THE SUB-ZERO TEMPERATURE CHROMATOGRAPHIC ISOLATION OF TRANSIENT INTERMEDIATES OF A MULTI-STEP CYCLE: PREPARATION OF THE SUBSTRATE-FREE OXY-FERROUS CYTOCHROME P_{450}*

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1. Introduction

In a preceding paper we described an application of sub-zero temperature chromatography to the purification of the substrate-bound oxy-ferrous compound ($Fe^{2+}_S-O_2$ or $m^{rs}_{O_2}$) of bacterial cytochrome P_{450} obtained after photochemical reduction of the ferric form [1]. The reducing system was retained on LH 20 Sephadex gel, and the ternary compound eluted at −20°C at which temperature it is almost indefinitely stable. The present paper describes a modification of this method for the preparation of the less stable binary substrate-free oxy-ferrous compound ($Fe^{2+} - O_2$ or $m^{r}_{O_2}$). The ternary $m^{rs}_{O_2}$ complex was first prepared and further isolated from the photochemical reducing system (proflavine and EDTA) and freed from its bound substrate camphor.

2. Materials and methods

Bacterial cytochrome P_{450} [2] as well as other reagents were obtained from the same source as described in the preceding paper [1] and spectrally controlled with the same equipment. The same mixed solvent was used, except that KCl and camphor were omitted.

2.1. *Preparation of the ternary $m^{rs}_{O_2}$ compound*

The substrate bound oxy-ferro cytochrome was prepared in the mixed solvent as previously described [1], except that KCl was omitted and the final concentration of camphor limited by dilution to 100 μM, this still being sufficient to saturate the ferric or ferrous cytochrome (final concentration 30 μM). The $m^{rs}_{O_2}$ was then rapidly brought to −40°C where it was stored before chromatography.

2.2. *Chromatography at −35°C*

Chromatography was performed at −35 ± 0.5°C with the equipment already described [3]. The LH 20 Sephadex gel was equilibrated at room temperature in the mixed solvent buffered with phosphate (protonic activity $pa_H = 7.4$) [4]. The gel was degased before packing and the column washed with the elution buffer while it was cooled to −35°C. At this temperature, the $pa_{H-35°C}$ is 7.7. The elution flow rate was adjusted to 0.3 ml min^{-1} with both a peristaltic pump and nitrogen pressure. At −35°C, the high viscosity of the medium of 90 cps [5] necessitated a stable pressure of 1.5 bar.

The gel bed was 10 cm high and the internal diameter of the column 2 cm. The penetration and elution speeds of the compounds were identical and 1.3 ml fractions were collected at −35°C. After collection, each tube was cooled to 77°K in liquid nitrogen to avoid any thermal decomposition of complex.

3. Results

3.1. *Retention properties of camphor on LH 20 Sephadex gel*

Figure 1 compares the elution volumes of camphor,

* Number 2 of a numbered series.

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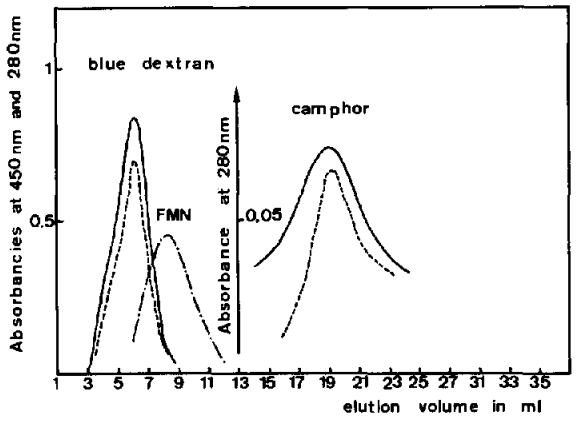

Fig.1. Comparative elution pattern of camphor, flavin mononucleotide (FMN) and blue dextran under different conditions of solvent and temperature. LH 20 Sephadex column (2 × 2.5 cm). 1 ml of a 6 mM camphor or 2.10^{-4} FMN solutions are applied to the column. The concentrations of camphor and FMN are respectively measured at 290 and 450 nm. (—) Elution in 0.05 M pH 7 phosphate buffer. (—·—) FMN in these conditions. (- - -) Elution in a 1 : 1 mixture of 0.1 M phosphate buffer and ethylene glycol at −20°C.

