The Acetylation State of Human Fetal Hemoglobin Modulates the Strength of Its Subunit Interactions: Long-Range Effects and Implications for Histone Interactions in the Nucleosome†

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Received September 13, 2000

ABSTRACT: The source of the 70-fold increased tetramer strength of liganded fetal hemoglobin relative to that of adult hemoglobin between pH 6.0 and 7.5 reported earlier [Dumoulin et al. (1997) *J. Biol. Chem. 272*, 31326] has been identified as the N-terminal Gly residue of the γ-chain, which is replaced by Val in adult hemoglobin. This was revealed by extending the study of the pH dependence of the tetramer−dimer equilibrium of these hemoglobins into the alkaline range as far as pH 9. From pH 7.5 to 9.0, the 70-fold difference in the association equilibrium constant between hemoglobins F and A lessened progressively. This behavior was attributed to the difference in the pK_a 8.1 of Gly-1(γ) compared to the pK_a 7.1 value of Val-1(β) of hemoglobins F and A, respectively. Evidence for this conclusion was obtained by demonstrating that natural hemoglobin F_1, which is specifically acetylated at Gly-1(γ) and hence unable to be protonated, behaves like HbA and not HbF in its tetramer−dimer association properties over the pH range studied. An increased degree of protonation of the γ-chain N-terminus of hemoglobin F from pH 9.0 to 8.0 is therefore suggested as responsible for its increased tetramer strength representing an example of transmission of a signal from its positively charged N-terminal tail to the distant subunit allosteric interface where the equilibrium constant is measured. An analogy is made between the effects of acetylation of the fetal hemoglobin tetramer on the strength of its subunit interactions and acetylation of some internal Lys residues within the N-terminal segments of the histone octamer around which DNA is wrapped in the nucleosome.

Differences in the functional properties of HbF[1] and HbA, such as O_2 binding and its pH dependence (alkaline Bohr effect) as well as the effects of allosteric regulatory molecules in the physiological pH range, control the important process involving the transfer of O_2 from the maternal to the fetal circulation (*1, 2*). The control of other important events such as pH fluctuations and CO_2 removal is not as well understood although the general buffering effect of hemoglobin as well as the identity of specific sites that react with CO_2 are known (*3, 4*). Fetal hemoglobin is a very effective inhibitor of sickle hemoglobin polymerization. Pharmacological agents such as hydroxyurea are efficient inducers of HbF synthesis and are currently used clinically (*5*).

A variable fraction of the γ-subunit of HbF is acetylated at its N-terminus. The ζ-subunit N-termini of two of the three human embryonic hemoglobins, which are the precursors of fetal hemoglobin during development, are completely acetylated, but in no case is the purpose of acetylation nor its effect on Hb structure known. Although there is some information available on the enzymes that either add (*6*) or remove (*7*) acetyl groups from N-terminal amino acid residues, the biological role of acetylation either at the N-terminus (N^{α}) or at internal Lys residues (N^{ϵ}) is not known. The HbF_1/HbF system provides the opportunity to address this question.

We reported recently that the lower response of HbF to the allosteric regulator 2,3-DPG relative to that of HbA was due, in large part, to the Glu(β) to Asp(γ) substitution at position 43 at the tetramer−dimer allosteric interface, the only amino acid substitution between these hemoglobins in this region (*8*). A recombinant Hb containing only this substitution but having all the other β-subunit amino acids including those at the DPG binding site had the same moderately increased intrinsic P_{50} of HbF as well as its decreased O_2 release in the presence of saturating DPG; i.e., the E43D(β) mutant was unable to perform the complete R to T transition typical of HbA even though the same DPG binding site was present in both. We also observed that this liganded recombinant Hb had a somewhat stronger tetramer−dimer interface, although it represented only a fraction of the tetramer strength of HbF itself. We also reported (*8*) that a recombinant Hb with Val-1(β) replaced by Gly, the amino acid at the N-terminus of the γ-subunit, also had a moderately increased tetramer strength at pH 7.5. This result was instrumental in our performing the studies described here.

