Keller, H., & Debrunner, P. G. (1980) *Phys. Rev. Lett. 45*, 68.
Koshland, D. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 98.
Kramers, H. A. (1940) *Physica (Amsterdam) 7*, 284.
Landauer, R., & Swanson, J. A. (1961) *Phys. Rev. 121*, 1668.
Larson, R. S., & Kostin, M. D. (1978) *J. Chem. Phys. 69*, 4821.
Levy, R., Karplus, M., & McCammon, J. A. (1979) *Chem. Phys. Lett. 65*, 4.
Lifshitz, I. M., Grosberg, A. Y., & Khokhlov, A. R. (1978) *Rev. Mod. Phys. 50*, 638.
Lumry, R., & Rosenberg, A. (1975) *Colloq. Int. C.N.R.S. 246*, 53.
McCammon, J. A., & Wolynes, P. G. (1977) *J. Chem. Phys. 66*, 1452.
McCammon, J. A., & Karplus, M. (1980) *CRC Crit. Rev. Biochem.* (in press).
Miner, C. S., & Dalton, N. N., Eds. (1953) *Glycerol*, Reinhold, New York.
Montgomery, J. A., Chandler, D., & Berne, B. J. (1979) *J. Chem. Phys. 70*, 4056.
Parak, F., Frolov, E. N., Mössbauer, R. L., & Goldanskii, V. I. (1980) *J. Mol. Biol.* (in press).
Prigogine, I. (1957) *The Molecular Theory of Solutions*, Interscience, New York.
Richards, F. M. (1979) *Carlsberg Res. Commun. 44*, 47.
Seidell, D. (1940) *Solubilities*, Vol. I, Van Nostrand, New York.
Sharrock, M., & Yonetani, T. (1980) *Biochim. Biophys. Acta* (in press).
Skinner, J. L., & Wolynes, P. (1978) *J. Chem. Phys. 69*, 2143.
Somogyi, B., & Damjanovich, S. (1975) *J. Theor. Biol. 48*, 393.
Weber, G. (1972) *Biochemistry 11*, 864.

Association of Alcohols with Heme Proteins: Optical Analysis and Thermodynamic Models†

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ABSTRACT: At concentrations lower than those causing denaturation, methanol, ethanol, and 1-propanol produce changes in optical absorption of alkaline ferricytochrome *c*. These changes arise from weak equilibrium associations characterized by dissociation constants at 25 °C of about 4 and 2 M, respectively, for the methanol– and 1-propanol–ferricytochrome *c* complexes. The difference spectra and temperature dependence of enthalpy and entropy changes accompanying formation of methanol and 1-propanol complexes, as well as changes induced in the EPR spectra, are different and suggest distinct binding modes. Considered in conjunction with related parameters from ferrihemoglobin and ferrimyoglobin, the spectral and thermodynamic data are consistent with models in which methanol is bound directly to the ferric ion of cytochrome *c*, methanol and ethanol are bound directly to the ferric ions of hemoglobin and myoglobin, and 1-propanol is bound to a hydrophobic region of cytochrome *c*. Both the absolute and alcohol-induced optical difference spectra of these proteins have been simulated, the former through summation of Gaussian bands and the latter as the difference between two such summations, one with parameters slightly altered from the other. This analysis reveals and characterizes previously unresolved structure, which is discussed in terms of electronic transitions of the heme group and changes caused by differing interactions of the heme with surroundings. Similarity between the difference spectra produced by IHP perturbation of ferrihemoglobin and that from the difference between absolute spectra of ferrimyoglobin and ferrihemoglobin suggests that, with ferrihemoglobin as reference, the conformations about the hemes of ferrimyoglobin and of ferrihemoglobin–IHP are in some way similar.

Methanol and ethanol at low concentrations react with ferric myoglobin and hemoglobin to form thermodynamically and spectroscopically well-defined complexes (Brill et al., 1976). There is a similarly well-defined complex of bacterial catalase with methanol, but the binding is much weaker. The site and nature of the association of the alcohol molecules with these high-spin ferric heme proteins have not been established, and analogous studies of reactions of small alcohols with low-spin ferric heme proteins have not been reported. In this paper we describe both new experimental results and an analysis of the data at hand which contribute information about contrasting responses to the formation of complexes by proteins.

The new data reported here are from ferricytochrome *c* in alkaline solution at moderately high ionic strength. This system was chosen to allow correlation of optical and EPR[1] results. In EPR investigation of the effects of alcohols, pH, and ionic strength, there were found, for example, methanol-induced transformations in cytochrome *c* above pH 10 (see below). The use of 0.3 M NaCl makes ionic strength essentially independent of changing contributions of buffer ions when pH is varied. It should also be noted that cytochrome *c* at neutral pH has a tendency to polymerize in the presence

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[1] Abbreviations used: EPR, electron paramagnetic resonance; IR, infrared; UV, ultraviolet; MCD, magnetic circular dichroism; IHP, inositol hexaphosphate.

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of organic solvents (Margoliash & Lustgarten, 1962). The time dependence of optical changes in ferricytochrome *c* initiated by alcohols at neutral pH is probably associated with a polymerization process. This precludes clear assignment of the observed response to alcohol complex formation, especially at high protein concentrations such as would be used in EPR studies.

Experimental Procedure

Horse heart cytochrome *c* (Sigma Chemical Co., Type III) was dissolved in 0.3 M NaCl and 0.01 M PO_4, pH 6.4, buffer. Dialysis against 0.01 M ferricyanide (in the same buffer, at 4 °C) was employed to convert all heme iron to the ferric valence state. The concentration of cytochrome *c* was determined spectrophotometrically after reduction (a_{550nm} = 27.6 mM^{-1} cm^{-1}; Margoliash & Frohwirt, 1959), and purity values ($A_{550nm}^{reduced}/A_{280nm}^{oxidized}$) were in the range 1.23–1.27.

Optical difference spectra were recorded with a Cary Model 14 spectrophotometer. Alcohol additions to the sample cell were matched in volume by buffer additions to the reference cell. Because of the nonadditivity of the volumes entering aqueous–alcohol mixtures, a protein concentration difference develops between sample and reference cells. Optical absorbance data were corrected for this effect, which was in the range 15–25% of the largest peak-to-trough difference absorbances. These measurements were carried out at pH 11.0 in 0.3 M NaCl and 0.01 M PO_4. At alkaline pH ferricytochrome *c* reduces spontaneously and, in order to minimize the amount of ferrous species present, two precautions were taken. First, the stock protein solution (prepared at pH 6.4) was diluted with a buffer of pH somewhat above 11 just before initiation of each experiment. Second, a small amount of ferricyanide, equivalent to the heme concentration, was added to maintain the metal in the higher valence state, as has been done for the cupric blue proteins (Brill et al., 1968). The heme concentration range in these experiments was 0.15–0.25 mM, and initial sample volume was 2.0 mL.

Stock alcohol solutions varied from about 30 to 50% alcohol by volume and contained 0.3 M NaCl and 0.01 M PO_4, pH 11.0. After the base line was recorded, appropriate alcohol stock and buffer additions (50–100 μL) were made to sample and reference cells, respectively, and the cells were inverted several times for mixing. Solutions were then allowed 15 min to come to equilibrium before difference spectra were recorded. This procedure was repeated 5 or more times, and after the last spectrum was recorded, pH values of the solutions were usually found to be within ±0.1 unit of 11.0.

Samples for EPR measurements were kept at high concentrations (~10 mM) by employing dialysis in formation of the alcohol complexes. For this purpose, a dialysis tube of small diameter was tied off such that an air bubble under slight positive pressure was formed, the sack was placed in appropriate buffer containing organic reagent, and rocking dialysis was carried out for 4 h at 4 °C. EPR spectra were recorded at liquid nitrogen temperature with a Varian 4500 X-band spectrometer.

Methods of Data Analysis and Results

Optical and Thermodynamic Changes in Ferricytochrome c. Methanol, ethanol, and 1-propanol induce changes in the optical absorption spectrum of alkaline ferricytochrome *c*. Difference absorption spectrophotometry was used to quantify the extent of reactions with methanol and 1-propanol, but the optical changes produced by ethanol at low concentrations are too small to permit the same measurements with the instrumentation available to us. In Figure 1 are shown difference

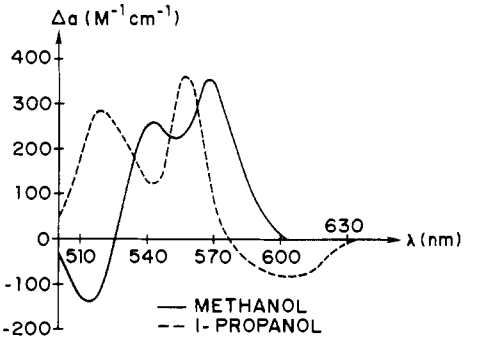

FIGURE 1: Optical difference spectra produced by the association of ferricytochrome *c* with methanol and 1-propanol vs. ferricytochrome *c* alone as reference. The limiting values of peak-to-trough absorptivity were used to scale the spectra which were recorded in 0.3 M NaCl, 0.01 M PO_4 buffer, pH 11.0, at 25 °C.

