Circular Dichroism Studies of Cyanate-Induced Conformational Changes in Hemoglobins A and S†

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ABSTRACT: Circular dichroism and difference spectroscopy have been used to study dilute aqueous solutions of oxygenated, deoxygenated, and carbamoylated deoxygenated hemoglobins A and S (HbA and HbS, respectively). The spectra of HbA and HbS, in comparable state of oxygenation or carbamoylation, are identical, strongly implying identical conformations about the heme groups of the respective proteins. The spectra of the oxygenated forms change little upon addition of KCNO, which is known to carbamoylate the NH_2 terminals of the individual chains (Cerami and Manning, 1971). The spectra of the deoxygenated forms, on the other hand, are markedly altered. The decreased magnitude of the 430-nm extremum with increased cyanate concentration can be used to calculate an addition curve which becomes asymptotic at a cyanate:heme molar ratio of approximately 10[3]. This conformational change occurs in the absence of O_2 and has been predicted (Njikam et al., 1973); it can also be demonstrated by difference spectroscopy techniques, whereby a comparable addition curve can be constructed from changes in the 555-nm absorption, while the 541-nm absorption remains invariant. The change described corresponds to the formation of a new conformation, corresponding to carbamoyldeoxyhemoglobin, carrying one carbamoyl group per chain. In the presence of a small quantity of oxygen, however, the above reported changes in CD are accompanied by a concomitant rise in the 415-nm peak—corresponding to the formation of oxyhemoglobin—while those in the difference spectra reflect not only a change in the 555-nm band but also a parallel one at 541 nm, confirming the formation of oxyhemoglobin. Thus the conformation achieved upon carbamoylation of deoxyhemoglobin has the higher oxygen affinity predicted by Nigen et al. (1974) for carbamoyldeoxyhemoglobin. Cyanate has been used (Cerami and Manning, 1971) as an antisickling reagent in vivo and in vitro, but, although it has been shown that it binds covalently to the NH_2-terminal residues of hemoglobin (Lee and Manning, 1973), its effect on hemoglobin conformation has not been previously shown nor has its mechanism of action been fully clarified. The results presented here show that the effect of cyanate on hemoglobin is the formation of a new conformation with heightened oxygen affinity. Since oxyHbS does not aggregate while deoxyHbS does, in a temperature-dependent fashion, the formation of carbamoyldeoxyHbS interferes with such aggregation in vitro in deoxygenated samples. In vivo, where there are generally low residual concentrations of O_2, the formation of oxyHb is favored by the higher O_2 affinity of carbamoyldeoxyHbS, and aggregation with concomitant red cell sickling is therefore disfavored.

The genetic variant HbS[1] differs from the normal HbA only in the $\beta 6$ position, where a valyl replaces the normal glutamyl residue (Pauling, 1955; Ingram, 1956). In the deoxygenated state HbS has a temperature (Murayama, 1957) and concentration-dependent tendency to aggregate, leading, in vivo, to "sickle cell crises" (Allison, 1957), and, in vitro, to gelation at high concentration (>200 mg/ml) (Harris, 1950; Singer and Singer, 1953; Murayama, 1962) and to pregelation aggregation at lower concentration (Wilson et al., 1974; Hartzband and Simons, 1974). The mechanisms of these aggregation steps are still under study (Murayama, 1962; Williams, 1973; Minton, 1973a,b, 1974; Hofrichter et al., 1974; Bookchin and Nagel, 1973; Whitin et al., 1975).

Several reagents have been tested for their ability to prevent, ameliorate, or reverse sickle cell crises. One of these, cyanate (Cerami and Manning, 1971), has been reported to increase the oxygen affinity of HbS (Kilmartin and Rossi-Bernardi, 1969; Diederich, 1972; May et al., 1972) as well as the survival of HbS-containing red cells (Gillette et al., 1971). There have been several studies (Lee and Manning, 1973; Njikam et al., 1973; Nigen et al., 1974; Williams et al., 1975) of the chemistry of the cyanate carbamoylation of hemoglobins, reportedly identical in HbS and in HbA,[2] as well as of the kinetics of the carbamoylation reaction itself. The reaction occurs more extensively at the NH_2 terminals of deoxy than of oxyhemoglobin, also favoring α over β chain NH_2 terminals in the deoxy but not the oxy case. Incubation with cyanate for 30 min at pH 7, the conditions employed here, leads to preferential carbamoylation of the amine terminal residues with essentially no lysyl side chain substitution (Stark, 1965; Njikam et al., 1973; Williams et al., 1975). Carbamoylation of the α chains (which is more rapid) increases the oxygen affinity of HbS without altering the minimum concentration at which gelling occurs, while carbamoylation of the β chains has an opposite effect—i.e., decreases the oxygen affinity—and leads to a raised minimum gelling concentration. Thus it has been concluded that two separate effects on deoxyHbS attributable to carbamoylation exist, one being independent of oxygen affinity while the other is not. The mechanism whereby these interfered with

