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Spectral Properties of the Oxyferrous Complex of the Heme Domain of Cytochrome P450 BM-3 (CYP102)

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Here we describe for the first time the formation of a complex of reduced CYP102 (cytochrome P450 BM-3) heme domain with molecular oxygen. To stabilize the oxycomplex, the experiments had to be done under argon atmosphere at cryogenic temperatures (−25°C) in the presence of 50% glycerol. The spectral properties of this species were different from those of another P450-type autosuffisant enzyme, i.e., the neuronal nitric oxide synthase. On the contrary, the oxyferrous complex of CYP102 possesses spectral properties similar to those of complexes of microsomal cytochromes P450, e.g., CYP2B4.

The oxyferrous complex ($Fe(II)O_2^{\cdot\cdot}$ or $Fe(III)O_2^{\cdot}$) of cytochrome P450 is up to now the last known intermediate of its catalytic cycle leading to the production of hydroxylated substrates (1, 2). It has been trapped and characterized for several isoforms by the use of subzero temperature absorbance spectroscopy (3–5).

The interest in the structure and function of flavohemoproteins increased after the finding that the enzyme forming a key endogenous regulating factor, i.e., the nitric oxide (nitric oxide synthase, NOS), is also a flavo-hemoprotein consisting of a flavin-containing reductase domain and of a heme-containing oxygenase domain (6). The structure and function of the oxygenase domain resemble those of cytochrome P450, as there is a thiolate-ligated heme, the reaction requires molecular oxygen, and the reduction is achieved by NADPH as electron donor. The formation of the oxyferrous complex of this enzyme has been recently described (7, 8), showing that the spectral properties of this compound differ from those of a prototypical cytochrome P450, LM2 (CYP2B4) (7).

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In this paper, the formation of the oxyferrous complex of another flavo-hemoprotein, cytochrome P450 BM-3 (CYP102), is described. This enzyme consists also of the P450 (heme) domain which binds the substrates (long-chain fatty acids) as well as of the reductase domain, both of known structures (9). As this enzyme is often taken as a model of microsomal P450s (because of its functional similarity), it should also be elucidated whether its reaction intermediate (the oxyferrous complex) resembles that of the flavo-hemoprotein NO synthase or that of the microsomal P450s. The aim of this paper was therefore to stabilize the oxyferrous intermediate of the CYP102 heme domain and to compare its properties with spectral data of the analogous complex of neuronal NO synthase and of microsomal CYP2B4.

EXPERIMENTAL

CYP102 and its isolated heme domain (residues 1–142) were prepared by the expression of the plasmid constructs with the appropriate gene/subgene in *Escherichia coli*. Details of the procedure have been described in a previous publication (10). The purified enzyme has been stored at temperatures below −50°C.

For an experiment, the enzyme was diluted to 1 μM final concentration with 50 mM K/PO_4 buffer (pH 7.4) and glycerol (to prevent freezing of the sample, the experiments were done in mixed organic solvent, glycerol/water 1/1 (v/v)). This solvent did not change the spectral properties of the enzyme; in its presence, the enzyme retained its activity (10). The substrate (palmitate, 20 μM final concentration) was then added and the reduction was achieved by addition of dithionite (230 μM final concentration), prepared in the same solvent. Argon was bubbled through the solutions prior to their use for at least 30 min in a glove box; also, all the handling of the samples was done under argon atmosphere. The reduction was followed spectrally as it was rather slow (complete within 45 min) (11, 12). After that, the temperature was lowered to −25°C and 2–5 ml of precooled oxygen was bubbled through the solution with a gas-tight syringe.

Cytochrome P450 LM2 (CYP2B4) was prepared from phenobarbital-treated rabbits by standard procedures (7). The experimental conditions used for low-temperature measurement were the same as those used for CYP102.

Neuronal nitric oxide synthase (holoenzyme) was purified from baculovirus-infected insect cells. It contained tightly bound BH_4 in the

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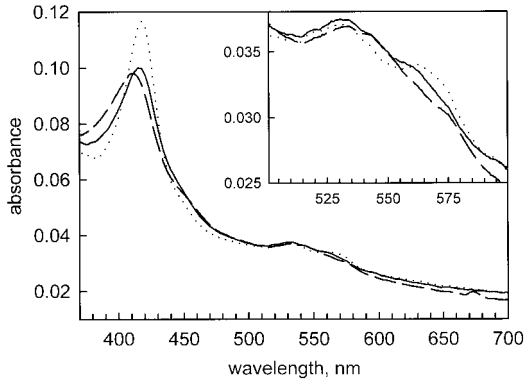

FIG. 1. Absolute spectra of the CYP102 heme domain at −25°C. Reduced form, dashed line; oxyferrous form, full line; reoxidized (ferric) form, dotted line. The spectrum of the oxyferrous form was taken immediately after addition of oxygen to the reduced form; the reoxidized spectrum was recorded 15 min later.

