PREPARATION AND PURIFICATION OF CARBAMYLATED INTERMEDIATES OF HUMAN HEMOGLOBIN

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SUMMARY: The binding of organic phosphate to the amino terminal groups of hemoglobin has been exploited to develop a chromatographic method for preparing gram quantities of specifically carbamylated intermediates of human hemoglobin. The method does not require subunit separation or modification of sulfhydryl groups and it removes hemoglobin carbamylated at the ϵ-NH_2 groups of lysine. It provides a separation of $\alpha_2^C \beta_2^C$ from $\alpha_2^C \beta_2$ and from $\alpha_2 \beta_2^C$ by taking advantage of the alteration in net charge that results from the binding of organic phosphates to the unmodified beta subunits within the tetramer.

The amino-terminal carbamylated derivatives of hemoglobin have proved to be useful for investigating the role of the amino-termini in carbon dioxide binding and in the regulation of oxygen affinity through the Bohr effect and organic phosphate binding (1,2,3). Studies on intact homozygous-S erythrocytes have shown that carbamylation of the intracellular hemoglobin by exposure to cyanate reduces sickle cell formation and extends the lifetime of the cells (4,5). Furthermore, carbamylation of purified hemoglobin-S can inhibit gel formation (6,7). Efforts to study the physico-chemical properties of these modified hemoglobins have been hampered by the difficulty of preparing tetramers specifically substituted at either the alpha or beta chain amino-termini (8). Although such specifically carbamylated intermediates of human hemoglobin have been prepared by recombination of carbamylated alpha and beta subunits (2,3), present methods require blocking of the sulf-

Abbreviations: DPG, 2,3-diphosphoglycerate; IHP, inositolhexaphosphate; PMB, p-hydroxymercuribenzoate.

hydryl groups followed by quantitative regeneration. Complete regeneration of both sulfhydryl groups of the beta subunit is not always achieved (9). In addition, present methods do not provide a means of separating the subunits reacted at ϵ-NH_2 lysines. For abnormal hemoglobins that are often unstable and available in small quantities a general method is required that offers a better yield than the above method, is faster and does not require sulfhydryl modification. In addition, there is no general method for preparing native subunits of hemoglobins from other species. A method of preparing specifically

Preparative Steps	Purpose	Results
HbA_o + KNCO.	Carbamylation	--
(A) DEAE chromatography, elution by pH gradient.	Removal of ϵ-lysine-reacted hemoglobin.	Fig. 2 and Table I
(B) CM-cellulose chromatography, elution by pH gradient.	Separation of NH_2-terminal 4-NCO, 2-NCO, and O-NCO Hbs	Fig. 3 and Table II
(C) CM-cellulose chromatography of Hb-2NCO in presence of IHP, elution by pH gradient.	Separation of $\alpha_2^c \beta_2$ from $\alpha_2 \beta_2^c$	Fig. 4
Analytical Steps		
PMB reaction followed by CM-cellulose chromatography, elution by pH gradient.	Separation of α, α^c, β, and β^c followed by NH_2-terminal analysis.	Fig. 5 and Table III

Figure 1. Summary of preparation and analysis of carbamylated intermediates of human HbA_o. Details of each step are given in the text and in the figures and tables indicated. See text for abbreviations.

carbamylated horse hemoglobin has been reported (10), but we have been unable to apply it to human hemoglobin. Figure 1 summarizes a method for preparing specifically carbamylated intermediates of human hemoglobin that does not require chain separation or sulfhydryl modification and is capable of yielding gram quantities of each intermediate.

Hemoglobin A_o was separated from minor hemoglobin components and organic phosphates by the method of Williams and Tsay (11). All preparative steps were carried out using liganded hemoglobin (O_2 or CO). Carbamylation was performed at 23°C using 0.1M[^{14}C]KNCO in 2-3 gm Hb/100 ml in 0.01M $Na(PO_4)$, pH 6.5. These conditions were chosen to provide a high yield of protein carbamylated at the NH_2-terminal valines (6,12). The reaction was allowed to proceed for 30 minutes and was then stopped by passing the mixture at 5°C through a column of Sephadex G-25 equilibrated with 0.05 M Tris buffer, pH 8.0.

