Kinetic Studies on the Successive Reaction of Neuronal Nitric Oxide Synthase from L-Arginine to Nitric Oxide and L-Citrulline†

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ABSTRACT: Rat neuronal nitric oxide synthase (nNOS) was heterologously expressed in *Escherichia coli* and purified. The conversion of L-arginine to N^{ω}-hydroxy-L-arginine and further to L-citrulline in one cycle of the reaction of the purified nNOS was measured with the reaction rapid quenching method using ^{3}H-L-arginine as the substrate. It was found that most of the produced ^{3}H-N^{ω}-hydroxy-L-arginine was successively hydroxylated to ^{3}H-L-citrulline without leaving the enzyme. From the analysis of time courses, the rate constants for each reaction step, and also for the dissociation of the intermediate, were estimated at various temperature in which the rates for the first and the second reactions were not much different each other but the rate for the dissociation of ^{3}H-N^{ω}-hydroxy-L-arginine from the enzyme was significantly slow. Under the steady-state reaction condition, almost all of the nNOS was estimated to be active from the amount of burst formation of L-citrulline in the pre-steady state. The rate constant for the dissociation of the product L-citrulline from nNOS was calculated from the combination of results of the rapid quenching experiments and the metabolism of L-arginine in the presence of an excess amount of substrate, which was the smallest among all the rate constants in one cycle of the nNOS reaction. The activation energies for all the reaction steps were determined from the temperature dependence of the rate constants, which revealed that the rate-determining step of the nNOS reaction in the steady state was the dissociation of the product L-citrulline from the enzyme.

The reaction of nitric oxide synthase is not simple and has been investigated by several laboratories over the past decade. Marletta and coworkers proposed that NO[1] and L-citrulline are produced from L-arginine by a two step reaction via an intermediate metabolite N^{ω}-hydroxy-L-arginine, which is formed in the initial hydroxylation reaction of the guanidino group of L-arginine (1, 2). That N^{ω}-hydroxy-L-arginine is produced from L-arginine during NOS reaction was demonstrated by Stuehr et al. in a system with purified iNOS and ^{14}C-L-arginine where 44-fold excess of nonradioactive N^{ω}-hydroxy-L-arginine was present over ^{14}C-L-arginine (*3*). It was found that the formation of 1 mol of N^{ω}-hydroxy-L-arginine from L-arginine consumed 1 mol of NADPH, but only 0.5 mol of NADPH were required for the formation of one mol of L-citrulline from N^{ω}-hydroxy-L-arginine (*3*). The reaction of NOS from L-arginine to N^{ω}-hydroxy-L-arginine and further to L-citrulline could not occur without NADPH but from N^{ω}-hydroxy-L-arginine the formation of L-citrulline and NO was observed in the system containing H_2O_2 without NADPH (*4*−*7*). It was an interesting observation that cytochrome P450 also catalyzed the formation of L-citrulline and NO from N^{ω}-hydroxy-L-arginine in the presence of NADPH but did not catalyze NO formation from L-arginine under the same conditions (*8*−*10*). The formation of L-citrulline and NO from N^{ω}-hydroxy-L-arginine was also detected in a reaction system containing the oxygenase and reductase domains of iNOS but not from L-arginine (*11*, *12*). There are various proposals concerning the reaction mechanism of two reactions of NOS, from L-arginine to N^{ω}-hydroxy-L-arginine and from N^{ω}-hydroxy-L-arginine to L-citrulline and NO (*6*, *7*, *13*−*15*). There are no reports, however, about the rate constants for the first and the second reactions during the conversion of L-arginine to L-citrulline and NO.

Very little N^{ω}-hydroxy-L-arginine has been detected in the steady-state reaction system converting L-arginine to L-citrulline and NO with purified NOS and NADPH: only about 1/50 of the formation of L-citrulline (*3*, *5*). There is a report that a large amount of N^{ω}-hydroxy-L-arginine was produced in a reaction of NOS induced in murine EMT-6 cells (*16*). It has been suggested that the reactions of NOS resemble those of cytochrome P450s (*17*, *18*). Several cytochrome P450s have been reported to catalyze multistep reactions, which is especially remarkable in the case of steroidogenic P450s (*19*−*21*). $P450_{11\beta}$ and $P450_{17\alpha}$ catalyze multistep reactions in which a part of the intermediate produced in the initial hydroxylation reaction is further

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[1] Abbreviations used are: NO, nitric oxide; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; $P450_{11\beta}$, cytochrome P450 having steroid 11β-hydroxylase activity; $P450_{17\alpha}$, cytochrome P450 having steroid 17α-hydroxylase activity; H_4B, (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; CaM, calmodulin; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

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metabolized without leaving the binding site of the P450 (*22*−*24*). The reactions that occur successively without the intermediate leaving the binding site are known as to the successive reactions. In the successive reactions of P450s, the dissociation of the final products from the enzymes plays an important role in the steady-state reaction (*22*−*24*).

