Experimental details

Instrumentation:

UV/Vis absorption and emission measurements were carried out on a SpectraMax M2 plate reader. Anaerobic work was performed in an anaerobic workstation produced by Don Whiteley Scientific.

Myoglobin (FeMb) was purchased from Sigma. The haem group was removed from FeMb using the acid/butanone method described previously to generate apoMb.[1, 2] ApoMb was reconstituted with a two times excess of cobalt protoporphyrin (CoPPIX) dissolved in a minimal amount of pyridine. Unbound CoPPIX was removed using a PD10 column from GE Healthcare.

pRSET plasmid encoding Cyan fluorescent protein (CFP) was purchased from Invitrogen. The manufactures instructions were followed for expression and purification of CFP.

Diethylamine diazen-1-ium-1,2-diolate (NONOate) was purchased from Sigma and dissolved in 50 mM phosphate (pH 7) to generate dissolved NO.[3] For dissociation constant (K_d) determinations and comparison of limit of detection (LOD) between FeMb and CoMb, the same NONOate solutions were used.

In a typical experiment a solution of Mb (15 μM) and CFP (0.65 μM) in 50 mM phosphate buffer (pH 7) was reduced using dithionite. A 12.9 mM NONOate solution was freshly prepared and serial dilutions were carried out to give a range of concentrations from 15 μM to 1.5 mM under anaerobic conditions. 10 μL of the respective NONOate solution was added to 90 μL of the reduced Mb/CFP solution in a 96 well plate to generate a dissolved NO calibration curve. Following incubation in the absence of oxygen for 15 minutes to allow full NO release, the solutions were excited at either 420 nm or 430 nm and CFP fluorescence was monitored at 480 nm. All experiments were carried out in a minimum of triplicate repetitions.

K_d of NO binding (Equation 1a and b) was determined from the following relation between the observed fluorescence F_{NO} intensity (Equation 2):

$$Fe^{2+}Mb + NO \rightleftharpoons Fe^{2+}Mb\text{-}NO \qquad \text{Equation 1a}$$

$$K_d = \frac{[Fe^{2+}Mb - NO]}{[Fe^{2+}Mb] + [NO]} \qquad \text{Equation 1b}$$

$$F_{NO} = F_0 - \frac{(F_0 - F_\infty)[NO]}{[NO] + K_d} \qquad \text{Equation 2}$$

Where [NO] is the concentration of free NO, F_0 and F_∞ are the emission intensities of the NO free and NO bound Mb respectively.

The limit of detection was determined from $3\sigma_X$ of the y-intercept from the concentration dependence.

References

1. F. W. Teale, *Biochim. Biophys. Acta*, 1959, **35**, 543.
2. F. Ascoli, M. R. Fanelli and E. Antonini, *Methods Enzymol.*, 1981, **76**, 72.
3. L. K. Keefer, R. Nims, K. Davies and D. Wink, *Methods Enzymol.*, 1996, **268**, 281.