Study of thermal stability of cytochrome P450 by differential scanning calorimetry

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Thermal denaturation of cytochrome P450 is shown to be a complex process which occurs in two steps. The first (about 50°C) takes place in several stages which can be attributed to denaturation of different regions in the cytochrome P450 with different stability. The second transition (about 90°C) is fully reversible and similar to those described for other hemoproteins.

Cytochrome P450 *Protein denaturation* *Differential scanning calorimetry* *Thermal stability* *Protein structure*

1. INTRODUCTION

It is generally accepted that the uniqueness of enzyme function is intimately connected with their conformation and stability. Differential scanning calorimetry (DSC) has proved its value as a tool in studying conformational transitions of biopolymers in many cases [1,2]. DSC very specifically records the heat capacity changes of a biopolymer solution as a function of temperature. Analysis of DSC thermograms leads to estimates of thermodynamic parameters of a thermal transition and to further information concerning the mechanism of protein denaturation [1–5].

Cytochrome P450 (P450) is a hemoprotein acting as a terminal oxidase in monooxygenation of a variety of non-polar endogenous and exogenous substrates, such as steroids or drugs [6,7]. Under the influence of different factors (e.g., heating, high concentration of a salt or an organic solvent) it denatures easily into its inactive form, cytochrome P420.

The DSC technique and the measurement of absorbance changes at 417 nm (Soret maximum of P450) offer a more detailed insight into the denaturation mechanism of P450.

2. MATERIALS AND METHODS

P450 was isolated from liver microsomes of phenobarbital-treated rats as in [8,9]. It was 10–20 μM, with a specific content of P450 up to 16.9 μmol/g protein. P450 preparations were placed in buffers as follows: 0.01 M potassium phosphate, 0.1 mM EDTA, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% Renex 690 (pH 7.4) [8] and 0.01 M Tris–acetate, 1 mM EDTA, 20% (v/v) glycerol (pH 7.4) [9].

DSC measurements were performed with a DASM-1M instrument [1] (USSR Academy of Sciences); the heating rate was 2 K/min.

Absorbance changes were followed with a Specord UV VIS spectrophotometer (Carl Zeiss, Jena) equipped with thermostated cell holders in a Perspex-made cell compartment with an aluminium foil. Both the sample and the reference cell were filled with the same sample of P450; the sample cell was then heated at a rate of 0.5 K/min up to 75°C in a thermostated water bath. The temperature was measured with ~0.1 K precision in both DSC and spectrophotometry.

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3. RESULTS AND DISCUSSION

A typical calorimetric curve of a P450 sample in buffer at pH 7.4 [8] is shown in fig. 1A. The course of the experimental curve indicates a multi-stage character of thermal denaturation. The two main regions where conformational transitions occurred were at about 50 and 95°C. The first region was apparently composed of several transitions at 40, 49.5, 54 and 60°C. This multi-stage character of the P450 denaturation was independent on the presence or absence of detergents (for buffer composition, see section 2).

