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OPTICAL SPECTROSCOPIC OBSERVATION OF A METASTABLE FORM OF SPERM WHALE MYOGLOBIN GENERATED BY RECONSTITUTION

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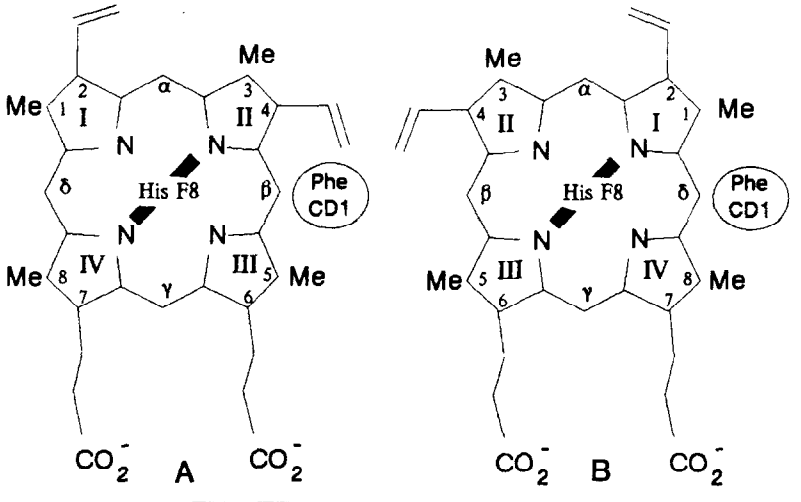
The optical spectrum of Sperm Whale myoglobin, which has been freshly reconstituted with iron protoporphyrin-IX, is shown to be different from that obtained from the native myoglobin, and from the reconstituted, incubated myoglobin (These last two have equivalent absorption spectra.). The effect is immediately evident as a shift of about +1 nm in the Soret band during incubation of freshly reconstituted metMb. Difference spectroscopy can be used to deconvolute changes in optical spectra occurring during and after Mb reconstitution into two components. The initial phase reflects incorporation of hemin into the protein matrix; this is already known to produce two forms, differing by relative hemin orientation. The rate of the second process follows the known pH dependance of iron protoporphyrin-IX reorientation. Presence of the second process indicates that the absorption spectrum of each of the two hemin-insertion Mb forms is unique, so interconversion between the two forms is monitored. Thus iron protoporphyrin-IX reorientation in proteins may be studied by visible spectroscopy.

Iron (III) protoporphyrin-IX (hemin) binding to hemoproteins, including hemoglobin (Hb) and myoglobin (Mb), is often heterogeneous. Heme-insertion isomers A and B, which are generated during reconstitution, differ by a formal 180° rotation about hemin the α,γ-meso axis (1). This is shown for the proximal side of Sperm Whale myoglobin (SWMb) relative to two key residues (2).



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Abbreviations: NMR, nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; CD, circular dichroism; hemin, ferriprotoporphyrin-IX; Mb, myoglobin; SWMb, Sperm Whale myoglobin; EqMb, equine myoglobin; λ_{max}, wavelength of local maximum absorbance; ε_{max}, molar absorbance at λ_{max}.

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The time course of hemin reorientation between A and B has been studied for metSWMb by La Mar, *et al.* using ^1H NMR spectroscopy (3). The hemin reconstitution product initially contains the two hemin-insertion isomers in equal amounts, then the mixture relaxes to the ratio 9:1 (A:B) which characterizes the native material. The rate for this is strongly pH dependent, exhibiting a minimum near pH 7. This behavior has also been detected by circular dichroism (CD) spectroscopy (4). No spectroscopic method other than NMR or CD has been reported for detecting the hemin-insertion isomers or their interconversion in solution. Optical absorption spectra of this reaction are reported to be complete either after seconds (5,6) or up to three hours (7), apparently depending on the degree of hemin aggregation before reconstitution.