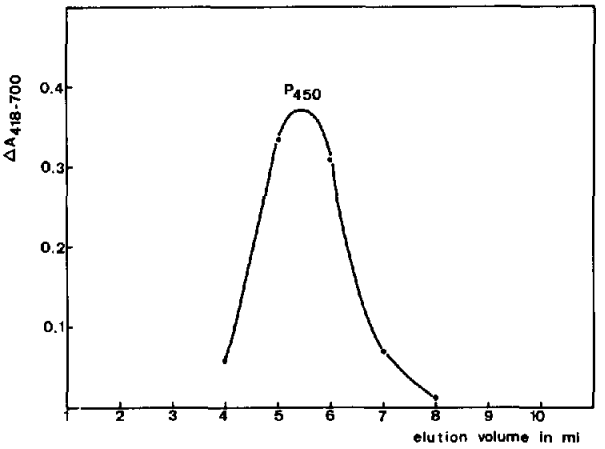

Fig.2. Elution pattern of a +20°C chromatography of cytochrome P_{450} in 0.05 M pH 7 phosphate buffer. LH 20 Sephadex column (2 × 2.5 cm). 1 ml fractions are collected. The camphor would be eluted at 20 ml (not shown on this figure, see fig.1).

flavin mononucleotide and blue dextran, eluted from a gel bed 2.5 cm high (internal diameter 2 cm) under various conditions of solvent and temperature.

Given the molecular weight of camphor, its elution volume should be of the order of 8–9 ml as in the case of flavin mononucleotide. However, the elution volume of camphor is 20 ml, either in pure aqueous buffer or in a mixed solvent at −20°C. These results suggest that camphor, like numerous other compounds [6] including proflavine [1] is retained abnormally on the LH 20 Sephadex gel. The retention is apparently not due to the same interaction that occurs in the case of proflavine since no temperature nor solvent effects were recorded.

We thus have rather favorable conditions since camphor, like proflavine will be retained on the column and be well resolved from cytochrome P_{450}. This property has been used to prepare camphor-free cytochrome P_{450}. A 1 ml solution of 0.05 M aqueous phosphate buffer containing ferric cytochrome P_{450} (8 μM) and camphor (600 μM) was applied to the same column. As shown in fig.2, the cytochrome is eluted in the void volume (5–6 ml), whereas camphor would be eluted at 20 ml (see fig.1). The cytochrome was absolutely free of camphor and its concentration in the best tube was 3.2 μM, that is only 2.5 times more diluted than the starting material. The yield after chromatography is nearly 100%.

Moreover, with a flow rate of 2 ml min^{-1}, the time required to obtain the cytochrome P_{450} is of the order of 3–4 min which makes it considerably shorter than the conventional method of preparing camphor-free cytochrome by G 10 Sephadex chromatography [7].

However, since the dissociation rate constant of camphor from the oxy-ferrous compound must be rather slow at low temperature, a bed gel 10–12 cm high was chosen in order to be sure that it was well separated from the protein at sub-zero temperature.

3.2. *Low temperature separation*

When prepared from the substrate-free ferric cytochrome, the oxy-ferrous compound $m^{I}_{O_2}$ decays much faster than the camphor-bound compound. The activation energy of the autoxidation process is rather small: 14.5 kcal mol^{-1} for $m^{I}_{O_2}$ in the hydro-organic mixture, and 19 kcal mol^{-1} for $m^{IS}_{O_2}$ in the same solvent (see fig.3).

From the Arrhenius plot of fig.3, a half-life time of 5 h may be extrapolated at −35°C which is why it was chosen for the chromatography. Using the

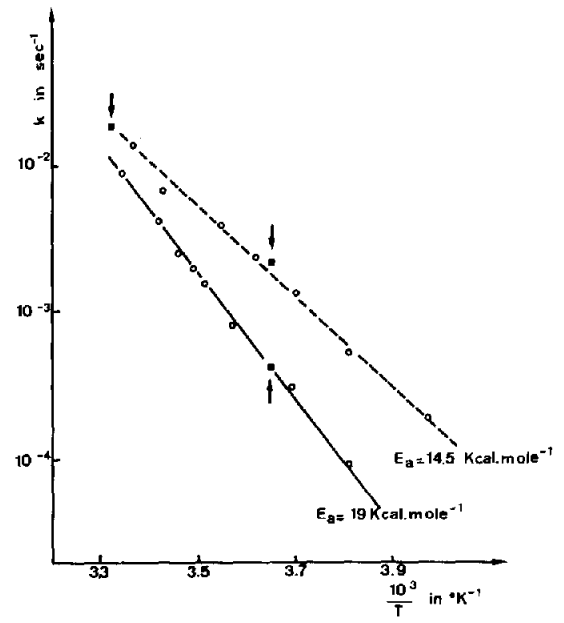

Fig.3. Comparative Arrhenius plots of the spontaneous decay rate constant of (——) camphor-bound ($m^{IS}_{O_2}$) and (- - -) camphor-free ($m^{I}_{O_2}$) oxy-ferrous cytochrome P_{450}. Solvent, 1 : 1 mixture of 0.1 M pH 7 phosphate buffer and ethylene glycol. The points indicated by arrows show the rate constants for the chromatographed camphor-free compound ($m^{I}_{O_2}$) just after chromatography and after chromatography and addition of camphor to the reaction medium.

experimental conditions defined in Materials and methods and a bed gel 10 cm high, the protein was eluted between tube 10 and 14. The maximal concentration obtained in tube 12 was 8.3 μM. The final recovery of the cytochrome was nearly 100%. The elution pattern was the same as in the case of camphor-bound oxy-ferrous compound ($m^{IS}_{O_2}$).

