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Hemin Is Kinetically Trapped in Cytochrome b_5 from Rat Outer Mitochondrial Membrane

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Cytochrome b_5 from the outer mitochondrial membrane of rat liver (OM cyt b_5) is substantially more stable to thermal and chemical denaturation than cytochrome b_5 from the endoplasmic reticulum of bovine liver (microsomal, or Mc cyt b_5). In contrast, the corresponding apoproteins have similar stability, suggesting stronger interactions between hemin and the polypeptide in OM cyt b_5. Whereas complete transfer of hemin from bovine Mc cyt b_5 to apomyoglobin at pH 5.2 takes less than 1 h, hemin transfer from OM cyt b_5 is unmeasurably slow. Coupled with the previously reported 1:1 ratio of hemin orientational isomers in OM cyt b_5, this finding suggests that the cofactor is kinetically trapped under physiologically relevant conditions. This conclusion is confirmed by ^{1}H NMR studies which show that the hemin isomeric ratio changes when the protein is incubated for several hours at 68°C. Interestingly, the orientational isomer favored in OM cyt b_5 is the form less favored in all other known cytochromes b_5.

Key Words: **cytochrome b_5; mitochondria; protein stability; heme.**

Two distinct isoforms of cytochrome b_5 have been identified in mammalian hepatocytes. One of these isoforms is anchored to the endoplasmic reticulum (microsomal or Mc cyt b_5) (1), and the other is anchored to the outer membrane of mitochondria (OM cyt b_5) (2). Cytochromes b_5 are anchored to the appropriate membrane via a *C*-terminal motif, whereas the hydrophilic *N*-terminal domain containing the bis-histidine coordinated heme cofactor extends into the cytoplasm (3–5). Mc cytochromes b_5 from a variety of sources have been isolated and their genes cloned or synthesized (6–8). By comparison, the only OM cyt b_5 that has been positively identified to date originates from rat liver. The gene coding for this 146-residue protein has recently been sequenced (9). A partial amino acid sequence of the water soluble fragment, which includes the heme binding domain of OM cyt b_5, was determined in 1983 (10). On the basis of that amino acid sequence, Rivera *et al.* constructed a synthetic gene for the expression of residues 1–92 of rat OM cyt b_5 (henceforth referred to as OM cyt b_5) (11). Comparison of the sequence encoded by the synthetic gene with that encoded by the recently cloned gene revealed a single error in the sequence of the synthetic gene: a switch from asparagine to aspartic acid at position 1. Because residue 1 is distant from the heme, this error is not likely to exert a significant influence on heme properties.

The three-dimensional structure of OM cyt b_5 has been determined by X-ray crystallography to a resolution of 2.7 Å (12). Comparison of this structure with that of bovine Mc cyt b_5 (13) demonstrated that the two proteins adopt very similar folds. In fact, the rms difference between the crystal structure coordinates of the backbone atoms in the two proteins is only 0.6 Å (residues 5–84). The high structural similarity is consistent with the 58% identity in amino acid sequence between the two proteins, as is the fact that the two proteins have essentially identical UV/vis and EPR spectra (11). Sequences corresponding to Mc cytochromes b_5 from different species are commonly more similar to one another. For example, the tryptic digests of bovine and rat Mc cyt b_5 share 93% sequence identity.

Electrochemical studies carried out with recombinant OM cyt b_5 (14, 15) have revealed that its reduction potential is significantly more negative ($E_0 = -102$ mV) than the reduction potential of the various Mc proteins ($E_0 = +5$ mV) (16, 17). Herein we present evidence demonstrating that OM cyt b_5 is also significantly more stable than its microsomal counterparts.

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The increased stability of the mitochondrial protein with respect to the Mc isoforms appears to be related to a markedly slow heme release.

MATERIALS AND METHODS

Materials. The tryptic fragment of bovine Mc cyt b_5, comprising residues 7–90 of the full-length protein, was obtained from bovine liver microsomes as described previously (18). Expression of recombinant rat OM cyt b_5 comprising residues 1–92 was performed according to the method of Rivera *et al.* (11). Guanidinium hydrochloride was purchased from Aldrich. Horse skeletal muscle myoglobin (Mb) was purchased from Calbiochem. Removal of hemin from Mb, Mc cyt b_5 and OM cyt b_5 was accomplished using the method of Teale (19). Apoprotein concentrations were determined using the absorbance at 280 nm, for which extinction coefficients of 10.6 $mM^{-1}cm^{-1}$ for apocyt b_5 (20, 21) and 16.0 $mM^{-1}cm^{-1}$ for apoMb (22, 23) have been determined previously.

