Apocytochrome $P450_{cam}$ Is a Native Protein with Some Intermediate-like Properties†

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ABSTRACT: Holo- and apocytochrome $P450_{cam}$ were studied by differential scanning calorimetry (DSC), limited proteolysis, second-derivative spectroscopy, circular dichroism, and size-exclusion chromatography. The holoprotein shows three folding units (domains) in DSC. The prosthetic group is related to the most unstable domain, which has a thermal transition at 41.9 °C. Compared with the holoprotein, apocytochrome $P450_{cam}$ has a reduced helix content. The protein is compact as judged by the Stokes radius and is still able to undergo a two-state transition. However, the enthalpy change at thermal melting is reduced from 980 kJ/mol for the holoprotein to 135 kJ/mol for the apo form. Parts of the molecule have a destabilized tertiary structure. This is indicated by second-derivative spectroscopy, circular dichroism in the near-ultraviolet region, and a high susceptibility to proteolytic digestion. Apocytochrome $P450_{cam}$ is considered a native protein with the extremely low stability of ΔG = 7.5 kJ/mol, thus showing at the same time intermediate-like properties. The importance of the properties for *in vivo* folding are discussed.

The P450 cytochromes are a superfamily of monooxygenases found in a wide variety of organisms and tissues from bacteria, plants, animals, and human beings. P450 cytochromes are involved in endogenous metabolic processes such as steroid hormone and bile acid biosynthesis and fatty acid metabolism. The enzymes are also involved in the solubilization, transformation, and detoxification of hydrophobic xenobiotics, drugs, and carcinogens. In view of the broad involvement of the enzymes in biochemical processes, there is much interest in the structure, function and engineering of P450 cytochromes.

Knowledge about the structure of P450 cytochromes is widely deduced from the bacterial camphor hydroxylase $P450_{cam}$.[1] The protein is soluble, in contrast to most membrane-bound P450 cytochromes. $P450_{cam}$ is the only cytochrome P450 enzyme for which the crystallographic structure has been determined with high resolution (Poulos et al., 1987). Therefore, the protein serves as a basis for sequence comparison and structure modeling within the superfamily (Gotoh & Fujii-Kuriyama, 1989; Nelson & Strobel, 1989).

When P450 cytochromes are studied, most attention is paid to induction, substrate specificity, and the interaction with components of the electron-transfer system (Waterman et al., 1986). However, little is known about the folding and stability of the enzyme. In biosynthesis, first the apocytochrome appears. Approximately 30 h later, the heme-containing, spectroscopically detectable, and enzymatically active enzyme occurs (DuBois & Waterman, 1979; Waterman & Estabrook, 1983; Waterman et al., 1986). Therefore, apocytochrome P450 formation is an important step in *in vivo* folding. Surprisingly, the biophysical properties of the cytochrome P450 apoprotein have not yet been studied. Apo-$P450_{cam}$ was originally prepared to enable the exchange of the prosthetic group. It was possible to reconstitute holo-$P450_{cam}$ from heme and apoprotein to a form having full enzymatic activity, original EPR signals, and on original absorption spectrum (Wagner et al., 1981).

Generally, apoproteins are less stable than holoproteins. Thus Gibbs energy change at holomyoglobin unfolding amounts to 34 kJ/mol at pH 5 and 31 °C, compared with 11 kJ/mol for the apoprotein (Griko et al., 1988). Apocytochrome *c* is already completely unfolded and does not show any thermal transition, in contrast to the holo protein (Privalov et al., 1989; Potekhin & Pfeil, 1989). However, knowledge about the stabilizing effect of prosthetic groups is mainly based on small globular proteins. Larger proteins having a molecular mass above 30 kDa consist, as a rule, of domains (Privalov, 1982). A domain structure of bacterial and microsomal cytochrome P450 was proposed in a preliminary scanning calorimetric study (Anzenbacher et al., 1982; Jung et al., 1985). Thus, the question arises whether a single domain of $P450_{cam}$ or the protein as a whole is destabilized upon removal of the prosthetic group.

The present paper reports properties of both holo- and apo-$P450_{cam}$ that characterize the stability on the basis of thermodynamic quantities as well as limited proteolysis.

EXPERIMENTAL PROCEDURES

Materials. Preparation of Holo- and Apocytochrome $P450_{cam}$. Recombinant $P450_{cam}$ was isolated from *Escherichia coli* strain TB1 grown in a 30-L fermentor according to the procedure described in detail by Jung et al. (1992). Protein separation was performed according to Gunsalus and Wagner (1978). The purity and spectra of $P450_{cam}$ were checked as described by Gunsalus and Wagner (1978).

Apo-$P450_{cam}$ was prepared by 2-fold heme extraction with butanone of holo-$P450_{cam}$ in 0.1 M histidine, pH 2.5, at 4 °C, following the protocol of Wagner et al. (1981). The final apo-$P450_{cam}$ solution in 0.1 M histidine, pH 8.0, and 20% (w/w) glycerol was stored as a stock solution. Immediately before use, the stock solution was subjected to chromatography on Sephadex G75 (1.5 × 50 cm) in the buffer of choice.

