Dithionite reduction kinetics of the dissimilatory Cu-containing nitrite reductase of *Alcaligenes xylosoxidans*; the $\text{SO}_2^-$ radical binds to the substrate binding type 2 Cu site before the type 2 Cu is reduced.$^8$

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Dithionite Reduction Kinetics of Cu-Nitrite Reductase
We report here the first detailed study of the dithionite reduction kinetics of a Cu-containing dissimilatory nitrite reductase (NiR). The reduction of the blue type-1 Cu (T1Cu) center of NiR preparations that contained both type 1 and type 2 copper atoms, followed biphasic kinetics. In contrast, NiR that was deficient in Type 2 Cu (T2DNiR), followed mono-phasic kinetics with a second order rate constant $T_{2D}^2k = 3.06 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. In all cases the SO$_2$ radical rather than S$_2$O$_4^{2-}$ was the effective reductant. The observed kinetics are compatible with a reaction mechanism in which the T1Cu of the fully loaded protein is reduced both directly by dithionite and indirectly by the type-2 Cu (T2Cu) site via intra-molecular electron transfer. Reduction kinetics of the T2Cu were consistent with SO$_2$ binding first to the T2Cu center and then transferring electron (112 s$^{-1}$) to reduce it. As SO$_2$ is a homologue of NO$_2^-$, the NiR substrate, it is not unlikely that it binds to the catalytic T2Cu site. Effects on the catalytic activity of the enzyme using dithionite as a reducing agent are discussed.

Reduction of the semi-reduced T1Cu(I)T2Cu(II) state followed either second order kinetics with $k_2 = 3.33 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ or first order kinetics with $52.6 \text{ s}^{-1} < T_{1\text{red}} k_1 < 112 \text{ s}^{-1}$. Values of formation constants of the T1Cu(II)T2Cu(II)-SO$_2$ and T1Cu(I)T2Cu(II)-SO$_2$ adducts showed that the redox state of T1Cu affected binding of SO$_2$ at the catalytic T2Cu center.

Analysis of the kinetics required the development of a mathematical protocol that could be applied to a system with two inter-communicating sites but only one of which can be monitored. This novel protocol, reported for the first time, is of general application.
Dissimilatory nitrite reductase play an important role in the cycling of nitrogen in the biosphere. Some microorganisms are able to synthesize ATP by a respiratory pathway in which nitrate is sequentially reduced via nitrite to NO, N₂O and N₂ in the process of denitrification. In catalyzing the reaction:

\[
\text{NO}_2^- + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}
\]  (1)

nitrite reductase has agricultural impact, since it leads to a significant loss of soil nitrogen to the atmosphere, and is of environmental importance because NO is a pollutant and N₂O is a potent greenhouse gas following CO₂ and CH₄ in its contribution to global warming. Two distinct families of nitrite reductase are known, one containing heme \textit{cd}, and a second family containing copper as their functional electron transfer and catalytic centers (1-3).

The Cu-containing dissimilatory nitrite reductases (NiRs) have been the subject of extensive study and structures of the enzymes from \textit{Achromobacter cycloclastes} (AcNiR) (4-6), \textit{Alcaligenes faecalis} (AfNiR) (7) and \textit{Alcaligenes xylosoxidans} (AxNiR) (8-10) have been determined. The enzymes are trimeric, with subunits having typical cupredoxin folds. Each monomer contains a type 1 copper center (His₂, Met, Cys). At each adjacent monomer interface, a type 2 copper center (His₃, H₂O) is located in a ~13Å deep cleft directly exposed to the solvent. The RMS deviation of the C\textsubscript{\alpha} backbone alignment between these structures is ~ 0.5 Å, but small differences in the ligand geometry of the type 1 Cu center result in the enzymes being green, AcNiR and AfNiR or blue, AxNiR. Despite the overall sequence identity of ~78%, surprisingly the surface charge of the blue and green enzymes is markedly different (9). The Cu ions in adjacent type 1 and type 2 sites are separated by12.6 Å and are ligated by neighboring amino acid residues,
Cys130 and His129 (AxNiR residue numbering). Pulse-radiolysis (11-14) and directed mutational studies (15, 16) have shown the type 1 Cu sites to have a role in electron transfer to the catalytic type 2 Cu site at which nitrite binding and reduction occurs. In the absence of substrate these centers are in redox equilibrium with observed rate constants for electron transfer ranging from 1400 to 450 s⁻¹.