We are interested in ascertaining absorption spectra differences in hemin-insertion isomers. Considering the large molar absorbance and narrowness of the metMb Soret band, detection of even a small shift in λ_{max} and/or ϵ_{max} should be possible. We expect some difference in λ_{max} or ϵ_{max} since A is distinguished from B by the hemin orientation. If such a difference is found between A and B, visible spectroscopy becomes a method whereby interconversion of hemin insertion forms can be monitored routinely and with high sensitivity. This is especially valuable for proteins which are difficult to obtain.

Herein we demonstrate that optical spectroscopy actually senses two processes during reconstitution of apoSWMb with hemin. The first process, insertion of hemin into the protein, is fast and may have multiple components (5-7). The rate of the second process exhibits a pH dependence consistant with redistribution of hemin-insertion isomers A and B. While this is a small change to follow directly, difference spectroscopy provides a transparent view.

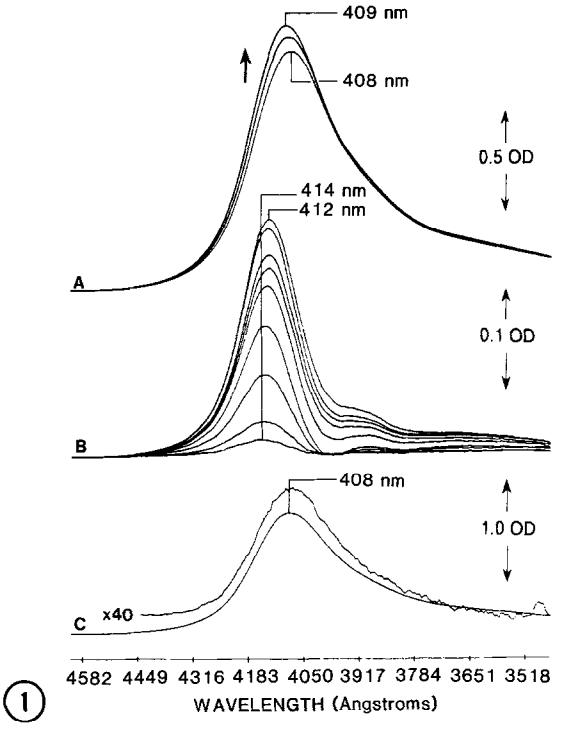
Experimental

SWMb was from Sigma; apoSWMb was obtained by the method of Teale (8). Hemin solutions were prepared fresh for each reconstitution by adding small amounts of NaOH to a H_2O suspension of hemin (Sigma) until no solid remained. Optical spectroscopy was performed in 50 mM phosphate/200 mM NaCl at pH 7.0 (unless otherwise noted) using 1 cm path length cells. Reconstitutions were performed by adding hemin solution directly to the optical cell containing a 5-fold excess of apoprotein. A 0.1 molar ratio of $K_3Fe(CN)_6$ was present in each run; this did not affect the reconstitutions significantly, but allowed for null difference spectra of control runs of native SWMb.

The spectrometer was a Cary 16; the cells were maintained at 25°C. A Macintosh Plus computer was employed to collect the data *via* a Keithely model 195A digital volt meter as the ADC and Mac488A bus controller from IOTech. Plots were executed on a Hewlett-Packard model 7470A plotter. Each run consisted of 2000 data points acquired over the ranges 750 to 351 nm in ten minutes (0.2 nm digital resolution) or 470 to 337 nm in three minutes (0.067 nm digital resolution). Times noted in the Figures indicate the start of each run. Data smoothing was performed as necessary by applying either three or seven point sliding window averages.