UV/Vis spectroscopy. UV/vis spectra were recorded on a Kontron UVIKON 9410 recording spectrophotometer with a thermostated cell compartment. Temperature within the cell was measured using an Omega Model HH200 thermometer with a T thermocouple (±0.2°C accuracy). Sample concentration ranged from 4 to 6 μM in potassium phosphate buffer, pH 7.0. (Sentence about reduction deleted) For thermal denaturation studies, spectra were measured in approximately 5°C increments, and samples were equilibrated for 15 min after reaching each desired temperature. The change in absorption at the Soret band λ_{max} as a function of temperature was measured to calculate the melting temperature (T_m).

Circular dichroism spectroscopy. CD measurements were performed on a Jasco J-710 spectropolarometer equipped with a Neslab RTE-111 circulating water bath and a Neslab RS232 remote temperature sensor. Protein samples (40–45 μM in 2 mM phosphate buffer, pH 7) were placed into a 0.5-mm cylindrical, water-jacketed cell. For thermal denaturation experiments, the mean residue ellipticity at 222 nm (θ_{222}) was recorded as a function of increasing temperature in increments of 5°C beginning at 30°C. Each sample was equilibrated for 15 min at each temperature. The melting temperature (T_m) was determined from the first derivative of the resulting θ_{222} vs temperature plot.

Chemical denaturation studies. Stock solutions of guanidinium hydrochloride (GdmCl) were prepared in 50 mM phosphate buffer, pH 7. The solutions with final protein concentration 5–6 μM and GdmCl concentrations from 0 to 6 M were kept at 20–C for 20 h before fluorescence and UV-vis spectra were recorded. Data were analyzed by the standard method for a two-state mechanism of dissociation (24, 25). The free energy of hemin dissociation ΔG_D° at 25°C and zero GdmCl concentration was estimated by extrapolation of the ΔG_D linear dependence on GdmCl concentration in the transition region to nondenaturating condition. Fluorescence measurements were obtained as a function of GdmCl concentration (a minimum of 15 solutions was used) using a PTI spectrofluorometer. Fluorescence spectra were recorded in the 300–400 nm range, with excitation at 280 nm, and represent an average of 5–7 scans. The fluorescence intensity at 340 nm was measured. For each sample, a spectrum of a suitable blank solution was subtracted. UV/vis spectra were recorded using the instrument described above.

Hemin transfer experiments. A 1.0 ml solution of apoMb (6–8 μM) in 50 mM sodium citrate/sodium phosphate buffer was equilibrated at 21°C in a 1 cm cuvette in the UV-vis spectrophotometer. An aqueous solution of cyt b_5 prepared in the same buffer (~5 μL; final concentration 5–6 μM) was added to the apoMb solution. Continuous measurements of the absorbance at 406 nm were initiated about 20 s from the time of addition of cyt b_5. The rate constants for hemin transfer at pH values of 5.2, 5.6, and 6.8 were calculated using the exponential equation for a first-order reaction (equation 1), where

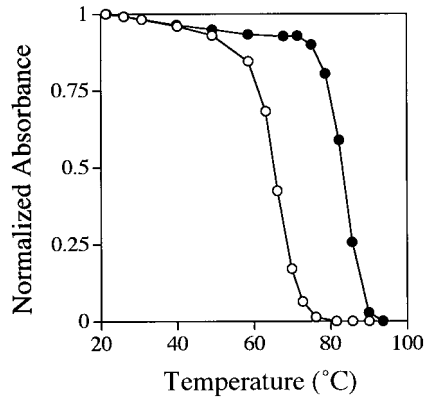

FIG. 1. Plots of normalized absorbance (Soret band) vs temperature for the tryptic fragment (residues 7–90) of bovine Mc cyt b_5 (○) and residues 1–92 of rat OM cyt b_5 (●). Data were acquired in 2 mM potassium phosphate buffer, pH 7.0 and normalized by setting the intensity of the Soret band of each 20°C spectrum to 1.0.

