



Thermodynamics of apocytochrome b_5 unfolding

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Abstract

Apocytochrome b_5 from rabbit liver was studied by scanning calorimetry, limited proteolysis, circular dichroism, second derivative spectroscopy, and size exclusion chromatography. The protein is able to undergo a reversible two-state thermal transition. However, transition temperature, denaturational enthalpy, and heat capacity change are reduced compared with the holoprotein. Apocytochrome b_5 stability in terms of Gibbs energy change at protein unfolding (ΔG) amounts to $\Delta G = 7 \pm 1$ kJ/mol at 25 °C (pH 7.4) compared with $\Delta G = 25$ kJ/mol for the holoprotein. Apocytochrome b_5 is a compact, natively protein. According to the spectral data, the cooperative structure is mainly based in the core region formed by residues 1–35 and 79–90. This finding is in full agreement with NMR data (Moore, C.D. & Lecomte, J.T.J., 1993, *Biochemistry* 32, 199–207).

Keywords: apocytochrome b_5 ; protein folding; scanning calorimetry; thermodynamics

At the folding of cytochromes, the apoprotein may represent an intermediate. Heme incorporation into the cytochromes b_5 and P450 proceeds several hours after the synthesis of the apoprotein (DuBois & Waterman, 1979; Shawver et al., 1984), and iso-1-cytochrome is supplied with the prosthetic group even after passage of the mitochondrial membrane (Dumont et al., 1991). Generally, apoprotein is less stable than the corresponding holoprotein, as shown in the example of myoglobin and cytochrome P450 (Griko et al., 1988; Pfeil et al., 1993), if not completely unfolded, as cytochrome c (Privalov et al., 1989). On the other hand, apoproteins have little tendency toward crystallization. Therefore, no X-ray structure of those altered protein states is available.

The aim of the present paper is to characterize the conformation of apocytochrome b_5 from rabbit liver by scanning calorimetric investigations and complementary techniques. Of particular interest is whether apocytochrome b_5 has a cooperative structure, and how stable this is compared with the holoprotein. The investigations will be performed on the fragment 1–90. For the tryptic fragment of the holocytochrome b_5 (from bovine liver), detailed structure is available based on X-ray structure

analysis (Mathews et al., 1972), NMR (Moore et al., 1991; Moore & Lecomte, 1993), and calorimetry (Pfeil & Bendzko, 1980).

Results

Apocytochrome b_5 is quite resistant against proteolytic cleavage below 25 °C at neutral pH, as tested by proteolytic enzymes having different specificity (data not shown; enzymes listed in Materials and methods). However, apocytochrome b_5 is less stable than the holoprotein at elevated temperature, as shown by thermolysin digestion in Figure 1.

In scanning calorimetry, apocytochrome b_5 displays a reversible two-state thermal transition at about 49 °C (Fig. 2). Compared with the holoprotein, the melting temperature, the denaturational enthalpy, and the heat capacity change (ΔC_p) are considerably reduced (Table 1). The Gibbs energy change at protein unfolding (ΔG), calculated using Equation 1, amounts to $\Delta G = 7 \pm 1$ kJ/mol at 25 °C and pH 7.4 for apocytochrome unfolding compared with $\Delta G = 25$ kJ/mol for the holoprotein (Pfeil & Bendzko, 1980):

$$\Delta G(T) = \Delta H[(T_{irs} - T)/T_{irs}] - \Delta C_p(T_{irs} - T) + T\Delta C_p \ln(T_{irs}/T). \quad (1)$$

The partial specific heat capacity (C_p) of apocytochrome b_5 amounts to $C_p = 1.57 \pm 0.12$ J/g/K at 20 °C, compared with $C_p = 1.32 \pm 0.09$ J/g/K for the holopro-

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet spectral region; ΔG , Gibbs energy change at protein unfolding; T_{irs} , thermal transition temperature; ΔH , denaturational enthalpy change at T_{irs} ; ΔC_p , heat capacity change at protein unfolding; C_p , partial specific heat capacity.

