Ligand-Dependent Aggregation of Chicken Hemoglobin A_I

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Summary

The hemoglobin A_I component of the white leghorn chicken may potentially provide an animal model for the in vitro aggregation behavior of human hemoglobin S. In solutions of low ionic strength, it has been found to undergo a striking loss of solubility upon deoxygenation, leading to the formation of macromolecular aggregates. This property is not shared by the other major chicken hemoglobin component, designated A_{II}. Compositional and NH_2-terminal sequence analysis indicate that extensive primary structural differences reside in the alpha chains of these two hemoglobins. The beta chains appear to be identical. Examination by electron microscopy suggests that the deoxyhemoglobin A_I forms microcrystalline arrays. The A_I component shows diminished reactivity with $^{13}CO_2$, as judged from ^{13}C NMR measurements.

Hemoglobin A_I from white leghorn chickens has been found to undergo ligand-dependent aggregation in vitro in a pattern similar to that of human hemoglobin S (1-5). Blood from immature chickens was collected in sodium citrate, hemolysates were prepared (6) and the two major chromatographic components A_I and A_{II} were isolated (Fig. 1). The indicated pools were homogeneous by polyacrylamide gel and cellulose acetate electrophoresis at pH 8.6. The two hemoglobin components were compared with respect to (1) reversible effects of ligand binding on aggregation under various conditions, (2) interaction with $^{13}CO_2$, (3) amino acid composition and NH_2-terminal sequences of the subunits, and (4) ligand-dependent microstructure.

Upon deoxygenation in a tonometer (8) unbuffered solutions of HbA_I but not HbA_{II} became turbid and opalescent. The uppermost curve (•) in Fig. 2 for the deoxy HbA_I in 0.05 M NaCl at pH 7.5 shows the strong decrease in transmission at 700 nm and deviation from Beer's law accompanying the

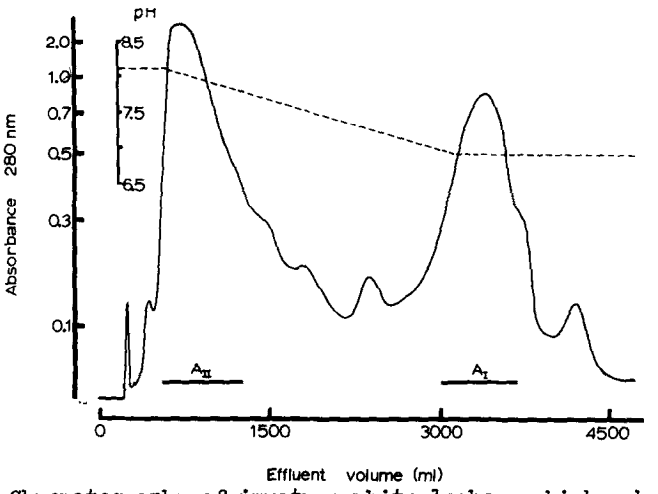

Figure 1. Chromatography of immature white leghorn chicken hemoglobin hemolysate on Sephadex DEAE A-50. Column dimensions were 5.5 x 60 cm. Elution was at 4° by 0.05 M Tris-HCl buffer, pH 8.1 to 6.9, as indicated by the dashed curve. Hemoglobin component A_{II} eluted from 560 ml to 1300 ml, and A_I from 3000 ml to 3700 ml, as shown by the underscoring. The nomenclature of the hemoglobins is based on their order of elution on cation exchange chromatography (7).