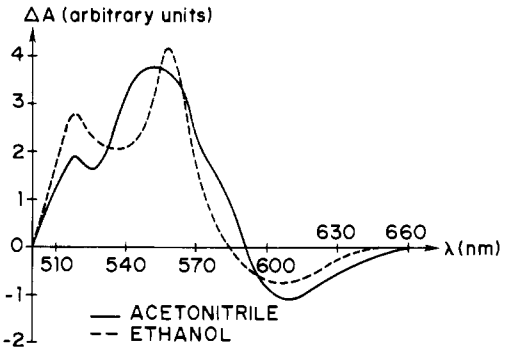

FIGURE 2: Optical difference spectra produced by the association of ferricytochrome *c* with ethanol and acetonitrile. Buffer conditions are as in Figure 1, but the absorptivities are scaled to arbitrary units.

spectra at pH 11 for the methanol and 1-propanol complexes of ferricytochrome *c* vs. alcohol-free protein. The changes produced by ethanol are very similar to those produced by 1-propanol (Figure 2). Preliminary data suggest that acetonitrile also forms a complex with alkaline ferricytochrome *c*, and the difference spectrum so induced, as seen in Figure 2, exhibits features which can be roughly described as a linear combination of the methanol- and propanol-induced alterations. The presence of isosbestic points indicates, in all four cases, that only two cytochrome *c* species are present in significant concentration. Scatchard-like plots of the methanol and 1-propanol reactions at 25 °C are linear and provide the dissociation constants, *K*, of the alcohol–ferricytochrome *c* complexes at this temperature and limiting values of absorbance change (in peak-to-trough difference absorbance used in the plot). In each case six titrations were performed and analyzed and the results averaged. In all cases, the data were fit by a simple equilibrium such that alcohol concentration enters as the first power.

The temperature dependence was determined by adding enough alcohol to bring the reaction to partial completion and then dropping the temperature from an initial value of 25 °C to a final value of 6 °C over a period of several hours while recording difference spectra at approximately 3 °C intervals. Spectra were recorded at a few temperatures as solutions returned to 25 °C; these were in agreement with those taken as the temperature was lowered. pH measurements at the conclusion showed a change of less than 0.1 unit in all cases. The dissociation constants at the various temperatures were calculated from

$$K(T) = \frac{[\Delta A_{lim} - \Delta A(T)][\text{alcohol}]}{\Delta A(T)}$$

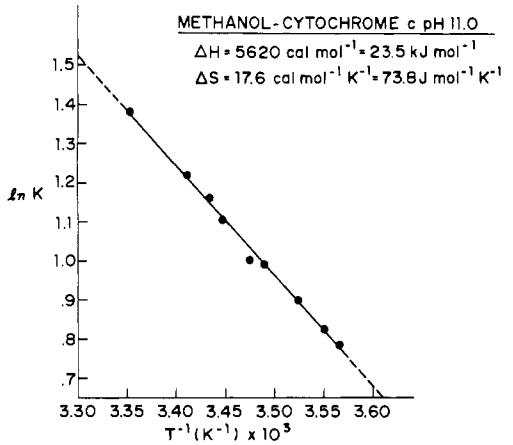

FIGURE 3: Temperature dependence of the dissociation constant of the methanol–ferricytochrome *c* complex expressed as ln *K* vs. $1/T$. The buffer was 0.3 M NaCl, 0.01 M PO_4, pH 11.0.

where ΔA_{lim} and $\Delta A(T)$ are respectively the limiting and observed (at temperature T) peak-to-trough difference absorbances. ΔA_{lim} can be determined in two ways: (1) as the product [cyt *c*]Δa_{lim}, where [cyt *c*] is the concentration of the protein in the cells and Δa_{lim} is the average value of limiting peak-to-trough difference absorptivities obtained from ΔA = 0 intercepts of the six Scatchard plots; (2) from

$$\Delta A_{lim} = \Delta A(25\ ^\circ C)\left[1 + \frac{K(25\ ^\circ C)}{[\text{alcohol}]}\right]$$

where $\Delta A(25\ ^\circ C)$ is the peak-to-trough difference absorbance observed in the temperature dependence experiment after addition of alcohol at 25 °C and $K(25\ ^\circ C)$ is the average value obtained from the slopes of the six Scatchard-like plots. ΔA_{lim} values from these two methods differed from their mean by 8% for methanol and 10% for 1-propanol; mean values were used in the formula above for $K(T)$.

Representative van't Hoff plots are shown in Figure 3 (methanol reaction) and Figure 4A (1-propanol reaction). In each case three temperature-dependence experiments were performed. For the two alcohols ln *K* is clearly a different function of $1/T$, linear for methanol and exhibiting significant positive curvature for 1-propanol. Linear dependence upon $1/T$ has been observed for the methanol complexes of ferric myoglobin and hemoglobin and for the ethanol complex of ferrihemoglobin (Brill et al., 1976). In order to test the possibility that curvature in the case of the 1-propanol–ferricytochrome *c* complex was the result of some unknown inaccuracy in ΔA_{lim}, we carried out calculations of $K(T)$ for four separate values of ΔA_{lim}, ranging from 0.5 to 1.5 of the average value obtained above; ln *K* remained a similarly curved function of $1/T$.

The averages of the ΔH and ΔS values obtained from linear least-squares analysis of the three methanol van't Hoff plots (subject to certain assumptions; Brill et al., 1976) are given in Table I. For 1-propanol, ΔH and ΔS are temperature dependent, and it is necessary to use a difference form of the van't Hoff equation to obtain these parameters:

$$\Delta H(\bar{T}_{i,i+1}) = -R\frac{\ln K(T_{i+1}) - \ln K(T_i)}{\frac{1}{T_{i+1}} - \frac{1}{T_i}}$$

$$\Delta S(\bar{T}_{i,i+1}) = \frac{\Delta H(\bar{T}_{i,i+1})}{\bar{T}_{i,i+1}} + R \ln K(\bar{T}_{i,i+1})$$

Here, $\bar{T}_{i,i+1}$ is the average temperature between successive data points i and $i+1$. The solid circles of Figure 4B show values of ΔH (as a function of $T = \bar{T}_{i,i+1}$) obtained in this manner from the data points of Figure 4A. The average values (from the three separate temperature-dependence experiments) of ΔH and ΔS for dissociation of 1-propanol at 23.4 and 8.6 °C (extremes of the temperature range) are given in Table I. Since heat capacity at constant pressure, C_p, and enthalpy of a process are related by

$$d(\Delta H)/dT = \Delta C_p$$

a linear, least-squares fit to the couples $(\Delta H, \bar{T})_{i,i+1}$ was carried out for each of the three sets of data available, one of which is shown in Figure 4B. The average of the three values of ΔC_p so obtained is 1.07 kJ mol^{-1} K^{-1} (255 cal mol^{-1} K^{-1}). Given ΔC_p and the values for the enthalpy and entropy changes for the process at some reference temperature, T_{ref}, ΔH and ΔS at any temperature can be calculated from

$$\Delta S = \Delta S_{ref} + \int_{T_{ref}}^{T} \Delta C_p \frac{dT}{T} = \Delta S_{ref} + \Delta C_p \ln (T/T_{ref})$$

$$\Delta H = \Delta H_{ref} + \int_{T_{ref}}^{T} \Delta C_p\, dT = \Delta H_{ref} + \Delta C_p(T - T_{ref})$$

In Table I are listed values of ΔH and ΔS at 0 and −5 °C extrapolated with these equations from the reference parameters at 8.6 °C; note the constancy of K at the lower temperatures.