In this study, we analyzed reactions of nNOS kinetically using the reaction rapid quenching method to obtain direct experimental evidence that N^{ω}-hydroxy-L-arginine is the intermediate in the conversion of L-arginine to L-citrulline and NO in one cycle of the reaction. We clarified that the reaction intermediate undergoes the hydroxylation reaction without leaving the enzyme. We determined the rate constants for the first and the second reactions, and also those for the dissociations of the intermediate and the product L-citrulline from the enzyme. To determine the activation energy of each reaction step, we measured the temperature dependence of the rate constants in one cycle of the reaction. We also measured the temperature dependence of the rate of L-citrulline formation from L-arginine in the steady state to obtain information on the rate-determining step in the steady state.

MATERIALS AND METHODS

Materials. L-Arginine and L-citrulline were obtained from Nacalai Tesque (Kyoto). N^{ω}-Hydroxy-L-arginine monoacetate was from LC Laboratories (Woburn, MA). NADPH was from Boehringer Mannheim (Mannheim, Germany). [2,3,4,5-^{3}H]-L-Arginine monohydrochloride and L-[ureido-^{14}C]-citrulline were from Amersham International (Amersham, UK). (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was from Dr. B. Schircks (Jona, Switzerland).

Protein Purifications. Plasmid containing rat nNOS cDNA in pBluescript SK(−) was kindly donated by Dr. Synder of Johns Hopkins Medical School, Baltimore (*25*), and pCWori was a gift from Dr. Dahlquist of Oregon University, Oregon (*26*). The construction of pCWnNOS and the protein expression were carried out according to the method of Roman et al. with some modifications (*27*). Briefly, *Escherichia coli* BL21 transfected with pCWnNOS was inoculated in Terrific Broth, containing 50 μg ampicillin/mL. After the cells were broken with pulsed sonication and a lysozyme, the supernatant was loaded on a DEAE-Sephacel column (2.5 × 40 cm, Pharmacia) and the nNOS was eluted with a salt gradient to 300 mM NaCl. The nNOS was adsorbed to a 2′,5′-ADP-Sepharose 4B column (1.5 × 20 cm, Pharmacia) and was eluted with NADPH solution. Finally, nNOS was purified by CaM-Agarose column chromatography (1.5 × 20 cm, Sigma). One band was detected by the SDS-PAGE for the purified nNOS. The sample was concentrated to 200 μM with a 100 kDa-Microsep (PALL FILRON) and frozen at 77 K. The concentration of nNOS was determined from the dithionite-reduced CO difference spectra, using the difference absorption coefficient $\Delta\epsilon$ (444−490 nm) = 91 mM^{-1} cm^{-1} (*28*).

Rat calmodulin was purified from *E. coli* BL21 transfected with a plasmid containing rat calmodulin cDNA, which was kindly donated by Drs. Hayashi and Taniguchi of the Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan (*29*). CaM was purified using Phenyl-Sepharose CL-4B column chromatography. The purified CaM was frozen at −80 °C at the final concentration of 100 μM.

Purification of [2,3,4,5-^{3}H]-L-Arginine. [2,3,4,5-^{3}H]-L-Arginine was purified with a HPLC cation-exchange column (7.5 × 75 mm, TOSOH SP-5PW). The following elution conditions were used; flow rate, 0.5 mL/min; column temperature, 22 °C; from 0 to 10 min, isocratic H_2O; 10−40 min, linear gradient from H_2O to 0.1 M ammonium hydroxide (pH 11.0); 40−50 min, isocratic 0.1 M ammonium hydroxide (pH 11.0); 50−60 min, linear gradient from 0.1 M ammonium hydroxide (pH 11.0) to H_2O. The purified [2,3,4,5-^{3}H]-L-arginine was eluted in the range from 19.6 to 21.6 mL and was lyophilized overnight. The dry powders were dissolved in a small volume of 5 mM HCl. The purified ^{3}H-L-arginine·HCl was stored at −80 °C.

