Nitric-oxide synthase (NOS) is a flavohemoprotein that has a cytochrome P450 (P450)-type heme active site and catalyzes the monooxygenation of \( \text{L-Arg} \) to \( \text{N}^\text{G}-\text{hydroxy-L-Arg} \) (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 and L-citrulline, occurs within the reaction in the first step of NO synthesis. Therefore, it was considered worthy to mutate Asp-314 and Thr-315 of neuronal NOS (nNOS) to study their effects on the catalytic turnover of nNOS.

In this study, we present data obtained for the Asp-314 → Ala and Thr-315 → Ala mutants of neuronal NOS, both of which had absorption maxima at 450 nm in the spectra of the CO-reduced complexes and studied NO formation rates and other kinetic parameters as well as the substrate binding affinity. The Asp-314 → Ala mutant totally abolished NO formation activity and markedly increased the rate of \( \text{H}_2\text{O}_2 \) formation by 20-fold compared with the wild type when L-Arg was used as the substrate. The NADPH oxidation and \( \text{O}_2 \) consumption rates for the Asp-314 → Ala mutant were 60–65% smaller than for the wild type. The Thr-315 → Ala mutant, on the other hand, retained NO formation activity that was 23% higher than the wild type, but like the Asp-314 → Ala mutation, markedly increased the \( \text{H}_2\text{O}_2 \) formation rate. The NADPH oxidation and \( \text{O}_2 \) consumption rates for the Thr-315 → Ala mutant were, respectively, 56 and 27% higher than for the wild type. When NHA was used as the substrate, similar values were obtained. Thus, we propose that Asp-314 is crucial for catalysis, perhaps through involvement in the stabilization of an oxygen-bound intermediate. An important role for Thr-315 in the catalysis is also suggested.

Nitric oxide synthase (NOS)\(^1\) is an important heme enzyme responsible for production of nitric oxide (NO) (Refs. 1–8 and references therein). NOS is composed of an oxidative domain and a reductase domain. The oxidative domain has an active site with a cytochrome P450 (P450)-like thiol-coordinated heme iron complex. The heme distal site of the oxidative domain should be one of the substrate binding site(s) and a site for molecular oxygen binding. The heme distal site must also play an important role in the activation of molecular oxygen before its reaction with the substrate. For P450s, it has been suggested that conserved Asp/Glu and neighboring Thr residues at the heme distal site are very important for the activation of molecular oxygen (9–12).

Fig. 1 shows a comparison between selected amino acid sequences of NOSs and P450s near the N-terminal site. Asp-314 and Thr-315 (numbered for neuronal NOS) are well conserved for all NOSs so far isolated. It is not unreasonable to match these amino acids of NOS with well conserved Glu/Asp and Thr residues (Glu-318 and Thr-319 of P450a or Asp-251 and Thr-252 of P450c) of P450s. These polar amino acids of P450s play a very important role in the activation of molecular oxygen (9–12). Therefore, it was considered worthy to mutate Asp-314 and Thr-315 of neuronal NOS (nNOS) to study their effects on kinetic parameters associated with activation of \( \text{O}_2 \).

In this study, we present data obtained for the Asp-314 → Ala and Thr-315 → Ala mutants of nNOS including rates for NO formation, \( \text{O}_2 \) consumption, \( \text{H}_2\text{O}_2 \) formation, NADPH oxidation, and cytochrome \( c \) reduction. On the basis of this work, we propose that Asp-314 and perhaps Thr-315 are crucially involved in the activation of molecular oxygen during the catalytic turnover of nNOS.
Roles of Asp-314 and Thr-315 in nNOS Function

RESULTS AND DISCUSSION

Fig. 2 shows optical absorption spectra of the wild type (A), the Asp-314 → Ala mutant (B), and the Thr-315 → Ala mutant (C). The ferric complex of the wild type had a Soret absorption peak at around 400 nm, which was shifted to 395 nm on addition of L-Arg as reported previously (15). On the other hand, for the ferric Asp-314 → Ala and Thr-315 → Ala mutants, the Soret peaks were observed at 440 and 395 nm, respectively, and were not altered on addition of L-Arg. The addition of L-Arg to imidazole-induced low spin complexes of wild type NOS changes the spin state to high spin, reflecting substrate binding to the active site (21). The addition of excess L-Arg clearly changed the spin state from imidazole-bound low spin (with a 427-nm absorption peak) to high spin (with a 395-nm absorption peak) for the Thr-315 → Ala mutant (not shown). The absorption peak observed at 395 nm for the Asp-314 → Ala mutant CO-reduced complex in the absence of L-Arg was located at 450 nm (– – –), the Asp-314 Ala mutant abolished the NO formation rate, 72.1 mol/min/mg NOS protein), the Thr-315 → Ala mutant (C) in the presence of 10 mM L-Arg. The Soret absorption peak of the CO-reduced complex of the wild type was located at 450 nm. In the absence of L-Arg, however, the Soret absorption peak of the CO-reduced complex of the Asp-314 → Ala mutant was located at 455 nm (– – –), suggesting that L-Arg binding/dissociation caused the absorption peak shift. Spectra were recorded at 25°C.

