β Chain Amino Termini of the Cat Hemoglobins and the Response to 2,3-Diphosphoglycerate and Adenosine Triphosphate*

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SUMMARY

The amino acid compositions of the isolated α and β chains from purified adult cat hemoglobins A and B have been determined. The data indicate that the α chains are similar but the β chains differ by about four substitutions. Amino-terminal analysis has shown the presence of the typical Val-Leu sequence in the cat α chains. However, the two β chains differ at their amino terminals. NH₂-terminal glycine is found in the α-β and a serine with a blocked α-NH₂ group occurs in this position in the B-β chain. The amino-terminal tryptic peptides of both β chains have been isolated and their sequences partially characterized.

The effects of 2,3-diphosphoglycerate and ATP on the oxygen saturation curves of isolated cat hemoglobins A and B (HbA and HbB) have been investigated. Cat HbA, with free β chain NH₂ termini, is sensitive to 2,3-diphosphoglycerate and ATP, whereas cat HbB, with blocked β chain NH₂ termini, is insensitive to these effector molecules.

The oxygen affinities of the hemolysates containing mixtures of HbA and HbB are changed by 2,3-diphosphoglycerate or ATP in proportion to the ratio of HbA to HbB in the hemolysate.

The work provides support for a role of the NH₂ terminus of the β chain in the control of oxygen binding in hemoglobin.

Phosphate esters such as 2,3-diphosphoglyceric acid and ATP have been found to interact with and to lower the oxygen affinities of human and other animal hemoglobins (1–3). This interaction involves 1 mole of 2,3-DPG per mole of hemoglobin tetramer, occurs preferentially with deoxyhemoglobin (4), and is accompanied by conformational alterations that can be measured by changes in the reactivity of the protein sulfhydryl groups (5, 6). Benesch, Benesch, and Yu (7) and Renthal et al. (8) have found that pyridoxal phosphate competes with 2,3-DPG for its binding site on hemoglobin by forming a Schiff’s base with the amino group of the NH₂-terminal residue of one of the β chains (7, 8). The β chain NH₂-terminal are thus presumed to play a role in the binding of phosphate and may be of significance with respect to the control of hemoglobin function.

The two major hemoglobins (A and B) of domestic cats (9) can be used to test this hypothesis since the amino groups of the NH₂-terminal residues of the β chain are blocked by substitution in HbB and are unblocked in HbA. Aside from the difference in the NH₂ terminal, the two hemoglobins are quite similar. They possess identical α chains and vary in only about 2 to 4 of their β chain residues. If the NH₂ terminus of the β chain plays a role in the binding of phosphates, the presence of the phosphate esters should cause a marked difference in the oxygen affinities of cat HbA and HbB.

NH₂-acetylated β chains appear to be a common feature in one or more major hemoglobins that are found in the blood of the Felidae. Data on the structures of the β chain NH₂ termini as well as on the effects of 2,3-DPG and ATP on the oxygen equilibria of domestic cat hemolysates and isolated hemoglobins are reported here.

EXPERIMENTAL PROCEDURE

Hemoglobin solutions were prepared as described earlier (10). HbA and HbB were isolated (10, 11) in relatively concentrated solutions (2 to 5%) from freshly prepared hemolysates by chromatography of 15% hemolysates on a column (2.5 x 12 cm) of BioRex-70 equilibrated with 0.05 M phosphate buffer, pH 6.50. After elution of HbB with the same buffer at 4°, the column was warmed to room temperature, and HbA was rapidly eluted with 0.14 M phosphate buffer, pH 7.0.

Globin was prepared from the purified hemoglobins by precipitation in acidic acetone at -20° (12). Separation of the α and β chains was carried out on carboxymethyl cellulose columns (13) essentially as described by Boyer et al. (14). Amino acid compositions of the chains was determined with a Spinco amino acid analyzer by the method of Spackman, Stein, and Moore (15). The Sanger reaction was used for NH₂-terminal analyses (10). Purified hemoglobins were digested with trypsin at 37° for 90 min. The digests were applied to a line on Whatman 3MM paper. The digests were applied to a line on Whatman 3MM paper.
for absorbance at 280 nm. The background absorbance of the acid buffer in the second chamber. The fractions were analyzed for absorbance at 280 nm, and elution was carried out using pyridine-isomyl alcohol-H2O solvent (17). The separated peptides were detected with ninhydrin and eluted with 5.7 N HCl into drawn tubes. The tubes were then sealed and the contents were heated at 110° for 18 to 22 hours to hydrolyze the peptides for amino acid analysis.