Assay of L-Citrulline Formation from L-Arginine in the Steady State. The reaction mixture contained 50 mM HEPES (pH 7.5), 2 mM $CaCl_2$, 20 μM H_4B, 0.1 mM DTT, 2 μM FAD, 2 μM FMN, 0.5 μM CaM, 50 μM L-arginine, and [2,3,4,5-^{3}H]-L-arginine (0.5 μCi/0.2 mL). Ten pmol of nNOS was added to 200 μL of the reaction mixture for the assays at 17 °C and 25 °C and 20 pmol of nNOS was used for those at 5 °C and 10 °C. After pre-incubation in a temperature-controlled water bath for one min, 10 μL of 5 mM NADPH was added to the reaction mixture for the initiation of the reaction. The reaction mixtures were incubated at 5, 10, 17, or 25 °C, up to 2.5 min. The reactions were stopped with vigorous mixing with 100 μL of a mixture (2:3, v/v) of 2-propanol and an aqueous solution containing 50 mM phosphoric acid, 50 mM sodium dihydrogenphosphate and 37.5 mM SDS. After the centrifugation at 400*g* for 10 min, the supernatant (300 μL) was loaded to the HPLC system consisting of a PU980 HPLC pump (JASCO Co., Tokyo), a column oven (CO-965 JASCO Co., Tokyo), an AS-8020 auto-sampler (TOSOH, Tokyo), and a Gilson 204 fraction collector with an ODS-column (4 × 250 mm, LiChrospher 100 RP-18, Cica-Merck, Tokyo). The HPLC system was operated using a mixture (23:77, v/v) of 2-propanol and an aqueous solution containing 20 mM phosphoric acid, 20 mM sodium dihydrogenphosphate, and 15 mM SDS as the eluent at a flow rate of 0.4 mL/min at the column temperature of 55 °C (*30*). The radioactivities of the separated metabolites were measured with a liquid scintillation counter (Aloka, LSC-700).

Reaction Rapid Quenching Experiments. Rapid quenching experiments were performed with a Unisoku MX-200. Figure 1 shows the flow path of the device. To test the rapid quenching device, the rate of alkaline hydrolysis of 2,4-dinitrophenyl acetate to dinitrophenol was measured (*31*). Two mM 2,4-dinitrophenyl acetate in reservoir A was mixed rapidly in mixer 1 with an equal volume of a certain concentration of NaOH (10 mM −1 M) in reservoir B and stored in the reaction coil. After a certain period, the solution was pushed out from the coil with H_2O in reservoir C and rapidly mixed in mixer 2 with 2 M glycine·HCl solution (pH 2.0) in reservoir D. The time course of the increase in the absorbance at 320 nm fit well with the first-order kinetics, and the rate constant was linearly dependent on the NaOH concentration (data not shown). The dead time of the device was 20 ± 5 ms, and the data in the rapid-quenching experiments could be reproduced with the time accuracy of ±5 ms.

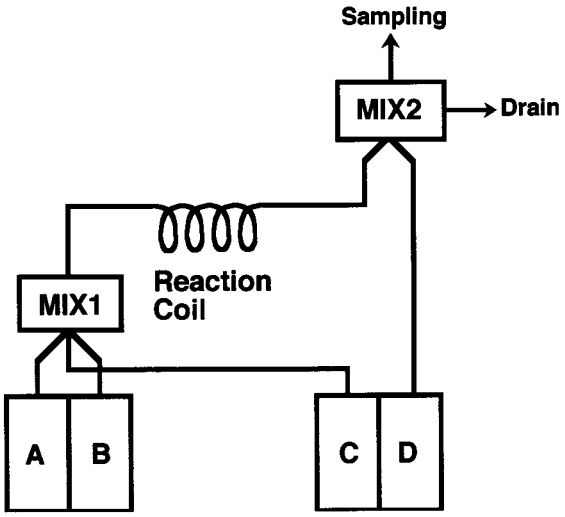

FIGURE 1: Schematic illustration of the reaction rapid quenching device used in the experiments. At reaction time zero, the reaction solution in A and the initiation solution in B are rapidly pushed into mixer 1 (MIX 1) with syringes driven by nitrogen gas pressure and stored in the reaction coil. After a certain period, the reacted solution in the coil is pushed out with the buffer solution (C) and mixed with the termination solution (D) in mixer 2 (MIX 2).

In the nNOS reaction, the reaction mixture in A contained 50 mM HEPES (pH 7.5), 2 mM $CaCl_2$, 10 μM H_4B, 0.1 mM DTT, 2 μM FAD, 2 μM FMN, 3 μM CaM, 0.6 μM of nNOS, and 3 pmol/90 μL of [2,3,4,5-^{3}H]-arginine (0.2 μCi). The initiation solution in B contained 50 mM HEPES, 2 mM $CaCl_2$, 10 μM H_4B, 0.1 mM DTT, 2 μM FAD, 2 μM FMN, 100 μM NADPH, and 0.4 mM L-arginine as the chaser. The solution in C was the HEPES buffer containing 2 mM $CaCl_2$. The termination solution in D was a mixture (74:26, v/v) of 2-propanol and an aqueous solution containing 100 mM phosphoric acid, 100 mM sodium dihydrogenphosphate, 80 mM SDS, 1 mM L-arginine, 1 mM N$^\omega$-hydroxy-L-arginine and 1 mM L-citrulline. The reaction was initiated by mixing the reaction mixture (90 μL) with the initiation solution (90 μL) in mixer 1. After being stored in the reaction coil for a period of time, the reacted solution was pushed out from the coil with solution C (200 μL) and mixed with the termination solution (200 μL) in mixer 2. The reaction coil was washed with solution C after each reaction. The reacted solution was shaken for 30 min vigorously and was centrifuged at 400*g* for 10 min. The metabolites in the supernatant were separated with the HPLC system as described above.

