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N-Terminal Processing and Amino Acid Sequence of Two Isoforms of Nitric Oxide Reductase Cytochrome P450nor from *Fusarium oxysporum*[1]

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Cytochrome P450nor (P450nor), a nitric oxide reductase involved in the denitrifying system of the fungus *Fusarium oxysporum*, revealed molecular multiplicity. Two isoforms of P450nor, termed P450norA (norA) and P450norB (norB), were isolated. They had distinct isoelectric points of 5.1 (norA) and 4.9 (norB). However, their catalytic, spectroscopic, and immunological properties were almost identical. Partial amino acid sequences, involving 263 amino acid residues of norA and 278 residues of norB among 404 and 402 residues, respectively, were determined. Corresponding sequences in the isoforms were identical, and all of the determined partial sequences of norA or norB coincided with the sequence deduced from the *CYP 55* gene or its cDNA. The amino acid sequence determination ruled out the possibility that there is a redox center in P450nor derived from amino acid residues, *e.g.*, quinonoid cofactors. The only difference between norA and norB was in their N-termini. The N-terminus of norA was a threonine residue, whereas that of norB was an *N*-acetylated alanyl residue and norB was shorter by 2 residues than norA. The results suggested that norA and norB may be the products of the same gene, but translated from different initiation codons. The hypothetical precursor of norA would have a presequence containing targeting and sorting signals for transportation to the intermembrane space of mitochondria. This is consistent with the results of a Western-blot analysis which showed that norA was recovered only in particulate fractions, whereas norB was in the soluble fraction. It is therefore likely that the intracellular localizations as well as the N-termini of norA and norB are different, owing to the differences in the translational initiation codons and co/post-translational processings.

Key words: amino acid sequence, amino-terminal processing, cytochrome P450nor, *Fusarium oxysporum*, P450nor isoforms.

Cytochrome P450nor (P450nor; P450 55A) is involved in fungal denitrification (*1, 2*) and acts as nitric oxide (NO) reductase (*3*). Unlike other P450s, P450nor is not a monooxygenase but catalyzes the following unique reaction without the aid of other proteinaceous components. Therefore, P450nor seems to receive electrons directly from NADH. Studies on the electron-transfer mechanism are under way (*4*).

$$2NO + NADH + H^+ \longrightarrow N_2O + H_2O + NAD^+$$

We have observed a characteristic intermediate spectrum upon reduction of the ferric P450nor-NO complex with NADH under anaerobic conditions, with a Soret peak at 444 nm (*4*). We speculated this intermediate arose from two-electron reduction of the ferric P450-NO complex. Though P450nor has a unique function, its primary amino acid sequence deduced from the cDNA shows that it belongs to the P450 superfamily (*5, 6*). It does not seem to contain a redox center other than 1 mol of protoheme, like other monooxygenase P450s.

P450nor was first isolated from *Fusarium oxysporum* (*3, 7*) and later from another denitrifying fungus, *Cylindrocarpon tonkinense* (*8*). It is of interest that P450nors of both fungi exhibit molecular multiplicity (*7, 8*). Two major isoforms of P450nor of *F. oxysporum*, P450norA (norA) and P450norB (norB), were initially termed as Fusarium P450A and Fusarium P450B, respectively (*7*), since their physiological functions had not then been identified. Two isoforms of *C. tonkinense* show a marked contrast in specificity for electron donors (*8*). P450nor1 is specific to NADH, whereas P450nor2 prefers NADPH to NADH as the electron donor. We isolated the respective cDNAs and genes for P450nor1 and P450nor2 (*9*), showing that they are the products of different genes. We expected that P450nor1 and P450nor2 of *C. tonkinense* would differ not only in electron-donor specificity but also in intracellular localization, because only the cDNA for P450nor1 contained a targeting-like presequence upstream of the N-termi-

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nus of the mature protein (*9*). In contrast, we have not been able to find out any difference in properties between norA and norB of *F. oxysporum*, except in isoelectric point (*7*). We could isolate only a single cDNA or gene for P450nor of *F. oxysporum* (*5, 10*). So it is of interest to clarify the mechanism of the molecular multiplicity of P450nor of *F. oxysporum*.