The binding of NO₂⁻ to oxidized NiR’s has been studied by crystallographic and spectroscopic methods. The diffusion of NO₂⁻ into crystals of AcNiR (6), and AxNiR (8, 9) resulted in the bound H₂O (or OH⁻) of the type 2 center being displaced by NO₂⁻ which bound to the Cu asymmetrically through both oxygen atoms. This is consistent with the extensive perturbation of the ¹H and ¹⁴N features of the ENDOR spectra of the type 2 centers of AxNiR (17), and Rhodopseudomonas sphaeroides NiR (RsNiR) (18) in the presence of NO₂⁻. Difference Cu EXAFS of AxNiR (19) showed an increase in the average His-Cu bond length of 0.08Å in the presence of NO₂⁻. These changes, together with the increase in redox potential of the type 2 site with NO₂⁻ bound may trigger internal electron transfer from the type 1 center during catalysis.

Comparative EXAFS, EPR, and Uv-Vis spectroscopy of reduced and oxidized AxNiR (20) showed that the reduced type 2 center lacked the liganded H₂O molecule seen in the oxidized state, and had assumed trigonal geometry similar to that of reduced superoxide dismutase. Additionally, the competitive inhibitor azide could no longer bind to the reduced site. These data, together with crystallographic data for AcNiR showing a low occupancy of NO₂⁻ in crystals of reduced enzyme (14), strongly suggest that NO₂⁻ does not bind to the reduced
center, consistent with the observation that reduction of AxNiR with ascorbate/PMS in the absence of NO$_2^-$ resulted in the inactivation of the enzyme. Based on these findings an ordered mechanism was proposed for AxNiR (20) in which NO$_2^-$ binds to the oxidized type 2 Cu center to trigger electron transfer from the reduced type 1 center.

Despite the wealth of structural information for these enzymes, few detailed kinetic studies have been undertaken. The enzymes are located in the periplasmic space and a number of putative electron donors have been identified and characterized, including cytochrome 551, and the cupredoxins azurin and pseudoazurin (see ref. 1). In the case of A. xylosoxidans two distinct azurins have been structurally characterized and shown to function as electron donors to AxNiR (21). However, we have recently shown that the kinetics of the reduced cupredoxin-dependent activity of both blue and green NiR's are unexpectedly complex (22). Under pseudo first-order conditions ([cupredoxin]/[NiR] = 16), progress curves for the reactions did not follow Michalis-Menten kinetics, and depending on the system under investigation, were bi- or tri-phasic. The reduction kinetics for NiR reacting with artificial electron donors are also complex. An early report of the reduction of AcNiR by ascorbate (23), showed biphasic kinetics, with only the first phase being faster in the presence of the mediator phenazine methosulfate. As part of a study to gain insight to electron flux in these systems we report a study of the transient kinetics of the reduction of AxNiR by dithionite ion.

**EXPERIMENTAL PROCEDURES**
Organism and Nitrite reductase purification

The organism used was *A. xylosoxidans* subsp. *xylosoxidans* (NCIMB 11015). Conditions of growth harvesting and purification of AxNiR from crude extracts that had been activated by the addition of CuSO$_4$ were as previously described (24). The type 2-deficient AxNiR (T2D NiR) was purified using similar procedures, from extracts that had not been activated by the addition of copper (19). Protein concentrations were determined by the microbiuret method and the copper content determined by inductively coupled plasma analysis carried out by Southern Science Ltd, Sussex Laboratory, Brighton, BN1 9PY, U.K. Ax NiR with a specific activity of 236 µmoles of NO$_2^-$ reduced min$^{-1}$ mg$^{-1}$ contained 6.3 Cu atoms per trimer, while T2D NiR with a specific activity of 20 µmoles of NO$_2$ reduced min$^{-1}$ mg$^{-1}$ contained 2.7 Cu atoms per trimer of the enzyme.