† Supported in part by NIH Grants HL-15157, HL-18819, and HL-58512.

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[1] Abbreviations: Hb, hemoglobin; HbF, fetal hemoglobin; HbA, adult hemoglobin; R state, liganded conformation; T state, unliganded conformation; K_a, equilibrium constant or tetramer−dimer association constant; K_d, tetramer−dimer dissociation constant.

10.1021/bi002157+ CCC: $20.00
Published on Web 01/18/2001

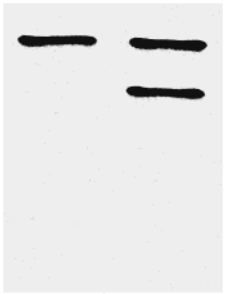

FIGURE 1: Isoelectric focusing of HbF_1 and HbF. Lane A contains purified HbF_1, and lane B contains HbF_1 and HbF prior to their separation on Mono S as described in the text. The anode is up, and the cathode is down.

EXPERIMENTAL PROCEDURES

Materials. Hemoglobin A (sometimes called HbA_o) was purified from the blood of a normal individual. After initial purification of the dialyzed hemolysate on CM-52 resin, a homogeneous HbA was obtained using an FPLC Mono S HR 5/5 column as described previously (*9*−*11*). Hemoglobin F (also referred to as HbF_o) and hemoglobin F_1 (its γ-Ac N-terminal counterpart) were purified from human postpartum umbilical cord blood. A fraction containing both hemoglobins, from which HbA had been removed on Bio Rex resin in Dr. Robert Bookchin's laboratory by Tania Balazs, was further purified by us on Mono S using a gradient of 10 mM sodium phosphate, pH 6.3, 0.5 mM EGTA, and 0.5 mM EDTA; the same buffer containing 1.0 M NaCl was in the second gradient chamber. Two peaks were collected—the first corresponding to HbF_1 and the second to HbF, consistent with the difference in their net charges.

Characterization of Hemoglobins. The isolated hemoglobins had the correct spectral properties with no evidence of oxidation to metHb. Isoelectric focusing on Resolve pH 6−8 range gels (*9*−*11*) was used to establish the purity of HbF_1, HbF, and HbA from the Mono S column (Figure 1). Amino acid analysis on a Beckman 6300 analyzer after acid hydrolysis overnight in 6 N HCl established that purified HbA did not contain any Ile whereas HbF contained eight Ile per tetramer. All other amino acids were within the theoretical range. HbF_1 and HbF were judged to be devoid of any contaminating HbA as confirmed by mass spectrometry, which was performed in the laboratory of Dr. Brian Chait at Rockefeller University using methods and instruments described previously for recombinant hemoglobins expressed in our laboratory (*9*−*11*).

Tetramer Strength (Tetramer−*Dimer Association Constant, Equilibrium Constant)*. These studies were performed on the liganded forms (either CO or oxy) of HbF_1, HbF, and HbA. Protein concentrations were accurately determined by amino acid analysis, as described previously (*9*−*11*). Samples (usually six to eight) of each hemoglobin in 100 μL of 150 mM Tris−acetate of varying pH at protein concentrations above and below the equilibrium constant were applied to a Pharmacia FPLC Superose-12 HR 10/30 column in the same buffer. The system was equipped with Pharmacia Director software, and the K_d (the dissociation constant) was calculated by the procedures described previously (*9*−*11*). The reciprocal, $1/K_d$, was then calculated to give the K_a value, which was plotted as a function of pH.