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[1] Abbreviations used: CD, circular dichroism; HbA and deoxyHbA, oxygenated and deoxygenated forms, respectively, of normal hemoglobin; HbS and deoxyHbS, oxygenated and deoxygenated forms, respectively, of sickle cell hemoglobin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

[2] While in corresponding states of carbamoylation, as well as oxygenation and deoxygenation, HbA and HbS have identical CDs, HbF appears to have a different carbamoylation profile (S. Zemel, C. E. Chapman, and E. R. Simons, unpublished data).

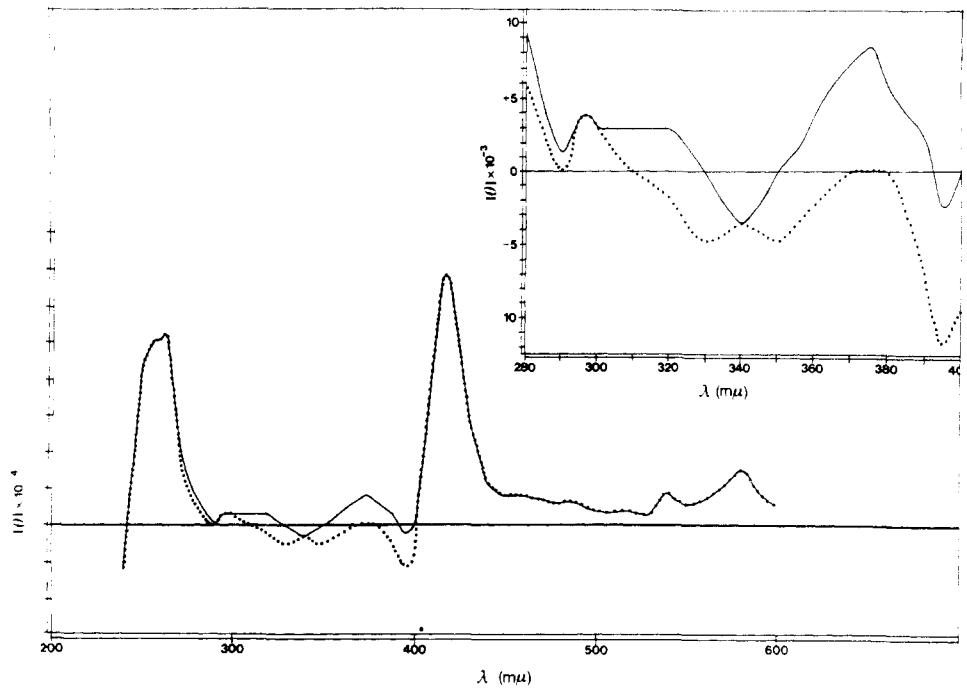

FIGURE 1: CD spectrum of oxyHb. (. . .) $[CNO^-] = 0$; (—) $[CNO^-] = 10$ mM. Inset: Ten times enlargement of ordinate scale.

sickling has been attributed to the increased oxygen affinity of the α chains (Nigen et al., 1974).

In connection with studies of the cyanate-induced decrease of deoxyHbS pregelation aggregation, we have investigated the conformational changes in deoxyhemoglobins attributable to cyanate addition. Utilizing CD and difference spectroscopy, we have been able to detect both of the effects previously attributed to cyanate and to demonstrate a mechanism for its action upon deoxyHbS aggregation.

Experimental Procedure

Blood samples from individuals homozygous and heterozygous for the sickle cell allele and from normal adults were donated by various clinical laboratories. These samples had been citrated or EDTA-treated and stored at 4 °C until ready for use.