RESULTS

The Identification of the Intermediate Metabolite in the nNOS Reaction. It is widely accepted that the NOS reaction has two steps and that the reaction intermediate is N$^\omega$-hydroxy-L-arginine, but there are no direct observations that L-arginine is converted to N$^\omega$-hydroxy-L-arginine in the first step reaction and metabolized subsequently to L-citrulline in one cycle of the reaction. We carried out rapid-quenching experiments to follow the metabolism of L-arginine to N$^\omega$-hydroxy-L-arginine and further to L-citrulline at 25 °C. The reaction solution in reservoir A contained 0.6 μM nNOS and 33 nM ^{3}H-arginine, where about 1/4 of the ^{3}H-L-arginine can be assumed to be complexed with nNOS (*3*). Once the reaction solution (90 μL) has been mixed with an equal

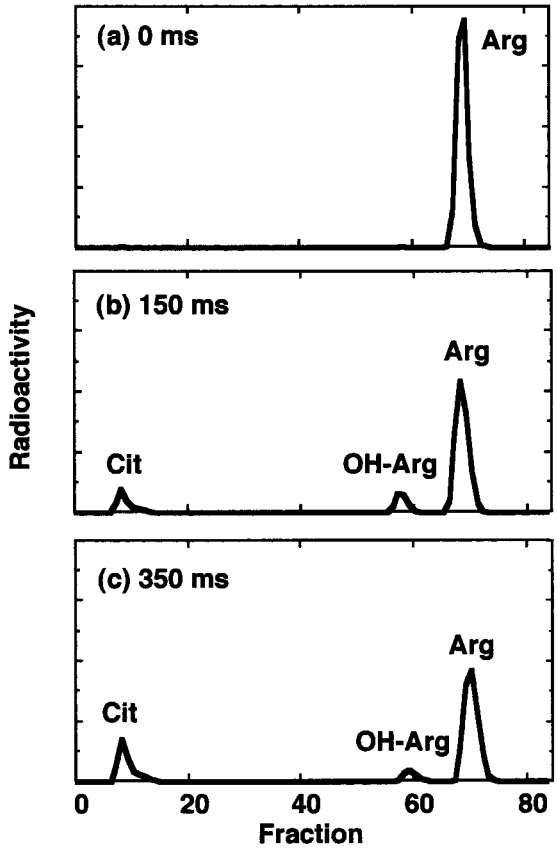

FIGURE 2: HPLC chromatograms of L-arginine and the metabolites in the nNOS reaction at 25 °C. ^{3}H-Arginine and the metabolites are separated by ion-pair chromatography using an ODS column. Chromatogram a, b, and c show the separation of the metabolites produced at the reaction time zero, 150 and 350 ms, respectively. Arg, OH-Arg, and Cit represent L-arginine, N$^\omega$-hydroxy-L-arginine, and L-citrulline, respectively. Details are in Materials and Methods.

volume of the initiation solution that contains 100 μM NADPH and 400 μM nonradioactive L-arginine, the free ^{3}H-L-arginine in the reaction solution cannot be metabolized because of the excess of nonradioactive L-arginine in the solution. There is almost no possibility that the released radioactive metabolites will rebind to nNOS and be metabolized by nNOS. In this reaction system, we can follow the metabolism of the ^{3}H-L-arginine bound to nNOS at the initiation time by assaying the amount of ^{3}H-substrate and the ^{3}H-metabolites only in one cycle of the reaction. Figure 2 shows the chromatograms of the ^{3}H-L-arginine and the ^{3}H-metabolites in the reaction solution at various reaction time points. One hundred and fifty ms after the reaction was initiated, two radioactive peaks in addition to ^{3}H-L-arginine could be detected (Figure 2b). The positions of the two peaks coincided with those of the authentic nonradioactive L-citrulline and N$^\omega$-hydroxy-L-arginine. Three hundred and fifty ms after the initiation, the radioactivity of N$^\omega$-hydroxy-L-arginine decreased and that of L-citrulline increased (Figure 2c). No other radioactive peaks were detected under these HPLC conditions and the summation of the radioactivities in these peaks remained constant in triplicate experiments. These results clearly show that ^{3}H-L-citrulline was formed from the produced ^{3}H-N$^\omega$-hydroxy-L-arginine.