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[1] Abbreviations: $P450_{cam}$, cytochrome $P450_{cam}$ = CYP101 according to Nebert et al. (1991); DSC, differential scanning microcalorimetry; CD, circular dichroism; UV, ultraviolet spectral region; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLCK, *N*-*p*-tosyl-L-lysinylchloromethane; TPCK, *N*-tosyl-L-phenylalanyl chloromethane; DNP, dinitrophenyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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Limited Proteolysis. Controlled proteolysis was performed according to Carrey's protocol (1989). 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 trysin from bovine pancreas (EC 3.4.21.4) (Merck).

Aliquots of holo- and apo-P450$_{cam}$ were incubated with the enzyme (w/w ratio 50:1 to 1000:1) for 0, 10, 20, 40, and 60 min in thermostated reaction vessels at 25 °C. The reaction was stopped by adding SDS and boiling. With respect to the different staining properties, 0.5-μg holo-P450$_{cam}$ and 2-μg apo-P450$_{cam}$ samples were subjected to standard SDS–PAGE (10% gel). The following marker proteins were used: phosphorylase B, bovine serum albumin, egg albumin, and carbonic anhydrase (Serva).

Differential Scanning Microcalorimetry. Scanning calorimetric measurements were performed on two instruments: the MicroCal MC-2D scanning calorimeter (MicroCal Inc., Northampton, MA) with the DA-2 data acquisition system and the DASM 1M microcalorimeter (SKB Puschino, USSR). The measurements were performed at a scan rate of 53.63 ± 0.34 s/K with the MC-2D and 59.18 ± 0.24 s/K with the DASM-1M calorimeter, using a protein concentration ranging from 1.2 to 4.5 mg/mL.

For the high-precision measurement of protein partial specific heat, the DASM 1M instrument was equipped with an XY recorder and a preamplifier and was interfaced with a PC-AT via the DAS8-PGA analog–digital board (Keithley Metrabyte Co., Taunton, MA). Special software was written in Turbo Pascal for data registration and treatment as well as for file transfer to the DA-2 software package (MicroCal Inc., Northampton, MA). For the calculation of the temperature dependence of the partial specific heat capacity of the protein, $C_{p,prot}$, the expression given by Privalov and Pothekin (1986) and Makhatadze and Privalov (1990) was used:

$$C_{p,prot} = C_{p,w}V^{\circ}_{prot}/V_w - M_{prot}\Delta C_{p,prot}/m_{prot} \quad (1)$$

where C_p and V represent partial specific heat capacity and partial specific volume of protein (subscript prot) and water (subscript w), respectively; m_{prot}, the mass of the protein; M_{prot}, the molar mass; and $\Delta C_{p,prot}$, the apparent heat capacity difference between the sample and the buffer. In the present paper, the partial specific volume of the protein was taken as being temperature independent. The temperature function $C_{p,w}/V_w$ was approximated by a polynomial expression. The partial specific heat capacity of unfolded protein was calculated according to Makhatadze and Privalov (1990). Polynomial expressions were derived from the tabulated values of the heat capacity increments of the amino acid residues reported (Makhatadze and Privalov, 1990).

All the solutions required for the sample preparation were carefully degassed. Proteins were used immediately after rechromatography on Sephadex G75 (1.5 × 50 cm). For low pH values and high salt content, the samples were equilibrated with the buffer system by dialysis.

Circular Dichroism. Circular dichroism measurements were carried out on a Jasco J600 spectropolarimeter calibrated with 1.104 mg/mL D-10-camphorsulfonic acid at an optical path length of 0.5 mm. Apo-P450$_{cam}$ and (substrate free) holo-P450$_{cam}$ were measured in a 10 mM sodium phosphate buffer, pH 8.0, at 22 °C. Over the near-ultraviolet range, the spectra were recorded at protein concentrations of 10–20 μM using a 10-mm path length. For the far-ultraviolet region, a path length of 1 mm was used, and the protein concentration was in the range of 1.0–3.1 μM.

Molar ellipticities [Θ] (deg cm^2 dmol^{-1}) were calculated using $[\Theta]_\lambda = 3300^*\Delta\epsilon$. The secondary structure content was determined according to the approaches of Provencher and Gloeckner (1981), Compton and Johnson (1986), and Yang et al. (1986).

Derivative Spectra. Second-derivative spectra, i.e., $d^2A/(d\lambda)^2$, were recorded on a UV 2101 PC double-beam UV–vis scanning spectrophotometer (Shimadzu, Japan). The standard instrument settings were 100 nm/min with a 0.5-nm sampling interval, slit width 0.5 nm, and $\Delta\lambda = 2.0$. Spectra were recorded at 25 °C with a 1-cm optical path length. The protein concentration was in the range of 6–14 μM.

Determination of Stokes Radius by Size-Exclusion Chromatography. Size-exclusion chromatography was performed on a Sephacryl S 300 superfine column (0.9 × 100 cm) at 4 °C using 20 mM HEPES buffer, pH 8.0. Calibration was performed with ferritin from horse spleen, catalase from bovine liver, aldolase from rabbit muscle, bovine serum albumin, albumin from egg, carbonic anhydrase from bovine erythrocytes, DNP-L-α-alanine (all from Serva), and blue dextran (Pharmacia). Retention volumes were normalized by the expression $K_{av} = (v_\infty - v_o)/(v_e - v_o)$, where v_o is the dead volume as determined by blue dextran; v_e, the elution volume of the corresponding protein; and v_∞, the elution volume of DNP-L-α-alanine.

