SI Appendix

Absorption spectra of label and nitrite reductase

Figure 5. *Alcaligenes faecalis* S-6 nitrite reductase absorption (solid line) and ATTO 655 emission (dashed line).
Calculation of Förster radius

$R_0 = 3.5$ nm for the ATTO 655 to type-1 Cu of NiR energy transfer. $R_0$ was calculated from the equation $R_0 = 0.211(J\kappa^2n^{-4}\Phi_0)^{1/6}$ Å (1), with a refractive index $n$ of 1.4, an orientation factor $\kappa$ of 2/3, corresponding to free rotation of both donor and acceptor (1) and a quantum yield of ATTO 655, $\Phi_D$, in the absence of the acceptor of 0.3 as given by the manufacturers. The spectral overlap integral $J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda)d\lambda$ was calculated from the fluorescence spectrum of the donor ATTO 655, $F_D(\lambda)$, as supplied by the manufacturer, and the measured absorption spectrum of NiR, $\epsilon_A(\lambda)$, as measured in the present study. The distance from ATTO 655 to type-1 Cu of NiR was estimated as $R = (d + 1)$ nm ± 0.5 nm = 3.9 ± 0.5 nm, where $d$ is the distance from the dye attachment point to type-1 Cu as derived from the protein crystal structure (2). As the structure of ATTO 655 is proprietary, a more precise estimation of the donor-acceptor distance is not possible.
Enzyme mechanism

Regarding the enzyme mechanism two pathways have been distinguished: “reduction first” (3, 4) and “binding first” (5). Recent investigations show that the enzyme operates according to a "random sequential mechanism" in which both pathways run in parallel (6). This is summarised in Scheme 2 where "A" denotes the "reduction first" pathway and "B" the "binding first" alternative. At saturating nitrite concentrations pathway "B" becomes the preferred pathway, while at high pH the Michaelis-Menten constant $K_M$ of this pathway becomes so large that "A" prevails. The choice of pH and nitrite concentration in the present study favour the lower pathway ("A") (6). Scheme 1 is obtained from Scheme 2 by considering only pathway "A" and by taking into account that the last step in the substrate conversion (i.e. the step OxRed.S $\rightarrow$ OxOx + P) is not rate limiting (6-9).

Scheme 2. Enzyme mechanism of NiR. "RedOx" denotes the state of the enzyme in which the type-1 site is reduced and the type-2 site oxidized and so on. R, S and P denote reductant, substrate and product, respectively. The states in red exhibit a high dye label fluorescence intensity, the others a low intensity. "A" and "B" denote the "reduction first" and the "binding first" pathway, respectively.
Fluorescence traces under aerobic and anaerobic conditions

Figure 6. Fluorescence traces of a labelled, immobilized single molecule of the H145A/L93C variant of NiR. This (colourless, since the type 1 site is permanently reduced) variant is enzymatically inactive and exhibits no turn-over. The top trace was measured in buffer only (20 mM Hepes, pH 7), the lower trace after addition of ascorbate/PES (see Methods section for details). Addition of nitrite has no effect on the appearance of the traces.
Figure 7. Autocorrelation graphs of fluorescence traces of six labelled and immobilized L93C NiR molecules at room temperature and 50 μM nitrite. The trace of a seventh molecule is left out because of excessive noise. For other experimental conditions: see Methods section.
Figure 8. Autocorrelation graphs of fluorescence traces of seven labelled and immobilized L93C NiR molecules at room temperature and 500 μM nitrite. For other experimental conditions: see Methods section.
Effect of bin size on autocorrelation graphs

Fig. 9. Autocorrelation graphs calculated with the use of bin sizes varying from 1 to 10 ms. The direct autocorrelation graph was calculated on the basis of the photon arrival times (10, 11). The latter procedure turned out to be time consuming in our hands, which is why binning was preferred. The bin size eventually used for the calculation of the correlation graphs was 1 ms. The graphs were calculated from the fluorescence time trace of a single immobilized and labelled NiR molecule in the presence of 5 μM nitrite. For other experimental conditions: see Methods section.
**Scheme 1 and Michaelis-Menten kinetics**

As stated in the main text, on the basis of the work by Qian and Elson (12) the general form of the autocorrelation function of the fluorescence intensity can be formulated as

\[
G(t) = A_1 \cdot \exp(\lambda_1 t) + A_2 \cdot \exp(\lambda_2 t),
\]

where \( \lambda_{1,2} = -\frac{1}{2} (k_1 \cdot S + k_2 + k_3 + k_{-3} \pm \sqrt{\Delta}) \),

\[
\Delta = (k_1 \cdot S - k_2 - k_3 + k_{-3})^2 - 4(k_2 - k_{-3})k_3
\]
and \( S \) is the substrate concentration.