Cyanate reacts both with ϵ-amino groups of lysine and with the four valine NH_2-termini of hemoglobin. The lysine-reacted protein was

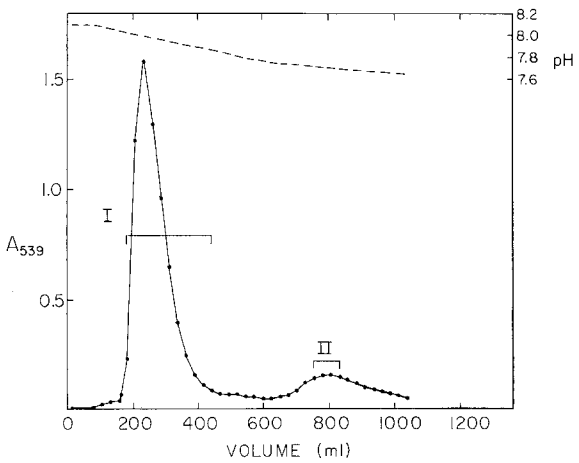

Figure 2. Chromatography at 5°C of carbamylated CO-hemoglobin on a 2.5 x 30 cm column of DEAE-cellulose (DE-52, Whatman). The column was initially equilibrated with 0.05 M Tris buffer, pH 8.1, and elution was accomplished with a pH gradient of 0.05 Tris, as shown. Results of analysis for ϵ-NH_2 carbamylated lysine and NH_2 carbamylated terminal valine are given in Table I.

Table I

Analysis of components isolated in Step A (Figure 2) for carbamylated lysines and NH_2-terminal valines (refs. 12,20). The extents of carbamylation of NH_2-terminal valines and of ϵ-NH_2 lysines were determined according to the analytical methods of Stark and Smyth (20).

Peak	Moles of carbamylated valine /mole of Hb_4	Moles of carbamylated lysine/mole of Hb_4
I*	2.3	0.0
II	2.3	0.5

* Analysis of Peak I was performed after subsequent separation (Fig. 3) and the data given here are computed from the concentration-weighted results in Table II, assuming no losses.

separated from the mixture by chromatography on DEAE cellulose (Figure 2). As shown, peak II contained lysine-reacted material whereas peak I is free of carbamylated lysine (see Table I). The faster moving peak (I) was then subjected to chromatography on CM-cellulose giving the elution pattern shown in Figure 3a, where it was found that peak III was nonradioactive, and was identified as being native hemoglobin tetramer by starch gel electrophoresis at pH 8.6 (13) and by measurement of the tetramer-dimer dissociation constant using sedimentation equilibrium methods (19). The extents of valine and lysine carbamylation for each of the three components are given in Table II. It is clear from these data that a separation has been obtained of partially NH_2-terminal valine-carbamylated hemoglobin (Peak II, Figure 3a) from fully NH_2-terminal valine-carbamylated protein (Peak I, Figure 3a). Since the NH_2-terminal valine analysis of these components (see Table IIa) indicated the possibility of less than complete reaction of the amino-termini, Peak I was subjected to further analysis by separation into alpha and beta subunits (14), as shown in Figure 3b. The components in this elution pattern were identified by amino acid analysis (15) and

