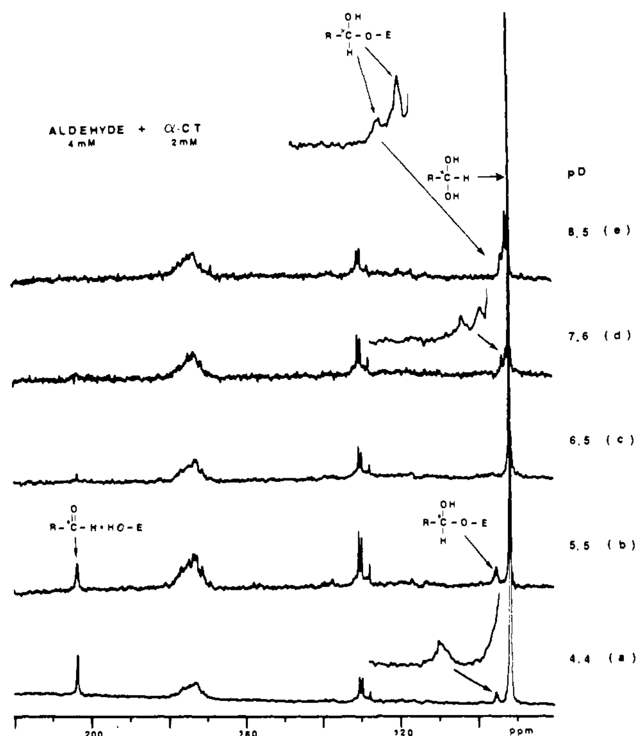
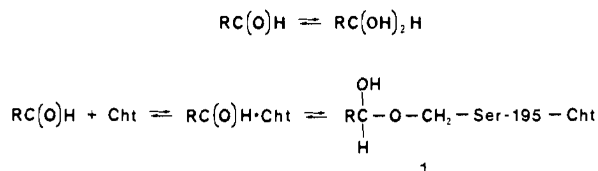


Figure 1. ^{13}C NMR, 50.32 MHz, proton gated decoupled spectra, 8K time domain data points, 7.1-μs pulse width (25 μs = 90° pulse) on an IBM WP-200 SY spectrometer. α-chymotrypsin was dissolved in 0.1 M sodium phosphate buffer solution in 99.9% D_2O containing 1 mM EDTA. To this solution was added the aldehyde dissolved in Me_2SO-d_6 so that the final concentration of Me_2SO was about 20% (v/v). *N*-acetyl-L-phenylalaninal 4 mM; fully active α-chymotrypsin 2mM. pD: (a) 4.4 (inset of hemiacetal, expanded region, 92–104 ppm), (b) 5.5, (c) 6.5, (d) 7.6 (inset of hemiacetal, expanded region, 92–104 ppm), (e) 8.5 (inset of hemiacetal, expanded region, 92–104 ppm).

Scheme I



tributed to the slower tumbling of the macromolecular complex.[3,9]

The chemical shifts and signal intensities for the two enzyme complex signals are pH dependent (Figure 1). The relative signal intensity of the noncovalent enzyme complex at 203.6 ppm increases at lower pH suggesting more of the inhibitor is noncovalently bound, which in turn explains why the inhibitor binds less tightly at low pH.

Significantly, at higher pH (>7) *two signals for the hemiacetal* have been observed, which are also pH dependent (insert, Figure 1c,d, expanded region of 110–80 ppm of Figure 1).

Kennedy and Schultz[10] and recently Shah and Gorenstein[4] have observed that K_i decreases only ~4-fold from pH 3.0 to 8.0 for the association of *N*-benzoyl-L-phenylalaninal and *N*-acetyl-DL-*p*-fluorophenylalaninal to Cht. This small variation in the binding constant was attributed to the binding of the aldehyde as the neutral hemiacetal throughout this pH range. The His_{57} imidazole likely functions as in the normal enzymatic mechanism to deprotonate Ser_{195} Oγ-H to initially yield the hemiacetal anion. This complex then rapidly picks up a proton to yield the neutral hemiacetal. While the two hemiacetal signals could be assigned to the neutral and anionic hemiacetals, they could also arise from slowly interconverting conformational isomers[7] or from interaction of the hemiacetal with an active site titrable group (such as His_{57}).

Recently it has been claimed that a tetrahedral intermediate (99 ppm) has been detected by ^{13}C NMR with pepsin and a ketone inhibitor.[11] Also a tetrahedral intermediate (98 ppm) has been detected by ^{13}C NMR with trypsin and a chloromethyl ketone specific inhibitor.[12] Very recently Gamcsik et al.[7] have studied the structure of the tetrahedral adduct of papain and an aldehyde by ^{13}C NMR. The results reported herein and taken together with the previous ^{13}C NMR studies indicate that ^{13}C NMR is an excellent probe for the detailed characterization of the protease complexes.

