Minireview

Update on Mechanism and Catalytic Regulation in the NO Synthases*

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Nitric-oxiđe synthases (NOSs, EC 1.14.13.39)1 oxidize l-arginine to nitric oxide (NO) and are interesting for several reasons. They are present in many life forms (1, 2), their gene regulation is complex (3), they are the only flavoheme enzymes reasons. They are present in many life forms (1, 2), their gene regulation (4, 5). Reviews are available on NOS biochemistry (6), regulation (7, 8), protein-protein interactions (9), and post-translational modifications (10). This minireview updates the NO biosynthetic mechanism and describes a global catalytic model that highlights the role of NO as an intrinsic regulator.

Mechanism of NO Biosynthesis

NOS is one of few heme-containing enzymes that make NO (11, 12). NOS holoenzymes a guanidino nitrogen of Arg and then oxidizes the N\textsuperscript{\textomega}-hydroxy-l-arginine intermediate (NOHA) to NO and l-citrulline (Scheme 1). The NOS flavoprotein domain first provides an electron (derived from NADPH) to the ferric heme (H\textsubscript{3}B) as a redox cofactor, and their electron transfer reactions are regulated by a Ca\textsuperscript{2+}-binding protein (calmodulin). In the past 5 years, crystal structures of NOS heme (oxygenase) domains and bacterial NOS-like proteins have shown how Arg, heme, and H\textsubscript{4}B bind in the active site (4, 5). Reviews are available on NOS biochemistry (6), regulation (7, 8), protein-protein interactions (9), and post-translational modifications (10). This minireview updates the NO biosynthetic mechanism and describes a global catalytic model that highlights the role of NO as an intrinsic regulator.

Each NOS Distributes Differently during Catalysis—Values of the three kinetic parameters (“k\textsubscript{f}, k\textsubscript{a}, k\textsubscript{d}”) differ significantly among NOSs (Table I), and this causes each NOS to distribute differently during steady-state NO synthesis. The enzyme distributions in Fig. 2B were derived from computer simulations of the global kinetic mechanism and mimic distributions estimated from actual experiments (17, 28, 29). NOSs distribute into five main forms during steady-state NO synthesis, namely the ferric, ferrous, ferrous-O\textsubscript{2} (or ferric-superoxo), ferric-NO, and ferrous-NO forms. For nNOS, a fast k\textsubscript{f}, k\textsubscript{a} relative to k\textsubscript{d} and k\textsubscript{a} causes it to exist predominantly as a ferrous-NO species. For eNOS the situation is reversed; a slow k\textsubscript{f}, relative to k\textsubscript{d} or k\textsubscript{a} causes it to exist predominantly as a ferric species. For iNOS, its moderately fast k\textsubscript{f} and fast k\textsubscript{a} create an enzyme distribution that is between the two extremes.

Knowing the distribution pattern helps to understand NOS...
NO Synthase Mechanism and Catalytic Regulation

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NOSs Have an Optimal Rate of Heme Reduction—With sufficient substrates the rate of NO biosynthesis is simply limited by \( k_r \) (Fig. 1). However, the relationship between \( k_r \) and the rate of NO release is modified by ferric heme-NO complex formation at the end of each catalytic event. The global kinetic model predicts that NOS will increase its NO release as a function of \( k_r \), but at a certain point the NO release will reach a maximum and then fall despite the enzyme working faster and faster (Fig. 3). The bell-shaped curves can be rationalized by considering how \( k_r \) impacts the ferric heme-NO product complex. Increasing \( k_r \) speeds its formation but also partitions more of it into the futile cycle (\( k_r' \)), which diminishes NO release via the productive pathway and reciprocally increases futile cycling and nitrate production (Fig. 3). The relationship between \( k_r \) and the rate of products release is also influenced by the \( k_r \) and \( k_{ox} \) values of each NOS. The approximate location of each NOS on its curve is indicated by an arrow in Fig. 3. Note that nNOS has evolved a near optimal \( k_r \), whereas eNOS and iNOS have sub-optimal rates.