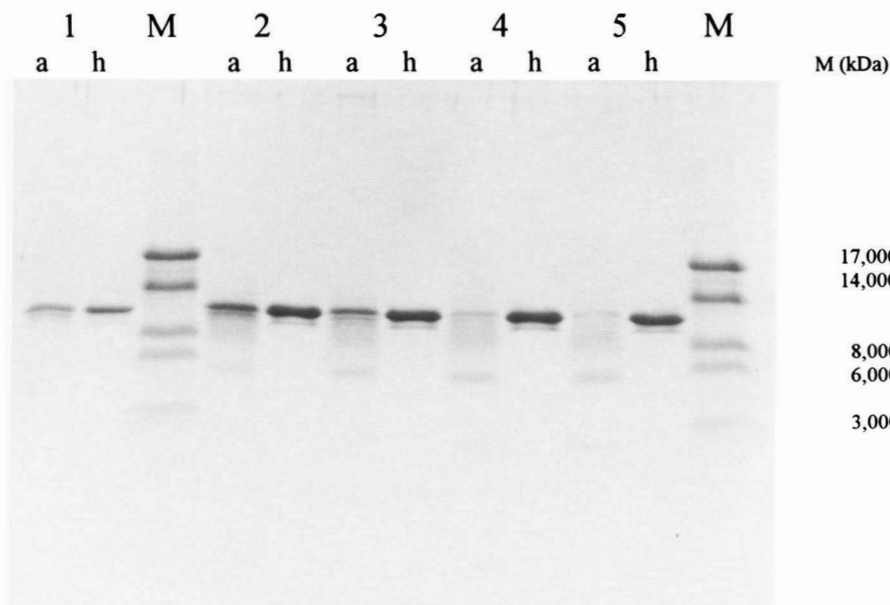


Fig. 1. Limited proteolysis of holo- (h) and apocytochrome b_5 (a) by thermolysin. Lane 1, without thermolysin treatment; lanes 2–5, after thermolysin treatment at 25, 30, 35, and 40 °C, respectively; M, molecular weight standard.

tein. The C_p of unfolded apocytochrome b_5 is expected to be 1.95 J/g/K at 20 °C, based on the increments of the amino acid residues according to Privalov and Makhatadze (1990) (C_p was calculated here without regard to the acetylated N-terminus). Because heat capacity increase originates from exposure of nonpolar groups to the solvent (Privalov & Makhatadze, 1992), apocytochrome b_5 seems to be less densely packed than the holoprotein. Nevertheless, Stokes's radius determined by size exclusion chromatography was found to be indistinguishable ($R = 19 \pm 1$ Å) for both the apo- and the holoprotein.

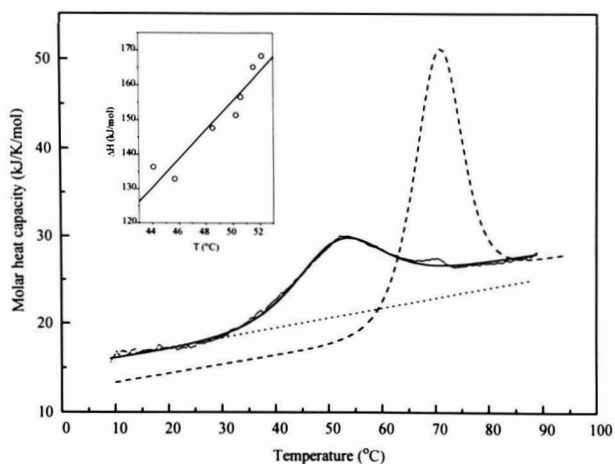


Fig. 2. Scanning calorimetric curves of holo- and apocytochrome b_5 at pH 7.4. Dashed line, holo- and apocytochrome b_5 (data from Pfeil & Bendzko [1980]). Raw data, apocytochrome b_5 . Solid line, two-state fit with $T_{irs} = 48.5 \pm 0.7$ °C, $\Delta H = 148 \pm 5$ kJ/mol, and $\Delta C_p = 4.2 \pm 0.5$ kJ/K/mol (the uncertainties refer to the data treatment of the single calorimetric recording). Dotted line, baseline used in the data treatment. **Inset:** ΔH versus transition temperature T_{irs} at thermal apocytochrome b_5 unfolding in the pH range 6–9.