For oxygen saturation studies, aliquots of HbA and HbB solutions were "stripped" of phosphates and other salts by passage through a column (1.5 x 30 cm) of Sephadex G-25 equilibrated with 0.1 M NaCl (3). The cyclohexylammonium salt of DPG was obtained from Boehringer Mannheim Corporation; it was converted to the free acid form by shaking the aqueous solution with Dowex 50-H+, and then neutralized with NaOH. ATP was obtained from P-L Biochemicals. All operations, including oxygen saturation measurements, were conducted within a few days after blood was drawn. Methemoglobin formation in this time was negligible.

RESULTS

Purification of Hemoglobins—A typical separation of the two cat hemoglobins by chromatography on a preparative Amberlite CG-50 column is illustrated in Fig. 1. Hemoglobin B is eluted first as a sharp peak followed by a broad band of HbA. The homogeneity of the fractions was confirmed by starch gel electrophoresis.

Separation of α and β Chains—An elution profile obtained from carboxymethyl cellulose column chromatography of a cat globin preparation is shown in Fig. 2. The two retarded peaks were identified as α and β chains, respectively, from tryptic peptide maps and from earlier hybridization data (18). This order of elution is similar to that obtained for rabbit (13) and sheep (14) globin chains separated under comparable procedures.

Amino Acid Compositions of Isolated Chains—Amino acid analyses of the β chains of hemoglobins A and B are presented in Table I. The values are given in residues of amino acid per molecule of protein and were calculated on the basis of the sum of 40 and 38 residues of lysine, glycine, and alanine in the A-β and B-β globins, respectively. The A-β chain clearly differs from the B-β chain in that it contains single additional lysyl and glycy1 residues as well as 1 less arginine and at least 1 less serine. The data also suggest that, in addition, the A-β chain might contain 1 more asparagine and threonine, 2 less serines, and 1 less leucine relative to the B-β chain. One tryptophan and methionine and 2 cysteines are present in the two β chains.

The amino acid compositions of the α chains isolated from hemoglobin A and B are also compared in Table I. They appear to be identical and the presence of 3 cysteine residues is unusual since no other α chain has been reported to contain more than 1 residue of this amino acid.

NH2-terminal Analysis—The results of amino-terminal analyses using Sanger's reagent are presented in Table II. The values are expressed on the basis of a molecular weight of 31,000 (per α3 unit) for globin. The expected number of NH2-terminal valyl residues is 2, but yields of 50% or less were obtained in these analyses. Only DNP-valine was recovered from either cat globin, and it was only about one-half of that obtained from human HbA treated in the same way. It appeared that in addition to NH2-terminal valine in one chain, another labile or blocked amino-terminal residue might be present in the other chain. In agreement with this, milder hydrolysis of the DNP globin from cat HbA in 10% HCl showed the presence of DNP-glycine in addition to DNP-valine. DNP-glycine is known to be very labile when hydrolyzed in 5.7% HCl (16). With DNP globin prepared from HbB, however, only DNP-valine could be detected even when hydrolysis was carried out in 10% HCl. The amount of DNP-valine recovered from this globin was almost equal to that obtained from cat HbA DNP globin and only about one-half of that found in the human. Qualitative end group analysis of the separated cat α and β chains of HbA showed that valine is present in the amino terminus of the α chain and that the β chain contains glycine in this position. Analysis of the isolated HbB α and β chains indicated that the DNP-valine was associated with the B-α chain, but no free α-amino group could be detected in the B-β chain.