The Effect of External NO—Binding solution NO to the NOS heme becomes significant at low micromolar NO concentrations. This equilibrium binding event differs from the reaction of newly generated NO with the ferric heme in the heme pocket. Solution NO binding is particularly important when the ferric form of a NOS predominates during steady-state catalysis (as for eNOS and iNOS). In this circumstance, solution NO binding increases the concentration of the ferric heme-NO species and alters the enzyme distribution pattern (Fig. 4A). It leads to greater production of the ferrous heme-NO complex. Increasing \( k_r \) speeds its formation but also partitions more of it into the futile cycle (\( k_r' \)), which diminishes NO release via the productive pathway and reciprocally increases futile cycling and nitrate production (Fig. 3). The relationship between \( k_r \) and the rate of products release is also influenced by the \( k_r \) and \( k_{ox} \) values of each NOS. The approximate location of each NOS on its curve is indicated by an arrow in Fig. 3. Note that nNOS has evolved a near optimal \( k_r \), whereas eNOS and iNOS have sub-optimal rates.

Interplay of Three Kinetic Parameters Determines Activity

NOS activity depends on interplay of \( k_r \), \( k_{ox} \), and \( k_{cat} \). With this in mind, we provide two examples of NOS catalytic behavior that could only be understood in the context of the global kinetic model and the three kinetic parameters.

More Is Less: the Case of S1412D nNOS—Both eNOS and nNOS contain a consensus sequence for Akt-dependent Ser phosphorylation in their C-terminal regulatory elements (8, 9). Phosphorylation at this Ser (or point mutation to Asp) caused a 3-fold increase in eNOS activity. However, identical point mutation in nNOS (S1412D) lowered its NO release rate by 30% (31). Analysis showed that S1412D nNOS actually has a faster \( k_r \) than wild type (Table I) and therefore a faster NO biosynthesis. Calmodulin mutants were used to slow down \( k_r \), in S1412D nNOS. This slowed down its NO biosynthesis but increased its NO release rate (31). This occurs because the S1412D mutation increased \( k_r \) beyond optimal in nNOS. Conversely, increasing \( k_r \) in eNOS by the same mechanism should increase its NO release because its basal \( k_r \) is below optimal (Fig. 3).

Less Is More: the Case of W409F nNOS—Most NOSs contain...
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TABLE I

Kinetic values for NOSs and selected heme proteins

| Protein       | $k_a$ | $k_d$ | $k_{ox}$ | Apparent $K_{O2}$ | Refs. |
|---------------|-------|-------|----------|-------------------|-------|
| nNOS          | 3–4   | 5     | 0.2      | 350               | 16, 30|
| iNOS          | 0.9–1.5 | 2     | 3        | 130               | 17, 29|
| eNOS          | 0.1   | 3     | 0.6      | 4                 | 17, 28|
| W409F nNOS    | 1.8   | 5     | NA       | NA                | 16, 32|
| S1412D nNOS   | 5.4   | 5     | NA       | 738               | 16, 31|
| Cyt P450BM3   | 99    | (15 °C) | NA      | 4                  | 42    |
| Mammalian Cyt P450 | NA   | NA   | 4–10 (10 °C) | 43 |
| Flavohemoglobin | 150 (37 °C) | 200–4000 (20 °C) | 12 (10 °C) | 60–90 (20 °C) | 44–46 |
| Myoglobin     | NA    | 40    | 10–2 (37 °C) | NA                | 47, 48|

*Calculated from a simulation of the global kinetic model.

Values of the $k_a$, $k_d$, and $k_{ox}$ parameters were determined at 10 °C unless otherwise noted. Apparent $K_{O2}$ values for NO synthesis were determined at 25 °C. $k_a$, rate of ferric heme reduction; $k_d$, dissociation rate of the ferric heme-NO complex; $k_{ox}$, rate of reaction between the ferrous heme-NO complex and approximately 140 μM O₂ (half air-saturated conditions). NA, not available; Cyt, cytochrome.