According to Scheme 1 the product formation rate, \( \frac{dP}{dt} \), is given by

\[
\frac{dP}{dt} = k_1 \cdot S \cdot P_{ss}(OR).
\]

The steady-state probability of the OR-state, \( P_{ss}(OR) \), can be calculated from (12)

\[
P_{ss}(OR) = \frac{k_1k_3}{\lambda_1\lambda_2}
\]

from which follows

\[
\frac{dP}{dt} = k_1 \cdot S \cdot P_{ss}(OR) = \frac{k_1k_2k_3 \cdot S}{\lambda_1\lambda_2}.
\]

With \( \lambda_1\lambda_2 = k_1S(k_2 + k_3) + k_2(k_3 + k_{-3}) \) (see Eq. S2) it can be seen that \( \frac{dP}{dt} \) follows Michaelis-Menten kinetics, i.e.

\[
\frac{dP}{dt} = V_{max} \cdot \frac{S}{S + K_M},
\]

with \( K_M = \frac{k_2(k_3 + k_{-3})}{k_1(k_2 + k_3)} \) and \( V_{max} = \frac{k_2k_3}{(k_2 + k_3)} \).
Alternative Schemes

The consequences of the use of an alternative for Scheme 1 have been investigated by considering Scheme 3.

Scheme 3. State "1" is the form of NiR in which the type-1 and the type-2 site are both reduced ("RR"). State "2" is the form in which the type-1 and the type-2 site are reduced and oxidized, respectively ("RO"). State "3" is the form in which the type-1 and the type-2 site are oxidized and reduced, respectively ("OR"). Conceivably this scheme might apply at very low nitrite concentrations or when the reduction of the type-1 site is very fast.

Denoting the fluorescence signal from state \( i \) by \( f(i) \) (12) the mean fluorescence signal is

\[
<f> = P_1^{SS} + P_2^{SS}
\]

with \( P_i^{SS} \) denoting the steady state probability of occupation of state \( i \). Following the formalism of ref. (12) the time correlation function becomes

\[
C_2(\tau) = P_{11}(\tau) P_1^{SS} + P_{12}(\tau) P_1^{SS} + P_{21}(\tau) P_2^{SS} + P_{22}(\tau) P_2^{SS} +
- ( P_1^{SS} )^2 - P_1^{SS} P_2^{SS} - P_2^{SS} P_1^{SS} - ( P_2^{SS} )^2
\]

The \( P_\beta(\tau) \) are bi-exponential functions with rate constants \( \lambda_1 \) and \( \lambda_2 \) so that
\[ C_2(\tau) = A_1 \exp(\lambda_1 \tau) + A_2 \exp(\lambda_2 \tau). \]

We further find after some algebra

\[ -2\lambda_{1,2} = (k_1S + k_2 + k_3 + k_{-3}) \pm \sqrt{\Delta} \quad (S5) \]

\[ \Delta = (k_1S - k_3 - k_{-3} - k_2)^2 - 4k_2k_3 \quad (S6) \]

and

\[ P_1^{SS} + P_2^{SS} = 1 - P_3^{SS} = 1 - k_1S k_3/(\lambda_1 \lambda_2). \quad (S7) \]

Retaining, as before, only \( \lambda_2 \) in the data analysis with

\[ -2\lambda_2 = (k_1S + k_2 + k_3 + k_{-3}) - \sqrt{\Delta} \quad (S8) \]

and using Eqs. (S8), and (S7) (instead of Eqs. (5) and (6) of the main text, respectively) to fit the data in Figs. 4A and 4C no satisfactory global fit could be obtained.

When using Eqs. (S7) and (S8) to fit the low concentration data in Figs. 4A and 4C (5 and 50 \( \mu \)M nitrite) and Eqs (5) and (6) of the main text to fit the high concentration data (500 and 5000 \( \mu \)M nitrite) in a single global fit, the fit is almost as good as the one reported in the main text with values for the rate constants that are similar to the values reported in the main text within the experimental uncertainty.

Reference List


