CIRCULAR DICHROISM AS A PROBE OF THE ALLOSTERIC R⇌T TRANSFORMATION IN HEMOGLOBINS AND MODIFIED HEMOGLOBINS

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SUMMARY

The circular dichroism spectra at pH 6.5 of a number of hemoglobins and modified hemoglobins have been recorded in the 280 nm region and interpreted in terms of shifts of the R⇌T allosteric transformation. Inositol hexaphosphate converts aquomet hemoglobin A(S) to the T form but the carbamlyated derivatives are unaffected by inositol hexaphosphate and remain in the R form. Fluorodinitrobenzene and dimethyl adipimidate modified hemoglobins are locked in an intermediate form, and inositol hexaphosphate has little or no effect. The circular dichroism in the 280 nm region is shown to be a useful diagnostic tool for chemical agents that affect the R⇌T allosteric transformation.

INTRODUCTION

The uv-vis absorption spectra, optical rotation and circular dichroism (CD) of hemoglobins (Hb) have been extensively investigated.(1-5) In particular, much experimental data have been accrued and discussion invoked concerning changes in the Soret region and its relationship to the changes at the metal site during the R⇌T allosteric transformation.(6-13) However, relatively little attention has been directed to the CD in the 280 nm region, in spite of the fact that characteristic changes are observed in this region upon R⇌T transformations. These absorptions are probably involved with the aromatic moieties tyrosine, C7(42)α and tryptophan C3(37)β.(7)

Oxy and aquomet HbA produce a weak positive ellipticity at 285 nm characteristic of the R quarternary structure even though there is good reason to expect subtle differences in the geometry of the metal environment as well as in the heme group itself. On the other hand, deoxy HbA gives a moderate negative peak at 287 nm characteristic of the T quarternary struc-

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ture which is accompanied by a distinctly non-planar five-coordinate iron environment. However, there are serious questions being raised as to whether or not the R⇄T allosteric transformation is necessarily linked to changes in the iron coordination sphere.(13-15)

Previous workers have shown that aquomet Hb shows spectral changes in the 280 nm region that can be associated with the R⇄T conformational change. (7) Therefore, we have used aquomet Hb for studies of the R⇄T transformation with chemical additives and molecular modifications.

Furthermore, the substitution 6βA-3 glu→val, (HbS), alters the physical properties of HbA in both the R (oxy, met) and T (deoxy) forms, especially the latter leading to the well known molecular aggregation that is the causative of erythrocyte sickling. The addition of various anions and chemical modifications to deoxy HbS has been shown by oxygen affinity studies, gelation experiments, and mechanical shaking to modify deoxy HbS to behave more like deoxy HbA.(16,17) We suspected that the addition of these anions and chemical modifications that modified the aggregation properties of HbS brought about changes in the R⇄T equilibrium, and therefore their effect could be followed by observing the circular dichroism in the 280 nm region.

METHODS AND MATERIALS

Hemoglobins A and S were purified by DEAE Sephadex chromatography.(18) The α and β chains were isolated by the p-hydroxy--mercuribenzoate method of Bucci and Fronticelli.(19) The purity of the separate fractions was checked by cellulose acetate electrophoresis. FDNB (1-fluoro-2,4-dinitrobenzene) hemoglobin was prepared by the method of DeBruin and Bucci.(20) DMA (dimethyl adipimidate) hemoglobin was prepared as described by Lockhart and Smith.(21) Methemoglobin was prepared by addition of a 1.5 molar excess of $K_3Fe(CN)_6$ followed by passing the mixture through a 1 x 35 cm column of G-25 Sephadex equilibrated with 50 mM Tris-0.1M NaCl (pH 7.5). Deoxyhemoglobin was prepared in a glove box by passing a stream of nitrogen across the solution of oxyhemoglobin. In most experiments a trace of sodium dithionite was added anaerobically in order to ensure complete deoxygenation. Inositol hexaphosphate (IHP) was purchased from P-L Biochemicals, Milwaukee, Wisconsin, and used without further purification. Circular dichroic measurements were performed with a Jasco J-40C automatic recording circular dichrograph using a cell path length of 1 mm. Raw CD curves and background curves were digitized then backgrounds were substrated out and averaged on the IBM 370/168. Final plots were made on a Calcomp 54" flat bed plotter.(23)

