*SPIN-STATE-DEPENDENT HEMOPROTEIN ULTRAVIOLET-ABSORPTION BANDS**

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The dependence upon magnetic moment of the Soret and visible absorption bands of hemoprotein complexes has been recognized for some time,[1, 2] and a quantitative description of this relation has been given.[3] This knowledge has been only indirectly helpful in elucidating the nature of the protein involvement in bonds to the iron of the heme group since the optical transitions belong to the prosthetic group and not to groups on the protein. The characteristic absorption bands of amino acid residues which could contribute atoms at positions 5 and/or 6 of the coordination sphere of iron in heme (coordinating pyrrole nitrogens at positions 1 through 4) are located in the ultraviolet (UV) where other amino acid residues and the peptide bond also have absorption bands. The spectroscopist is confronted with the problem of observing a change in the absorption band of the amino acid residue coordinated to the iron ion against a total absorbance which increases sharply as the wavelength decreases from 240 mμ. However, the technique of difference spectroscopy can be used to discriminate against the contributions of residues which are electronically unperturbed. We report here, using spectra from horse heart ferrimyoglobin (MetMb) as examples, a new class of spin-state-dependent protein absorption bands in respiratory pigments and heme enzymes which are manifested by the appearance of a characteristic difference spectrum upon the formation of various complexes and compounds from free ferric hemoproteins.

Materials.—MetMb: MetMb, obtained from Nutritional Biochemicals Corporation, was used without further purification. Each aliquot of the MetMb stock solution was oxidized with a slight molar excess of $K_3Fe(CN)_6$ to ensure complete formation of MetMb, dialyzed with four changes of buffer, and centrifuged to remove denatured protein. The Soret-to-protein absorbance ratio (purity criterion) was equal to or greater than 5.0 for all preparations. The calculation of heme concentration is based[1] on $a_{409\ m\mu} = 188\ mM^{-1}\ cm^{-1}$ at pH 6.4 and[4] $a_{410\ m\mu} = 152\ mM^{-1}\ cm^{-1}$ at pH 8.4.

Ligands: KCN and NaN_3 were of highest purity commercially available; 3 *M* stock solutions of cyanide or azide were made with distilled H_2O. Since aqueous cyanide solutions rapidly decompose and since azide solutions form a precipitate after standing for 12 hr, fresh stock solutions were made each day.

Peroxides: H_2O_2, analytical reagent grade, was obtained from Mallinckrodt Chemical Works. EtOOH was synthesized by a modification of the methods of Minkoff[5] and Harris.[6] Our product, obtained from vacuum distillation, had a refractive index, $n_D^{20} = 1.3808$, which compares very favorably with the literature values[5, 7] of 1.3810 and 1.3800.

Methods.—The absorption spectra were recorded with a Cary model 14 spectrophotometer. A tandem cell arrangement was used which provided for cancellation of the absorbance of the ligand as well as the protein. (Either equal volumes of solution were added, reagent to the "sample" hemoprotein and buffer to the "reference" hemoprotein, or a dilution correction was made to the base line.) There are stringent experimental conditions, optical and thermodynamic, which must apply before the detection of the difference bands is possible. Analysis of these requirements[4] establishes two criteria for the successful application of this technique to a particular complex:

$$(1)\quad K_{d\epsilon L}(\lambda_m) < \frac{A_m}{b},$$

where K_d is the apparent dissociation constant of the ligand-hemoprotein complex; $\epsilon_L(\lambda_m)$ is the molar absorptivity of the ligand at the shortest wavelength, λ_m, which is to be recorded (usually 210 mμ); A_m is the absorbance in the reference beam which results in maximum slit width at λ_m (about 2.0 absorbance units at 210 mμ); and b is the optical path (0.05 or 1.0 cm for the experiments reported here).

(2) $100\ K_d >$ saturation concentration of ligand,

where the factor 100 arises from considerations of hemoprotein dilution and extent of complex formation.