Isolation of Hemoglobin. All preparative procedures were carried out in a cold room at 4 °C. Red blood cells were separated from platelets and other blood components by centrifugation at 2125 rpm in an International Clinical Centrifuge (Model CL) for 15 min. They were then lysed as previously described (Briehl and Ewert, 1973). The resulting hemolysate was dialyzed for 24 h against a large volume of 0.05 M Tris buffer titrated to pH 8.1 with 0.05 M HCl. The dialysate was concentrated under nitrogen in an Amicon ultrafiltration apparatus equipped with a Diaflo UM 10 membrane.

The sample of dialyzed hemolysate was placed on a column of microgranular Whatman DE 52 equilibrated and developed with 0.05 M Tris, pH 8.1 (Briehl and Ewert, 1973). When a clear separation of the hemoglobin components present in the sample had been effected and the fast-running band had penetrated into the lower portion of the column, the column was split. Nonhemoglobin-containing material was then removed in order to obtain a more concentrated eluate, and the hemoglobin fractions were eluted separately with buffer: 3 parts NaCl, 0.1 M, to 1 part 0.1 M Tris, pH 7.0. The column eluates were immediately reconcentrated by ultrafiltration or in a Minicon B 15 (Amicon) and dialyzed against 0.1 M Tris, pH 7.0. These concentrated eluates were used immediately or frozen. Experimental results were the same in either case. Hemoglobin concentration was determined for all solutions in the oxy state on the basis of the millimolar heme extinction coefficient ϵ_{541} 13.8, using an equivalent molecular weight of 16 000 g (Antonini and Brunori, 1971). The absence of methemoglobin and of hemichromes (Rachmilewitz, 1974) was confirmed spectrophotometrically.

Electrophoresis. Identification of the hemoglobin components present in the hemolysates was made by electrophoresis on cellulose acetate sheets (Gelman) in 0.026 M boric acid buffer, pH 8.6 (ionic strength 0.57), against known standards. The electrophoresis was run at 500 V, 3.2 mA, for 2.5 h or until a good separation was achieved. This procedure was also used to verify the homogeneity of column-purified hemoglobin samples.

Deoxygenation. The deoxygenation procedure is critical. Many investigators have relied on chemical reducing agents, such as sodium dithionite, to ensure the complete deoxygenation of hemoglobin solution since they do not absorb in the visible and Soret regions of the spectrum. However, the use of such reagents can result in the degradation of hemoglobin by side reactions, accompanied by slow spectral changes (Dalziel and O'Brien, 1957a,b). Other investigators have prepared deoxyHb by the deoxygenation of the oxyHb solutions with N_2 at low Hb concentrations, but this method of deoxygenation results in the denaturation of hemoglobin and the formation of significant quantities of met-Hb (Antonini and Brunori, 1971), thereby introducing large errors into spectral measurements. To avoid these problems, we have deoxygenated in an ice bath, in very concentrated hemoglobin solutions, using N_2:CO_2 (95:5) gas mixture. The gas mixture was passed over, but never bubbled through, the Hb solution to avoid denaturation at air–water interfaces (Asakura et al., 1973, 1974; Roth et al., 1975). Since the pH is also critical in these studies, it was measured before and after each experiment, and maintained at 7.0.

Dilute solutions were prepared by anaerobic transfer (using N_2-purged syringes) of the concentrated solution to sealed cuvettes containing deoxygenated buffer (pH 7.0). Great care