Rapid Quenching Experiments for the nNOS Reaction. The time course of ^{3}H-L-arginine metabolism in one cycle of the nNOS reaction was measured at 25 °C with the rapid-

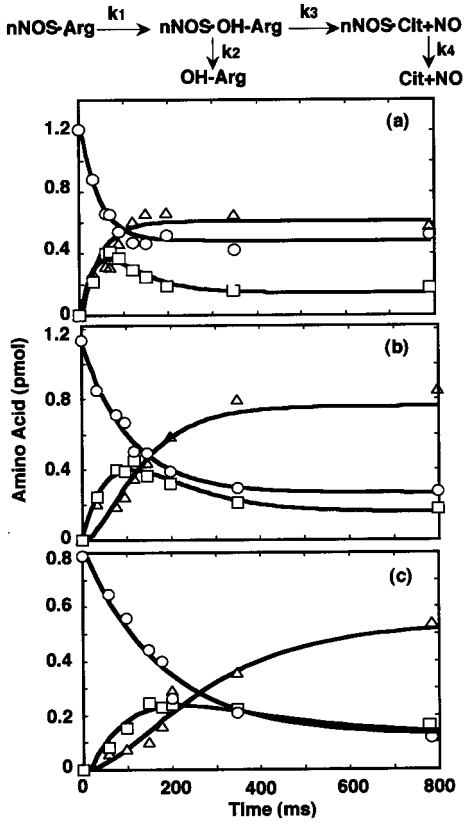

FIGURE 3: The time course of ^{3}H-L-arginine metabolism in the rapid quenching experiments of nNOS. The circles, squares, and triangles represent the amounts of ^{3}H-L-arginine, ^{3}H-N$^\omega$-hydroxy-L-arginine and ^{3}H-L-citrulline, respectively. The reactions a, b, and c were carried out at 25, 17, and 10 °C, respectively, as described in Materials and Methods. The curves were drawn using the rate constants obtained by the best fittings of the data to the following rate eqs 1−3 (ref *22*).

$$[\text{nNOS·Arg}] = A\text{e}(-k_1 t) \quad (1)$$

$$[\text{nNOS·OH-Arg}] + [\text{OH-Arg}] = (A(k_1 - k_2)/a)\text{e}(-k_1 t) - (Ak_1k_3/ab)\text{e}(-bt) + Ak_2/b \quad (2)$$

$$[\text{nNOS·Cit}] + [\text{Cit}] = (-Ak_3/a)\text{e}(-k_1 t) + (Ak_1k_3/ab)\text{e}(-bt) + Ak_3/b \quad (3)$$

where $a = -k_1 + k_2 + k_3$ and $b = k_2 + k_3$. Arg, OH-Arg, and Cit represent L-arginine, N$^\omega$-hydroxy-L-arginine, and L-citrulline, respectively. *A* represents the concentration of ^{3}H-L-arginine complexed with nNOS at reaction time zero.

quenching device. The amount of ^{3}H-L-arginine rapidly decreased to a flat level in 200 ms. ^{3}H-N$^\omega$-Hydroxy-L-arginine increased for 50 ms and then decreased gradually. ^{3}H-L-Citrulline increased till 200 ms as shown in Figure 3a. It must be noted that the summation of the amounts of ^{3}H-L-arginine, ^{3}H-N$^\omega$-hydroxy-L-arginine, and ^{3}H-L-citrulline was almost constant throughout the reaction (0−2000 ms) where the recovery of the ^{3}H radioactivity was 100 ± 13% of that of ^{3}H-L-arginine at reaction time zero. The time course of ^{3}H-L-arginine metabolism did not change at all when nonradioactive N$^\omega$-hydroxy-L-arginine was used as the chaser instead of nonradioactive L-arginine. The decrease in ^{3}H-N$^\omega$-hydroxy-L-arginine and the simultaneous increase in ^{3}H-L-citrulline clearly show the conversion to ^{3}H-L-citrulline. The curves in the figure were drawn using rate constants obtained with the computer software, Kaleida graph (Albelck Software), to fit the observed points to eqs 1−3 in the figure legend for the reaction scheme shown in the upper part of Figure 3. In the reaction scheme, it is assumed that a part of the produced ^{3}H-N$^\omega$-hydroxy-L-arginine leaves nNOS, and the rest is metabolized further into ^{3}H-L-citrulline. The rate constants for the conversion of ^{3}H-L-arginine to ^{3}H-N$^\omega$-hydroxy-L-arginine, for the conversion of ^{3}H-N$^\omega$-hydroxy-L-arginine to ^{3}H-L-citrulline, and for the dissociation of ^{3}H-N$^\omega$-hydroxy-L-arginine from nNOS, k_1, k_3, and k_2, respectively, are listed in Table 1. Since the amounts of ^{3}H-N$^\omega$-hydroxy-L-arginine and ^{3}H-L-citrulline detected in rapid-quenching experiments are not those of free ^{3}H-N$^\omega$-hydroxy-L-arginine and free ^{3}H-L-citrulline in the solution but are the sum of those bound to nNOS and the unbound forms, we cannot calculate the rate constant, k_4, for the dissociation of ^{3}H-L-citrulline from nNOS, which will be discussed later. We also performed rapid-quenching experiments for the nNOS reaction at 17 and 10 °C. Figure 3, b and c, show the results at 17 and at 10 °C, respectively, where the decrease of ^{3}H-L-arginine continues till about 800 ms and the maximum of ^{3}H-N$^\omega$-hydroxy-L-arginine is at about 200 ms at 10 °C. The theoretical curves follow quite well with the observed points. The values of rate constants, k_1, k_2, and k_3 at 17 and 10 °C are listed in Table 1.