Magnetic susceptibility data are available for complexes of many of the hemoproteins with fluoride, cyanate, thiocyanate, azide, and cyanide. Application of the two criteria show that the cyanide complexes are most satisfactory, that azide complexes are partially acceptable, but that the other complexes are refractory to this technique at present. Somewhat different problems arise when the method is applied to peroxide compounds of the hemoproteins. However, a systematic study has been possible[4] and a brief summary of the results will be given here.

Experimental Conditions.—All solutions were equilibrated at room temperature before being used. The cell compartments of the spectrophotometer were maintained at 25 ± 1°C. Since hydrocyanic and hydrazoic acids are weak acids, the hemoprotein solutions were buffered so that the pH did not shift more than 0.2 pH unit upon addition of cyanide or azide. In these experiments the concentration of either of these two ligands is at least 3 mM, and consequently the extent of formation is at least 95 per cent for all complexes except horseradish peroxidase (HRP)-N_3. For the compounds the concentration of peroxide which was used was the minimum needed to achieve full formation, as indicated by the Soret absorbance. Although a tandem cell arrangement was not used, the absorbance of the required peroxide concentration was either negligible or quantitatively accounted for.

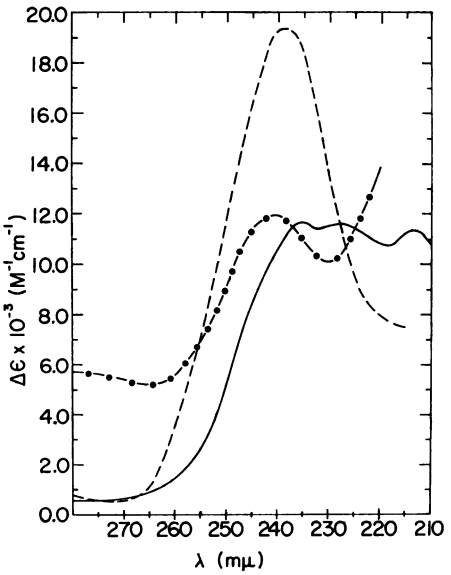

FIG. 1.—UV difference spectra of cyanide and azide complexes of metmyoglobin and metmyoglobin compound III. For MetMb-CN (———) and MetMb-N_3 (-·-·-), the heme concentration was 99 μM, the path length was 0.05 mm, the pH was 6.7 (0.3 *M* phosphate buffer), and the cyanide and azide concentrations were 30 mM. These ligand concentrations produce greater than 95% formation of the complexes. For MetMb compound III (– – – –), the H_2O_2/heme ratio was 4.5, the heme concentration was 4.9 μM, the path length was 1.0 cm, and the pH was 8.4 (0.17 *M* borate buffer). The Soret band indicates that compound III is fully formed after 45 min from the time of addition of H_2O_2.

Results.—The difference spectrum (210–280 mμ) of the MetMb-CN complex versus free MetMb is shown in Figure 1 (*solid line*). The complex was formed at two different heme concentrations (5 μM and 99 μM, 20-fold difference in path) with identical resultant spectra which were independent of time. The difference spectrum, with maxima at 214, 227, and 235 mμ, is the superposition of more than two difference bands. The average difference absorptivity at 235 mμ is $11.7^{+0.3}_{-0.4}$ mM^{-1} cm^{-1}, where +0.3 and −0.4 specify the range of difference absorptivity values.

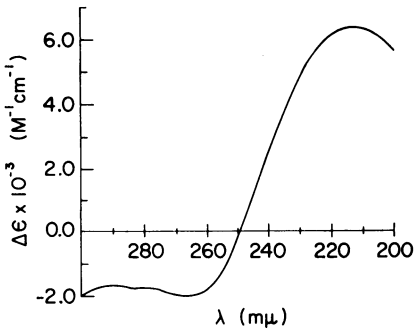

FIG. 2.—UV difference spectrum of biscyanoferriprotoporphyrin. The complex was formed in borate buffer, pH 9.5, with a ratio of cyanide to heme equal to 130. The heme concentration was 23 μM.

