Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 162-168

ON THE DOMAIN STRUCTURE OF CYTOCHROME P450 102 (BM-3): ISOLATION AND PROPERTIES OF A 45-kDa FAD/NADP DOMAIN

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Received June 17, 1994

SUMMARY: Cytochrome P450 102 is a catalytically self-sufficient monooxygenase isolated from barbiturate-induced *Bacillus megaterium*. The enzyme contains FAD, FMN, and heme in a single polypeptide chain of 1048 residues, and each of the cofactors is believed to be located in a separate domain. In the present study we have used exhaustive endogenous proteolysis to produce a 45 kDa fragment of the cytochrome. This fragment bound the 2',5'-adenosine diphosphate moiety of NADP(H) strongly, with approximately the same dissociation constant as in the native enzyme, and contained only FAD (0.93 equivalents per polypeptide, ε_{453nm} = 11,200 $M^{-1}cm^{-1}$). Reduction of the flavin by sodium dithionite proceeded quite slowly to yield $FADH_2$, but no stable semiquinone species was produced upon air re-oxidation. In contrast, NADPH rapidly reduced this FAD/NADP(H) domain aerobically to produce the FADH• semiquinone radical. At a 75:1 molar ratio of the FAD/NADP(H) domain to the P450 102 heme domain, no laurate hydroxylase activity was observed. Gas-phase sequence analysis showed the presence of two major sequences beginning at Phe^{646} (403 residues, MW 45,033) and Asp^{652} (397 residues). These data are in agreement with the crystal structures of related enzymes and closely define the boundary of the FAD/NADP$^+$ domain in P450 102.

Cytochrome P450 refers to a superfamily of versatile oxidative enzymes found in animals, plants, and microbes (1-6). In mammals, the monooxygenase system of the endoplasmic reticulum is composed of NADPH-cytochrome P450 reductase (M_r ~78,000) and various isoforms of cytochrome P450 (M_r ~56,000). The P450 hemeproteins serve to metabolize a wide variety of endogenous and foreign compounds in the presence of molecular oxygen and reducing equivalents from the reductase. The reductase contains one equivalent each of FAD and FMN (7,8) and utilizes NADPH as the proximal electron donor. Electron transfer is understood to proceed NADPH → FAD → FMN → heme (8-10).

Cytochrome P450 102 (BM-3), from the bacterium *Bacillus megaterium*, is a catalytically self-sufficient monooxygenase known to model the mammalian microsomal P450 system (11,12). Sequence alignments reveal that P450 102 is structurally homologous to

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The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/94 $5.00

mammalian P450s, P450 reductases, and nitric oxide synthases (13,14). In relation to the mammalian P450s, the strongest structural homology exists with the fatty acid omega hydroxylases. Accordingly, it has been found that P450 102 efficiently catalyzes the ω-2 oxidation of fatty acids in the presence of NADPH and molecular oxygen (11,15-17); however, the enzyme can use a variety of substrates, analogous to the behavior of the microsomal P450 monooxygenase system (15).

P450 102 contains FAD, FMN, and heme in a single polypeptide chain of 1048 residues (holoenzyme M_r ~119,500) (14,15,18). Sequence alignments and proteolysis studies have shown that each of the cofactors resides in a separate domain (9,12,19-21). FAD and NADP(H) are bound near the COOH-terminus, FMN is bound centrally in the sequence, and heme, substrate, and oxygen are bound by the heme-domain in the NH_2-terminal region of the primary structure. Tryptic proteolysis results in cleavage of the polypeptide at Arg^{471} to produce two fragments, one of which contains FAD and FMN (M_r 66,000) and the other contains heme (M_r 55,000) (19). The reductase/flavin domain can be cleaved further at Lys^{593} to produce an M_r 52,000 fragment that contained FAD and only traces of FMN (9). This 52 kDA fragment was reported to possess the characteristics of a folded domain.

