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

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Received 6 August 1976

1. Introduction

Many enzyme-substrate intermediates, too short lived to be obtained at sufficient concentrations, can be stabilized in fluid mixed solvents at sub-zero temperatures [1,2] and then accumulated and analysed by various physical techniques. However, such intermediates may be contaminated by an excess of substrates or reaction products, interfering with physicochemical investigation and forbidding their use as 'pure' reactants in subsequent processes. Purification can be achieved by column chromatography at subzero temperatures, a technique already developed in this laboratory [3].

By this method, the labile membrane bound microsomal cytochrome P_{450} was obtained in high yield, free from other proteins [3], and ternary enzyme-substrates intermediate compounds of bacterial luciferase were separated from an excess of free flavin mononucleotide [4,5].

For a slightly different purpose, a similar procedure has been successfully used to purify the unstable oxygenated compound of bacterial cytochrome P_{450} from the reducing system used for its preparation. While low temperature inhibits the spontaneous decay of the protein component, the LH 20 Sephadex gel shows an abnormal affinity towards the reducing agent (proflavine) which is thus retained on the column.

The present paper describes the purification of the ternary oxy-ferrous camphor-bound compound ($Fe_S^{2+} - O_2 = m_{02}^{rs}$) at −20°C. The following paper will present the adaptation of this method to the preparation of the much less stable camphor-free oxy-ferrous compound ($Fe^{2+} - O_2 = m_{02}^{r}$) of the same cytochrome.

*Number 1 of a numbered series.

2. Materials and methods

Bacterial cytochrome P_{450} was prepared in the presence of camphor according to a method already described [6], and was generously provided by the laboratory of Dr I. C. Gunsalus. Chemicals were purchased from Merck, except proflavine sulfate which was a Calbiochem compound. LH 20 Sephadex was purchased from Pharmacia Fine Chemicals and all reagents were used without further purification. Absorption spectra were recorded with an Aminco Chance DW 2 or Beckman Acta III spectrophotometer, equipped for low-temperature measurements [7].

2.1. *Solvent*

The solvent used throughout the procedure (preparation of the complex, equilibration and elution of the column) was a 1/1 mixture of 0.1 M sodium phosphate buffer and ethylene glycol, containing 3 mM camphor and 100 mM KCl as final concentrations. The protonic activity (pa_H) of this medium given either as previously described [8] or controlled by direct measurement with special electrodes [9], is 7.8 at −20°C.

2.2. *Preparation of the compound*

The ternary oxygenated compound (m_{02}^{rs}) was pre-

pared as follows: 0.2 ml of a stock solution of cytochrome P_{450} (m^{os}) in 0.1 M phosphate buffer pH 7, was diluted with 0.3 ml of 0.1 M pH 7 phosphate buffer saturated with camphor and 0.5 ml of ethylene glycol. The final concentration of m^{os} was of the order of 35 μM. KCl, EDTA and proflavine sulfate were added at final concentrations of 10^{-1}, 10^{-2}, 10^{-5} M respectively. The mixture was deoxygenated by 30 min equilibration with pure nitrogen, and the photosensitized reduction was performed by irradiation at +10°C.

Formation of m^{rs} was followed spectrophotometrically in the visible region. At the end of the reduction, the oxygenated compound m^{rs}_{02} was formed by addition of 0.5 ml of oxygen saturated elution buffer with concomitant bubbling of O_2. The compound was then promptly cooled to −40°C in order to avoid its conversion into the ferric m^{os}.

2.3. *Low temperature chromatography*

Chromatography was performed with an apparatus already described [3], using a column with an internal diameter of 2.5 cm. Sephadex LH 20 was degased under vacuum before packing in order to avoid irregularities in flow rate due to the eventual formation of microbubbles. The temperature was maintained at −20 ± 0.5°C during elution and the flow rate of the column, regulated with both a peristaltic pump and a nitrogen pressure (0.2 bar), was 0.4 ml min^{-1}; the penetration speed of the compounds was identical to the elution speed and 1.3 ml fraction were collected at −20°C.

3. Results

3.1. *Choice of experimental conditions*

The various redox states of cytochrome P_{450}, namely ferric (m^{o}), substrate bound ferric (m^{os}), substrate bound reduced (m^{rs}) and substrate bound oxy-ferrous (m^{rs}_{02}) were systematically tested in the mixed hydro organic solvent described under Materials and methods [10]. No denaturation, nor any gross change in the ϵ values of the absorption spectra of these various states was found, and the cytochrome was shown to retain fully its hydroxylating activity (P. Debey and L. Eisenstein, unpublished observations).