The Stokes radii of the calibration proteins were taken from Rogers et al. (1965), Ackers (1970), and Corbett and Roche (1984) and calculated from the diffusion coefficient (Andrews, 1965).

Coefficients. The concentration of apo-P450$_{cam}$ was determined according to Wagner et al. (1981), using an extinction coefficient of $E^{280} = 48$ mM^{-1} cm^{-1}. Holo-P450$_{cam}$ concentration was determined by using $E^{417} = 115$ mM^{-1} cm^{-1} (oxidized form; Ullah et al., 1990). The molecular weight of apo-P450$_{cam}$ was taken as $M = 46\,202$, based on the analytical data from Ullah et al. (1990) and the molecular weight of protoporphyrin IX. The partial specific volume of apo-P450$_{cam}$ was calculated on the basis of the amino acid composition (Poulos et al., 1987) as $V_{spez} = 0.724$ mL g^{-1}.

RESULTS

Spectral and Electrophoretic Properties of Apocytochrome P450$_{cam}$. Apo-P450$_{cam}$ was prepared by butanone extraction of the prosthetic group from the holoprotein according to Wagner et al. (1981) with high purity. The absorption spectrum (not shown) does not exhibit the characteristic Soret band at 417 nm except traces which do not exceed 1% of the holoprotein in the sample. The apo-P450$_{cam}$ used in this paper forms almost exclusively one band in SDS–polyacrylamide electrophoresis (see Limited Proteolysis below).

Solubility and Stokes Radius of Apocytochrome P450. Apo-P450$_{cam}$ has limited solubility in conventional buffers except the histidine–glycerol system originally reported by Wagner et al. (1981). Solutions containing about 2 mg/mL of the protein as required for high-precision scanning calorimetry can be prepared in 10 mM potassium phosphate or 20 mM HEPES at pH 7–8. However, on storage of the protein or on addition of 0.1 M KCl, the solution becomes opalescent,

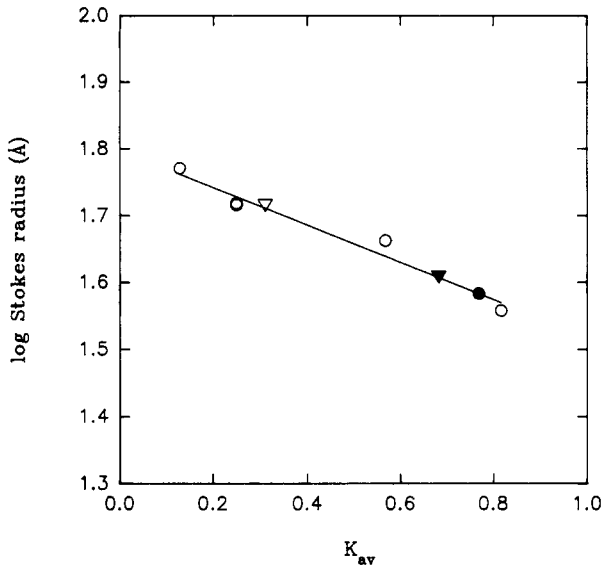

FIGURE 1: Stokes radius *versus* K_{av} determined by size-exclusion chromatography: ● = holo-P450$_{cam}$, R_S = 38.3 Å; ▼ = apo-P450$_{cam}$, R_S = 40.6 Å; ▽ = aggregates of the apoprotein; ○ = calibration proteins (see Materials and Methods).

thus indicating the formation of soluble aggregates. Therefore, all investigations reported below were made on apo-P450$_{cam}$ immediately after rechromatography of the histidine–glycerol-containing stock solution on Sephadex G 75 at 4 °C using the final buffer system.

Compared with the holoprotein, apo-P450$_{cam}$ is slightly expanded as judged by the Stokes radius determined by size-exclusion chromatography (Figure 1). The stokes radius of apo-P450$_{cam}$ amounts to 41 ± 1 Å compared with 38 ± 1 Å for the holoprotein. The observed increase of the Stokes radius of the apoprotein of approximately 6% compared with the holo form is on the order of magnitude usually reported for proteins being in the molten globule state (Ptitsyn, 1987; Kuwajima, 1989; Christensen & Pain, 1991). When apo-P450$_{cam}$ is stored at 4 °C over several days, aggregates form. In size-exclusion chromatography, a new peak becomes visible. The peak position in the chromatogram corresponds to a molecular mass of 250 ± 30 kDa probably due to hexamers of apo-P450$_{cam}$.

Circular Dichroism. Circular dichroism spectra of holo- and apo-P450$_{cam}$ (Figures 2 and 3) show marked differences. In the far-ultraviolet region apo-P450$_{cam}$ has less pronounced molar ellipticity than the holo form, thus indicating a loss of secondary structure on removal of the heme group from cytochrome P450. Analysis of the far-ultraviolet CD spectrum according to the approaches of Provencher and Gloeckner (1981), Compton and Johnson (1986), and Yang et al. (1986) gives values ranging from 33 to 38% for the helix content and from 33 to 37% for the antiparallel β-sheet and turns. According to the X-ray structure analysis, the helix content (α and $3_{10helix}$) of holo-P450$_{cam}$ amounts to 53.3%, and the content of residues involved in antiparallel β-sheet and turns is 25.4% (Poulos et al., 1987).