Alcohol-Induced EPR Spectral Changes. Changes in EPR spectrum of ferricytochrome *c*, both of large and small magnitude, can be induced by perturbation with alcohols at alkaline pH. Figure 5 shows the spectrum of ferricytochrome *c* at pH 11.0 in 0.3 M NaCl and 0.01 M PO_4; it is similar to the spectrum seen at pH 7 in being composed of broad resonances. However, the *g* values are altered and the shape suggests the presence of more than one paramagnetic species. The existence of multiple forms of ferricytochrome *c* at alkaline pH is well documented (Brautigan et al., 1977; Gupta & Koenig, 1971). 1-Propanol causes slight spectral changes with an enhancement of the resonance centered at $g = 2.21$ (as seen in Figure 6A)

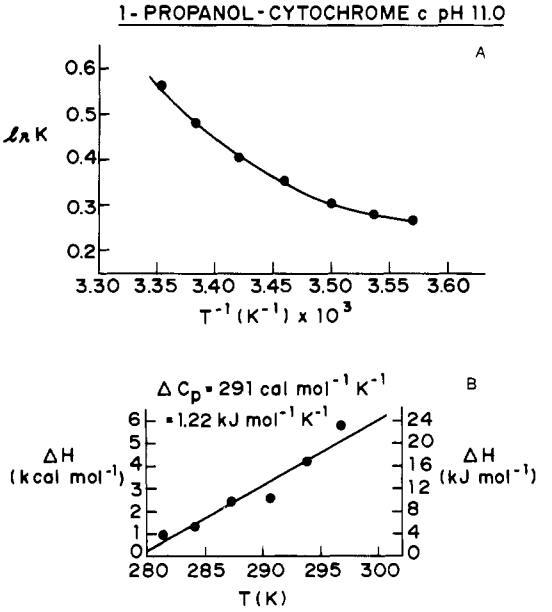

FIGURE 4: Temperature dependence of the dissociation constant of the 1-propanol–ferricytochrome *c* complex (A) and standard enthalpy change as a function of temperature (B). The deviation from linearity in part A is expressed as a temperature dependence of the enthalpy change in part B. The buffer conditions were as in Figure 3.

Table I: Thermodynamic Parameters Characterizing the Dissociation of Alcohol Complexes of Heme Proteins

heme protein	pH	alcohol	temp (°C)	K (M)	ΔG		ΔH		ΔS		obsd range[a] (°C) of constant ΔH ($\Delta C_p = 0$)	ref
					kJ mol^{-1}	cal mol^{-1}	kJ mol^{-1}	cal mol^{-1}	J mol^{-1} K^{-1}	cal mol^{-1} K^{-1}		
ferricytochrome *c*												
horse heart	11.0	methanol	25	3.6	−3.2	−760	24	5800	92	22	7–25	this paper
		1-propanol	25	2.3								
			23.4	2.1	−1.8	−440	22	5200	79	19	ΔC_p = 1.07 kJ mol^{-1} K^{-1} = 255 cal mol^{-1} K^{-1}	this paper
			8.6	1.5	−1.0	−230	6	1400	24	5.8		this paper
			0[b]	1.5	−0.9	−210	−3.2	−760	−8.5	−2.0		
			−5.0[b]	1.5	−1.0	−230	−9	−2000	−28.5	−6.8		
ferrimyoglobins												
sperm whale skeletal	6.3	methanol	25	0.17	4.4	1000	15	3700	37	9	10–33	Brill et al. (1976)
horse heart	6.3	methanol	25	0.22	3.8	900	25	6000	72	17	10–33	Brill et al. (1976)
ferrihemoglobins												
human	6.3	methanol	25	0.045	7.3	1700	25	6000	60	14	10–32	Brill et al. (1976)
		ethanol	25	0.20	4.1	1000	20	4700	52	13	6–34	Brill et al. (1976)
horse	6.3	methanol	23	0.041	7.5	1800	21	4900	45	11	6–34	Brill et al. (1976)
		ethanol	23	0.22	3.6	900	17	4000	45	11	8–33	Brill et al. (1976)

[a] Each temperature is either a limit of the range of measurements or a temperature at which ln K vs. $1/T$ begins to show curvature. [b] See text for method of extrapolation for 0 and −5.0 °C entries.

Table II: Band Analysis of the Absolute Absorption Spectra of Ferric Horse Heart Myoglobin and Human Hemoglobin at pH 6.3 and Horse Heart Cytochrome *c* at pH 11.0, and the Corresponding Difference Spectra of Several Alcohol Complexes

band identification	*myoglobin*, Fe(III), aquo			*hemoglobin*, Fe(III), aquo			myoglobin, methanol difference			hemoglobin, ethanol difference		
	energy (cm^{-1})	width (cm^{-1})	oscillator strength[b]	energy (cm^{-1})	width (cm^{-1})	oscillator strength	energy difference (cm^{-1})	width difference (cm^{-1})	oscillator strength difference	energy difference (cm^{-1})	width difference (cm^{-1})	oscillator strength difference
16 × 10^3 cm^{-1} (625 nm)	15 619	409	69	15 860	375	147	−37	−28	0	26	−5	6
	15 841	428	75				103	21	2			
α	16 970	701	226	17 230	700	232	0	0	0	0	0	0
β	18 360	450	101	18 560	450	105	16	5	0	16	0	−1
20 × 10^3 cm^{-1} (500 nm)	19 910	990	1087	20 000	1000	990	53	0	−18	25	0	−7
22 × 10^3 cm^{-1} (455 nm)	21 778	573	360	21 800	610	332	13	−13	−14	7	−4	−6
23 × 10^3 cm^{-1} (435 nm)	22 975	485	629	22 980	530	647	nv[a]	nv	nv	nv	nv	nv

band identification	cytochrome *c*, Fe(III), pH 11.0			cytochrome *c*, methanol difference			cytochrome *c*, 1-propanol difference		
	energy (cm^{-1})	width (cm^{-1})	oscillator strength	energy difference (cm^{-1})	width difference (cm^{-1})	oscillator strength difference	energy difference (cm^{-1})	width difference (cm^{-1})	oscillator strength difference
16 × 10^3 cm^{-1} (625 nm)	16 200	1100	46	nv	nv	nv	nv	nv	nv
α	17 790	670	255	−146	0	−62	35	−42	47
β	19 085	850	766	−112	62	90	45	−52	−29
21 × 10^3 cm^{-1} (475 nm)	20 990	600	260	nv	nv	nv	nv	nv	nv
23 × 10^3 cm^{-1} (435 nm)	23 270	1120	2095	nv	nv	nv	nv	nv	nv

[a] nv signifies not varied in fitting the difference spectrum. [b] $10^4 f_i$.

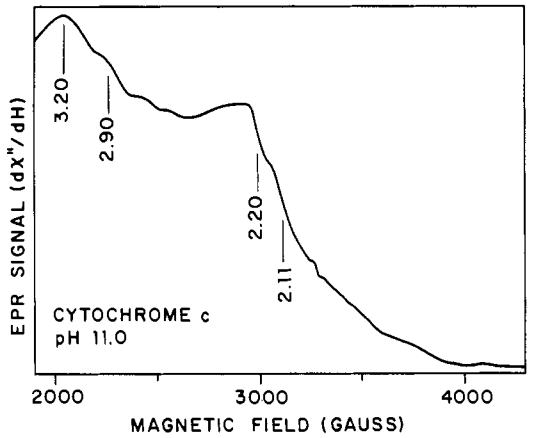

FIGURE 5: EPR spectrum of a frozen solution of horse heart ferricytochrome *c* at liquid nitrogen temperature in 0.3 M NaCl, 0.01 M PO_4 buffer, pH 11.0.

but alters little the broad line widths and overall shape of the unperturbed spectrum. In contrast, parts B–D of Figure 6 show that methanol induces large spectral changes in a significant fraction of macromolecules present, in the form of transitions from broad- to narrow-lined structures composed of two distinct sets of EPR resonances. The relative amplitude of members of each set is very much a function of ionic strength, pH, and alcohol concentration; just above pH 11, in the presence of methanol, a third narrow-lined species appears with *g* values identical with those of certain hydroxide forms of myoglobin. This same set of *g* values has been noted with ferricytochrome *c* in the absence of alcohol at "very high" pH (Brautigan et al., 1977).

