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Association of Methanol and Ethanol with Heme Proteins†

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ABSTRACT: The behavior of ferrihemoglobin and ferrimyoglobin in widely varying concentrations of the lowest four alcohols has been studied by optical and electron paramagnetic resonance absorption spectroscopy. Methanol and ethanol, at concentrations too low to cause general conformational destabilization of the protein, produce both optical and electron paramagnetic resonance absorption spectral changes in ferrihemoglobin. These changes arise from equilibrium associations, characterized by dissociation constants at 25 °C of about 40 and 200 mM, respectively, for the methanol-ferrihemoglobin and ethanol-ferrihemoglobin complexes so formed. Other optical spectral changes appear when the methanol concentration exceeds 3.5 M and the ethanol, 1.0 M. At concentrations lower than 0.5 M, 1- and 2-propanol produce spectral changes of this second kind. At room temperature no optical evidence has been found that the propanols associate with ferrihemoglobin in the manner of methanol and ethanol. Methanol and ethanol at low concentration have specific effects, characterized by electron paramagnetic resonance spectral differences, upon ferric α_{SH} chains. All four alcohols, over a wide range of concentrations, reduce the symmetry of electron paramagnetic resonance spectra from frozen solutions of ferrihemoglobin; even at the high end of this concentration range, none of the alcohols reduces the symmetry of electron paramagnetic resonance spectra from frozen ferrimyoglobin. Ferrimyoglobin and catalase association with methanol is measureable optically; the binding is about five and sixty times weaker, respectively, for these two proteins as compared with ferrihemoglobin.

There is considerable information available on the effects of alcohols on protein stability and reactivity obtained under experimental conditions designed to alter the structure of water and weaken hydrophobic bonds (Kaminsky and Davison, 1969; Herskovits et al., 1970; Tan and Lovrien, 1972; Anusiem and Lumry, 1973). With regard to denaturation, for example, an increase in the length of the hydrocarbon chain increases the effectiveness of the alcohol, and branching decreases it; methanol, ethanol, 2-propanol, and 1-propanol at concentrations of about 12, 6, 4, and 3 M, respectively, are required for half-denaturation (based upon Soret absorbance) of myoglobin and cytochrome *c* (in acetate, 0.1 M, pH 5.7, 25 °C) (Herskovits et al., 1970). In other experimental systems, effects produced by alcohols can be attributed to complex formation. Thus, the influence of alcohols on the thermal transition of ribonuclease has been interpreted in terms of the hydrophobic

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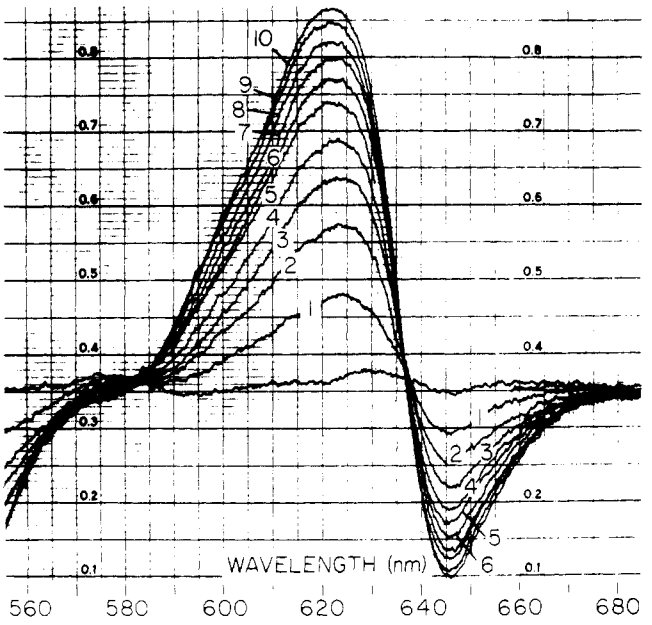

FIGURE 1: Difference absorption spectra, human ferrihemoglobin plus ethanol vs. the ferrihemoglobin alone as reference, 0.033 M phosphate buffer, pH 6.3 (at 24 °C), 30.2 °C. The initial ferrihemoglobin (heme) concentration was 33.4 μM and volume, 26.0 ml. Ethanol was added in ten 50-μl increments to the sample cell, and buffer similarly to the reference cell; 10.0-cm path length cells and a 0–0.1 absorbance slide wire were used.

bonding of the alcohol molecules to the denatured protein (Schrier et al., 1965), but there is no direct experimental evidence for this association at the present time and other explanations can be offered (Tan and Lovrien, 1972). Similarly, the observation that straight-chain alcohols (n = 6–12) at very low concentrations ($<10^{-3}$M) greatly increase the rate of denaturation of ferrihemoglobin can be explained by the formation of complexes of the alcohols with the protein, but no binding of these alcohols to the protein has been detected either by equilibrium dialysis or absorption spectrophotometric measurements (Cassatt and Steinhardt, 1971). Octanol, decanol, and dodecanol do bind to bovine serum albumin (Ray et al., 1966). Ethanol increases both the maximum velocity and Michaelis constant of D-amino acid oxidase. Ethanol also produces, in the visible and ultraviolet absorption spectra of this enzyme, changes which are characterized by several well-defined isosbestic points, the latter indicating an equilibrium between the original protein and another species formed by the addition of the alcohol (Õhama et al., 1973).