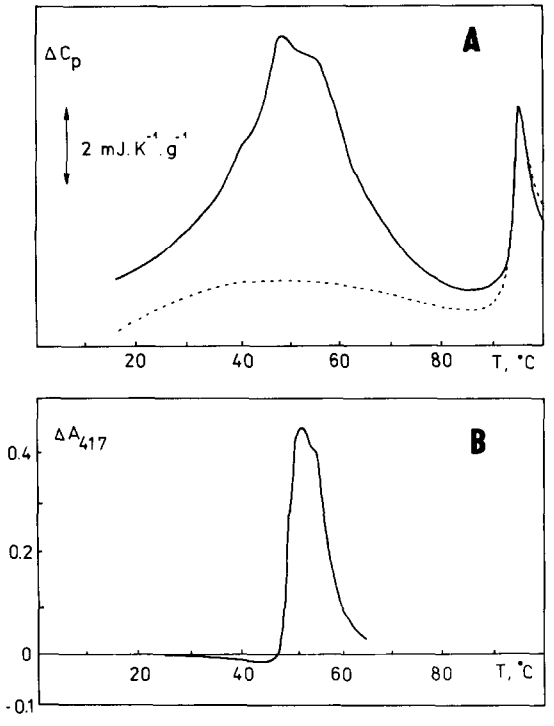

Fig. 1. Thermal denaturation of purified rat liver microsomal P450: (A) DSC thermal denaturation endotherms of heated solution of P450. 10 μM P450 in buffer with glycerol and detergent. Reference cell was filled with the same buffer without P450. (——) native P450; (---) P420 (second run, after thermal denaturation of P450); (B) Absorbance changes at 417 nm induced by heating 5 μM P450 in buffer with glycerol and detergent. Reference cell was filled with the same sample of P450 but kept at 15°C.

Absorbance changes at 417 nm (A_{417}) are shown in fig. 1B. There was an initial decrease in absorption caused by thermal perturbation of the heme chromophore [10] and by interconversion of the low-spin form of ferric P450 with a Soret maximum at 417 nm to a high-spin form with Soret absorption at shorter wavelengths [11]. This was followed by a sharp increase in A_{417} with the midpoint at 49.5°C, corresponding to the maximum on the calorimetric curve. Such a coincidence is valid for a simple two-state process [1]. However, from the shape of the transition curve it is evident that the process is more complex. With increasing temperature, several maxima can be distinguished both on the calorimetric and the optical curves. This implies that the transition at 49.5°C is not simple but that several concomitant processes take place.

The course of both experimental curves can be interpreted as a denaturation of different regions of P450 with different stabilities [1,2]. These effects are not common for globular proteins and were described for more complicated structures composed of various domains; e.g., Bence-Jones protein [12] or meromyosin [1].

The T_m-values (temperatures of transition) found here are very close to the temperatures where thermal inactivation of rabbit P450-mediated hydroxylations, as in [13]. These authors reported that inactivation takes place between 40 and 58°C. Transition curves of rabbit P450 based on temperature-induced conformational changes studied by circular dichroism also indicate T_m-values of about 45°C for soluble P450 [14].

The reversibility of P450 denaturation was tested by repeating the calorimetric experiment with the same P450 samples which were left to cool, and a second curve was then registered (fig. 1A, ---). The thermal denaturation was completely reversible for the second transition (about 90°C) but the first was irreversible. To compare various modes of P450 inactivation, the P450 sample was converted to the inactive form, P420, by adding solid KSCN up to 1 M. This treatment is known to lead to complete formation of an inactive P420 [15]. The calorimetric behavior of P420 was essentially the same as of the thermally denatured P450. The second (reversible) transition was shifted up by 10°C due to the stabilizing effect of the inorganic salt present in the solution.

Table 1

Transition temperatures T_m and effective enthalpies of reversible transition ΔH^{eff} for the thermal denaturation of purified rat liver microsomal P450

System	T_m (°C)	ΔH^{eff} (kJ/mol)
P450, buffer with glycerol [9]	82.6	1050.0
P450, buffer with glycerol and detergent [8]	95.9	1130.2
P420, buffer with glycerol and detergent [8]	95.9	821.8
P420, buffer with glycerol, detergent and 1 M KSCN	106.0	795.0

The reversibility of the second transition permitted us to calculate the effective (van 't Hoff) enthalpy of this process using the approximate formula [16,17] $\Delta H^{eff} = 4RT_m^2/\Delta T_{1/2}$, where $\Delta T_{1/2}$ is the half-width of the transition peak. The calculated values of ΔH^{eff} together with the corresponding T_m are presented in table 1. The ΔH^{eff}-values are in the same range as those found for other hemoproteins, e.g., hemoglobin with its derivatives (0.8–1.6 MJ/mol) [18].

In conclusion, the thermal denaturation of P450 was shown to be a complex process which occurred in two stages. The first (about 50°C) took place in several steps which could be attributed to denaturation of different regions in the P450 molecule with different stability. The second transition (about 90°C) was fully reversible and similar to those described for other hemoproteins.

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