SUBZERO TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME *P*-450

O-Dealkylation of 7-ethoxycoumarin coupled to single turnover

Kristoffer K. ANDERSSON, Pascale DEBEY and Claude BALNY
Unité 128 de l'INSERM, BP 5051, 34033 Montpellier Cédex, France

Received 28 March 1979

1. Introduction

We reported with intact microsomes the formation and partial stabilization of a ferrous–oxy intermediate of cytochrome *P*-450 in fluid mixed solvents at subzero temperature [1]. The data compare well with those from stopped flow measurements on purified cytochrome above 0°C [2] and steady state recordings on intact microsomes [3].

The subzero temperature experiments offered two advantages, particularly with intact microsomes:

(1) Uncoupling of the oxygen reaction from the other steps of the cycle;
(2) Therefore single turnover of enzyme since the first electron is blocked at $\lesssim -20$°C [4,5].

The decomposition of this ferrous–oxy compound was also shown to be accompanied by the discharge of possible 'leaks' of oxidizing species, indirectly detected by the oxidative luminescence of luminol [5].

Here we use the same temperature programme to show that enzymatic *O*-dealkylation of the substrate 7-ethoxycoumarin occurs during the single redox cycle of cytochrome *P*-450. This substrate was mainly chosen for the easy fluorometric detection of its product 7-hydroxycoumarin (umbelliferone, 7-OH coumarin) [6].

2. Materials and methods

2.1. *Materials*

Hepatic microsomes prepared according to [7] from untreated rabbits were stored in 20% glycerol at −30°C for up to 2 months and freshly thawed just before use.

The cytochrome *P*-450 and *P*-420 contents were estimated as the ferrous–CO adduct by the method in [8]. The microsomes contained about 1.5 nM *P*-450/mg protein, $< 10\%$ in the form of *P*-420. *O*-dealkylation of 7-ethoxycoumarin was measured according to [6] with the substrate generously supplied by Dr Ullrich. Ethylene glycol was from Riedel de Haen and buffers were reagent grade from the usual suppliers.

2.2. *Experimental procedures*

Absorption spectra were measured with an Aminco Chance DW 2 spectrophotometer thermostable at subzero temperatures [9] and fluorescence emission in an Aminco-Bowman spectrofluorimeter similarly equipped. The final hydro-organic reaction medium contained 0.1 M Tris acetate buffer ($pH_{20°C}$ 8.4), 45% (v/v) ethylene glycol, $\leqslant 2\%$ glycerol and 500 μM ethoxycoumarin. The proton activity is strongly temperature dependent: at −30°C, $pa_H = 10.35$ [10]. The two reaction vessels at 1.5–3.5 mg microsomal protein/ml were deoxygenated by argon bubbling at 4°C and the cytochrome reduced at 5°C by anaerobic addition of deoxygenated NADPH to 50 μM final conc. After full reduction, the cuvettes were cooled to −30°C, respectively, in the spectrophotometer and fluorimeter and oxygen admitted by 10 s bubbling.

The fluorescence excitation was at 385 nm to minimize NADPH interference; product formation was calibrated by adding commercial 7-hydroxycoumarin (Merck) at the end of each experiment.

Number 7 of a numbered series

3. Results

3 1. *The effect of solvent and temperature on the steady state* O-*dealkylation of 7-ethoxycoumarin*

Prior to low temperature experiments, we studied the effects of various parameters on the steady state *O*-dealkylating activity of aerobic microsomal suspensions

The rate of *O*-dealkylation measured at 20°C decreases as a function of ethylene glycol concentration, in a way parallel to the progressive inhibition of other microsomal activities [4] and overall electron flow, moreover, the temperature effect is the same whether microsomes are suspended in aqueous or hydro-organic medium.

Since preliminary experiments on the microsomal (P D , unpublished results) as well as camphor-free or bound bacterial cytochrome *P*-450 [11] showed that high pa_H greatly stabilize the intermediate oxy–ferrous compound, we also studied the effect of pa_H on steady state activity At room temperatures and 45% (v/v) ethylene glycol, the *O*-dealkylation activity decreases by a factor of 1 5 when the starting pa_H of the buffer increases from 7 4–8 4 The latter conditions (Tris (pH 8 4)/ethylene glycol 45%) have thus been used for further experiments, we have checked that under those conditions, no denaturation occurs before, during, or after a cooling heating cycle.