Our laboratory has been interested in cytochrome P450 102 as a tool to study structure-function relationships in the mammalian microsomal P450 monooxygenase system. In the present report, we have used exhaustive proteolysis to produce an M_r 45,000 fragment of P450 102. We have studied the sequence localization, cofactor binding, spectral properties, and electron-transfer properties of this fragment and show that this COOH-terminal piece of P450 102 closely defines the structural boundary for the FAD/NADP(H) domain of the enzyme.

MATERIALS AND METHODS

Growth and Harvest of *Bacillus megaterium*: Growth, induction with 5 mM sodium pentobarbital, harvest, and breakage of *Bacillus megaterium* ATCC 14581 were performed, as described (15).

Preparation of the 45 kDa FAD/NADP(H) Domain of Cytochrome P450 102: *B. megaterium* cell-free extract was treated with 1 μg DNAse (Sigma Chemical Co.) per mL solution overnight at 4°C. The resulting solution was submitted to dialysis against 0.1 M potassium phosphate, 0.1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, pH 7.4 at 4°C. After a few days of dialysis at 4°C the dialysate became cloudy due to bacterial growth. Following incubation for 7 days, the mixture was clarified by filtration (Millex GV, 0.22 μm). The yellowish filtrate was submitted to affinity chromatography on adenosine-2',5'-diphosphate agarose, as described (15). Intact P450 102 was purified as described (15). Protein concentration was estimated with the bicinchoninic acid (BCA) assay (22) with crystalline bovine serum albumin as standard. SDS-PAGE was carried out after the method of Laemmli (23), as modified (24).

Flavin Analyses and Extinction Coefficient Determinations: Flavin cofactors (FAD and FMN) were analyzed by high performance liquid chromatography with an isocratic mobile phase (92.5% of 50 mM ammonium acetate buffer, pH 4.5, to 7.5% of acetonitrile (v/v)) and a Beckman Ultrasphere ODS column (4.6 x 250 mm, 5 μ particles) at ambient temperature,

as described (15). Absorption spectra were recorded with a Kontron Uvikon scanning spectrophotometer at 25°C.

Kinetics Experiments: Reactions were carried out at 25°C in 0.1 M potassium phosphate buffer, pH 7.4 . Laurate hydroxylase activity was measured as the substrate-dependant rate of NADPH oxidation. Reaction mixtures contained 90 μM laurate and 0.1 mM NADPH; the following proteins, when present, were added at the indicated final concentrations: cytochrome P450 102 (14.6 nM), P450 102 heme domain (residues 1-470; 14.6 nM), and P450 102 M, 45,000 flavin domain (1.1 μM). Reactions were carried out at least in duplicate. Chemical reduction was by addition of small quantities of solid sodium dithionite (final concentration ~600 μM); reduction was monitored at 452.5 nm and at 30°C. Reduction by NADPH was accomplished with 29.4 μM coenzyme (Sigma Type I) at 25°C and at the same wavelength.

Protein Sequence Analysis: The 45 kDa flavin domain polypeptide was submitted to reversed-phase HPLC, as described (25). The fraction eluting at 55% of 3:1 acetonitrile:2-propanol (containing 0.1% trifluoroacetic acid) was collected and was brought to dryness under reduced pressure. Gas-phase sequencing was carried out on an Applied Biosystems Model 470A instrument, with on-line PTH-amino acid determination via a Model 120A analyzer. The sample was applied to the glass filter disk in a minimal volume of neat, distilled trifluoroacetic acid. Standard programs, as supplied by the manufacturer, were utilized for sequencing and PTH-amino acid analysis.

RESULTS AND DISCUSSION

Isolation of a 45 kDA Fragment of Cytochrome P450 102: The genus *Bacillus* is well-known for the ability to secrete non-specific proteases. We made use of this property to digest *B. megaterium* cell-free extract extensively. The cell-free extract was not totally acellular, and the few viable cells remaining were permitted to grow in the extract during dialysis and promote hydrolysis of proteins present. When the digested cell-free extract was applied to the affinity stationary phase, a yellow band was seen to bind tightly to the top of the column. This was in contrast to the golden-orange band that would normally have been seen with undigested cell-free extract (15). The bound material was eluted with chromatographic properties identical to intact cytochrome P450 102, thereby indicating similar binding affinity for the 2',5'-ADP portion of NADP(H). Because P450 102 binds to 2',5'-ADP agarose tighter than any other cellular protein (15), it was believed that the yellow material represented a flavin-containing portion of the monooxygenase responsible for pyridine nucleotide binding. The final preparation was free of affinity eluant (adenosine-2'-monophosphate), and SDS-PAGE analysis (data not shown) revealed the presence of two closely-spaced bands of nearly equal intensity. Relative to calibration standards, the molecular weight proved to be 45 kDa.