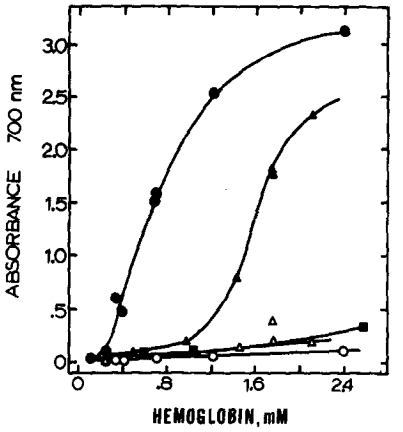

Figure 2. Concentration-dependent absorbance at 700 nm of chicken A_I hemoglobin under various conditions, at pH 7.50 ± .07, 25°, and 2 mm path-length cell. • Hb, 0.05 M NaCl; ▲ Hb ($\alpha_2^c\beta_2^c$) 0.05 M NaCl; Δ HbO_2 ($\alpha_2^c\beta_2^c$), 0.05 M NaCl; o HbO_2, 0.05 M NaCl; ■ Hb, 0.1 M phosphate buffer. Concentrations, expressed as heme content, were determined as the cyanoferri derivative, assuming a millimolar extinction coefficient of 11.1. In all cases, the oxy-determinations were made following the deoxy measurements.

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aggregation. The lowest curve (o) shows the small absorbance of the solution of the HbA_IO_2 regained after air had been readmitted into the tonometer. The corresponding curves (▲) and (Δ) refer to the deoxy- and oxy-forms of HbA_I at the same pH and salt concentration, but following treatment (9) of

the HbA_IO_2 with a tenfold molar excess of recrystallized NaNCO at 37° and pH 6.9 for two hours. Loss of reactive NH_2-terminal groups was measured by automated Edman degradation on a Beckman model 890C Sequencer. Homocitrulline derived from lysine residues was measured (10). The results shown in Fig. 2 apply to a preparation containing on the average per tetramer 1.4 modified termini each of α- and β-chains, designated as $\alpha_2^C\beta_2^C$. Less than 2 lysine residues on the average were modified per tetramer. The results in Fig. 2 show that with this cyanate modified preparation the onset of aggregation was at higher concentration and the development appeared weakly inhibited. The time required after deoxygenation to achieve a constant level of turbidity, typically on the order of 30 minutes with the unmodified HbA_I, was now greater than 90 minutes. The aggregation was less readily reversed on readmission of O_2.

The effect of various buffers on the aggregation was observed qualitatively. Higher ionic strength generally decreased aggregation. For example, it was almost completely inhibited in 0.1 M phosphate at pH 7.5, Fig. 2. The tendency to aggregate was restored by dialysis into 0.05 M NaCl. At comparable concentrations, inhibition of aggregation relative to NaCl was observed for phosphate, Tris-HCl, and bicarbonate buffers. Stoichiometric addition of inositol hexaphosphate had the same effect. An analogous effect of Tris buffer on HbS gelation has been reported (11). HbA_{II} was soluble over the range of conditions examined.

The comparison between HbA_I and HbA_{II}, readily observed in terms of solubility in the deoxy form, could be extended for the dissolved species by ^{13}C Fourier transform nuclear magnetic resonance spectroscopy with CO_2, carbonate and bicarbonate isotopically enriched in ^{13}C. The samples were prepared and the ^{13}C spectral measurements were made as previously described for studies of carbamino formation in human hemoglobin (6,12,13). Fig. 3 shows spectra expressed in parts per million upfield of (external) CS_2, with HbA_I represented in the upper set and HbA_{II} in the lower set. Spectra A and D contrast