This paper reports optical and EPR[1] absorption measurements on ferrihemoglobin and ferrimyoglobin in the presence of the lowest four alcohols at a wide range of concentrations. Both the optical and magnetic measurements reveal formation of complexes of methanol and ethanol with ferrihemoglobin. Association of methanol with ferrimyoglobin and catalase is shown by optical means.

Experimental Section

Materials

Human hemoglobin was prepared from whole human blood. Erythrocytes were washed three times and lysed, and then the ghosts were centrifuged out. Horse hemoglobin was purchased as the lyophilized powder and dissolved in 0.1 M phosphate buffer. Ferricyanide was used to oxidize the heme iron to the ferric valence state. The solutions were then dialyzed, first to remove ferricyanide, then against EDTA to remove extraneous metal ions, and finally several times against 0.033 M phosphate buffer prepared with deionized water. The amount of alkaline low-spin form was minimized by keeping the pH at 6.3–6.4 (at 25 °C).

α and β chains of human hemoglobin were prepared with regenerated sulfhydryl groups (designated α_{SH} and β_{SH}) by the method of Geraci et al. (1969) with minor modifications. Electrophoretic patterns of ferrous α_{SH} and β_{SH} preparations and of normal ferrihemoglobin, obtained by polyacrylamide gel electrophoresis, exhibited the appropriate relative mobilities (De Renzo et al., 1967). The α_{SH} and β_{SH} chains were converted to the ferric form with ferricyanide and the latter reagent was removed by passage over a Bio-Gel P-2 column.

Ferrihemoglobin was "stripped" (organic phosphate removed) by dialysis twice against 0.5 M NaCl in 0.01 M phosphate buffer, pH 6.3, first for 4 h and then overnight. The NaCl was removed by dialysis against 0.01 M phosphate, pH 6.3, and then 0.033 M phosphate, pH 6.3, each three times.

Preparation of sperm whale skeletal muscle and horse heart muscle ferrimyoglobin, obtained from both Nutritional Biochemical Corp. and Sigma Chemical Co., started with ferricyanide treatment and continued as above for ferrihemoglobin.

Catalase from *Micrococcus lysodeikticus* was prepared as before (Brill and Sandberg, 1968).

The spectrophotometric purity value, as defined as PV $\equiv A_{\text{Soret peak}}/A_{\text{peak at 275–280 nm}}$, was in the range 5.0–6.0 for the several ferrihemoglobin preparations, 4.2–5.2 for the ferrimyoglobin preparations, and 0.81 for the catalase, where the values 6.0, 5.4, and 0.92 correspond to maximum purity of the proteins in the order given. The PV of the single "stripped" ferrihemoglobin preparation was 4.85.

The concentrations of the proteins were determined from measurements of the peak Soret absorbance on suitably diluted solutions. The average absorptivities per heme employed in these determinations are: 166 for human[2] and 179 for horse ferrihemoglobin, 157 for sperm whale and 188 for horse heart ferrimyoglobin, all at pH 6.3; 103 for bacterial catalase at pH 7.

Methods

A Cary Model 14 recording spectrophotometer was used for measuring optical absorption spectra.

The EPR measurements were carried out at 4.2 K with a 160-ml liquid helium dewar described elsewhere (Brill et al., 1971). The sample was situated within a fused quartz tail that is inserted into the Varian 4531 general-purpose cavity which is part of the Varian 4500 X-band spectrometer. All spectra were taken as the first derivative of the microwave absorption. One hundred kilohertz field modulation of amplitude (peak-to-peak) 2–4 G was employed. The levels of microwave power (5–6 mW at the cavity) were nonsaturating. The scanning magnetic field was monitored by a Varian F-8A fluxmeter with the ^{1}H NMR radio frequency determined by a Hewlett-Packard 5246L electronic counter; the field markers shown

[1] Abbreviations used are: EPR, electron paramagnetic resonance; EDTA, (ethylenedinitrilo)tetraacetic acid.

[2] Heme analysis (pyridine hemochromogen) and absorption measurements on human ferrihemoglobin in 0.033 M phosphate buffer, pH 6.3, carried out in this laboratory, give the following peak absorptivities (mM^{-1} cm^{-1}): 4.1 at 631 nm, 9.2 at 501 nm, and 166 at 405.5 nm. We thank Mr. B. W. Turner for his assistance with this determination.

TABLE I: Characterization of Methanol and Ethanol Complexes of Hemeproteins.[a]

Heme Protein	Thermodynamic Parameters: K (M)	ΔG^b kJ mol^{-1}	ΔG^b cal mol^{-1}	ΔH^b kJ mol^{-1}	ΔH^b cal mol^{-1}	ΔS^b J mol^{-1} K^{-1}	ΔS^b cal mol^{-1} K^{-1}	Optical Difference Spectra Parameters: Isosbestic Points (nm)	Peaks λ (nm)	Peaks a (mM^{-1} cm^{-1})	Troughs λ (nm)	Troughs a (mM^{-1} cm^{-1})
A. Methanol Complexes												
Ferrihemoglobins												
Human	0.045	7.3	1700	25	6000	60	14	637, 583	622	0.39	646	−0.21
Horse[c]	0.041	7.5	1800	21	4900	45	11	637, 572	622	0.38	645	−0.18
											544	shoulder
											519	−0.45
Ferrimyoglobins												
Sperm whale	0.17	4.4	1000	15	3700	37	9	634, 587, 495	615	0.16	660	−0.06
											640	−0.06
skeletal											523	−0.41
Horse heart	0.22	3.8	900	25	6000	72	17	632, 588, 497	613	0.17	655	− .07
									490	0.07	640	− .10
Micrococcus Catalase[d]	2.2	−1.9	−500	19	4500	69	17	635, 560, 515, 398	614	1.0	522	− .39
									500	shoulder	655	−0.3
									415	12.6	540	−0.3
											378	−4.3
B. Ethanol Complexes												
Ferrihemoglobins												
Human	0.20	4.1	1000	20	4700	52	13	637, 583, 495, 416, 405	622	0.26	646	−0.13
									410	0.5	546	−0.15
											520	−0.15
											420	−0.3
											396	−2.4
Horse[c]	0.22	3.6	900	17	4000	45	11	638, 583, 490, 458	623	0.33	646	−0.10
											546	−0.21
											520	−0.24