The ternary oxy-ferrous compound (m^{rs}_{02}) could be

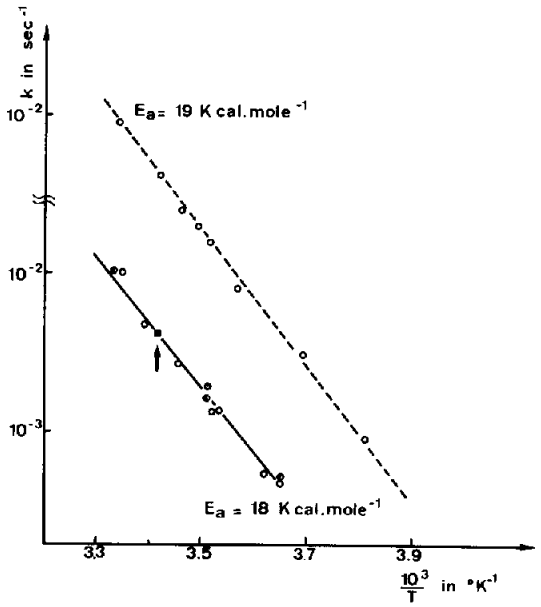


Fig.1. Arrhenius plots of the autoxidation rate constant of m^{rs}_{02} under various conditions of solvents. (——) 0.05 M phosphate buffer, ○ pH = 7, ⊙ pH = 7.4, containing 0.1 M KCl and 3 mM camphor as final concentrations. (- - -) mixture of 0.1 M phosphate buffer pH 7 and ethylene glycol (volume ratio 1:1). The experimental point represented by ■ and indicated by the arrow is the decay constant of the purified m^{rs}_{02} as obtained after low temperature chromatography on LH 20 Sephadex gel.

formed as in water (see Materials and methods) and its autoxidation ($m^{rs}_{02} \rightarrow m^{os}$) was found to be similarly first order [11]. The rate constant and activation energy (respectively 18 kcal mol^{-1} in water and 19 kcal mol^{-1} in mixed solvent) were found to be practically unaltered by the presence of the organic solvent, which does not exert any noticiable stabilizing effect [10] (fig.1). The rate constant of spontaneous decay as extrapolated to −20°C, was 2.4×10^{-5} sec^{-1}, yielding to a half time of 7 h, sufficient to perform chromatography without appreciable autoxidation of the m^{rs}_{02} compound.

3.2. *Low temperature chromatography*

1.5 ml of m^{rs}_{02} stabilized at −40°C were applied on the top of the column precooled at −20°C. With a bed gel height of 10 cm and a flow rate of 0.4 ml min^{-1}, the time required to obtain the pure complex was 30 min. The maximum concentration in the eluate

