ALKALI DENATURATION OF HUMAN HEMOGLOBIN: POSSIBLE ROLE OF DISULFIDE FORMATION

by

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One of the most striking differences between normal human adult hemoglobin, $\alpha_2\beta_2$, and normal human fetal hemoglobin, $\alpha_2\gamma_2$, is the markedly greater resistance of the latter to denaturation by alkali. Although the sequences of the β and γ chains differ at over thirty amino acid positions (Schroeder et al., 1963), these differences have not yet been related to the great difference in alkali resistance of the two hemoglobins.

Adult hemoglobin (Hb A) has six sulfhydryl groups per molecule of $\alpha_2\beta_2$, two of which are "reactive" and four of which are "non-reactive" (Allison and Cecil, 1958). Fetal hemoglobin (Hb F) has four sulfhydryls, two reactive and two non-reactive (Allison et al., 1960). Of the non-reactive sulfhydryls two occupy the 104th position from the N-terminus of each α chain (Hb A and Hb F) and, in the case of Hb A, the others occupy position 112 of each β chain. In both Hb A and Hb F the reactive sulfhydryls occupy the 93rd position from the N-terminus of the β and γ chains, respectively (Goldstein et al., 1961).

The data herein reported suggests that the formation of disulfide bonds plays a significant role in the process of alkali denaturation of hemoglobin. It is proposed that the increased resistance to alkali denaturation exhibited by Hb F may be due, at least in part, to the presence of only one non-reactive sulfhydryl per half-molecule.

Methods and Materials

Hemoglobin samples were prepared from whole blood as previously described (Itano and Singer, 1958). Hb F was purified from cord blood hemoglobin by column chromatography (Allen et al., 1958).

Carbonmonoxyhemoglobin A (HbCO A) and oxyhemoglobin A (HbO_2 A) were reacted with an eight-fold molar excess of $HgCl_2$ and pCMB according to Cecil and Snow (1962). Bound mercury was determined by coupling with dithizone after acid hydrolysis (Irving et al., 1949). HbCO A was reacted with a sixty-fold molar excess of iodoacetamide (INH_2) according to Guidotti and Konigsberg (1964); prolonged reactions were carried out at pH 9 in the buffers of Cecil and Snow (1962).

Rate of alkali denaturation was measured in two ways, by precipitation of denatured protein with acidic $(NH_4)_2SO_4$ (Haurowitz, 1929) at various time intervals (HbCO and HbO_2) and secondly by continuous recording of absorbance at a specific wavelength (HbO_2) (Haurowitz et al., 1954). When rate of change of absorbance at 250 mμ of HbCO in alkali was measured, complete UV and visible spectra were also recorded at various stages of the reaction.

Results and Discussion

Figure 1 shows the results of alkali denaturation of HbCO samples in 0.10 N NaOH as determined by the precipitation technique. Untreated HbCO A and HbCO A with 1.8 moles Hg^{+2}/mole Hb have identical curves. Point readings of HbCO A- INH_2 in which only the reactive sulfhydryls were blocked gave similar results. HbCO A with 6.1 moles Hg^{+2}/mole Hb was more resistant to denaturation than HbCO F. Untreated

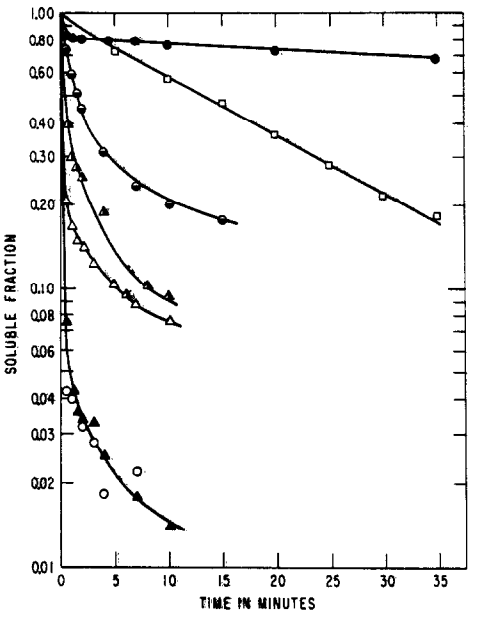

Fig. 1. Alkali denaturation of HbCO in 0.10 N NaOH, 20° C. The reaction mixture contained 5 ml 0.20 N NaOH, 4 ml H_2O and 1 ml HbCO (3-5%). At specified intervals 1 ml was pipetted into 2 ml acidic 50% $(NH_4)_2SO_4$, mixed, filtered, and absorbance of the soluble fraction measured at 538 mμ. Zero time concentrations were determined by adding HbCO to 33% saturated neutral $(NH_4)_2SO_4$. ▲ untreated A, ○ A/$HgCl_2$(1.8), Δ A-pCMB (3.7), ◭ A-INH_2, 17 hrs at pH 9, ◒ untreated A in presence of 1.4 M 2-mercaptoethanol (1.5 N NaOH, pH 13), □ F_{II} (Itano et al., 1964), ● A-$HgCl_2$(6.1).