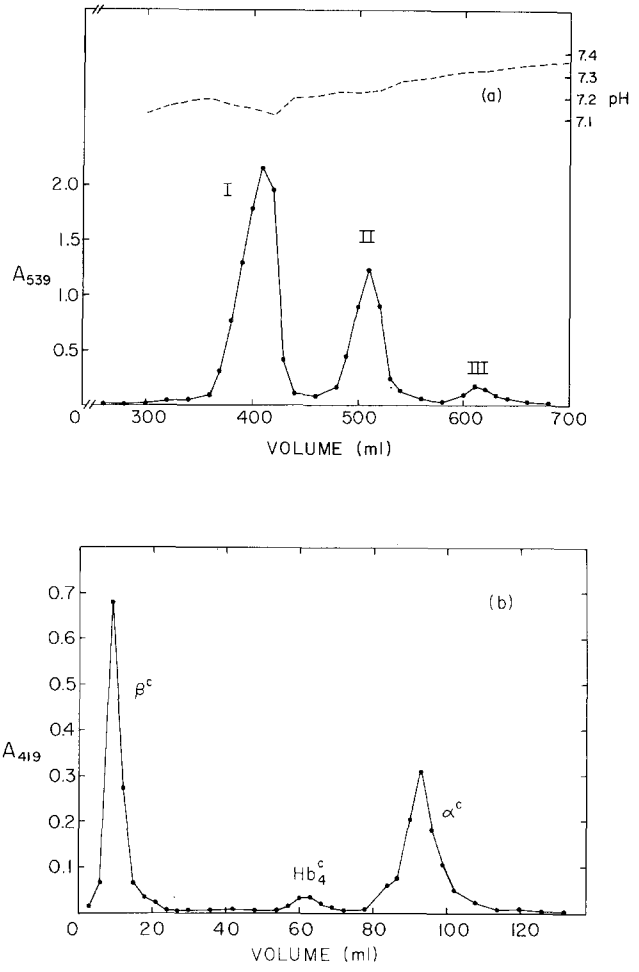

Figure 3. (a) Chromatography at 5°C of NH_2-terminal carbamylated CO-hemoglobin on a 1.5 x 30 cm column of CM-cellulose (CM-52, Whatman). Column was equilibrated initially with 0.01 M sodium phosphate buffer, pH 6.5, and elution was accomplished with a pH gradient between that buffer and 0.01 M sodium phosphate buffer, pH 8.0, as shown. Results of analysis of all components for ϵ-NH_2 carbamylated lysine and NH_2 carbamylater terminal valine are given in Table IIa.

(b) Analysis of peak I, Fig. 3a, by separation of the subunits following reaction with PMB according to the method of Bucci and Fronticelli (14). Results of radioactive analysis for extent of NH_2-terminal carbamylation given in Table IIb. Chromatography was performed on a 1.5 x 20 cm column of CM-cellulose equilibrated initially with 0.01M sodium phosphate buffer, pH 6.1. Elution was accomplished with a gradient between that buffer and 0.015M Na_2HPO_4.

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starch gel electrophoresis at pH 8.6 (13). Radioactive analysis of $N^{14}CO$ gave the results shown in Table IIb. Since the separation method

Table IIa

Analysis of all components isolated in Step B (Figure 3a) for carbamylated lysines and NH_2-terminal valines (refs. 12,20).

Peak	Moles of carbamylated valine /mole of Hb_4	Moles of carbamylated lysine/mole of Hb_4
I	2.9	0.0
II	1.6	0.0
III	0.0	0.0

Table IIb

Radioactive analysis* of Peak I (Figure 3a) after subunit separation (Figure 3b).

Components	Moles of $N^{14}CO$/subunit
alpha subunit	1.1
beta subunit	1.1
Hb_4	0.9

* Duplicate aliquots were dried on filter-paper discs and counted in PPO-toluene (4 g/l). The apparent specific radioactivity of the reagent KNCO was measured in the reaction mixture by the same technique used for the samples. Channels-ratio quench correction was applied throughout.

used in Figure 3b was shown to distinguish between α, α^c, β, β^c (superscript means carbamylated NH_2-terminal valine) and tetramer, it is concluded that peak I in Figure 3a is carbamylated at all four amino-termini although the results of analysis for valine hydantoin (Table IIa) yielded somewhat less than one carbamyl group per polypeptide chain. When step A (Figure 1) was omitted an elution pattern similar to that shown in Figure 3a was obtained but the corresponding peak I contained 2.63 moles of carbamylated NH_2-valine and 1.10 moles of carbamylated ϵ-NH_2 per mole of tetramer.