Optical Band Analysis. Heme states of ferric myoglobin, hemoglobin, and cytochrome *c* are affected so slightly by formation of complexes with alcohols that the energy levels differ little from those of unperturbed heme proteins. Therefore, the possibility that optical difference spectra of the perturbed vs. unperturbed heme proteins could be characterized by small changes in the parameters [energy, width, and oscillator strength; see, e.g., Brill (1977)] of the unperturbed absorption bands was investigated. Gaussian bands were found to give a better fit than Lorentzian bands. Absolute absorption spectra are then expressed by

$$a_{\bar{\nu}} = (9.24 \times 10^4)\sum_i \frac{f_i}{w_i} \exp[-(\bar{\nu} - \bar{\nu}_i)^2/(2w_i^2)]$$

where $a_{\bar{\nu}}$ is the absorptivity (mM^{-1} cm^{-1}) at wavenumber $\bar{\nu}$ ($\bar{\nu} = 1/\lambda$), the index *i* refers to the band centered at $\bar{\nu}_i$, f_i is the oscillator strength, and w_i (cm^{-1}) is the half-width of band *i*. In Table II are listed band parameters for the absolute spectra of ferric myoglobin and hemoglobin at pH 6.4 and ferricytochrome *c* at pH 11.0 and the changes in these parameters which attend formation of several alcohol complexes. [The spectra of alcohol complexes of ferric myoglobin and hemoglobin come from an earlier study (Brill et al., 1976), and we thank the authors for providing this information in more detail than was published.] Note that while in general the bands for myoglobin differ little from those for hemoglobin, there is a splitting of the 16.0×10^3 cm^{-1} level in the former protein. The absolute spectrum of ferrimyoglobin at pH 6.4 can be fit by a single Gaussian band at 15.7×10^3 cm^{-1}, but the structure of the difference spectrum with methanol in this region requires the existence of two bands. Note also that for both ferric myoglobin and hemoglobin it was necessary to place bands at 21.8×10^3 and 23.0×10^3 cm^{-1} and for ferricytochrome *c* at 21.0×10^3 and 23.3×10^3 cm^{-1}, bands which are unre-

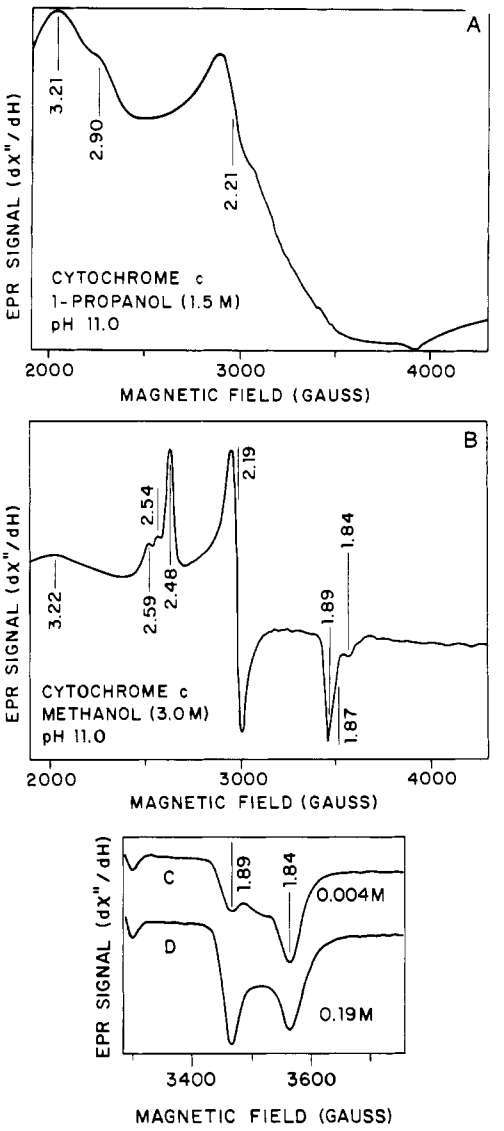

FIGURE 6: Effect of perturbants on the frozen solution EPR spectrum of horse heart ferricytochrome *c*. (A) 1.5 M 1-propanol; (B) 3.0 M methanol; (C, D) detail of high-field resonances at fixed methanol concentration, 5 M, with 0.004 and 0.19 M NaCl, respectively. Other conditions: pH 11.0, 0.01 M PO_4 buffer.

solved in the absorption spectra. MCD spectra provide independent evidence for bands in this region (Vickery et al., 1976a,b). Although near-IR and Soret bands contribute to absorptivity within the region of interest, parameters which describe these bands were only approximated and therefore are not listed in Table II. (For example, Soret band parameters were chosen for an accurate description of the lower energy half of the absorption peak, that half which borders the spectral region of interest.) For the 29 energies at 250-cm^{-1} increments, it was possible, by repeated adjustment of the band parameters, to match calculated and observed absorptivities within 2% accuracy for all but three amplitudes of ferrimyoglobin and five of ferrihemoglobin from 14.5×10^3 to 21.5×10^3 cm^{-1} (690–465 nm), and for all but five ferricytochrome *c* amplitudes from 16.5×10^3 to 23.5×10^3 cm^{-1} (625–425 nm). The fit was better than 1% for more than half of the points for each of the three proteins. From 14.5×10^3 to 23.5×10^3 cm^{-1}, the root mean square absorptivity difference (mM^{-1} cm^{-1}) was 0.34 for ferrimyoglobin, 0.31 for ferrihemoglobin, and 0.15 for ferricytochrome *c*. It should be noted from Table II that there is a reduction in transition energy of the 16×10^3 cm^{-1}, α, β, and (20–23) $\times 10^3$ cm^{-1} bands from

Table III: Comparison of Observed and Simulated Difference Spectra of Several Alcohol Complexes of Heme Proteins

heme protein	pH	alcohol	troughs: energy (cm^{-1}) obsd	troughs: energy (cm^{-1}) simulated	troughs: absorptivity (M^{-1} cm^{-1}) obsd	troughs: absorptivity (M^{-1} cm^{-1}) simulated	peaks: energy (cm^{-1}) obsd	peaks: energy (cm^{-1}) simulated	peaks: absorptivity (M^{-1} cm^{-1}) obsd	peaks: absorptivity (M^{-1} cm^{-1}) simulated	isobestic: energy (cm^{-1}) obsd	isobestic: energy (cm^{-1}) simulated
ferrimyoglobin, horse heart	6.3	methanol	15 260	15 050	−57	−38	16 310	16 450	170	175	15 820	16 050
			15 630	15 750	−100	−106	20 410	20 700	70	52	17 010	17 120
			19 160	19 150	−390	−394					20 120	20 330
ferrihemoglobin, human	6.3	ethanol	15 480	15 330	−130	−100	16 080	16 100	260	270	15 700	15 630
			18 320	18 450	−150	−166					17 150	16 970
			19 230	19 300	−150	−150					20 200	20 450
ferricytochrome *c*, horse heart	11.0	methanol	18 080	18 130	+225	+183	17 570	17 100	350	355	19 020	19 100
			19 460	19 600	−138	−140	18 420	18 350	255	188		
		1-propanol	16 600	16 640	−82	−125	17 950	17 830	360	397	17 330	17 100
			18 450	18 600	+125	+148	19 270	19 330	285	283		

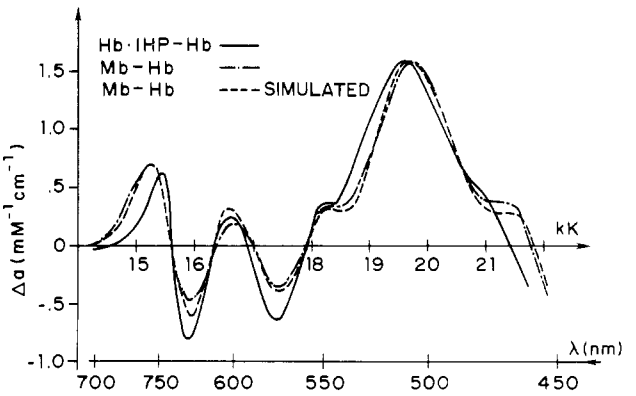

FIGURE 7: Simulated difference spectra of horse heart myoglobin vs. human hemoglobin as reference and the measured difference spectrum of human hemoglobin with IHP vs. human hemoglobin as reference. Two methods were used to obtain the former spectra. The first was a point-for-point subtraction of the two absorption spectra, and the second was a subtraction of the Gaussian simulations to the absorption spectra. All spectra were from heme proteins in the ferric state in solutions near pH 6.4. The IHP-induced spectrum is replotted from data by Perutz et al. (1974). The simulation shown includes, in order to improve the fit to the IHP-induced spectrum, a change in oscillator strength of 5% in all bands of one of the proteins (such as would result from a corresponding small error in determining heme concentration).

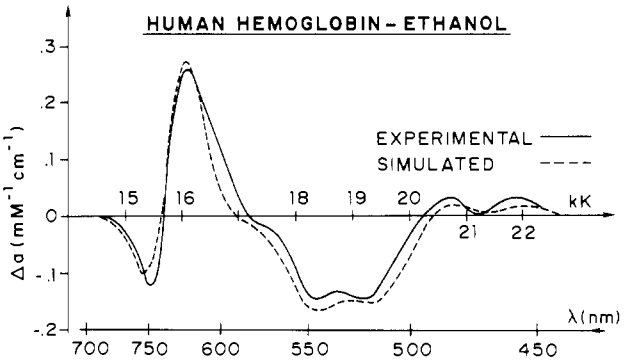

FIGURE 8: Optical difference spectra produced by the association of human hemoglobin (ferric) with ethanol vs. human hemoglobin (ferric) as reference and the corresponding difference spectrum simulated through small changes in the human hemoglobin absorption band parameters. The parameters were obtained through a fit of the absolute absorption spectra. The limiting values of peak-to-trough absorptivity were used to scale the spectra which were recorded in 0.033 M PO_4 buffer, pH 6.4, at 25 °C.

