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PREPARATION AND CONFORMATIONAL CHARACTERIZATION OF Cr(III)HEMOGLOBIN

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SUMMARY

Chromium(III) substituted hemoglobin has been prepared. Circular dichroism spectra in the UV region have been recorded in the presence and absence of the allosteric affector inositol hexaphosphate. The reactivity with bromthymol blue and p-mercuribenzoate has been measured. All data indicate a T state (or T state-like) structure, whereas an R structure would be expected from the chromium stereochemistry. Similarities to cobalt(III) hemoglobin suggest that the chromium derivative also exists as an internal hemichrome. Thus, despite major tertiary structure differences, "denatured" hemichromes may have a quaternary structure quite similar to deoxyhemoglobin.

INTRODUCTION

Despite numerous studies, the role of the heme active site in controlling the cooperativity of oxygen binding in hemoglobin is not fully understood. The precise causes of the change in heme geometry upon ligand binding, and the relationship between metal spin-state, heme tension and oxygen affinity remain unclear. In the original Perutz model (1,2) the small decrease in the ionic radius of iron accompanying the spin-state transition upon oxygen binding is amplified by stereochemical constraints on the porphyrin into a relatively large movement of the proximal histidine relative to the heme, triggering further conformational changes. This theory predicts that the allosteric state may be largely governed by the position of the metal with respect to the porphyrin plane. Warshel

Abbreviations used: Hb, hemoglobin; CrPPIX, chromium(III)-protoporphyrin IX; DMF, N,N-dimethylformamide; IHP, inositol hexaphosphate; BTB, bromthymol blue; PIPES, piperazine-N, N'-bis[2-ethane sulfonic acid].

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has suggested (3) that the spin-state change is more a consequence of ligand binding than a major controlling factor, and that heme geometry and ligand-porphyrin nonbonded interactions should be emphasized. This model predicts that five coordinate derivatives should (generally) be T state, and six coordinate derivatives R state.

The substitution of other metals for the iron in hemoglobin has played an important role in testing theories of Hb allostery (4). In this communication we report the preparation of a new derivative, chromium(III) hemoglobin. This derivative is expected to be six coordinate, with the metal lying in the mean porphyrin plane. Thus, current theories predict that Cr(III)Hb should be R state. Therefore, we have investigated the conformational state of Cr(III)Hb via several independent methods previously used to characterize the allosteric states of Hbs (5-10).

MATERIALS AND METHODS

Chromium(III)protoporphyrin IX (CrPPIX) was prepared using a modification of the method described by Adler et al.(11) for the preparation of chromium(III) ms-tetraphenylporphin. 200 mg protoporphyrin IX (Sigma Chemical Co.) were reacted with anhydrous $CrCl_2$ (Alfa-Ventron) in 150 ml DMF. The DMF solution was deaerated by bubbling with prepurified nitrogen, and brought to reflux. Portions of a deaerated solution of DMF saturated with $CrCl_2$ were then added with an addition funnel. The course of the reaction was followed by taking aliquots and observing the disappearance of the protoporphyrin Soret band with the simultaneous appearance of the CrPPIX Soret at 445 nm. Upon completion of the reaction, the solvent was flash evaporated. The residue was redissolved in 0.2 N KOH and centrifuged for 20 min. at 18,200 x g. The supernatant was removed, adjusted to pH 3.0 with 5 N HCl, and the resulting precipitate was washed free of acid with distilled water and air dried. The resulting CrPPIX chloride was dissolved in methanol and chromatographed on a column (2.5 x 20 cm) of Polyamid CC 6.6 (Brinkman Instruments, Inc.). After evaporation of the methanol, CrPPIX was dissolved in 0.1 N KOH, precipitated with HNO_3 (final pH 3.0), filtered and air dried. The presence of a characteristic metalloporphyrin spectrum and total absence of any free protoporphyrin absorption bands at reaction completion, in conjunction with the elution of CrPPIX as a single band on the highly resolving (12) Polyamide column indicated a pure preparation.

Apohemoglobin was prepared by the acid/butanone method (13) using human hemoglobin (Sigma Type IV). Cr(III)Hb was reconstituted by dropwise addition of a 1.5 fold excess of CrPPIX in 0.1 M Tris base to the globin in 50 mM Tris buffer, pH 8.5, in an ice bath. After overnight storage at 4°C, the solution was centrifuged briefly to remove a small amount of precipitate. Following a change of buffer to 10 mM phosphate, pH 6.6, on a Biogel P-2 column (Biorad Labs.), the Cr(III)Hb was separated from unreacted CrPPIX and simultaneously concentrated by chromatography on a CM-cellulose (Whatman CM32) column (1.5 x 10 cm). CrHb thus obtained displayed an absorbance ratio, A_{445}/A_{275} = 1.23. CrHb concentrations were determined using an extinction coefficient of 45.2 $mM^{-1}cm^{-1}$ (monomer basis) at 275 nm.