As originally reported by Huntley and Strittmatter (1972), the circular dichroism spectra of apocytochrome b_5 and the holoprotein are different both in the far-UV and in the near-UV regions (Figs. 3, 4). In apocytochrome b_5 , distinct secondary and tertiary structure content is present. Using CONTIN (Provencher & Glöckner, 1981; Johnson, 1990), 21% α -helix and 44% β -sheet were determined, compared with 38% α -helix and 27% β -sheet for the holoprotein as derived from the X-ray structure analysis (Matthews, F.S. & Durley, R.C.E., 1991, PDB file 3B5C). In contrast to previous findings (Schnellbacher & Lumper, 1971), apocytochrome b_5 also shows a characteristic spectrum in the near-UV region, but less band intensity than the holoprotein.

Further details on apocytochrome b_5 structure can be derived from second derivative spectra. As shown in Figure 5, the second derivative spectra of holo- and apocytochrome b_5 are rather similar in the spectral region of

Table 1. Thermodynamic quantities of holo- and apocytochrome b_5 at pH 7.4

	Holo ^a	Apo ^b	Remarks
T_{irs} (°C)	69.5	48.5 ± 0.5	
ΔH (kJ/mol)	329	149.3 ± 6.7	At T_{irs}
ΔC_p (kJ/K/mol) ^c	6.0	4.2 ± 0.6	
ΔC_p (kJ/K/mol)		4.2 ± 0.5	
ΔG (kJ/mol)	25	7.1 ± 1.1	At 25 °C
C_p (J/K/g)	1.32	1.57 ± 0.12	At 20 °C

^a Data from Pfeil and Bendzko (1980).

^b Mean value and standard deviation from seven calorimetric recordings measured on independently prepared samples.

^c From H versus T_{irs} of single calorimetric recordings measured in the pH range 6–9.

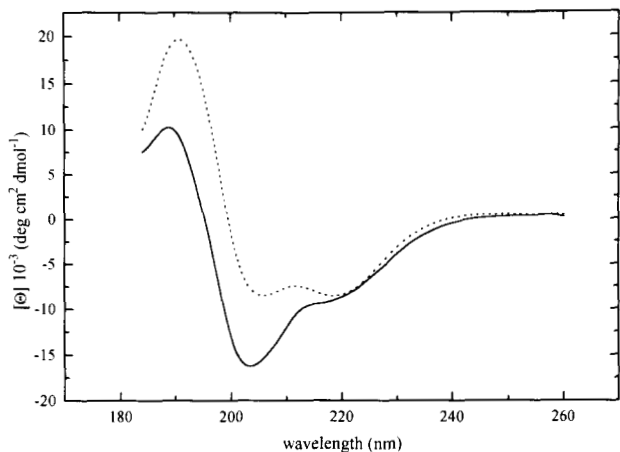


Fig. 3. CD spectrum of apocytochrome b_5 (solid line) and the holoprotein (broken line) in the far-UV region, pH 7.4.

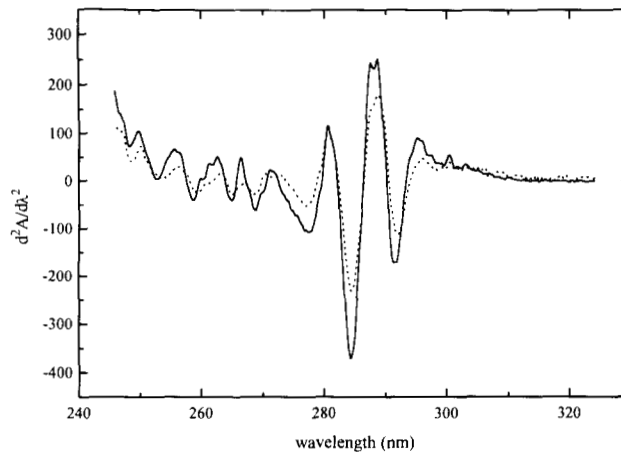


Fig. 5. Second derivative spectrum of apocytochrome b_5 (solid line) and the holoprotein (broken line) in potassium phosphate, pH 7.4.