ΔA_t is the increase in absorbance at time t, ΔA_{eq} is the increase in absorbance at equilibrium, and k equals the rate constant for hemin transfer.

$$\Delta A_t = \Delta A_{eq}(1 - e^{-kt}) \quad [1]$$

^1H NMR spectroscopy. ^{1}H NMR spectra of OM cyt b_5 were recorded on a Varian Unity Inova NMR spectrometer operating at a frequency of 399.92 MHz. The protein solution (1.5 mM in a 5 mm tube) was exchanged with perdeuterated sodium phosphate buffer (μ = 0.10 M; pH 7.0, not corrected for the isotope effect), followed by equilibrating the sample at the appropriate temperature and then cooling to room temperature. Spectra were acquired at 25°C with 1024 scans over a 20 kHz spectral width, with an acquisition time of 0.4 s and water presaturation during a relaxation delay of 1.5 s.

RESULTS AND DISCUSSION

Thermal denaturation studies. We initially examined the thermal stability of OM cyt b_5 by monitoring change in absorbance at the Soret band to indicate loss of the cofactor from the protein. The intensity of the Soret band decreases slightly between room temperature and about 75°C, but above 75°C it decreases sharply and is replaced by a broad band centered near 395 nm. These changes result from conversion of low-spin bis-His coordinated hemin to a high-spin species. Similar temperature-dependent spectral changes have been reported for bovine (26), rabbit (27–29) and rat (24) Mc cytochromes b_5. For the Mc proteins, however, the temperature at which the spectral changes become apparent is lower. Analysis of a plot relating temperature-dependent changes in the intensity of the Soret band at 412 nm (Fig. 1) indicates that the midpoint of thermal denaturation (T_m) for OM cyt b_5 is 83.6°C in 2 mM potassium phosphate buffer (Table 1). By comparison, the T_m value we have measured for the tryptic fragment of Mc cyt b_5 (Fig. 1) in 2 mM phosphate buffer is 65.2°C. This latter value is similar to

TABLE 1

Thermal Denaturation Data for Apo- and Holocytochromes b_5 at pH 7.0

Protein	Buffer	T_m (°C) UV/vis	T_m (°C) CD	Reference
Mc cyt b_5 (7–90)[a]	2 mM phosphate	65.2	66.0	This work
	100 mM phosphate	67.2		26
	5 mM MOPS	67.4		7
Mc cyt b_5 (1–90)	5 mM MOPS	73.2		7
Mc cyt b_5 (1–104)	5 mM MOPS	73.1		7
OM cyt b_5 (1–92)	2 mM phosphate	83.6	≥82	This work

[a] Numbers in parentheses refer to the length of the protein.

values previously reported for the tryptic fragments of bovine (7, 26) (Table 1) and rabbit (28) Mc cyt b_5. Thermal denaturation data have also been reported for fragments of bovine Mc cyt b_5 containing residues 1–90 and residues 1–104 (7). The T_m values for these two proteins are nearly identical (Table 1), and about 6°C higher than for the trypsin-solubilized fragment. Nonetheless, these longer fragments of Mc cyt b_5 are still significantly less stable than the recombinant OM cyt b_5 comprising 92 residues.

T_m values for OM cyt b_5 and the tryptic fragment of Mc cyt b_5 derived from the temperature dependence of mean residue ellipticity at 222 nm (θ_{222}) in circular dichroism (CD) spectra are nearly identical to those determined by electronic spectroscopy under the same conditions (Table 1). Although complete denaturation of OM cyt b_5 was not observed even at 90°C, the highest temperature we could reach, analysis of the available data indicate that the T_m value of this protein is ≥82°C. The results of this study suggest that protein unfolding and heme release are coupled in both proteins. Similar results were previously obtained in chemical denaturation studies of the tryptic fragment of rabbit cyt b_5 (30), which was also followed by both UV/vis and CD.