Table 1: Summary of Properties of Purified Natural Hemoglobins Used in This Study

	hemoglobin		
	A	F	F_1
N-terminal residue (β, γ)	Val-1(β)	Gly-1(γ)	Ac Gly1-(γ)
pK_a of N-terminus (β or γ)	7.1[a]	8.1[b]	unable to be protonated
FPLC Mono S elution order	#3	#2	#1
Isoelectric focusing	one band	one band	one band
Mass spectrometry[c]			
α	15126 ± 3 (15126)[d]	15126 ± 3 (15126)	15126 ± 3 (15126)
β	15868 ± 3 (15868)	−	−
γ	−	15995 ± 7 (15995)	−
γ^{Ac}	−	−	16042 ± 7 (16037)

[a] From ref *3*. [b] From ref *4*. [c] From ref *10*. [d] Mass values in parentheses are theoretical.

The data from pH 6.0 to 7.5 for HbA and HbF reported earlier as K_d values (*9*) were expressed as K_a values. We chose this manner of data presentation since it better suits the objectives of this report. At pH values from 7.5 to 9.0, the acetate anion concentration would be progressively lower, but this did not affect the K_a values since acetate, unlike chloride, binds very weakly to hemoglobin. Furthermore, we also found that the difference in K_a between HbF and HbF_1 (see below) could not be explained by buffer effects.

RESULTS AND DISCUSSION

Hemoglobins. The experiments described here were all performed with natural hemoglobins that were extensively purified from human blood and characterized. The purity of each of the three hemoglobins—A, F, and F_1—was established according to the criteria listed in Table 1.

Previous Results on Tetramer Strength of Hemoglobins A and F. An increased extent of tetramer dissociation of liganded HbA to dimers with decreasing pH has been described in detail by Antonini et al. (*12*) and later by Atha and Riggs (*13*) and by Chu and Ackers (*14*). Our results for HbA and HbF in the pH range of 6.0−7.5 (*10*) (see below) showed essentially the same profile although HbF had an approximately 70-fold increase in tetramer strength over this pH range. The slopes of these profiles are between 1 and 2, indicating that about this number of protons is absorbed per dimer pair upon tetramer dissociation, or the same number of protons is released per tetramer upon association of dimers. The amino acid side chain(s) responsible for this effect in this pH range has (have) not yet been identified.

Relative Extent of Tetramer Formation at Higher pH. Under slightly alkaline conditions, both Atha and Riggs (*13*) and Chu and Ackers (*14*) observed a decreased association for HbA. We investigated the association behavior of HbA, HbF, and HbF_1. As shown in Figure 2 at a single hemoglobin concentration, all three hemoglobins formed the tetrameric structure to approximately the same extent at pH 9.0. With decreasing pH from pH 9.0 to 7.5, HbF became increasingly tetrameric whereas HbF_1 and HbA showed the opposite behavior. The Hb concentration in the experiments in Figure 2 was 5×10^{-8} M (expressed as tetramer), which is in the same range as the H^+ concentration from pH 7.5 to 8.0,

