A 4-METHOXYBENZOATE MONOOXYGENASE SYSTEM FROM *PSEUDOMONAS PUTIDA*. CIRCULAR DICHROISM STUDIES ON THE IRON–SULFUR PROTEIN

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1. Introduction

A monooxygenase system (O-demethylating) was isolated from *P. putida* grown on 4-methoxybenzoate as the sole carbon source and could be separated into two components: an iron-containing flavoprotein with FMN as flavin component (= a conjugated iron–sulfur protein, electron paramagnetic resonance (EPR) at $\bar{g} \geqslant 1.95$) and a second iron–sulfur protein [1–4]. The iron-containing flavoprotein acts as the NADH-iron–sulfur protein oxidoreductase, whereas the iron–sulfur protein probably acts as the terminal oxidase capable of hydroxylating various substrates. Both the aliphatic CH-bond of different para- and meta-substituted benzoic acid derivatives and the aromatic ring are attacked by this enzyme [5].

The physicochemical properties of the iron-sulfur protein differ considerably from those of other well-known iron–sulfur proteins of the ferredoxin type, like adrenodoxin or putidaredoxin, which function as intermediate electron carriers between a flavin-dependent reductase and a cytochrome *P*-450 containing terminal oxidase [6,7].

The main difference is the following: the iron-sulfur protein of the 4-methoxybenzoate monooxygenase shows an EPR spectrum in the reduced state with a mean *g*-value† of $\bar{g} = 1.90$ [8] which is distinctly lower than that of the iron–sulfur proteins of the ferredoxin type. For the latter proteins mean *g*-values of $\bar{g} \geqslant 1.95$ are characteristic [9,10]. Differences also exist with regard to the redox potential. While the iron–sulfur proteins of the ferredoxin type possess low redox potentials, i.e. less than −235 mV [11], the redox potential of the iron–sulfur protein of the 4-methoxybenzoate monooxygenase was determined to be about +5 mV at pH 7.8.

In this communication we report on interactions of the iron–sulfur protein of the 4-methoxybenzoate monooxygenase with different substrates of this enzyme system. These findings confirm our hypothesis that this iron–sulfur protein is the terminal oxidase within this enzyme system.

2. Methods

Bacterial growth conditions and the preparation of the iron–sulfur protein have been described previously [8]. Protein was determined according to the method of Lowry et al. [12]. The circular dichroism (CD) measurements were performed with a Cary Model 61 spectropolarimeter at room temperature.

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† Mean *g*-value $\bar{g} = 1/3\,(g_x + g_y + g_z)$.