tryptophan and tyrosine (band positions 291.8, 288.6, 284.4, and 280.5 nm). On the other hand, holo- and apocytochrome b_5 differ in the spectral region of phenylalanine (band positions 266, 262, 255.8, and 249.8 nm). When comparing apocytochrome b_5 at pH 7.4 with the same protein in 6 M guanidine hydrochloride (Fig. 6), the opposite effect can be observed, i.e., high similarity in the phenylalanine region and differences in the spectral region of tyrosine and tryptophan. It can be concluded that tryptophan and tyrosine in apocytochrome b_5 are in a natively-like environment, whereas phenylalanine is in an exposed, unfolded-like situation.

Discussion

Cytochrome b_5 is a membrane protein (Spatz & Strittmatter, 1971). However, most studies on the structure and folding of the protein were performed on the tryptic frag-

ment (residues 1–90), i.e., the protein devoid of its membrane anchor (Kinemage 1).

There is evidence for the presence of apocytochrome b_5 at in vivo folding (Shawver et al., 1984). Furthermore, on the tryptic fragment it was shown by NMR (Moore & Lecomte, 1990, 1993; Moore et al., 1991) that apocytochrome b_5 has a hydrophobic core region. Current interest is focused on protein folding concerns, in particular the stage at which intermediates occur that are able to undergo a cooperative transition. Thus, the question arises, does apocytochrome b_5 show the cooperativity of a folded protein conformation, or does cooperativity occur only after incorporation of the prosthetic group?

Scanning calorimetry can provide evidence for the presence of a cooperative structural transition. As shown in Figure 2 and Table 1, apocytochrome b_5 is less stable than the holoprotein, but is still able to undergo a two-state thermal transition. At the same time, the C_p of

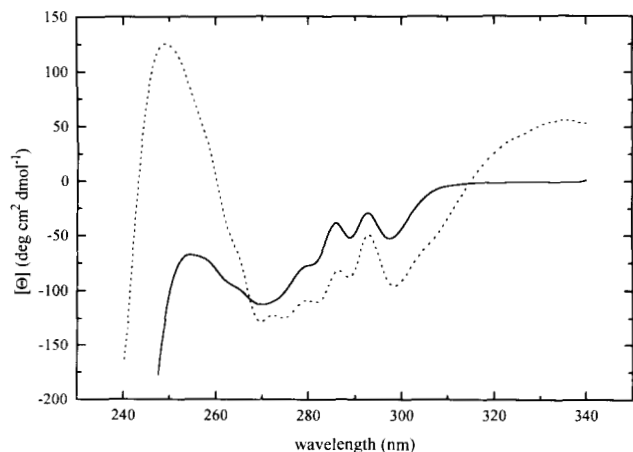


Fig. 4. CD spectrum of apocytochrome b_5 (solid line) and the holoprotein (broken line) in the near-UV region, pH 7.4.

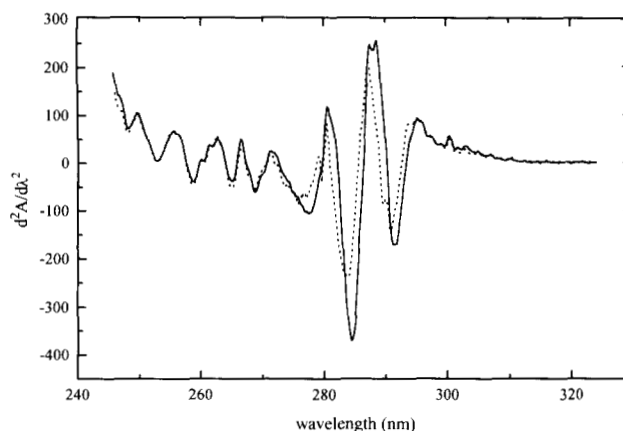


Fig. 6. Second derivative spectrum of apocytochrome b_5 in 50 mM potassium phosphate, pH 7.4 (solid line), and in 6 M guanidine hydrochloride (broken line).