OxyHb was prepared by dithionite reduction of metHb followed by immediate removal of dithionite by gel filtration. Zn(II)Hb was prepared as previously described (14,15). All hemoglobin derivatives were stripped of phosphates by column chromatography on AG11A8 ion retardation resin (Biorad Labs.) and subsequent gel filtration.

Circular dichroism (CD) spectra were recorded on a JASCO J-40 automatic recording spectropolarimeter, interfaced with a Digital PDP 11/34 computer which provided instrument control and signal averaging. Spectra were drawn on a Tektronix 4662 interactive digital plotter after baseline subtraction on a Tektronix 4051 graphics system. Kinetic measurements were made with a Dionex (Durrum) stopped flow system. UV-visible absorption spectra were recorded on a Cary 118 spectrophotometer.

RESULTS AND DISCUSSION

The UV-visible spectrum of Cr(III)Hb is shown in Fig. 1. Noteworthy are the relatively intense bands in the 300-400 nm region. These extra transitions are seen not only in Cr(III)Hb but also in CrPPIX $(H_2O)_2$ and CrPPIX(imidazole)$_2$ and are thus characteristic of Cr(III) porphyrins. The incorporation of CrPPIX into globin is accompanied by a red-shift of the CrPPIX spectrum and a decrease in the α/β absorbance ratio.

The CD spectrum of Hb in the 287 nm region is attributed (5) to tyrosine C7(42)α and tryptophan C3(37)β, and has been shown to be diagnostic of the quaternary state (5,6). In particular, metHb in the presence of IHP, and deoxyHb, derivatives known to possess the T structure, exhibit moderately negative ellipticities in this region, whereas phosphate stripped metHb and oxyHb show weak

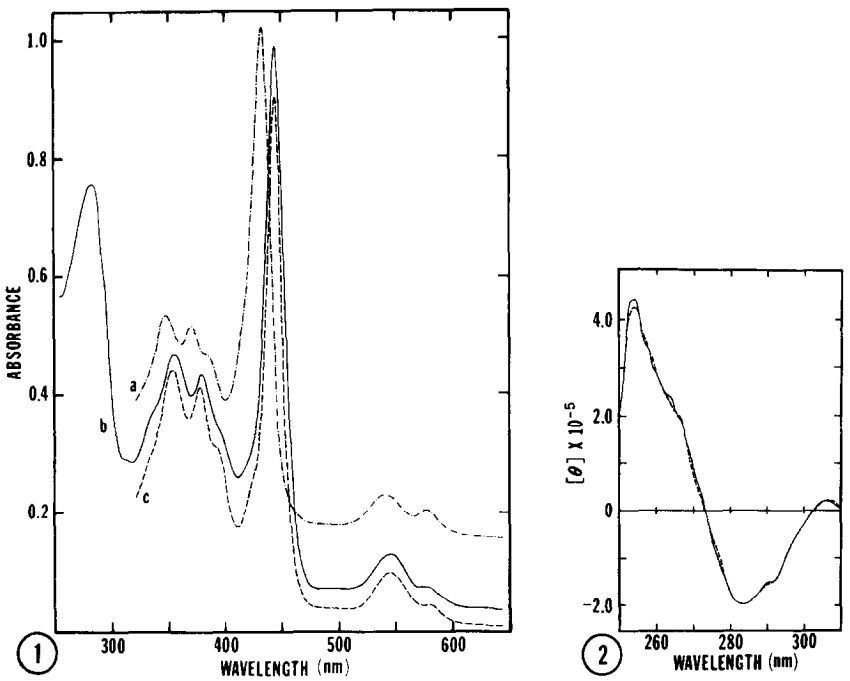

FIGURE 1: UV-visible absorption spectra. (a) 15.3μM CrPPIX in H_2O, pH 7.0 (b) 4.2μM (tetramer) Cr(III)Hb, 50mM PIPES, 100mM NaCl, pH 6.5. Addition of IHP produced no spectral changes. (c) 15.3μM CrPPIX in 2 M imidazole. Spectra have been displaced vertically for clarity.

FIGURE 2: UV region CD spectra of a 4.7μM (tetramer) solution of Cr(III)Hb in the presence (---) and absence (——) of IHP. 50mM PIPES, 100mM NaCl, pH 6.5. Molar ellipticities are in terms of tetramers.

positive ellipticities indicative of the R state (5,7). The CD spectrum of Cr(III)Hb in the ultraviolet region is shown in Fig. 2. The moderatively negative maximum at about 285 nm is similar to that observed previously for T state Hbs. The addition of IHP ($\geq$ 4 fold molar excess) which causes an R → T conversion in metHb (5) produced no major changes in this spectrum, nor in the Soret CD, nor in the UV-visible absorption spectrum. Of course, no changes would be expected for a T state Hb.