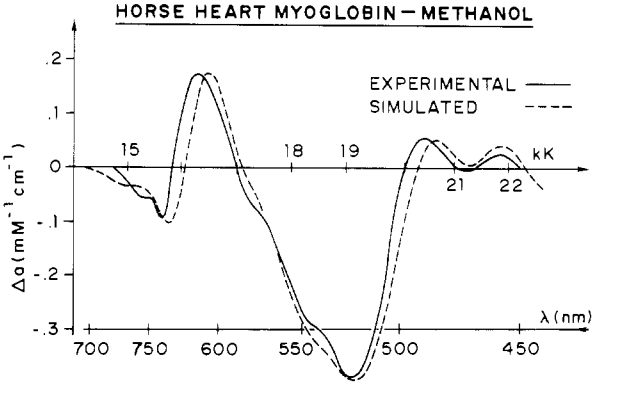

FIGURE 9: Optical difference spectra which correspond to association of horse heart myoglobin (ferric) with methanol vs. horse heart myoglobin (ferric) and the corresponding simulated difference spectrum. Details are as in Figure 8.

ferric hemoglobin to myoglobin; shifts displayed by the α and β bands are greater than those of the others.

An independent test of the accuracy of Gaussian band analysis of ferric myoglobin and hemoglobin is a comparison of the simulated difference spectrum with the spectrum obtained by subtracting the experimental absolute spectrum of one from that of the other. As shown in Figure 7, simulated and measured spectra are essentially identical. Included also in this figure is the experimental difference spectrum induced by addition of IHP to hemoglobin (Perutz et al., 1974), which shows striking similarity to those just mentioned. A plausible physical basis for this similarity will be discussed later.

In Table III prominent features of observed and simulated difference spectra for the several alcohol complexes are compared. Fits of the difference spectra are seen to be significantly poorer than those of the absolute spectra. This is especially true with the alcohol complexes of ferricytochrome *c* because of different rates of autoreduction and/or reoxidation in the sample and reference cells (caused by the presence of alcohol in the former). In effect, a very small amount of a third component (ferrocytochrome *c*) with an intense, narrow band near 18×10^3 cm^{-1} is introduced into the difference spectra and produces distortion in that region. Figures 8 and 9 give a spectral comparison of the simulated and observed difference spectra for ferrihemoglobin–ethanol and ferrimyoglobin–methanol.

Salient features of the difference parameters of Table II are as follows: (1) formation of methanol and ethanol complexes has little effect upon the α and β bands of ferric myoglobin and hemoglobin; (2) formation of the methanol complex of ferrimyoglobin and the ethanol complex of ferrihemoglobin increases the energies of all the bands (16×10^3, 20×10^3, and 22×10^3 cm^{-1}) which strongly involve the metal orbitals (the average of the two bands of ferrimyoglobin at 16×10^3 cm^{-1} is considered in this context); the magnitudes of these

shifts are about half those found between ferric hemoglobin and myoglobin; (3) formation of the methanol complex of ferricytochrome *c* decreases the energies of the α and β bands; these decrements are much greater than the increments found with 1-propanol; (4) formation of the complex of 1-propanol and cytochrome *c*, in contrast to all the other complexes, is accompanied by narrowing of the α and β bands; these narrowings are in proportion to the unperturbed widths.

Discussion

Thermodynamic Analysis of Heme Protein–Alcohol Interactions. In Table I are listed thermodynamic parameters which characterize the dissociation of alcohol complexes of ferric myoglobin and hemoglobin as well as ferricytochrome *c*. Dissociation of the 1-propanol complex of ferricytochrome *c* is unique among the reactions listed in having a nonzero change in specific heat, ΔC_p. The other reactions exhibit constant enthalpy changes over all, or nearly all, of the ranges of temperature at which measurements were made. Positive values for ΔC_p are commonly found for transfer of hydrocarbon groups from an organic (hydrocarbon, alcohol, etc.) solvent to an aqueous phase, but these values are normally much smaller than 1.1 kJ mol^{-1} K^{-1}. Two other observations are in order before analyzing in detail various contributions to the thermodynamic parameters. First, there are the large values of enthalpy change (at 25 °C), ranging from 13 to 25 kJ mol^{-1}, which immediately rule out hydrogen bonding as the prime factor in holding alcohol–protein complexes together. Second, the temperature independence of enthalpy changes for all but the reaction of ferricytochrome *c* and 1-propanol requires that the exposure of hydrophobic groups does not significantly change when alcohol complexes form or dissociate and hence excludes hydrophobic bonding as a dominant factor (except in the case of cytochrome *c*–propanol).

The discussion which follows deals with the reaction

$$\text{protein·alcohol} \rightarrow \text{protein} + \text{alcohol}$$

The observed entropy difference, $\Delta S_{measured}$, can be expressed as

$$\Delta S_{measured} = \Delta S_{mixing} + \Delta S_{freedom} + \Delta S_{solvation} + \Delta S_{structure}$$

where $\Delta S_{mixing} \equiv$ entropy of mixing, which is 33.4 J K^{-1} per mol of alcohol entering the aqueous buffer, $\Delta S_{freedom} \equiv$ entropy change associated with the translation and increased rotation of the freed molecules, $\Delta S_{solvation} \equiv$ entropy change associated with the interaction of water molecules with hydrophobic groups on the protein and the alcohol, and $\Delta S_{structure} \equiv$ entropy change arising from local and conformational changes in the protein. In the complex, the alcohol molecule loses between half and all of the rotational entropy it has when free (values listed in Table IV) and there is a gain in entropy (internal) from low-frequency vibrations of the biomolecular system, which Paige (1977) considers to be about 50 J mol^{-1} K^{-1}. Thus, for the dissociation of water, methanol, ethanol, and 1-propanol (total solution entropies listed in Table IV) from protein complexes, $\Delta S_{freedom}$ is in the range 15–20, 50–75, 65–110, and 85–140 J mol^{-1} K^{-1}, respectively. If the process involves the substitution of a water molecule for an alcohol, i.e.

$$\text{protein·alcohol} + H_2O \rightarrow \text{protein·}H_2O + \text{alcohol}$$

the ΔS_{mixing} term is unchanged but the $\Delta S_{freedom}$ term becomes the difference between those of alcohol and water and is in the range 30–60 (methanol), 45–95 (ethanol), and 65–125 (1-propanol) J mol^{-1} K^{-1}. $\Delta S_{solvation}$ for the introduction of pure liquid methanol into an aqueous phase is −29 J mol^{-1} K^{-1}, which balances ΔS_{mixing}; corresponding values for ethanol and 1-propanol are −46 and −54 J mol^{-1} K^{-1}. All of the entropy values given in this paragraph are for 25 °C.

Table IV: Entropies of Liquids at 25 °C[a,b]

	entropy					
	total (measd)		translational (calcd)[c]		rotational = total − translational	
liquid	J mol^{-1} K^{-1}	cal mol^{-1} K^{-1}	J mol^{-1} K^{-1}	cal mol^{-1} K^{-1}	J mol^{-1} K^{-1}	cal mol^{-1} K^{-1}
water	70.0	16.7	62	15	8	2
methanol	127.0	30.3	68	16	59	14
ethanol	161.0	38.4	73	17.5	87.5	21
1-propanol	192.0	45.9	77	18	115	27.5

[a] *Handbook of Chemistry and Physics* (1978–1979). [b] *Landolt-Börnstein Tabellen* (1962). [c] From S_{trans} (per mole) = $2.5R + R \ln [(2\pi RMT)^{3/2} v_f N^{-4} h^{-3}]$. v_f, the free volume per mole, is estimated from $RT_{boiling}/(2.7 \times 10^4$ atm) (Fowler & Guggenheim, 1949) and is about 1 cm^3 in all four cases. M is the molecular weight. N is the Avogadro number.