[a] Unless indicated otherwise, parameters are from solutions buffered with phosphate, 0.033 M, pH 6.3, and T = 25 °C. [b] The assumption used in arriving at the values for ΔG, ΔH, and ΔS is stated in the Discussion. [c] T = 23 °C. [d] Phosphate 0.010 M, pH 7.0.

in Figures 3–5 are in gauss. The klystron frequency was measured with a Hewlett-Packard X532B frequency meter.

Optical Studies

Hemoglobin. Methanol, ethanol, 1-propanol, and 2-propanol all cause changes in the optical absorption spectrum of ferrihemoglobin. Difference absorption spectrophotometry was used to measure the progression of the reactions of methanol and ethanol with ferrihemoglobin as a function of alcohol concentration. Figure 1 shows the difference spectra from ethanol–human ferrihemoglobin (in 0.033 M phosphate) mixtures (vs. the free hemeprotein to which appropriate additions of buffer have been made). Isosbestic points indicate that only two hemoglobin species are present in significant concentration. On the basis that the reaction obeys the simplest (all concentrations entering to the first power) equilibrium relation, a least-squares fit to the sequence of measured peak-to-trough values of difference absorbance was performed to obtain the best values for the dissociation constant and the limiting peak-to-trough difference absorptivity, at each of several temperatures and ferrihemoglobin concentrations. For human ferrihemoglobin with ethanol, the standard error in dissociation constant was less than ±9%, and in difference absorptivity, less than ±3% for every experiment. At 25 °C, for six ferrihemoglobin (heme) concentrations spanning the range from 15 μM to 150 μM, the best dissociation constants were within ±15% of the average value, and the best difference absorptivities were within ±3% of the average. The optical changes are quantitatively reversed upon decreasing the alcohol concentration (by dilution). Similar consistency was found for the reaction of human ferrihemoglobin with methanol and for horse ferrihemoglobin with both alcohols. The values for the dissociation constants at room temperatures are given in Table I along with several parameters which characterize the difference spectra. These studies at low-alcohol concentrations suggest that the reaction is a reversible association, proportional to the concentrations of ethanol (or methanol) and free ferrihemoglobin.

The optical data for the reactions of methanol and ethanol with ferrihemoglobin have also been analyzed by a method analogous to that of Scatchard (1949). In the present case the quantity measured (difference absorbance, ΔA) is proportional to the extent of protein complex formed rather than the average number of small molecules bound. The Scatchard plot then becomes:

$$\frac{\Delta A}{[\text{alcohol}]} = \frac{A}{K} - \frac{\Delta A}{K} \qquad (1)$$

where K, the dissociation constant of the ferrihemoglobin–alcohol complex, and A, the limiting difference absorbance

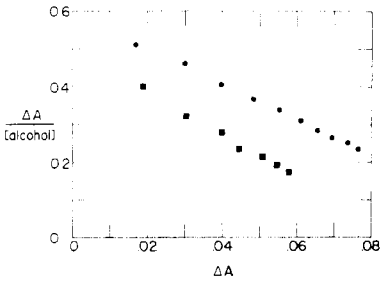

FIGURE 2: Scatchard-related plots of optical titration data. The points ● are obtained from the data of Figure 1. The points ■ are from sperm whale skeletal ferrimyoglobin, 0.033 M phosphate buffer, pH 6.3, 25 °C; the initial ferrimyoglobin concentration was 16.1 μM and volume, 26.0 ml; methanol was added in 50-μl increments; 10.0-cm path length cells and a 0–0.1 absorbance slide wire were used.

(rather than the number of binding sites on the protein), can be determined from the best straight line through the experimental $\Delta A/[\text{alcohol}]$, ΔA couples. Such an analysis presupposes that the alcohol binding sites on the protein act independently; other assumptions do not lead to a linear relation between $\Delta A/[\text{alcohol}]$ and ΔA. The possibility that a relation more complicated than linear applies was tested by seeking the exponent m in

$$\frac{\Delta A}{[\text{alcohol}]^m} = \frac{A}{K} - \frac{\Delta A}{K} \qquad (2)$$

which gives the best (least-squares) fit to the difference absorbance titration data. In Table II are summarized the results from all the experiments so analyzed; m is clearly one, in support of the conclusion of the preceding paragraph. A plot of $\Delta A/[\text{alcohol}]$ vs. ΔA for the data in Figure 1 is shown in Figure 2.

In principle, the number of independent alcohol binding sites can be determined by titrating, with low alcohol, a solution in which the ferrihemoglobin concentration is comparable with or exceeds the dissociation constant of the complex. The latter condition immediately rules out this determination for the ethanol reaction and indicates that even for the five-times more tightly bound methanol complex the stoichiometric ratio will be difficult to measure.