apocytochrome b_5 below the transition is enhanced compared with the holoprotein. This indicates parts of non-polar residues, being buried in the holoprotein, exposed to the solvent in the apoprotein (Kinemage 1). Nevertheless, apocytochrome b_5 possesses a hydrophobic core: the C_p is significantly lower than the value expected for the unfolded protein, and apocytochrome b_5 unfolding is accompanied by a characteristic $\Delta C_p = 4.2 \pm 0.6$ kJ/K/mol. Protein stability expressed by ΔG amounts to 7 ± 1 kJ/mol for apocytochrome b_5 , compared with $\Delta G = 25$ kJ/mol for the holoprotein at neutral pH and 25 °C.

The reduced stability of apocytochrome b_5 compared with the holoprotein is also reflected by the higher susceptibility to thermolysin digestion at an elevated temperature (Fig. 1).

Holocytochrome b_5 has a unique structure (Rossmann & Argos, 1975). Residues 1–35 and 79–90 form a core region consisting of an α -helix and five β -sheets (the amino acids of rabbit liver cytochrome b_5 are numbered here according to the sequence alignment given by Ozols [1989]) (Kinemage 1). This core region serves as the basis for a four-helix bundle that encloses the heme group, which is fixed in the axial position by H 43 and H 67. At the same time, the three tyrosine residues (Y 10, Y 11, Y 34) and the only tryptophan (W 26) are located within the core region, whereas the three phenylalanine residues are within (F 39, F 62) or adjacent to (F 78) the helical parts. As shown by second derivative spectroscopy (Fig. 5), the spectral band positions of tyrosine and tryptophan are almost identical in holo- and apocytochrome b_5 . At the same time, holo- and apocytochrome b_5 are rather different in the spectral region of the phenylalanine residues. On the contrary, apocytochrome b_5 shows the same phenylalanine spectrum in the absence and in the presence of 6 M guanidine hydrochloride, whereas the spectral band position of tryptophan and tyrosine is significantly changed only after the addition of 6 M guanidine hydrochloride (Fig. 6).

Second derivative spectroscopy is rather sensitive to local conformational changes (Pfeil et al., 1993), and unfolding is usually accompanied by a spectral shift toward a shorter wavelength of about 2.5–2.7 nm for tyrosine and tryptophan and 1.5–1.7 nm for phenylalanine (Ruckpaul et al., 1980). Thus, the conformational changes accompanying removal of the prosthetic group from cytochrome b_5 concern mainly the helical part, but not the core region formed by residues 1–36 and 80–90. This result is further supported by the CD spectra of apocytochrome b_5 (Figs. 3, 4). These indicate a loss of α -helix content in apocytochrome b_5 compared with the holoprotein. The tertiary structure, at least in the region of the tyrosine and tryptophan residues in the near-UV region, does not seem to be affected.

The findings presented in this paper are in full agreement with the NMR studies presented by Moore and Lecomte (1990, 1993) and Moore et al. (1991): residues 36–78, forming the four-helix bundle in the holoprotein,

do not seem to contribute to the enthalpy and ΔC_p at thermal unfolding of the apoprotein. These parts of the molecule appear to be uncoupled in the cooperative thermal unfolding transition. Nevertheless, the stability of apocytochrome b_5 is marginal and does not exceed about 3 kT.

Apocytochrome represents an intermediate in vivo folding that exhibits nativelike properties that are distinctly different from those of the molten globule state (Ptitsyn, 1992). Apocytochrome b_5 bears resemblance in this respect to apomyoglobin (Griko et al., 1988; Cocco & Lecomte, 1990), apocytochrome P450 (Pfeil et al., 1993), and the more recent findings made on a folding intermediate of subtilisin BPN' (Eder et al., 1993).