In this study we show that norA and norB of *F. oxysporum* differ in N-terminal structure as well as in intracellular localization. We determined partial amino acid sequences of norA and norB, which indicated that they are products of the same gene.

MATERIALS AND METHODS

Isolation of P450nor Isoforms—NorA and norB were purified from the denitrifying cells of *F. oxysporum* as described elsewhere (*3, 7*). Their homogeneity was checked by SDS-PAGE and isoelectric focusing. The latter was performed on Ampholine PAG plates pH 4.0–6.5 (Pharmacia Biotech, Sweden).

Protein Modification—Protoheme was removed from P450nor by the acid-acetone method (*11*). Three milliliters of concentrated P450nor solution (39 mg protein) was mixed with 30 ml of cold 0.3% HCl solution in acetone, and the mixture was left overnight at −20°C. The white precipitate resulting from centrifugation at 10,000×*g* for 30 min was washed twice with cold acetone and dried *in vacuo*. The obtained apoprotein was suspended in 2 ml of 1.0 M Tris-HCl (pH 8.6) containing 8.0 M urea and 0.2% EDTA. The suspension was flushed with helium gas and sealed with a rubber stopper. The apoprotein solution was reduced by incubating overnight with β-mercaptoethanol (0.1 ml) at room temperature under stirring, and then carboxymethylated by adding 1 ml of an iodoacetic acid solution (250 mg/ml, adjusted to pH 8.6 with KOH) and allowing the mixture to stand for 20 min in the dark under anaerobic conditions. Then the reaction mixture was dialyzed twice against 5 liters of distilled water. The modified apoprotein was lyophilized and stocked at −20°C until use.

Cyanogen Bromide Degradation—The modified protein (5 mg) was dissolved in 1 ml of 70% formic acid, and about 10 mg of cyanogen bromide (CNBr) (100-fold molar excess of Met residues) was added to the solution. The reaction mixture was incubated on a rotary shaker at 25°C for 24 h under anaerobic conditions (flushed with helium gas), and then diluted with 9 volumes of distilled water and lyophilized.

Tryptic Digestion—The *S*-carboxymethylated apoprotein or its peptides cleaved with CNBr (CNBr-peptides) were dissolved in a small volume of 8 M urea. The solution was diluted with 3 volumes of 50 mM potassium phosphate (pH 7.5), containing TPCK-trypsin (Sigma, USA) at an enzyme/substrate ratio of 1 : 100 (w/w). The digestion was performed at room temperature for 3 h.

Separation of Peptides—Peptides resulting from CNBr-degradation or tryptic digestion were separated at room temperature by reverse-phase liquid chromatography with a Tosoh HPLC (high-performance liquid chromatography) system consisting of CCCP pumps, a model SC-8010 system controller, and a model UV-8010 detector, equipped with a VP-304-1251 column (4.6×250 mm) for separation of CNBr-peptides or a VP-318-1251 column (4.6× 250 mm) for separation of tryptic peptides (Sensyu Kagaku, Tokyo). Peptides were adsorbed to the column equilibrated with distilled water containing 0.1% trifluoroacetic acid, and then eluted with a linear gradient of acetonitrile. Peptides were monitored by measuring the absorbance at 220 nm. Each isolated peptide was dried, dissolved in distilled water, and subjected to amino acid sequence determination with an Applied Biosystems 477A protein sequencer.

Structure Determination of the N-Terminus of norB—*S*-Carboxymethylated norB was dissolved in 0.01 N formic acid, and treated with pepsin at 37°C for 4 h at a pepsin/protein ratio of 1 : 100 (w/w). The reaction mixture was then passed through an SP-Sepharose (Pharmacia Biotech) column (10×25 mm) equilibrated with 0.01 N formin acid. The flow-through fraction was collected and lyophilized. The peptides thus obtained were further purified by HPLC (*12*) to obtain an acidic peptide fraction (P-1). A portion of the P-1 fraction was hydrolyzed with 6 N HCl at 110°C for 24 h, and the amino acid composition of the resulting hydrolysate was analyzed with a Hitachi L8500 amino acid analyzer. Another portion of the P-1 fraction was treated with acylamino acid releasing enzyme (*13*) (Takara, Japan), and an acylamino acid and the residual peptide were separated by HPLC. The structure of the acylamino acid fraction was analyzed by the use of an electron impact ionization mass spectrometer, model Hitachi M-80B.

