THE FERROPROTEIN COMPONENT OF A METHYLENE HYDROXYLASE*

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Reports of Kimura and Suzuki (1965a,b), Omura, et al. (1965a,b), and Cushman and Gunsalus (1966a,b) have implicated ferroproteins in the hydroxylation of methylene groups of steroids and of mono-terpenoids respectively by adrenal and microbial enzyme systems. In each case the iron bearing subunit contains similar amounts of acid labile iron and sulfur. There are absorption maxima near 280 (only partly due to aromatic amino acid content), 320, 415, and 455 in the oxidized form; the three bands at longer wavelength are bleached on reduction either by specific flavoprotein reductases or by dithionite, leaving a residual maximum at 545 mu. The iron and sulfide content, the absorption spectra, and the molecular size resemble spinach (Tagawa and Arnon, 1962) and alfalfa (Keresztes-Nagy and Margoliash, 1966) iron proteins. In contrast, the methylene hydroxylase linked ferroproteins have an E'_0 at pH 7 of about +100 mv (Kimura and Suzuki, 1965; Cushman and Gunsalus, 1966), 500 mv more positive than the spinach enzyme (Tagawa and Arnon, 1962).

The present communication concerns the purification, properties, and role of the ferroprotein of Pseudomonas putida in a 2-bornanone-5-exo-hydroxylase system. The EPR spectra of this ferroprotein (putida redoxin) containing sulfur isotopes 32 and 33 are shown in the accompanying paper (DerVartanian, et al., 1966).

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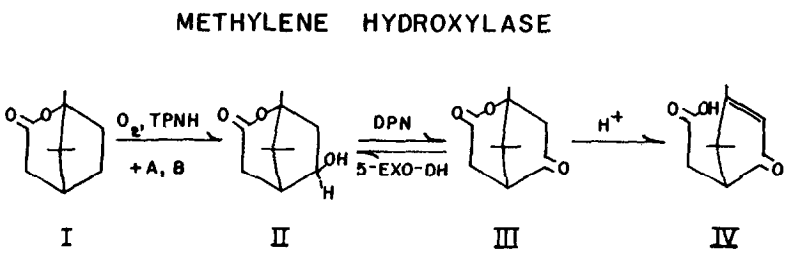
Chemicals. Pyridine nucleotides, substrates, and enzymes for assay, i.e., glucose-6-phosphate dehydrogenase, were purchased from the usual sources in the highest purity available. The 2-bornanone-5-exo-alcohol dehydrogenase (5-exo-ADH) was a 20-fold purified preparation, obtained by a modification of the procedure of Paisley (1961), donated by Dr. Richard L. Prairie of this laboratory. The FMN-linked DPNH dehydrogenase (E_1) was prepared according to Trudgill, et al. (1966) by Mr. Rene DuBus. Adrenodoxin was a gift from Professor T. Kimura, of St. Paul's University, Tokyo (1965). Camphor-1,2-lactone was synthesized according to Sauers (1959).

Hydroxylase Assay. Scheme I depicts the reaction sequence for measuring hydroxylation of the 5 methylene carbon of (+)-camphor or its 1,2 lactone



Scheme I. Methylene Hydroxylase Assay Components and Reaction Sequence

(Hedegaard and Gunsalus, 1965). The reaction is quantitated by the spectrum of the eneone chromophore IV after the alcohol formed by hydroxylation has been oxidized to the ketolactone III by a specific secondary alcohol dehydrogenase (Paisley, 1961) and the lactone ring opened on acidification with metaphosphoric acid, which also serves to deproteinize the reaction mixture. The chromophore IV is extracted quantitatively by isoamyl alcohol and read at λ max 227 mμ (E = 14,000) (Cushman, 1966).

This coupled assay can be run in a fixed time incubation as shown in Table I. Since either DPNH or TPNH serves as substrate for the flavoprotein in fraction A which reduces the ferroprotein (fraction B), the latter is em-

TABLE I

Bornane-5-exo-Hydroxylase Assay Components

System		Activity
	mM or mU[1]	U x 10^3
1. Complete[2]		21.3
2. - S = 1,2-lactone	6	0
3. - O_2	0.2 Atm	0.5
4. - TPNH Gen[3]	1	2.8
- 4 + TPNH	5	19.5
- 4 + DPNH	5	18.0
5. - Putida redoxin (E_B)	23	0
6. - Fraction E_A	24	0.5
7. - 5-exo-ADH	400	7.0
8. - DPN	5	0

[1]A mU = 1 nanomole of product IV formed per minute
[2]Components 1 through 8 plus 50 mM Tris chloride buffer, pH 7.4, in 0.4 ml
[3]Per tube of 0.4 ml: TPN 1 mM; glucose-6-phosphate 5 mM; yeast glucose-6-phosphate dehydrogenase 0.6 units (Boehringer)

Reaction started with glucose-6-phosphate, 0.02 ml; incubated 10 min, 30°; stopped by adding 0.8 ml 1% metaphosphoric acid. Chromophore IV extracted with 2 ml isoamyl alcohol, 5 mm cell, 1 U = 33 O.D., 227 mu.