HbCO A in the presence of 2-mercaptoethanol exhibited intermediate resistance. If more than two but fewer than six sulfhydryls of Hb A were blocked, resistance to alkali denaturation was increased but intermediate to that of untreated Hb A and Hb A in which all six sulfhydryls were blocked. The results with HbO_2 samples, as determined

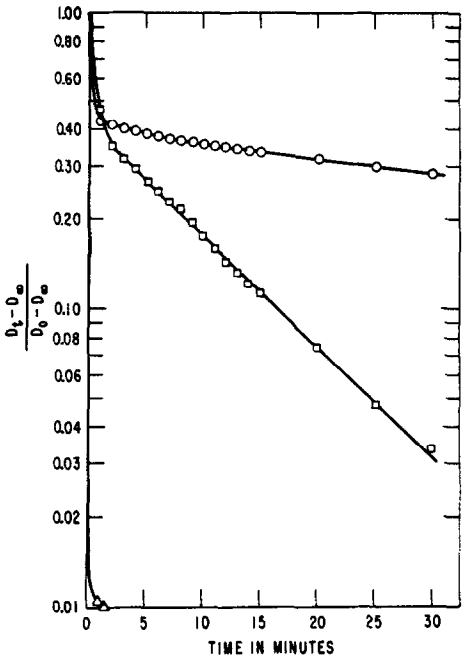

Fig. 2. Increase in absorbance at 250 mμ of HbCO in 0.10 N NaOH at 25° C.

Equal volumes of 0.1% HbCO in H_2O and 0.20 N NaOH were mixed, saturated with CO, and the increase in absorbance recorded with a Cary Model 14 until there was no further change (D∞). D_o was determined by mixing equal volumes of 0.1% HbCO and phosphate buffer pH 6.0, μ = 0.2. Δ untreated A, □ F_{II}, ○ A-$HgCl_2$(5.5).

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by either method, paralleled these findings.

Figure 2 gives the results of absorbance changes of HbCO in 0.10 N NaOH at 250 mμ. Absorbance at 250 mμ increased as that at 538 mμ decreased. The order of rates determined at 250 mμ paralleled those determined at 538 mμ.

Alkali denaturation of hemoglobin is characterized not only by changes in the visible spectrum, i.e., changes in the heme environment,

but also by changes in solubility. Unquestionably more than one mechanism is involved in this phenomenon. However, one aspect can be discussed on the basis of the data obtained, namely, the possible role of formation of disulfide bonds.

Protein sulfhydryls have long been known to be labile in alkali. It was suggested by Riggs in 1956 that hemoglobin sulfhydryls are oxidized aerobically to disulfides in alkali. If this is indeed the case then it would be impossible to regain the native conformation of the molecule merely by returning to neutral pH. If the sulfhydryl groups could be protected either by prior reaction or by high concentrations of such reagents as mercaptoethanol, the restrictive effect of disulfide bonds on reassumption of the native conformation could be circumvented.

Does the data presented in Fig. 2 offer any evidence of disulfide formation? Four amino acids must be considered in interpreting absorbance changes in the 240-260 mμ region, namely, phenylalanine, tryptophan, tyrosine, and cysteine (Greenstein and Winitz, 1961). Neither phenylalanine nor tryptophan exhibit an increase in absorbance at 250 mμ (D_{250}) when the pH is raised from 6 to 13. On the other hand, there is over a ten-fold increase in D_{250} of tyrosine in alkali. However, this change in spectrum occurs immediately and is constant even after sixty minutes (Robinson, unpublished results). Cysteine, saturated with CO, likewise shows a significant change in spectrum in alkali (Benesch and Benesch, 1955; Robinson, unpublished results). There is about a one hundred-fold increase in D_{250} initially, but after longer exposure to alkali the absorbance decreases and the final spectrum is similar to that of cystine.

Hermans (1962) reported that of the twelve tyrosyl residues in HbCO A, four titrate abnormally and that ΔD_{245} increased with time

between pH 11.4 and 12.3 but was independent of time at pH 12.3. Whether this is also true of the tyrosyl residues of Hb F has not been reported. However, at pH 13 it seems reasonable to assume that $\Delta D_{245-250}$ would not be time dependent if this Δ were due solely to ionization of abnormal tyrosyl residues. On the other hand, since there is a 2:1 ratio of tyrosine to cysteine in Hb A and a 2.5:1 ratio in Hb F, it is not possible, without further experimentation, to attribute the curves shown in Fig. 2 solely to changes in cysteinyl residues.

If one assumes that the increased resistance to alkali denaturation exhibited by Hb A with three to six sulfhydryls blocked is due to an increased resistance to disulfide formation, the most likely disulfide linkage in untreated Hb A would seem to be between the non-reactive sulfhydryl of the α chain and the non-reactive sulfhydryl of the β chain. This is supported by the fact that the rate of denaturation of Hb A is not changed when only the reactive sulfhydryls are blocked and by the fact that denaturation occurs above the pH of dissociation into half-molecules (Hasserodt and Vinograd, 1959; Gottlieb, unpublished results). If interchain disulfide linkages between non-reactive sulfhydryls do play a significant role in the process of alkali denaturation, the presence of only one non-reactive sulfhydryl per half-molecule in Hb F may be significant.

Anaerobic studies in special silica apparatus of treated and untreated Hb A and Hb F are planned as are experiments to determine the location of the cysteinyl residues in samples in which more than two sulfhydryls are blocked. Detection of disulfide groups in alkali denatured Hb A and Hb F will be attempted.

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