Subcellular Fractionation—The denitrifying cells of *F. oxysporum* (10 g in wet weight) was disrupted on a cold mortar, as reported (*14*), by grinding with quartz sand (10 g) and 10 ml of a sucrose medium (0.8 M sucrose, 2 mM EDTA, 0.1% bovine serum albumin, 3 mM phenylmethylsulfonyl fluoride, 3 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 10 mM Tris-HCl, pH 7.2). The slurry was mixed with another 30 ml of the medium and centrifuged at 1,500×*g* for 15 min to remove quartz sand and undisrupted cells. The resulting supernatant was fractionated by differential centrifugations firstly at 12,000×*g* for 30 min, and secondly at 150,000×*g* for 45 min. The 12,000×*g* precipitate (large particle fraction) was washed twice with the sucrose medium. Then the large particle fraction and the 150,000×*g* precipitate, which usually contains microsomes, were each exposed to hypotonic treatment by incubating in a medium consisting of 0.4 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.2 for 24 h, and centrifuged at 150,000×*g* for 45 min. The resulting supernatants were analyzed by isoelectric focusing and immunoblotting with anti-P450norA polyclonal antibodies (*5*).

RESULTS

Purification and Characterization of P450nor Isoforms of F. oxysporum—A highly purified P450nor preparation with a specific content of 20 nmol/mg, which was obtained by purification at pH 7.2 (*3*), contained several isoforms. They could be separated by further purification at pH 6.0, as reported (*7*). Two major components of P450nor isoforms, norA and norB, were thus isolated. The pI values of norA and norB were 5.1 and 4.9, respectively (Fig. 1). The isoforms could not be immunologically discriminated (Fig. 1). The catalytic turnover numbers of the isoforms for NADH-NO oxidoreductase activity were almost identical

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irrespective of the assay conditions employed (data not shown). Absorption spectra of ferric, ferrous, and CO-bound ferrous forms (*3, 7*) were also identical (data not shown). So far as examined, therefore, we could not find any difference between norA and norB except for the slight difference in pI value. As shown in Fig. 2, the elution profiles on HPLC of CNBr-peptides of both isoforms were also almost identical, except for some minor peaks.

Determination of Amino Acid Sequences—Partial amino acid sequences of norA and norB were determined utilizing

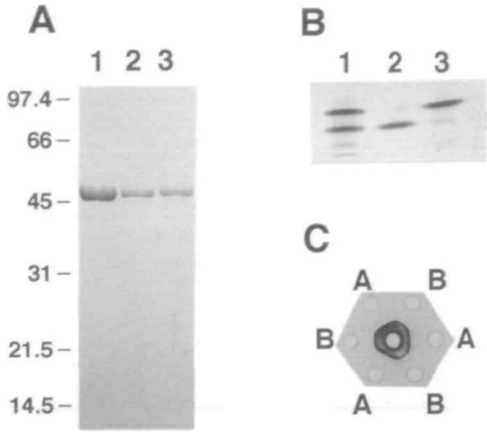

Fig. 1. **Comparison of purified P450nor isoforms.** A: SDS-PAGE. Lane 1, purified P450nor preparation before separation of isoforms (20 μg protein); lane 2, isolated norB (10 μg); lane 3, isolated norA (10 μg). B: Isoelectric focusing. Each lane corresponds to the same sample as in A. Protein, 2 μg each. C: Double immunodiffusion test. Each well contained anti-norA antibody (center), norA (A), or norB (B). The antibody was previously prepared (*5*).

