Hemoglobin Abruzzo [β143 (H21) His→Arg]

CONSEQUENCES OF ALTERING THE 2,3-DIPHOSPHOGLYCERATE BINDING SITE*

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SUMMARY

Hemoglobin Abruzzo is an abnormal human hemoglobin with a substitution at a residue known to be involved in the binding of 2,3-diphosphoglyceric acid. It has increased oxygen affinity and reduced heme-heme interaction in the absence of organic or inorganic phosphate cofactors. In inorganic phosphate buffers the Bohr effect and heme-heme interaction are normal, but the oxygen affinity remains higher than that of hemoglobin A. CO combination in inorganic phosphate buffers the Bohr effect and heme-heme interaction are normal, but the oxygen affinity remains higher than that of hemoglobin A. CO combination in inorganic phosphate is more strongly autocatalytic than in normal hemoglobin and a slower rate of oxygen dissociation is observed. Although many of the functional differences of this variant may be attributed to the high oxygen affinity of the mutant β chains, the interactions between subunits are also affected by the histidine to arginine substitution at β143. Stripped hemoglobin Abruzzo appears to be significantly more dissociated than hemoglobin A. Kinetic studies indicate that interaction with organic or inorganic phosphates decreases its subunit dissociation. In all of the functional properties examined, hemoglobin Abruzzo is more sensitive to the allosteric influence of organic and inorganic anions than is hemoglobin A.

The metabolic intermediate 2,3-diphosphoglycerate plays an important physiological role as an allosteric regulator of the oxygen affinity of human and most mammalian hemoglobins (1). Additionally, it has been found that small anions as well as organic phosphates can lower the oxygen affinity of hemoglobin. These cofactors are believed to bind preferentially to deoxyhemoglobin and thereby shift the conformational equilibrium toward the low affinity (T) conformation (1, 2). The allosteric mechanism involved has been under intensive investigation. X-ray crystallographic studies have shown that β143 His is involved in the binding of DPG and inositol hexaphosphate by normal human hemoglobin (2, 3). The following results concern the changes in hemoglobin function which occur due to the substitution of arginine for histidine at the β143 position in Hb Abruzzo (4). Both the kinetics and equilibria of ligand binding by Hb Abruzzo [β143 (H21) His→Arg] are described. Particular emphasis is given to the effects of small anions and organic phosphates on the functional properties of this hemoglobin variant.

MATERIALS AND METHODS

Hemolysates from the blood of a patient with Hb Abruzzo were prepared by the ammonium sulfate procedure and freed of ions by passage through a column of mixed ion-exchangers (5). Since the patient was homozygous, more than 95% of the hemoglobin was of the Abruzzo type (4) and no further purification was performed. Starch gel electrophoresis was done using the method of Poulik (6). At pH 8.6 starch gel electrophoresis of hemoglobins A and Abruzzo showed that Hb Abruzzo has a lower anodic mobility than HbA, consistent with the amino acid substitution of Hb Abruzzo (4). At pH 8.6, β143 His of HbA should be uncharged and β143 Arg of Abruzzo should carry a full positive charge. Concentrated stock solutions of DPG were prepared by dissolving the pentacyclohexylammonium salt of 2,3-diphosphoglyceric acid in water, converting the salt to the free acid with Dowex 50-X8, and neutralizing the acid with NaOH. Concentrated IHP solutions were made by dissolving phytic acid (Sigma Chemical Co.) in water and adjusting the pH with concentrated phosphoric acid. Bis-Tris buffer was prepared by neutralizing 0.1 M NaOH with 1 M HC1. Oxygen equilibria were measured spectrophotometrically by the method of Rossi-Fanelli and Antonini (7). Rapid mixing experiments were performed with a stopped flow apparatus (8) and flash photolysis of CO hemoglobin was done in an apparatus previously described (9).

RESULTS

Oxygen Equilibria—Fig. 1 shows oxygen binding curves for Hb Abruzzo and HbA in various buffers at neutral pH at 20°. When Hb Abruzzo is stripped of anions, it shows a higher oxygen affinity, \( p_{50} = 0.8 \text{ mm Hg} \), and reduced heme-heme interaction, \( n = 2.0 \), relative to normal human hemoglobin under these conditions.