Acknowledgment. Support of this work by NIH (GM-17575) is gratefully acknowledged. Purchase of the NMR spectrometer was assisted by an NSF departmental equipment grant.

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Carboxylate Heme Complexes as Models for Hemoglobin J Altgeld Gardens (β-F8-His → Asp)

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Received January 30, 1984

One of the more intriguing mutant hemoglobins discovered in recent years is Hb J Altgeld Gardens (β-F8-His → Asp) (Hb J AG), where the proximal histidine of the normal β-subunits is replaced by Asp.[1] Surprisingly, this hemoglobin (Hb) is reported to exhibit normal O_2 affinity, only a slightly decreased Hill coefficient, and "normal" absorption spectra of the oxy, met, and cyanmet derivatives. Hb J AG is the only functional Hb known where the F8 residue is other than histidine. Since it is not apparent how the nearly normal functionality of Hb J AG can be reconciled with the mechanisms currently thought to operate in Hb[2] and since samples of this Hb are not presently available, it was thought that a study of carboxylate heme complexes might provide some insight into the molecular mechanism employed by this unusual Hb.

A titration of heme diester with potassium propionate is shown in Figure 1. Tight isosbestic points were not observed but the close approach to isosbesty in the visible region indicates that the visible spectrum of the intermediate complex(es) is similar to that of the final product. Plots of log $[(A - A_0)/(A_\infty - A)]$ vs. log [propionate] (see ref 4) at either 592 or 558 nm were nonlinear with slopes of approximately 1 and 2 during the early and latter stages, respectively, of the titrations. Additional evidence that the final complex is 6-coordinate comes from the close similarity between the spectra of the carboxylate complexes and those of the heme diphenolate complexes[5] and the dihydroxyl complex.[6] Also the acetate complex is probably low spin[7] whereas a 5-co-

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(7) Determined by Evans method[8,9] in DMF-d_7 using 3% *tert*-butyl alcohol and 9% toluene as the references. The Δ frequency observed with the acetate heme diester complex (0.0 H_z) was within experimental error, the same as that of cyanohem*in* diester (0.3 H_z) (one unpaired electron) and significantly less than that of chlorohem*in* diester (2.0 H_z) (five unpaired electrons), all measured under the same conditions. NMR spectra were taken with a Perkin-Elmer R-32 spectrometer.

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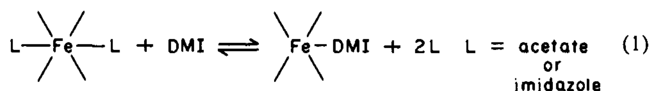
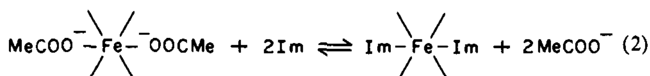
ordinate complex would be expected to be high spin. It follows that the intermediate complex is the 6-coordinate monocarboxylate–heme–DMF (DMF = *N,N*-dimethylformamide) complex and DMF is subsequently replaced by another carboxylate. An estimate of the product of the two association constants (neglecting DMF) gave a value of 10^6 M^{-2}, essentially the same as that determined for imidazole under the same conditions.

Verification that carboxylates and imidazole are nearly equivalent in ligand strength was obtained by titrating both the diacetate and diimidazole heme diester complexes with 1,2-dimethylimidazole (DMI). Both titrations gave excellent isosbesty; the only product formed was the 5-coordinate 1,2-dimethylimidazole heme complex:



The equilibrium constant in DMF at 25 °C for the reaction where L = acetate was 2.1×10^{-3} M, and where L = imidazole was 1.7×10^{-4} M. Combining these two reactions gives reaction 2 with an equilibrium constant of 12.



The addition of CO to the diacetate heme diester complex in DMF resulted in the appearance of a two-banded visible spectrum typical of 6-coordinate CO–heme complexes. This spectrum was remarkably similar to the spectrum of the DMF–heme–CO complex,[3] engendering the suspicion that very little, if any, carboxylate–heme–CO complex was present. When CO was added to a toluene solution of the 18-crown-6-potassium diacetate heme diester complex, the resulting spectrum had a Soret peak at 419 nm and a broad peak centered at 552 nm. This spectrum was identical with the spectrum obtained from heme diester plus CO in pure toluene and analogous to that reported[10] for deuteroheme–CO in benzene. An upper limit for the association constant for acetate to heme–CO in toluene is estimated to be 20 M^{-1}. Chang and Dolphin[11] also reported that they were unable to prepare carboxylate–heme–CO complexes.

