Nitric Oxide Synthesis Capabilities of Cytochrome P450 1A2 and NADPH-Cytochrome P450 Reductase from N^G-Hydroxy-L-Arginine

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NO was formed from N^G-hydroxy-L-arginine by cytochrome P450 1A2 (P450 1A2) with turnover numbers of 0.6 - 1.2 nmol/nmolP450/min and 62 pmol/nmolP450/min in peroxide-supported shunt and reconstituted systems, respectively. A Glu318Ala mutation of P450 1A2 enhanced the shunt-reaction activity up to 7.3-fold, whereas the mutation abolished the activity with the reconstituted system. Involvement of H_2O_2 or superoxide anion in the NO synthesis is also suggested.

Nitric oxide (NO) plays significant roles in brain, muscle and blood vessels and contributes to many important functions.[1-3] Nitric oxide synthase (NOS) has a cytochrome P450 (P450)-type active site.[1-3] NOS catalyzes the monooxygenation of L-Arg to N^G-hydroxy-L-arginine (NHA) according to the normal P450-type reaction in the first step of NO synthesis. However, there is some controversy as to how the second step of the reaction, from NHA to NO, occurs within the P450 domain of NOS. It appears to be a one-electron oxidation culminating in the release of NO, but this is not a normal P450 reaction.[4-6] However, detailed studies using purified P450 have not yet been reported. In the present study, we examined whether or not rat liver P450 1A2 (P450 1A2) and/or NADPH-cytochrome P450 reductase has a capability of NO synthesis from NHA.

Site-directed mutagenesis, DNA sequencing, expression of wild-type and mutant P450 1A2 proteins in yeast (*Saccharomyces cerevisiae*), and subsequent purification of expressed P450 1A2 proteins were carried out as described previously.[7-9] NO concentrations were spectrophotometrically determined as NO_2^- by using the "NO Detection Kit" of Cayman Laboratories (Ann Arbor, MI).

P450 1A2 synthesizes NO from NHA in the presence of cumylhydroperoxide (CHP) or *tert*-butylhydroperoxide (TBHP), but in the absence of NADPH-cytochrome P450 reductase. Table 1 summarizes the catalytic values derived from these hydroperoxide-supported shunt reactions. TBHP is more capable of supporting the reaction than CHP under these conditions. A Glu318Ala mutation at the putative distal site of P450 1A2 markedly increased the k_{cat} values with CHP, whereas the same mutation markedly decreased the K_m value for the NO synthesis reaction supported by TBHP (Table 1). Thus, for the Glu318Ala mutant, the catalytic activity of the shunt reaction in terms of k_{cat}/K_m is 5.8-7.3-fold higher than the wild type. For the Glu318Asp mutant, a similar tendency was observed on addition of CHP, while the catalytic parameters obtained on addition of TBHP were different. The k_{cat} value of the Thr319Ala mutant with TBHP was lower than for the wild-type enzyme, while that with CHP was comparable. The catalytic activity in terms of k_{cat}/K_m of the Thr319Ala mutant with CHP was higher than for the wild-type enzyme.

P450 1A2 produced NO from NHA with a turnover number of 62 pmol/nmolP450/min in the reconstituted system, which was composed of P450 1A2 and NADPH-cytochrome P450 reductase. However, surprisingly, the reductase alone can synthesize NO from NHA at a turnover number of 26 pmol/nmolP450/min (Table 1). Taking this into account, the Glu318Ala mutant has a very low activity when the activity due to the reductase has been subtracted from the total activity. The activities of the Glu318Asp and Thr319Ala mutants were comparable to the wild type value (Table 1). Cytochrome b_5 inhibited the reaction.

Table 1. Catalytic activities toward N^G-hydroxy-L-Arg (NHA) with P450 1A2 enzymes

Enzyme	CHP[a]			TBHP[a]			Reconstituted[b]	
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	obs.	calc.[c]
Wild type	0.63	0.41	1.5	1.2	0.21	5.7	62	36
Glu318Ala	2.3	0.22	11	1.4	0.043	33	31	5
Glu318Asp	1.0	0.18	5.6	0.43	0.88	0.49	58	32
Thr319Ala	0.46	0.11	4.2	0.23	0.060	3.8	53	27
WT+cyt.b_5[d]							31	5
Reductase[e]							26	< 1
Control[f]							< 1	

[a] Kinetic values were obtained for P450 1A2 enzyme, 0.5 μM; dilauroyl-L-α-phosphatidylcholine (DLPC), 80 μM; CHP, 2.5 mM or TBHP, 10 mM; temperature, 25 °C. Values described here are means of four separate experiments and experimental errors are within 20%. Units of k_{cat} and K_m are expressed by nmol/nmolP450/min and mM, respectively. The k_{cat} values expressed by nmol/mgP450/min can be obtained by multiplying 20. The K_m values described here are associated with NHA affinity, but not with CHP or TBHP affinity, in the reaction. Catalytic activities toward NHA in the H_2O_2-supported system without the reductase were less than 0.1 nmol/nmol P450/min.
[b] Turnover numbers were obtained from product concentrations measured at 3 h reaction time. Units of the values are expressed by pmol/nmolP450/min. Turnover numbers expressed by pmol/mgP450/min can be obtained by multiplying 20. Experimental errors are within 20%. P450 1A2 enzyme, 0.5 μM; NADPH-cytochrome P450 reductase, 1.5 μM; NADPH, 1.0 mM; NHA, 2.0 mM; DLPC, 80 μM; temperature, 25 °C.
[c] Calculated values were obtained by subtracting the turnover number with the reductase alone from the observed values.
[d] Wild-type P450 1A2, 0.5 μM; NADPH-cytochrome P450 reductase, 1.5 μM; NADPH, 1.0 mM; NHA, 2.0 mM; cytochrome b_5, 0.5 μM; DLPC, 80 μM; temperature, 25 °C.
[e] NADPH-cytochrome P450 reductase, 1.5 μM; NADPH, 1.0 mM; NHA, 2.0 mM; DLPC, 80 μM; temperature, 25 °C. Unit should be expressed by pmol/3 nmol reductase/min.
[f] NADPH, 1.0 mM; NHA, 2.0 mM; DLPC, 80 μM; temperature, 25 °C.