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FIG. 1. Oxygen equilibrium curves in different media for Hb Abruzzo (open symbols) and HbA (closed symbols) at pH 7, 20°C, and 3 to 5 mg/ml. O₂ concentrations in mm Hg. ○, 0.1 M bis-Tris; ▽, 0.2 M potassium phosphate; △, 0.1 M bis-Tris, 5 × 10⁻⁴ M DPG; □, 0.1 M bis-Tris, 5 × 10⁻⁴ M IHP.

Fig. 2. Diagrammatic representation of the shifts in oxygen affinity induced by addition of organic and inorganic phosphate cofactors to solutions of Hb Abruzzo and HbA. Conditions as in Fig. 1. Hatched bars show results obtained with Hb Abruzzo.

ditions (p½ = 1.8 mm Hg, n = 2.4). Fig. 1 also shows that in the presence of inorganic phosphate, DPG, or IHP, the extent of cooperative subunit interactions in Hb Abruzzo is increased to normal levels. The relative shifts in oxygen affinity brought about by various anions are of interest because of the involvement of βι43 in the binding of DPG and IHP. Fig. 2 shows that both inorganic phosphate and DPG lower the oxygen affinity of Hb Abruzzo significantly more than they lower the oxygen affinity of HbA. In contrast, IHP causes only a slightly greater shift in the affinity of the abnormal hemoglobin. Titrations of the oxygen affinity of Hb Abruzzo versus cofactor concentration indicated that the cofactor concentrations used in Figs. 1 and 2 were saturating. At lower (nonsaturating) concentrations of DPG or IHP the Hill plots of oxygen binding were very nonsymmetrical with n values lower at 20% saturation than at 80% saturation. Similar Hill plots have been reported for normal hemoglobin in the presence of less than saturating amounts of DPG (10).

Fig. 3 shows the pH dependence of the oxygen affinities of Hb Abruzzo and HbA in inorganic phosphate buffer from pH 5.4 to pH 8.5. The abnormal hemoglobin in inorganic phosphate buffer shows a slightly higher oxygen affinity and an increased alkaline Bohr effect relative to HbA over this pH range. In borate buffer above pH 9 the oxygen affinity of Hb Abruzzo is sharply increased; and, as is also shown in Fig. 3, this increased affinity is associated with decreased heme-heme interaction. These features may reflect an instability of the tetramer at high pH, since at netural pH, Hb Abruzzo appears to be more dissociated than normal hemoglobin.

**Oxygen Kinetics**—The rate of oxygen dissociation from Hb Abruzzo was measured by rapid mixing of air-equilibrated hemoglobin solutions with buffers containing sodium dithionite. Fig. 4 shows the effect of inorganic phosphate and IHP on the kinetics of oxygen dissociation. Table I summarizes the rates observed and presents data on HbA for comparison. At pH 7 the dissociation of oxygen from stripped Hb Abruzzo in 0.05 M bis-Tris buffer shows an autocatalytic time course. The initial rate is about 0 s⁻¹. The final rate, about 17.5 s⁻¹, is close to the rate of 22 s⁻¹ that is observed throughout the process of deoxygenation for normal hemoglobin under these conditions. The autocatalytic character seen in bis-Tris buffer is not apparent when Hb Abruzzo is present in inorganic phosphate buffer or in bis-Tris buffer with IHP. In inorganic phosphate at pH 7.0 the oxygen dissociation from Hb Abruzzo can be described by a single exponential function with a rate slightly lower than that observed with HbA in this buffer. In the presence of IHP, the reaction becomes faster and slightly biphasic, although not as markedly heterogeneous as deoxygenation of HbA in the presence of IHP (11). As shown in Table I, the pH dependence of the rate of oxygen dissociation from Hb Abruzzo (in inorganic phosphate and 2% borate) is similar to that of normal hemoglobin.

**Carbon Monoxide Kinetics**—The process of CO combination with Hb Abruzzo was studied by both flash photolysis and rapid mixing techniques. The effects of various buffers on the CO combination velocity constant are summarized in Table I. Fig. 5 shows the large effect of inorganic phosphate on the time course of CO recombination with Hb Abruzzo after complete flash photolysis of the CO derivative in the presence of dithionite.