Table 1: Rate Constant for Each Step of a Single Cycle Reaction of nNOS

	reaction temperature		
rate constant (s^{-1})[a]	25 °C	17 °C	10 °C
k_1	25 ± 2.0	11 ± 3.0	7.0 ± 1.0
k_2	4.4 ± 0.5	2.4 ± 0.6	1.6 ± 0.5
k_3	19 ± 3.0	12 ± 1.0	6.0 ± 1.0
k_4[b]	2.7 ± 0.3	0.81 ± 0.02	0.32 ± 0.4

[a] The rate constants were obtained by best fitting the obtained data to the rate equations in the legend for Figure 3. [b] The values were estimated by combining results of rapid-quenching experiments with those from steady state kinetics. The details were in the text.

Temperature Dependence of nNOS Activity in the Steady State. The activity to produce L-citrulline from L-arginine was measured at 25, 17, 10, and 5 °C in the steady state conditions in the presence of 50 μM of L-arginine. The amount of N$^\omega$-hydroxy-L-arginine detected in the steady state was less than 5% of that of L-citrulline in the reaction time from zero to 10 min at each temperature, which was in the error range of the amount of L-citrulline formation. No radioactive peaks other than N$^\omega$-hydroxy-L-arginine and L-citrulline were detected in the steady state. The L-citrulline production activity was increased about 10-fold by the temperature rise from 5 to 25 °C. The turnover number of nNOS for L-citrulline formation at 25, 17, 10, and 5 °C was 136 ± 5, 44 ± 6, 27 ± 2, and 13 ± 3 nmol/min/nmol of nNOS, respectively. The logarithm of the turnover number vs the inverse of the observed temperature (in Kelvin) shows a linear relationship, from which we estimated the activation energy, 19 ± 1.9 kcal/mol, for the nNOS reaction for L-arginine using the Arrhenius equation (Figure 4).

Quantification of Active nNOS. It is quite important to quantify the concentration of active nNOS in the reaction solution for the kinetic analysis. The concentration of active nNOS can be estimated from the burst formation of L-citrulline in the pre-steady state in the presence of excess amount of L-arginine in solution where most of the active nNOS can be assumed to be complexed with L-arginine, if

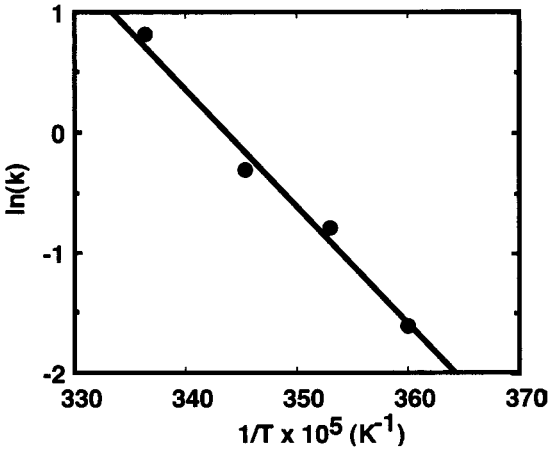

FIGURE 4: Temperature dependence of the rate constant of nNOS mediating L-citrulline formation from L-arginine in the steady state. The reactions were performed as outlined in Materials and Methods.