Flavin Composition and Optical Properties: Flavin analysis by reversed-phase HPLC showed the presence of 0.93 equivalents of FAD per 45 kDa polypeptide. No FMN was detected. The near stoichiometric flavin content and avid nucleotide binding properties suggested that the purified polypeptide represented a tightly-folded FAD/NADP(H) domain of P450 102. This assertion was further supported by the observation that the domain

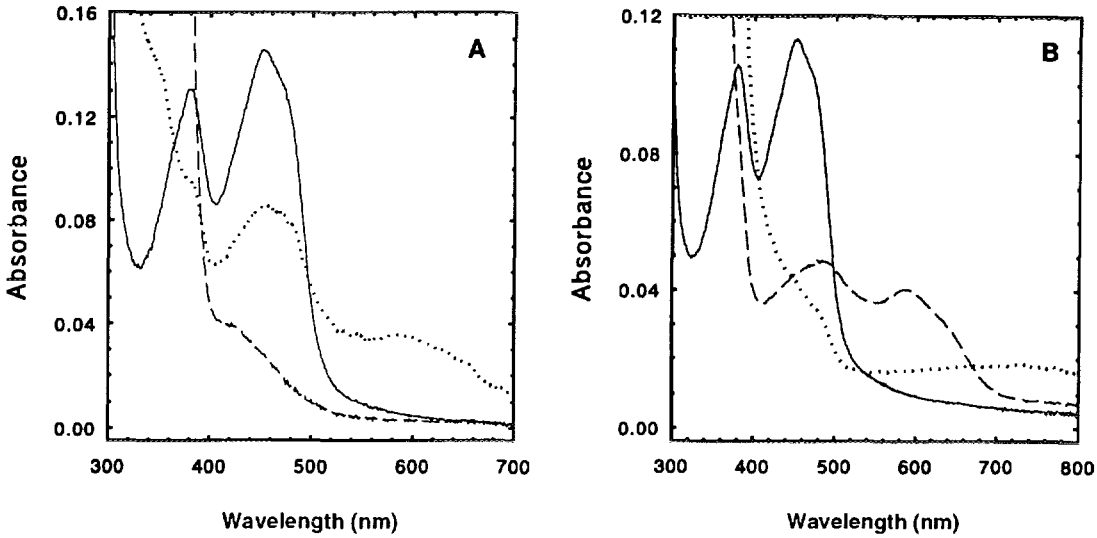

Fig. 1. Spectral Characterization of Redox States in the 45 kDa P450 102 FAD/NADP Domain. Panel A shows the oxidized spectrum (———) of the domain and that which resulted after extensive treatment with sodium dithionite (- - - -); the remaining spectrum (. . . .) was obtained transiently during air re-oxidation of the fully-reduced domain. Panel B shows the oxidized spectrum (———) and that which resulted immediately after addition of an excess of NADPH (- - - -); the remaining spectrum (. . . .) was obtained upon addition of an excess of sodium dithionite.

appeared to be a limit digest which resisted further proteolysis during preparation. Also, note that the ω-3 residue, W^{1046}, must still have been present due to its presumably essential role in FAD binding (13).