* Gel filtration experiments of E. Chiancone, personal communication.
A range of values is given for cases where the initial and final apparent rates were clearly different. The CO combination velocity constants were estimated on the basis of the CO concentration dependence of CO binding as measured under pseudo-first order conditions. All experiments were done at 20° except those marked with an asterisk, which were done at 22° and with 0.01 M NaCl in the bis-Tris buffer (from Ref. 26). Saturating concentrations of organic phosphates were used.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer</th>
<th>pH</th>
<th>k (s⁻¹)</th>
<th>( I'(x) \times 10^{-4} \ s^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb Abruzzo</td>
<td>0.05 M bis-Tris</td>
<td>7</td>
<td>9-17.5</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>0.2 M phosphate</td>
<td>7</td>
<td>24</td>
<td>( \sim 0.6-2.1 )</td>
</tr>
<tr>
<td></td>
<td>0.05 M bis-Tris + IHP</td>
<td>7</td>
<td>69-45</td>
<td>( \sim 0.6-1.25 )</td>
</tr>
<tr>
<td></td>
<td>2% borate</td>
<td>9.2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>HbA</td>
<td>0.05 M bis-Tris</td>
<td>7</td>
<td>22</td>
<td>2.3-3.2*</td>
</tr>
<tr>
<td></td>
<td>0.2 M phosphate</td>
<td>7</td>
<td>35</td>
<td>( \sim 1.6-3.3 )</td>
</tr>
<tr>
<td></td>
<td>0.05 M bis-Tris + IHP</td>
<td>7</td>
<td>62-81</td>
<td>( \sim 0.8-1.3* )</td>
</tr>
<tr>
<td></td>
<td>2% borate</td>
<td>9.2</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Oxygen Bohr effect and values of \( n \) at 50% saturation of Hb Abruzzo (3 to 5 mg/ml) in 0.2 M potassium phosphate (open symbols) and 2% sodium borate (closed symbols) at 20°. The continuous line without experimental points shows the Bohr effect for HbA under the same conditions.

As with HbA, the CO recombination is markedly biphasic. In inorganic phosphate the magnitude of the fast phase of CO recombination with Hb Abruzzo is reduced, and the time course of the slow phase takes on an autocatalytic character that is more pronounced than in HbA. Fig. 6 shows that for both HbA and Hb Abruzzo the percentage of the fast phase of CO combination after full flash photolysis is dependent on protein concentration. In 0.05 M bis-Tris at pH 7, the percentage of the fast phase observed with Hb Abruzzo is large relative to that observed with HbA. As with HbA, the percentage of the fast phase does not depend on the CO concentration (from 33 to 145 \( \mu \)M CO). This implies that in Hb Abruzzo, as in HbA, the R→T transition is fast relative to the process of ligand binding. Fig. 6

Fig. 4. Time course of oxygen dissociation from Hb Abruzzo after rapid mixing with buffers containing dithionite. Symbols represent solutions in 0.05 M bis-Tris (○), 0.2 M inorganic phosphate (△), and 0.05 M bis-Tris + IHP (□). Hb Abruzzo at 37 \( \mu \)M (heme) before mixing, 20°, pH 7. Observation wavelength was 460 nm.

Fig. 5. Time course of CO combination after complete flash photolysis of Hb Abruzzo in 0.05 M bis-Tris (○) and 0.2 M inorganic phosphate (△). CO at 50 \( \mu \)M, Hb Abruzzo at 6.8 \( \mu \)M (heme) at 20°, pH 7. Observation wavelength was 437.5 nm.

Fig. 6. Effect of 1 mM IHP (filled symbols) on the protein concentration dependence of the percentage of the fast phase of CO combination observed after complete flash photolysis of the CO derivatives of Hb Abruzzo (○, △) and HbA (□, ■) in 0.05 M bis-Tris, pH 7, 20°. The CO concentration was 50 \( \mu \)M and observation wavelength was 437.5 nm.
shows that IHP reduces the percentage of the fast phase, this
effect being much more pronounced in the case of the abnormal
hemoglobin. Even larger effects of IHP have been reported in
flash photolysis experiments with Hb Bethesda, β145 Tyr→His
(12) and carboxypeptidase-digested hemoglobin (13).