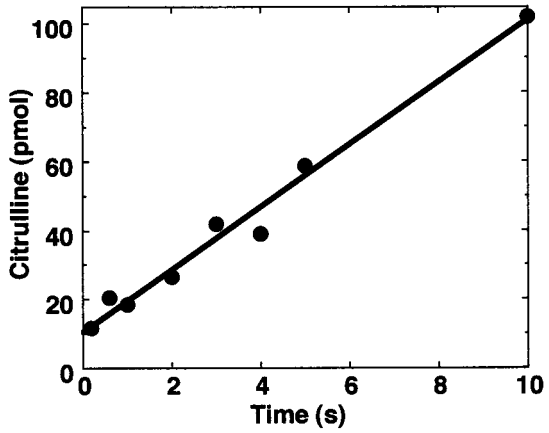

FIGURE 5: Estimation of the concentration of active nNOS in the steady state. The reaction was carried out at 25 °C with 7 μM L-arginine and 6 pmol nNOS. The value where the line crosses the *y*-axis shows the active concentration of nNOS in the steady state. The details are described in the text.

the formation of L-citrulline takes much less time than a single cycle of the enzyme reaction (*32*−*34*). We used the rapid quenching device to follow the burst and the subsequent production of L-citrulline at 25 °C in the time range from zero to 10 s in the presence of 7 μM of L-arginine containing 0.5 μCi ^{3}H-L-arginine and 6 pmol nNOS as shown in Figure 5. The time course of L-citrulline formation shows a linear increase from which we obtain the turnover number of 92 ± 15 nmol/min/nmol of nNOS. The value at the point where the line crosses the *y*-axis must indicate the amount of L-citrulline formed in the pre-steady state, which is equal to the active nNOS concentration. The value is 8.5 ± 2.0 pmol, almost equal to the amount of nNOS in solution, showing that almost all the NOS is active in this experiment.

DISCUSSION

That a small amount of N$^\omega$-hydroxy-L-arginine has been detected in the steady-state reaction of nNOS, converting L-arginine to L-citrulline, and NO does not necessarily mean that N$^\omega$-hydroxy-L-arginine is the intermediate metabolite in the reaction. It might occur as a by-product in the reaction. The consumption of 1.5 mol of NADPH would suggest two step monooxygenase reactions for the formation of one mol each of L-citrulline and NO from L-arginine, showing that there must be some intermediate metabolite in the reaction. Furthermore, nNOS metabolizes N$^\omega$-hydroxy-L-arginine into L-citrulline with the consumption of 0.5 mol of NADPH per mol of L-citrulline produced (*3*, *13*, *14*). There are no contradictions chemically in the scheme proposed for the NOS reaction to convert L-arginine to N$^\omega$-hydroxy-L-arginine and further to L-citrulline and NO. These studies suggest that N$^\omega$-hydroxy-L-arginine is the intermediate metabolite in the NOS reaction. Strictly speaking, however, there is no direct evidence that L-arginine is converted to N$^\omega$-hydroxy-L-arginine and further to L-citrulline in one cycle of the reaction.

To identify the intermediate metabolite in one cycle of the reaction, two conditions must be satisfied. It is necessary to terminate the reaction long before a single cycle is completed. The turnover number of nNOS for the conversion of L-arginine to L-citrulline and NO was 136 mol/min/mol at 25 °C in the steady state, which corresponds to a single cycle reaction of 441 ms (60000 ms/136). The reaction rapid-quenching device used in this experiment, Unisoku MX-200, is good enough for analyzing a single cycle of the nNOS reaction. To identify the intermediate, it is also necessary to separate the metabolites from the substrate and to quantify them at high sensitivity. We used ion-pair chromatography for the separation of L-arginine, N$^\omega$-hydroxy-L-arginine, and L-citrulline with an ODS-column using a low pH buffer containing 2-propanol and SDS (*30*). In this condition, the recovery of the metabolites was about 60%. The high sensitivity in the quantification was accomplished by using radioactive ^{3}H-L-arginine as the substrate where one can detect changes in the range of 0.02 pmol. The real usefulness of ^{3}H-L-arginine in the rapid quenching experiments is that only the metabolism of ^{3}H-L-arginine bound to nNOS at the initial stage is detectable in one cycle of the reaction in the presence of an excess amount of nonradioactive L-arginine in the reaction solution. The results of Figures 2 and 3 are the first direct experimental evidence that N$^\omega$-hydroxy-L-arginine is the intermediate metabolite in the two step monoxygenation reaction from L-arginine to L-citrulline and NO.