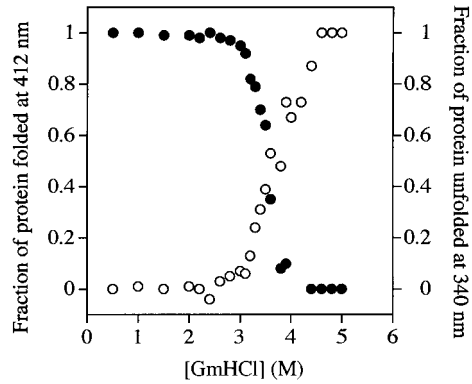

FIG. 2. GmHCl-induced denaturation of rat OM cyt b_5. Fractions of folded and unfolded protein were monitored by UV/Vis (●) and fluorescence (○) spectrophotometry. All measurements were performed in 50 mM potassium phosphate buffer, pH 7.0, and 25°C.

Chemical denaturation studies. Whitford and coworkers recently examined the stability of the fragment of bovine cyt b_5 comprising residues 1–104 in both the holo (31) and apo (32) forms. UV/vis and fluorescence spectroscopy were used as probes of guanidinium hydrochloride (GdmCl)-mediated denaturation of the holoprotein. Single cooperative transitions with midpoints at 3.0 M GdmCl were observed with both spectroscopic probes (Table 2), leading to the conclusion that disruption of the hydrophobic core around the invariant residue Trp-26 and of the heme binding domain occur as part of the same cooperative transition (31).

GdmCl-mediated denaturation of OM cyt b_5 also occurs with a single cooperative transition with a midpoint near 3.6 M GdmCl, using both UV/vis and fluorescence spectroscopy as probes (Fig. 2; Table 2). The free energy of unfolding of OM cyt b_5 determined by extrapolating the data to 0 M GdmCl indicates that it is about 10 kJ/mol (~2.5 kcal/mol) more stable than the Mc protein.

The OM and Mc apocytochromes b_5 are both considerably less stable than the respective holoproteins, as

TABLE 2

Thermodynamic Data for GdmCl-Mediated Denaturation of Apo and Holocytochromes b_5 at pH 7.0

Protein	UV/vis Data C_m (M)	UV/vis Data ΔG° (kJ/mol)	Fluorescence Data C_m (M)	Fluorescence Data ΔG° (kJ/mol)
Mc apocyt b_5 (1–104)[a,b]			1.6	11.6 ± 1.5
OM apocyt b_5 (1–92)[c]			1.2 ± 0.2	13.1 ± 0.8
Mc cyt b_5 (1–104)[a,d]	3.05 ± 0.1	27.5 ± 3.3	2.99 ± 0.1	25.5 ± 3.5
OM cyt b_5 (1–92)[c]	3.64 ± 0.1	37.7 ± 3.2	3.51 ± 0.1	38.5 ± 4.8

[a] In 30 mM MOPS buffer.
[b] Ref. (32).
[c] In 50 mM sodium citrate/sodium phosphate buffer.
[d] Reference 31.

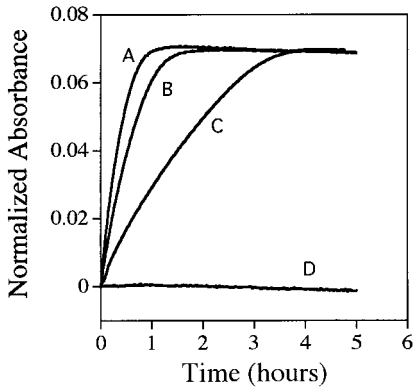

FIG. 3. Changes in absorbance as a function of time in hemin transfer experiments for bovine Mc cyt b_5 at pH 5.2 (A), pH 5.6 (B), and pH 6.8 (C), and for rat OM cyt b_5 at pH 5.2 (D). All experiments were conducted at 25°C in 50 mM sodium phosphate/citric acid buffer.

determined by fluorescence spectroscopy, but are essentially identical in stability to one another (Table 2). These results indicate that the greater stability of OM cyt b_5 stems from more favorable interactions between the hemin cofactor and the polypeptide. This result is consistent with the findings from a recent report that equilibrium constants for chemical denaturation of myoglobin mutants correlate almost exclusively with hemin affinity, rather than with stability of the apoproteins (33).