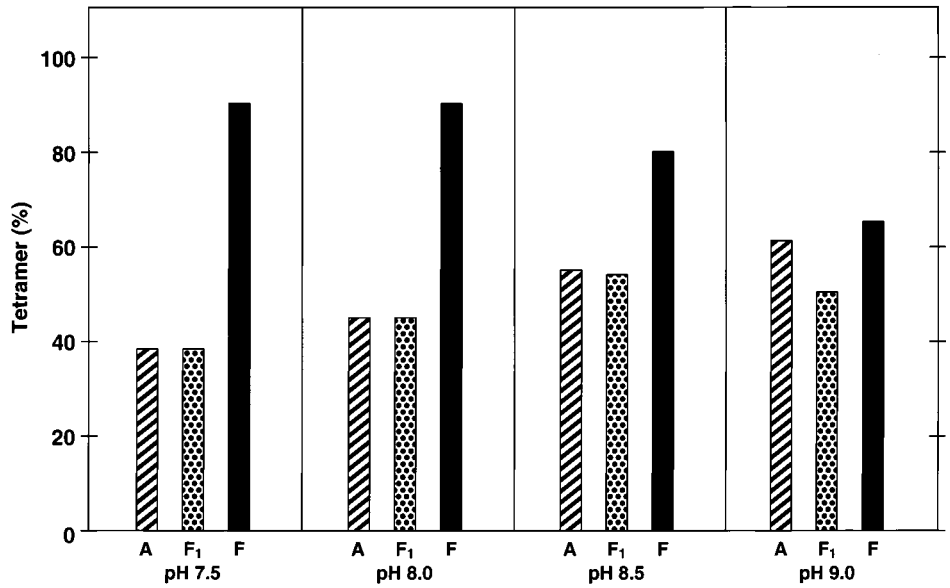

FIGURE 2: Relative amounts of tetrameric hemoglobins F, F_1, and A. The percent tetramer was determined on Superose-12 as described previously (*7*, *9*). The elution buffer was 150 mM Tris−acetate, pH 7.5, 8.0, 8.5, or 9.0. At each pH, the column was calibrated with the cross-linked tetrameric Hb and the mutant dimeric Hb. The concentration for each Hb was 5.0×10^{-8} M (as tetramer).

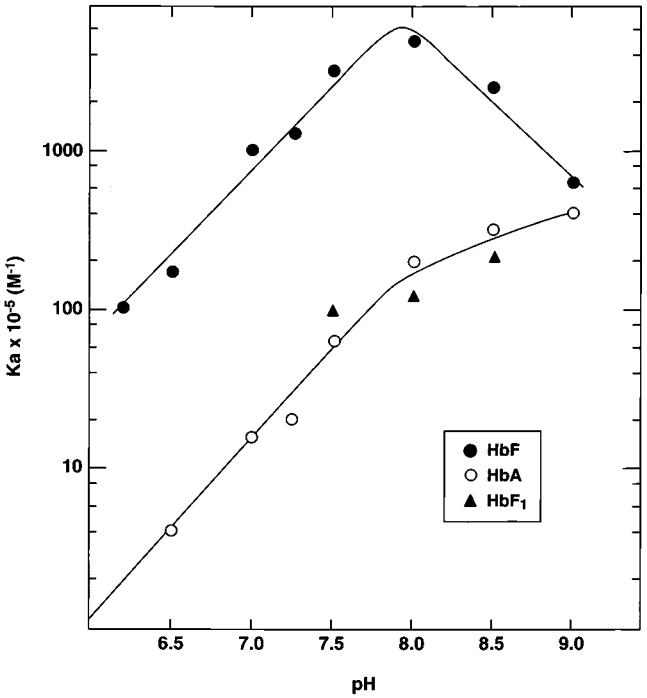

FIGURE 3: Association constants (K_a) of hemoglobins F, F_1, and A as a function of pH. The constants were calculated as described in the text. The buffer used for the Superose-12 column (*7*, *9*) was 150 mM Tris−acetate adjusted to the appropriate pH with acetic acid.

suggesting that protonation may be responsible for the selective tightening of the HbF structure, although alternative explanations such as a more general electrostatic effect involving subunits are not excluded (see below). We studied this behavior in more detail by measuring the association constants (K_a) over a larger pH range, as described next.

Tetramer−Dimer Association Constants. The effect of pH on the equilibrium constants, K_a, for HbF, HbF_1, and HbA was determined as shown in Figure 3. Whereas the K_a values for HbA proceeding from pH 9.0 to 8.0 continued to decrease moderately, reflecting the behavior shown in Figure 2, a dramatic increase was found for HbF. K_a values for these hemoglobins converged at pH 9.0. The negative slope of the HbF profile from pH 8 to 9 generally approximates the positive slopes for both hemoglobins A and F in the pH range of 6.0−7.5. In contrast, the naturally acetylated HbF_1 variant, which has the same amino acid sequence as HbF except for acetylated Gly-1(γ), which removes its positive charge, behaves just like HbA in the pH 7.5−9 range (Figure 3). This result clearly shows that the positive charge of Gly-1(γ) acquired during its protonation from pH 9.0 to pH 8.0 is mainly responsible for the increased tetramer strength of HbF.