Whether or not substitution of water is involved, $\Delta S_{freedom} + \Delta S_{solvation} + \Delta S_{structure} = \Delta S_{measured} - \Delta S_{mixing} = 59$ J mol^{-1} K^{-1} for methanol dissociation from ferricytochrome *c*. If methanol is assumed to be totally exposed to solvent before dissociation, as is suggested by ΔC_p strictly = 0 (i.e., temperature-independent enthalpies), this entropy change is within both ranges (methanol replaced or not replaced by water). However, it is plausible that part of the methanol could be masked by protein groups before dissociation such that a fraction of the portion of $\Delta S_{solvation}$ connected with the alcohol (−29 J mol^{-1} K^{-1} for an entire methanol) can be included in the above formulation. (Exposure of an entire buried methanol to the aqueous phase would account for less than 0.1 of the ΔC_p found in the case of propanol, and it is possible that a small contribution could exist and be lost in the noise.) In this case, $\Delta S_{freedom} + \Delta S_{structure} = 59 - \lambda(-29)$ J mol^{-1} K^{-1}, which, for $\lambda > 1/5$, is within the range found for dissociation without water substitution. There is little evidence for an iron–hydroxyl link in this enzyme at pH 11, and it is more likely that a protein-contributed group replaces methanol as a ligand. $\Delta S_{measured} - \Delta S_{mixing}$ averages 20 J mol^{-1} K^{-1} for the methanol complexes of ferric myoglobin and hemoglobin and 15 J mol^{-1} K^{-1} for ethanol complexes of ferrihemoglobin. These are relatively small values, falling below even the ranges for water substitution, and suggest that the alcohols replace water as a ligand and retain much of their rotational entropy in the complexes. Availability of this rotational freedom is supported by the fact that ligands of greater bulk than ethanol can be accommodated at the iron binding site of myoglobin and hemoglobin (Antonini & Brunori, 1971). For the three dissociations discussed, there can be negative contributions from $\Delta S_{solvation}$, $\Delta S_{structure}$, or both, but the former must be small in view of the temperature-independent enthalpies in contrast to the ferricytochrome *c*–1-propanol case to be discussed below.

For models of dissociation of 1-propanol from ferricytochrome *c* with and without exchange for a water molecule, $\Delta S_{solvation} + \Delta S_{structure} = \Delta S_{measured} - \Delta S_{mixing} - \Delta S_{freedom}$ is within the range −15 to −75 and −35 to −90 J mol^{-1} K^{-1}, respectively, at 25 °C. A negative entropy change may be interpreted in terms of exposure of hydrophobic portions of the protein and the propanol molecule when the complex is dissociated and masking of these areas when alcohol is bound. Furthermore, it has been suggested (Brants, 1964) that heat capacity changes may be accounted for by differences in the

interaction of hydrophobic groups with bulk water as would be expected if masking were present. This is reflected in the large value of ΔC_p, which is much larger than that found for the transfer of a propanol from the organic to the aqueous phase. Thus, both entropy and heat capacity changes point toward the same kind of model for dissociation of propanol from ferricytochrome *c*, that associated with a conformational change which enhances hydrophobic exposure. It might be suggested that decrease in $\Delta S_{solvation} + \Delta S_{structure}$ upon alcohol dissociation is mainly due to a decrease in the number of conformations allowed, i.e., remove denaturant and the protein refolds. However, the heat capacity change suggests exposure of hydrophobic regions, an unlikely occurrence during refolding.

The magnitude of the heat capacity change can be quantitatively interpreted in terms of transfer of hydrocarbons to the aqueous phase. With $\Delta C_p = 33N_H$ J mol^{-1} K^{-1} (Gill & Wadsö, 1976), N_H, the number of hydrogens exposed, is 32 when propanol dissociates from cytochrome *c* and 0 for methanol. The number of exposed propanol hydrogens is 7 to 8; hence, about 24 hydrogens in the protein are unmasked. The dissociation of a single propanol is not likely to expose 24 protein hydrogens unless a conformational change occurs simultaneously. (Linked dissociation of two or more propanols is ruled out by the titration behavior.) With $\Delta S_{solvation} = -21.5 - 5.9N_H$ J mol^{-1} K^{-1} (Gill & Wadsö, 1976), one finds that $\Delta S_{structure}$ is 120–175 J mol^{-1} K^{-1} for bound alcohol not replaced by water and 135–195 J mol^{-1} K^{-1} otherwise. These large positive values indicate a gain in conformational entropy upon propanol release.

The model just developed requires several hydrophobic residues to be exposed to the aqueous phase and remain so when methanol but not propanol is bound. ORD (Myer, 1968) and NMR (Gupta & Koenig, 1971) suggest heme–polypeptide separation and hence polypeptide exposure at alkaline pH.

Analysis of Band Parameters. Three points of major significance in the spectral analysis are (1) previously unresolved absorption bands in ferrihemoglobin, ferrimyoglobin, and ferricytochrome *c* are uncovered by Gaussian band analysis of absolute spectra, (2) alcohol-induced difference spectra of the aforementioned proteins can [apart from (3), next] be simulated by small variations of band parameters obtained from such an analysis (thereby reinforcing its validity), and (3) fine structure, within an absolute Gaussian band, can be revealed through the constraint of reproducing difference spectra. While the literature provides other kinds of evidence for the presence of absorption bands and hidden structures mentioned in (1) and (3), their existence is required and parameters necessary for their description are quantified by the absolute and difference spectra themselves. This reemphasizes the importance of optical absorption and difference spectroscopy in the investigation and quantification of changes induced in absorption bands through protein perturbations, especially when the changes are minute.

Absolute Absorption Spectra. Gaussian fits to the optical absorption spectra of ferrihemoglobin, ferrimyoglobin, and ferricytochrome *c* require two bands in the region (21.0–23.5) $\times 10^3$ cm^{-1}, previously unresolved from the shape of these spectra but contributing significant intensity. Indirect evidence provided by both spectral techniques (Vickery et al., 1976a,b; Eaton & Hochstrasser, 1968) and theoretical calculations (Zerner et al., 1966) can be found in the literature to support their presence.

Identical heme structure and ligands bound in positions 5 and 6 imply that spectral differences between ferric hemoglobin and myoglobin must originate with different interactions between hemes and the amino acids which surround them. This view is supported by, for example, CD measurements (Nagai et al., 1969), amino acid differences in the heme pocket (Takanao, 1977; Dickerson & Geis, 1969; Dayhoff & Eck, 1968), and X-ray diffraction (Perutz et al., 1974; Ladner et al., 1977; Takano, 1977). Construction of space-filled models of the two proteins by McLachlan (Perutz, 1970) shows the interior of myoglobin to be rigid because of tightly packed side chains, whereas hemoglobin appears more loosely packed. In studies of infrared ligand stretch frequencies [as reviewed by Rifkind (1973)] and CO recombination (Alberding et al., 1978), the "tight" heme pocket of myoglobin has been noted. Furthermore, resonance Raman data indicate smaller heme core size in low-spin species and are consistent with greater iron out-of-planarity in high-spin species of myoglobin as compared with hemoglobin (Asher & Shuster, 1979).

When band centers of ferric hemoglobin and myoglobin are compared, a trend of greater displacement of the α, β, and Soret bands (those associated with π–π^* transitions) than the 16×10^3, 20×10^3, and $(20–23) \times 10^3$ cm^{-1} bands (those suggested to be of mixed or charge-transfer origin) is discovered. π–π^* transition energy changes would be affected most by interactions of the porphyrin ring with the protein environment while charge-transfer and mixed transition changes are influenced as well by the metal coordination. The decrease in energies of π–π^* transitions from ferric hemoglobin to myoglobin may then be viewed within the framework of the nonpolar interactions between heme and the two globins. In view of the large number of established heme–globin van der Waals contacts (Antonini & Brunori, 1971) and strong dependence upon separation distance of the accompanying forces, an observable effect caused by difference in protein conformation upon the energy levels of the system can be expected. One should also note that perturbations of electron distribution within metal-containing porphyrins through both solvent alteration (Boucher, 1970) and side-chain substitution (Falk, 1964) are known to shift α, β, and Soret bands in the same direction. Changes in iron out-of-planarity as well as porphyrin structural perturbations could produce the observed smaller shifts in mixed or charge-transfer transitions.

Simultaneous examination of all differences in band parameters between ferric horse heart myoglobin and human hemoglobin can be seen in the difference spectrum of Figure 7. This difference spectrum (horse heart myoglobin – human hemoglobin) shows a striking resemblance to that produced by addition of IHP to human hemoglobin [(IHP + human hemoglobin) – human hemoglobin]. Furthermore, Soret changes follow this trend. Perutz et al. (1974) supply the pH dependence of the IHP-induced difference spectrum at intervals throughout the range of 5–7.5, and the pH 6.5 spectrum matches more closely the simulated one, which is composed of components recorded at pH 6.4, than any of the others. In addition, spectra induced with IHP showed pronounced variation over the pH range indicated above. Taken together, these results imply that, when viewed from human hemoglobin as reference, conformation about hemes in horse heart myoglobin and human hemoglobin–IHP is in some way similar. It appears that conservative amino acid substitution between the two proteins produces a compensatory effect, such that substitution in the vicinity of the heme is not the direct cause of the difference between ferric hemoglobin and myoglobin absorption bands. Substitution may, however, contribute to the difference in overall conformation between the two proteins, which seems to be the cause of the variations in spectra

and heme–globin interactions.

