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PARTICULAR ABILITY OF LIVER P450s3A TO CATALYZE THE OXIDATION OF N$^{\omega}$-HYDROXYARGININE TO CITRULLINE AND NITROGEN OXIDES AND OCCURRENCE IN NO SYNTHASES OF A SEQUENCE VERY SIMILAR TO THE HEME-BINDING SEQUENCE IN P450s

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ABSTRACT : Liver microsomes from rats pretreated with various inducers of P450 isoforms exhibit very different abilities to catalyze the oxidation of N$^{\omega}$-hydroxy-L-arginine (NOHA) by NADPH and O_2 with formation of citrulline and nitrogen oxides. Treatment of rats with dexamethasone, a classical inducer of P450 3A, leads to a spectacular 7-fold increase of the activity found for untreated rats, while induction by phenobarbital causes a much lower increase of this activity and induction by 3-methylcholanthrene or clofibrate decreases it. Specific inhibitors of P450s3A as troleandomycin and dihydroergotamine strongly inhibit NOHA oxidation whereas metyrapone, an inhibitor of other P450 subfamilies, was without effect. These data show the particular ability of P450s of the 3A subfamily to catalyze the second step of the oxidation of L-arginine by NO synthases (NOS). This analogy between NOSs and P450s3A is further substantiated by a protein sequence comparison which shows that a 9-aminoacid segment present in all NOSs exhibits a strong similarity with the sequence mainly responsible for heme binding in P450s3A which is well conserved in all P450s. This segment contains all the structural factors which are thought to be crucial for heme binding in P450s.

The endogenous formation of nitric oxide, NO, has become recently a subject of considerable interest because of its multiple biological functions (1). A class of enzymes called NO synthases (NOS) catalyze the formation of NO from L-arginine in two steps. The first one is an N-oxygenation of L-arginine with consumption of NADPH and O_2 and formation of N$^{\omega}$-hydroxy-L-arginine (NOHA), and the second one an oxidative cleavage of the C=N bond of NOHA which also consumes NADPH and O_2 and leads to citrulline and nitrogen oxides including NO (2) (eq. 1). During these last few months, two kinds of results establishing a strong relationship between NOSs and P450s have been

Abbreviations : P450s : the updated recommended nomenclature for P450s (10) is used; P450cam is the trivial name for P450 101 ; NOS : nitric oxide synthase ; NOHA : N$^{\omega}$-hydroxy-L-arginine ; TAO : troleandomycin ; DEX : dexamethasone ; 3MC : 3-methylcholanthrene ; PB : phenobarbital ; CLO : clofibrate ; SKF 525A : 2-diethylaminoethyl-2,2-diphenylvalerate.

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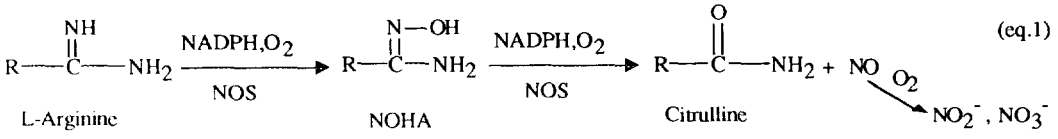
published. The first ones showed that brain and macrophage NOS were P450-like hemeproteins the iron(II)-CO complexes of which exhibited a Soret peak around 450 nm (3-6), whereas the second ones indicated that some liver microsomal P450s involved in the metabolism of xenobiotics catalyzed the second step of the reaction catalyzed by NOSs which is the monooxygenation of NOHA to citrulline and nitrogen oxides including NO (eq. 1) (7). In a more general manner, liver microsomal P450s were found to catalyze the oxidative cleavage of C=N bonds of compounds containing a -C(NH_2)=NOH function by NADPH and O_2 with formation of the corresponding derivatives containing a -C(NH_2)=O function and of nitrogen oxides (7,8). However, although the NOS carboxyl-terminal domain which contains binding sites for NADPH, FAD, and FMN has been found homologous to NADPH-P450 reductases (9), no sequence homology has been reported to date between NOS isoforms and P450s (4).