The Unusual O₂ Response of NOSs—Apparent $K_{O2}$ values differ among the NOSs and in some cases are much higher than those reported for related monoxygenases (Table I). This is because NOS interacts with O₂ in two ways; the ferrous enzyme binds O₂ during NO biosynthesis, and the ferrous heme-NO species reacts with O₂ in the futile cycle ($k_{ox}$). Both NOSs have slow heme reduction rates which contribute to their specificity.

Some Implications

Why Do NOSs Have Slow Heme Reduction Rates?—Other flavoheme enzymes have faster $k_a$ values compared with the NOSs (Table I), suggesting that NOSs are under a unique selective pressure. Their slow $k_a$ is understandable, because increasing $k_a$ beyond optimal will partition more NOS into the futile cycle and ultimately convert it into an NO oxygenase (Fig. 3). However, the slow $k_d$ makes it difficult for NOS to couple oxygen activation to substrate oxidation (19, 33). This is because delivery of the second electron to the heme must be rapid enough for the enzyme to generate the heme-oxy species that will react with Arg or NOHA before autoxidation of ferric-superoxy species occurs (18). NOS solves this dilemma by utilizing H₄B as a source of the second electron. H₄B delivers the second electron about 3–30 times faster than can the NOS flavoprotein, and this difference is sufficient to minimize superoxide release from the heme and so enable coupled oxygen activation (20, 23). Thus, heme-N0 binding imposes a kinetic constraint on NOS heme reduction that impacts its coupled oxygen activation. NOS overcomes this problem by using two kinetically distinct sources of electrons: a slow electron transfer from the flavoprotein to minimize ferrous heme-NO formation and futile cycling, and a fast reduction by H₄B at the kinetically sensitive step in its oxygen activation cascade.

**FIG. 4.** Effect of NO concentration on iNOS enzyme distribution pattern and product release rates in the steady state (A and B) and enzyme distribution pattern of W409F nNOS during the steady state (C). A and B, simulations were done as described in Fig. 2 but factored in the presence of solution NO at the indicated concentrations. C, the structure was adapted from the crystal structure of mouse iNOS oxygenase (1DWX) and highlights a hydrogen bond between Trp-409 and the heme-thiolate ligand (Cys-415) in nNOS. The enzyme distribution shown in the pie graph was calculated by simulation using kinetic parameters from Table I and reaction conditions described in the legend of Fig. 2.
interactions combine to determine the apparent $K_m$ for $O_2$. The oxygen concentration dependence of $k_{ox}$ becomes particularly important if the ferrous heme-NO species builds up in the steady state. This situation occurs naturally for nNOS but not for eNOS, and so the apparent $K_m$ for $O_2$ of eNOS is low and reflects almost entirely the $O_2$ concentration response of its NO biosynthetic reaction. The high apparent $K_m$ for $O_2$ of nNOS holds for nNOS activity in whole animals and tissues (34, 35), suggesting it is intrinsic to the enzyme. Solution NO binding to the ferric heme also increases the apparent $K_m$ for $O_2$ and NO (28, 29). A low $K_m$ also increases the apparent turnover number for nNOS activity in whole animals and tissues (34, 35), suggesting that NO binding to the enzyme contributes to its productive and futile catalytic cycles. Although $k_{ox}$ and $k_{dis}$ values for the three kinetic parameters differ among NOSs throughout the animal kingdom? Are the kinetic parameters of some NOSs set to favor futile cycling instead of NO release? Bacterial NOSs may be configured this way (39). What is the physical basis of the three kinetic parameters? Presently, we only know that the NOS flavoprotein determines $k_{ox}$ (40, 41) whereas the oxygenase domain determines $k_{ox}$ and $k_{dis}$ (17). The wide variation in $k_{ox}$ and $k_{dis}$ values among heme proteins (Table I) suggests that interesting structure-function relationships remain to be explored. NO function in signaling and cytotoxicity may also be linked to its productive and futile catalytic cycles. Although written as nitrate, the identity of the futile cycle product depends on the oxidation mechanism of the ferrous heme-NO complex and could be the cytosolic molecule peroxynitrite. It will be interesting to see how NOS product release, which differs according to the environment and kinetic parameters of a NOS, correlates with its physiologic roles.

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