In order to observe the spectral changes which occur when cyanide is the only ligand bound to the heme group, we have recorded the difference spectrum (shown in Fig. 2) of biscyanoferriprotoporphyrin versus hematin in 0.04 *M* borate buffer, pH 9.5. Hematin at 1 mM concentrations exists in polymer forms, and biscyanoferriprotoporphyrin exists as a monomer.[8] Thus the difference measurement is complicated by the presence of polymeric forms. In the region 200–300 mμ, the difference spectrum has one maximum, located at 213 mμ.

The dotted curve in Figure 1 is the difference spectrum (220–280 mμ) of MetMb-N_3 complex versus free MetMb. Since the difference spectrum is time-dependent, it is recorded immediately after the azide is added. The magnitude of the time-dependent difference absorbance increases at lower wavelengths and appears to be proportional to the azide absorptivity. Inasmuch as the absorptivity of azide is much greater than that of cyanide, we have been able to record the difference spectrum of MetMb-N_3 only with a short path length (0.05 cm) and high heme concentration (99 μM). The difference spectrum contains one resolved difference band, at 240 mμ, with a difference absorptivity of 11.9 mM^{-1} cm^{-1}.

Within the pH range 8–9, MetMb reacts with H_2O_2 to give a stable intermediate, defined as compound III.[9] The difference spectrum (210–280 mμ) of MetMb compound III versus free MetMb at pH 8.4 is shown in Figure 1 (*dashed line*). Reactions with H_2O_2 and EtOOH give identical spectra. The difference spectrum contains one maximum at 238 mμ with $\Delta a = 19.3^{+0.4}_{-0.4}$ mM^{-1} cm^{-1}.

Similar difference spectra have been observed for complexes and compounds of other ferric hemoproteins, i.e., bacterial micrococcus catalase (BMC), HRP, and horse ferrihemoglobin (MetHb).[4] A summary of the wavelengths of the maxima in the difference spectra for complexes and compounds of these four hemoproteins is given in Table 1.

Discussion.—The difference spectra of all the low-spin complexes and compounds versus free hemoprotein (high-spin) exhibit maxima at about 240 mμ. While more complicated explanations can be advanced, we infer that the behavior of one particular transition is responsible for this band. This difference band cannot arise from metal d-d absorption alone because the wavelength is too short and the intensity too great. Note also that the 240-mμ band is absent in biscyanoferriprotoporphyrin versus hematin. It follows that the transition is associated with a group common to all four hemoproteins. Presumably, the amino acid residue in coordination position 5, known from X-ray diffraction analysis to be histidine in MetMb and MetHb, is the one involved.[12] We suggest then that HRP and BMC also have histidine in position 5 since all four hemoproteins exhibit this particular spectroscopic feature.

Azide has the interesting property of producing low-spin complexes with MetMb and MetHb, and a high-spin complex with BMC. There are *no bands* in the UV

TABLE 1

SUMMARY OF DIFFERENCE SPECTRA MAXIMA AND SPIN TYPE FOR COMPLEXES AND COMPOUNDS OF HEMOPROTEINS, AND FOR BISCYANOFERRIPROTOPORPHYRIN

Complex or compound	Wavelengths of Maxima in the Difference Spectrum[a] 250–230 (mμ)	230–220 (mμ)	220–210 (mμ)	Spin state[b]
BMC-CN	236	[e]	[f]	Low[h]
-N_3	—[c]	—	[f]	High[h]
Compound I	240	224	—	Intermediate, if ferric
Compound II	248	—	[f]	Low[h, i]
HRP-CN	235	225	[f]	Low
Compound I	[d]	[d]	—[d, g]	Intermediate, if ferric
Compound II	238	—	—[g]	Low[i]
MetHb-CN	235	223	213	Low
-N_3	241	—	—	Low
Compound III	238	—	—	[j]
MetMb-CN	235	227	214	Low
-N_3	240	—	[f]	Low
Compound III	238	—	—[g]	Low[i]