(eq.1)

In order to further precise the analogy between NOS and the various members of the P450 superfamily, we have studied the abilities of different liver microsomal P450s to catalyze the oxidation of NOHA to citrulline and nitrogen oxides, and compared the protein sequences of the NOS isoforms with those reported for P450s. This communication shows that the P450s of the 3A subfamily are particularly prone to catalyze this oxidation of NOHA. It also indicates the existence of an aminoacid sequence present in all NOS isoforms described so far which is very similar to that involved in heme binding in P450s3A and other P450s. These very analogous sequences of NOSs and P450s contain the structural components which are known to play a key role in heme binding by P450s.

MATERIALS AND METHODS

Chemicals : N^{ω}-hydroxy-L-arginine was synthesized according to a previously described method (11) using N^{δ}-benzyloxycarbonyl-L-ornithine (Sigma) as starting material and displayed physical and spectroscopic characteristics identical to those reported (11). SKF 525A was a gift from Smith, Kline and French laboratories. All the other chemicals used were of the highest purity commercially available.

Preparation of rat liver microsomes : Male Sprague-Dawley rats (200-250g) were provided laboratory chow and water *ad libitum*. After 10 days of adaptation, animals were treated either with 3MC (20 mg/kg, in corn oil, i.p. for 3 days), PB (80 mg/kg, in 0.9% saline, i.p. for 4 days), DEX (100 mg/kg, in corn oil, i.p. for 3 days), CLO (500 mg/kg, in corn oil, i.p. for 3 days) or TAO (500 mg/kg, in corn oil, i.p. for 3 days). Control animals were treated with corn oil (0.5 ml). Microsomes were prepared as reported (12) and stored at -80°C until use. Protein concentrations were determined by the method of Lowry (13) with bovine serum albumin as standard. Cytochrome P450 contents were determined as described by Omura and Sato (14).

Incubation procedures : Standard incubations (total volume:0.5 ml) were performed at 37°C in phosphate buffer (0.1M, pH 7.4, 0.1 mM EDTA) containing NOHA (100 µM) and

rat liver microsomes (0.5 nmol P450). After preincubation for 1 min at 37°C, the reaction was started by addition of 500 nmol NADPH. Incubations were stopped by adding 0.5 ml cold acetonitrile and cooling at 0°C before centrifugation at 3000 rpm for 20 min at 4°C. Formation of nitrite ions (15) and citrulline (16) were routinely measured by previously described spectrophotometric methods.

RESULTS AND DISCUSSION

Oxidation of NOHA by liver microsomes from rats treated with classical P450 inducers

It was previously reported that the oxidation of NOHA by NADPH, O_2, and rat liver microsomes with formation of citrulline and NO_2^- (one of the products of oxidation of NO *in situ*) was not catalyzed by any NOS which could have been present in these microsomes as N^{ω}-methyl- and N^{ω}-nitro-arginine, two good inhibitors of NOS, were unable to inhibit the reaction (7). This result is in agreement with previous literature data which showed the existence of an NOS activity in rat liver only after treatment of rats by immunostimulating agents like lipopolysaccharides (17). Moreover, this microsomal oxidation of NOHA was almost completely suppressed by classical inhibitors of P450s like CO and miconazole (7). In order to determine which liver microsomal P450s were involved in this oxidation of NOHA, the activities of liver microsomes from rats pretreated with classical inducers of the main xenobiotic-metabolizing P450 subfamilies (3MC, PB, DEX, and CLO as inducers mainly for the 1A, 2B, 3A, and 4A subfamily respectively(18)) were studied. As shown in Fig. 1, treatment of rats with 3MC or CLO led to NOHA