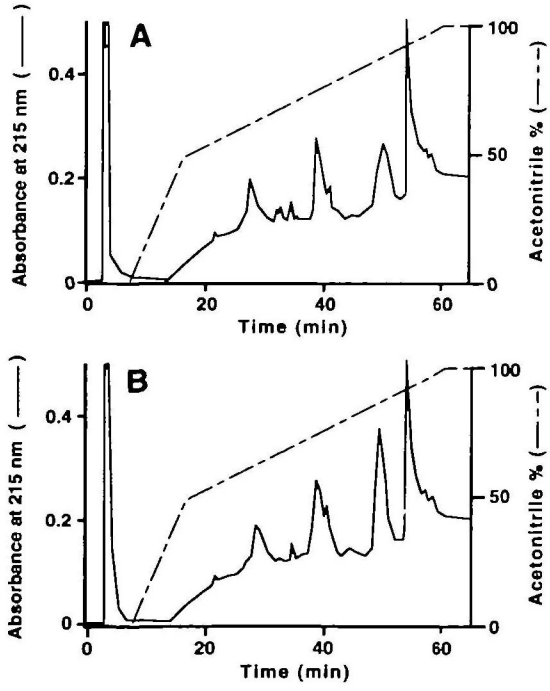

Fig. 2. **The CNBr-peptide map of P450nor isoforms.** CNBr-peptides of norA (A) or norB (B) were prepared and analyzed by HPLC as described in "MATERIALS AND METHODS."

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cDNA 1 MASGA PSFPF SRASG PEPPA EFAKL RATNP VSQVK LFDGS LAWLV TKHKD
P450A TMASGA PSFPF SRASG PEPPA EFAKL RATNP ----- LFDGS LAW-- ----D
P450B  #SGA PSFPF SRASG PEPPA EFAK- -ATNP VSQVK ----- ----- -----

  51 VCFVA TSEKL SKVRT RQGFP ELSAS GKQAA KAKPT FVDMD PPEHM HQRSM
     VCFVA T---- ----- -QGFP ELSAS GK--- ----- ---*D PPEH* HQRS*
     ----- ----- ----- ----- ----- ----- -AKPT FVDMD PPEHM HQRSM

 101 VEPTF TPEAV KNLQP YIQRT VDDLL EQMKQ KGCAN GPVDL VKEFA LPVPS
     VEPTF TPEAV KNLQP YIQRT ----- --*KQ KG@AN GP--- --EFA LPVPS
     VEPTF TPEAV K---- ----T VDDLL EQMKQ KG@AN GPVDL VKEFA LPVPS

 151 YIIYT LLGVP FNDLE YLTQQ NAIRT NGSST AREAS AANQE LLDYL AILVE
     YIIYT LLGVP FNDLE YLT-- ----- ----- ----- ----- ----- -----
     YIIYT LLGVP FNDLE YLTQQ NAIR- ----- --EAS AANQE LLDYL AILVE

 201 QRLVE PKDDI ISKLC TEQVK PGNID KSDAV QIAFL LLVAG NATMV NMIAL
     ----- ----- ---L@ TEQVK PGNID K---- ----- ----- ----V N*IAL
     QRLVE PKDDI ISKL@ TEQVK PGNID K---- ----- ----- ----- --IAL

 251 GVATL AQHPD QLAQL KANPS LAPQF VEELC RYHTA SALAI KRTAK EDVMI
     GVATL AQHPD QLAQL KA--- ----- -EEL@ RYHTA SALAI KR--- ---*I
     GVATL AQHPD QLAQL KANPS LAPQF VEEL@ RYHTA SAL-- ----- ----I

 301 GDKLV RANEG IIASN QSANR DEEVF ENPDE FNMNR KWPPQ DPLGF GFGDH
     GDKLV RANEG IIASN QSANR DEEVF ENPDE FN*NR KWPPQ DPLGF GFGDH
     GDKLV RANEG IIASN QSANR DEEVF ENP-- ----- ----- ----- -----

 351 RCIAE HLAKA ELTTV FSTLY QKFPD LKVAV PLGKI NYTPL NRDVG IVDLP
     R@IAE HLAKA ELTTV FSTLY QKFPD LKVAV ----I NYTPL NR--- -----
     -@IAE HLAKA ELTTV FSTLY QK--- --VAV PLGKI NYTPL N-DVG IVDLP

 400 VIF 403
     ---
     VIF
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Fig. 3. **Amino acid sequences of P450nor isoforms.** The sequences of norA and norB were determined as described in "MATERIALS AND METHODS." #, *N*-acetylalanyl residue; *, modified methionyl residue; @, *S*-carboxymethylcysteinyl residue; –, not determined.