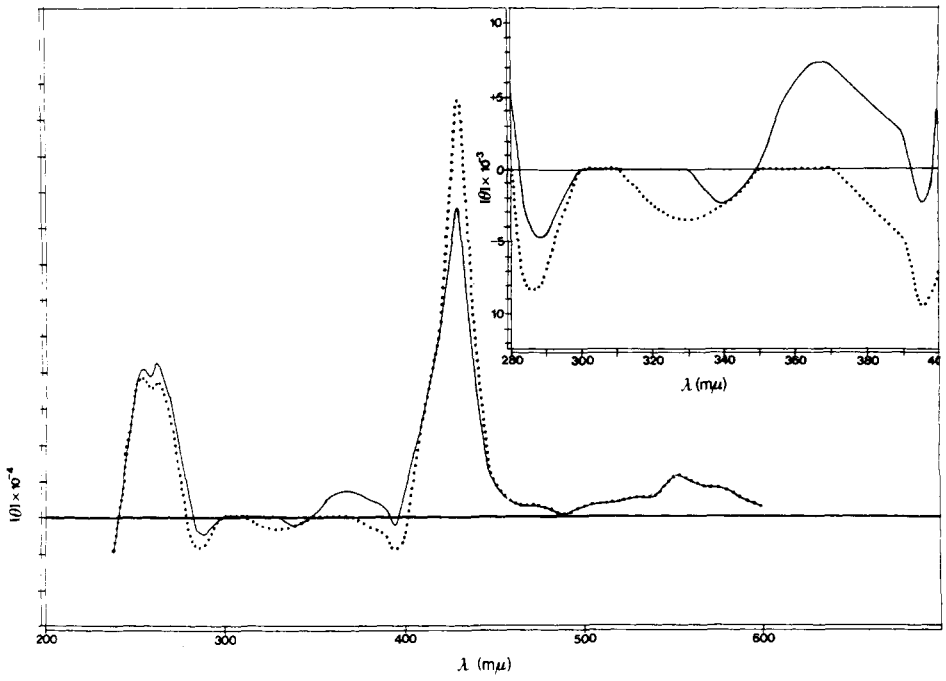

FIGURE 2: CD spectrum of deoxyHb. (. . .) $[CNO^-] = 0$; (—) $[CNO^-] = 10$ mM. Inset: Ten times enlargement of ordinate scale.

was taken to ensure that solutions were not exposed to air during transfer since deoxygenated hemoglobins reoxygenate very readily. Using this method of deoxygenation, spectral measurements (both absorption and circular dichroism) did not change significantly with time.

The extent of deoxygenation was judged spectrally before and after experiments. Deoxygenation was judged complete when the absorption bands at 541 and 577 nm were completely replaced by a band at 555 nm (Antonini and Brunori, 1971), $\epsilon_{555}/\epsilon_{571}$ 1.35. Spectra were obtained before and after each experiment to ensure the absence of denatured and of metHb.

Circular Dichroism. Measurements of circular dichroism were made on a Cary 61 spectropolarimeter at 6 and at 27 °C. All spectra of deoxygenated Hb solutions were observed using the 1-cm path length quartz cuvettes. For oxyHb, these cells or round cells of 1-mm path length were utilized. Solutions of deoxyHb at a concentration appropriate for the circular dichroism measurements were prepared by dilution of stock solutions with deoxygenated buffer (as above). After CD measurements of this solution were completed, the same protein solution was equilibrated with air for several minutes and read again.

Carbamoylation. KNCO (1.0 M) in H_2O or in 0.1 M Tris, pH 7.0, was freshly prepared, just before use. For addition to deoxyHb the cyanate solution was deoxygenated as described above. Anaerobic addition to 2.0 ml of hemoglobin solution was performed with a gas-tight Hamilton syringe. In order to maintain comparability with previous studies (Njikam et al., 1973; Nigen et al., 1974 Williams et al., 1975), the final concentration of cyanate attained was 10–18 mM. Incubation proceeded for 30 min (as in Nigen et al., 1974; Williams et al., 1975) before each spectrum was taken, but no changes in the Soret region were observed after 5 min, nor did any further changes in this spectrum appear when the sample was observed for over 2 h. It should be noted that this does not imply that no further carbamoylation of hemoglobin occurs, as indeed modification, e.g., of lysines, continues (Nigen et al., 1974; Stark and Smyth, 1963), nor that equilibrium has been reached, but merely that carbamoylations affecting the Soret CD spectral region are complete after 5 min. Further carbamoylation does not appear to affect the 410–440 nm CD region.[3]

For analysis purposes, the carbamoylated hemoglobin was isolated according to Williams et al. (1975). Only N-terminally modified hemoglobin was detectable; upon analysis, this contained one carbamoyl group per heme, as measured by valine hydantoin (Lee and Manning, 1973).[4] No hemoglobin partially or totally carbamoylated on the lysine ϵ-NH_2 groups was present, as indicated by the absence of more slowly elutable absorption peaks. Thus, as observed earlier by others, under these experimental conditions (Njikam et al., 1973; Nigen et al., 1974; Williams et al., 1975), only N-terminal carbamoylation is detectable.

