Dual Effects of the Substrate and Pterins on the Kinetics of CO Binding to Neuronal Nitric Oxide Synthase: A Laser Flash Photolysis Study

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Derivatives of pterin, an important cofactor for neuronal nitric oxide synthase, markedly changed the kinetics of CO binding to the enzyme-bound heme. In the absence of the substrate, L-Arg, CO binding was dependent on the CO concentration for pterin complexes, whereas in the presence of L-Arg, it became CO-independent for most pterin complexes. This suggests dual effects of substrate and pterin binding on the kinetics and access of CO to the ligand channel.

Nitric oxide (NO), a key signalling molecule, is the final product of the sequential oxidation of the terminal guanidino nitrogen of L-Arg and is produced by NO synthase (NOS) via the intermediate N^G-hydroxy-L-Arg (NHA).[1–4] The mechanisms of the two monooxidations by NOSs are similar to that of cytochrome P450 in that the electrons critical for catalysis are supplied by NADPH through the FAD- and FNM-bound reductase domain. However, in contrast to the P450 system, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H4B) is essential for catalysis. H4B delivers electrons to the oxygen-bound heme complex of NOS, activating the molecular oxygen and the cleavage of the O–O bond,[5] but it is not yet clear how H4B regulates the access of O_2 to the heme active site in the presence of L-Arg.

Examination of the O_2 binding kinetics even with sophisticated kinetic methods such as laser flash photolysis is difficult because of the high rate of autoxidation of the heme in NOS. Instead, studies of CO binding kinetics are useful for exploring the structure and characteristics of the ligand access channel and the heme distal side of NOS,[6–10] because CO does not cause enzyme oxidation. Therefore, to understand the effects of H4B on the structure and dynamics of the ligand access channel, in the present study we examined the kinetics of CO binding to the isolated heme-bound oxygenase domain (nNOSox) of neuronal NOS by laser flash photolysis in the presence of L-Arg and various pterin derivatives (Supplementary Figure S1).

The flash photolysis curve associated with the binding of CO was composed of only one phase, and the rate constants obtained in the absence of L-Arg were dependent on the concentration of CO (Supplementary Figure S2). However, in the presence of H4B and the other pterin derivatives except for 6-methyl-B, the CO-dependent rate constants were smaller than in the absence of pterins (Table 1). This suggested that these pterin derivatives hampered CO binding. Interestingly, the presence of 6-methyl-B enhanced the rate constants by 60%, and of the seven pterin derivatives studied here, 6-methyl-B is the only one lacking a 1,2-dihydroxypropyl side chain.

The crystal structure of the heme active site of nNOS in the presence of H4B and the substrate NHA is available from Protein Data Bank (PDB number: 1LZX) (Supplementary Figure S3). The 1,2-dihydroxypropyl side chain of H4B forms (i) hydrogen bonds with the main-chain carbonyl groups of Ser334 and Phe691; and (ii) hydrophilic and/or hydrophobic interactions with both Ser334 and Phe691 within the pterin pocket.[16] It is likely that the CO access channel in the 6-methyl-B-bound complex was wider than that in other pterin-bound complexes. This may be a main reason why the CO binding rate was high for the 6-methyl-B complex. Raman et al.[17] show that the stereo-specific recognition of H4B by NOS is dictated by the substitution at the C6 position. Importantly, the N-terminal hairpin hook required for the dimerization of NOS proteins interacts with the H4B dihydroxypropyl side chain and forms the access channel to the substrate.[17,18]

The binding of L-Arg largely closes CO access channel since CO binding rate became CO-independent irrespective of the presence of H4B or H2B (Table 1). However, in contrast to the singly type of binding in the H4B-bound enzyme, in the presence of L-Arg, two types of CO binding were observed. Specifically, in the presence of 4-amino-B or 5-methyl-B, CO concentration-dependent rate constants were observed, whereas in the presence of H4B, H2B, 4-amino-H2B, 6-methyl-B, or L-sepiaptein or in the absence of pterins, CO concentration-independent binding was observed (Table 1: Supplementary Figure S4). These dual effects of the substrate and pterins on the kinetics of the CO binding to NOS have not been previously observed. The kinetics of CO-independent CO binding in the presence of L-Arg and other biopterin derivatives may be due to partial closure of the CO access channel by these compounds so that the CO molecule cannot move from the heme plane all the way to the protein surface after flash photolysis. In contrast, the 4-amino group in 4-amino-B and the 5-methyl group in 5-methyl-B may allow the CO binding channel to open so that the CO molecule can move to the protein surface and contact the solvent. L-Sepiapterin has the same amino group at the fourth position as 4-amino-B, but 4-amino-B has a double bond in the B ring of the pterin structure that may cause the effect of the 4-amino group on the CO binding kinetics.