To characterize further the NH2-terminal sequences in the α and β chain, the cat globins were subjected to a single step of the Edman degradation and then reacted with Sanger's reagent. DNP-leucine was present in the hydrolysates of globins A and B. DNP-phenylalanine was also found in the hydrolysate of the cat HbA globin in an amount equivalent to that of the DNP-leucine. Since all known mammalian α chains begin with a Val-Leu sequence, these results suggested that the penultimate amino acids in the A-α and B-α chain are leucine, and that the corresponding amino acid is phenylalanine in the A-β chain. Only a trace of DNP-phenylalanine could be detected in the globin derived from hemoglobin B, indicating that a significant difference occurs at the N terminus of A-β and B-β. The amino group of the B-β chain was probably blocked and hence unreactive to the Edman reagent.

Isolation and Characterization of Amino-terminal Peptides of β Chains—A clear difference between the tryptic peptides maps of

Fig. 1 (left). Chromatography of an hemolysate of cat red blood cells on a preparative column of Amberlite CG-50. Approximately 6 g of hemoglobin were applied and elution was carried out at 4° with 0.05 M sodium phosphate buffer, pH 6.50, as described in the text.

Fig. 2 (right). Separation of the α and β chains of cat hemoglobin A. Globin (400 mg) was applied to a carboxymethyl cellulose column (0.47 meq per g) column (2.5 x 40 cm) and eluted at 6 ml fractions at a flow rate of about 20 ml per hour with a gradient made up of 350 ml of 0.01 M pyridine-0.113 M formic acid buffer, pH 2.7, in the mixing chamber and 0.1 M pyridine-1.13 M formic acid buffer in the second chamber. The fractions were analyzed for absorbance at 280 nm, and background absorbance of the gradient.
HbA and HbB is found in one of the acidic peptides (20). This
difference is easily seen when the tryptic digest is separated by
paper electrophoresis at pH 6.5 (Fig. 3). The electrophoretic
patterns of the isolated chains are also included to show that
the peptides in question are found in the respective $\alpha$ chains. They
were eluted from the paper and further purified by paper chromatog-
raphy.

The amino acid compositions of these peptides are presented
in Table III. They appear to be similar to the amino terminal
peptides in question are found in the respective $\alpha$ chains. They

| Amino acid compositions of $\alpha$ and $\beta$ chains from cat hemoglobins A and B |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | A-\(\alpha\)     | B-\(\alpha\)     | A-\(\beta\)      | B-\(\beta\)      |