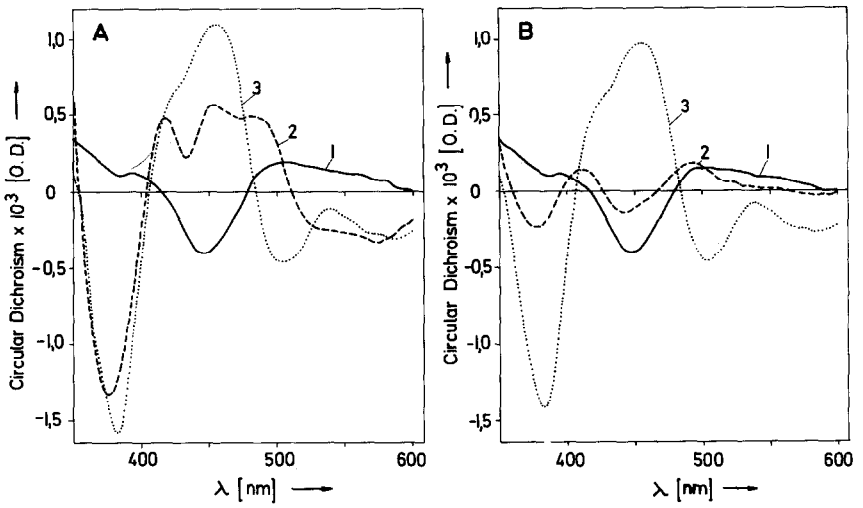

Fig. 1. CD absolute spectra of the iron–sulfur protein (g = 1.90) in the oxidized and reduced state in presence of different substrates. Experiments were done in dual compartment spectropolarimeter cells as described under 'Methods': A) Curve 1, the front compartment of the cuvette contained 21 mg oxidized iron–sulfur protein in a total volume of 3 ml 50 mM potassium phosphate buffer pH 8.0, the rear compartment contained the substrate 12 μmol 4-methoxybenzoate, 60 μmol 4-ethoxybenzoate, 60 μmol *N*-methyl-4-aminobenzoate, or 60 μmol 4-hydroxybenzoate in a total volume of 3 ml 50 mM potassium phosphate buffer pH 8.0. Curve 2, after mixing the oxidized iron–sulfur protein of the front compartment with the substrate solution of the rear compartment. Curve 3, after reduction of the enzyme–substrate solution in the 2 compartments with a minute amount of sodium dithionite; B) The experiments of the curves 1–3 were identical to those of fig. 1A but as substrate, 60 μmol benzoate, 60 μmol 4-methylbenzoate, or 60 μmol 4-trifluoromethylbenzoate, was used.

The spectropolarimeter cells used for absolute spectrum measurements were dual compartment silica cells. The light path in each compartment was 1.0 cm. 3 ml of the oxidized enzyme solution was pipetted into the front compartment of the cuvette. An equal volume of substrate solution in the same buffer was added to the rear compartment. After recording the CD spectra of the oxidized enzyme, the content of the two compartments were mixed and the spectra of the enzyme–substrate complex measured. The spectra of the reduced enzyme–substrate complex were determined after addition of a minute amount of sodium dithionite to the enzyme–substrate solution. Details of the assays are given in the legend to fig. 1.

3. Results and discussion

The optical spectra of the iron–sulfur protein show a small shoulder at 570 nm and absorptions at 322 nm and 455 nm in the oxidized form and at 375 nm, 412 nm and 518 nm in the reduced state. In contrast to other iron–sulfur proteins, there is no significant peak at 411 nm in the absorption spectrum of the oxidized protein at room temperature.

After addition of substrate to the oxidized enzyme only insignificant changes in the visible region of the absolute spectrum could be observed because of the lower sensitivity of this method. In the substrate difference spectrum, however, significant alterations were obtained, indicating interactions between enzyme and substrate [4].

To confirm these findings CD measurements were performed. We could show that the CD spectra of the oxidized iron–sulfur protein are also influenced by the addition of substrates. As presented in fig. 1, the CD studies showed that, depending on the substrate added, two different types of enzyme-substrate binding spectra are observed within the optical absorption region of the chromophore. These CD spectra are also quite different from those of other iron–sulfur proteins of the ferredoxin type [13]. The first type of CD spectra is obtained by the addition of, for instance, 4-methoxybenzoate, *N*-methyl-4-aminoben-

zoate, or 4-hydroxybenzoate to the oxidized iron–sulfur protein (fig. 1A). These substrates are characterized by an oxygen or nitrogen containing ligand in para-position to the carboxy group. Substrates without such a ligand (for instance: 4-methylbenzoate, 4-trifluoromethyl-benzoate, or benzoate) give rise to the second type of CD spectra (fig. 1B). Each of the two enzyme–substrate binding spectra is distinct from those of the oxidized and reduced enzyme. An enzyme–substrate complex could not be observed by CD studies with the enzyme after reduction by dithionite since the CD spectra of the reduced enzyme are identical in presence and absence of the substrate.

These spectral changes provide additional evidence that the iron-sulfur protein (2 Fe + 2 S††/spin) interacts with these substrates and therefore should be the terminal oxidase. The enzyme has a mol. wt. of 120 000 as estimated by filtration on Sephadex G 100, and is a dimer containing two subunits with a molecular weight of about 52 000 each as estimated by SDS disc electrophoresis.

Earlier findings [5] demonstrating that the iron–sulfur protein, which is highly unstable in the presence of oxygen, can partially be stabilized by addition of 4-methoxybenzoate, and the observation that the reduction rate of the iron–sulfur protein by the iron-containing reductase is increased in presence of substrates, confirm the conclusion that this protein is the terminal oxidase of this enzyme system.

In view of these results it is difficult to place the described enzyme in one of the four categories of iron–sulfur proteins recently proposed by the Commission on Biochemical Nomenclature [14].

†† *S* = acid labile sulfur.

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