Reagents and Solutions

Sodium dithionite and NaCl were purchased from BDH while Tris and HEPES were purchased from Sigma. All chemical were used as supplied and solutions were prepared in Milli-Q water. Sodium dithionite solutions were prepared in oxygen-free 50 mM HEPES buffer, pH 7.4, in a glove box with oxygen concentration less than 1ppm maintained by circulation of N$_2$ gas over BASF catalyst columns. Dithionite concentrations were determined from the absorbance at 315 nm using a molar extinction coefficient of 8043 M$^{-1}$ cm$^{-1}$. In a typical kinetic experiment, first a concentrated stock solution of dithionite was prepared in 50 mM HEPES buffer and its ionic strength adjusted to 0.1M with NaCl. All other working
solutions were then prepared by dilution of the stock solution with 50 mM HEPES buffer, I = 0.1M (NaCl).

Stopped-flow studies

The reduction of NiR using dithionite as a reducing agent was recorded at 595 nm by monitoring the decay of the blue color of the type 1 Cu centers of the enzyme. The kinetic traces were acquired at 25 °C using a Hi-Tech SF-61 stopped-flow rapid-scan spectrophotometer (High Tech Scientific, Salisbury, Wilts., UK.) in the single wavelength mode of the machine. The mixing and reaction chamber of the stopped-flow spectrophotometer was installed in an anaerobic glove box with oxygen level less than 1ppm. The temperature of the reaction mixture was controlled with a Techne-400 circulator (Techne (cambridge) Ltd., oxford, Cambridge.) UK. A pseudo first order condition was maintained by keeping dithionite in excess over the NiR concentration. Typically, syringe A of the stopped flow contained 11 µM NiR while syringe B contained 0.3 – 2.2 mM dithionite solution. The rate constants were determined by fitting the kinetic traces to exponential functions using KinetAsyst-3.

RESULTS AND DISCUSSION

Kinetics of Reduction of AxNiR by Dithionite: Sodium dithionite is widely used as a reductant in assays for nitrite reductase activity, either directly, or in the presence of the mediator Methyl Viologen. The major path for electron flow under these conditions is via the type 1 Cu site. However, studies of mutated NiR from
several sources have shown that when the type 1 center of NiR is non-functional
[e.g. AfNiR-Met150Glu (5) or AxNiR-His139Ala (16)], Methyl Viologen or dithionite
can support a decreased, but significant, level of activity (5~20%) by the direct
reduction of the type 2 site. Other than the determination of the apparent $K_m$ for
dithionite (25), there has been little published work on artificial electron donors in
these systems.

The kinetics of reduction of AxNiR by dithionite ion were studied under the
pseudo first-order conditions with $[S_2O_4^{2-}] > [NiR]$ and the reaction was monitored
by recording the bleaching of the blue color of NiR at 595 nm in a stopped-flow
spectrophotometer. Unusual kinetics were observed since, the reaction exhibited
two phases (Fig. 1). The initial rapid phase was associated with an amplitude of ~
10% of the total absorbance change.

**Fig. 1 and 2**

Although the observed rate constants of the fast and slow phases of the reaction
$k_{obs1}$ and $k_{obs2}$, respectively, were dependent on dithionite concentration, neither
constant was linearly related to $[S_2O_4^{2-}]$ (Fig. 2). The reduction of a number of
proteins has been shown to involve a prior dissociation of dithionite to form $SO_2^{•-}$
as the effective reductant in which case a square root dependence on $[S_2O_4^{2-}]$ is
expected. In the present case, when $k_{obs1}$ and $k_{obs2}$ were plotted against the square
root of $[S_2O_4^{2-}]$, a linear plot was observed for $k_{obs2}$ only (Fig. 3). This shows that the
slow step ($k_{obs2}$) of the reduction process is consistent with Eq. 2, where the
monomeric $SO_2^{•-}$ radical of dithionite is the reacting species; $K_{DT}$ is the equilibrium
constant for the dissociation of $S_2O_4^{2-}$ to $SO_2^-$. It also shows that the fast step ($k_{obs1}$) is a more complex process since although $k_{obs1}$ was dependent on $[S_2O_4^{2-}]$, neither plot was linear (Fig.2, Fig.3).

\[
k_{obs2} = k_2 K_{DT}^{1/2}[S_2O_4^{2-}]^{1/2}
\]  

