Resolution of *p*-cresol methylhydroxylase into catalytically active subunits and reconstitution of the flavocytochrome

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Received 17 May 1982

p-*Cresol methylhydroxylase* *Flavocytochrome* *Resolution–reconstitution* *Flavin* *Heme*

1. INTRODUCTION

Cells of *Pseudomonas putida* (NCIB 9869) grown on 3,5-xylenol contain a flavocytochrome which converts *p*-cresol into *p*-hydroxybenzaldehyde [1,2]. The isolated enzyme requires an added electron acceptor, such as phenazine methosulfate, for the reaction, which proceeds by 2 dehydrogenations and a hydration step, catalyzed by a single protein. It consists of 2 subunits of equal M_r (57 000) [1,2], one which contains a *c*-type cytochrome, the other 8α-*O*-tyrosyl FAD in covalent linkage [2–4].

We have now succeeded in separating the flavoprotein and heme protein subunits by isoelectric focusing. The separated flavoprotein retained 2% of the catalytic activity of the holoenzyme toward *p*-cresol. Reconstitution of the holoenzyme was achieved by titrating the flavoprotein subunit with the cytochrome. To our knowledge, this is the first instance of the reversible resolution of a flavocytochrome into its component subunits.

2. MATERIALS AND METHODS

The organisms were grown and the enzyme isolated as in [1,4]. Catalytic activity was determined in the phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol assay spectrophotometrically at 25°C in the presence of 50 mM Tris buffer (pH 7.6) saturating *p*-cresol (1.2 mM) and varying concentrations of PMS, activity being calculated by extrapolation to infinite concentration of the dye.

Isoelectric focusing was conducted in the Pharmacia model FTE 3000 flat bed apparatus at 4°C. Sephadex G-200 (3 g) was suspended in 96.5 ml H_2O and 6.45 ml Biolyte 4-6 (Bio-Rad Labs.) ampholyte was mixed in to give a thick slurry. A portion of this was poured onto a galss plate to yield a layer of 7.1 × 12.8 × 0.15 cm. From the partially dried plate a 0.8 × 6.5 cm strip was removed, mixed with 0.5 ml solution of 1:16 diluted Biolyte, containing 6 mg *p*-cresol methylhydroxylase, and the resulting slurry was replaced on the plate. Current was applied initially at a constant 25 W (500–600 V) and after 30 min rose to the limiting value of 1200 V and the power fell to 13 W. After 3 h the 2 focused, colored bands were scraped off and protein was quantitatively eluted from each by repeated extraction with 2 ml portions of 0.05 M Tris-HCl buffer (pH 7.6). Each solution was concentrated 3-fold, the cytochrome subunit in an Isco electrophoretic concentrator, and the flavoprotein by ultrafiltration through an Amicon membrane. This treatment, followed by passage through a small column of Sephadex G-50 (equilibrated with 0.05 M Tris-HCl (pH 7.6)) served to remove all ampholytes.

3. RESULTS

Fig.1. illustrates the separation of the flavoprotein and cytochrome *c* subunits by isoelectric focusing on Sephadex G-200. The pI-value of the flavoprotein was found to be pH 5.0, whereas that of the cytochrome pH 4.4. Fig.2 represents the absorption spectra of the separated subunits, recovered from the gel as in section 2. The main part is the heme protein subunit and the inset shows the spectrum of the flavoprotein. The absence of heme

Published by Elsevier Biomedical Press

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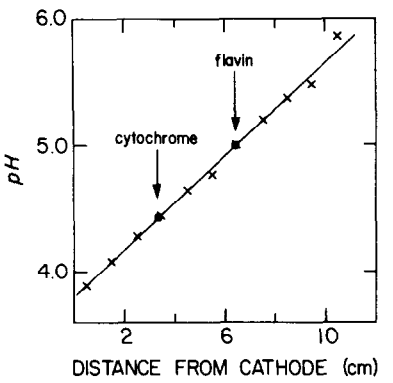


Fig.1. Isoelectric focusing of *p*-cresol methylhydroxylase. Conditions were as in section 2: (×) experimentally determined pH-values on samples scraped off the plate at the end of the experiment, suspended in water, and centrifuged to remove the Sephadex; (•) colored zones where the 2 subunits were located.

from the latter is evident from the spectrum, while the absence of flavin from heme protein was ascertained by lack of fluorescence following trichloroacetic acid precipitation and SDS solubilization, sodium dithionite reduction and reoxidation, conditions which cleave the flavin–tyrosine bond revealing the potential fluorescence of this type of covalently bound flavin [4].

The mM extinction coefficient of the cytochrome

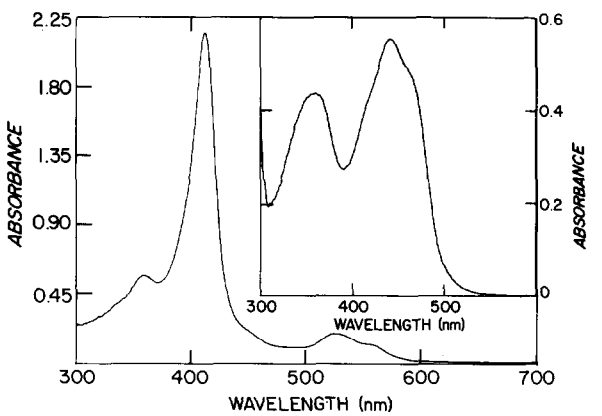


Fig.2. Absorption spectra of the heme protein (main part) and flavoprotein (inset) subunits.

subunit in the reduced form was determined to be $\varepsilon_{552} = 26.9$ by titration with dithionite, while that of the oxidized flavoprotein was found to be $\varepsilon_{440} = 11.3$ by titration of the reduced form with ferricyanide.