Materials and methods

The tryptic fragment of rabbit cytochrome b_5 was prepared as described by Pfeil and Bendzko (1980) and was characterized as the fragment 1–90 by mass spectrometry. The apoprotein was obtained using the butanone extraction method (Teale, 1959) as described by Pfeil et al. (1993). All measurements were performed on apocytochrome immediately after rechromatography on a 60-cm Sephacryl S-100 HiLoad column (Pharmacia, Sweden) in 50 mM potassium phosphate buffer, pH 7.4. When necessary, buffer change was made using a 1 × 10-cm Sephadex G25 column.

The protein concentration was determined spectrophotometrically at 280 nm using $\epsilon_{280\text{nm}} = 10.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Strittmatter, 1960). The molecular weight of apocytochrome b_5 was taken as $M_r = 10,359$, according to the sequence (Ozols, 1989). The partial specific volume was assumed to be 0.713 mL/g based on the amino acid composition.

Scanning calorimetry was carried out on a Privalov-type microcalorimeter DASM 1M modified for automated data acquisition. Data treatment was made using the DA2 and ORIGIN software packages (MicroCal, Northampton). The baseline treatment was performed by linear extrapolation of the initial slope of the calorimetric recording. The curve fit was carried out using the two-state model with ΔC_p . The protein concentration was in the range of 0.8–2.5 mg/mL, and the heating rate was 1 K/min.

Controlled proteolysis was performed according to Carrey's (1989) protocol. The following enzymes were used without further purification: carboxypeptidase A (EC 3.4.17.1; Miles), TLCK-treated α -chymotrypsin from bovine pancreas (EC 3.4.21.1; Sigma), clostripain from *Clostridium histolyticum* (EC 3.4.22.8; Boehringer), papain from *Papaya carica* (EC 3.4.22.2; Serva), pepsin from porcine stomach (EC 3.4.23.1; Worthington), pronase P from *Streptomyces griseus* (EC 3.4.24.4; Serva), protease from *Staphylococcus aureus* V8 (EC 3.4.21.19; Miles), and TPCK-treated trypsin from bovine pancreas (EC 3.4.21.4; Merck).

Limited proteolysis at elevated temperature was carried out using thermolysin (EC 3.4.24.4; Boehringer, lot 13054120-30). Apo- and holocytochrome b_5 were incubated with thermolysin in a ratio of 250:1 (w/w) in a thermostated vessel for 10 min, and the reaction was stopped with EDTA. SDS-PAGE was conducted in a discontinuous Tris-Tricine system (Schägger & von Jagow, 1987) on 16% gel. The molecular mass was identified relative to the Pharmacia myoglobin fragment standard.

CD spectra were measured on a JASCO 720 spectropolarimeter. Measurements were made in 50 mM sodium phosphate, pH 7.4, at about 0.6 mg/mL and 0.1-mm optical pathlength for the far-UV region, and 1.6 mg/mL and 10-mm optical pathlength in the near-UV region. Secondary structure content was determined using CONTIN (Provencher & Glöckner, 1981; Johnson, 1990).

Second derivative spectra were obtained on a KONTRON 930 UV-VIS spectrophotometer at 0.1 nm resolution and 10 nm/min scan rate. The spectra were concentration normalized on a molar basis, and each three to six spectra from independent preparations were superimposed.

The determination of Stokes radius was performed by size exclusion chromatography on a Sephadex G75 column (1 × 120 cm) in 50 mM potassium phosphate, pH 7.4. The calibration was made using carbonic anhydrase from bovine erythrocytes, myoglobin from horse, cytochrome c from horse heart, trypsin inhibitor from bovine lung, DNP-L- α -alanine (all substances from Serva), and blue dextran (Pharmacia). The Stokes radii of the proteins were taken from Rogers et al. (1965), Ackers (1970), and Corbett and Roche (1984).

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