Results

The CD spectra of oxy- and deoxyhemoglobin are shown in Figures 1 and 2, respectively, and agree well with those published for HbA (Sugita et al., 1971). As shown, the CD spectra of oxyHbA and oxyHbS are indistinguishable, as are those of the corresponding deoxygenated species, deoxyHbA and deoxyHbS.[2] The oxygenated form is marked by CD extrema at 415 nm, the Soret band ($[\theta]$ = 100 000 deg cm^2 dm^{-1} heme), and at 260 nm, by a slightly weaker (71 500 deg cm^2 dm^{-1} heme) complex aromatic band. The inset shows minor but reproducible fine structure in the 290 to 390 nm region.

[3] We thank Dr. J. Manning, Rockefeller University, New York, N.Y., for the valine hydantoin assays.

[4] Multiple attempts to isolate carbamoylhemoglobin, following the technique of Williams et al., 1975, but on hemoglobin rather than CO-hemoglobin (the compound they used), yielded samples which contained some methemoglobin and hemichromes. CO, on the other hand, is not quantitatively removable. The chromatographically isolated carbamoyldeoxyhemoglobin was therefore unsuitable for CD measurements, which are more sensitive to low concentrations of methemoglobin or hemichrome formation than absorption spectroscopy; they were also very difficult to deoxygenate, due to their higher oxygen binding capability explained herein. Dr. Manning's assay showed them to be fully carbamoylated on the N-terminal residues, i.e., one valine hydantoin per heme.

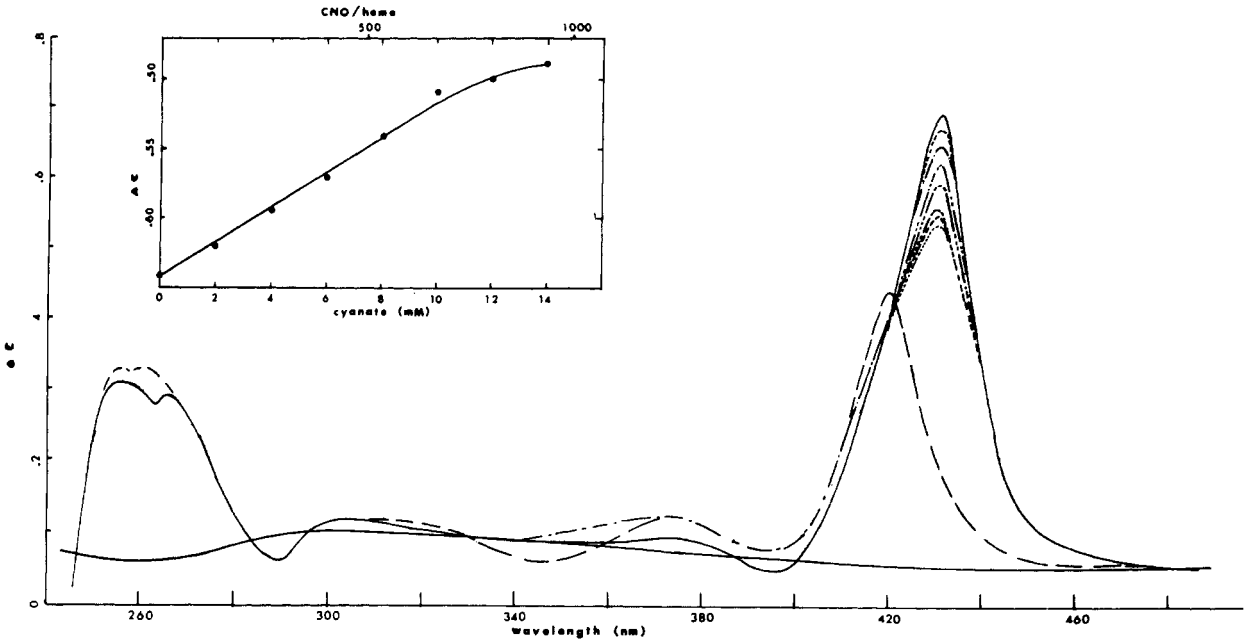

FIGURE 3: Representative traces of CD upon addition of KCNO to deoxyHb. [KCNO] = 0 (—), 2 (- - -), 4 (-·-), 6 (-··-), 8 (-···-), 10 (-·····-), 12 (— — —), 14 mM (·····). Reoxygenated after 14 mM KCNO (- - -). Inset: Calculated curve of $\Delta\epsilon_{430}$ as function of [KCNO] corrected for dilution. [Hb] = 1.5×10^{-5} M; 1-cm optical path; T = 6 °C; $\Delta\epsilon$ = recorder reading when full range = 0.05°. Some tracings have been omitted for clarity.