The apparent second order velocity constant for CO binding
to Hb Abruzzo in rapid mixing experiments is plotted in Fig. 7
as a function of the fractional saturation with CO. In inorganic
phosphate buffer the apparent “on” constant changes by a
factor of about 3.5 as successive CO molecules are bound. When
IHP is added to the reactants, there is less difference between
the initial and final rates of CO binding.

**Discussion**

The preceding results indicate that substitution of arginine for
histidine at the β143 position shifts the hemoglobin tetramer somewhat toward the high affinity “R” conformation character-
istic of liganded hemoglobin. The functional effects of this sub-
stitution are of particular interest because of the involvement of
β143 His in the binding of organic and inorganic phosphates (2,
3). As has been demonstrated by this study, the properties of Hb
Abruzzo differ appreciably in the presence and absence of anions.
For a detailed discussion of previous studies of anion interactions
with normal and variant hemoglobins we refer the reader to two
excellent reviews (see Refs. 14 and 15).

In bis-Tris buffer Hb Abruzzo shows reduced cooperativity and
increased oxygen affinity relative to HbA. Equilibrium studies
show that the oxygen affinities of Hb Abruzzo and HbA differ
most greatly at low levels of saturation. This appears to be
largely attributable to the high oxygen affinity of the abnormal
β chains of Hb Abruzzo. Properties of the isolated chains are
described in detail in the following article. In bis-Tris buffer at
neutral pH, the kinetic data on oxygen dissociation from Hb
Abruzzo differ significantly from data for HbA. The apparent
initial rate of deoxygenation is about one-half as fast as the ap-
parent final rate, which is somewhat slower than that observed
throughout the process of oxygen dissociation from HbA. If Hb
Abruzzo is indeed more dissociated from HbA, then at a concen-
tration of 37 μM (heme concentration prior to mixing) some
dimers would be present. In their absence the initial and final
rates might be even more different. The kinetics of CO combina-
tion are also changed in Hb Abruzzo; and the large fast phase of
CO combination, after flash photolysis at low protein con-
centration, suggests greatly increased subunit dissociation relative
to CO-HbA. Previous studies have shown that subunit
dissociation of the CO derivative of HbA is reflected in the
kinetics of CO combination after complete flash photolysis (13).

In contrast to its behavior in bis-Tris buffer, Hb Abruzzo in
inorganic phosphate buffer shows a normal level of cooperativity.
The Bohr effect is also normal although the oxygen affinity re-
 mains higher than that of HbA. The differences between HbA
and Hb Abruzzo at pH 9.2 in borate buffer may be due to in-
stability of the Hb Abruzzo tetramer at high pH or to differences
in interactions of the two hemoglobins with the borate ion. The
rate of oxygen dissociation conforms to the kinetics expected for
a first order process when measured at 437.5 nm. The time course
was not investigated at other wavelengths where dimer to
tetramer aggregation would be apparent as a slow “drift” phase
(16, 17). The rate of oxygen dissociation, which is slower for the
abnormal hemoglobin than for HbA, appears to be the major
kinetic contribution to the higher oxygen affinity of Hb Abruzzo
since the “on” constant (for CO) is less than or equal to that for
HbA. From the kinetics of CO binding it appears that the process
of ligand combination is affected by the substitution, with
greater differences between the apparent initial and final rates of
CO binding to Hb Abruzzo in inorganic phosphate buffer than
those observed in studies with normal hemoglobin. In normal
hemoglobin in inorganic phosphate buffer the initial and final
rates differ only by a factor of 2 (9). The factor of nearly 4 ob-
erved with Hb Abruzzo may indicate that there is a greater dif-
ference between the CO reactivities of the “T” and “R” forms
of Hb Abruzzo than for HbA. IHP reduces the difference be-
 tween the initial and final rates of CO binding to Hb Abruzzo.
This is also true in experiments with HbA. The final rate appears
to be attained at a somewhat lower degree of CO saturation in
the presence of IHP.