We considered the possibility that the fast $k_{obs1}$ step of the reduction process involved a rapid pre-equilibration between the enzyme and $S_2O_4^{2-}$ (or its monomeric $SO_2^-$ radical) to form an adduct (Eq. 3), followed by the rate-limiting electron transfer step, Eq. 4. The $X_r$ in these equations is $S_2O_4^{2-}$ or $SO_2^-$. 

\[
NiR_{ox} + X_r \rightleftharpoons NiR_{ox}X_r \quad \text{....................... (3)}
\]

\[
NiR_{ox}X_r \rightarrow k \quad NiR_{r} + X_{ox} \quad \text{................. (4)}
\]

In this case $k_{obs1}$ will follow saturation kinetics and the straight-line equations 5a (if $X_r = S_2O_4^{2-}$), or 5b (if $X_r = SO_2^-$) apply. $K_{ND}$ is the equilibrium constant for formation of the $NiR_{ox}X_r$ adduct.

\[
\frac{1}{k_{obs1}} = \frac{1}{kK_{ND}[S_2O_4^{2-}]} + \frac{1}{k} \quad \text{....................... (5a)}
\]

\[
\frac{1}{k_{obs1}} = \frac{1}{kK_{ND}K_{DT}^{1/2}[S_2O_4^{2-}]^{1/2}} + \frac{1}{k} \quad \text{....................... (5b)}
\]
The plot of $1/k_{obs1}$ versus $1/[S_2O_4^{2-}]$ was not linear, but the plot of $1/k_{obs1}$ versus $1/[S_2O_4^{2-}]^{1/2}$ was (Fig. 4). This result indicated that the SO$_2$** radical rather than the S$_2$O$_4^{2-}$ species was the effective reducing agent. More importantly, it also showed that the fast phase of the reaction ($k_{obs1}$) involved formation of an adduct between NiR and SO$_2$** before electron transfer occurred.

**Fig. 3 and 4 here**

The dependence of $k_{obs1}$ and $k_{obs2}$ on the $[S_2O_4^{2-}]$, discussed above, shows that the slow phase of the reaction follows simple bimolecular kinetics, while the initial fast phase follows a more complicated path of saturation kinetics. It also identifies the SO$_2$** radical as the effective reductant for both the fast and slow phases of the reaction. This finding contrasts with data for AcNiR where $k_{obs} = a [S_2O_4^{2-}] + b [S_2O_4^{2-}]^{1/2}$, consistent with both S$_2$O$_4^{2-}$ and the SO$_2$** radical apparently being effective donors (23). In the present case, our data do not fit this plot.
**Scheme for the Reduction of AxNiR by Dithionite:** What are the specific reactions of dithionite with AxNiR that are responsible for the observed kinetics of decay of the blue color in two different ways? Studies of the H139A mutant form of AxNiR in which type 1 center is reduced and it is no longer in redox equilibrium with the type 2 centers (16) due to an elevated redox potential in excess of 700 mV show that the oxidized type 2 Cu centers do not contribute to the $A_{594}$. Thus, in our studies the decrease in absorbance at 595 nm observed during the two phases of the reaction, is assigned to reductive reactions of the type 1 Cu center only. In other words the T1Cu center is reduced in two different ways.

Earlier pulse radiolysis studies of AxNiR showed that the type 1 and type 2 Cu ions were in redox equilibrium and that the redox potentials of the two centers were very similar with mid-point potentials of 240 and 230 mV respectively. If the same equilibration occurs on dithionite reduction of the enzyme then the two phases of the reduction kinetics can be explained by Scheme 1, which assumes the three active sites of the enzyme (where each site is composed of a pair of T1Cu and T2Cu centers), function independently.

