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Sensitive and Selective Detection of Nitric Oxide Using an H−NOX Domain

Elizabeth M. Boon and Michael A. Marletta*

Department of Chemistry, Department of Molecular and Cellular Biology, and Division of Physical Sciences, Lawrence Berkeley National Laboratory, University of California, Berkeley, Berkeley, California 94720-1460

Received May 25, 2006; E-mail: marletta@berkeley.edu

The H−NOX (*H*eme-*N*itric *O*xide or *OX*ygen binding domain) family is a newly discovered family of heme proteins with significant homology to soluble guanylate cyclase (sGC), a well-characterized and conserved eukaryotic nitric oxide (NO) receptor.[1] The crystal structure of a very stable H−NOX domain from the thermophile *Thermoanaerobacter tengcongensis* (*Tt* H−NOX) has been solved to 1.77 Å resolution.[2] This H−NOX has several exploitable properties: it is (i) easily overexpressed and purified, (ii) stable to oxidation, and (iii) amenable to mutagenesis. Furthermore, specific amino acids that contribute to discrimination between NO and O_2 as ligands are known. A distal pocket tyrosine (Y140 in *Tt* H−NOX) has been shown to be the key molecular switch for O_2 versus NO ligand discrimination in the H−NOX family.[3] Mutagenesis has demonstrated that removing this tyrosine from members of the family that bind O_2 significantly decreases their affinity for this ligand while maintaining high affinity for NO. Likewise, introducing this tyrosine into the distal pocket of members of the H−NOX family that do not bind O_2 results in proteins that now bind O_2.

The overall properties of *Tt* H−NOX make it an attractive target for development as an NO sensor. Detection of NO at low concentration is an especially challenging problem.[4] The best currently available technologies depend on expensive and specialized chemiluminescent[5] instrumentation or on porphyrin-based electrodes.[6] Protein[7−9] (e.g., oxyglobin) and small molecule[10,11] (e.g., DAF) detectors of NO (or its decomposition products) are also in use, and more recently, fluorescence detection of NO has been achieved with a copper complex.[12] However, due to the limitations of low sensitivity and specificity or complicated instrumentation, these systems are not generally useful, especially in biological settings. Physiological concentrations of NO are in the low nanomolar range, thus, a simple spectroscopic method for detection of NO at these concentrations, without competition from other small diatomic ligands such as O_2, will find immediate application. Here we present data demonstrating that the Y140F mutant of *Tt* H−NOX (*Tt* Y140F) is a sensitive and selective NO sensor.

Untagged *Tt* Y140F is purified from a standard *E. coli* expression system with an unoptimized yield of greater than 25 mg/L.[13] As purified, *Tt* Y140F is in the ferrous oxidation state, as demonstrated by formation of a stable complex with CO ($\lambda_{max} = 424$ nm) but not with CN^-. Y140F is extremely stable; after more than 5 h in air at 20 °C, there was no change in the absorption spectrum. At 37 °C, the rate of oxidation in air was measurable, but extremely slow (0.009 hr^{-1}). In fact, *Tt* Y140F is extremely recalcitrant to oxidation, even with chemical oxidants; significant oxidation (∼75%) with ferricyanide occurs only after reaction with a large molar excess (100-fold) at 42 °C for 1 h. *Tt* Y140F does not have any measurable affinity for O_2, even under 1 atm of pure O_2. Importantly, however, in the presence of NO, ferrous *Tt* Y140F forms a six-coordinate complex with a sharp Soret absorbance maximum at 420 nm. After 5 h in air at 20 °C, there was no change in the electronic spectrum of the Fe^{II}-NO complex. The rate of NO dissociation from the heme was determined to be $(2.0 \pm 0.3) \times 10^{-4}$ s^{-1}, using a previously described dithionite/CO trap for dissociated NO.[14,15] This rate is comparable to other dissociation rates determined for proteins in the H−NOX family. Thus, *Tt* Y140F is a selective and stable trap for NO, even in the presence of O_2.

To determine if *Tt* Y140F is a quantitative trap for NO, the extinction coefficients of purified *Tt* Y140F with and without bound NO were determined by HPLC using myoglobin as a heme standard.[14,16] *Tt* Y140F has an extinction coefficient of $\epsilon_{424} = 154 \pm 3$ mM^{-1} cm^{-1} as purified and $\epsilon_{424} = 184 \pm 4$ mM^{-1} cm^{-1} as the Fe^{II}−NO complex. Thus, NO can be quantified by the change in absorbance at 424 nm using $\Delta\epsilon_{424} = 30$ mM^{-1} cm^{-1}. Therefore, in theory, NO can be detected and quantified between concentrations of 300 nM and 30 μM using *Tt* Y140F as a trap, with simple electronic absorption detection.

Figure 1 illustrates the results of a titration of *Tt* Y140F with NO.[17] *Tt* Y140F is a quantitative trap for NO. As NO is added to the solution of *Tt* Y140F, the Soret maximum shifts to a shorter wavelength with an extinction decrease. NO binding to *Tt* Y140F can be followed from the direct spectrum (Figure 1a) or from the difference spectrum (Figure 1b), where the spectrometer is blanked against the starting sample of purified *Tt* Y140F, so that as NO is added to the solution and binds to *Tt* Y140F, the change in absorbance between *Tt* Y140F with and without NO bound is recorded. Figure 1c is a plot of the change in absorption at 424 nm versus the concentration of NO added to the solution. There is a 1:1 linear response until the amount of NO added exceeds the concentration of *Tt* Y140F in solution (in this example, 1.1 μM *Tt* Y140F). Figure 1d is a plot of the concentration of NO added to the solution versus the concentration of NO calculated from the change in absorbance at 424 nm. If *Tt* Y140F is trapping all of the NO added to solution, the slope of this line should be 1.00. For the titration illustrated here, the slope is 1.01; the average slope of six such titrations is 1.06 ± 0.15.[10]