Results

Figure 1A illustrates a typical reconstitution of hemin into apoSWMb at pH 7.0. A process after hemin insertion is evident since the initial and final λ_{max} are not identical. This is demonstrated very clearly by difference spectroscopy, as shown in Figure 1B. Difference spectra from runs taken several hours after reconstitution are characterized by a λ_{max} nearly 5 nm higher than the Soret band. Hemin insertion into apoMb is also evident in the earliest difference spectra, manifested in a gain in absorbance at the shorter wavelength side of the Soret band (see below), and a less shifted λ_{max} for difference spectra. Initial incorporation of hemin into the protein can be



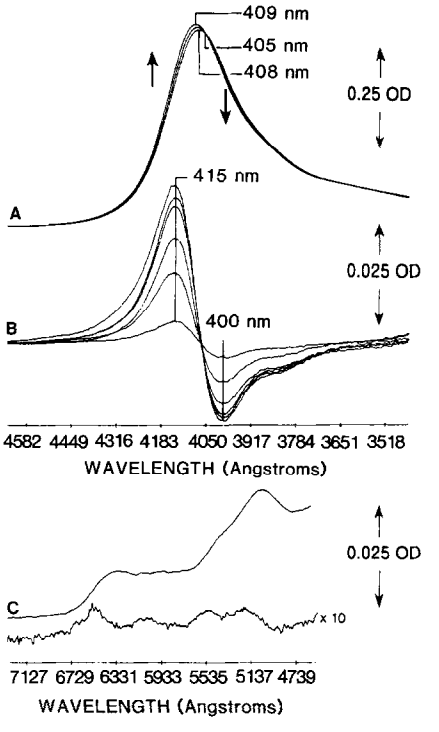


Figure 1. **Optical spectra for reconstitution of apoSWMb with protohemin IX at pH 7.0.** (A) Soret region (from bottom) at 109, 560, and 4200 min after reconstitution. Note the shift from an initial λ_{max} of 408 nm to the accepted value of 409 nm after several hours. (B) Difference spectra of Soret region generated by subtracting spectra corresponding to (from top) 19, 24, 37, 62, 109, 289, 560, 1695, 2800 min from the spectrum corresponding to 4200 min after reconstitution. Note that the later difference spectra have a constant λ_{max}, and that the first several difference spectra show λ_{max} shifted to shorter wavelenth. (C) Top: Difference spectrum corresponding to 19 - 13 min after reconstitution; bottom: Spectrum at 77 min after reconstitution. Note that the initial reconstitution product grows in at nearly the same absorbance as it appears.

Figure 2. **Optical spectra for reconstitution of apoSWMb with protohemin IX at pH 8.1.** (A) Soret region (from bottom) at 40, 535, and 2670 min after reconstitution. Note the shift from an initial λ_{max} of 408 nm to the accepted value of 409 nm after several hours. (B) Difference spectra of Soret region generated by subtracting spectra corresponding to (from top) 2, 20, 40, 97, 535, 1141 min from the spectrum corresponding to 2670 min after reconstitution. Note that the difference spectra have a constant λ_{max}. (C) Top: 740 to 465 nm region 2670 min after reconstitution; bottom: 740 to 465 nm region of the difference spectrum 2670 - 25 min. Note that there are four transitions in the difference spectrum, none of which are at the same wavelength as peaks in the reference spectrum.

demonstrated as in Figure 1C. So the result of early reconstitution is a product with λ_{max} = 408 nm; after several hours λ_{max} shifts to 409 nm, the reported value for metSWMb (9).

Figures 2A and 2B are analogous to Figures 1A and 1B, but for pH 8.1; at this pH hemin aggregation is minimal (7) so contributions from longer-time hemin insertion to the optical spectra are less than at pH 7. The earliest spectra have a λ_{max} of 408 nm, and after incubation 409 nm. This time, however, difference spectra reflecting the second process have both positive and negative features, with an isosbestic at 405 nm. The loss of absorbance at 400 nm and the isosbestic are likely masked in Figure 1 by dissociation of hemin aggregates with subsequent insertion into apoMb. Absorption bands other than the Soret also show shifts in λ_{max}. Figure 2C