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Average or extrapolated values</th>
<th>Integral values</th>
<th>Average or extrapolated values</th>
<th>Integral values</th>
<th>Average or extrapolated values</th>
<th>Integral values</th>
<th>Average or extrapolated values</th>
<th>Integral values</th>
<th>Differences (A-(\beta)-B-(\beta))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>10.04 \pm 0.07</td>
<td>10</td>
<td>10.10 \pm 0.23</td>
<td>10</td>
<td>11.83 \pm 0.21</td>
<td>12</td>
<td>11.14 \pm 0.16</td>
<td>11</td>
<td>+1</td>
</tr>
<tr>
<td>Histidine</td>
<td>9.89 \pm 0.09</td>
<td>10</td>
<td>9.60 \pm 0.12</td>
<td>10</td>
<td>6.02 \pm 0.11</td>
<td>0</td>
<td>6.06 \pm 0.17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>2.80 \pm 0.06</td>
<td>3</td>
<td>2.88 \pm 0.04</td>
<td>3</td>
<td>2.88 \pm 0.06</td>
<td>3</td>
<td>3.48 \pm 0.10</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.48 \pm 0.20</td>
<td>10-11</td>
<td>10.75 \pm 0.11</td>
<td>11-10</td>
<td>12.79 \pm 0.15</td>
<td>18</td>
<td>17.23 \pm 0.48</td>
<td>17-18</td>
<td>(+1)</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.32 \pm 0.24</td>
<td>10</td>
<td>9.36 \pm 0.40</td>
<td>9-10</td>
<td>2.48 \pm 0.13</td>
<td>2</td>
<td>1.71 \pm 0.30</td>
<td>2</td>
<td>(+1)</td>
</tr>
<tr>
<td>Serine</td>
<td>12.98 \pm 0.23</td>
<td>13</td>
<td>12.54 \pm 0.20</td>
<td>12-13</td>
<td>9.95 \pm 0.40</td>
<td>10</td>
<td>11.59 \pm 0.41</td>
<td>11-12</td>
<td>-1(-2)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.22 \pm 0.12</td>
<td>7</td>
<td>7.29 \pm 0.09</td>
<td>7</td>
<td>10.65 \pm 0.43</td>
<td>11</td>
<td>10.77 \pm 0.32</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>5.65 \pm 0.48</td>
<td>5-6</td>
<td>4.98 \pm 0.06</td>
<td>5</td>
<td>3.67 \pm 0.16</td>
<td>4</td>
<td>3.75 \pm 0.14</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>6.07 \pm 0.03</td>
<td>6</td>
<td>6.41 \pm 0.17</td>
<td>6</td>
<td>13.07 \pm 0.11</td>
<td>13</td>
<td>11.77 \pm 0.15</td>
<td>12</td>
<td>(+1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>21.93 \pm 0.01</td>
<td>20</td>
<td>20.52 \pm 0.15</td>
<td>20-21</td>
<td>15.09 \pm 0.12</td>
<td>15</td>
<td>15.12 \pm 0.16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.91 \pm 0.03</td>
<td>3</td>
<td>2.47 \pm 0.22</td>
<td>3</td>
<td>1.62 \pm 0.30</td>
<td>2</td>
<td>1.77 \pm 0.12</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>11.17 \pm 0.01</td>
<td>11</td>
<td>11.74 \pm 0.16</td>
<td>11</td>
<td>15.12 \pm 0.06</td>
<td>15</td>
<td>15.12 \pm 0.16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>1.75 \pm 0.06</td>
<td>2</td>
<td>1.65 \pm 0.08</td>
<td>2</td>
<td>1.15 \pm 0.10</td>
<td>1</td>
<td>1.00 \pm 0.09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.91 \pm 0.02</td>
<td>1</td>
<td>1.01 \pm 0.06</td>
<td>1</td>
<td>2.04 \pm 0.04</td>
<td>2</td>
<td>1.62 \pm 0.10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>14.27 \pm 0.01</td>
<td>14</td>
<td>14.43 \pm 0.15</td>
<td>14</td>
<td>17.06 \pm 0.22</td>
<td>17</td>
<td>17.48 \pm 0.46</td>
<td>17-18</td>
<td>(-1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.84 \pm 0.11</td>
<td>4</td>
<td>3.44 \pm 0.12</td>
<td>4</td>
<td>2.06 \pm 0.11</td>
<td>2</td>
<td>2.86 \pm 0.08</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.93 \pm 0.19</td>
<td>9</td>
<td>8.70 \pm 0.30</td>
<td>9</td>
<td>9.81 \pm 0.10</td>
<td>10</td>
<td>9.71 \pm 0.25</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.60 \pm 0.02</td>
<td>2</td>
<td>1.73 \pm 0.04</td>
<td>2</td>
<td>1.16 \pm 0.04</td>
<td>1</td>
<td>1.04 \pm 0.02</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>147-143</td>
<td>150-154</td>
<td>148-145</td>
<td>148-147</td>
<td>156-154</td>
<td>145-140</td>
<td>148-147</td>
<td>145-140</td>
<td>-2(-4)</td>
</tr>
</tbody>
</table>

- Averages of duplicate hydrolysates prepared at 110°C. Calculated on the basis of 10 lysine, 6 glycine, and 21 alanine residues in A-\(\alpha\) and B-\(\alpha\), and lysine + glycine + alanine = 40 and 38 for A-\(\beta\) and B-\(\beta\), respectively.
- Obtained by extrapolation at zero time.
- Average of 72-hour values only.
- Determined spectrophotometrically (19).

As indicated above, this amino acid was detected at
the amino-terminal position of the intact A-\(\beta\) chain with San-
gers reagent. On the other hand, the corresponding B-\(\beta\) pep-
tide stained blue with ninhydrin, gave negative results with
Sanger's reagent, and showed no change in the amino acid com-
position after one step of Edman degradation. The NH-termin-
al group of this peptide therefore behaved like that of the intact
B-\(\beta\) chain and exhibited properties of an NH2 blocked amino-
terminal residue. The electrophoretic mobility of the B-\(\beta\)
péptide is consistent with a net charge of -2 at pH 6.5, com-
pared with a -1 for the A-\(\beta\) peptide, and is in accord with the
absence of a free amino group in the B-\(\beta\) peptide. The A-\(\beta\)
and B-\(\beta\) peptides were then further characterized by examining
the products of their chymotryptic digests. The products were
eluted from the paper and further purified by paper chromatog-
raphy. Both peptides yielded free leucine and a peptide containing serine,
alanine, glutamic acid, and lysine. A dipeptide containing
glycine and phenylalanine was also found in the digest of the A-\(\beta\)
chains.
Aminoterminal analysis of cat globins and their isolated subunits