TABLE I. **N-terminal amino acid sequence of intact norA.** The first 12 residues from the N-terminus was determined. Data were from two independent experiments (3.0 and 0.5 nmol of norA were used, respectively).

Cycle	PTH-amino acid (yield) (nmol)		cDNA
	Experiments		
	1	2	
1	Thr (0.594)	Thr (0.105)	–
2	Met (2.083)	Met (0.248)	Met
3	Ala (1.969)	Ala (0.301)	Ala
4	Ser (0.561)	Ser (0.043)	Ser
5	Gly (1.447)	Gly (0.167)	Gly
6	Ala (1.732)	Ala (0.227)	Ala
7	Pro (0.818)	Pro (0.122)	Pro
8	Ser (0.130)	Ser (0.024)	Ser
9	Phe (0.708)	Phe (0.095)	Phe
10	Pro (0.359)	Pro (0.067)	Pro
11	Phe (0.384)	Phe (0.075)	Phe
12	Ser (0.120)	Ser (0.014)	Ser

TABLE II. **Amino acid composition of the N-terminal acyl peptide (P-1) derived from norB.**

Amino acid	Yield (nmol)	Number of residues
Ser	3.5	2
Gly	2.1	1
Ala	4.2	2
Phe	2.1	1
Pro	2.2	1

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their tryptic and CNBr-degraded peptides, as described in "MATERIALS AND METHODS." Sequences of 263 among 404 amino acid residues of norA, and 278 among 402 residues of norB were determined, as shown in Fig. 3. All of the sequences could be aligned on the amino acid sequence deduced from the cDNA (*5*), and the determined amino acid sequences of the peptides completely coincided with that deduced from the cDNA. These results indicate that norA and norB are the products of the same gene, *CYP 55A1*. When both sequences are combined, 358 residues among 404 (or 402) amino acid residues of P450nor had been determined by the amino acid sequencing.

P450nor of *F. oxysporum* contains 8 Tyr and 2 Trp residues in its total amino acid sequence deduced from the cDNA and these might be modified by dimerization or oxidation to form a quinonoid cofactor such as topaquinone (TPQ) or tryptophan-tryptophan quinone (TTQ) (*15*). However, all of the Tyr and Trp residues were identified in the peptide sequencing, which ruled out the possibility that such a redox center exists in P450nor.

N-Terminal Structures of P450nor Isoforms—The N-terminal amino acid sequence of intact norA was determined (Table I), and was longer by two amino acid residues than that previously determined (*5*). The N-terminal Thr residue lay on the N-terminal side of the Met residue that was previously thought to correspond to the initial codon of the open reading frame (ORF) (*5*). This indicates that the Met residue is not the starting residue of the ORF, at least in the case of norA.

In contrast, we could not detect any PTH-amino acids by the Edman degradation of norB, suggesting that its N-terminus is blocked. In such a case an acidic peptide is expected to form after proteolysis. A candidate (P-1) was isolated after the pepsin-digestion of norB, as described in "MATERIALS AND METHODS." The amino acid composition of P-1 (Table II) was consistent with the sequence of the heptapeptide initiated from the second Ala residue in the previously postulated ORF. P-1 was treated with an acylamino acid-releasing enzyme, and the resulting N-terminal amino acid residue was identified as *N*-acetylalanine by HPLC comparison with an authentic sample (*12*) and by mass-spectral (Fig. 4) analyses. The remaining portion of P-1 was sequenced (Table III), and its sequence coincided with that of the hexapeptide expected from the cDNA. So the N-terminus of norB was determined to be as indicated in Figs. 3 and 5.

Subcellular Fractionation—The denitrifying cells were disrupted under hypertonic conditions and the cell-free extracts were fractionated by differential centrifugation. Each fraction was examined for the presence of P450nor isoforms. As shown in Fig. 6, norB was detected only in the soluble fraction, whereas the large particle and microsomal fractions contained only norA. Without the hypotonic treatment, norA could not be detected in the precipitate fractions (data not shown).