The near-ultraviolet CD spectrum of holo-P450$_{cam}$ is characterized by a positive Cotton effect which is absent in the apoprotein. The CD spectrum of the apoprotein resembles the molten globule state (Ptitsyn, 1987; Kuwajima, 1989; Christensen & Pain, 1991), showing that the optically active arrangement of the aromatic side chains may be disrupted owing to their exposure to the solvent.

Second-Derivative Spectroscopy. Second-derivative spectroscopy is a sensitive tool for studying the environmental

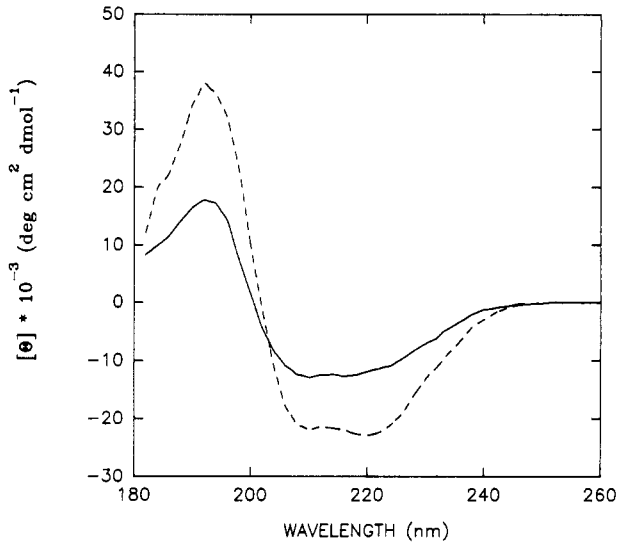

FIGURE 2: Circular dichroism spectra of apo-P450$_{cam}$ (solid line) and the holoprotein (broken line) in the far-UV region in 10 mM potassium phosphate, pH 8.0, at 22 °C, measured at both 1 and 3 μM protein concentration; optical path length, 1 mm.

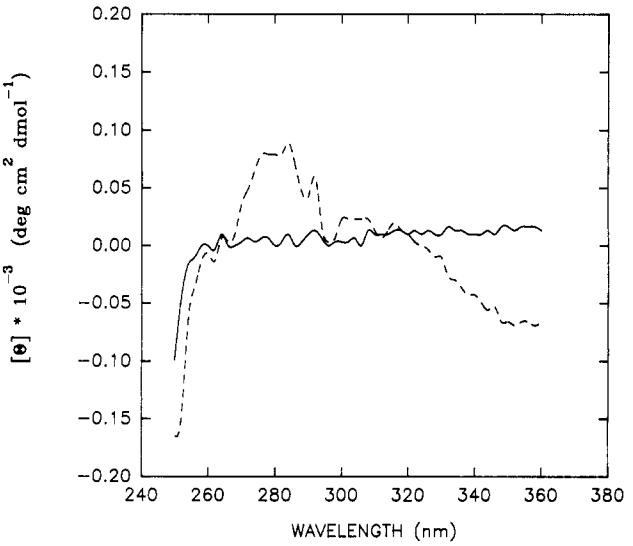

FIGURE 3: Circular dichroism spectra of apo-P450$_{cam}$ (solid line) and the holoprotein (broken line) in the near-UV region in 10 mM potassium phosphate, pH 8.0, at 22 °C Concentration, 11 μM; optical path length, 10 mm.

effects on aromatic amino acid residues. The method was successfully applied to cytochrome P450 (Ruckpaul et al., 1980). Comparing second-derivative spectra of (microsomal) cytochrome P450 with a mixture of the corresponding amino acids, the band positions of the amino acid mixture were found to be shifted to shorter wavelengths (blue shift). Similarly, the positions of minima of the second-derivative spectrum of apo-P450$_{cam}$ show a slight blue shift compared with the minima of the holoprotein (Figure 4; see, e.g., the minima at 260 nm). More remarkable, however, is the presence of double maxima (e.g., at 275–280 nm and at 296–312 nm). This finding is consistent with the presence of amino acids within a different environment. This could be interpreted as part of the aromatic amino acid residues being buried as in a folded structure and another part being exposed to the solvent as in an unfolded state.

Limited Proteolysis. Holo-P450$_{cam}$ is more resistant to proteolytic digestion in vitro than the apo form. This holds true for all the tested proteolytic enzymes: carboxypeptidase A, TLCK–chymotrypsin, clostripain, papain, pepsin, pronase,