In the NOS reaction, tetrahydrobiopterin (H_4B) is essential to give a high NO production rate. The addition of H_4B to the CHP- or TBHP-supported shunt systems and the reconstituted system markedly hampered NO synthesis (Table 2). NOS inhibitors such as N^G-nitro-L-Arg derivatives and N^G-monomethyl-L-Arg did not inhibit the NO synthesis reaction with P450 1A2 supported by CHP or TBHP, but N^G-nitro-L-Arg slightly inhibited NO production in the reconstituted system (Table 2). Surprisingly, some of the NOS inhibitors slightly

Table 2. Relative catalytic activities (%) of the shunt reactions supported by CHP, TBHP and of the reconstituted system in the presence of various effectors

effector	CHP[a]	TBHP[a]	Reconstituted[b]
no addition	100	100	100
40 μM H_4B	2	7	16
2 mM N^G-nitro-L-Arg	123	169	40
2 mM N^G-nitro-L-Arg methyl ester	128	105	70
2 mM N^G-monomethyl-L-Arg	167	93	89
30% CO	130	98	45
75% CO	113	105	50
25 μg/ml catalase	115	116	9
75 μg/ml catalase	214	149	7
25 μg/ml superoxide dismutase	15	57	4
75 μg/ml superoxide dismutase	2	52	4

[a] Relative catalytic activities were obtained for wild-type P450 1A2, 0.5 μM; NHA, 2.0 mM; dilauroyl-L-α-phosphatidylcholine (DLPC), 80 μM; CHP, 2.5 mM or TBHP, 10 mM; temperature, 25 °C. Experimental errors are within 20%.
[b] Values were obtained for wild-type P450 1A2, 0.5 μM; NADPH-cytochrome P450 reductase, 1.5 μM; NADPH, 1.0 mM; NHA, 2.0 mM; DLPC, 80 μM; temperature 25 °C. Experimental errors are within 20%.

increased the rate of NO synthesis by the shunt reactions. Similarly, CO, an inhibitor of the P450 reaction, did not inhibit the shunt reaction, while the reaction in the reconstituted system was about 50% inhibited by the addition of CO (Table 2). Since the NO synthesis reaction seems to be associated with active oxygen species, we examined the effects of catalase and superoxide dismutase on the turnover numbers (Table 2). Catalase stimulated NO synthesis in the CHP- or TBHP-assisted shunt systems, whereas it markedly inhibited NO synthesis in the reconstituted system. Superoxide dismutase markedly inhibited NO synthesis in both the CHP- and reductase-supported systems. The NO synthesis reaction supported by TBHP was also inhibited by superoxide dismutase, but the effect was less marked.

It appears that NADPH-cytochrome P450 reductase can synthesize NO from NHA (Table 1). It is also known that the reductase constantly produces H_2O_2.[10-11] NOS itself produces H_2O_2 under suboptimal conditions, i.e., low concentrations of L-Arg or H_4B, in the presence of calmodulin.[12] Further, addition of H_2O_2 to NOS causes NO production from NHA.[5] The addition of catalase to the reconstituted system markedly inhibits NO production by P450 1A2 from NHA. Therefore, there is a possibility that H_2O_2 produced from uncoupled reaction of O_2 with NOS, facilitates the second step for the production of NO. H_2O_2 is hydrophilic, while TBHP and CHP are hydrophobic. Therefore, only alkyl hydroperoxides might be able to reach the hydrophobic heme active site in P450, and induce the synthesis of NO from NHA.

Uncoupling the microsomal P450/NADPH-cytochrome P450 reductase system also produced the superoxide anion, which is comparable to H_2O_2 under certain conditions.[13] Strong inhibition of NO production by superoxide dismutase, for the reductase-supported P450 1A2 system suggests that superoxide produced from the reductase and/or from P450 Fe^{3+}-O-O^- works to produce NO from NHA for the reductase-assisted system. However, the effect of superoxide dismutase on the shunt system remains to be elucidated.

The present findings in the reconstituted system are in accordance with those observed previously, in that Glu318 is involved in the activation of the O_2 molecule and/or in the cleavage of the O=O bond.[14] We therefore suggest that the normal P450-type reaction is also involved in the production of NO from NHA. The Glu318Ala mutation enhanced the activity, while the Thr319Ala mutation decreased the activity of the peroxide-assisted shunt reaction. The role of these distal amino acids in the catalytic function of the peroxide-assisted shunt reaction may be different from their role on the reductase-assisted reaction.[15]

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