Hemin transfer studies. The results described above led us to postulate that the increased stability of OM cyt b_5 with respect to Mc cyt b_5 results from more favorable interactions between the cofactor and the polypeptide in the OM protein. Moreover, since protein unfolding in cyt b_5 is concomitant with hemin release, it appears that slow hemin release from OM cyt b_5 contributes to its enhanced stability. Therefore, we turned our attention to measuring rates of hemin release from OM and Mc cytochromes b_5. Kinetics of heme dissociation from cytochromes b_5 were studied by measuring first-order rate constants (k_{-h}) for transfer of hemin from cyt b_5 to apomyoglobin, using experiments based on a method developed to measure hemin release from myoglobin (Mb) mutants (34) and other hemoproteins (35). Addition of an equimolar amount of horse apoMb to a solution containing Mc cyt b_5 results in distinct time-dependent changes in the UV-Vis spectrum of the solution. The last spectrum obtained in this series is identical to that obtained from a solution of horse Mb. The plot shown in Fig. 3C demonstrates that at pH 6.8 Mc cyt b_5 transfers its hemin to apomyoglobin quantitatively in 6 h ($k_{-h} = 0.52\ h^{-1}$), whereas at pH 5.2 (Fig. 3A), hemin transfer is complete within 1 h ($k_{-h} = 3.1\ h^{-1}$). Similar results have been reported for the trypsin-solubilized form of Mc cyt b_5 (26, 36) and for the lipase-solubilized fragment which comprises residues 1–93 (36) at pH 7 (0.23–0.3 h^{-1}) and pH 5.0 (6.8 h^{-1} trypsin-solubilized fragment; 4.2 h^{-1} lipase-solubilized fragment (36)). In sharp contrast, hemin transfer from OM cyt b_5 is exceedingly slow (Fig. 3D). In fact, the UV/vis spectrum obtained after 12 h of mixing OM cyt b_5 and apoMb at pH 5.0 is identical to the spectrum of OM cyt b_5 recorded at the beginning of the experiment. The findings from this study corroborate the idea that the enhanced stability of OM cyt b_5 is in large part due to slow hemin release, hence indicating that hemin in OM cyt b_5 is kinetically trapped at physiologically relevant temperatures. More compelling evidence demonstrating this idea was obtained from ^{1}H NMR spectroscopy.

^{1}H NMR studies. Rat OM cyt b_5 is known to be heterogeneous as a result of heme being bound in two orientations related to each other by a 180° rotation about the porphyrin α–γ-meso axis (Fig. 4) (11). This is a well-known property of cytochromes b_5 and other hemoproteins (37). Heme orientational disorder differs widely amongst cytochromes b_5; 9:1 (A:B) in bovine Mc, 20:1 in chicken Mc, 1.6:1 in rat Mc, and 1:1 in rat OM cyt b_5 (37–39). When apocytochromes b_5 are reconstituted with hemin, the resultant holoprotein contains a mixture of isomers A and B in an approximately 1:1 ratio. As time proceeds, the ratio of isomer A to isomer B in the Mc isoforms increases until an equilibrium mixture is reached (40, 41). In the case of bovine Mc cyt b_5, the half-life for the transformation of isomer B into A is 13 h at pH 7.0 and 24°C.

The product of overexpression of OM cyt b_5 in *E. coli* is the holoprotein, which is purified as a statistical (1:1) mixture of isomers (11). No change in this ratio is observed even after the protein is exposed to room temperature for extended periods of time. This observation, together with the remarkably slow hemin transfer from OM cyt b_5 to apoMb noted above, strongly suggested that the hemin in oxidized OM cyt b_5 is kinetically trapped.

Taking advantage of the remarkable thermal stability of OM cyt b_5, we attempted to accelerate the rate of heme reorientation by incubating the protein at temperatures significantly above room temperature. In order to measure heme orientation ratio, the area corresponding to the peaks originating from hemin methyl groups A5Me and B8Me (Fig. 4) were evaluated from ^{1}H NMR spectra obtained after incubating the protein at different temperatures. The resonances originating from A5Me and B8Me were chosen because A5Me and B8Me are located in similar chemical environments in the two isomers, therefore possessing similar chemical shifts. This choice of signals is also aimed at eliminating potential differences in apparent signal intensity that result from possible differences in excitation power.