With the heme protein solution at room temperature, as the concentration of methanol is raised beyond 3.5 M, and ethanol, beyond 1.0 M, optical changes appear which indicate that several processes are occurring. Spectral changes of the latter kind are produced by 1- and 2-propanol at concentrations under 0.5 M, and would tend to obscure evidence of a simultaneous simple equilibrium reaction if it were taking place. Within the latter limitation, optical difference absorption measurements at room temperature indicate that the propanols do not associate with ferrihemoglobin in the manner of methanol and ethanol.

The temperature dependence of the alcohol–ferrihemoglobin association reaction was determined by optical difference spectrophotometry in two ways. In one method the progression of the reaction was quantitated with successive additions of alcohol, this experiment being performed at several fixed temperatures. In the other method, alcohol was added to bring the reaction to partial completion; the temperature was then dropped from an initial value of about 33 °C to a final value of about 4 °C over a period of several hours, and difference spectra were taken at various temperatures on the way down. The measured $\ln K$ vs. $1/T$ relation provides single, well-defined values of enthalpy and entropy difference over the range

TABLE II: Values of m for Best Fit to $\Delta A/[\text{Alcohol}]^m = A/K - \Delta A/K$ from Representative Sets of Data.

Protein	Alcohol	Heme (mM)	Temp (C°)	Best m
Human ferrihemoglobin	Methanol	0.036	25	1.00
		0.144		1.10
	Ethanol	0.038	25	1.00
		0.082		0.95
		0.149		1.05
		0.033	30	1.00
Horse ferrihemoglobin	Methanol	0.019	23	1.00
	Ethanol	0.020	23	0.95
Sperm whale ferrimyoglobin	Methanol	0.140	7	1.05
		0.016	25	0.95
		0.084		0.95
		0.163		0.90
		0.162	33	1.10

33–10 °C for the dissociation of both the methanol and ethanol complexes of the human and horse proteins (Table I). (The assumption used in arriving at these thermodynamic parameters is stated in the Discussion.) Below 10 °C the equilibria exhibit a more complicated temperature dependence.

The long-term stability of human ferrihemoglobin in 0, 0.2, 0.5, and 1.0 M methanol and ethanol was observed by recording absolute visible (700–450 nm) absorption spectra. The solutions were 80 μM in heme, buffered with 0.033 M phosphate, pH 6.3, and left at room temperature (23–24 °C). Spectra taken 1–2 h, 1 day, and 2 days after the addition of the alcohols were identical. The protein in 1.0 M methanol and ethanol was found to be stable for a very long period of time; spectra of these solutions taken at 32 days had not changed at any wavelength by more than two divisions on a scale where the maximum absorbance (at 500 nm) was 81 divisions, and at 69 days had not changed by more than four divisions. The controls (no alcohol) were slightly less stable than the 1.0 M alcohol solutions, their spectra changing by as much as three scale divisions and showing some loss of resolution in the troughs after 32 days. In 0.2 and 0.5 M methanol and ethanol the protein was significantly less stable than in 0 and 1.0 M; haze, sediment, and, in the ethanol solutions, some pink color were present after 19 days, and 32 days after the addition of the alcohols the spectrum of the original complex was discernible in only one of the four solutions.

Myoglobin. Both methanol and ethanol also cause changes in the optical absorption spectra of sperm whale skeletal muscle and horse heart muscle ferrimyoglobin. The changes produced by ethanol, however, are too small to quantitate. Measurements similar to those described above for ferrihemoglobin were carried out on the methanol–ferrimyoglobin system (Table I). For sperm whale ferrimyoglobin concentrations spanning the range from 16 to 160 μM, the dissociation constants were within ±6% of the mean and the limiting difference absorptivities were within ±2% of the mean. (Dissociation constants and difference absorptivities were determined by means of the Scatchard method of plotting discussed above.) Similar spreads were found in the related parameters from horse heart ferrimyoglobin experiments. Dilution data indicate that these reactions are quantitatively reversible.

The temperature dependence of the dissociation constants for the methanol complexes of the two myoglobins was determined as for hemoglobin. The thermodynamic parameters obtained are listed in Table I.

Table II lists the values of the parameter m, as defined in

TABLE III: Characterization of the EPR Spectra from Free (Aquo Complexes) Ferrimyoglobin and Ferrihemoglobin.

Hemeprotein	Buffer	$g_z^{effective}$	"g"$_{crossover}$[a]	Line width[b]
Ferrimyoglobin (sperm whale skeletal)	33 mM PO_4, H_2O, pH 6.3	2.00	5.88	30
Ferrihemoglobin (human)	33 mM PO_4, H_2O, pH 6.3	2.00	5.84	38
stripped	33 mM PO_4, H_2O, pH 6.3	2.00	5.86	38
α_{SH} chains (human)	33 mM PO_4, H_2O, pH 6.3	2.00	5.84	40
β_{SH} chains (human)	33 mM PO_4, H_2O, pH 6.3	2.00	5.84	41

[a] "g"$_{crossover} \equiv h\nu/\beta H_{crossover}$ where $H_{crossover}$ is the field strength at which the first derivative of the absorption is zero. Computer analysis shows that $g_{H \text{ in heme plane}}^{effective} \simeq 1.01$"$g$"$_{crossover}$. [b] Peak-to-trough, of low-field resonance (G).