3.3. *Purity of the $m^{r}_{O_2}$ compound.*

Figure 4 shows the visible UV spectrum at −45°C of the contents of tube 12 diluted 10 times. It is obviously not contaminated by proflavine. Since the binding of camphor has no measurable effect on the Soret and visible bands of the oxygenated compound, the recording of the optical spectrum alone does not allow one to ascertain the absence of bound camphor. Furthermore free camphor may only be detected by its absorption at 290 nm (ϵ = 0.03 mM^{-1}) and is thus obscured by the higher absorption of the protein at the same wavelength. The absence of free and bound camphor was therefore tested by the autoxidation rate of the compound at different temperatures.

The spontaneous decay of the chromatographed compound was monitored at different temperature in the presence of methyl viologen, which is necessary in order to obtain a reproducible autoxidation rate

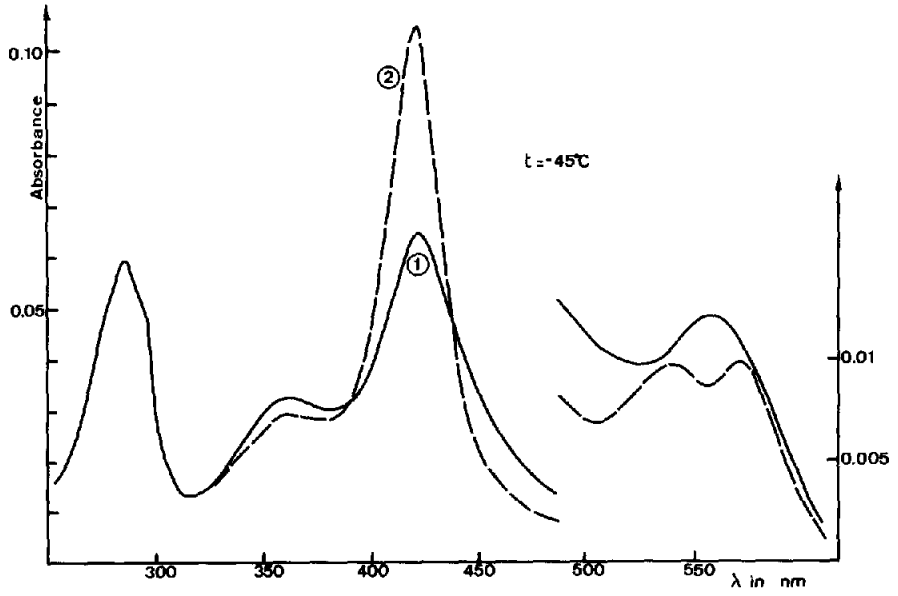

Fig.4. UV-visible optical spectra of the chromatographed $m^{I}_{O_2}$ compound (1) just after chromatography and (2) after thermodecomposition. Both spectra are recorded in the 1 : 1 mixture of 0.1 M phosphate buffer and ethylene glycol at −45°C. 10 times dilution.

(P. Debey, unpublished observations). As shown on the fig.4 the values obtained for the chromatographed compound are in excellent agreement with those obtained when $m^r_{O_2}$ is prepared from the camphor-free ferric cytochrome (m^o). After decomposition the m^o spectrum is given in the fig.4. Furthermore, when camphor is added to the $m^r_{O_2}$ solution before decomposition, the autoxidation rate constant falls on the Arrhenius plot for $m^{rs}_{O_2}$ decay (fig.3).

4. Discussion

In a previous study [1], the camphor-bound oxy-ferro cytochrome was separated from the exogenous reducing system (proflavine-EDTA) as well as from its side reaction products with O_2.

In the present paper, the oxy-ferrous cytochrome was prepared in the presence of saturating concentrations of camphor, ensuring a higher stability of the complex. In the subsequent chromatography the $m^{rs}_{O_2} \rightarrow m^r_{O_2}$ conversion was achieved during the elution. The $m^r_{O_2}$ compound, separated in the same preparative step both from the exogenous redox components and from its bound substrate, underwent a negligible spontaneous decay at −35°C. Camphor and proflavine were retained non specifically on the column, theoretically allowing very short column to be used. However at −35°C the dissociation rate constant of camphor is reduced and this imposes the use of a higher gel bed (10–12 cm).

It is worth mentioning the fact that the retention of camphor may be used to prepare the substrate-free ferric protein at room temperature, where a very short column is sufficient. The advantages of this procedure are the rapid elution (4 min) and the low dilution (nearly twice) of the substrate-free ferric cytochrome, both factors leading to its increased stability [7].

Stock solutions of pure and concentrated camphor-bound and camphor-free oxy-ferrous compounds may be stored at low temperature (77°K), without appreciable decay. This allows one to undertake comparative investigation on their respective decomposition mechanisms and products.

Acknowledgements

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