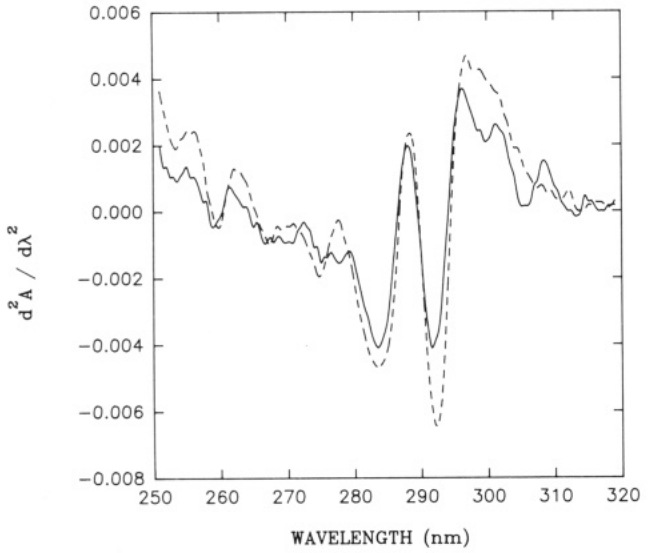

FIGURE 4: Second-derivative spectra of holo- and apo-P450$_{cam}$: solid line, apo-P450$_{cam}$ in 10 mM potassium phosphate buffer, pH 7; broken line, holo-P450$_{cam}$ in the presence of the same buffer, saturated with camphor (≈40 μM). The spectra are concentration-normalized.

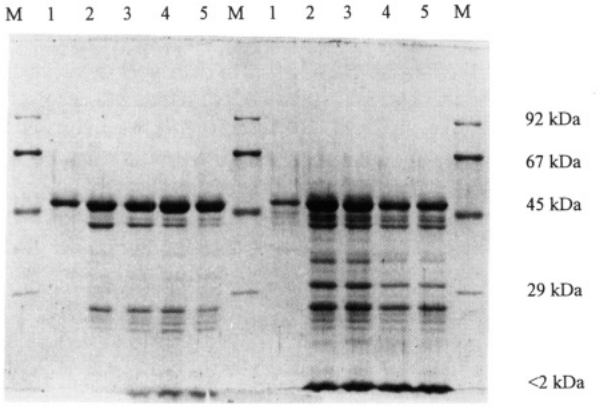

FIGURE 5: Electrophoretic separation of proteolytic fragments produced from holo-P450$_{cam}$ (lanes 1–5) and apo-P450$_{cam}$ (right-handed lanes 1–5) under the influence of TPCK-treated trypsin (w/w protein: trypsin ratio = 500:1). Incubation times were 0, 10, 20, 40, and 60 min. M = molecular weight standards (for more details, see Materials and Methods).

protease V8, and TPCK–trypsin (for details, see Materials and Methods). Electrophoresis patterns obtained by papain and trypsin treatment of the holo- and the apoprotein are shown in Figures 5 and 6 as representative examples. Some fragments are formed from holo-P450$_{cam}$. However, the main part of the protein remains uncleaved. More intense bands of fragments are produced from apo-P450$_{cam}$, and the original band of the protein disappears subsequently. At the same time, small fragments running within the bromophenol front are produced.

There are not only quantitative differences in the intensity of the electrophoretic pattern from apo- and holo-P450$_{cam}$ proteolytic cleavage. Additional bands corresponding to larger fragments of about 30–40-kDa size occur, e.g., on trypsin digestion of the apo form. Similarly, further bands are detectable on the apo form when proteolytic enzymes with different cleaving sites are used, i.e., chymotrypsin, which is preferably specific for aromatic residues, clostripain, which is specific for arginine, and protease, which is V8 specific for glutamate and aspartate (data not shown). Thus, it can be concluded that parts of the polypeptide chain of apo-P450$_{cam}$ which are buried in the holoprotein are exposed.

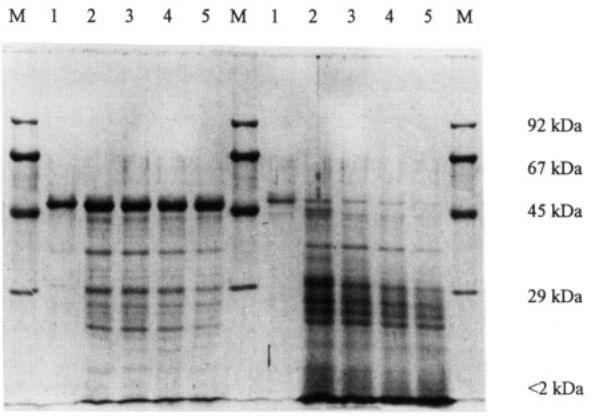

FIGURE 6: Electrophoretic separation of proteolytic fragments produced from holo-P450$_{cam}$ (left-hand lanes 1–5) and apo-P450$_{cam}$ (right-hand lanes 1–5) under the influence of papain (w/w protein: papain ratio = 200:1). Incubation times were 0, 10, 20, 40, and 60 min. M = molecular weight standards (for more details, see Materials and Methods).

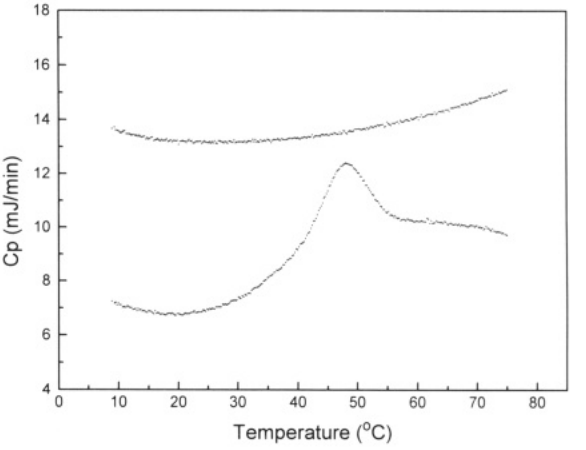

FIGURE 7: Scanning calorimetric recording of holo-P450$_{cam}$ along with the baseline (1 cal = 4.184 J). Buffer used: 20 mM HEPES, pH 8.0, and 1 mM KCN. Protein concn: 1.82 mg/mL.