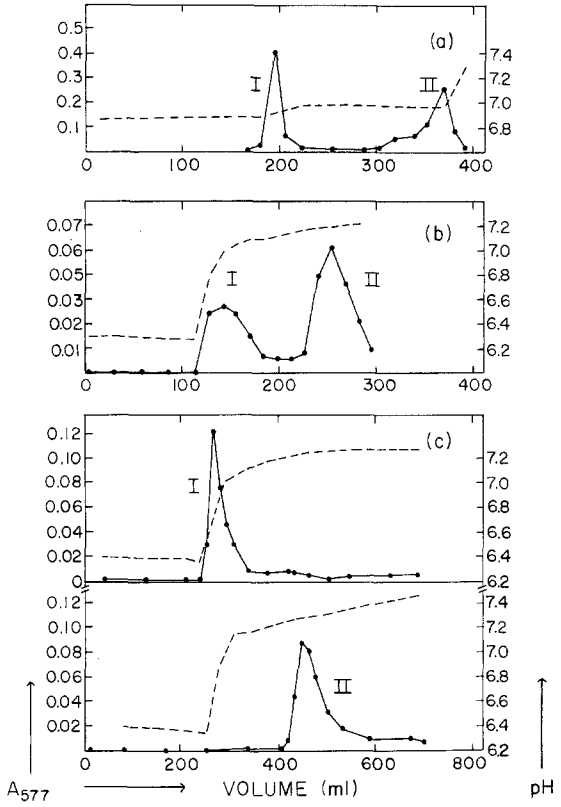

Figure 4. (a) Anaerobic chromatography at 5°C of 2 NCO-NH_2-terminal deoxy hemoglobin (Peak II, Fig. 3a) on a 3 x 30 column of CM-cellulose (CM-52, Whatman). Spectrophotometric analysis of the eluted hemoglobin revealed about 35% O_2 saturation. The column was equilibrated initially with 0.01 M sodium phosphate buffer, pH 6.8, in the presence of 10^{-4}M IHP and 10^{-4}M sodium phosphate containing 10^{-4}M IHP and 10^{-4}M EDTA.

(b) Chromatography at 5°C of 2 NCO-NH_2-terminal oxy hemoglobin (Peak II, Fig. 3a) on a 3 x 30 cm column of CM-cellulose (CM-52, Whatman). The column was equilibrated initially with 0.01 M sodium phosphate buffer, pH 6.3, in the presence of 10^{-4}M IHP and 10^{-4}M EDTA. Elution was accomplished with a pH gradient between that buffer and 0.015M Na_2HPO_4 which contained 10^{-4}M IHP and 10^{-4}M EDTA. Results of analysis of each component after separation of the subunits following reaction with PMB (14) are shown in Figs. 5b, c and Table IIIb.

(c) Analysis of 2 NCO-NH_2-terminal hemoglobin separated as deoxy hemoglobin into $\alpha^S_2\beta_2$ (Peak I, Fig. 4a) and $\alpha_2\beta^S_2$ (Peak II, Fig. 4a). Each component was oxygenated, concentrated and re-chromatographed as oxyhemoglobin as in Fig. 4b. Compare 4c and 4b where the pH values of the elution maxima of respective fast and slow components are seen to be similar.

Further separation of the mixture of $\alpha_2^c \beta_2$ and $\alpha_2 \beta_2^c$ (Peak II, Figure 3a) was achieved by taking advantage of the known binding site of organic phosphates to hemoglobin. Arnone (16) has shown that both DPG and IHP bind to the amino terminus of the beta chain. Chromatography of Peak II on CM-cellulose in a buffer containing 10^{-4}M IHP gave the results shown in Figure 4. Separation of $\alpha_2^c \beta_2$ from $\alpha_2 \beta_2^c$ was obtained by chromatographing either deoxy- or oxy- hemoglobin in the