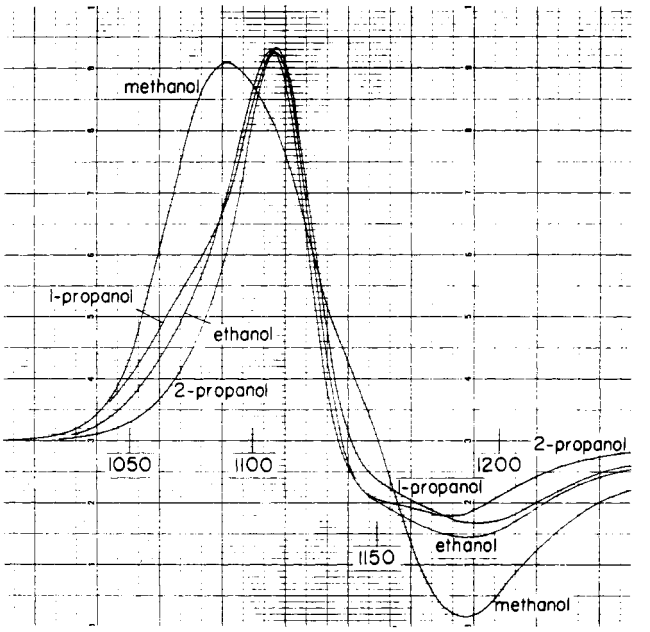

FIGURE 3: Low-field EPR spectra of 1.2 mM (heme) ferrihemoglobin in the presence of alcohols at 0.10 M. Microwave frequency, 9.27 GHz. The recorder gain setting was adjusted in each case so as to maintain about the same peak heights. In this figure, and in Figures 4 and 5, the spectra shown were traced from the original records.

eq 2, which give the best fit to the data from several myoglobin–methanol experiments. A plot of ΔA/[alcohol] vs. ΔA for one of these experiments is shown in Figure 2.

Catalase. Measurements and the analysis of the data with catalase–methanol were analogous to those for hemoglobin and myoglobin (Table I). Ethanol appears to cause an optical change in catalase, but the effect is too small for quantitative measurements.

EPR Studies. EPR spectra from ferrimyoglobin, ferrihemoglobin, and α_{SH} and β_{SH} chains in buffered aqueous solutions provide reference for comparison of the effects of organic cosolvents. Characterization of the spectra from the free hemeproteins is given in Table III. Note that only two g values are observed. The apparently wholly axial g tensors exhibited by frozen solutions of these hemeproteins in the high-spin ferric form is a curious feature which they do not share with other high-spin ferric hemeproteins or with some abnormal hemoglobins (Ehrenberg, 1962; Ehrenberg and Estabrook, 1966; Rein et al., 1968; Hayashi et al., 1966; Bemski and Nagel, 1968; Morita and Mason, 1965; Peisach et al., 1971). However, EPR studies of single crystals of ferrimyoglobin and its fluoride complex have revealed small degrees of rhombic character (Kotani and Morimoto, 1967). EPR spectra from catalase show a large rhombic splitting which, however, is variable (Peisach et al., 1971); this variation interferes with the establishment of a reference spectrum and catalase EPR data will not be reported below.

Methanol, ethanol, and 2-propanol, at concentrations up to 1 M, produce little if any EPR-detectable structural change in ferrimyoglobin. 1-Propanol at 0.1 M and above produces significant additional absorption in the "wings" of the low-field band of this hemeprotein, and the peak-to-trough line width increases somewhat (e.g., at 0.1 M the line width is 33 G), but the spectrum shows no rhombic character and the g values remain unchanged. These observations, while limited to levels of solvent which in general are low compared with those employed in denaturation studies, reflect the destabilization order of alcohols reported by Herskovits et al. (1970).

Even at concentrations under 0.1 M, methanol, ethanol, 1-propanol, and 2-propanol all cause structural changes in ferrihemoglobin, as evidenced by the EPR spectra (Figure 3). At a level of ethanol which would be mildly denaturing at room temperature, new features of the low-field EPR spectrum begin to appear. In the presence of any of the four alcohols some ferrihemoglobin is converted to low-spin form as evidenced by new EPR absorption in the characteristic magnetic field region. While the extent of this conversion has not yet been quantitated, the increase in high-field absorption is clearly small (just detectable) for ethanol as compared with the other three alcohols.

The ferrihemoglobin EPR spectra change with increasing methanol and ethanol concentrations in such a way as to raise the question as to whether the magnetic data are consistent with equilibrium reactions. A cell was therefore constructed (of Teflon) such that the volume of ferrihemoglobin solution in the sensitive part of the resonant cavity is fixed, independent of variations in extent of filling (except for the possibility of bubbles in the sensitive region). Apart then from changes in the total intensity of the low-field absorption which arise from some conversion to low-spin form, the low-field region of the EPR spectra (from solutions of equal ferrihemoglobin concentration) could then be analyzed in the same way as optical absorption spectra from solutions of equal path length and concentration. A set of superimposed EPR spectra from solutions of equal ferrihemoglobin concentration and increasing ethanol, obtained with the constant volume cell, is shown in Figure 4. Note that at three magnetic field strengths, in the latter figure, the signal remains at approximately constant amplitude as the concentration of ethanol is increased. In analogy with isosbestic points, these "points" of approximately equal slope indicate that two species account for most of the ferrihemoglobin present. With the assumption that a reaction takes place obeying an equilibrium relation in which each concentration enters to the first power, computations of a nonlinear least-squares fit to the measured signal amplitude differences (at a single field strength close to either a peak or a trough of the EPR spectrum from alcohol-free ferrihemoglobin) give standard errors in the range 10–16%. This result suggests that the progression of EPR spectral change with increasing methanol and ethanol concentration reflects the extent of association of the alcohols to ferrihemoglobin at a