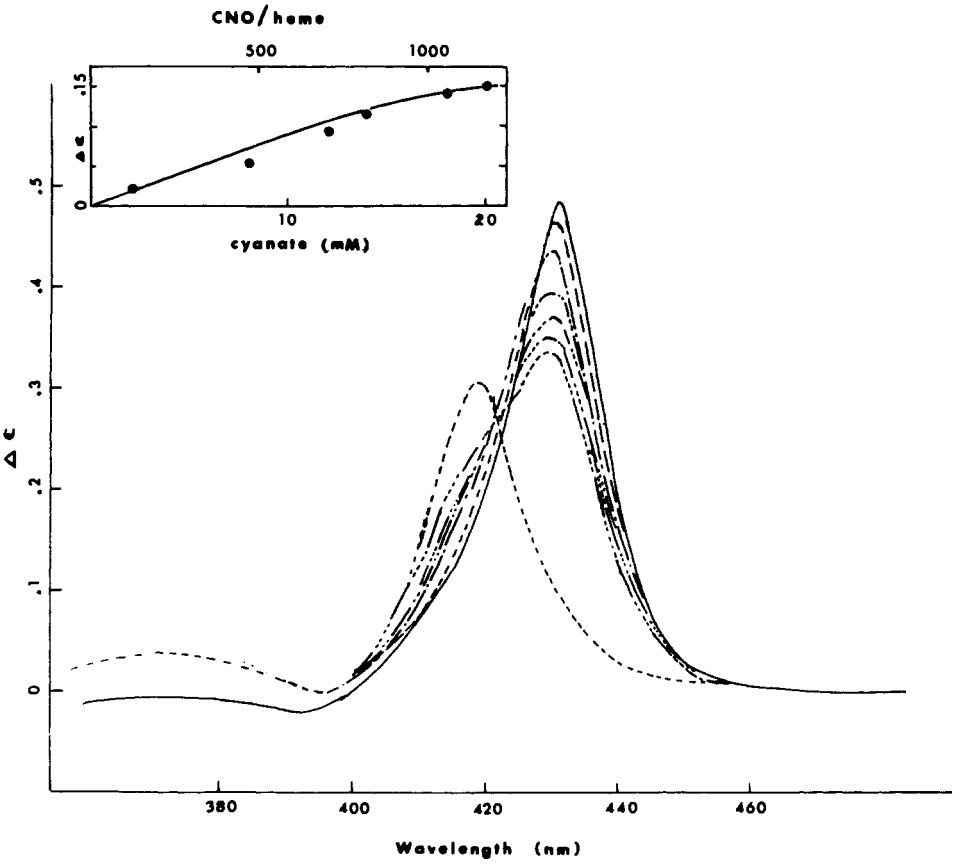

FIGURE 4: Representative traces of CD upon addition of KCNO to 82% deoxyHb; 18% oxyHb. [KCNO] = 0 (—), 4 (- - -), 8 (—·—), 12 (—··—), 16 (—···—), 18 (—·····—), 20 mM (—·····—). Reoxygenated after 20 mM KCNO (- - -). Inset: Calculated curve of $\Delta\epsilon_{430}$ as function of [KCNO] corrected for dilution. [Hb] = 1.6×10^{-5} M; 1-cm optical path; T = 6 °C; $\Delta\epsilon$ = recorder reading when full range = 0.05°. Some tracings have been omitted for clarity.

It is only in this latter region that changes are observed upon addition of KCNO to a final concentration of 10 mM (Figure 1), the concentration employed in previous studies of the carbamoylation of individual and recombined hemoglobin chains (Lee and Manning, 1973; Njikam et al., 1973; Nigen et al., 1974; Williams et al., 1975).