Weighed samples (0.2 to 0.3 pmole) of dry DNP-globins were hydrolyzed under the conditions given in the table. The DNP-amino acids were chromatographed and eluted from the paper with 1% NaHCO₃ and quantitated spectrophotometrically by their absorbance at 340 nm. Residues of DNP product per molecule of globin were calculated on the basis of a molecular weight of 31,000 for both cat and human globins. The results of qualitative analyses for DNP amino acids in the isolated α and β chains are also included.

<table>
<thead>
<tr>
<th>DNP-globin</th>
<th>Ratios (&lt;5 M HCl, 110°C, 16 hours) of DNP-amino acids to DNP-globin</th>
<th>Ratios (10 M HCl, 110°C, 4 hours) of DNP-amino acids to DNP-globin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Valine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Human</td>
<td>0.97⁺</td>
<td>0.77⁺</td>
</tr>
<tr>
<td>Cat HbA</td>
<td>0.57</td>
<td>0.44</td>
</tr>
<tr>
<td>Cat HbB</td>
<td>0.51</td>
<td>0.28</td>
</tr>
<tr>
<td>A-α</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>B-α</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>A-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-β</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁺ The expected number of NH₂-terminal valine per αβ unit for human globin is 2; the yields obtained were 50% or less.

Fig. 3. A diagrammatic representation of the tryptic peptide patterns obtained for hemoglobins A and B and their isolated subunits by electrophoresis at pH 6.4 on Whatman No. 3MM paper for 1.5 hours at 2500 volts. The shaded bands indicate the positions of the β₁T-1 peptides in HbA, HbB, and their β chains. The positions of phenylalanine and lysine under these conditions are also shown.

To determine that serine was in fact the amino-terminal residue and to characterize the blocking groups at the NH₂ terminus of the B-β chain, 0.2 pmole of the B-βT-1 peptide was digested first with chymotrypsin and then with carboxypeptidase A. The enzymes were denatured after about 3 hours by heating to 95°C for 5 min. After adjusting the pH 3.5, the digestion mixtures was applied to a column (0.7 x 3 cm) of Dowex 50-X2 (H⁺) to remove any amino acids or peptides containing a free amino group. The column was washed with two 1-ml aliquots of distilled water and the combined eluate was lyophilized. The material was washed with two 1-ml aliquots of distilled water, applied along a 3-cm line on Whatman No. 1 paper along with samples of authentic N-acetylserine (Calbiochem). After descending chromatography overnight with a butanol-acetic acid-water (5:7:3) solvent the paper was air-dried and cut into two strips. One-
half of the unknown was stained with ninhydrin and the other by chlorination (21). Negative results were obtained with ninhydrin but the chlorination stain showed the presence of a compound exhibiting the same mobility as the authentic N-acetylsereine ($R_s$ 0.63). Both the unknown and authentic N-acetylsereine along with a paper blank were eluted from the paper with 5.7 M HCl and hydrolyzed for 12 hours at 110°C. The known and unknown samples contained a ninhydrin-positive compound that cochromatographed with authentic $[^4]$C]serine. Thus, the amino-terminal position of the $\beta$ chain is occupied by a serine.

To further establish that the blocking group is an acetyl group, methanolysis of the hemoglobins (22) followed by distillation and gas chromatographic analysis of the products have been carried out. The results of these analyses will be reported in a later communication.