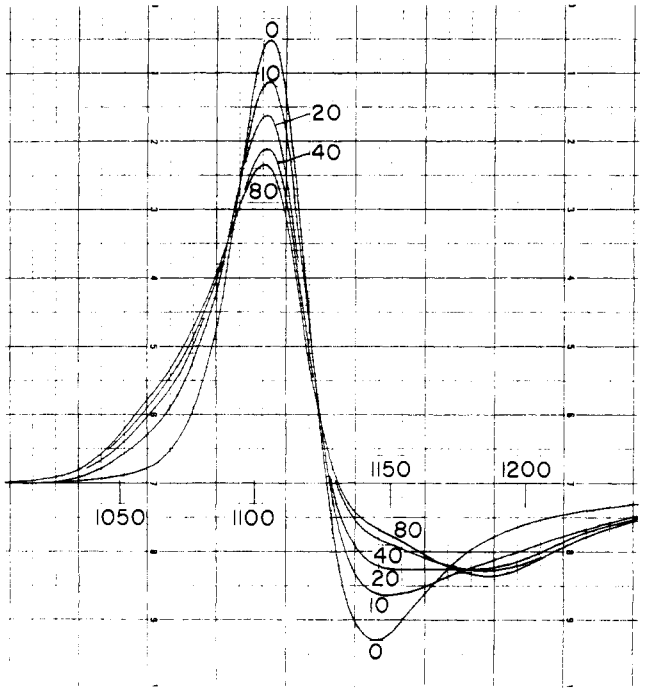

FIGURE 4: Low-field EPR spectra of 0.50 mM (heme) ferrihemoglobin in the presence of ethanol at concentrations of 0, 10, 20, 40, and 80 mM. (For pictorial clarity, the spectra taken with ethanol at 30 and 60 mM have not been included.) The same sample cell and identical instrument settings were employed in obtaining all of the spectra. Microwave frequency, 9.23 GHz.

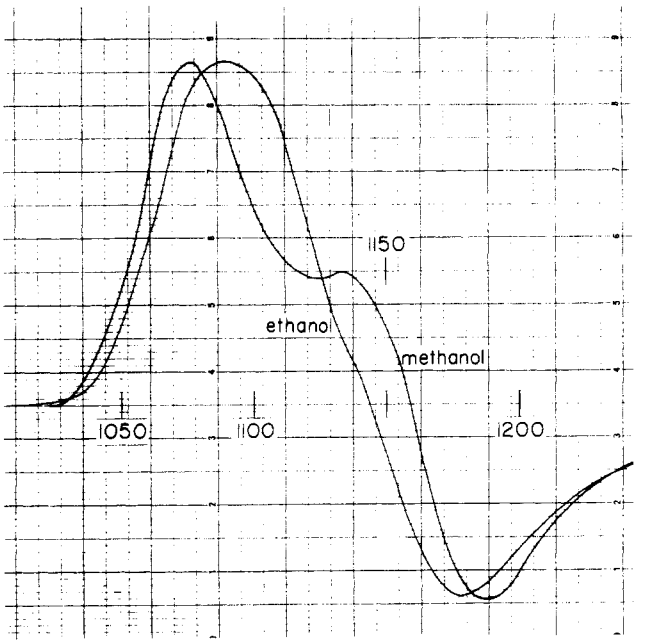

FIGURE 5: Low-field EPR spectra of 0.88 mM (heme) ferric α_{SH} chains (from human hemoglobin) in the presence of methanol and ethanol at 86 mM. Microwave frequency, 9.23 GHz.

temperature near freezing. Extrapolation of the optically derived behavior down from 4 °C to freezing is not well defined. Qualitatively one finds that the EPR-determined dissociation constants of 0.7 mM for the methanol complex and 20 mM for the ethanol complex are, respectively, on the low side and within the range of the corresponding extrapolated optical data.

Other experiments, in which alcohol has been added to hemoglobin derivatives, have shown: (1) Additions of methanol and ethanol to solutions of stripped ferrihemoglobin produce the same EPR spectra as when added to the unstripped protein. (2) Methanol and ethanol markedly affect the EPR spectra of ferric α_{SH} chains (Figure 5). Methanol produces significant broadening of the low-field spectrum of ferric β_{SH} chains. Complex EPR spectral changes accompany successive additions of ethanol to ferric β_{SH} chains and the low-field resonance is ultimately narrowed. (3) The presence of heavy water influences the EPR changes produced in ferrihemoglobin by ethanol and the thermodynamic parameters of both the methanol and ethanol reactions (B. W. Castleman, to be published).