Scheme 1

$$
T_1Cu(II)T_2Cu(II) \xrightarrow{k_1[S_2O_4^{2-}]} T_1Cu(II)T_2Cu(I) \xleftarrow{k_5} T_1Cu(I)T_2Cu(II) \xrightarrow{k_2[S_2O_4^{2-}]} T_1Cu(I)T_2Cu(I) \xrightarrow{k_3[S_2O_4^{2-}]} T_1Cu(I)T_2Cu(I)
$$
In this scheme, the initial reaction is the spectroscopically silent reduction of the type 2 Cu center in a relatively fast step \( (k_1) \), followed by even faster redox equilibration \((k_4 \text{ and } k_5)\) to yield the two partially-reduced redox species of the enzyme \( T_1\text{Cu(II)}T_2\text{Cu(I)} \) and \( T_1\text{Cu(I)}T_2\text{Cu(II)} \). The formation of the latter species is responsible for the observed decrease in absorbance. This reaction sequence \((k_1, k_4 \text{ and } k_5)\) is responsible for the fast phase of the kinetics, with \( k_1 \), which is far smaller than the equilibration rate \((k_4 + k_5)\), being the rate limiting step. The enzyme in the two semi-reduced states is then further reduced in relatively slow steps \((k_3 \text{ and } k_2 \text{ respectively})\) to yield the fully-reduced species \( T_1\text{Cu(I)}T_2\text{Cu(I)} \). This reaction constitutes the slower phase of the reaction kinetics.

A detailed mathematical analysis of this scheme is available as Supplementary Data. According to Eq. IVc of the Supplementary Data,

\[
k_{\text{obs}1} = k_1[S_2O_4^{2-}] \tag{6}
\]

Thus a plot of \( k_{\text{obs}1} \) versus \([S_2O_4^{2-}]\) should yield a linear line with slope equal to \( k_1 \). However, as discussed above, this plot is nonlinear, (see Fig. 2), while a plot of \( 1/k_{\text{obs}1} \) versus \( 1/[S_2O_4^{2-}]^{1/2} \) gives a straight line, Fig. 4, and equation 5b applies. This indicates that \( SO_2^- \) radical first binds to NiR and then transfers an electron to reduce the Type-2 Cu center. At this point it is important to mention that in subsequent discussion, the rate constants \( ^{\text{T1ox}}k_1 \text{ and } ^{\text{T1red}}k_1 \) or equilibrium constants \( ^{\text{T1ox}}K_{\text{ND}} \text{ and } ^{\text{T1red}}K_{\text{ND}} \) have the same meaning as \( k_1 \) (or \( K_{\text{ND}} \)) but designate the oxidation state of the type 1 center. According to Eq. 5b, the y-intercept of the
linear line in Fig. 4 is equal to $1/k_1$ which gives a value of $k_{1}^{T1ox} = 112 \text{ s}^{-1}$ for the rate of reduction of the type 2 center. Slope of the line is equal to $1/k_1^{T1ox} K_{ND} K_{DT}^{1/2}$ which, together with the literature value of $K_{DT} = 1.6 \times 10^{-9} \text{ M}$ (24), yields the value of $K_{ND}^{T1ox} = 5.58 \times 10^5 \text{ M}^{-1}$, equilibrium constant for formation of the $T1Cu(II)T2Cu(II).SO_2^{•-}$ adduct of the enzyme. The observed kinetics are unable to define the exact position at which $SO_2^{•-}$ binds to NiR. However, as both $SO_2^{•-}$ and $NO_2^- $ are anionic species and structural analogues with angular structures; the O-S-O angle in $SO_2$ being $129^\circ$ while the O-N-O angle in $NO_2^-$ range from 120 to $130^\circ$, it is likely that $SO_2^{•-}$ binds directly to the solvent-accessible type-2 Cu atom. The value of $1/k_1^{T1ox} K_{ND}$, which in enzyme kinetics is equivalent to the Michaelis constant, $K_m$, is equal to $1.79 \times 10^{-6} \text{ M}$. Surprisingly, this value is at least 20 times smaller than the apparent $K_m$ of $3.6 \times 10^{-5} \text{ M}$ of the biological substrate $NO_2^-$ of NiR determined from the steady-state kinetic studies. This difference indicates that $SO_2^{•-}$ binds to the substrate-binding site (type-2 Cu) tighter than the substrate $NO_2^-$ does. In this context it is important to note that the tighter binding of $SO_2^{•-}$ is obtained for the oxidized $T1Cu(II)T2Cu(II)$ state of the enzyme while the Michaelis constant of $NO_2^-$ is reported for the turnover conditions of the enzyme where it may bind to a different redox state of the enzyme, see below.