The rate constants of the two step reactions, k_1, k_2, and k_3 could be estimated by best fitting the observed data to the rate equations in the legend for Figure 3, which are derived from the reaction scheme in the upper part of the figure. It becomes clear that the rates for the first and the second reactions are not much different each other but that for the dissociation of the intermediate ^{3}H-N$^\omega$-hydroxy-L-arginine from the enzyme is significantly slow (Table 1). We cannot estimate the rate constant for dissociation of the product ^{3}H-L-citrulline only from the data of the rapid quenching experiments. This reaction scheme is the same as that for the P450$_{17\alpha}$ reaction, which catalyzes the conversion of progesterone to androstenedione via 17α-hydroxyprogesterone as the intermediate metabolite where a part of the intermediate metabolite is metabolized without leaving the enzyme (*22*). In the analysis of P450$_{17\alpha}$ mediating progesterone metabolism, the rate constant of the product dissociation was estimated by combining the results of rapid-quenching experiments with those from the steady-state kinetics, which was the smallest rate constant in one cycle of the reaction of P450$_{17\alpha}$. The L-citrulline production activity in the steady state in this study can be expressed with the

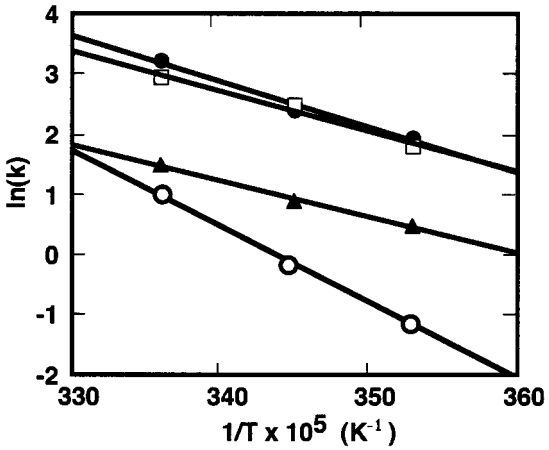

FIGURE 6: Temperature dependence of the rate constant of each step of the nNOS reaction in a single cycle of the reaction. The closed circles, closed triangles, open squares, and opened circles represent the rate constants for k_1, k_2, k_3, and k_4, respectively. The details are described in the text.

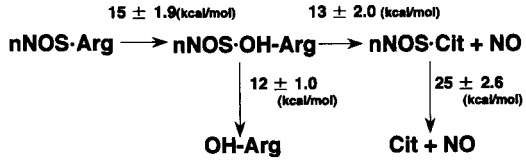

FIGURE 7: The activation energy for each reaction step of nNOS catalyzing successive reaction converting L-arginine to L-citrulline and NO. The activation energy was obtained from the slope of the lines in Figure 6.

same equation as that used for the successive reaction of $P450_{17\alpha}$, $v = k_1k_3k_4[Et]/((k_1 + k_2 + k_3)k_4 + k_1k_3)$ where k_4 is the rate constant for the dissociation of L-citrulline from the enzyme and [Et] represents the total concentration of the enzyme in the steady-state reaction (*22*, *35*). The dissociation rate constant of L-citrulline, k_4 at 25 °C, was calculated as $2.7 \pm 0.3\ s^{-1}$, which is, apparently, the smallest rate constant in the scheme. The values of k_4 at 17 and 10 °C were also calculated and are listed in Table 1. In this calculation, we assumed that all the nNOS in the reaction solution is active for the catalysis. The active concentration of the enzyme can be determined from the burst formation of the product L-citrulline, which corresponds to the amount of L-arginine bound to nNOS before the initiation of the reaction, if the burst formation is significantly faster than one cycle of the nNOS reaction in the steady state. The smallest rate constant of k_4 in the reaction scheme ensures the condition for the estimation of the active concentration of the enzyme (*32*−*34*).

The rate constants, k_1, k_2, and k_3 at 10 °C increased about 3-fold with the temperature increase to 25 °C, but k_4 increased about 8-fold. The logarithm of the rate constants are plotted against the reverse of the observed temperature (Figure 6). The obtained rate constants fit well with the Arrhenius equation, $k = Ae(-Ea/RT)$ where k and Ea are the observed rate constant and the activation energy for the reaction, respectively. We estimated the activation energy for each reaction step from Figure 6. The values are listed in Figure 7. As expected from the slopes of the lines, the values of activation energy for k_1, k_2, and k_3 are almost the same but that for the dissociation of L-citrulline from nNOS is about twice larger. It is remarkable that the activation energy for k_4 (24.8 ± 2.6 kcal/mol) almost coincides with the value for the activation energy of the reaction in the steady state (19 ± 1.9 kcal/mol). That the activation energy is highest for the dissociation of the product is not unreasonable, because the dissociation of the product must significantly perturb the enzyme structure. The activation energy for the conformation change in P450d induced by substrate binding was reported to be 16.4 kcal/mol (*36*). The highest activation energy and the smallest rate constant for the dissociation of the product L-citrulline from nNOS clearly shows that the dissociation is the rate-determining step in the steady state reaction.

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