[a] From ref. 4.
[b] Assignments made on basis of magnetic susceptibility data from refs. 1, 2, and 10.
[c] No difference band observed.
[d] Broad difference band with flat top extending from 233 mμ to the limit of measurement (215 mμ).
[e] Absorbance increases so rapidly in the region 230–220 mμ that a superimposed maximum may be obscured.
[f] Measurement does not extend below 220 mμ.
[g] Measurement does not extend below 215 mμ.
[h] Based upon data from catalases other than BMC.
[i] Since the oxidation state of compounds II and III is one equivalent above the ferric hemoproteins,[9, 11] the magnetic susceptibilities of these compounds indicates that they are essentially low-spin regardless of whether the iron is tri- or tetravalent.
[j] Data not available.

difference spectrum of BMC-N_3 (ligand at a concentration in excess of that required for 95% complex formation) versus free BMC. All the low-spin versus high-spin UV difference spectra, including the azide complexes of MetMb and MetHb, have one or more bands. We therefore infer that a necessary condition for the appearance of the difference bands reported here is a difference in spin state.

In the region of the UV covered in these studies, electronic transitions within the coordination sphere may be classified as either ligand or charge-transfer, but such transitions are rarely entirely one type or the other. The difference band with a maximum at 213 mμ for biscyanoferriprotoporphyrin most likely arises from a transition from a cyanide π orbital to a mixed metal-ligand orbital. This charge-transfer band has been observed in simpler iron-cyanide complexes, e.g., ferric hexacyanide.[13] Where experimental conditions permit measurements down to 210 mμ, the peak at 213 mμ is observed for the cyanide complexes of the four hemoproteins, but not for the azide complex or the compounds.

The Soret band is an example of a transition which is essentially ligand (porphyrin π-π^*) in character. However, the π system is affected by a change in spin state, and spectral changes take place. The locus of points which relates the wavelengths of the Soret band maxima of a series of complexes (of MetHb and MetMb) with the corresponding magnetic susceptibilities is a straight line where the Soret maximum of the low-spin state occurs at a longer wavelength than that of the high-spin state.[2]

Histidine has an absorption maximum[14] at 211 mμ with an absorptivity of 5900 M^{-1} cm^{-1}. The transition responsible for this band has not been assigned, but surely involves the imidazole ring. The wavelength of the band maximum should not differ greatly between free histidine and histidine bound to iron in high-spin hemoproteins, but there is no experimental evidence which bears directly upon

this. Since the electronic structure of the imidazole which contributes a nitrogen to the coordination sphere of iron must be perturbed by a change in spin state, spectral transitions associated with the ring will be influenced. The only band which is consistently found in difference spectra of low-spin versus high-spin hemoproteins is the one at about 240 mμ. This spectral change could arise from a shift in the imidazole absorbance to longer wavelength, analogous to that of the porphyrin Soret, or from the appearance of a charge-transfer transition from imidazole to a metal-imidazole molecular orbital.

The corresponding long-wavelength difference bands of MetMb and MetHb agree in location (cyanide, 235 mμ; azide, 241 and 240 mμ; peroxide, 238 mμ). A similar correlation is not observed for the bands at about 225 mμ, the origin of which is therefore less certain and will not be discussed in this paper.

Spectra not displayed here will be presented in forthcoming publications, together with additional experimental details, data related to the measurement of dissociation constants, a quantitative relation among the intensities of the difference bands in the 235–248-mμ region, and discussions of the relations of the observations in these studies to the structures of hemoprotein complexes and compounds.[4] We suggest that the extension of this technique to ferrous hemoproteins and model complexes may be informative.

Definitions and abbreviations: Complex, a hemoprotein structure which results from the equilibrium binding of a ligand in coordination position 6; compound, a hemoprotein structure which results from reaction with a peroxide; BMC, bacterial micrococcus catalase; HRP, horseradish peroxidase; MetMb, horse heart ferrimyoglobin; MetHb, horse ferrihemoglobin.

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