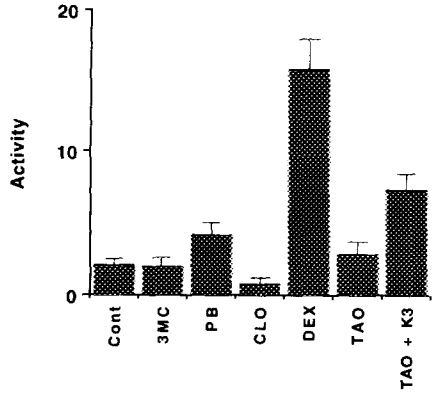


Fig.1. Effect of treatment of rats with several inducers on the NADPH-dependent microsomal oxidation of NOHA to citrulline and nitrogen oxides.

Activities in nmol NO_2^- formed per nmol P450 per 30 min ; mean values ± SD from 3 to 6 experiments (P450 contents of the different liver microsomes : 1.1, 2.0, 1.9, 2.4, 1.7 and 2.1 nmol P450 per mg protein respectively for control rats and rats treated with DEX, 3MC, PB, CLO and TAO). An almost identical variation of the activities as a function of rat induction was found upon measurement of citrulline.

The results obtained from TAO-treated rats correspond to the activity measured either directly on liver microsomes, where P450s3A are mostly present under an inactivated form due to a TAO-derived metabolite bound to P450 iron (TAO), or on the same microsomes but after their treatment with 2.5 eq. of ferricyanide (10 min at 0°C) in order to regenerate P450s3A under their catalytically active form (according to ref.21) (TAO + K_3).

oxidation activities slightly or markedly lower than that found for untreated rats. Treatment with PB led to a marked increase(2-fold) of this activity. However, the most spectacular increase (7-fold by comparison with untreated rats) was observed after treatment of rats with DEX. In fact, the level of microsomal activity towards NOHA appears related, at least from a qualitative point of view, to the previously reported amount of P450s3A in the different microsomes (18,19), which is relatively low in microsomes of untreated or 3MC-treated or CLO-treated rats, and increases either slightly or dramatically after treatment with PB or DEX respectively. Troleandomycin (TAO) is another strong inducer of P450s 3A in rats although these P450s are recovered in that case under an inactivated form with a TAO-derived metabolite strongly bound to P450 iron (20). This explains the relatively low activity observed with microsomes from rats treated with TAO (Fig. 1). As previously reported (20), it is possible to regenerate, at least in part, P450s3A under their catalytically active form after dissociation of the P450-iron-metabolite complex upon treatment of TAO-treated rat microsomes with ferricyanide. Accordingly, Fig. 1 shows that the activity of TAO-treated rat microsomes towards NOHA dramatically increased after preincubation of the microsomes with ferricyanide.

All these results indicate that P450s3A are particularly active for NOHA oxidation and could be mainly responsible for this reaction in rat liver microsomes. This is confirmed by results observed with various P450 inhibitors on NOHA oxidation by DEX-treated rat liver microsomes (Table 1). Metyrapone, a classical inhibitor of P450s 2B, was found inactive even when used at relatively high concentrations (100 μM) whereas two specific inhibitors of P450s3A, TAO and dihydroergotamine (21), were found to inhibit strongly NOHA oxidation (90 and 60% inhibition) at relatively low concentrations (20 μM). SKF

Table 1. Effects of various P450 inhibitors on the oxidation of NOHA by rat liver microsomes

Conditions	Activity[b]	(%)
Complete system[a]	16.0 ± 2	100
+CO ($CO:O_2$=1:3)	1.5 ± 0.2	9
+ metyrapone (10μM)	15.4 ± 2	96
" (100μM)	16.3 ± 2	102
+ TAO[c](10μM)	3.0 ± 0.6	19
" (20μM)	1.3 ± 0.3	8
+ dihydroergotamine		
(20μM)	6.6 ± 1.2	41
(50μM)	4.2 ± 0.8	26
+ SKF 525A (10μM)	16.3 ± 2	102
" (100μM)	2.2 ± 0.4	14

a) DEX-pretreated rat liver microsomes (1μM P450) + 100μM NOHA + 1mM NADPH in aerobic pH7.4 phosphate buffer ; b) nmol NO_2^- (nmol P450)$^{-1}$.(30 min)$^{-1}$; c) TAO was preincubated with the complete system without NOHA for 5 min before the addition of NOHA to start the reaction. We have checked that this preincubation of microsomes with NADPH alone for 5 min before NOHA addition did not change the activity.

