Abstract

Overproduction of nitric oxide (NO) by inducible NO synthase (iNOS) has been implicated in the pathogenesis of many diseases. iNOS is active only as a homodimer. Dimerization of iNOS represents a potentially critical target for therapeutic intervention. In this study, we show that intracellular iNOS forms dimers that are ”undisruptable” by boiling, denaturants, or reducing agents. Undisruptable (UD) dimers are clearly distinguishable from the easily dissociated dimers formed by iNOS *in vitro*. UD dimers do not form in *Escherichia coli*-expressed iNOS and could not be assembled *in vitro*, which suggests that an *in vivo* cellular process is required for their formation. iNOS UD dimers are not affected by intracellular depletion of $H_4B$. However, the mutation of Cys-115 (critical for zinc binding) greatly affects the formation of UD dimers. This study reveals insight into the mechanisms of *in vivo* iNOS dimer formation. UD dimers represent a class of iNOS dimers that had not been suspected. This unanticipated finding revises our understanding of the mechanisms of iNOS dimerization and lays the groundwork for future studies aimed at modulating iNOS activity *in vivo*.

Nitric oxide (NO) is an important signaling and cytotoxic molecule that is synthesized from l-Arg by isoforms of NO synthase (NOS) (1-3). As a signaling molecule, NO is produced by two constitutive calcium ($Ca^{2+}$)-dependent isoforms: neuronal NOS and endothelial NOS. $Ca^{2+}$-activated calmodulin binds to and transiently activates constitutive NOS dimers. Due to the transient nature of elevated $Ca^{2+}$ levels, the activity of produced NO is short-lived. As an agent of inflammation and cell-mediated immunity, NO is generated by a $Ca^{2+}$-independent cytokine-inducible NOS (iNOS) that is widely expressed in diverse cell types under transcriptional regulation by inflammatory mediators (2-4). Even at basal $Ca^{2+}$ levels, calmodulin is tightly bound to iNOS. For this reason, iNOS is notably distinguished from the constitutive isoforms for its production of relatively large amounts of NO (5). iNOS has been implicated in the pathogenesis of many diseases, some of which include Alzheimer's disease, tuberculosis, asthma, glaucoma, inflammatory bowel disease, arthritis, stroke, and septic shock (6, 7). Such wide-based implication has in turn produced an interest in understanding the regulation of NO synthesis by iNOS with the intrinsic goal of developing therapeutic strategies aimed at selective modulation of iNOS activity (6-8).

The human iNOS gene contains 26 exons and encodes a protein of 131 kDa (9, 10). Human iNOS has three domains: (*i*) an amino-terminal oxygenase domain that binds heme, tetrahydrobiopterin ($H_4B$) and l-Arg; (*ii*) a carboxyl-terminal reductase domain that binds FMN, FAD, and NADPH; and (*iii*) an intervening calmodulin-binding domain that regulates electron transfer between the oxygenase and reductase domains (3, 9, 11). iNOS, similar to other NOSs, is active only as a homodimer in which the subunits align in a head-to-head manner, the aminoterminal oxygenase domains forming the dimer interface (12).

Posttranslational subunit dimerization of iNOS represents a potentially critical locus for therapeutic interventions aimed at regulating its activity. There have been a large number of studies addressing the mechanisms of iNOS dimerization (3, 12-15), but most of these studies were performed *in vitro* under dictated experimental conditions and by using either recombinant protein or partial domains. Very few studies have addressed iNOS dimerization *in vivo* using cultured or primary cells. In this study, we show that intracellular iNOS forms dimers that are ”undisruptable” by heat, SDS, strong denaturants, and/or reducing agents. These dimers are clearly distinguishable from the easily dissociated dimers formed by

iNOS *in vitro*. This unexpected finding revises our understanding of the mechanisms of iNOS dimerization.