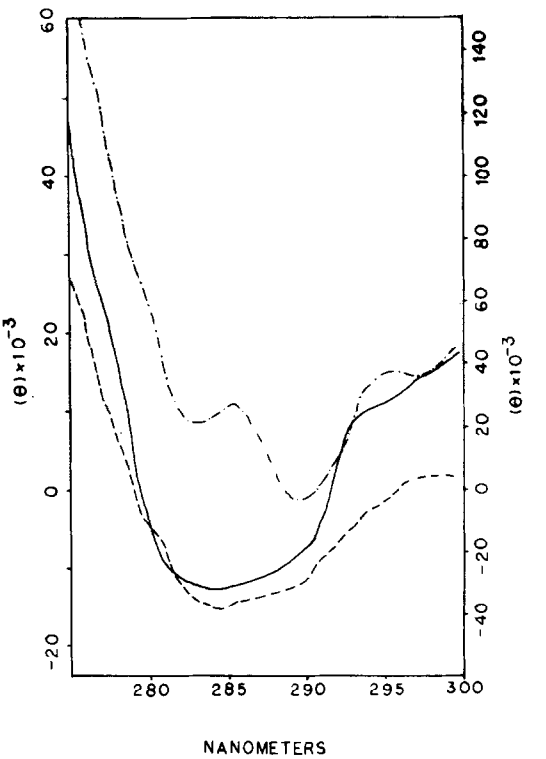

FIGURE 1: CD spectra of a 60 μM (tetramer) solution of aquomet HbS in the presence and absence of IHP and deoxy HbS in 50 mM bis TRIS of pH 6.5.(—·—·) stripped aquomet HbS, (——) stripped aquomet HbS + four moles IHP per mole of Hb,(— — —) deoxy HbS. Ellipticities are reported in terms of tetramers. The right ordinate refers only to deoxy HbS.

RESULTS AND DISCUSSION

We have recorded the CD spectra of stripped oxy, deoxy and aquomet HbS and HbA and find them essentially identical in the 280 nm region at a pH of 6.5. However, if [IHP, 2,3-diphosphoglycerate (2,3DPG) and other phosphates] effector molecules are not rigorously excluded, the oxy CD spectrum appears different from either the oxy or deoxy form. The CD differences between the R and T forms are clearly seen. The characteristic spectrum of the T form of deoxy HbA or HbS is readily generated from stripped aquomet Hb upon adding an excess (>1 mole) of IHP/Hb (Fig. 1). It has been shown that 2,3 DPG and IHP bind in the β chain pocket generated in the T or deoxy form and there-

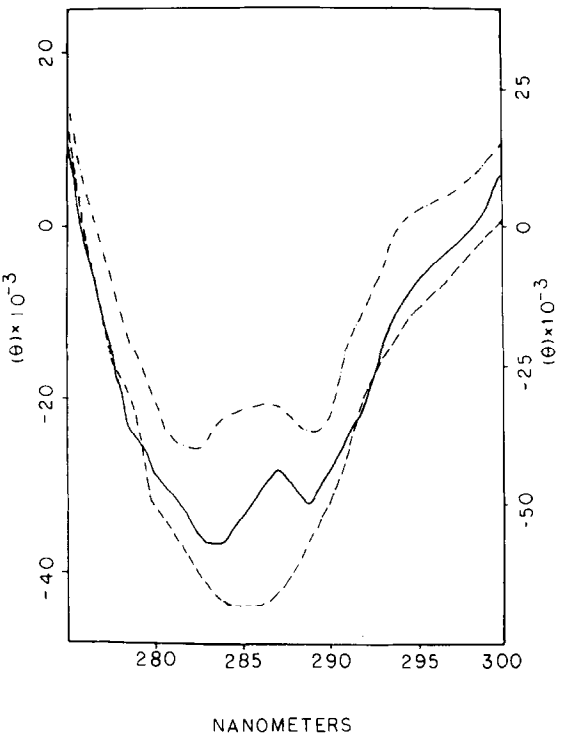

FIGURE 2: CD spectra of FDNB HbA in 50 mM bis TRIS of pH 6.5). (—·—) stripped aquomet FDNB HbA 64 μM (tetramer) (——) stripped aquomet FDNB HbA 64 μM (tetramer) + four moles IHP per mole of Hb, (— — —) deoxy FDNB HbA 94 μM (tetramer). Ellipticities are reported in terms of tetramers. The oxy and carbnmonoxy CD spectra of FDNB HbA also showed negative ellipticities in this region but were omitted for clarity. The right ordinate refers only to deoxy FDNB HbA.

fore these modifiers stabilize the T form.(24,25) We have also investigated the effect on the R⇄T transformation of aquomet Hb by addition of a variety of straight chain and cyclic phosphates and they all develop a negative ellipticity characteristic of the T form. However, the addition of a positively charged spermine molecule not only inhibits the R⇄T conformational change upon the addition of IHP to aquomet Hb, but in fact displaces IHP and converts the T form to the R form. Since HbS aggregates only in the deoxy or T form it might be possible to find charged species less toxic than spermine to reverse or inhibit HbS aggregation and deduce this property from the 280 nm CD spectra.