Table I summarizes the various parameters obtained for the wild type and mutant enzymes. The NO formation rate, 72.1 mol/min/mg NOS protein, obtained for the wild type is comparable to the value previously reported (1–8, 16). In the presence of L-Arg, the NADPH oxidation and O₂ consumption rates obtained for the wild type are essentially coupled to the rate of NO formation, considering that oxidation of 1.5 mol of NADPH and consumption of 2 mol of O₂ generates 1 mol of NO.

More H₂O₂ was generated with the mutants both in the absence and presence of the substrate compared with the wild type (Table I). In the P450cam system, mutations at the first layer of residues above the heme generate more H₂O₂ than those at the second and third layers with uncoupled substrates (22). Uncoupling occurs when mutations are introduced so as to alter water distribution in the P450cam active site and/or the stability of the oxygenated heme intermediate. Altered polarity at the heme pocket caused by mutation of nNOS Asp-314 and Thr-315 residues would favor charge separation at the iron, promoting the release of H₂O₂ or O₂. Thus, it is suggested that Asp-314 and Thr-315 must be located close to the heme.

The Asp-314 → Ala mutant abolished the NO formation
Roles of Asp-314 and Thr-315 in nNOS Function

Table I
Catalytic activities of the wild type and mutant nNOSs

|            | NO formation | NADPH oxidation | O2 consumption | H2O2 formation | Cytochrome c reduction |
|------------|--------------|-----------------|---------------|----------------|------------------------|
| Wild type  |              |                 |               |                |                        |
| - l-Arg    |              |                 |               |                |                        |
| + NHA      | 104 ± 8.5    | 106 ± 5.2       | 135 ± 14      | 20.9 ± 3.9     |                        |
| Asp-314 → Ala |            |                 |               |                |                        |
| - l-Arg    | 9.9 ± 2.7    | 153 ± 7.7       | 200 ± 6.9     | 17.9 ± 0.6     | 5.503 ± 70             |
| + NHA      |              |                 |               |                |                        |
| Thr-315 → Ala |            |                 |               |                |                        |
| - l-Arg    |              |                 |               |                |                        |
| + NHA      |              |                 |               |                |                        |

NO formation and other rates are expressed by μmol/min/μmol NOS heme. These values were obtained at 25 °C as described under "Experimental Procedures." Data are expressed as the mean ± S.D. of at least three separate experiments.

To examine whether or not Asp-314 is involved in the second reaction from NHA to NO, the tendency of the catalytic values with NHA was similar to that with l-Arg (Table I). Thus, it is suggested the Asp-314 is also involved in the second reaction from NHA to NO.

The rates of NO formation, NADPH oxidation, and O2 consumption obtained for the Thr-315 → Ala mutant with l-Arg were 23–56% higher than the wild type. The Thr-315 → Ala mutation increased the formation of H2O2 by 11-fold compared with wild type in the presence of l-Arg. These results differ from the observations made for the Thr-252 → Ala mutants of P450cam and P450BM3, which exhibited low monooxidation activities, less than 15% those of the wild type values, and NADH oxidation and O2 consumption rates 25–48% those of the wild type values (10, 11, 23). The NO formation rate with NHA for the Thr-315 → Ala mutant of nNOS was 17% higher than with l-Arg (Table I) but was similar to the NADPH oxidation rate. Thus, the role of Thr-315 of nNOS may be different from that of Thr-252 of P450cam (10, 11). H2O2 produced by the Thr-315 → Ala mutant of nNOS may in part be used to facilitate the second reaction with NHA and generate extra NO, since NO is known to be generated from NHA and H2O2 with nNOS even in the absence of NADPH (27). A hydrogen atom of a water molecule near Ala-315 in the Thr-315 → Ala mutant of nNOS may also directly serve as a hydrogen bond donor in the acid-base catalysis required for O–O bond scission (12, 24–26, 28). Otherwise, the Thr-315 → Ala mutation may alter the conformation and/or orientation of the substrate to allow easier access to active oxygen for more efficient catalysis to occur in the active site.