According to Eq. VIIf of the Supplementary Data, $k_{obs2}$ of the slow phase is given by Eq. 7, where $K_{NR} = k_4/k_5 = T_1Cu(I)T_2Cu(II)/ T_1Cu(II)T_2Cu(I)$.

$$
k_{obs1} = \frac{(k_3 + k_2 K_{NR})}{(1 + K_{NR})} K_{DT}^{1/2} [S_2O_4^{2-}]^{1/2} \quad \text{......... (7)}$$
This equation clearly shows that the value of \( k_3 + k_2 \frac{K_{NR}}{1+K_{NR}} \) can be evaluated from the slope of the plot of \( k_{obs1} \) versus \( [S_2O_4^{2-}]^{1/2} \), provided the values of \( K_{DT} \) is known. The literature value of \( K_{DT} = 1.6 \times 10^{-9} \) M was used to derive a value of \( k_3 + k_2 \frac{K_{NR}}{1+K_{NR}} \) which was calculated to be \( 5.56 \times 10^6 \) M\(^{-1}\)s\(^{-1}\). This value is used below to calculate the value of the second order rate constant \( k_2 \).

**Kinetics of the Reduction of Type 2 Depleted AxNiR by Dithionite:** As discussed above, Scheme-1 successfully explains the observed kinetics, but it does not provide an answer to the important question as to why the fast phase of the reduction process \( (k_1) \) should be assigned to the reduction of type-2 Cu rather than type-1 Cu. This question was addressed by a separate kinetic study of type-2 depleted NiR, T2DNiR. This species of AxNiR essentially lacks Cu in the type 2 center and has low catalytic activity, attributed to residual occupancy of Cu in the catalytic center. Small angle x-ray scattering studies have shown that T2DAxNiR is trimeric (19) and the crystal structure of T2DAcNiR has a similar structure to enzyme with a full complement of occupied Cu sites (6). T2DNiR reacted with \( S_2O_4^{2-} \) in apparently a single exponential process which corresponds to the slower reaction observed with NiR, indicating that the fast phase of the reduction kinetics is associated with the type 2 center. The dithionite dependence of the observed rate constant for T2DNiR (\( T^{2D}_{obs}k_{obs} \)) is consistent with Eq. 8.

\[
T^{2D}_{obs}k_{obs} = T^{2D}_k K_{DT}^{1/2}[S_2O_4^{2-}]^{1/2} \quad \text{.................................................. (8)}
\]
The slope of the plot of $T^{2D}k_{obs}$ versus $[S_{2}O_{4}^{2-}]^{1/2}$, (see Fig. 5), together with the $K_{DT} = 1.6 \times 10^{-9}$ was used to calculate the value of the second order rate constant $T^{2D}k = 3.06 \times 10^{6}$ M$^{-1}$ s$^{-1}$.

**Fig. 5**

If we assume that $T^{2D}k$ is independent of the absence/presence and redox state(s) of type-2 Cu, then $T^{2D}k = k_3$. This value and the value of $5.56 \times 10^{6}$ M$^{-1}$ s$^{-1}$ calculated above for the composite rate $\{k_3 + k_2 K_{NR} / (1 + K_{NR})\}$ enables the value of $k_2$ to be calculated, provided the value of $K_{NR}$ is known. Although values of $K_{NR}$ determined from pulse radiolysis studies are reported in the literature (10-14), for reasons discussed below, we determined the value of $K_{NR}$ from the amplitudes of the slow and fast phases where $K_{NR} = \text{Amp}_{\text{fast}} / \text{Amp}_{\text{slow}} = 0.0902$. Using this value for the equilibrium constant and $k_3 = T^{2D}k$, the value of $k_2$ for the reduction of T1Cu(II)T2Cu(I) was calculated to be $3.33 \times 10^{7}$ M$^{-1}$ s$^{-1}$.