TABLE 2

Properties of Putida Redoxin (Fraction B)

Sp. Act., U/mg (III-30A)	1.20
M. Wt. x 10^{-3}	
Sed. Vel.	11.2
Sed. Eq.	10.0
A. A. Anal.(2 phe)	15.4
E'_o (pH 7.0), mv	96
λ max, E x 10^{-3}	per 15 x 10^3 M. W.
277 mu	24.2
320 "	12.1
415 "	7.75
455 "	7.0
$S^=$ acid labile	1.80
Fe	1.95
Cysteine	>2

ployed because the alcohol dehydrogenase is specific for DPN, which can thus serve as oxidizing agent for conversion of alcohol to ketone (Cushman, 1966). With purified hydroxylase fractions the assay is run in two steps to avoid activities carried by the 5-exo-ADH which is isolated from the same extracts (Paisley, 1961). Thus the DPN and 5-exo-ADH are omitted from the reaction vessel during the hydroxylation, the enzymes are inactivated by heat, and the accumulated alcohol oxidized to ketone by adding these reagents in a second 10 minute incubation at 30° before the reaction is stopped by adding the deproteininzing agent, etc., see Table I.

Enzymes and Purification. Pseudomonas putida strain CIB was grown in mineral salts medium with 10 mM glucose and 6 mM (+)-camphor as carbon source, according to Conrad, et al. (1965). A kilogram of cell paste was suspended in 1.5 liters of 50 mM Tris chloride buffer, pH 7.5, containing 10 mM β-mercapto-ethanol (hereafter termed buffer) and an extract prepared by treatment with a Branson Sonic Oscillator, centrifugation, and DNAase and RNAase treatment as described by Trudgill, et al. (1966) to yield about a liter of extract containing 45 g of protein at a specific activity of 0.01. The liter of extract was added to a DEAE cellulose column (5.5 x 50 cm, 1 liter), washed with 2 liters of buffer, and linear gradient elution accomplished with 2 liters of buffer in each of 2 vessels, the second made 0.8 M with KCl. Fraction A (containing a reductase flavoprotein) eluted between 0.1 and 0.2 M and Fraction B (putida redoxin) between 0.28 and 0.32 M KCl; both were concentrated by adding cold, deaerated, saturated ammonium sulfate and redissolved to give 40 mg protein per ml. The activity recovered was about 50% of A and 30 to 40% of B, at specific activities, respectively, 0.05 and 0.15.

The ammonium sulfate concentrated B fraction, volume 20 ml, was added to a Biogel P60 column, 4 x 90 cm, and displaced with buffer. The putida redoxin activity is displaced immediately following the main protein peak and contains ca 20% of the starting units, sp. act. 1.2. More than 90% of this protein traveled with the activity as a single band in disc electrophoresis.

Results

The oxygenase ferroprotein for (+)-camphor 5 methylene hydroxylation is separated from a reductase-containing fraction A in essentially homogeneous form, as illustrated in Figure 1; its properties are shown in Table 2.

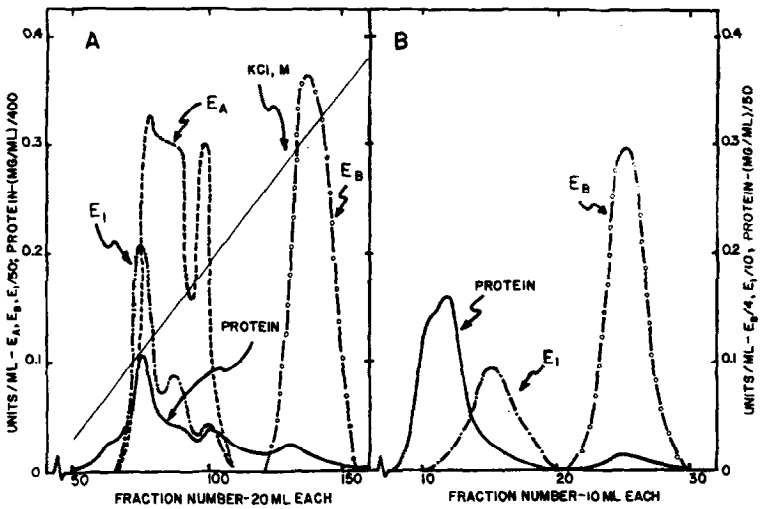


Figure 1. Methylene Hydroxylase Ferroprotein Purification by Column Chromatography

A. DEAE cellulose column (5.5 x 50 cm, 1 liter) 45 g protein as DNAase-RNAase treated sonic extract, hydroxylase sp. act. 0.01 (= 1 uM product formed/min/mg protein). Gradient 2 liters 0.05 M Tris chloride buffer, pH 7.5, containing 10 mM β-methylmercaptanol plus 2 liters 0.8 M KCl in same buffer.