Interpretation. For HbF, at pH 9.0 tetramer strength approaches that of HbA (and HbF_1) (Figure 3) since most of the positive charge at Gly-1(γ) or Val-1(β) is neutralized. With decreasing pH to about 8.0, Gly-1(γ) absorbs protons ($pK_a = 8.1$), causing HbF tetramers to become more associated. From pH 9.0 to 8.0, HbA tetramer strength does not increase since Val-1(β) with a pK_a of 7.1 (*3*) does not become significantly protonated. HbF_1 behaves like HbA in the alkaline pH range since the γ-Ac N-terminus cannot be protonated (at any pH). At pH values below 8.0, tetramer association for hemoglobins A and F weakens with the same profile down to pH 6.0. However, the increased tetramer strength of HbF acquired between pH 9.0 and 8.0 relative to HbA persists in the pH 6−8 range so that at physiological pH the concentration of $\alpha\gamma$ dimers from HbF will always be less than the concentration of $\alpha\beta$ dimers from HbA. Hence, HbF has more buffering capacity because of its enhanced ability to absorb protons. This difference may contribute to the different functional responses of each type of hemoglobin tetramer, e.g., their alkaline Bohr effects, which has long been considered to be essential for the transfer of O_2 from maternal HbA to fetal HbF (*1*, *2*); i.e., more of the protons released upon oxygenation of T state tetramers of HbA would be reabsorbed by the $\alpha\beta$ dimers than would occur with the lower concentrations of $\alpha\gamma$ dimers from HbF. Therefore, the net proton release during oxygenation of tetramers would be greater for HbF than for HbA.

Three major conclusions from the results in Figure 3 are as follows:

(1) The increased buffering capacity of fetal hemoglobin to absorb protons may help offset the low pH of fetal blood due to its higher CO_2 content (*1*).

(2) Just as the Bohr effect, which is considered to have a physiological role in the O_2 transfer between HbA and HbF (*1, 2, 18*), is due to the reversible protonation of a small number of specific sites in the T and R state tetramers [the N-terminus of the α-subunits and the C-terminus of the *β*- or *γ*-subunits (*18*)], the specific protonation of a different site, Gly-1(*γ*), confers a selective tetramer strength to HbF. In this instance, however, it is the tightening of an interface in the R state that is involved rather than its rearrangement, which occurs in the alkaline Bohr effect involving R and T states.

(3) Protonation of the N-terminus of the *γ*-subunit affects the allosteric $\alpha_1\gamma_2$ interface, which is a considerable distance away. This may represent an example of transmission of a signal between remote parts of the protein as discussed below.

Long-Range Effects or Electrostatic Coupling between Remote Sites. In earlier reports (*19*−*21*), particularly those on Hb Felix (*19*) where the A-helix of the *γ*-subunit with eight substitutions linked to the remaining helices of the *β*-subunit was expressed with the α gene, the recombinant hybrid Hb had a greatly increased strength at its tetramer−dimer interface, which approached that of HbF. This allosteric interface is distant from the replacement sites, so the existence of long-range remote effects in hemoglobin was suggested. The present report describes the simple reversible protonation of a single site. The N-terminus is at the end of a three residue nonhelical segment (small tail in gray at top of Figure 4) before the A-helix, which begins at residue 4 of both *β*- and *γ*-subunits. It is the N-terminus at this site in HbF where the protonation that generates tetramer strength occurs. In the deoxy HbF structure, Frier and Perutz (*22*) showed that this tail protrudes deeper into the central cavity than does the corresponding N-terminal tail for deoxy HbA; the structure of oxy HbF has not been solved, but it is assumed to have the same property. Subunit association with α-chains making up the allosteric interface takes place at the FG corner (the G-helix is in gold in the middle of Figure 4) and at the C-helix (at the bottom in Figure 4) of the *γ*-subunit. These regions are remote from the N-terminus where the protonation occurs leading to strengthening of the allosteric interface. We have used the term "electrostatic coupling" between distant side chains of opposite charge in hemoglobin to produce changes in tetramer strength and function (*8*). The details of the transmission of the effects of the protonation of Gly-1(*γ*) in HbF to its $\alpha_1\gamma_2$ allosteric interface remain to be elucidated. It is possible that the central cavity of the hemoglobin tetramer into which the charged N-terminal tails protrude and through which part of the allosteric interface passes provides the medium for the transmission of such a signal by some unknown mechanism.