Differential Scanning Microcalorimetry. The thermal unfolding of (holo) P450 cytochromes so far reported is nonreversible (Anzenbacher et al., 1982; Jung et al., 1985). Calorimetric investigations are rendered more difficult due to the occurrence of an exothermic heat effect within the transition region which makes any determination of calorimetric values hazardous (data not shown). The heat effect seems to be related to the axial ligand position of heme, since it can be suppressed by cyanide. Reproducible scanning calorimetric investigations of the holoprotein can be performed on cyanide complexation of the free ligand positions of heme. However, the thermal transition is not fully reversible, as judged by reheating. With heat denaturation of the cyanide complex, part of the protein forms soluble, heme-containing associates ($M_r \approx 300$ kDa, monitored by size-exclusion chromatography) that show in the absorption spectrum a slightly reduced Soret band. Heat denaturation in the absence of cyanide leads to more drastic aggregation and complete loss of the characteristic absorption spectrum.

A scanning calorimetric recording of camphor-free holo-P450$_{cam}$ at pH 8 in the presence of 1 mM KCN is shown in Figure 7. The holoprotein undergoes thermal denaturation with a significant melting peak at about 48 °C. The cooperative ratio, CR = $\Delta H^{cal}/\Delta H^{vH}$, amounts to 3.8, thus indicating a multistate transition. The melting peak can be resolved by deconvolution into three subtransitions, as shown

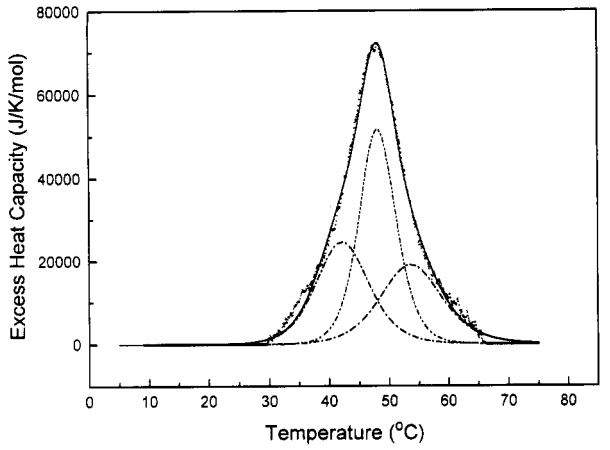

FIGURE 8: Deconvolution of the excess heat capacity function of holo-P450$_{cam}$ in the presence of KCN (data from Figure 7; baseline treatment using cubic splines). The results of deconvolution are T_{trs1} = 42.3° and ΔH_1 = 285 kJ/mol, T_{trs2} = 48.1° and ΔH_2 = 421 kJ/mol, and T_{trs3} = 53.7 °C and ΔH_3 = 260 kJ/mol.

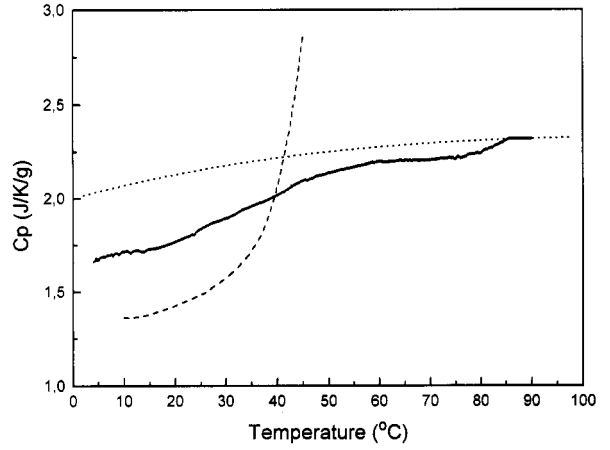

FIGURE 9: Partial specific heat of apo-P450$_{cam}$ at pH 8.0 (raw data) and holo cytochrome P450 at pH 8.0 (dashed line) compared with the heat capacity function of unfolded apo-P450$_{cam}$ (dotted line).

in Figure 8. As the mean value of six independent measurements, it follows T_{trs1} = 41.9 ± 1.0 °C and ΔH_1 = 296 ± 15 kJ/mol, T_{trs2} = 47.8 ± 0.7 °C and ΔH_2 = 405 ± 38 kJ/mol, and T_{trs3} = 54.3 ± 0.6 °C and ΔH_3 = 279 ± 34 kJ/mol.