B. Biogel P60 column (4 x 90 cm, 1 liter) 800 mg protein E_B from DEAE column after $AmSO_4$ precipitation (flow rate 0.05 column volumes/hour).
E_1 DPNH dehydrogenase flavoprotein of ketolactonase system (Trudgill, et al., 1965); E_A, E_B methylene hydroxylase components--B = putida redoxin, A = putida reductase flavoprotein containing fraction.

Excess thiol and disulfide, derived principally from β-mercapto-ethanol of the buffer, can be removed from the ferroprotein by dialysis for 4 hours versus two changes M/20 NH_4HCO_3 without loss of activity to give a preparation with the oxidized and reduced spectra shown in Figure 2. The extinctions of the absorption bands are given in Table 2. The molecular weight

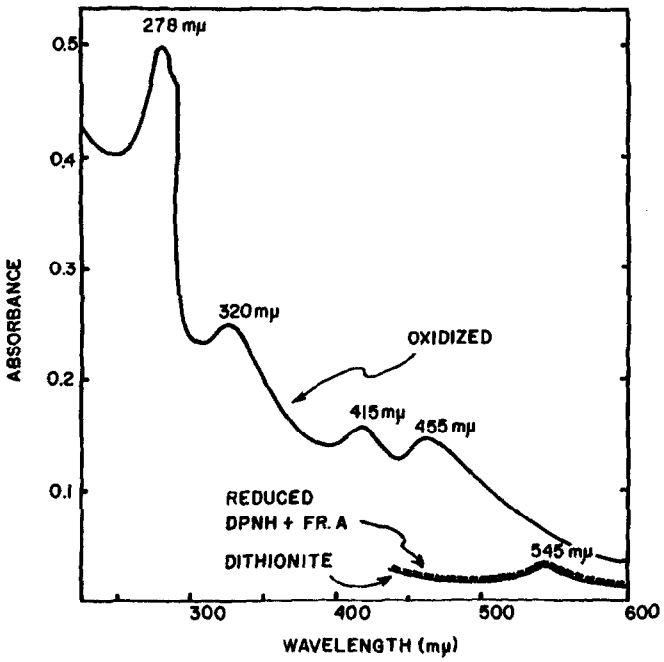


Figure 2. Absorption Spectra of Oxidized and Reduced Putida Redoxin

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of the protein as estimated from equilibrium centrifugation, amino acid analysis (nearest whole integer for two mols phenylalanine), and retention on gel filtration is 12 to 15 x 10^3. This protein contains 2 mols each of acid labile iron and sulfide; the alkyl sulfide (cysteine) content is yet to be established (Table 2).

The specific activity of the putida redoxin preparation can be increased by removal of the contaminating metals via formation of the alkyl mercury salt, passage through a chelex column, and replacement of the iron accompanied by displacement of the alkyl mercurial with dithiothreotol followed by adsorption and elution from a DEAE column (Tsibris, et al., 1967).

A DPNH dehydrogenase flavoprotein, E_1, which catalyzes the electron donor reaction for an earlier mixed function oxidation in the camphor pathway, that is, the ketolactonase system (Conrad, et al., 1965; Trudgill, et al., 1965), is present in fraction A recovered from DEAE and Biogel P60 columns. The

purified E_1 (Trudgill, et al., 1965) does not replace fraction A in the reduction of putida redoxin or hydroxylation. Since E_1 is specific for DPNH (inactive with TPNH) whereas the hydroxylation proceeds equally well with either DPNH or TPNH as reducing agent, one is led to conclude that the putida redoxin reductase flavoprotein must be separate from the E_1 flavoprotein. In preliminary experiments, adrenodoxin (Kimura and Suzuki, 1965) did not replace putida redoxin (E_B) in the bornanone methylene hydroxylase. However, in view of the lability of ferroproteins, a single experiment with Kimura's enzyme should not be taken as definitive until one establishes that this enzyme has not been degraded in our hands.

Data on the mechanism of the methylene hydroxylation reaction, the number and binding of the components, and purification of the fraction A component(s) will be published subsequently.

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