525A, which is able to inactivate P450s3A by P450-iron-metabolite complex formation but less efficiently than TAO (M. Delaforge and D. Mansuy, in preparation), was not inhibitory at 10 μM but led to a 86% inhibition of NOHA oxidation at 100 μM.

Comparison of the aminoacid sequences of P450s and NOSs

The above data show that P450s from the 3A subfamily are particularly prone to catalyze the second step of the NOS-dependent oxidation of arginine. It was thus tempting to compare the aminoacid sequences of P450s3A (as well as of the other reported P450s) with those described so far for NOSs in order to find common sites either for the binding of NOHA or for the binding of the heme. In fact, the segments involved in the binding of substrates in the different P450s are generally ill-defined whereas the peptide responsible for heme binding is widely admitted (22). The peptide surrounding the proximal cysteinate in P450cam and the corresponding segment in rat P450 3A1 are shown in Figure 2. The structural factors which are important for heme binding by this proximal domain have been determined on the basis of the crystallographic structure of P450cam (23) and of the aminoacids that are conserved throughout the P450 superfamily (22). They include the presence of (i) an absolutely conserved cysteinate (Cys 357 in P450cam), bound to the heme iron (at position 8 of the sequence alignment in Figure 2), followed by a hydrophobic aliphatic aminoacid at position 9 and a glycine at position 10 which is also found in all P450s known to date

Role in heme binding	cysteinate protection			hairpin turn initiation		interaction with heme propionate		proximal cysteinate		close contact to the heme
P450 cam	**F**	G	H	**G**	S	**H**	L	C_{357}	**L**	**G**
P450 (consensus)	**F**	G/S	x	**G**	x	**R**	x	**C**	**hy**	**G**
P450 3A1	**F**	G	N	**G**	P	**R**	N	C_{443}	**I**	**G**
	.		:	.	:	:		:	:	:
mm NOS	**W**	R	N	**A**	P	**R**	-	C_{194}	**I**	**G**
baec NOS	**W**	R	N	**A**	P	**R**	-	C_{186}	**V**	**G**
huvec NOS	**W**	R	N	**A**	P	**R**	-	C_{184}	**V**	**G**
rb NOS	**W**	R	N	**A**	S	**R**	-	C_{415}	**V**	**G**
Relative position in the segment	1	2	3	4	5	6	7	8	9	10

Fig.2. Comparison of the 10-aminoacid sequence responsible for heme binding in P450s with a highly-conserved cysteine-containing peptide in NO synthases.

Aminoacid sequences were drawn from the following references : P450cam (23), P450 3A1 (29), mouse macrophage (mm) NOS (24, 25), rat brain (rb) NOS (9), bovine aortic endothelial cell (baec) NOS (26, 27) and human vein endothelial cell (huvec) NOS (28). The segment called P450 (consensus) is drawn from the compilation of a great number of P450 sequences (22). The one-letter code for aminoacids is used ; aminoacid positions with low consensus are indicated by x ; hy means a hydrophobic aliphatic aminoacid (L, I, V or A). For the alignment between P450 3A1 and macrophage NOS, sequence identity is denoted by ":" and conservative aminoacid substitution by "."

and is in close contact to the heme, (ii) an aminoacid at position 6 which interacts with a heme propionate (His 355 in P450cam which is replaced by an arginine in all microsomal P450s), and (iii) a phenylalanine at position 1, which is conserved in all P450s and which probably protects the proximal cysteinate from protonation and/or oxidation by creating a hydrophobic pocket (23).