Fig. 4 shows that isolated cat HbA and HbB have identical oxygen affinities over a wide range of pH and temperature values when measured in a 0.1 to 0.2 M phosphate buffer. "Stripped" HbA and HbB exhibit the same low oxygen affinities when measured in the presence of 0.1 to 0.2 M orthophosphate. However, the two hemoglobins have different oxygen affinities when they are stripped and measured in a 0.05 M bis-tris buffer (25) with no phosphate added (Fig. 5). HbA and HbB both increase in oxygen affinity upon stripping, but the affinity of HbA increases more. Definite lowering of oxygen affinity of stripped HbA was observed when 2,3-DPG or ATP was added in the range of $1 \times 10^{-4}$ to $1 \times 10^{-2}$ M. Maximum effect on lowered affinity was observed at about $1 \times 10^{-3}$ M of added DPG or ATP. Under these conditions, the oxygen affinity of stripped HbB was unchanged. In the presence of 1 to $5 \times 10^{-4}$ M 2,3-DPG or ATP, the oxygen affinity of stripped HbA is decreased to that of the "unstripped" preparations while the oxygen affinity of stripped HbB is unchanged. Thus, the two hemoglobins clearly respond differently to these concentrations of the organic phosphates. More recently we have also found altered reactivity of the NH$_2$ groups in the presence of 2,3-DPG for deoxy-HbA but not with deoxy-HbB.

Since cat blood contains the two hemoglobins in ratios that vary greatly from animal to animal, it was of interest to investigate the effects of the stripping procedure on the oxygen affinities of hemolysates containing different proportions of HbA and HbB. Fig. 6 shows that stripping increases the oxygen affinity of both hemolysates with HbA:HbB ratios of 9 and 1, although the effects are not as large as those seen with the human hemolysate. Furthermore, the oxygen affinity of the hemolysate with an HbA:HbB ratio of 9 increases more upon stripping than does the oxygen affinity of the hemolysate having an HbA:HbB ratio of 1. With the addition of $5 \times 10^{-4}$ M 2,3-DPG or ATP, the oxygen affinity of the stripped hemolysate with an HbA:HbB ratio of 9 is lowered to $p_O$ values observed in the presence of 0.1 M phosphate, but the oxygen affinity of the hemolysate with the HbA:HbB ratio of 1 is only partially lowered. In the case of the latter hemolysate, $p_O$ values can be completely lowered only when measured in higher concentrations of phosphate (0.1 to 0.2 M).

The shape of the oxygen saturation curve of the stripped 1:1 hemolysate in the presence of added organic phosphate reflects a response that is to be expected from an equal mixture of a phosphate-sensitive hemoglobin and a phosphate-insensitive hemoglobin: at lower $p_O$ values the curve is similar to that of the stripped hemolysate, and at higher $p_O$ values, the curve approaches that of the unstripped sample. Presumably, HbB which is insensitive to the action of the organic phosphates, combines preferentially with oxygen at lower partial pressures, and HbA with its phosphate induced lowered oxygen affinity combines with oxygen at higher pressures. Blood having an HbA:HbB ratio of 9 is more sensitive to the effects of low concentrations of phosphates because it contains more of the phosphate-sensitive HbA.

**DISCUSSION**

These studies support earlier data (11) to show that the structural differences in the two cat hemoglobins are confined exclusively to their $\beta$ chains. The discovery that one of these substitutions, serine for glycine, occurs in the NH$_2$-terminal position of the $\beta$ chain, and further that the amino group of the serine in B-$\beta$ is blocked with an acetyl group accounts for a unit charge difference between the two $\beta$ chains. The relative electrophoretic mobilities of the two hemoglobins are consistent with a decrease in +2 charge units in HbB.

