Abstract

Human inducible nitric oxide synthase (iNOS) is active as a dimer of two identical subunits. Each subunit has an amino-terminal oxygenase domain that binds the substrate l-Arg and the cofactors heme and tetrahydrobiopterin and a carboxyl-terminal reductase domain that binds FMN, FAD, and NADPH. We previously demonstrated that a subdomain in the oxygenase domain encoded by exons 8 and 9 is important for dimer formation and NO synthesis. Further, we identified Trp-260, Asn-261, Tyr-267, and Asp-280 as key residues in that subdomain. In this study, using an *Escherichia coli* expression system, we produced, purified, and characterized wild-type iNOS and iNOS-Ala mutants. Using H_2O_2-supported oxidation of N^{ω}-hydroxy-l-Arg, we demonstrate that the iNOS mutants' inabilities to synthesize NO are due to selective defects in the oxygenase domain activity. Detailed characterization of the Asp-280–Ala mutant revealed that it retains a functional reductase domain, as measured by its ability to reduce cytochrome *c*. Gel permeation chromatography confirmed that the Asp-280–Ala mutant exists as a dimer, but, in contrast to wild-type iNOS, urea-generated monomers of the mutant fail to reassociate into dimers when incubated with l-Arg and tetrahydrobiopterin, suggesting inadequate subunit interaction. Spectral analysis reveals that the Asp-280–Ala mutant does not bind l-Arg. This indicates that, in addition to dimerization, proper subunit interaction is required for substrate binding. These data, by defining a critical role for Asp-280 in substrate binding and subunit interactions, give insights into the mechanisms of regulation of iNOS activity.