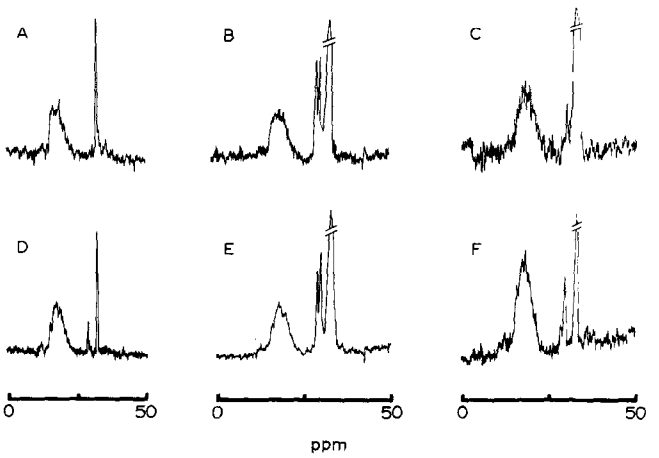

Figure 3. 15.1 MHz ^{13}C NMR spectra of chicken hemoglobins A_I (top), A_{II} (bottom) in 0.05 M NaCl under various conditions. (Except C, which was done at 25.2 MHz.) Temperature was 30°.
A, HbA_I pH 7.00, 7 mM carbonates; B, HbA_I pH 8.66, 68 mM carbonates; C, HbA_I CO pH 8.12, 67 mM carbonates; D, HbA_{II} pH 7.28, 13 mM carbonates; E, HbA_{II} pH 8.40, 70 mM carbonates; F, HbA_{II} CO pH 7.47, 29 mM carbonates; Hemoglobin concentration was between 6 to 12 mM.

the two proteins at neutral pH and relatively low levels of bicarbonate. Under these conditions HbA_I (Fig. 3A) shows no detectable carbamino signal, whereas HbA_{II} (Fig. 3D) shows a single carbamino resonance at 29.3 ppm. Spectra B and E show that at more alkaline pH values and higher carbonate levels the two proteins develop strong resonances at 29.2 ppm and also at 28.4 ppm upfield of CS_2, presumably representing the α-amino and ϵ-amino adducts respectively (13). Spectra C and F contrast HbA_I CO with HbA_{II} CO, respectively, under conditions of pH and carbonate levels that enhance relatively the carbamino formation by HbA_I CO to make the resonances at 29.2 ppm visible in both spectra. For comparable conditions the resonance at 29.2 ppm is much more pronounced in HbA_{II} CO, and that at 28.4 ppm is also more readily elicited. Any specific effect of either protein upon the bicarbonate-carbonate resonance near 32 ppm was not apparent in these studies.

Many points of primary structural difference between HbA_I and HbA_{II} appear to reside in the α-chain. The α- and β-subunits of HbA_I were separated on Biorex 70 according to Matsuda et al. (14). The composition of the

Table I

Amino Acid Composition of A_I α and β Subunits

Amino Acid	α Mol %	α Mol per 141 Amino Acids	β Mol %	β Mol per 142 Amino Acids
Lysine	7.42	10.4	7.12	10.0
Histidine	3.89	5.5	4.84	6.9
Arginine	3.17	4.5	4.34	6.2
Aspartic	9.29	13.1	9.05	12.8
Threonine	4.97	7.0	4.91	7.0
Serine	6.41	9.0	5.13	7.3
Glutamic	11.30	15.9	8.62	12.2
Proline	3.74	5.3	3.85	5.5
Glycine	6.26	8.8	5.70	8.1
Alanine	12.74	18.0	10.61	15.1
Cysteine	0.86	1.2	2.07	2.9
Valine	7.63	10.8	8.26	11.7
Methionine	2.45	3.5	1.07	1.5
Isoleucine	1.58	2.2	4.27	6.1
Leucine	10.08	14.2	12.82	18.2
Tyrosine	3.38	4.8	1.64	2.3
Phenylalanine	4.82	6.8	5.70	8.1

The purified subunits were hydrolysed for 24, 48 and 72 hrs. in 6 N HCl at 110°. Cysteine was determined as cysteic acid (15). The α and β subunits were assumed to have 141 and 142 non-tryptophane residues respectively as in HbA_{II}.

α-chain (Table I) differs markedly from that of the α-chain of HbA_{II}, whereas that of the β-chain is not readily distinguished from the reported composition of HbA_{II} (16,17). The previously reported differences between the proteins (18,19,20) in glutamic acid, lysine and histidine contents are ascribable to the differences in the α-chains. Automated Edman degradation confirmed for

21 residues of the β-chain of HbA_I the NH_2-terminal sequence reported by Matsuda et al. (17) for the β-chain of HbA_{II}. The NH_2-terminal sequence of the HbA_I α-chain was found to be H_2N-Met-Leu-Thr-Ala-Glu-Asp- extending a previous report by Moss and Thompson (19). Complete sequencing of the α-chain is in progress.

The HbA_I isolated here appears to differ from that described (22) earlier in which an acetylated NH_2-terminal group was reported, an assumption that formed the basis of a previous tentative correlation (12) with the very slight tendency of the component to form a carbamino adduct at low concentrations of CO_2.

