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Studies on the Reaction of Nitric Oxide with the Hypoxia-Inducible Factor Prolyl Hydroxylase Domain 2 (EGLN1)

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The hypoxic response in animals is mediated via the transcription factor hypoxia-inducible factor (HIF). An oxygen-sensing component of the HIF system is provided by Fe(II) and 2-oxoglutarate-dependent oxygenases that catalyse the posttranslational hydroxylation of the HIF-α subunit. It is proposed that the activity of the HIF hydroxylases can be regulated by their reaction with nitric oxide. We describe biochemical and biophysical studies on the reaction of prolyl hydroxylase domain-containing enzyme (PHD) isoform 2 (EGLN1) with nitric oxide and a nitric oxide transfer reagent. The combined results reveal the potential for the catalytic domain of PHD2 to react with nitric oxide both at its Fe(II) and at cysteine residues. Although the biological significance is unclear, the results suggest that the reaction of PHD2 with nitric oxide has the potential to be complex and are consistent with proposals based on cellular studies that nitric oxide may regulate the hypoxic response by direct reaction with the HIF hydroxylases.

Introduction

In animals, the cellular responses to hypoxia involve an extensive gene array that is regulated by the α,β-heterodimeric hypoxia-inducible transcription factor (HIF); for reviews, see Refs. [1–4]. Both stability and transcriptional activity of the HIF-α subunit are regulated by its posttranslational hydroxylation. Hydroxylation of either of two prolyl residues in the N- and C-terminal oxygen-dependent degradation domains (NODD and CODD, respectively) of human HIF-α promotes its binding to the von Hippel–Lindau protein elongin B/C complex, thus signalling for the degradation of HIF-α via the

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Abbreviations used: PHD, prolyl hydroxylase domain-containing enzyme; HIF, hypoxia-inducible factor; 2OG, 2-oxoglutarate; CODD, C-terminal oxygen-dependent degradation domain; NODD, N-terminal oxygen-dependent degradation domain; GSNO, *S*-nitrosoglutathione; EPR, electron paramagnetic resonance; PDB, Protein Data Bank; MS, mass spectrometry; MMTS, methyl methanethiosulfonate; TOF, time-of-flight; LC, liquid chromatography.

ubiquitin–proteasome pathway. The HIF prolyl hydroxylations are catalysed by a set of closely related HIF prolyl hydroxylase domain-containing enzymes (PHDs or EGLNs), of which the most important in humans is PHD2. HIF activity is also regulated by a separate mechanism involving asparaginyl hydroxylation in its C-terminal transactivation domain. The HIF hydroxylases are members of the ubiquitous Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase superfamily. The dependence of the PHDs on molecular oxygen as a co-substrate, together with other biochemical properties, enables them to act as an oxygen-sensing component for the HIF system.[1–4]

Nitric oxide is an important signalling molecule involved in diverse physiological functions including vasodilation, the immune response, and neurotransmission.[5,6] Studies on the effect of NO (or NO donors/transfer reagents) on the HIF system have suggested that, at least in some cell types, it has a bimodal profile with respect to oxygen availability.[7] Under normoxia, NO donors/transfer reagents stabilise HIF-1α and promote HIF-mediated transcriptional activity in cultured cells[8] and in macrophages during bacterial infection.[9] In contrast, under hypoxic conditions, NO and reactive nitrogen species antagonise HIF activity.[10,11] There is evidence that the effect of NO on the hypoxic response system is related to HIF-α prolyl hydroxylation.[10,11] A recent study has reported that NO donors inhibit PHD2 catalysis.[12] Here, we report biochemical and biophysical studies on the reaction of PHD2 with NO. The results reveal that the reaction of PHD2 with NO has the potential to be complex, involving reactions at both the Fe(II) centre and more than one cysteinyl residue.

Results

Previous work has shown that NO donors (e.g., *N*-acetyl-*N*-nitroso-tryptophan) inhibit PHD2 catalysis.[12] Because NO donors may react to give different products, or react at different rates, compared to NO itself, we initially investigated the effect of "free" NO on the activity of the catalytic domain of PHD2 (residues 181–426; tPHD2 hereafter). Freshly prepared NO solutions (ranging from 3 to 50 μM) were added to tPHD2 (4 μM) under near-anaerobic (PO_2 < 0.1 ppm) conditions for an hour prior to activity assays. The NO-treated tPHD2 was then assayed by using a 2OG decarboxylation assay under standard aerobic conditions. The results reveal that, under our standard assay conditions, NO inhibits tPHD2 in a dose-dependent manner with an IC_{50} of 10 μM (see Fig. 1a).

To test whether NO binds to Fe(II) at the PHD2 active site, as has been observed for some other 2OG oxygenases and related enzymes,[13,14] we then performed electron paramagnetic resonance (EPR) experiments (Fig. 1b). The spectrum of tPHD2 alone revealed a small feature at $g = 4.3$, characteristic of a small amount of ferric iron present in the sample, as observed previously.[15] Anaerobic addition of dissolved NO resulted in the formation of an EPR signal in the $g = 4$ region, typical of the [FeNO],[7] $S = 3/2$ signal previously observed for NO binding to the ferrous active site of other non-heme iron oxygenases.[16–18] Compared to the tPHD2–Fe(II) complex, the tPHD2–Fe(II)–2OG complex was apparently unable to or has a very much reduced ability to bind NO at its active-site Fe(II) with only a small feature appearing in the $g = 4$ region (at least within the timescale of our analyses). This observation is supportive of NO binding to the active-site Fe (II) because in the PHD2–Fe(II)–2OG complex, the 2OG binds to the Fe(II). No such signal with a substantial intensity was detectable when *S*-nitrosoglutathione (GSNO) (250 eq) was added under near-anaerobic conditions (Fig. 1b), raising the possibility that NO itself and NO donors may react differently with PHD2 under the experimental conditions.

We then attempted to obtain a crystal structure of a tPHD2–Fe–NO complex. tPHD2 crystallises in the presence of particular inhibitors that complex to its active-site metal [either Fe(II) or a surrogate metal].[19] Because NO and NO donors may react differently, we attempted to obtain structures of the tPHD2 with both NO and an NO donor, GSNO. Pre-grown tPHD2–Fe(II)–inhibitor crystals were exposed to NO gas under anaerobic conditions, whilst tPHD2–Zn(II)–inhibitor crystals were soaked with 50 mM GSNO prior to cryo-cooling in liquid nitrogen. Structural models were generated by molecular replacement using a reported tPHD2 structure [Protein Data Bank (PDB) ID: 2G19] (Table S1). NO binding to the Fe(II) was not observed in either of the structures obtained. Given that 2OG appeared to block NO binding to Fe(II) as observed by EPR, it is possible that the inhibitor used to induce tPHD2 crystallisation also blocks NO binding, though we cannot also rule out the possibility of crystal packing effects. However, both data sets had positive difference density extending from the side chain of Cys302, suggesting the addition of an NO group to this residue (Fig. 1c), and a different mode of NO delivery to PHD2.

The tPHD2SNO–Fe(II)/Zn(II)–inhibitor structures were initially refined using the stereochemical restraints for an *S*-nitrosocysteine residue as described for the crystal structure of blackfin tuna myoglobin (PDB ID: 2NRM; 1.09 Å resolution[20]) with the C–S–N–O dihedral angle unrestrained. Attempts to refine the structural models imposing planar geometric restraints for the *cis*/*trans*-SNO dihedral angles were not productive because the O of the SNO group could not be fitted within the experimental electron density. The exact chemical nature of *S*-nitroso tPHD2 adducts is therefore