyl residue which is less accessible to the reagent when hemoglobin is in the deoxy conformation.

DISCUSSION

Amidination of hemoglobin results in a rather specific increase in oxygen affinity with minimal effects on cooperativity and alkaline Bohr effect. We propose that this increase in oxygen affinity is due to amidination of 1 specific lysyl residue, lysine C5(40)(r, oxy (R) and deoxy (T) conformations toward the high affinity form. The Hill plots for extensively amidinated samples are somewhat sigmoid in character, indicating cooperative binding of oxygen. The Hill plots for extensively amidinated samples are somewhat sigmoid in character, indicating cooperative binding of oxygen.

Extensively amidinated hemoglobin has a very high oxygen affinity, comparable to that of myoglobin or of isolated hemoglobin chains (Table II). The ligand-binding curves are, however, definitely sigmoid in character, indicating cooperative binding of oxygen. The Hill plots for extensively amidinated samples are somewhat sigmoid in character, indicating cooperative binding of oxygen. The Hill plots for extensively amidinated samples are somewhat sigmoid in character, indicating cooperative binding of oxygen.
affinity may possibly reflect an effect of extensive amidination on the oxygen affinity of the R form of hemoglobin, in addition to an effect on the allosteric equilibrium between T and R forms.

The change in oxygen affinity is proportional to the extent of amidination of lysyl residues, and is not related to the extent of amidination of NH$_2$-terminal valyl residues (Fig. 2). The $p_{50}$ for oxygen decreases progressively with amidination of up to 60 to 70% of the lysyl residues. Further amidination causes only a minimal additional increase in oxygen affinity (Fig. 2, Table II). These data indicate that certain residues which are more susceptible to amidination than the bulk of the lysines are responsible for the increased oxygen affinity upon amidination. It is apparent that the NH$_2$-terminal valyl residues are not the residues in question, since nearly maximal effect on oxygen affinity is seen upon amidination of only 8 to 10% of the valyl o-amino groups.

Further amidination to an extent of 28% does not appreciably affect the oxygen affinity (Fig. 2).

The reactive lysyl residues which are responsible for the effect of amidination on oxygen affinity reside in the o chain. Hybrid hemoglobin molecules, containing approximately 3 amidinolysyl residues per chain, were prepared from native hemoglobin and from hemoglobin which had been amidinated in the tetrameric state. The oxygen-binding curves of native hemoglobin (Hb$\beta\beta$) and of hybrid hemoglobin containing amidinated $\alpha$ chains (Hb$\alpha\beta\beta$) are superimposable in stripped hemoglobin and in the presence of 2 mM DPG. The oxygen-binding curves of amidinated hemoglobin (Hb$\alpha^\lambda\beta^\lambda$) and of hybrid hemoglobin containing amidinated $\alpha$ chains (Hb$\alpha^\lambda\beta$) are likewise superimposable in the presence and absence of DPG. Hybrid hemoglobin molecules containing 11 amidinolysyl and 0.3 amidinovalyl residues per chain were similarly prepared. The oxygen-binding curves of Hb$\alpha\beta$ and of Hb$\alpha^\lambda\beta^\lambda$ are superimposable in the absence of DPG. The oxygen-binding curves of Hb$\alpha^\lambda\beta^\lambda$ and of Hb$\alpha^\lambda\beta$ are likewise superimposable in the absence of DPG. In the presence of DPG, however, Hb$\alpha^\lambda\beta^\lambda$ has a higher oxygen affinity than Hb$\alpha\beta$, and Hb$\alpha^\lambda\beta$ has a lower oxygen affinity than Hb$\alpha^\lambda\beta^\lambda$. The significance of this result will be discussed below.

These reactive lysyl residues of the o chain appear to be amidinated to a greater extent in the oxy conformation than in the deoxy conformation, although the over-all extent of amidination of hemoglobin is the same in both conformations. Amidination of oxyhemoglobin to an extent of 26% lowers the $p_{50}$ from 1.75 to 1.08 mm Hg. Amidination of deoxyhemoglobin to an extent of 28% only lowers the $p_{50}$ to 1.45 mm Hg (Table V). It therefore appears as if the key lysyl residues are less accessible to the reagent in the deoxy conformation, implicating lysine C5(40)o in the deoxy conformation, but is free in the oxy conformation (2).

The extent of amidination of a number of lysyl residues of the o chain was determined in a hemoglobin sample which contained 1.2 amidinolysyl residues per o chain. This represents an average of 0.11 amidino group at each of the 11 lysyl positions. We found the following extent of amidination (Table IX): lysine 7, 0.11; lysine 11, 0.13; lysine 16, 0.16; lysine 40, 0.40; lysine 56, 0.03; lysine 60-61 (average), 0.10; lysine 90-99-127-139 (average), 0.05. Lysine C5(40)$_\alpha$ is amidinated 2.5 times as extensively as is lysine A14(16)$_\alpha$, and 3 times as extensively as any other lysyl residue in the o chain. We consider it unlikely that lysine A14(16)$_\alpha$ is involved functionally in the effect of amidination on oxygen affinity, since the variant hemoglobin I (lysine A14(16)$_\alpha$ to glutamate) has no apparent abnormal properties (25).