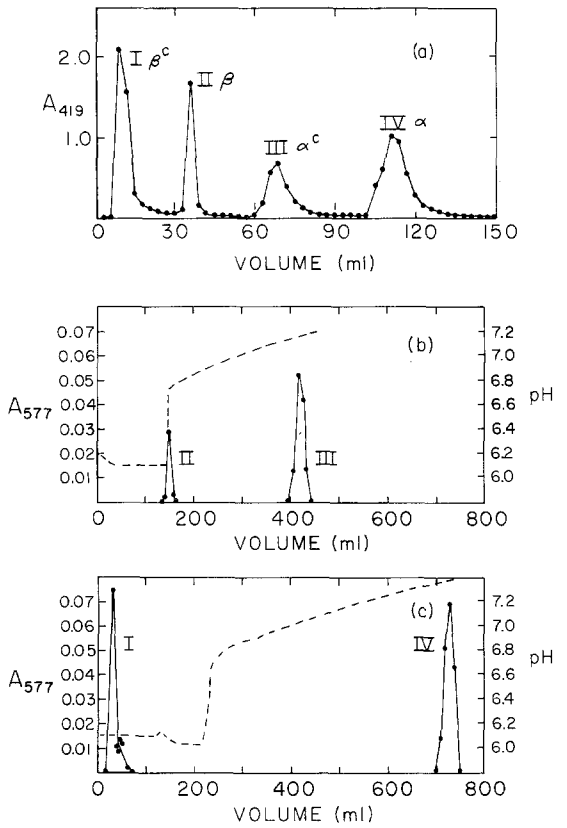

Figure 5. (a) Analysis of Peak II, Fig. 3a, by separation of the subunits after reaction with PMB (ref. 14). Results of radioactive analysis for extent of carbamylation of NH_2-terminal valine are given in Table IIIa.

(b) Analysis of Peak I, Fig. 4b, by subunit separation as in Fig. 5a.

(c) Analysis of Peak II, Fig. 4b, by separation of subunits as in Figure 5a. Results of NH_2-terminal valine analysis for components I-IV in Figs. 5b, c are given in Table IIIb.

Table IIIa

Radioactive analysis (as in Table IIb) of unfractionated 2-NCO-hemoglobin A isolated in Step B (Peak II, Figure 3a) after separation into alpha and beta subunits (Figure 5a).

Peak	Subunit*	Moles $N^{14}CO$/subunit
I	beta	1.0
II	beta	0.04
III	alpha	1.2
IV	alpha	0.01

* Identified by amino acid analysis (refs. 12,15) and starch gel electrophoresis at pH 8.6 (13).

Table IIIb

Analysis for NH_2 carbamylated terminal valines (refs. 12,20) of fractionated 2-NCO-hemoglobins isolated in Step C (Figure 4b), after separation into alpha and beta subunits (Figure 5b,c).

Peak	Subunit	Moles of carbamylated valine/subunit
I	beta	0.82
II	beta	0.04
III	alpha	0.82
IV	alpha	0.01

* Identified by starch gel electrophoresis at pH 8.6 (13).

presence of the organic phosphate. To demonstrate that the same carbamylated intermediates are separated in liganded and in unliganded hemoglobin, the isolated deoxyhemoglobin peaks (Figure 4a) were individually re-chromatographed as oxyhemoglobin and gave the results shown in Figure 4c. It can be seen that these components, originally separated as deoxyhemoglobin, when rechromatographed individually as oxyhemoglobin, elute at pH values closely similar to those for the two components in the mixture which was fractionated as oxyhemoglobin (Figure 4b). Since the binding of IHP would alter significantly the net charge of hemoglobin,

these results further suggest that IHP probably binds with the same stoichiometry to liganded and unliganded hemoglobin and probably to the same site (16). The identities of the two components isolated as oxyhemoglobin (Figure 4b) were established by NH_2-terminal valine analysis of the subsequently isolated alpha and beta subunits of each component. The results are given in Figure 5 and in Table III and show that each elution boundary in Figure 4b is a pure component and that this method allows one to prepare and isolate $\alpha^c_2\ \beta_2$ and $\alpha_2\ \beta^c_2$. The following control experiments confirm that it is the binding of IHP to the unmodified beta subunits and not the alpha subunits within the tetramer that results in the separations shown in Figures 5a,b. Chromatography of Peak II, Figure 3, in the absence of IHP yields a single elution boundary as does chromatography in the presence of IHP of Peak I, Figure 3, or of native hemoglobin.

A scheme similar to that shown in Figure 1 has been used successfully to separate the carbamylated intermediates of HbS and will be reported elsewhere together with a characterization of their properties (18,19).

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