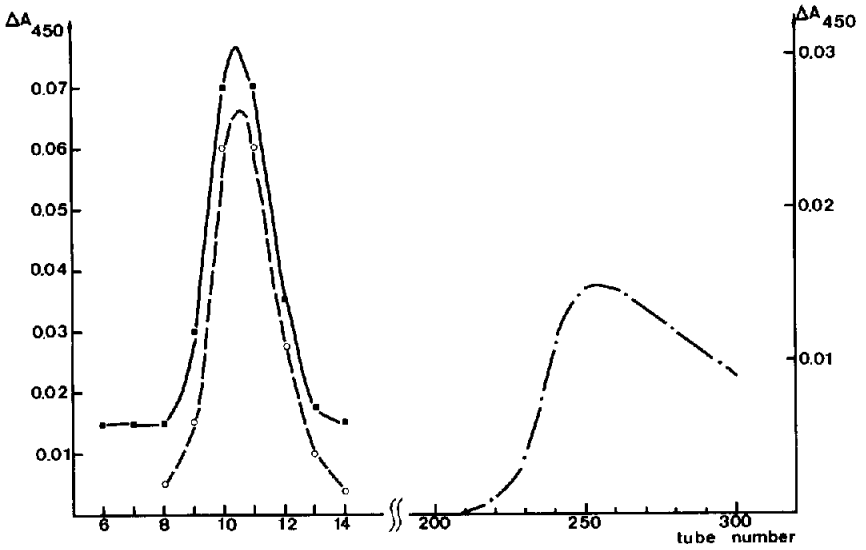


Fig.2. Elution pattern of the sub-zero temperature chromatographic separation of oxy-ferrous cytochrome P_{450} (m_{02}^{IS}) and proflavine Solvent and concentrations as described under Materials and methods. Temperature −20°C. Pressure 0.2 bar. Flow rate 0.4 ml min^{-1}. 1.3 ml fractions are collected. The concentrations of the various components are measured as follows (after 10-fold dilution for the determination of the protein concentration): (-o-o-) absorbance at 418 nm; (x ——x) absorbance at 280 nm; (—·—·—) absorbance at 450 nm (proflavine) (this elution profile is just indicative).

was 10 μM in 1.3 ml (best tube), only 3 times less that of the starting solution. The overall recovery of the chromatography was nearly 100%. These values were highly reproducible, and the elution pattern is shown on the fig.2.

It is important to note the abnormal elution volume of proflavine (250–350 ml). Given a molecular weight of 534.6 and the above geometry of the column, the elution volume should theoretically be 20–30 ml. This is actually the elution volume obtained with flavin mononucleotide, which has a very similar molecular weight (mol. wt. 478).

Some characteristic features of this aspecific retention were further studied using a short LH 20 column of 2 cm internal diameter and 2.5 cm bed gel height and different elution conditions. Figure 3 shows the elugraphe of proflavine in pure aqueous 0.05 M pH 7 phosphate buffer. The elution volume is of the order of 90 ml, independent of the ionic strength up to 1 M NaCl, whereas it is slightly less at −20°C in the mixed solvent (60 ml).

Furthermore we can see that the addition of 30% dioxane after a 140 ml elution with aqueous buffer decreases significantly the retention of the proflavine. However the elution of the proflavine is never complete.

All these observations strongly suggest that the interaction between the LH 20 Sephadex gel and the proflavine are of hydrophobic nature.

3.3. *Purity of the complex*

Figure 4 shows the UV visible absorption spectrum of the elution peak tube after 10-fold dilution on purely aqueous buffer (so that the proportion of ethylene glycol falls down to 5%), measured at 2°C immediately after chromatography and corrected for camphor absorption.

From this spectrum the absence of contaminating proflavine is apparent. Furthermore there is no noticeable change of the 280 nm absorption after decomposition of the complex into m^{OS} (spectrum 2, fig.4).

From this spectrum, the ratio of the absorbancies at 392 nm and 280 nm may be measured A_{392}/A_{280} = 1.48, in good agreement with the previous value of 1.47 obtained by Yu and Gunsalus [6].

The solution of pure compound may be stored frozen at 77°K, or more conveniently in fluid state down to −35°C, without any appreciable reoxidation.

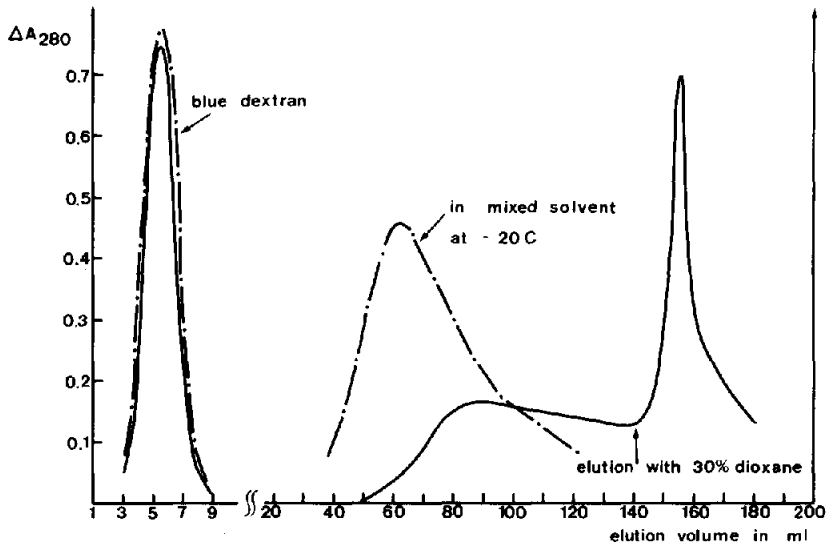
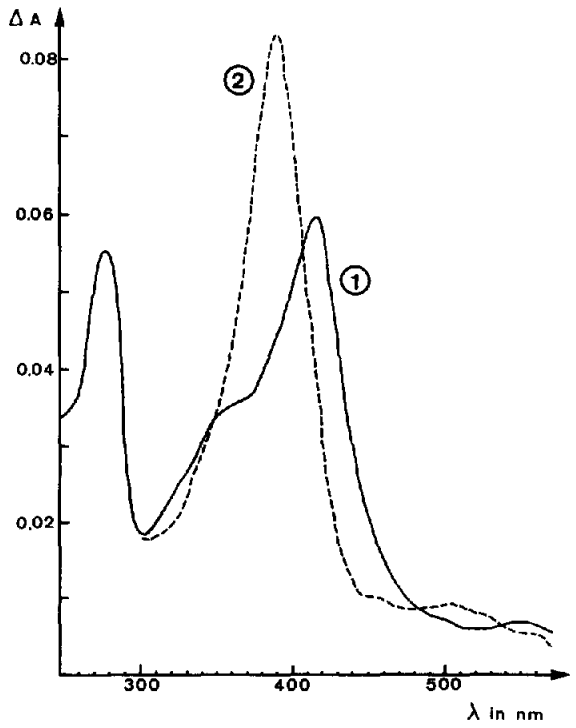