During the review process, an x-ray crystallographic study of the truncated oxidative domain (residues 115–498, equivalent to residues 337–720 of nNOS) of inducible NOS (iNOS) was published (34). It was claimed that l-Arg itself works as a proton donor to cleave the O–O bond of molecular oxygen for the reaction l-Arg to NHA because only one polar amino acid, Glu-371, exists in the active-site cavity, and this is in contact with l-Arg. However, the structure of half (residues 1–336) of the active site.

To examine whether or not Asp-314 is involved in the second reaction from NHA to NO, the same catalytic parameters were obtained for NHA as the substrate (Table I). For wild type nNOS, the NO formation rate with NHA was similar to the NADPH oxidation rate, implying that most of the reaction from NHA to NO is of the P450-type. For the Asp-314 → Ala mutant, the parameter obtained for the Asp-314 → Ala mutant were lower than observed with the wild type by 35 and 41%, respectively. Note that this mutant retained its cytochrome c reductase activity, indicating that the structure of the reductase domain is well preserved. The kinetic values obtained for the Asp-314 → Ala mutant are reminiscent of Thr-252 mutants of P450cam. This residue of P450cam is located at the heme distal site and is suggested to be important in the activation of molecular oxygen (9–11). The Thr-252 → Ala mutation of P450cam abolished monooxygenase activity and markedly increased the H2O2 formation rate. The O2 consumption rate obtained for this mutant of P450cam was 48% that of the wild type value, whereas the NADH oxidation rate was comparable to that of the P450cam wild type. Similarly, kinetic values obtained for the Asp-314 → Ala mutant of nNOS also appear to correspond with kinetic parameters obtained for the Thr-268 → Ala mutant of cytochrome P450BM3 (23). However, the results obtained for the Asp-314 → Ala mutant of nNOS differ from the kinetic parameters obtained for the Asp-251 → Ala and Asp-251 → Asn mutants of P450cam, (or the Glu-318 → Ala mutant of P450d) in that the monooxidation activity, O2 consumption, and NADH oxidation rates with the P450cam mutants were extremely low, less than 1% that of the wild type values; therefore no marked increase in the rate of H2O2 formation was detected (24–26). In the P450 reaction cycle, protonation of dioxegen is required before the O–O bond can be cleaved and the active oxygen species responsible for the monooxidation reaction can be generated (9–13, 23–26). The distal-site threonine is thought to stabilize the oxygen-bound intermediate through the formation of a hydrogen bond to the iron-bound oxygen. H2O2 formation by the Thr mutants of P450cam and P450BM3 could be caused by breakdown of a putative iron-peroxo complex before the formation of the high-valent iron-oxo species, since the loss of the Thr hydroxyl group reduces the stability of the oxygen-bound intermediate (9–11, 23). An extra bound water molecule in the Thr mutants of P450cam and P450BM3 may be responsible for un-coupling catalysis by supplying a proton and aiding the release of water and H2O2. Therefore, we conjecture that for nNOS, the carboxylate proton of Asp-314 is involved in the second reaction from NHA to NO, the same catalytic parameters were obtained for NHA as the substrate (Table I). For wild type nNOS, the NO formation rate with NHA was similar to the NADPH oxidation rate, implying that most of the reaction from NHA to NO is of the P450-type. For the Asp-314 → Ala mutant, the tendency of the catalytic values with NHA was similar to that with l-Arg (Table I). Thus, it is suggested the Asp-314 is also involved in the second reaction from NHA to NO.

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heme iron, switching the spin state. In fact, the x-ray crystal structure shows that the heme of the truncated iNOS oxidative domain is orientated to face the vacant space and is exposed in places (34). In P450s, amino acids in contact with the substrate differ from those contributing to O–O scission; for NOS, the same situation also appears likely (9–14, 23–26).

In summary, the results of this study suggest that Asp-314 is crucially involved in the stabilization of an oxygen-bound intermediate in nNOS catalysis and that Thr-315 is located close to the heme. Since the nNOS reaction is composed of two stepwise monooxidations, further studies to clarify how the Asp-314/Thr-315 residues contribute to these two steps remains to be carried out.

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