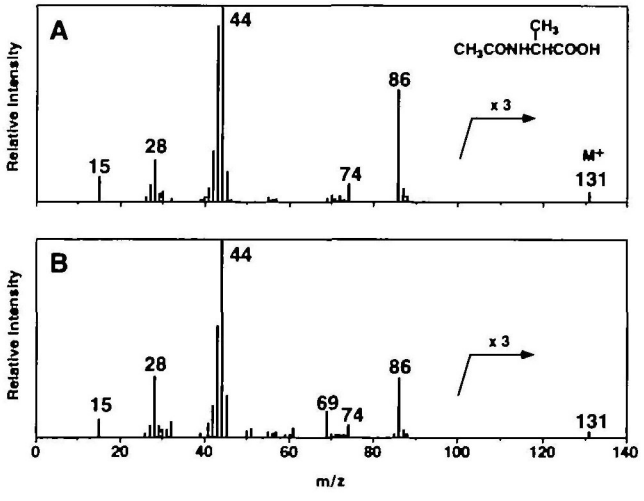

Fig. 4. **Mass spectrum of the N-terminal amino acid from norB.** A: Authentic *N*-acetylalanine. B: The N-terminal acylamino acid of norB.

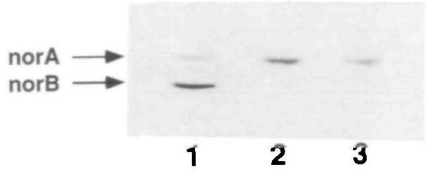

Fig. 6. **Distribution of P450nor isoforms among subcellular fractions.** P450nor isoforms in each subcellular fraction were detected by immunoblotting after isoelectric focusing. Lanes: 1, soluble (150,000 × *g* supernatant) fraction (20 μg protein); 2, large particle (12,000 × *g* precipitate) fraction (7 μg protein); 3, microsomal (150,000 × *g* precipitate) fraction (5 μg protein). The precipitate fractions were analyzed after hypotonic treatments ("MATERIALS AND METHODS").

TABLE III. **Amino acid sequence of the residual peptide generated by the digestion of P-1 with the acylamino acid releasing enzyme.**

Cycle	Amino acid	Yield (nmol)
1	Ser	2.04
2	Gly	4.96
3	Ala	6.91
4	Pro	5.20
5	Ser	1.34
6	Phe	2.49

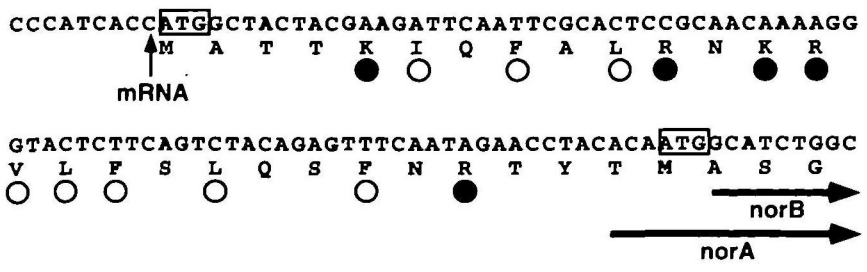

Fig. 5. **Nucleotide and deduced amino acid sequences of *CYP 55A1* gene around the putative presequence.** Boxes indicate possible translational initiation sites. Bold arrows indicate the N-termini of mature norA and norB, respectively. mRNA indicates the transcriptional initiation site previously determined (*10*). Open circle, hydrophobic amino acid residue; closed circle, positively charged amino acid residue.

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DISCUSSION

Two major isoforms of P450nor of *F. oxysporum*, norA and norB, were shown to be products of the same gene. So far as examined, they could not be discriminated in enzymatic and spectroscopic properties or in primary structure, except in the structure of N-terminus. The N-terminus of norB was acetylated, whereas that of norA was free. This can explain the slight difference between them in pI value. Amino acid sequencing detected all of the Tyr and Trp residues that are encoded in the ORF of cDNA. The result is important in that it rules out the possibility that P450nor contains a redox center derived from Tyr or Trp residues. Nitroblue tetrazolium (NBT)/glycinate assay (*16*) was also negative in the case of P450nor (data not shown). The present results along with the previous metal analysis (*7*) imply that P450nor contains only 1 mol of protoheme as a redox center, like other monooxygenase P450s, although P450nor seems to receive electrons directly from NAD(P)H (*3, 4, 8*).