The NH$_2$ terminal analysis of the cat hemoglobins indicates that this end of the cat $\alpha$ chain is occupied by the same Val-Leu sequence that has been found in all of the other mammalian $\alpha$ chains. The amino-terminal sequences of the A-$\beta$ and B-$\beta$ chains appear to be Gly-Phe-Leu(Ser, Ala, Glu)Lys, and N-acetylseryl-Phe-Leu(Ser, Ala, Glu)Lys, respectively, and are different from $\beta$-$\gamma$ sequences in other animal hemoglobins. Although Masai (24) detected DNP-methionine in addition to DNP-valine and DNP-glycine in his crystalline cat hemoglobin preparation, we have been unable to detect NH$_2$-terminal methionine in either cat HbA or HbB. This apparent contradiction may be
due to differences in animals and would suggest that at least one more hemoglobin type other than HbA and HbB may be found in some cats. The fact that he found an amino-terminal Val-Leu sequence as well as an NH$_2$-terminal glycine residue suggests that his preparation contained a hemoglobin similar if not identical with our HbA. The presence of N-acetylserylserine at the amino terminus of the $\beta$ chain of cat HbB is of special interest. To the best of our knowledge, this is the only instance in which a major mammalian hemoglobin component has been shown to possess a blocked amino group on either chain. A minor component of human fetal blood, hemoglobin F, contains N-acetylglycine on the amino terminus of the $\gamma$ chain (25). Similarly, an amino-terminal valine that is blocked by a hexose moiety (26, 27) is found in the $\beta$ chain of a minor component, hemoglobin A$_{0}$, of adult human blood. The acetylation of the NH$_2$-terminal amino group seems to be a more general phenomenon in the hemoglobins of the lower vertebrates. For example, N-acetylserylserine is found in the carp $\alpha$ chain (28), and N-acetylserylserine in the frog species, Rana esculenta (29). Another feature about cat $\beta$-chains is that they both contain phenylalanine at the penultimate amino-terminal position. The NH$_2$-terminal sequences Gly-Phe and acetyl-Ser-Phe found in the $\beta$ chains of HbA and HbB, respectively, are clearly different from any other presently known $\beta$ chain structures.

The occurrence of HbB, with its $\beta$ chain terminus specifically blocked, provides a molecule that can be examined to assess the involvement of this group in the control or modulation of oxygenation properties. Kilmartin and Roccia Bernardi (30) have recently examined the effects of blocking the $\alpha$-amino groups of horse hemoglobin with cyanate. They report that blocking the terminal amino groups of the $\alpha$ chains with this reagent reduces the alkaline Bohr effect by 25% and appears to decrease CO$_2$ binding. In contrast, however, the Bohr effect retains its full value when only the $\alpha$-amino groups of the $\beta$ chains are blocked and no differences could be detected in the oxygenation characteristics of this molecule compared to unmodified hemoglobin. Similarly, the absence of the free $\alpha$-amino group in the $\beta$ chain of cat HbB causes no apparent effect on the oxygen affinity, Bohr effect or Hill constant, when they are measured in 0.1 to 0.2 M phosphate buffer. Under these conditions, the same values are found for cat HbA, in which the NH$_2$-terminal groups are free, as they are for HbB in which the $\beta$ chain amino groups are blocked. However, in buffers of low ionic strengths, the oxygen affinity of HbB is lower than that of HbA, and low concentrations of effectors such as DPG and ATP, alter the oxygen binding of HbA but not of HbB.

During the course of this work, Bunn and Bierhel (31) reported similar findings using the $N$-acetylated minor human fetal hemoglobin F$_1$, and have also implicated a role for histidine 143-$\beta$ (32) in the binding of 2,3-DPG. More recently, Perutz (33) has found that a molecular model of 2,3 DPG will fit into the central cavity of the model of hemoglobin in such a way that the charged phosphate groups can form salt bridges with $\alpha$-amino groups of the $\beta$ chain NH$_2$-termini as well as with lysine residues at 82-$\beta$ and histidine residues at position 143-$\beta$. Our data on the effects of 2,3-DPG on cat hemolysates as well as on the isolated cat hemoglobins indicate that cat HbB behaves similarly to pyridoxal phosphate-modified human hemoglobin (7, 8) and to human fetal hemoglobin F$_1$ and suggest that the NH$_2$-terminal positions of the $\beta$ chains participate in the binding of organic phosphate compounds to hemoglobin. R. F. Bunn has also obtained results with the cat hemoglobins that are similar to ours. It is of interest to note here that fingerprints of cat HbA and HbB differ in $\delta$T-14, the peptide that includes position 143-$\beta$. The peptide from HbA gives a positive Paulus reaction for histidine but the corresponding peptide from HbB apparently does not. Details on this as well as other studies on the structure of the cat hemoglobins will be presented in a later communication.

REFERENCES

β Chain Amino Termini of the Cat Hemoglobins and the Response to 2,3-Diphosphoglycerate and Adenosine Triphosphate
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