Although a complete structural explanation for these functional
differences is not possible without detailed crystallographic data,
some inferences may be made. On the basis of x-ray crystallo-
graphic studies, β143 His was shown to be involved in DPG and
IHP binding to normal deoxyhemoglobin (2, 3). The same resi-
due is also involved in small anion binding (3). Substitution of
arginine for histidine does not remove positive charge from the
binding site so that decreases in anion binding on this account
are not expected. The pK of arginine in solution is much higher
than for histidine, and thus the β143 position in Hb Abruzzo may
be “available” for anion binding at higher pH values than in
normal hemoglobin. The extra length and greater flexibility of the
arginine side chain might be expected to make anion inter-
actions “easier” and more influential in that the positively
charged guanidium group of arginine may be more accessible.
Since the arginines at the two β143 positions are relatively close
to the other positively charged groups in the central cavity where
DPG and IHP are bound, it is possible that repulsive electro-
static interactions are present in stripped Hb Abruzzo that are
absent in stripped HbA. Charge effects may produce changes in
the oxygen affinity of the protein through effects on both the
tertiary and quaternary structures. The tertiary effects are ap-
parent from studies of the isolated chains as described in the
following article. As to the quaternary effects, repulsive inter-
actions would partially destabilize formation of the deoxy con-
formation and thereby result in increased oxygen affinity. If the
dissociation into subunits is greatly increased this might also
contribute to increased oxygen affinity (18). Alternatively, the
extra length and flexibility of the arginine side chain might allow
this residue to form salt bridges that directly strengthen the
liganded conformation and thereby result in increased oxygen
affinity. Analogy with Hb Little Rock, β143 His→Gln, where the

![Fig. 7. Apparent CO combination velocity constant observed in
rapid mixing experiments for Hb Abruzzo in 0.2 M inorganic
phosphate buffer (O) and in 0.05 M bis-Tris, 7 mM IHP (x) as a
function of the extent of CO binding (Y), CO at 50 μM and Hb
Abruzzo at 5 μM (heme) before mixing, 20°, pH 7. Observation
wavelength was 437.5 nm.](image-url)
oxy conformation is thought to be stabilized by hydrogen bonding between β143 Gln and α139 Asp (19), would suggest this alternative. However, our results suggest an increased subunit dissociation in Hb Abruzzo, indicating that attraction between subunits is decreased instead of increased.

The cationic groups of normal β chains, which form salt bridges with the anionic groups of DPG and IHP, include the NH₂-terminal valines, the histidine groups at β2 and β143, and β82 lysine (2, 3). That these positively charged groups are not equally involved in binding of small anions was indicated by x-ray crystallographic work (2, 3) and is consistent with the data summarized below. In Lemur hemoglobin, the presence of lysine at β2 does not seem to affect chloride binding but does reduce effects of DPG binding (20). Hb Leiden presents another variation; a deletion in the polypeptide chain at position p6 or 7 decreases both organic and inorganic anion interactions (21). Furthermore, the substitution of arginine for β143 His in Hb Abruzzo has quite different effects than substitution of arginine for β2 His in Hb Deer Lodge. Another example of the somewhat obvious fact that the cationic groups of the binding site are not equivalent is the reduced DPG affinity of Hb Little Rock (β143 His→Gln) and the apparently unchanged affinity of this variant for chloride and inorganic phosphate (19). It should be noted, however, that anion binding effects may be reduced without chemical alteration of any of the residues known to be involved in the binding site. This has been shown in studies of the functional properties of carboxypeptidase-digested hemoglobins (22, 23). The interaction of small anions with Hb Abruzzo has been measured directly in an NMR study of chloride binding (24).

The preceding results on the kinetics of oxygen dissociation and on the kinetics of CO combination with Hb Abruzzo clearly indicate that both organic and inorganic phosphates interact with the liganded form of this hemoglobin as well as with its unliganded form. The addition of organic phosphates to the fully liganded form of normal human hemoglobin produces spectral changes which appear to reflect changes in quaternary and tertiary structure (25). Kinetic studies indicate that oxygenated tetramers (but not dimers) of normal human hemoglobin can bind IHP (26). The large effects of both organic and inorganic phosphates on the kinetic and equilibrium properties of Hb Abruzzo appear to be due to the tendency of these anions to overcome the destabilization of the deoxy (tertiary or quaternary) conformation that is caused by the presence of arginine at the β143 (H21) position.

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