By looking for segments containing a cysteine and highly conserved among the NOSs from macrophages (24,25), brain (9) or endothelial cells (26-28) described so far, we found a segment of 9 aminoacids which exhibits most of the characteristics of the 10-aminoacid, heme-binding consensus peptide of P450s (Fig. 2). Its terminal CI(or V)G region is almost identical to that of P450s, CI(V, L or A)G. As in most P450s, the aminoacid at position 6 in all NOSs is an arginine which could be involved in the binding of a heme propionate. The first aminoacid of the NOS segment is always a tryptophan which could play a role equivalent to that of the conserved phenylalanine in P450s. Finally, the NAP intermediate segment in NOSs is highly similar to the corresponding segment (NGP) in P450 3A1 (and 3A2) but not in other P450s. Only the central glycine of this segment (position 4) is strongly conserved among P450s, probably because it favors the initiation of the hairpin turn allowing the phenylalanine to lie in close proximity of the proximal cysteine (23). In NOSs, an alanine is found in the place of the conserved glycine. This alanine could still play the role of the glycine with the help of the following proline. Anyhow, this specific similarity further highlights the peculiar analogy between NOSs and P450s from the 3A subfamily (see the high sequence analogy between P450 3A1 and mmNOS in fig.2). The main difference between the putative heme-binding peptides in NOSs and in P450s is the absence of one aminoacid (position 7 in Fig. 2) between the arginine and the cysteine in NOSs. Molecular graphic modeling experiments (Insight II and Discover programs from Biosym Biotechnologies), however, suggest that this one-aminoacid deletion still allows the key interactions between the heme and the proximal region (J.P. Renaud and D. Mansuy, to be published). Briefly, starting from the crystallographic structure of P450cam, the 10 aminoacids of the proximal segment (FGHGSHLCLG) were replaced by the 9 corresponding aminoacids of NOSs (WRNAPRCIG), keeping constant the position of the cysteinate and of the following glycine and superimposing the aryl group of the N-terminal tryptophan to that of the corresponding phenylalanine of P450cam, and then subjecting the new structure to energy minimization. Interestingly, in this model, the arginine at position 2 in NOSs was found suitably located to create a salt bridge with the second heme propionate.

Naturally, many experiments using for instance site-directed mutagenesis on NOSs remain to be carried out to confirm the importance of this 9-aminoacid segment for heme binding. However, the occurrence of this short sequence in all NOSs described to date[1] and its high analogy with the heme-binding region in P450s make it a good candidate for heme-binding in NOSs.

[1] During the preparation of this manuscript, a sequence of 14 aminoacids comprising the 9 aminoacids shown in Fig. 2 was found conserved in 3 NOSs and proposed to contain the proximal cysteine but without comparison with any P450 proximal region (6).

CONCLUSION

Rat P450s3A are particularly prone to catalyze the second step of the NOS-dependent oxidation of arginine, the oxidative cleavage of the C=N bond of NOHA by NADPH and O_2 with formation of citrulline and nitrogen oxides. This greater activity of P450s3A in liver microsomes could be related to the ability of these P450s to oxidize positively charged compounds like amines at their cationic sites (30), as the guanidinium function of NOHA is also cationic at pH 7.4. Moreover, a 9-aminoacid segment present in all NOSs described so far exhibits a striking similarity with the heme-binding region of P450s 3A. It contains the structural components which were found crucial for the binding of the heme in P450 cam.