3 2 *Low temperature* O-*dealkylation by the oxy compound of ferrous cytochrome* P-*450*

The optical characteristics of the compound(s) obtained after 10 s oxygen bubbling at −30°C are given in fig 1 They are essentially similar to spectra already described on rat liver microsomes at lower pa_H [1], except that the two maxima at 430 nm

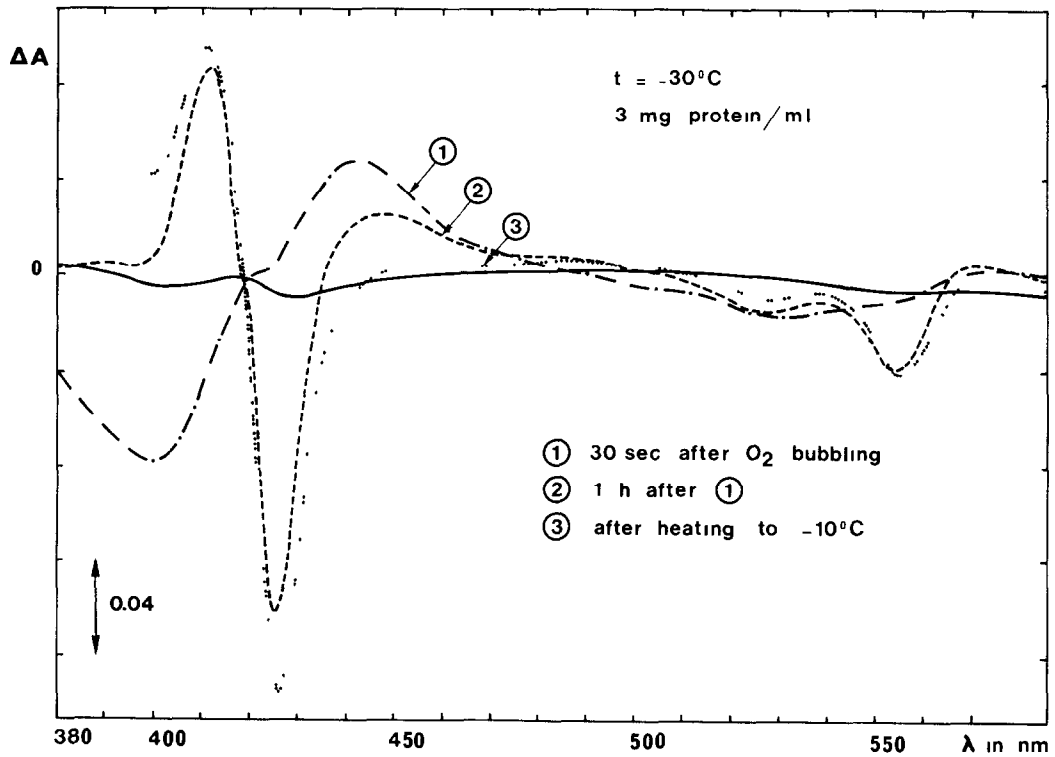

Fig.1 Differential optical spectra of the microsomal oxy–ferro cytochrome *P*-450 at −30°C Microsomal suspension (3 mg protein/ml) in 45% (v/v) ethylene glycol–0 1 M Tris acetate buffer $pa_{H,20°C}$ = 8 5, 50 μM NADPH and 500 μM ethoxycoumarin Baseline Fe^{2+} minus Fe^{2+} recorded at −30°C Spectra (1) 30 s after oxygen bubbling at −30°C, (2) after 1 h at −30°C, (3) after heating to −10°C (5 min)

and 445 nm are less resolved. $\Delta\epsilon_{440-490}$ between $(Fe^{2+} \cdot O_2)$ and (Fe^{2+}) is estimated to 12 ± 3 mM^{-1} and $\Delta\epsilon_{440-490}$ between (Fe^{3+}) and (Fe^{2+}) to -3 mM^{-1} at 30°C.

The maximum at 440 nm decreases slowly with time, while cytochrome b_5 gets progressively oxidized. After 1 h at −30°C a subsequent heating for 5 min at −10°C leads to a further reoxidation of both cytochromes (spectrum 3, fig.1), which is however not complete since a full transformation into Fe^{3+} would give a negative absorption in the 440–450 nm region. However high temperature incubation was not prolonged longer to avoid enzyme recycling through reduction of the ferric cytochrome.

Figure 2 describes the parallel fluorometric recordings on an aliquot of the same suspension. The −30°C spectra before and after full reduction are a control that no substrate was transformed during the anaerobic reduction stage. The progressive increase of fluorescence in presence of 7-ethoxycoumarin after oxygen bubbling at −30°C parallels the decomposition of the optical intermediate compound (fig.2, curve a). Subsequent heating to −10°C further increases the fluorescence intensity measured at −30°C. The fluorescence increase is calibrated at −30°C by addition of known concentrations of the fluorescent product 7-OH-coumarin.