CD spectra of deoxygenated hemoglobin, on the other hand (Figure 2), exhibit a larger Soret peak at 430 nm (155 000 deg

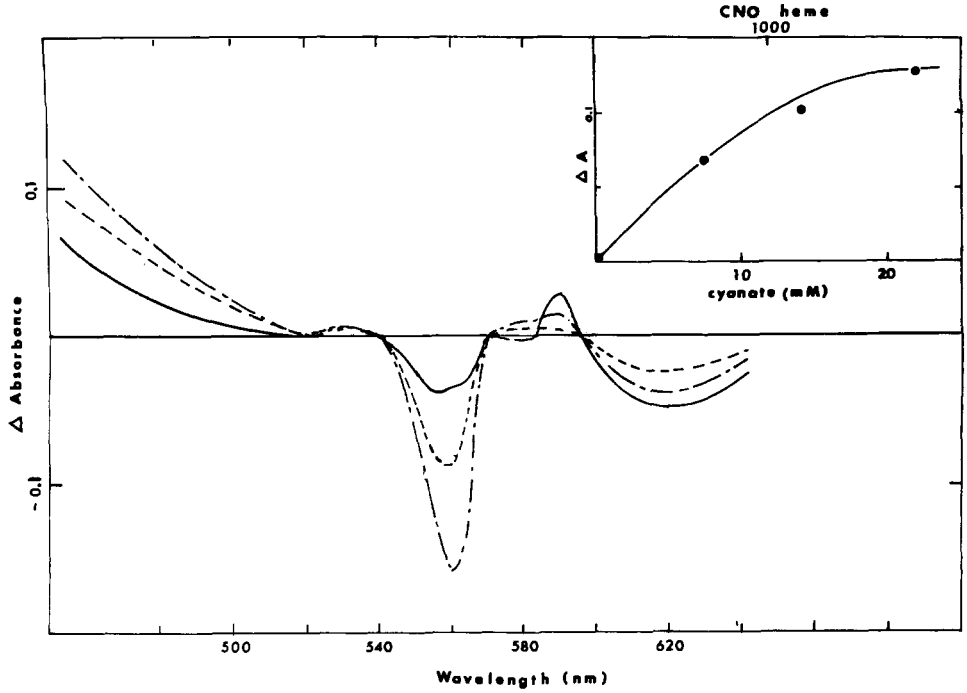

FIGURE 5: Representative difference absorbance tracing, KCNO added to deoxyHb. [KCNO] = 7.28 mM (—), 14.6 mM (- - -), 21.9 mM (—·—). Inset: Calculated curve for ΔA_{555nm} as function of KCNO. [Hb] = 1.2×10^{-5} M; 1-cm optical path length; 6 °C.

$cm^2\ dm^{-1}$ heme), and a smaller though better resolved pair of aromatic peaks (51 000 deg $cm^2\ dm^{-1}$ at 254 nm, 48 000 deg $cm^2\ dm^{-1}$ at 264 nm). The addition of KCNO to the same 10 mM final concentration led to the previously discussed minor changes in the 290–390 nm region, which are independent of the state of oxygenation of the hemoglobin. In addition, carbamoylation of deoxyhemoglobin (Figure 2) led to a 25% decrease in the Soret peak, unaccompanied by any wavelength shift, as well as to a proportionally smaller (10 and 12%) increase in the shorter wavelength bands at 254 and 264 nm, respectively. These spectral changes indicate that carbamoylation of deoxyHb is accompanied by a change in conformation affecting the Soret spectral region. The carbamoylated deoxyHb spectrum is not identical with that of the oxygenated species, however, differing in both the magnitude and the location of the Soret peak.