REFERENCES

1. Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Pharmacol. Rev. **43**, 109-141.
2. Stuehr, D.J., Kwon, N.S., Nathan, C.F., Griffith, O.W., Feldman, P.L. and Wiseman, J. (1991) J. Biol. Chem. **266**, 6259-6263.
3. White, K.A. and Marletta, M.A. (1992) Biochemistry, **31**, 6627-6631.
4. Stuehr, D.J. and Saito, M.I. (1992) J. Biol. Chem. **267**, 20547-20550.
5. Klatt, P., Schmidt, K. and Mayer, B. (1992) Biochem. J. **288**, 15-17.
6. Mc Millan, K., Bredt, D.S., Hirsch, D.J., Snyder, S.H. Clark, J.E. and Masters, B.S.S. (1992) Proc. Natl. Acad. Sci. USA **89**, 11141-11145.
7. Boucher, J.L., Genet, A., Vadon, S., Delaforge, M., Henry, Y. and Mansuy, D. (1992) Biochem. Biophys. Res. Commun. **187**, 880-886.
8. Andronik-Lion, V. Boucher, J.L., Delaforge, M., Henry, Y. and Mansuy, D. (1992) Biochem. Biophys. Res. Commun. **185**, 452-458.
9. Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H. (1991) Nature **351**, 714-718.
10. Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Loper, J.C., Sato, R., Waterman, M.R., and Waxman, D.J. (1991) DNA **10**, 1-14.
11. Wallace, G.C. and Fukuto, J.M. (1991) J. Med. Chem. **34**, 1746-1748.
12. Kremers, P., Beaune, P., Cresteil, T., De Graeve, J., Columelli, S., Leroux, J.P. and Gielen, J. (1981) Eur. J. Biochem. **118**, 599-606.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275.
14. Omura, T. and Sato, R. (1964) J. Biol. Chem. **239**, 2370-2378.
15. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. **126**, 131-138.
16. Ceriotti, G. and Spandrio, L. (1965) Clin. Chim. Acta **11**, 519-522.
17. Knowles, R.G., Salter, M., Brooks, S.L., and Moncada, S. (1990) Biochem. Biophys. Res. Commun. **172**, 1042-1048.
18. Okey, A.B. (1990) Pharmac. Ther. **45**, 241-298.
19. Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.K., and Kaminsky, L.S. (1982) Biochemistry, **21**, 6019-6030.
20. Pessayre, D., Descatoire, V., Konstantinova Mitcheva, M., Wandscheer, J.C., Cobert, B., Level, R., Benhamou, J.P., Jaouen, M., and Mansuy, D. (1981) Biochem. Pharmacol. **30**, 553-558.
21. Delaforge, M., Rivière, R., Sartori, E., Doignon, J.L., and Grognet, J.M. (1989) Xenobiotica **19**, 1285-1295.
22. Gotoh, O., and Fujii-Kuriyama (1989) in Frontiers in Biotransformation, Ruckpaul K. ed., Akademie Verlag, Berlin, Vol. 1, 195-243.

23. Poulos, T.L., Finzel, B.C., and Howard, A.J. (1987) J. Mol. Biol. **195**, 687-700.
24. Lyons, C.R., Orloff, G.J., and Cunningham, J.M. (1992), J. Biol. Chem. **267**, 6370-6374.
25. Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T., and Nathan, C. (1992) Science **256**, 225-228.
26. Lamas, S., Marsden, P.A., Li, G.K., Tempest, P., and Michel, T. (1992) Proc. Natl. Acad. Sci. USA **89**, 6348-6352.
27. Sessa, W.C., Harrison, J.K., Barber, C.M., Zeng, D., Durieux, M.E., D'Angelo, D.D., Lynch, K.R., and Peach, M.J. (1992) J. Biol. Chem. **267**, 15274-15276.
28. Marsden, P.A., Schappert, K.T., Chen, H.S., Flowers, M., Sundell, C.L., Wilcox, J.N., Lamas, S., and Michel, T. (1992) FEBS Lett. **307**, 287-293.
29. Gonzalez, F.J., Nebert, D.W., Hardwick, J.P., and Kasper, C.B. (1985) J. Biol. Chem. **260**, 7435-7441.
30. Smith, D.A., and Jones, B.C. (1992) Biochem. Pharmacol. **44**, 2089-2098.