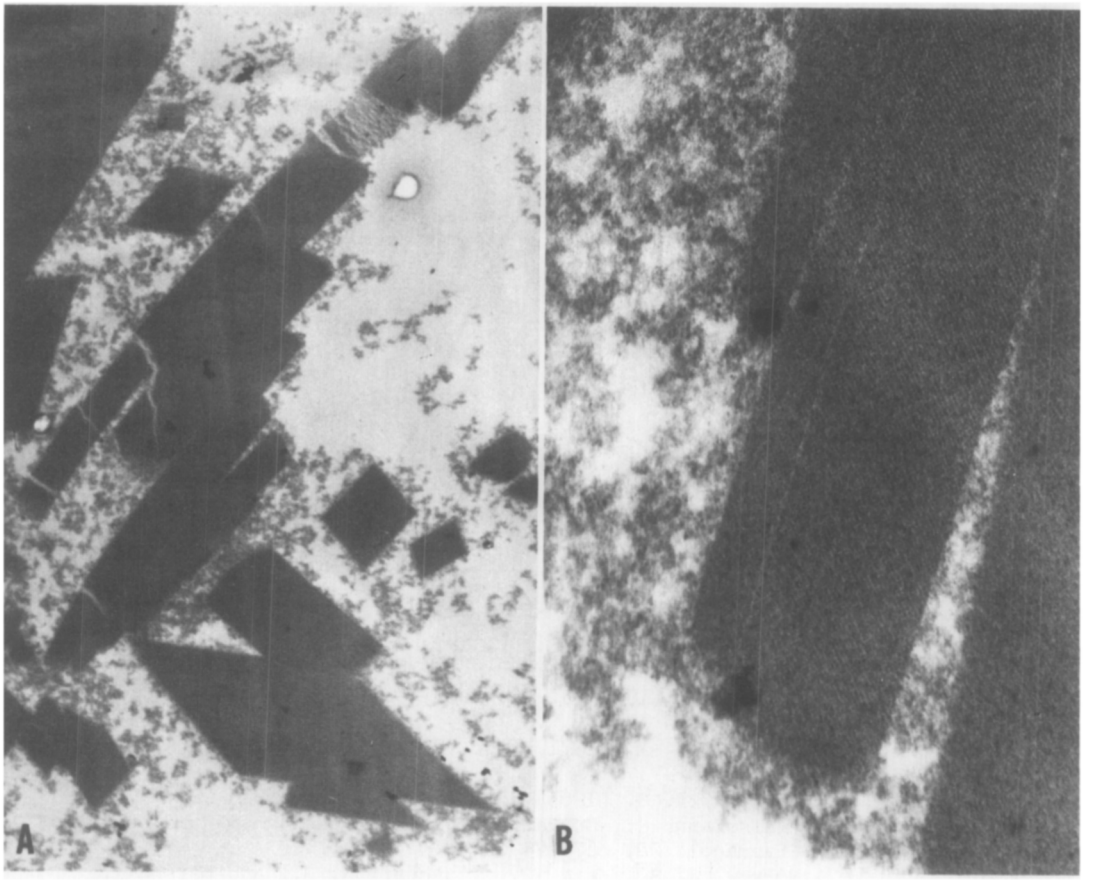

Figure 4. Electron micrographs of deoxy HbA_I X13,000 (a) and X100,000 (b). Deoxyhemoglobin was fixed in 1% glutaraldehyde for 12 hrs. at 4°, postfixed 1 hr. in 1% OsO_4 and embedded in Epon (24). Thin sections were stained with lead citrate. The rhomboid array seen in (b) has spacings of 90 to 110 Å in both dimensions.

Deoxygenation of HbA_I under conditions that lead to aggregation (Fig. 2) produces suspensions that appear to contain molecular order under the polarizing microscope and that scintillate when stirred. Under conditions of preparation for electron microscopy crystalline arrays are formed (Fig. 4). The nature of the aggregates under various conditions is under study to define the points of similarity and contrast with the properties of human HbS (1-5,9,23).

Acknowledgements

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