The conformational change described above can be followed as a function of cyanate concentration. Figure 3 shows a representative CD spectrum obtained upon addition of deoxygenated KCNO to deoxyHbS at 6 °C. The change in ellipticity, $\Delta\epsilon$, shown in the inset of Figure 3, becomes asymptotic at an approximate KCNO:heme ratio of 1000:1. In order to verify that this new conformation may correspond to the higher affinity species postulated by Nigen (Nigen et al., 1974), the same titrations were repeated with hemoglobin preparations which had intentionally been partially oxygenated. The extent of oxygenation was determined spectroscopically by the method of Benesch (Benesch et al., 1965). A representative CD spectrum, obtained with an 18% saturated deoxyHbS sample, is shown in Figure 4. Even though *no additional oxygen* was added, the decrease in the 430-nm deoxyHb peak already observed for totally deoxyHb (Figure 3) is accompanied by an increase in the 415-nm band when some oxygen is present. The ellipticity differences of either band are shown in the inset of Figure 4; $\Delta\epsilon$ becomes asymptotic to the CNO:heme ratio at approximately 1000 CNO:heme. This is approximately the same concentration of cyanate obtained in Figure 3 for fully deoxygenated hemoglobin and corresponds by quantitative analysis to one residue of valine hydantoin per heme.[4]

Although the CD data described above are considerably more accurate, we have also attempted to follow the conformational changes associated with carbamoylation of deoxyhemoglobin by difference spectroscopy. For this purpose the 500 to 600 nm region of the spectrum was easier to use to study conformational changes than the Soret bands themselves since the latter overlap considerably (Antonini and Brunori, 1971). A representative difference curve for totally deoxyHbS is shown in Figure 5, with an inset of the calculated change in ΔA at 555 nm, the deoxyHb maximum (Antonini and Brunori, 1971), which becomes asymptotic at an approximate CNO:heme ratio of 1000:1. There is no parallel change in the absorbance at the oxyHb maxima, 541 and 575 nm. Thus the difference spectra, corroborating the CD results, indicate a conformational change upon carbamoylation of fully deoxygenated hemoglobin. A parallel set of difference spectra obtained upon addition of KCNO to partially oxygenated hemoglobin leads, again in corroboration of the CD data, to the parallel reduction of the absorbance of deoxyHb at 555 nm and increase of the absorbance of oxyHb at 541 and 575 nm.

Discussion

The use of cyanate in the treatment of sickle cell disease (Gillette et al., 1971) has been attributed to the carbamoylation of the N-terminal residues, which has been postulated to lead to two separable effects, the dominant one in vivo being the increased oxygen affinity of carbamoylated chains (Nigen et al., 1974). No study of the conformational changes accompanying the carbamoylation of hemoglobin has, however, been reported to date. The results presented here document such conformational changes, in vitro in dilute solutions of deoxygenated hemoglobin, by means of circular dichroism measurements corroborated by difference spectroscopy.

Upon addition of cyanate to oxygenated hemoglobin solu-

tions, no changes in the Soret band at 415 nm or in the 260-nm region are observed. This correlates with Njikam's findings (Njikam et al., 1973) that carbamoylation occurs predominantly in the deoxygenated hemoglobin. In contrast, the addition of cyanate to fully deoxygenated hemoglobin leads to conformational changes which affect the heme environment as evidenced by a decrease in the Soret peak at 430 nm and to an increase in smaller bands at 254 and 264 nm, but there is no evidence of the appearance of a 415-nm band characteristic of a true deoxyHb conformation. This conformational change, which must correspond to the oxygen-independent effect predicted earlier (Nigen et al., 1974), is dependent upon the concentration of cyanate up to a ratio of 1000 KCNO per mole of heme in dilute solutions of hemoglobin, a concentration which corresponds to full carbamoylation of each N-terminal of the hemoglobin molecule (i.e., one valine hydantoin per heme group).

On the other hand, if oxygen is present even at low, 20%, concentration, the observed conformational change is more complex: as cyanate is added, the characteristic deoxyHb CD peak at 430 nm still decreases but an oxyHb peak at 415 nm begins to appear, even though *no* further oxygen is added to the system. The carbamoyldeoxyHb must therefore be more readily oxygenated than its noncarbamoylated counterpart. This explains the reported higher oxygen affinity of carbamoylated blood (Kilmartin and Rossi-Bernardi, 1969; Cerami and Manning, 1971; May et al., 1972; Diederich, 1972) as being due to the more ready oxygenation of carbamoyldeoxyHb because a conformational change has already taken place.

We have therefore been able to utilize circular dichroism measurements, confirmed by less sensitive difference spectroscopy, to show that the effect of cyanate upon deoxyHb can indeed be separated into two components, one corresponding to a conformational change of the hemoglobin moiety for which no oxygen is required, and the other to the more ready oxygenation of this new conformation by an oxygen present in the system.

Acknowledgments

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