Alternatively, the pH effect on the tetramer−dimer equilibrium could be due to general electrostatic effects involving charges within the different subunit types. Even though it could be fortuitous that HbA and HbF_1 have similar K_a values between pH 8.0 and 9.0 considering the 39 of 146 amino acid differences between *β*- and *γ*-subunits, the large difference in K_a values for HbF and HbF_1 in the same pH range cannot be explained in this context since they have the same sequence. Also, it is conceivable that the acetyl group in HbF_1 simply forces the central cavity and the allosteric interface into more open conformations. Such a mechanism can be assessed when the structure of HbF_1 is solved.

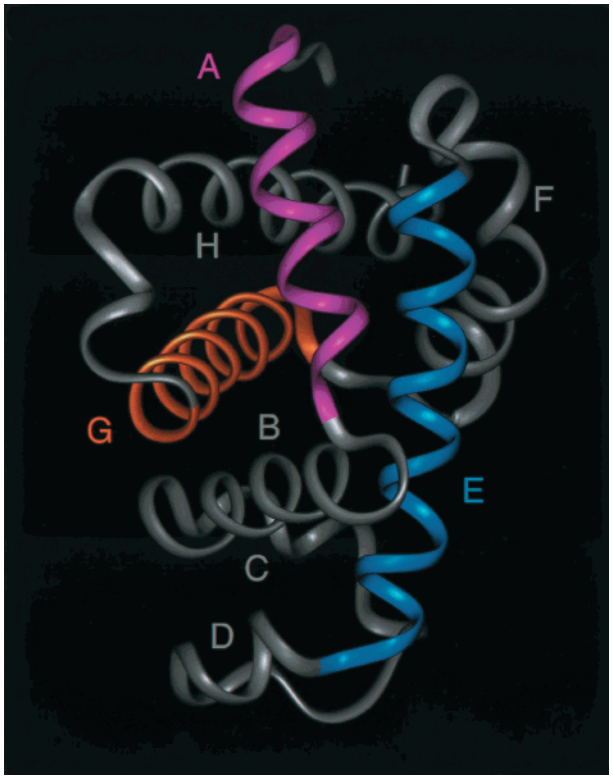

FIGURE 4: Structure of hemoglobin A *β*-subunit. The locations of the site of protonation corresponding to Gly-1 of the *γ*-subunit as well as the designations of the helices comprising the allosteric interface are described in the text.

Reichard et al. (*23*) used the term cross-talk to describe the long-range relationship between the two allosteric sites, i.e., the activity site and the specificity site in ribonucleotide reductase. The manner in which these sites correspond with one another is involved in the regulation of this important enzyme.

Analogy with Acetylation of the Histone Octamer in the Nucleosome. The crystal structure of the nucleosome where DNA is wrapped around the histone octamer shows that the lysine residues that are subject to acetylation are located on N-terminal tails distant from the subunit interfaces between helical portions of the histones (*24*). It has been known for many years that acetylation of these lysine residues is strongly correlated with activation of transcription (*25*), but the mechanism is not known although there is evidence that such acetylation leads to less compact nucleosome and chromatin structures (*26*). The effects of N-terminal acetylation on loosening the distant subunit interfaces of the fetal hemoglobin tetramer and the interfaces of the histone octamer may occur by a common mechanism.

ACKNOWLEDGMENT

We gratefully acknowledge the expert assistance of Roger Avelino in preparing the manuscript.

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BI002157+