The heat capacity profile of apo-P450$_{cam}$ is quite different from that of the holoprotein. As shown in Figure 9, the partial specific heat capacity of apo-P450$_{cam}$ is enhanced compared with the holo form. However, the partial specific heat capacity of apo-P450$_{cam}$ below 25 °C does not reach the value expected for the completely unfolded protein according to Makhatadze and Privalov (1990). As judged by the partial specific heat capacity values, apo-P450$_{cam}$ can be assumed to be partly unfolded. On the other hand, the marginal transition of the protein can be approximated (with some uncertainty due to the baseline problem) as a two-state transition with a heat capacity change (Figure 10, main panel), thus giving T_{trs} = 50 ± 2 °C, ΔH = 135 ± 10) kJ/mol, and ΔC_p = (3.2 ± 0.5) kJ/K/mol (mean of five measurements). Neglecting that the transition is not fully reversible, this corresponds to a ΔG of about 7.5 ± 1.0 kJ/mol at 25 °C:

$$\Delta G(T) = \Delta H[(T_{trs} - T)/T_{trs}] - \Delta C_p(T_{trs} - T) + T\delta C_p \ln(T/T_{trs}) \quad (2)$$

When the buffer was changed to 0.1 M histidine, pH 8.0, with 20% (v/v) glycerol, as originally recommended by Wagner

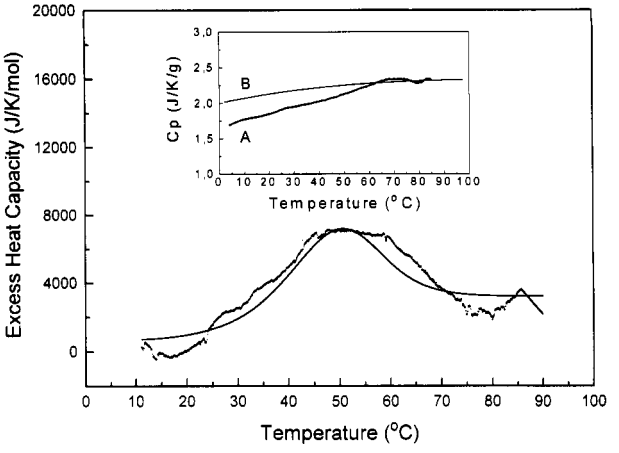

FIGURE 10: Two-state fit of the molar heat capacity function of apo-P450$_{cam}$ at pH 8.0 (raw data from Figure 9; baseline treatment by linear extrapolation). Inset: Partial specific heat capacity of apo-P450$_{cam}$ in 20 mM glycine at pH 2.5 (A) compared with the heat capacity of the unfolded protein (B).

et al. (1981), both the heat capacity function and the thermodynamic quantities were found to be unchanged (data not shown). The two-state approximation used in data treatment was chosen as the simplest possible model. Approximation by two independent transitions (deconvolution gives T_{trs1} = 47 °C and T_{trs2} = 55 °C) is also possible. However, it seems not to be justified. No significant reduction in the standard deviation of the curve fit can be obtained despite the use of more parameters in the procedure.

At acid pH no thermal transition of apo-P450$_{cam}$ is visible. At pH 2.5 the partial specific heat increases monotonously, approaching the value of the unfolded protein at about 60 °C (Figure 10, inset).

DISCUSSION

As a rule, proteins having a molecular mass above 30 kDa are composed of two or more cooperative units (domains; Privalov, 1982). On various P450 cytochromes from bacteria and higher animals a nonreversible multistate thermal transition was reported, consistent with the existence of a domain structure of the protein (Anzenbacher et al., 1982; Jung et al., 1985). Difficulties in quantitative analysis of the melting profiles could be avoided in the present paper by complexation of the heme iron. Probably the axial ligand binding positions of the heme iron become accessible during unfolding and may cause misfolding. Protection of these positions by cyanide, which is one of the strongest ligands, enables a precise determination of the excess heat capacity functions of the holoprotein suitable for the determination of thermodynamic quantities. According to deconvolution of the excess heat capacity of the protein (Biltonen & Freire, 1978; Freire & Biltonen 1978a,b; Privalov & Potekhin, 1986), holo-P450$_{cam}$ consists of three folding units. The overall transition of the holoprotein could be resolved into three two-state transitions at 41.9, 47.8, and 54.3 °C, respectively.

The thermal transition of cytochrome P450$_{cam}$ is accompanied by a substantial decrease in the helix content (Nölting et al., 1992). At the same time, half-conversion at thermal denaturation of the substrate-free holoprotein was found at about 42 ± 1 °C by means of circular dichroism in the far-UV and Soret regions [see Table 2 in Nölting et al. (1992)]. The half-transition temperature coincides with the value for the lowest subtransition obtained by deconvolution (41.9 °C) on the cyanide complex. It can be concluded that the prosthetic

group is located within the most thermolabile domain of holo-$P450_{cam}$.

The prosthetic group of cytochrome $P450_{cam}$ is located in a central position of the macromolecule (Poulos et al., 1987). The heme group is involved in a hydrogen bond network with helix K and aromatic side chain interactions such as with Phe350 (Yoshikawa et al., 1992). Thus, it is not surprising that substantial conformational changes occur on removal of the prosthetic group. In fact, as follows from the CD spectra, the helix content of the apo protein amounts to 33–38% compared with about 53% for the holoprotein (Poulos et al., 1987). The Stokes radius of apo-$P450_{cam}$ is only slightly (about 6%) increased compared with the holoprotein. At the same time, apo-$P450_{cam}$ is much more susceptible to protease digestion than the holoprotein. Recently, an exposed trypsin-sensitive loop region for holo-$P450_{cam}$ leading to 20- and 26-kDa fragments was described (Tsokos et al., 1992). Apo-$P450_{cam}$ can be cleaved by different proteolytic enzymes varying in specificity toward acidic, basic, and aromatic residues. Here, a wide spectrum of fragments, in particular, those having a molecular mass larger than 30 kDa, can be observed. Therefore, parts of the molecule which are buried in holo-$P450_{cam}$, including hydrophobic regions, seem to be accessible for proteolytic cleavage in the apoprotein. This finding is further supported by the optical investigations. Second-derivative spectra of apo-$P450_{cam}$ may be interpreted as resulting from the presence of aromatic residues in both a folded and an unfolded environment. Circular dichroism in the near-UV region does not show significant tertiary interaction.