Amidination of hemoglobin to the extent of 1.2 lysyl residues per chain results in amidination of lysine C5(40)$_\alpha$ in 40% of the $\alpha$ chains. The increase in oxygen affinity seen for this hemoglobin sample is 40% of the over-all difference in oxygen affinity between control and totally amidinated hemoglobin (Fig. 2). We therefore conclude that there exists a direct cause and effect relationship between amidination of lysine C5(40)$_\alpha$ and an increase in oxygen affinity of the tetrameric hemoglobin. Since lysine C5(40)$_\alpha$ is known to participate in a salt bond with histidine HC3(146)$_\beta$ which stabilizes the deoxy (T) conformation, we propose that amidination of the e-amino group prevents formation of the salt bond, thus shifting the position of allosteric equilibrium toward the high affinity oxy (R) conformation.

Kilmartin and Hewitt (26) conclude that, since at pH 8.5 des (Arg-141o) hemoglobin lacks cooperativity ($n = 1$), the salt bridge involving lysine 40o is too weak to stabilize the deoxy conformation in the absence of the salt bridges involving arginine 141o and the imidazole group of histidine 146o. We have shown that amidination of lysine 40o, which we propose to prevent formation of the salt bridge between lysine 40o and histidine 146o, does not appreciably affect the cooperativity of oxygen binding. Although the degree of sigmoidicity of the oxygen equilibrium curves is apparently decreased in the more extensively amidinated samples, the Hill plots in all cases have slopes greater than 2.0 at 50% ligand saturation. Since the accuracy of polarographic measurement of very low oxygen concentrations ($p_{50} < 1$ mm Hg) is poor, it is difficult to determine whether the altered shape of the oxygen equilibrium curves and Hill plots is due to a change in heme-heme interaction, or is simply due to instrumental error. In any case, even the exhaustively amidinated hemoglobin is still highly cooperative in its oxygen binding. Amidination of lysine 40o is thus proposed to result in a destabilization of the deoxy (T) conformation to an extent which permits the T to R transition to occur at a lower ligand saturation than seen in native hemoglobin. This destabilization is not so great as to prevent the formation of the deoxy quaternary conformation, however, so cooperativity of ligand binding is retained ($n > 2$). Our results are therefore consistent with the proposal of Perutz and TenEyck (27) that selective removal or blockage of salt bridges leads to a specific increase of oxygen affinity and reduction of the Hill coefficient.

The opening and closure of the salt bridges involving the imidazole of histidine HC3(146)$_\beta$ and the amino group of valine NA(1)$_\alpha$ accounts for most, or possibly all, of the alkaline Bohr effect (27). Amidination of lysine HC3(40)$_\alpha$ causes a very minimal reduction of the Bohr effect (Table III) while presumably preventing the formation of the salt bond between lysine 40o and the carboxyl of histidine 146o. It is therefore probable that the salt bridges between the imidazole of histidine 146o and aspartate FU1(94)$_\beta$ and between valine 1o and the carboxyl of arginine HC3(141)$_\alpha$ remain intact in amidinated hemoglobin.

Decovalylenhemoglobin binds DPG on the dyad axis in the central cavity so that its acidic groups are coordinated to the imidazole groups of histidine H21(143)$_\alpha$ and the amino groups of valine NA 1(1)$_\beta$ of both $\beta$ chains and to the e-amino group of lysine EF6(82)$_\beta$ of one of the $\beta$ chains (27). The ability of DPG to lower the oxygen affinity decreases progressively as more amino groups are amidinated (Table III). Amidination of 29% of the amino groups of hemoglobin reduces to about 75% the ability of DPG to lower oxygen affinity (Table IV). This effect is due to amidination of residues in the o chain, since Hb$\alpha^\lambda\beta$ exhibits the same DPG effect as native Hb, whereas Hb$\alpha^\lambda\beta$ exhibits the same DPG effect as amidinated hemoglobin. Amidination of 100% of the lysine and 28% of the valine residues reduces to about 35% the ability of DPG to lower oxygen affinity (Table IV). This effect is due in part to amidination of residues in the $\beta$ chain, presumably...
valine 1β and lysine 82β, since the DPG effect of Hbaβ* is reduced to about 60% that of native hemoglobin. The DPG effect of Hbaαβ is reduced to about 75% of control, regardless of whether 29 or 99% of the amino groups of the α chain are amidinated. The reduction of the ability of DPG to stabilize the deoxy conformation of hemoglobin upon amidination of the α chains may be directly due to diminished binding of DPG, or may be an indirect result of destabilization of the deoxy conformation by prevention of the salt bridge involving lysine 40α.

The reagent methyl acetimidate exhibits great selectivity toward reaction with the ε-amino group of lysine 40α in the oxy conformation of hemoglobin. Since the majority of the lysyl residues of hemoglobin, including lysine 40α, are exposed on the surface of the molecule, it is difficult to explain this selectivity on steric grounds. It therefore appears likely that the positively charged methyl acetimidate interacts with a carboxylate group on the surface of the molecule which is in the immediate vicinity of lysine 40α, thus facilitating nucleophilic attack on the imidester by the ε-amino group of lysine 40α. It is of interest to note that amidination of about 60% of the amino groups of hemoglobin, presumably using the uncharged reagent O methylisourea, resulted in only a slight increase in the oxygen affinity (28). The extent of modification of lysine 40α under these conditions is not known. Since the structure of ε-aminocaproyllysine is very similar to that of homoarginine, one might predict that neither derivative would be able to form the salt bridge with histidine HC3(146) in deoxyhemoglobin. Thus, comparable effects on oxygen affinity should result from comparable extents of modification of lysine 40α. The fact that the oxygen affinity of guanidinated hemoglobin is only slightly increased strongly suggests that lysine 40α has not been extensively modified, and emphasizes the selectivity of amidination for this residue.

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