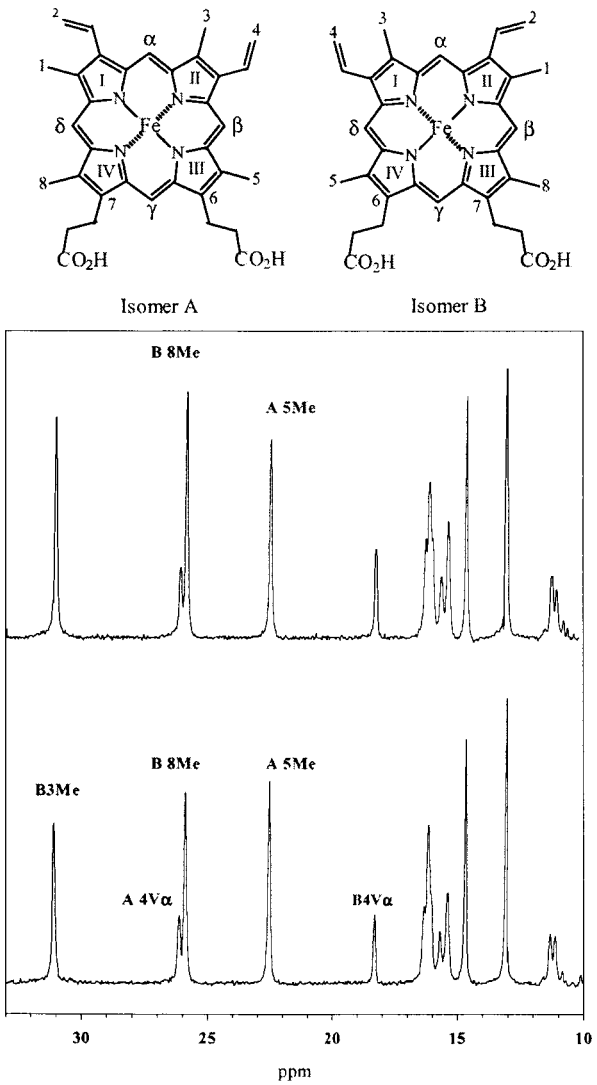

FIG. 4. ^{1}H NMR spectra acquired at 25°C from samples containing OM cyt b_5 incubated at 25°C for 24 h (bottom) and OM cyt b_5 incubated at 68°C for 7 h (top).

The ^{1}H NMR spectrum of OM cyt b_5 (Fig. 4, lower trace) was obtained after the protein was purified and exchanged into deuterated phosphate buffer. The heme isomer ratio A:B = 1:1 is in agreement with a previously measured value (11). The spectrum obtained after incubation of the protein at 65°C for 3.5 h indicate that the heme isomer ratio A:B is 1:1.2. The spectrum obtained after the protein was incubated for an additional 3.5 h at 68°C (Fig. 4, upper trace) shows that the hemin isomer ratio A:B is 1:1.3. Incubation of the protein for an additional 5.5 h at 68°C did not have any further effect on the isomer ratio, indicating that the hemin isomer ratio dictated by the thermodynamic equilibrium had been achieved. To the best of our knowledge, OM cytochrome b_5 is the first example of a hemoprotein in which hemin orientational isomer B is favored at equilibrium and is the first *b*-type hemoprotein in which the hemin has been shown to be kinetically trapped at physiologically relevant temperatures.

CONCLUSION AND FUTURE DIRECTIONS

Despite their very similar three-dimensional structures, the two isoforms of cyt b_5 in the hepatocyte are localized to specific subcellular membranes and exhibit markedly different redox potentials, stabilities and hemin affinities. It appears that nature has tailored the properties of OM and Mc cyt b_5 to serve distinct roles in the cell, although the function of the OM protein is presently unclear. The more negative reduction potential and markedly higher stability of the rat OM protein relative to the Mc isoforms is intriguing, as comparison of the OM and Mc crystal structures revealed only minor differences in their structural properties. Consequently, this case represents an unparalleled opportunity to systematically examine how protein sequence modulates stability, hemin affinity and redox potential in the cytochromes b_5. In this context, a systematic study in which the structure of one isoform serves as a template for designing mutants of the other, coupled with detailed biophysical characterization, molecular dynamics simulations, and crystallography, is likely to yield the desired information.

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