Optical spectra of the FAD/NADP(H) domain in various redox states are shown in Fig. 1. The oxidized spectrum was that of a typical flavoprotein with λ_{max} values at 380 and 452.5 nm; the ratio of $A_{452.5nm}/A_{380nm}$ = 1.08 and $A_{276nm}/A_{452.5nm}$ = 6.5 . Quantitative flavin and polypeptide analyses showed the extinction coefficient at 452.5 nm to be 11,200 $M^{-1}cm^{-1}$. The oxidized spectrum was similar to that reported for the 52 kDa FAD domain isolated by Peterson and coworkers (9) and the spectrum of the FAD moiety in rabbit microsomal NADPH-cytochrome P450 reductase (26). The rate of dithionite reduction of the FAD was rather slow with a $t_{½} \approx 2.3$ min at 30°C. The $FADH_2$ species showed a broad maximum at 420 nm, characteristic of a neutral hydroquinone. Upon re-oxidation, the semiquinone form was seen transiently, but was not air stable as is seen with mammalian P450 reductases (8). Ultimately, the spectrum of the oxidized flavoprotein was recovered. However, reduction of the 45 kDa FAD/NADP(H) domain with NADPH yielded significantly different results, as shown in Fig. 1B. Electron-transfer to FAD was extremely fast and the spectrum produced appeared to be the FADH• semiquinone, with λ_{max} values at 488 nm, 589 nm, and 645 nm (shoulder). Since NADPH is an obligate two electron donor, the FAD semiquinone must have resulted from either reaction with molecular oxygen or electron transfer to an equivalent of oxidized flavin domain. This interpretation is in harmony with data on the complete holoenzyme (27). Addition of dithionite to this mixture resulted in an unusual spectrum with

shoulders at 455 nm and 483 nm, and a very broad band at 728 nm. This long wavelength species appeared to indicate a charge-transfer interaction between reduced pyridine nucleotide and the FAD hydroquinone.

Reconstitution Studies of P450 102 Heme Domain and 45 kDa FAD/NADP(H) Domain: Relative to the complete P450 102 holoenzyme, the P450 102 heme domain showed only negligible laurate-dependent NADPH oxidation activity. The 45 kDa FAD/NADP domain exhibited some NADPH-oxidase activity, but this amounted to a rate only about 15% of the laurate-hydroxylase rate found with the intact cytochrome. This value was quite similar to the rate observed with rabbit tryptic microsomal NADPH-cytochrome P450 reductase. However, incubation of a 75:1 molar ratio of the 45 kDa FAD/NADP domain to P450 102 heme domain showed no significant increase in NADPH oxidation rate over the 45 kDa domain alone. The results of others (28) have shown that functional monooxygenation could be reconstituted if the proper redox partners were combined. Thus, the FAD/NADP domain, while retaining native capacities to bind FAD and NADPH, lacked the ability to promote electron transfer to the P450 102 heme domain. These findings are in agreement with the reported (9,10) vectorial nature of electron transfer in P450 102 and the microsomal P450 monooxygenase system: NADP $\rightarrow$ FAD $\rightarrow$ FMN $\rightarrow$ heme.

Sequence Analysis of the 45 kDa FAD/NADP Domain: Gas-phase sequencing of the 45 kDa domain showed the presence of two major sequences: Phe-Val-Asp-Ser-Ala-Ala-Asp-Met-Pro-Leu--- and Asp-Met-Pro-Leu-Ala-Lys-Met-His-Gly-Ala---. A minor sequence of Leu-Gln-Phe-Val-Asp-Ser-Ala-Ala-Asp-Met--- was also detected. With reference to the known primary structure of P450 102 (14), these sequences corresponded to cleavages at -Gln^{645}Phe-, -Ala^{651}Asp-, and -Ser^{643}Leu-, respectively, as indicated in Fig. 2. Assuming that