It was reported that H2B and L-sepiapterin compete with H4B for the same binding site on endothelial NOS (eNOS).[19] For nNOS, it is likely that these pterins share the same site or binding orientation. In addition, 4-amino-B binding to nNOS has been reported to cause allosteric changes in the protein that are indistinguishable from the effect of H4B.[20,21] Crane et al.[22] showed that a hydrogen bonding interaction between the pterin 3,4-amide and a heme propionate group is essential for the ability of H4B to influence heme reactivity. Specifically, 4-amino-H4B binds the active site of the NOS oxygenase domain with the same geometry as H4B and binds with higher affinity than H4B.[20,21,23] Poulos et al.[24] explain that the interdependence of pterin and L-Arg binding is mediated by the series

Table 1. Rate constants of concentration-dependent ($\times 10^6$ $M^{-1}\,s^{-1}$) and -independent (s^{-1}) CO recombination with nNOSox in the presence and absence of L-Arg and pterin derivatives[a,b]

	−L-Arg	+L-Arg
No addition	1.0	201 (CO-independent)
+H4B	0.59	101 (CO-independent)
+H2B	0.52	160 ± 41 (CO-independent)
+4-amino-B	0.66	0.62
+4-amono-H2B[c]	0.42	150 ± 48 (CO-independent)
+5-methyl-B	0.75	0.85
+6-methyl-B	1.62	136 ± 60 (CO-independent)
+L-sepiapterin	0.50	139 ± 18 (CO-independent)

H4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; H2B, 7,8-dihydro-L-bioptein; 4-amino-B, 4-amino-3,4-dihydro-L-biopterin; 5-methyl-B, (6*R*)-5-methyl-5,6,7,8-tetrahydro-L-biopterin; 6-methyl-B, (6*R*/*S*)-6-methyl-5,6,7,8-tetrahydro-L-biopterin dihydrochloride; 4-amino-H2B, 4-amino-3,4,7,8-tetrahydro-L-biopterin; L-sepiapterin, 6-lactoyl-7,8-dihydro-L-pterin.
[a]Concentration of the substrate, L-Arg was 10 mM, and those of pterin and pterin derivatives were up to 100 μM. The concentrations were sufficient to cause the maximal effects on the CO binding rates. Solutions in the absence of H4B consisted of 8 μM nNOSox, 2 mM 2-mercaptoethanol, 1% glycerol, and 50 mM Tris-HCl (pH 7.5), whereas those in the presence of H4B consisted of 8 μM nNOSox, 10 to 100 μM H4B, 1 mM dithiothreitol, 1% glycerol, and 50 mM Tris-HCl (pH 7.5). Flash photolysis experiments were repeated at least three times, and the average values are presented. Experimental errors were within 20% except for those noted in Table 1. See Refs. 10–15 and Supplementary Materials for protein purification and laser flash photolysis experiments. [b]All pterin derivatives were purchased from Schirks Laboratories (Jona, Switzerland). Other reagents of the highest quality available from Wako (Osaka, Japan) and were used without further purification. [c]CO dependence was observed only in the presence of high CO concentrations.

of H-bonds which are conserved in all NOS isoforms and by the binding of the pterin co-factor and L-Arg hydrogen bond to the same heme propionate. Studies of the interaction of 4-oxo- and 4-aminopteridine with nNOS by Kostonis et al.[16] suggested that the 4-oxopteridine binds more tightly to the enzyme because of enhanced hydrophilic interactions with both the propionic acid group of the heme and the iminium group of the guanidine in Arg367 (Arg375 in inducible NOS and Arg367 in eNOS). Crane et al.[22] further showed that an amino substitution at the 4-position of the pterin ring enhances binding because it makes protonation more favourable. A crystal structure of 5-methyl-B-bound inducible NOS revealed that there are small changes in conformation of the bound pterin and in its interactions with the protein.[25] These previous results emphasize the important role of the 4- and 5-positions of the pterin ring in binding to nNOSox. They also agree with our findings that the CO binding rates are CO-dependent only in the presence of L-Arg and either 4-amino-B or 5-methyl-B and that the rates in the presence of other pterins are CO-independent.

In conclusion, we show for the first time that substrate and pterins have dual effects on the kinetics of the CO binding to the heme of nNOSox. It appears that H4B not only donates electrons to the O_2-bound heme but also plays an important role in modulating the O_2 (and CO) access channel.

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