Discussion

From the experiments reported above we conclude that methanol and ethanol combine with ferrihemoglobin, and methanol combines with ferrimyoglobin and catalase, to form complexes that are stable and have well-defined optical absorption spectra. We further find, from exploration of the effects of varying the concentrations of alcohol and protein, that these systems can be characterized by a function of the concentrations at equilibrium, namely:

$$K = K([c_i]) = \frac{[\text{free sites}][\text{free alcohol}]}{[\text{occupied sites}]}$$

where K is a constant at fixed temperature (and pressure). With the use of activity coefficients, γ_i, the "standard" Gibbs energy change is

$$-\Delta G^\circ = RT \ln \{K(\gamma_i)K([c_i])\}$$

where the standard states for reactants and products correspond to concentrations of 1 mol/l. at the experimental pH and ionic strength. Liquid water, which may play a role in the reactions (see below), is an exception, being assigned an activity of unity. For the alcohol–hemeprotein equilibria under consideration, either the γ_i are independent of the $[c_i]$ or depend upon the $[c_i]$ in such a way that $K(\gamma_i)$ is independent of $[c_i]$. It follows that

$$-\Delta G^\circ = RT \ln (K(\gamma_i)) + RT \ln K([c_i])$$

where both terms on the right are concentration independent and ΔG° is uncertain to the extent $RT \ln K(\gamma_i)$. While the function $K([c_i])$ remains the same, the number K is found to vary with temperature. Above 10 °C

$$\frac{d}{d\left(\frac{1}{T}\right)} \ln K = \text{constant}$$

Since

$$\ln K([c_i]) = \frac{\Delta S}{R} - \frac{\Delta H}{RT} - \ln K(\gamma_i)$$

the preceding equation requires that $\ln K(\gamma_i)$ is either temperature independent or else a linear function of $1/T$. The latter constraint and the required independence of $\ln K(\gamma_i)$ upon the $[c_i]$ are (nonuniquely) satisfied by setting $\ln K(\gamma_i)$ equal to zero, as we have done in arriving at the phenomenological values of ΔG°, ΔH, and ΔS of Table I, but one cannot rule out a contribution from the neglected term. If ΔH were determined independently (calorimetric measurement), ΔG° and ΔS would still remain uncertain.

It is not necessary for an alcohol molecule to be bound to or near a heme group in order to produce a spectroscopic change in that group. A priori, it is conceivable that the binding of two or more alcohol molecules is required to produce the effect. The latter process is ruled out by the close fit of the data to the Scatchard relation. In ferrimyoglobin this means that the binding of a single alcohol molecule induces the entire spectroscopic change in the one heme group present; however, there could be independent binding sites for this alcohol molecule, provided all such sites are identical in K and Δa (absorptivity change). In ferrihemoglobin and catalase, with four heme groups per protein molecule, the adherence of the data to the Scatchard relation indicates that one alcohol molecule induces the entire spectroscopic change in however many hemes it affects—1, 2, 3, or 4. The information available at the present time does not permit statements as to how many of the four hemes in hemoglobin and catalase are ultimately perturbed in their spectra by bound alcohol, how many alcohol molecules must be bound to produce the maximal spectral change, nor whether or not there are independent alcohol binding sites which affect the same heme.

The entropy changes accompanying the dissociation of the methanol complexes suggest that the alcohol binding sites on the proteins are normally occupied by water. In the latter situation, apart from possible conformational changes in the proteins, the entropy change will be the difference between the entropies of the methanol and water in solution, or about 62 J mol^{-1} K^{-1} (15 cal mol^{-1} K^{-1}). The average of the five ΔS values in Table I for dissociation of methanol complexes is 57 J mol^{-1} K^{-1} (14 cal mol^{-1} K^{-1}). Only two ΔS values are available in Table I for the dissociation of ethanol complexes, and these are smaller than the entropy difference between ethanol and water in solution, 91 J mol^{-1} K^{-1} (22 cal mol^{-1} K^{-1}).

From the x-ray diffraction determined structures, the same groups (porphyrin, histidine imidazole, and water) are known to be coordinated to the ferric ion in the acid forms of ferrimyoglobin and ferrihemoglobin (Kendrew et al., 1960; Perutz, 1970). The EPR spectra from frozen solutions of these two hemeproteins are much the same; both are axial, one shifted and broadened by about 8 G with respect to the other. In contrast, the EPR spectra from frozen solutions of the methanol complexes of ferrimyoglobin and ferrihemoglobin differ greatly. The ferrimyoglobin EPR does not change, and the ferrihemoglobin EPR exhibits rhombic distortion when methanol is added. (Similar observations apply when ethanol is added; however, ethanol binds so weakly to ferrimyoglobin at 25 °C that there may not be sufficient complex present even in frozen solution to contribute significantly to the EPR signal.) We attribute the appearance of rhombic character in the EPR of the methanol complex of ferrihemoglobin to a lowering of ferric ion coordination symmetry rather than to a separation of axial g values from heme to heme, although there may well be some small shifting of average equatorial g values. The change in coordination symmetry which occurs upon methanol (and ethanol) association with ferrihemoglobin is probably the result of the introduction, or modification, of a specific coupling between the heme group and the protein or, less likely, due to a more general protein conformational change. In view of the identical nature of the groups immediately fixed to the iron in myoglobin and hemoglobin, the contrasting EPR behavior of ferrimyoglobin and ferrihemoglobin in the presence of alcohols is seen to arise from one or more of the differences in the structures of the protein moieties.

There are two interesting aspects to the stability of ferrihemoglobin in methanol and ethanol. First, there appears to be a maximum stability at concentrations in the neighborhood of 1 M, and, second, at 1 M methanol and ethanol, the protein is at least as stable as in the alcohol-free buffer. We cannot yet rule out bacterial contamination in the 0.2 and 0.5 M solutions, although two sets of experiments gave the same results: controls and 1 M alcohol solutions, ferrihemoglobin stable for months; 0.2 and 0.5 M solutions, for weeks. It may be that complete complexation with methanol and ethanol imparts to ferrihemoglobin a stability which the partially complexed protein does not have, or that there is a solvent effect on conformational stability which is not monotonic in alcohol concentration. In any case, a 1 M solution of methanol or ethanol in 0.033 M phosphate buffer, pH 6.3, is an exceedingly favorable environment for ferrihemoglobin.