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                            ↓ ↓      ↓
102   (B. meg)     STLSLQFVDSAADMPLAKM..............HGA |FSTNVVASKE |LQQPGSARS |TRHLEIELP |K.E.ASYQEGD |HLGVI |PRN
                   640                                                                                          706
NOS    (Human)     YTSNVTWDPHHYRLVQD..SQPLDLSKALSSMHAKNV |FTMRLKSRQN |LQSPTSSRA |TILVELSCE |DGQGLNYLPGE |HLGVC |PGN
                   698                                                                                          779
FpT    (Human)     YELVVHTDIDAAKVYMGEMGRLKSYENQKPPFDAKNP |FLAAVTTNRK |LNQ.GTERH |LMHLELDIS |DSK.IRYESGD |HVAVY |PAN
                   244                                                                                          325
FpT   (Rabbit)     YELVLHTDIDVAKVYQGEMGRLKSYENQKPPFDAKNP |FLATVTTNRK |LNQ.GTERH |LMHLELDIS |DSK.IRYESGD |HVAVY |PAN
                   245                                                                                          326
FpD    (Human)                        RWPRAALALESPDI... |KYPLRLIDRE |IISHDT.R. |RFRFALPPP |QH.ILGLPVGQ |HIYLS |.A.
                                      1                                                                         56
NR    (Barley)     ....................VAGAPIALSSPRE... |KVPCRLVDKK |ELSHDV.R. |LFRFALPSS |DQ.VLGLPVGK |HIFVC |.A.
                                       643                                                                      697
FNR (Spinach)      ASDVEAPPPAPAKVEKHSKKMEEGITVNKFKP..KTP |YVGRCLLNTK |ITGDDAPGE |TWHMVF..S |HEGEIPYREGQ |SVGVI |PDG
                   58                                                                                           137
                                                         └───Fβ1───┘             └───Fβ2───┘             └─Fβ3─┘
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Fig. 2. Sequence Alignment of the Initial Portion of the Cytochrome P450 102 FAD/NADP(H) Domain with Homologous Regions of Related Flavoprotein Oxidoreductases. Abbreviations and database codes for the aligned proteins are 102, cytochrome P450 102 (PIR A34286); NOS, macrophage nitric oxide synthase (SwissProt P35228); FpT, NADPH-cytochrome P450 reductases (PIR A60557 and A25505); FpD, NADH-cytochrome b_5 reductase (PIR B26616); NR, nitrate reductase (PIR S17453); and FNR, ferredoxin-NADP reductase (SwissProt S00438). Boxed areas indicate secondary structures derived from the crystal structure of spinach FNR (13); regions prefixed with **F** are associated with FAD binding. Arrows (↓) show the first residues beyond the sites of proteolysis in cytochrome P450 102 determined in the present study. Dots (...) indicate gaps introduced to achieve optimal alignment.

the COOH-terminus was intact for each fragment, the expected molecular weights were calculated to be ≈45,000 g/mol. These data confirmed our hypothesis that the FAD/NADP domain contained an unaltered COOH-terminus. Furthermore, because the 45 kDa domain contained stoichiometric FAD and retained native NADP(H) binding characteristics, the sequence results demonstrated that the domain boundary must be no more than at Asp^{652} (397 residues). This finding was in excellent agreement with the structure of the 45 kDa domain predicted with reference to other FAD-binding oxidoreductases, notably flavodoxin-NADP reductase for which the three-dimensional structure is known (13; see Fig. 2). According to this alignment which is in general agreement with the work of others (29,30), the first β-stand of the FAD-binding β-barrel in P450 102 is Phe^{662}Ser-Thr-Asn-Val-Val-Ala-Ser-Lys-Glu^{671}. Our results showed that the fragment beginning at Asp^{652}, only 10 residues away from this sequence, was sufficient to function as a holo FAD/NADP(H) binding domain.

Thus, a precise definition for the limits of the FAD/NADP(H) binding domain of cytochrome P450 102 has been determined. Reconstitution and redox experiments showed that the 45 kDa FAD/NADP domain of P450 102 was not sufficient to function as a P450 reductase, but instead likely served as the initial electron-transfer complex. Future experiments are aimed at further elucidation of structure-function correlations in this important P450 model system.

ACKNOWLEDGMENTS

We wish to thank Dr. Thomas L. Poulos who kindly provided the recombinant cytochrome P450 102 heme domain used in these experiments. The fine technical assistance of Mr. Siamak Kazemi and Ms. Lynn Freck in the initial stages of this investigation is also gratefully acknowledged. This work was supported by NIH Grant GM10339.

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