In a previous study (*5*) we reported that the N-terminus of norA is the Ala residue just downstream of the second Met residue. The discrepancy between the previous and present results might be a result of the difference in protease inhibitors added when the cells were disrupted. Only phenylmethylsulfonyl fluoride was employed in the previous studies to block internal peptidases; if it had not been completely effective, apparent isoforms of P450nor might have been generated (*7*). There is another in-frame ATG codon in the gene (*10*), 81 bases 5′-upstream from the previously postulated initiation codon discussed above, as shown in Fig. 5. The initiation codon for norA is thus expected to be the first, upstream ATG codon. The hypothetical N-terminal presequence consists of 26 amino acid residues with a cluster of positively charged amino acid residues in the N-terminal half followed by a hydrophobic stretch in the C-terminal half. The sequence of the putative presequence is characteristic of the targeting and sorting signals for mitochondrial proteins that are localized in the intermembrane space (*17*).

The N-terminus of norB was identified as an *N*-acetylalanyl residue. This is consistent with the general observation that many cytosolic proteins which are N-terminally acetylated contain an Ala or Ser residue at the N-terminus (*18, 19*). The *N*-acetylation is considered to occur by a post- or co-translational modification after removal of the initial Met residue. So it seems that norB is a cytosolic protein and is translated from the second initiation codon in the putative ORF of the gene. These considerations were further supported by the distribution of norA and norB among subcellular fractions (Fig. 6). As was expected, norB was recovered only in the soluble fraction, whereas norA was mainly recovered in particulate fractions. A portion of norA was recovered in the microsomal fraction. This might be because fungal mitochondria become smaller when the cells are exposed to denitrifying conditions (Suzuki, S., Takaya, N., Maruo, F., and Shoun, H., unpublished data). It can be concluded from these results that norA and norB of *F. oxysporum* are the products of the same gene. However, their translational initiation points are different, which results in differences in their intracellular localization and in the structure of their N-termini. Examples of both mitochondrial and cytoplasmic proteins being encoded by a single nuclear gene are known with several yeast proteins (*20-23*).

It is of interest to compare the present results with those on P450nor isoforms of *C. tonkinense*. We showed that two isoforms of P450nor of *C. tonkinense*, P450nor1 (nor1) and P450nor2 (nor2), are the products of different genes, *CYP 55A2*, and *CYP 55A3* (*9*). Their intracellular localizations might be different; the ORF of nor1 contains a targeting-like presequence, whereas that of nor2 does not. We recently showed that the denitrifying systems of *F. oxysporum* and *C. tonkinense* are localized in mitochondria (*14, 24*). The results also indicate that one of two isoforms of P450nor, norA in the case of *F. oxysporum* and nor1 in the case of *C. tonkinense*, is localized in mitochondria. The other isoform of P450nor, norB (*F. oxysporum*), and nor2 (*C. tonkinense*), might be localized in the cytosol. The toxicity of NO might be so great that it must be scavenged both at mitochondria and in the cytosol.

Although P450nor is involved in denitrifying systems of fungi, it is not associated with the respiratory chain, unlike nitrate and nitrite reductases, since it receives electrons directly from NAD(P)H. It is likely to be a detoxifying enzyme. Another role of P450nor might be as an electron sink for anaerobic growth. Two isoforms of P450nor would be necessary for cells to be sufficiently protected from NO. It is intriguing that two isoforms of P450nor are produced in different ways in the cells of *F. oxysporum* and *C. tonkinense*. The specificity for electron donors, NADH and NADPH, of the P450nor isoforms also differs between the two fungal species. There is no difference in specificity between norA and norB of *F. oxysporum* (more specific for NADH), whereas nor1 and nor2 of *C. tonkinense* are more specific for NADH and NADPH, respectively. This implies some difference in anaerobic energy metabolism between the two fungal species. Further studies on the intracellular localization of the isoforms are under way utilizing spectroscopic and histochemical analyses.

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