The addition of O_2 to dicarboxylate–heme complexes in DMF at −55 °C results in the appearance of a spectrum essentially identical with that previously observed for the DMF–heme–O_2 complex.[3] Since O_2–heme complexes oxidize rapidly in toluene solution even at low temperature, an alternative experiment was designed whereby a perturbation of the equilibrium between DMF and another weak ligand coordinated trans to O_2 could be detected. 1,2,4,5-Tetramethylimidazole (TMI) was chosen as the other weak ligand because it forms only a 5-coordinate heme complex even at low temperature, and the spectra of the TMI-heme complexes are distinctive.[3]

In DMF solution at −55 °C the concentrations of acetate and TMI were adjusted such that at least 95% of the heme complex formed was the diacetate complex (Table I). After the addition of O_2 the spectrum obtained was indistinguishable from that obtained in an identical solution except that it contained no acetate (3 and 4 in Table I). Analysis of the spectrum for the two components was consistent with a mixture containing 72% TMI–heme–O_2 and 28% DMF–heme–O_2. The equilibrium position of the reaction, TMI–heme–O_2 + DMF ⇌ DMF–heme–O_2 + TMI, is unaffected by a relatively high concentration of acetate, and, therefore, carboxylates are weak ligands trans to O_2 as well as to CO.

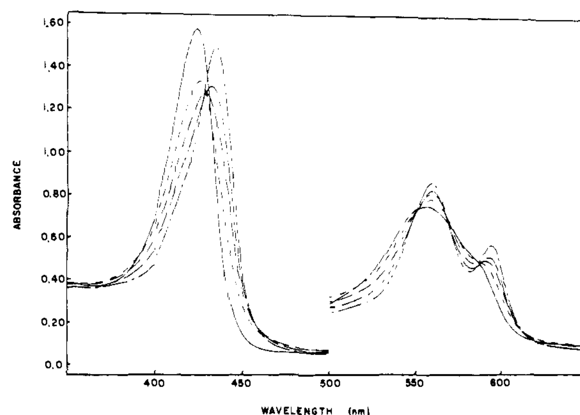


Figure 1. Titration or protoheme dimethyl ester (9.7 μm) with potassium propionate in DMF. Protohemin dimethyl ester was reduced by the addition of a 3–5-fold excess of $Na_2S_2O_4$ in 5 μL of degased water per 10 mL of DMF solution.[3] Potassium propionate was added via syringe in degassed aqueous solution; concentrations are (—) O, (---) 0.39, (– –) 0.65, (–·–) 1.9, and (- - -) 3.2 mM. Wavelengths of maxima in the final spectrum are 434, 558, and 592 nm. Cell path length was 0.2 cm from 350 to 500 nm, 1.0 cm from 500 to 700 nm. Spectra were recorded on a Hewlett-Packard 8450A spectrophotometer with 10-s scan times.

Table I. Spectral Parameters of Complexes Formed in Competition between Acetate, DMF, and TMI for Coordination to Heme and to Heme–O_2[a]

ligand (concn)	wavelength at maxima		α/β^b
	deoxygenated	plus O_2	
(1) DMF (solvent)	424, 554	410, 538, 572	1.19
(2) 1 + K O_2CCH_3 (3.5 mM)	434, 558, 592	410, 538, 572	1.06
(3) 1 + TMI (10.2 mM)	426, 556	412, 542, 574	0.93
(4) 1 + K O_2CCH_3 (3.5 mM), TMI (10.2 mM)	434, 558, 592	412, 542, 574	0.93

[a] Low-temperature (−55 °C) spectra were obtained as previously described.[3] Protohemin dimethyl ester (14 μM) was reduced with H_2/Pd and the solution decanted from the Pd. [b] α/β refers to the ratio of absorbance of the long wavelength visible peak (α) to the shorter wavelength visible peak (β) of the O_2 complexes.

Although these results provide a basis for understanding some of the properties of Hb J AG, the reported cooperativity[12] appears to be unreconcilable with the results presented here and the mechanism currently considered to operate in normal hemoglobins.[2] This dilemma has prompted us to consider the possibility that the heme in hemoglobins can coordinate under some circumstances with the distal (E7) histidine. Coordination with F8 would be favored in low-affinity (T) conformations, whereas coordination to E7 could occur in high-affinity (R) conformations. Thus, superimposed upon the conformational equilibrium would be an equilibrium involving movement of the heme between proximal and distal residues. If such were the case, substitution of the F8 His by Asp might have relatively little effect on the allosteric equilibrium since carboxylates and imidazole are approximately equally strong ligands. However, since carboxylates are exceedingly weak ligands trans to O_2, oxygenation of the β-subunits in Hb J AG would occur only when the heme is coordinated to the E7 His.

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(12) The Hill coefficient Hb J AG is reported to be 2.3 compared to 2.8 for Hb A.[1] It should be noted that the person from whom Hb J AG was obtained also has β-thalassemia minor and, therefore, Hb J AG is her only major hemoglobin.[1] She is now in her early 30's and reportedly in good health. It is unlikely that a human could function normally without a highly cooperative hemoglobin.[13]

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