Fig.3. Comparative elution volumes of proflavine and blue dextran under different conditions of solvent and temperature. LH 20 Sephadex column (2 × 2.5 cm). Equilibration of the column is performed with the elution medium. 1 ml of a 3×10^{-4} M proflavine solution is applied to the column. 1 ml fractions are collected (——) Elution in 0.05 M pH 7 phosphate buffer (followed by the addition of 30% dioxane). (- - - -) Elution in a 1:1 mixture of 0.1 M phosphate buffer and ethylene glycol at −20°C.



The spontaneous decay of purified m^{rs}_{02} at higher temperatures was absolutely identical to the autodecomposition of the non-chromatographed compound (see fig.1).

4. Discussion

Already used to separate an oxygenated intermediate compound of luciferase from excess of flavin mononucleotide [4,5], low temperature chromatography on LH 20 Sephadex is now utilized to prepare the ternary oxy-ferro compound of cytochrome P_{450} in the absence of the chemicals used to reduce it, as well as of their oxidation products.

Fig.4. UV visible absorption spectra of the chromatographed compound, (1) before, and (2) after autoxidation. 0.1 ml of the elution peak tube are rapidly diluted at 2°C in 0.9 ml of 0.05 M phosphate buffer containing 0.1 M KCl and 3 mM camphor. The decomposition is then performed by heating and the spectrum of the product again taken at 2°C (spectrum 2).

Such a purification takes advantage of the stability of the compound at −20°C (the extrapolated value of its spontaneous decay rate constant is 2.4×10^{-5} sec^{-1} at −20°C), and of the aspecific retention of proflavine on the LH 20 gel, which could be related to the already noticed retention of several aromatic compounds [12]. Such a property could permit the use of very short columns, and thus reduce the elution time.

The above described procedure may be applied to many other compounds or multicomponents systems, involving at one step a chemical reduction. Similarly although not benefiting of the same specific retention, dithionite and its oxidation products could be separated at low temperature from a rather unstable macromolecular component.

It is very important to note that the complex is also completely purified from hydrogen peroxide, which can be detected at rather high concentration in the initial solution and is due to the rapid reoxidation of proflavine by the excess oxygen added to form the oxy-ferro cytochrome.

For reactions of oxygen metabolism, the absolute absence of excess oxido-reduction agents and decomposition products is especially required because they could interact with short-lived oxidizing species or protein intermediates, likely to be produced during the oxygen activation process. In the case of cytochrome P_{450}, stock solutions of rather concentrated and *pure* m^{rs}_{02} may now be utilized as starting material to study the further steps of the hydroxylation cycle. Such a study is now under way in this laboratory.

Acknowledgements

The authors wish to thank Dr I. C. Gunsalus for the generous gift of bacterial cytochrome. We are grateful to Mrs E. Begard for her technical assistance. This work was supported by grants from the INSERM, the DRME (Contrat n° 75/353), the DGRST (Contrat n° 75-7-0745), the CNRS (ERA 262) and the Fondation pour la Recherche Médicale Française.

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