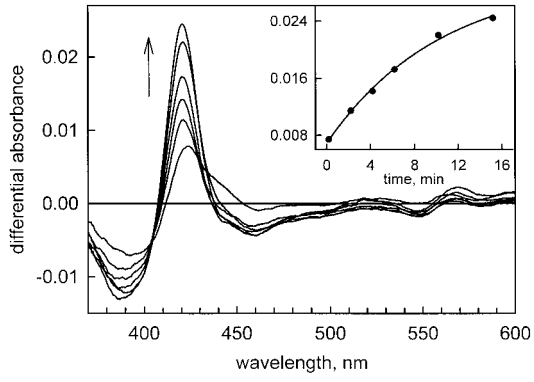

FIG. 2. Reoxidation of the Fe(II)O_2 complex following the spectral change at −25°C. Difference spectra obtained by subtraction of the spectrum of the reduced form from the spectrum of the reduced oxygenated form. Inset: Time course of absorbance change at 421 nm.

ratio of 0.5 per heme (7). The final concentration of the enzyme used for measurement was 3 μM in a 50 mM K/PO_4 buffer (pH 7.4) containing 1 mM CHAPS, 0.5 mM EDTA, and 1 mM 2-mercaptoethanol. The experimental procedure was the same as described for the CYP102 except that ethylene glycol was used as a cryosolvent.

The spectra were taken using a Cary 3E (Varian) spectrophotometer equipped with a double sample compartment, making it possible to work at low temperatures using a Haake F3-Q bath filled with ethanol. Formation of ice and condensation of water were prevented by a flow of cold dry nitrogen between the sample cuvette and double quartz windows, and a flow of warm nitrogen between the double windows.

RESULTS AND DISCUSSION

Under anaerobic and cryogenic conditions, it was possible to observe the reduced oxygenated complex of the CYP102 heme domain for a couple of minutes. The appearance of this new species resulted in a shift of the Soret band from the position characteristic for the reduced enzymes (411 nm) to higher wavelengths (416 nm, Fig. 1). Its subsequent reoxidation then led to a sharp Soret band at 418.5 nm.

Figure 2 documents the presence of the oxyferrous intermediate by difference spectra, obtained by subtracting the spectrum of the reduced form from that after addition of oxygen. The kinetics of reoxidation were followed at 421 nm (the λ_{max} of the difference spectrum). They could be fitted to a single exponential with a time constant of $k = 0.1$ min^{-1}.

The oxyferrous complex was detected only with the CYP102 heme domain, as the holoenzyme reoxidized quickly to the ferric resting state, as shown previously by Sevrioukova and Peterson (12). Under their experimental conditions (5°C, presence of arachidonic acid), the rate of autodecomposition (reoxidation) of the reduced oxycomplex of CYP 102 had been estimated to be on the order of $k = 6000$ min^{-1} (13), i.e., 4 to 5 orders of magnitude faster than under our conditions. In the same paper, the authors claimed that they were able to observe the formation of a new species which differed from the spectra of the oxycomplex of other P450s. As the spectral data were not given, we tried to stabilize and examine the properties of the reduced oxygenated complex of CYP102. The comparison of data obtained with P450 LM2 (CYP2B4) and with the neuronal NO synthase is given in Fig. 3. For the oxyferrous complex of the NO synthase, we followed also the kinetics of reoxidation, which gave a first-order rate constant of 0.15 min^{-1}, well comparable to the value obtained for the CYP102.

The difference spectra show a minimum at about 392 nm followed by a maximum between 420 and 430 nm, characteristic for all oxyferrous complexes; however, the complex of nitric oxide synthase exhibits a clear minimum at about 458 nm (see also (7)), which is not seen in the spectra of the CYP102 or microsomal P450 (CYP2B4). The origin of this difference has been discussed in (7) and it has been suggested to stem from the properties of the reduced state of the NO synthase rather than from its oxygen complex. As it is present in

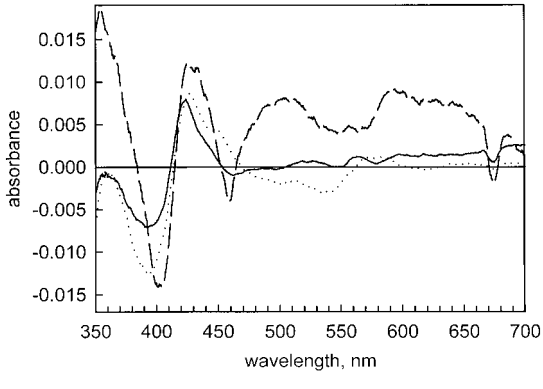

FIG. 3. Comparison of the of oxyferrous forms of different P450 enzymes and of neuronal NO synthase. The difference spectra were constructed by subtracting the spectra of the reduced enzymes from that of their oxyferrous forms. CYP102 (1 μM), full line; CYP2B4 (1 μM), dotted line; nNOS (3 μM), dashed line.

both the spectra of the holoNOS and of the oxygenase domain alone, it seems to be characteristic for this particular enzyme (NO synthase) and not for the flavo-hemoproteins in general (in such a case, it should also appear in the spectra of oxyferrous CYP102).

The similar spectral properties of the last known reaction intermediate, i.e., of the oxyferrous complex, suggest that the flavo-hemoprotein CYP102 (BM-3) is a better model for microsomal P450s than for other flavo-hemoproteins, such as NO synthase.

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