The reaction with bromthymol blue has been used previously as a probe of hemoglobin conformation (8). The rate of reaction of deoxyHb has been found to vary between two and four times that of R state

derivatives depending on experimental conditions (8). The reaction of Hb with BTB is heterogeneous with many binding sites per molecule, an observation also made for Mn(II)Hb (16). This also appears to be the case for Cr(III)Hb as evidenced by the observation of multiphasic kinetics. However, the diagnostic phase of the reaction is the fast binding of BTB at a single site unique to the T structure (9). The inhibition of BTB binding below pH 7.5 by IHP (9) suggests that these binding sites are the same or in close proximity, and become unavailable in the R structure. Table I compares the rates of reaction of BTB with Cr(III)Hb and three other derivatives. In accord with previous findings, the R derivatives oxyHb and phosphate-free metHb react at comparable rates. ZnHb, a derivative which has been demonstrated to be T state (17,18) reacts almost twice as fast, and shows a greater total absorbance change. Cr(III)Hb reacts even faster than ZnHb, but displays about the same total absorbance change.

The reaction of p-mercuribenzoate with the β93 cysteine of Hb has been used as an indicator of the quaternary state (7,10). T state derivatives typically react 80-100 times slower than R state Hbs. Preliminary experiments indicate that Cr(III)Hb reacts at least an order of magnitude slower than the T state derivatives ZnHb and metHb + IHP. Thus, by four independent criteria (u.v. CD, bromothymol reactivity, cysteine reactivity, and IHP effects) Cr(III)Hb

TABLE I

Apparent First Order Rate Constants for the Reaction of Hemoglobin Derivatives with Bromthymol Blue

Derivative	HbO_2	Hb^+	Cr(III)Hb	ZnHb
$k^{app}(sec^{-1})$	14	12	31	23

Reaction in 50mM Tris, 100mM NaCl, pH 7.5. Hemoglobin concentration 4.4μM (tetramer). BTB concentration 20.8μM. 20°C.

adopts a "T" state-like conformation. This conformation is not predicted by the current stereochemical theories of Hb allostery.

The trigger mechanism of hemoglobin allostery (1,2) equates an in-plane metal with the R state. High-spin Cr(III) has an ionic radius of 0.62 Å (19), which is smaller than that of high-spin Fe(III) and would therefore be expected to fit nicely into the porphyrin plane. Furthermore, since Cr(III) complexes are almost universally hexacoordinate (20), Cr(III)Hb would be expected to be a rather close analogue of metHb and thus exhibit the R quaternary structure. The present results, however, are obviously inconsistent with an R state for Cr(III)Hb. One conceivable explanation for this apparent contradiction of current Hb theory and experiment is that Cr(III)Hb exists in some altered state which closely resembles deoxyHb. One possibility for such denatured protein is an internal hemichrome, in which both HisF8 and HisE7 are coordinated to the metal. Such a conformation has been previously observed for Co(III)Hb (21,22). Like Cr(III)Hb, the cobalt derivative exhibits a negative ellipticity at 287 nm which is even more pronounced than that of deoxyCoHb (23). Co(III)Hb was found unable to bind most anions (22). Similarly, we have seen that the visible absorption spectrum of Cr(III)Hb shows no changes indicative of ligand binding in the presence of 0.1 M N_3^- (50 mM Tris, pH 7.5). Furthermore, as seen in Fig. 1, the UV-visible spectrum of Cr(III)Hb is virtually identical to that of bisimidazole CrPPIX. It is totally unaffected by exposure to 0.5 M salicylate which is known to convert native metHb to the internal hemichrome (24), implying that the hemichrome structure is preformed in Cr(III)Hb.

On the basis of these results, it is reasonable to assign a hemichrome structure to Cr(III)Hb. A particularly striking feature of these results is the observation of structural parallels between "denatured" hemichromes and the native T state Hb. The parallelism

is maintained in three independent probes; circular dichroism, BTB binding rates, and β93 sulfhydryl reactivity. Each of these methods measures a different property and indeed probes a different region of the Hb molecule. It is likely then, that although the tertiary structure of internal hemichromes must differ from that of deoxyHb, the quarternary structures of these derivatives are surprisingly similar. It may be noted that in the process of binding the E7 histidine to the heme iron, the E helix must certainly be displaced in the direction observed (25) for the R → T transition.

A T-like structure of Hb hemichromes may have interesting physiological implications. The lifetime of the Hb molecule in vivo is governed by the balance between oxidative and reductive mechanisms, and hemichrome formation. The latter can result in the eventual precipitation of Hb within the erythrocyte. Thus, factors which bias the allosteric equilibrium toward the T state might well contribute to hemichrome formation and its consequences. This possibility is under active investigation.

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