In agreement with the former conclusion, the partial specific heat capacity values given in Figure 9 also indicate exposure of nonpolar residues. Thus, holo-$P450_{cam}$ has a partial specific heat capacity of $C_p = 1.36 \pm 0.06$ J/K/g, which corresponds to values commonly found for compact globular proteins (Privalov & Makhatadze, 1992). C_p of apo-$P450_{cam}$ amounts to $C_p = 1.71 \pm 0.06$ J/K/g at pH 8.0 and $C_p = 1.78 \pm 0.08$ J/K/g at pH 2.5, less than the $C_p = 2.07$ J/K/g expected for the fully solvated polypeptide chain of the protein calculated according to Makhatadze and Privalov (1990). The C_p values refer to 10 °C with respect to the extended transition region of apo-$P450_{cam}$ (the protein achieves maximal stability at about 8 °C).

On the other hand, apo-$P450_{cam}$ is still able to undergo a cooperative thermal transition which is accompanied by a heat capacity increase. However, the enthalpy change is reduced to about 135 kJ/mol compared with about 980 kJ/mol for the holoprotein, i.e., even less than the transitional heat of the weakest domain of the latter. The overall stability amounts to $\Delta G = 7.5 \pm 1.0$ kJ/mol, i.e., much less than the 25–60 kJ/mol usually found for globular proteins (Pfeil, 1986). In this respect, apo-$P450_{cam}$ can be considered as a protein with rather marginal stability.

In the case of cytochrome *c*, removal of the prosthetic group leads to complete unfolding (Privalov et al., 1989; Potekhin & Pfeil, 1989). On the other hand, apomyoglobin remains in a compact state, able to undergo a cooperative melting transition. The existence of hydrophobic cores in apomyoglobin was shown by both calorimetry (Griko et al., 1988) and NMR (Cocco & Lecomte, 1990). The properties of apo-P450 bear resemblance to apomyoglobin. Apo-P450 is characterized by the existence of a secondary structure, compactedness, a heat capacity lower than for the unfolded protein, and the ability to undergo a cooperative thermal transition accompanied by an increase in heat capacity (Griko et al., 1988; Haynie & Freire, 1993). Finally, the protein can be reconstituted with heme to give a fully active enzyme (Wagner et al., 1981). Therefore, similarly to what was clearly stated by Privalov and co-workers for apomyoglobin (Griko et al., 1988), apo-P450 can be considered as a "native" protein.

The extraction of the prosthetic group from cytochrome $P450_{cam}$ is accompanied by the loss of the characteristic domain structure. At the present stage, the remaining core cannot be assigned to distinct regions of the three-dimensional structure of the holoprotein (Poulos et al., 1987). On the other hand, parts of apo-$P450_{cam}$ seem to be highly mobile, as shown by the accessibility to proteolytic cleavage, by the absence of the CD spectrum in the near-UV region, and by the high partial heat capacity. These properties may be a prerequisite for the uptake of the prosthetic group under *in vivo* conditions. In this respect, apo-$P450_{cam}$ can be considered as intermediate-like (Kim & Baldwin, 1990), even if the properties are only a consequence of the marginal stability of apo-P450, which amounts to about $3kT$. Therefore, the term "molten globule" will be avoided here. The term refers to a compact protein state with intact secondary structure which is at the same time liquidlike and fluctuating (Dolgikh et al., 1981; Ptitsyn, 1987, 1992; Kuwajima, 1989). The underlying phase transition concept (Shaknovich & Finkelstein, 1989; Finkelstein & Shaknovich, 1989) is not applicable to apo-$P450_{cam}$ in the neutral pH region, since it assumes (i) the presence of a homogeneous phase and (ii) the absence of any further first-order thermal transition (Haynie & Freire, 1993). However, at acid pH the heat capacity function seems to be consistent with the smooth molten globule–coil transition (Ptitsyn, 1992).

In summary, apo-$P450_{cam}$ is a compact "native" protein. Due to its extremely low stability, apo-$P450_{cam}$ has both native-like and intermediate-like properties and does not show the domain structure of the holoprotein. Apo-$P450_{cam}$ occurs much earlier in *in vivo* folding than the holoprotein (DuBois & Waterman, 1979; Waterman & Estabrook, 1983; Waterman et al., 1986). It is protease-sensitive and has a tendency toward aggregation. Having in mind that apo-P450 is synthesized *in vivo* about 30 h before the holoprotein occurs, the question arises how the highly degradable and aggregation-sensitive protein resists in the living cell. Possibly, the protein needs protection by chaperones (Ellis & van der Vies, 1991; Wickner et al., 1991; Gething & Sambrook, 1992). Since denaturant or extreme pH is not required in studying apo-$P450_{cam}$, the protein could be of particular interest in folding studies.

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