Similarities exist in structural differences between hemoglobin and myoglobin, and those induced by IHP in ferric and ferrous hemoglobin ("oxy → deoxy"-like) (Perutz et al., 1974; Perutz, 1970; Perutz & TenEyck, 1971). Myoglobin and IHP–hemoglobin exhibit increased iron out-of-planarity over hemoglobin. The myoglobin heme pocket is tighter; in the β chian, the oxy → deoxy transition lessens the distance between porphyrin and E helix and blocks position 6. Other evidence centers on the stabilizing influence on tertiary structure of the hydrogen bond between tyrosine H23 (H22) in myoglobin and α and β HC2 in hemoglobin and the main chain carboxyl. This hydrogen bond is intact in ferric myoglobin and ferrous deoxyhemoglobin; however, ferric hemoglobin shows significantly more motion in the vicinity of the carboxyl-terminal ends (Watson, 1969; Ladner et al., 1977).

Difference Spectra and Structural Differences: Alcohol Complexes of Ferric Myoglobin and Hemoglobin. Binding of methanol to ferrimyoglobin and ethanol to ferrihemoglobin raises transition energies of all visible bands except α (remains unchanged); shifts at 16×10^3 and 20×10^3 cm^{-1} are about twice as large as that of the β band. Consistent with this difference is alteration of d orbital energy levels; such changes affect charge transfer or mixed bands more than mainly π–π^* bands. The most direct manner in which d level changes could be affected is through ligand exchange, and the spectral differences do appear to follow the correlation between absorptivity and transition energy found in several high-spin derivatives of ferrimyoglobin by Smith & Williams (1970).

Spectral changes induced by association with alcohols are not large compared to those induced by exchange of water for other high-spin ligands; the alcohol-induced changes are even less than those found between hemoglobin and myoglobin. The small magnitude of change can be taken to suggest that the alcohols do not bind to the iron but at a position which induces a conformational change that affects heme–globin interactions. However, the alcohols have certain properties in common with water, and their similarity to the hydrophobic environment in the proximity of the iron does not argue for large spectral changes with their replacement of water bound in position 6. The two factors which are most important in the binding process, the electronic structure of the hydroxyl portion of the alcohol and the orientation allowed in the hydrophobic crevice, may be similar to those found with bound water. Hydrophobic ligands much larger than ethanol can be accommodated within the crevice, and the thermodynamics of dissociation suggest rotational freedom of the two smallest alcohols in the bound state.

Association of similar ligands (methanol and ethanol) at a site common to both proteins makes reasonable the great similarity of the difference spectra induced by these alcohols.

The methanol complexes of horse heart and sperm whale ferric myoglobin both exhibit a splitting in their difference spectra near 16×10^3 cm^{-1} which is not observed in either the methanol or ethanol complexes of human or horse heart ferrihemoglobin. The fit of the absolute spectra of horse heart myoglobin and human hemoglobin shows a significant increase in the width of the "band" near 16×10^3 cm^{-1} of the former over that of the latter. These results suggest a structural difference between ferric myoglobin and hemoglobin which might be considered somewhat unexpected since the latter is composed of two chains, which are in many ways dissimilar, and therefore might be thought to cause a more diffuse spectrum. The width variation and splitting of the difference spectra appear to point toward some structural inequivalence in myoglobin which is more pronounced than the chain inequivalence found with hemoglobin. Alternatively, the latter inequivalence might act to smooth out different spectral responses of the two chains to the alcohol.

There is much evidence for a structural difference between hemoglobin and myoglobin in the interaction of the ligand bound in position 6 with the globin structure adjacent to it. Examination of CO infrared stretch frequencies in ferrous myoglobin and hemoglobin in comparison with that of protoheme monopyridine monocarboxyl (Alben, 1978) shows similarities in the latter two complexes; both exhibit one band, whereas myoglobin exhibits two. The latter two bands, along with changes in vibrational frequencies in mutant hemoglobins, can be interpreted in terms of differing extents of interaction of bound CO with the distal imidazole or the group substituted for it in that position (Caughey, 1970). Greater interaction in myoglobin is expected for the tighter crevice previously discussed. Yoshida et al. (1975) report low-temperature optical absorption splitting in the aquo and methanol complexes of ferrimyoglobin which appears to parallel that seen near 16×10^3 cm^{-1} in the room-temperature difference spectrum. Ferric hemoglobin and protoheme do not exhibit such low-temperature splitting, and lack of a corresponding room-temperature difference spectral splitting in the methanol and ethanol complexes of ferrihemoglobin is again in accordance with a less constrained heme environment. The authors suggest that a hydrogen bond from the distal imidazole to the sixth position ligand (water or methanol) causes the splitting and that the latter is affected by both the pH and the presence of phosphate ions. A related origin of low- and room-temperature spectral features could be further examined by measuring the difference spectra produced with variation in pH and phosphate at room temperature. Furthermore, it is possible that normal absence of the 16×10^3 cm^{-1} methanol-induced splitting in the difference spectrum of hemoglobin may be altered by IHP. This will be investigated.

Correlations between Alcohol-Induced Conformational and Spectral Alterations in Alkaline Ferricytochrome c. The presence of distinctly different modes of association of methanol and 1-propanol with ferricytochrome *c* as described thermodynamically is paralleled by distinctly different alterations induced by these alcohols in both optical and EPR spectra. More specific characteristics of these alterations can be interpreted with consistency within the framework of a model which suggests conformational change with hydrophobic association of 1-propanol and heme–ligand exchange with little or no conformational change induced by methanol. Even allowing for distortions in alcohol-induced optical difference spectra due to differential rate of autoreduction with and without alcohol and the third species absorption which it introduces, significant differences in peak and trough positions connected with unique sets of band parameter changes dominate the spectra. However, similarity in the limiting magnitude of the optical changes would not have been expected and may be accidental. A large alteration in conformation could exert spectral influence on the heme as the cumulative effect of small changes in porphyrin–amino acid interactions, in some more specific singular alteration, or both. A lack of conformational change would, however, appear to dictate a more specific singular alteration and would be consistent with ligand replacement at the iron, though a more subtle conformational alteration with compensating thermodynamic changes cannot be ruled out.

Further support for specific and cumulative effects on ferricytochrome *c* is found in the band parameter alterations

Table V: g Values Obtained from Frozen Solution EPR Spectra of Various Heme Systems at Liquid Nitrogen Temperature

g values	crystal field parameters[a]	pH	buffer or solvent	perturbant or ligand	heme system	ref
2.93, 2.25, 1.31	2.55, 0.634	7.0	0.01 M PO_4, 0.3 M NaCl	none added	ferricytochrome c	this work
3.22, 2.10, -		11.0	0.01 M PO_4, 0.3 M NaCl	none added	ferricytochrome c	this work
2.48, 2.19, 1.90	6.87, 0.608	11.0	0.01 M PO_4, 0.3 M NaCl	3.0 M MeOH	ferricytochrome c	this work
2.60, 2.21, 1.83	5.60, 0.580	11.0	0.01 M PO_4, 0.3 M NaCl	3.0 M MeOH	ferricytochrome c	this work
2.54, 2.2, 1.86		~12	~0.07 M PO_4, 0.3 M NaCl	3-5 M MeOH	ferricytochrome c	this work
2.53, 2.14, 1.82	6.59, 0.516	8.0	H_2O	none added (OH^-)	ferric hemoglobin (hydroxide)	Hollocher & Buckley (1966)
2.54, 2.17, 1.85	6.56, 0.539	12.8	0.06 M KCl	none added (OH^-)	ferric myoglobin (hydroxide)	Gurd et al. (1967)
2.56, 2.18, 1.85[b,c]	6.42, 0.539	very high	H_2O	none added (OH^-)	ferricytochrome c	Brautigan et al. (1977)
2.61, 2.21, 1.84[d]	5.81, 0.559		toluene	p-nitrophenoxide:N-methylimidazole	heme	Tang et al. (1976)
2.43, 2.15, 1.92[d]	8.67, 0.535		toluene	methoxide:N-methylimidazole	heme	Tang et al. (1976)

[a] Crystal field parameters [tetragonal energy (Δ/λ), in units of spin–orbit coupling constant, and rhombic ratio (V/Δ)] were calculated by the method of Taylor (1977). [b] This spectrum was taken at 4 K. [c] Other sets of g values distinctly different from these may be obtained with ferricytochrome c at very high pH. [d] Recorded at 95 K.

induced by the two alcohols. Changes in the width of the α and β bands are more prominent with 1-propanol than with methanol, and this may be connected with changes in vibronic coupling associated with variation in heme–apoprotein interactions caused by protein conformational differences. Furthermore, the decreases in width of the α and β bands are in exact proportion to the widths themselves; the decreased structural entropy of the 1-propanol complex appears to be expressed in a balanced narrowing of the levels of the porphyrin. With methanol the transition energies are shifted to a greater extent than with propanol and, in fact, much greater than that found with alcohol association in ferric hemoglobin and myoglobin. This may at first seem at odds with the ligand-exchange hypothesis in ferricytochrome c. However, with the latter it is likely that a protein-donated ligand rather different than methanol participates in the exchange, while the similarity between water, methanol, and ethanol, those ligands exchanged with myoglobin and hemoglobin, has been previously discussed. Furthermore, ligand exchange among both low- and high-spin ligands in myoglobin and hemoglobin does produce noticeable shifts in α- and β-band centers, though the latter changes are less pronounced than those observed at 16×10^3 and 20×10^3 cm^{-1} (Smith & Williams, 1968, 1970).