To demonstrate the facility of *Tt* Y140F as a sensor for biological sources of NO, it was used to quantify the specific activity of recombinate murine inducible nitric oxide synthase (iNOS) in comparison with the oxy-hemoglobin (HbO_2) assay, as described previously.[18] Following the change in absorbance at 424 nm using 2.4 μM *Tt* Y140F in an assay with 0.5 μg iNOS, a specific activity of 0.85 ± 0.05 μmol/min/mg was measured. When 6 μM HbO_2 was used and when the change in absorbance was followed at 401 nm, the specific activity measured for 0.5 μg iNOS was 0.75 ± 0.05 μmol/min/mg.

In summary, *Tt* Y140F is an excellent sensor of NO. It is practical and inexpensive to produce from standard *E. coli* expression systems. It is stable to heat (up to 70 °C) and oxidation, and does not bind O_2. It freezes well and can be stored for long periods of time. It is tolerant of all salt and buffer conditions tested so far. As purified, with no additional preparation, *Tt* Y140F is a quantitative trap for NO. In the absence of a trap for dissociated NO, the Fe^{II}−

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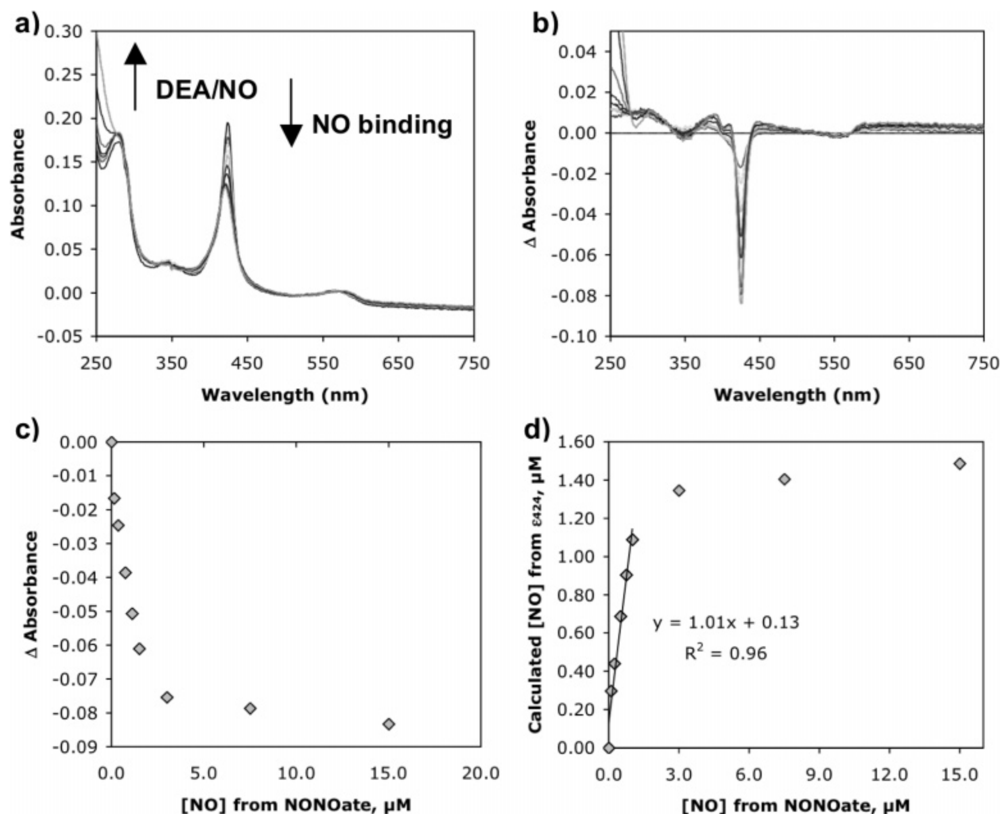

Figure 1. *Tt* Y140F is a quantitative NO sensor. Panel (a) illustrates the change in absorbance of a solution of 1.1 μM ferrous *Tt* Y140F, as purified, as NO is added to solution. As NO binds to *Tt* Y140F, the Soret absorbance maximum shifts and the extinction coefficient decreases. Panel (b) illustrates the same experiment, except the change in absorbance is measured because the spectrometer was blanked against 1.1 μM *Tt* Y140F before addition of NO. Panel (c) is a plot of this change in absorbance at 424 nm plotted against the concentration of NO added to the solution. There is a linear response with NO until the concentration of NO added exceeds the concentration of *Tt* Y140F in solution. Panel (d) is a plot comparing the concentration of NO measured from the change in absorbance of *Tt* Y140F versus the concentration of NO added from the DEA/NONOate, showing a 1:1 correlation.

NO complex is stable for over 5 h in solution at room temperature, and in the presence of a trap for dissociated NO, the rate of dissociation is slow. Finally, *Tt* Y140F does not require specialized equipment. There is no need to reduce or otherwise treat the sample, anaerobic conditions are unnecessary, and detection and quantification is accomplished using a standard absorbance measurement and the Beer−Lambert law. The change in absorbance at 424 nm is simply divided by the change in extinction coefficient at 424 nm (30 μM^{-1} cm^{-1}) to obtain the concentration of NO (assuming a path length of 1 cm).

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