The flavoprotein subunit, separated and isolated as in section 2, was found to have ~2% of the catalytic activity of the holoenzyme (140 ± 12 *vs* 7450 ± 300 mol *p*-cresol . min^{-1} . mol protein^{-1}) but their K_m-values for PMS were essentially the same (4.6 ± 0.62 mM for the flavoprotein and 4.17 ± 0.40 mM for the holoenzyme).

Fig.3 illustrates the reconstitution of the holoenzyme. The experimental conditions are given in its legend: recombination occurred within 5–15 min after addition of the cytochrome *c* subunit to the flavoprotein in the presence of all components of the assay mixture. The maximal rate obtained by extrapolation from the data shown was 1270 mol substrate oxidized . min^{-1} . mol flavoprotein^{-1} at a fixed (2.21 mM PMS, whereas the unresolved

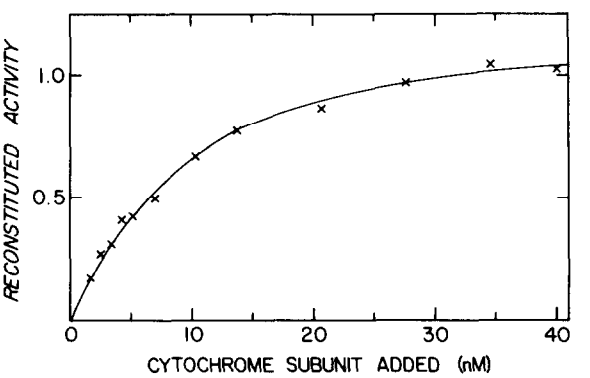


Fig.3. Reconstitution of the holoenzymes. Each of a series of spectrophotometric cuvettes contained in 3 ml 50 mM Tris-HCl buffer (pH 7.6 at 25°C), 1.2 mM *p*-cresol, 2.21 mM PMS, 95 μM 2,6 dichlorophenolindophenol and 5.31 nM flavoprotein subunit. The latter was added last and the absorbance change at 600 nm due to the basal activity of the flavoprotein subunit was monitored for 1–2 min, at which time varying amounts of the cytochrome subunit were added to give 1.73–40 nM. The rate increased gradually and a constant value was produced after 5–15 min. From this rate, the rate given by the flavoprotein alone was subtracted and the difference is plotted on the ordinate as mol substrate oxidized . min^{-1} . mmol flavoprotein^{-1} at 25°C.

enzyme gave 2590 mol . min^{-1} . mol flavocytochrome^{-1}. However, this is not the maximum reconstitution obtainable, since the optimal conditions for recombination have not been defined. The K_d–value for the reassociation of the subunits (fig.3) may be calculated from the relation:

$$K_d = \frac{1}{K_{eq}} = \frac{[\text{flavoprotein}]\,[\text{cytochrome}]}{[\text{flavocytochrome}]}$$

and was found to be ~6 nM, indicating a rather tight interaction between the subunits.

4. DISCUSSION

Although a number of flavocytochromes have been isolated and extensively studied from microbial sources, reversible dissociation into flavoprotein and cytochrome subunits has not been achieved with any of these, probably because dissociation requires drastic conditions in most cases. *p*-Cresol methylhydroxylase seems rather unique in that its 2 subunits seem to be held primarily by electrostatic interaction, since procedures such as isoelectric focusing or chromatofocusing suffice to separate them and mere mixing of the components reestablishes the catalytically active quaternary structure. This offers an unusual opportunity to study the individual functions of the component subunits of this complex flavoprotein, as well as the effect of one subunit on the catalytic properties and stability of the other. There is no question, however, that the flavocytochrome behaves as a single protein prior to resolution, since they accompany each other as a single functional unit through extensive purification [1,2].

It is interesting to note in this context that the isolated flavoprotein subunit has only ~2% of the catalytic activity of complex toward *p*-cresol. This is not ascribable to lesser reactivity of PMS, the electron acceptor, with the flavin than with the cytochrome *c* in the intact flavocytochrome (assuming that electrons flow from substrate → flavin → cytochrome → PMS), not only because the K_m for PMS does not change materially when the subunits are dissociated, but also because reoxidation of the flavin and subsequent steps are not rate-limiting in either form of the enzyme, as judged by the fact that the same kinetic isotope effect was found in both the flavoprotein and the flavocytochrome [5]. The low activity of the isolated flavoprotein cannot be due to the presence of contaminating cytochrome, since cytochrome *c* would have been detected by its intense Soret band at concentrations far lower than those required to account for the activity, and no indication of heme contamination was seen in any of the preparations of the flavoprotein subunit (cf. fig.1). Since the K_m for *p*-cresol is also very similar in the 2 forms [5], it seems possible that combination with the cytochrome may increase the rate of electron transfer between the substrate and the covalently-bound flavin.

ACKNOWLEDGEMENTS

We are grateful to Dr D.J. Hopper for a supply of the enzyme. This investigation was supported by program project HL-16251 from the National Institutes of Health, by grant no. PCM 81-19609 from the National Science Foundation, and by the Veterans Administration.

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