The changes induced in EPR spectra are ultimately connected with alterations in coordination geometry and electron distribution within ligands bound to the iron. Spectral changes in ferricytochrome c caused by 1-propanol, as measured by both line widths and resonance positions, are small, as might be expected for small changes in the energy levels of iron induced through altered porphyrin–amino acid interactions or ligand geometry. The methanol-induced changes are much more drastic as determined by both aforementioned criteria, and this, coupled with the hypothesized lack of conformational change, is consistent with ligand replacement at the iron. Furthermore, the crystal field parameters of the resonance associated with methanol binding (both sets) are more similar to those associated with bound oxygen (hydroxide forms of hemoproteins and a tyrosine–heme–imidazole model complex) than any others produced with ferricytochrome c save one. The latter crystal field parameters are associated with a third distinctly different set of EPR resonances believed to be the hydroxide form of the heme protein, which can be produced both with and without methanol as the pH is raised above 11.0. The above-mentioned g values and crystal field parameters are listed in Table V.

Taken together, both spectral and thermodynamic parallels can be drawn among the systems methanol– and ethanol–ferrihemoglobin, ethanol–ferrimyoglobin, and methanol–ferricytochrome c which are consistent with little conformational change accompanying ligand replacement at the iron by the corresponding alcohol. However, the spectral and thermodynamic characteristics of the interaction between 1-propanol and ferricytochrome c are in contrast with those above and are consistent with a completely different mode of interaction which involves large protein conformational changes.

It is interesting to note that acetonitrile produces alterations in EPR spectra which are similar to those found with 1-propanol, but with species conversion to a greater degree. Because of its size, this perturbant would be less sterically hindered than propanol in possible modes of association with the iron; the CN group would exhibit different hydrogen/iron bonding characteristics from that of OH. The optical difference spectrum induced by acetonitrile combines features of both methanol- and propanol-induced spectra.

Unique Methanol-Induced EPR Transition in Alkaline Ferricytochrome c. With increasing concentration, methanol causes two successive, distinct alterations in EPR spectra from ferricytochrome c at pH 11, the second characterized by a changing ratio of first derivative amplitudes of the two sets of narrow-lined resonances; variations of pH and ionic strength also change this ratio. The latter spectral changes may be associated with two important characteristics of ferricytochrome c, the large number of exterior charged groups (Dickerson, 1972) and the structural stabilization provided by heme–apoprotein and other hydrophobic interactions (Fisher et al., 1973). Methanol can interact with the protein directly through ligand binding or indirectly through altering water structure. Changes in the aqueous environment can affect both hydrophobic and charge interactions of the protein and lead to alteration of conformation. The initial spectral changes (broad- to narrow-lined resonances) appear to be associated with methanol binding, and those of the second, especially considering the high concentration of alcohol over which the ratio of peak heights vary, are probably associated with alteration of water structure. This assignment is supported by interrelated changes in the ratio of peak heights produced by variation of methanol concentration, pH, and ionic strength with the latter two capable of altering protein conformation through overall charge and charge shielding effects. Similarly, the spectrophotometric pK of the "conformation-

sensitive" band at 695 nm in a modified form of ferricytochrome *c* is very dependent upon ionic strength (Pettigrew et al., 1976).

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References

Alben, J. O. (1978) *The Porphyrins* (Dolphin, D., Ed.) Vol. III, Academic Press, New York.

Alberding, N., Chan, S. S., Eisenstein, L., Fraunfelder, H., Good, D., Gunsalus, I. C., Nordlung, T. M., Perutz, M. F., Reynolds, A. H., & Sorensen, L. B. (1978) *Biochemistry 17*, 43.

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin and Their Reaction with Ligands*, North-Holland Publishing Co., Amsterdam.

Asher, S. A., & Schuster, T. M. (1979) *Biochemistry 18*, 5377.

Boucher, L. J. (1970) *J. Am. Chem. Soc. 92*, 2725.

Brants, J. F. (1964) *J. Am. Chem. Soc. 86*, 4302.

Brautigan, D. L., Feinberg, B. A., Hoffman, B. M., Margoliash, E., Peisach, J., & Blumberg, W. E. (1977) *J. Biol. Chem. 252*, 574.

Brill, A. S. (1977) *Transition Metals in Biochemistry*, p 144, Springer-Verlag, West Berlin.

Brill, A. S., Bryce, G. F., & Maria, H. J. (1968) *Biochim. Biophys. Acta 154*, 342.

Brill, A. S., Castleman, B. W., & McKnight, M. E. (1976) *Biochemistry 15*, 2309.

Caughey, W. S. (1970) *Ann. N.Y. Acad. Sci. 174*, 148.

Dayhoff, M., & Eck, R. (1968) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, D.C.

Dickerson, R. (1972) *Sci. Am. 226*, 58.

Dickerson, R., & Geis, I. (1969) *The Structure and Action of Proteins*, Harper and Row, New York.

Eaton, W. A., & Hochstrasser, R. M. (1968) *J. Chem. Phys. 49*, 985.

Falk, J. (1964) *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam.

Fisher, W., Taniuchi, H., & Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188.

Fowler, R., & Guggenheim, E. A. (1949) *Statistical Thermodynamics*, p 336, Cambridge University Press, Cambridge, England.

Gill, S. J., & Wadsö, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2955.

Gupta, R. K., & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134.

Gurd, F., Falk, K., Malmström, B., & Vänngård, T. (1967) *J. Biol. Chem. 242*, 5724.

Handbook of Chemistry and Physics (1978–1979) 59th ed., CRC Press, Cleveland, OH.

Hollocher, T., & Buckley, L. (1966) *J. Biol. Chem. 241*, 2976.

Ladner, R., Heidner, E., & Perutz, M. (1977) *J. Mol. Biol. 114*, 385.

Landolt-Börnstein Tabellen (1962) 6th ed., Vol. II, Sect. 2b, Springer-Verlag, West Berlin.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570.

Margoliash, E., & Lustgarten, J. (1962) *J. Biol. Chem. 237*, 3397.

Myer, Y. P. (1968) *Biochemistry 7*, 765.

Nagai, M., Sugita, Y., & Yoneyama, Y. (1969) *J. Biol. Chem. 244*, 1652.

Paige, M. I. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 449.

Perutz, M. F. (1970) *Nature (London) 228*, 726.

Perutz, M. F., & TenEyck, L. F. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 295.

Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., & Slade, E. F. (1974) *Biochemistry 13*, 2187.

Pettigrew, G., Aviram, I., & Schejter, A. (1976) *Biochem. Biophys. Res. Common. 68*, 807.

Rifkind, J. (1973) *Inorganic Biochemistry* (Eichhorn, G., Ed.) Elsevier, Amsterdam.

Smith, D., & Williams, R. J. P. (1968) *Biochem. J. 110*, 297.

Smith, D., & Williams, R. J. P. (1970) *Struct. Bonding (Berlin) 7*, 1.

Takano, T. (1977) *J. Mol. Biol. 110*, 537.

Tang, S. C., Koch, S., Papaefthymiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A., & Holm, R. H. (1976) *J. Am. Chem. Soc. 98*, 2414.

Taylor, C. P. S. (1977) *Biochim. Biophys. Acta 491*, 137.

Vickery, L., Nozawa, T., & Sauer, K. (1976a) *J. Am. Chem. Soc. 98*, 343.

Vickery, L., Nozawa, T., & Sauer, K. (1976b) *J. Am. Chem. Soc. 98*, 351.

Watson, H. (1969) *Prog. Stereochem. 4*, 299.

Yoshida, S., Iizuka, T., Nozawa, T., & Hatano, M. (1975) *Biochim. Biophys. Acta 405*, 122.

Zerner, M., Gouterman, M., & Kobayashi, H. (1966) *Theor. Chim. Acta 6*, 363.