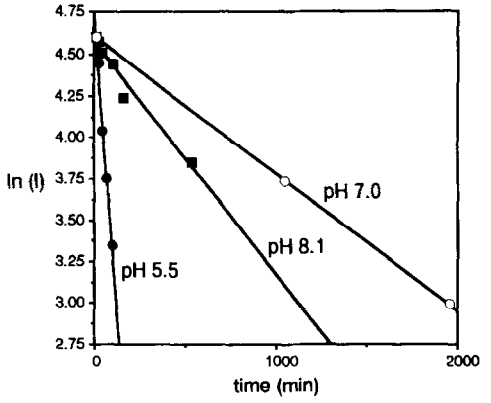


Figure 3. Plots of ln (Intensity) for the difference spectra from Figures 1B and 2B, and for pH 5.5. These only are for differences at long times so that contribution from hemin incorporation is minimized. λ = 415 nm. The intensities are multiplied by factors to allow direct comparison of the three runs. Time = 0 is taken as the time of the initial difference spectrum for each series; the later data points are then referenced to this time.

presents a longer wavelength region for the pH 8.1 reconstitution. Features with altered λ_{max} values are seen in the difference spectrum for each of the four metSWMb transitions (10).

Since reconstitution at pH 7.0 exhibits a slower rate for the reaction shown in Figures 1 and 2, and since pH 7 is near the minimum for interconversion of A and B (3), we performed a reconstitution at pH 5.5. In this case the form of the difference spectra (not shown) was very similar to those in Figure 1. Time dependencies for the difference spectra are shown in Figure 3 for each of the reconstitutions. The process occurring after hemin insertion is significantly slower at pH 7.0 than at pH 5.5 or 8.1. Caution is required lest these slopes be used as a quantitative evaluation of the rate constants at these pH values: At least at pH 5.5 and 7.0 these data contain considerable interference from hemin insertion into apoMb.

Discussion

Changes in optical absorption spectra obtained during reconstitution of hemin into apoSWMb reflect a process in addition to insertion into the globin. Spectra acquired minutes after reconstitution are not equivalent to native metSWMb; a spectrum identical to native metSWMb can only be obtained after incubation for several hours at pH 7. Since reconstitution of SWMb produces a metastable form based on hemin *disorder* (i.e., the presence of significant amounts of both A and B (1)), and since loss of this disorder follows the pH dependence of the process monitored here (Figure 3), we propose that optical spectroscopy of freshly reconstituted metSWMb reflects the presence of hemin disorder, and that subsequent loss of this disorder is manifested as in Figures 1 and 2. We anticipate that absorption spectroscopy can be used to observe interconversion of hemin-insertion isomers in other hemoproteins.

Hemin disorder is most dramatically shown by ^{1}H NMR spectroscopy (1,3), although CD spectroscopy provides a qualitative alternative (4). It now appears that optical absorption spectroscopy also allows detection of dirsorder loss or gain. The spectral changes are subtile, so

demands on instrumentation are stringent. Computer interfacing is an aid for difference spectroscopy, but digital resolution (data points per nm) must be sufficient. Likewise, the detector must be stable during the experiments, which can last for several days.

Optical absorbance detection of hemin-insertion isomer redistribution must presently be regarded as qualitative, or semi-quantitative at best. A rate constant for intensity change at some wavelength can be conveniently extracted from difference spectra, but whether the value is due solely to interconversion of A and B remains in question. At pH 8.1, where interference from hemin insertion is minimized, we extract the apparent rate constant 1.4×10^{-3} min^{-1} from Figure 3. This value is not far from the NMR result of about 0.8×10^{-3} min^{-1} for loss of disorder (3). However, the Soret region is complex (11); contribution(s) to the difference spectra by each species may not be resolved with certainty. As details of hemin substituent conformations and positions of the hemin-insertion isomers relative to the protein residues become available, the sign and magnitude of the spectral band shifts may become interpretable.

Preliminary experiments have demonstrated similar absorbance changes for horse Mb (metEqMb). The methods outlined here have also shown utility for monitoring reconstitution processes in cases where interconversion between hemin-insertion forms is too fast to observe conveniently by NMR spectroscopy. Further, we are exploring ways of removing interference from slow insertion of hemin into apoMb at neutral and low pH. Details of these studies involving protoporphyrin-IX itself, and metal-substituted porphyrins, will appear elsewhere.

Acknowledgments

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