The CD spectra of stripped aquomet HbA (HbS) in which both the α and β chains are carbamylated is that characteristic of the R form. In this instance the spectra is independent of IHP. Since carbamylation is known to effect the environment of the N terminus of the β chains which is crucial to the binding of IHP in the deoxy or T form the success of cyanate treatment of sickle cell crises can be understood on this basis.

Neer, et al., modified the amino terminus of the alpha chains of hemoglobin with fluorodinitrobenzene (FDNB) and brought about a number of changes in the properties of the molecule including a loss in cooperativity. (26) They proposed that the modified Hb (DNP-Hb) was locked in an altered conformation independent of ligands.(26) The uv CD of aquomet DNP-Hb in the presence and absence of IHP and deoxy DNP-Hb is shown in Fig. 2. The CD of the stripped aquomet form of DNP-HbA shows a distinct negative ellipticity which is probably due to the environment of the dinitrophenyl group on the amino terminus of the α chain. The addition of IHP to stripped aquomet DNP-HbA produces a moderate decrease in ellipticity in the 280-290 nm region but did not produce the characteristic T form of the CD spectra. However, deoxygenation of the oxy form (R') gave rise to the CD spectrum characteristic of the T form. In this case IHP cannot drive the R' form of aquomet DNP-HbA to the T form due to the molecular constraints imposed by the dinitrophenyl modification but deoxygenation of the DNP derivative can bring about the allosteric transformation to T.

From the crystallographic data of Perutz, et al., on liganded Hb's the two α chains are sufficiently separated such that there are no close interchain contacts.(27) However, it is possible (supported by models) that if an α_1 chain N terminus is modified by FDNB the α chains are within sufficiently close proximity that the DNP group can interact with, e.t., tyrosine (HC2)140 of the α_2 chain. Such an interaction might effect the ellipticity at 285 nm as observed and is subject to other experimental tests which are underway.

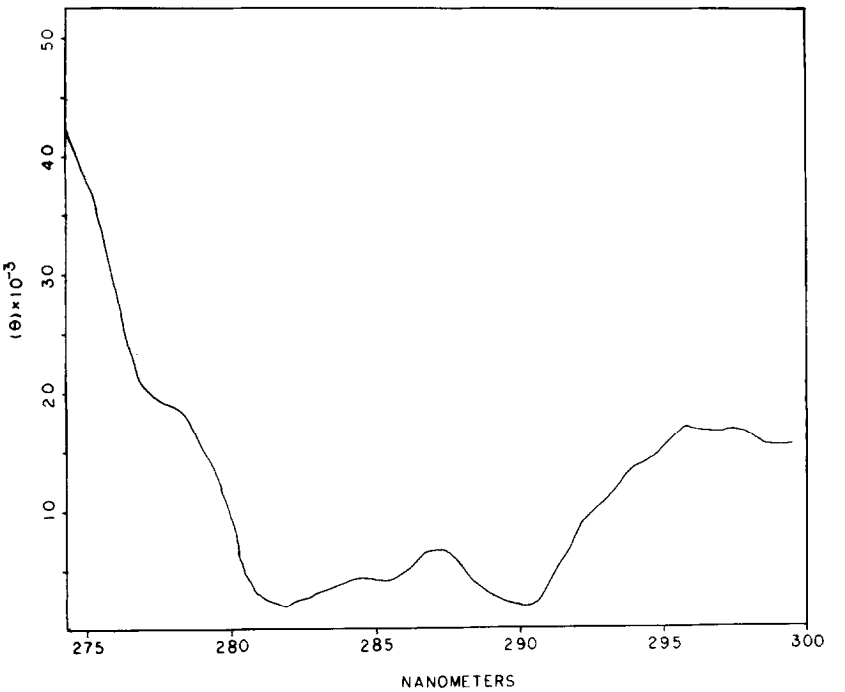

FIGURE 3: CD spectrum of a 60 μM (tetramer) solution of stripped aquomet DMA HbA in 50 mM bis TRIS of pH 6.5. Addition of IHP produced no spectral change.

Dimethyl adipimidate is a bifunctional cross-linking agent which is highly specific for amino groups and has been shown to produce a decrease in the ability of erythrocytes to sickle *in vitro*. The uv CD spectra of stripped aquomet DMA treated HbA or HbS is shown in Fig. 3. The spectrum shows a positive ellipticity similar though not identical to stripped aquomet HbA or HbS. Addition of IHP to aquomet DMA-HbA or aquomet DMA-HbS produced no change in the 280-290 nm CD region indicative of no shift toward the T form. It appears this sickling decrease is again due, at least in part, to the inhibition of the formation of the T form of HbS.

We have shown that the CD in the 280 nm region can be used as a useful probe for chemical additives and modifying agents that may alter the R⇄T allosteric equilibrium. This technique may prove very useful in the classification of chemical anti-sickling agents.

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