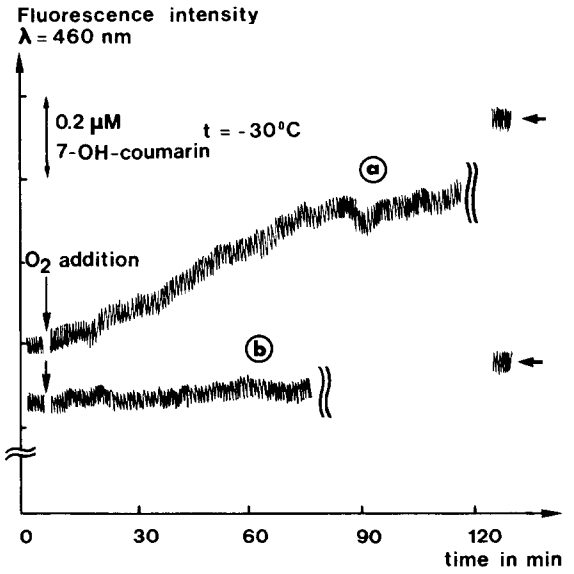

Fig.2. Evolution of the fluorescence intensity at −30°C (λ_{exc} = 385 nm, λ_{em} = 460 nm) after oxygen addition on completely reduced microsomal suspension. (a) in presence, (b) in absence of 7-ethoxycoumarin. Conditions as in fig.1. Arrows indicate the final levels recorded at the same temperature after heating 5 min to −10°C. Intensity expressed in 7-OH-coumarin concentration.

In the experiment of fig.1,2 (4.5 μM cytochrome), ~80% of the oxy-compound is decomposed into Fe^{3+} after heating to −10°C, while 0.55 μM product 7-OH-coumarin are formed. The yield of product formation is thus 15%. Several experiments with different preparations lead to a 10–20% yield.

Control experiments showed that neither spectral change nor fluorescence increase occur when argon is bubbled instead of oxygen, when only cytochrome b_5 is reduced or when CO is added prior to oxygen.

4. Discussion

The above experiments clearly show that the single formation–decomposition cycle of an intermediate involving both oxygen and the fully reduced microsomal cytochrome *P*-450, is coupled to the *O*-dealkylation of 7-ethoxycoumarin. The control experiments, already discussed previously [1,5] show again that hydroxylation requires absolutely the full reduction of the microsomal components including non-liganded cytochrome *P*-450 (Fe^{2+}), before oxygen addition.

It is worth noting that at −10°C the electron flow does not proceed at a detectable rate, so that only one turnover is observed. The present method offers a simplified alternative to the elegant method set up at room temperature [12] to measure elementary turnover at room temperature.

High pa_H has been used to slow down the decomposition and allow a direct comparison with the already observed liberation of oxidizing species [5] which can be considered as a side reaction competing with the presently observed enzymatic activity.

The 10–20% yield of product formation is quite satisfactory owing to the fact that:

(1) Various forms of *P*-450 have been detected in liver microsomes [13] which may not all be functional in *O*-dealkylation;

(2) Maximal substrate binding has been estimated to 70% [14];

(3) The oxy-compound can be partly decomposed via non-product, forming side reactions such as H_2O_2 production [15,16]

Further subzero temperature experiments are under way to study in more details the number and properties of functional oxy-intermediates involved in hydroxylation

Acknowledgements

The authors greatly acknowledge many helpful and stimulating discussions with P Douzou and I. C. Gunsalus They thank Dr V Ullrich for his gift of purified substrate 7-ethoxycoumarin This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (Unité U 128), the Délégation Générale à la Recherche Scientifique et Techniques (contrat no 78 7 0332), the Fondation pour la Recherche Médicale Française, and the Centre National de la Recherche Scientifique

References

[1] Bégard, E , Debey, P and Douzou, P (1977) FEBS Lett 75, 52–54
[2] Guengerich, F P., Ballou, D P and Coon, M J (1976) Biochem Biophys Res Commun 70, 951–956
[3] Estabrook, R W , Hildebrandt, A G , Baron, T , Netter, K T and Leibman, K (1971) Biochem Biophys Res Commun 47, 132–139
[4] Debey, P , Balny, C and Douzou, P (1973) Proc Natl Acad Sci USA 70, 2633–2636
[5] Debey, P , Balny, C and Douzou, P (1974) FEBS Lett 46, 75–77
[6] Ullrich, V and Weber, P (1972) Hoppe Seyler's Zeit Physiol Chem 353, 1171–1177
[7] Van der Hoeven, T A and Coon, M J (1974) J Biol Chem 249, 6302–6310
[8] Nishibayashi, H and Sato, R (1967) J Biol Chem 61, 491–496
[9] Maurel, P , Travers, F and Douzou, P (1974) Anal Biochem 57, 555–563
[10] Douzou, P , Hui Bon Hoa, G , Maurel, P and Travers, F (1976) in Handbook Biochem Molec Biol (Fasman, D ed) 3rd edn, pp 520–538, CRC Press, Cleveland, USA
[11] Lipscomb, J D , Sligar, S G , Namtvedt, M J and Gunsalus, I C (1976) J Biol Chem 261, 1116–1124
[12] Ihlefeld, H and Diehl, H (1976) FEBS Lett 64, 111–115
[13] Guengerich, F P (1977) J Biol Chem 252, 3970–3979
[14] Ebel, R E , O'Keeffe, D H and Peterson, J A (1978) 253, 3888–3897
[15] Nordblom, G D and Coon, M J (1977) Arch Biochem Biophys 180, 343–347
[16] Lipscomb, J D , Sligar, S G , Namtvedt, M J and Gunsalus, I C (1976) J Biol Chem 251, 1116–1124