The question arises as to whether or not association with alcohols affects the reactivities of hemeproteins.[3] It has been found that both methanol and ethanol weaken the binding of cyanide to ferrimyoglobin; 1 M methanol increases the dissociation constant of the cyanide complex about sevenfold (M. E. McKnight, to be published). Further investigation along this line is under way.

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[3] Not under consideration here are the well-known roles of methanol and ethanol as substrates in the peroxidatic reactions catalyzed by catalase (Brill, 1966).

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Interaction of [^{3}H]Bongkrekic Acid with the Mitochondrial Adenine Nucleotide Translocator†

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ABSTRACT: Chemical labeling by ^{3}H and biosynthetic labeling by ^{14}C of bongkrekic acid (BA) are described. In the rat liver cell, mitochondria are the only subcellular particles to bind [^{3}H]BA with high affinity. The high affinity sites for BA in mitochondria are located in the inner membrane. High affinity binding sites for BA are only displayed at pH below 7; they amount to 0.15–0.20 nmol/mg of protein in rat liver mitochondria and to 1.1–1.3 nmol/mg of protein in rat heart mitochondria. These values are similar to those found for the high affinity atractyloside binding sites and for the carboxyatractyloside binding sites. The kinetic parameters for BA binding to rat heart mitochondria at 20 °C are $K_d = 10\text{–}40 \times 10^{-9}$ M, $k_{+1} = 0.7 \times 10^5$ M^{-1} s^{-1}, $k_{-1} = 1.4 \times 10^{-3}$ M s^{-1}. Binding assays carried out with rat heart mitochondria, under equilibrium conditions, showed that the amount of BA bound to high affinity sites increases with temperature and reaches the maximum value of 1.1–1.3 nmol/mg of protein at 32–35 °C. At lower temperatures, and under equilibrium conditions, a significant fraction of high affinity sites remains masked and is not titrated by BA; these masked BA sites are revealed by addition of micromolar concentrations of ADP or by energization of the mitochondria. Carboxyatractyloside added to rat heart mitochondria preloaded with [^{3}H]BA is able to displace part of the bound [^{3}H]BA. Displacement of the bound BA is enhanced by simultaneous additions of carboxyatractyloside plus ADP, or by energization of the mitochondria. The synergistic effect of carboxyatractyloside and ADP on displacement of bound [^{3}H]BA is also observed in isolated inner membrane vesicles from rat liver mitochondria. When BA is preincubated with rat heart mitochondria before addition of [^{14}C]ADP for assay of ADP transport, the inhibition of ADP transport is a mixed-type inhibition. When BA is preincubated with the mitochondria together with a very small concentration of ADP (less than 0.5 μM), the inhibition of [^{14}C]ADP transport is markedly increased (up to ten times) and it becomes typically uncompetitive, which suggests the formation of a ternary complex, carrier–ADP–BA. The transition from a mixed-type inhibition, with high K_i value, to an uncompetitive type of inhibition, with low K_i value, upon addition of ADP, is explained by an ADP-induced conformational change of the ADP translocator.

Bongkrekic acid (BA)[1] is an inhibitor of ADP transport in mitochondria (Henderson and Lardy 1970; Henderson et al., 1970). Similarly to atractyloside and carboxyatractyloside which also inhibit ADP transport (for review, see Vignais et al., 1973b), BA prevents the oxidative phosphorylation of extramitochondrial ADP (Welling et al., 1960) but not the phosphorylation of intramitochondrial ADP (Kemp et al., 1970; Klingenberg et al., 1970). However, BA differs from atractyloside and carboxyatractyloside in virtue of some unique features. (1) Its inhibitory effect is not immediate (Henderson and Lardy, 1970); the lag time following addition of BA and the potency of inhibition depend on temperature and pH; in particular inhibition by BA is markedly increased at pH below 7 (Kemp et al., 1971). (2) Inhibitory efficiency of BA is enhanced by preincubation of mitochondria with micromolar amounts of ADP or ATP (Kemp et al., 1970, 1971). (3) BA decreases the amount of ADP or ATP required to elicit maximal contraction of mitochondria (Stoner and Sirak, 1973). (4) Addition of BA to heart mitochondria previously equilibrated with nonsaturating concentration of [^{14}C]ADP results in a significant increment of [^{14}C]ADP binding (Erdelt et al., 1972; Klingenberg and Buchholz, 1973).

The pH effect can readily be explained by assuming that BA interacts with the ADP translocator from the inside of the inner mitochondrial membrane and that it must be protonated to enter this membrane. Since the average pK of the carboxylic groups of BA is of the order of 5.5 (Lijmbach, 1969), the pH of the medium must be sufficiently low to allow a significant fraction of BA to be protonated. The other effects, especially the synergistic effect of ADP, are most probably inherent to the mechanism itself of ADP transport. Further insights into the mechanism of ADP transport can be obtained by binding studies using radioactively labeled BA. This paper reports data on the binding properties of [^{3}H]BA to mitochondria. These include the dependence of the binding parameters on the membrane environment of the ADP translocator and on the energy state of mitochondria. Parallel experiments have been

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[1] Abbreviations used are: BA, bongkrekic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.