**Pre-equilibrium Binding of SO$_2$••• to NiR:** It is important to note that the value of $\{k_3 + k_2 K_{NR} / (1 + K_{NR})\}$ computed above, assumes that both the T1Cu(II)T2Cu(I) and T1Cu(I)T2Cu(II) redox states of NiR react with the SO$_2$••• radical in a bimolecular rate-determining step. The analysis presented above indicates that the type-1 Cu center reacts in this manner, and thus T1Cu(II)T2Cu(I) or $k_3$ may show similar kinetic behavior. However, as the type 2 Cu follows saturation kinetics, it is likely that the reaction of T1Cu(I)T2Cu(II) or $k_2$ follows saturation kinetics as well. Under such conditions $k_2$ becomes a first-order rate constant and it is replaced by $T^{1\text{red}}k_1$. 
(first-order rate constant for the reduction of type 2 Cu in the redox state where type 1 Cu is reduced).

This situation has been considered in the mathematical analysis as a “special case” and Eq. VIIIle of the Supplementary Data applies. According to this equation, a plot of 

$$\frac{1}{[k_{\text{obs}2}-\{xK_{\text{DT}}^{1/2}[S_{2}O_{4}^{2-}]^{1/2}\}]}$$

versus 

$$\frac{1}{[S_{2}O_{4}^{2-}]^{1/2}}$$

should yield a straight line with y-intercept equal to 

$$\frac{1}{xK_{\text{NR}}^{1/2}}$$

and slope equal to 

$$\frac{1}{xK_{\text{NR}}^{1/2}}k_{1}^{\text{red}}K_{\text{ND}}^{1/2}$$

where 

$$x = 1/(1+K_{\text{NR}})$$

Such a plot is shown in Fig. 6. A linear line is obtained, which indicates that like the T1Cu(II)T2Cu(II) redox state, T1Cu(I)T2Cu(II) may also react through the adduct formation. However, it is important to note that compared to the slope of this plot, the y-intercept is very sensitive to extreme values of 

$$k_{\text{obs}2}$$

and 

$$[S_{2}O_{4}^{2-}]$$

For this reason, the use of the intercept value for calculating the absolute value of 

$$k_{1}^{\text{red}}$$

was avoided, and the limiting values of 

$$k_{1}^{\text{red}}$$

and 

$$K_{\text{ND}}^{\text{red}}$$

were determined from the slope of the plot. If the T1Cu(I)T2Cu(II) and SO$_2$$^-\text{•}$ interaction is not affected by the redox state of T1Cu, then 

$$K_{\text{ND}}^{\text{ox}} = K_{\text{ND}}^{\text{red}} = 5.58 \times 10^5 \text{ M}^{-1}$$

Using this value, 

$$k_{1}^{\text{red}}$$

was calculated to be 52.6 s$^{-1}$ which is nearly half of 

$$k_{1}^{\text{ox}} = 112 \text{ s}^{-1}$$

computed above. Similarly, if it is assumed that 

$$k_{1}^{\text{ox}} = k_{1}^{\text{red}}$$

then 

$$K_{\text{ND}}^{\text{red}}$$

is calculated to be 2.62 $\times 10^5$ M$^{-1}$ which is, as expected, nearly half the value of 

$$K_{\text{ND}}^{\text{ox}} = 5.58 \times 10^5 \text{ M}^{-1}$$

Considering the redox equilibrium

$$\text{T1Cu(I)T2Cu(II)} \rightleftharpoons \text{T1Cu(II)T2Cu(I)}$$

and the electrostatic interaction between T1Cu(I)T2Cu(II) and SO$_2$$^-\text{•}$, compared to that of T1Cu(II)T2Cu(II) and SO$_2$$^-\text{•}$, the values of 

$$k_{1}^{\text{red}}$$

and 

$$K_{\text{ND}}^{\text{red}}$$

lower than 

$$k_{1}^{\text{ox}}$$

and 

$$K_{\text{ND}}^{\text{ox}}$$

respectively, are not unexpected. In fact, these values for 

$$k_{1}^{\text{red}} = 52.6 \text{ s}^{-1}$$

and 

$$K_{\text{ND}}^{\text{red}} = 2.62 \times 10^5 \text{ M}^{-1}$$

are the lower and 

$$k_{1}^{\text{ox}} = 112 \text{ s}^{-1}$$

and 

$$K_{\text{ND}}^{\text{ox}} = 5.58 \times 10^5 \text{ M}^{-1}$$
$10^5 \text{ M}^{-1}$ are the upper limits of these constants. Difference in the values of $T_{1\text{red}} K_{\text{ND}}$ and $T_{1\text{ox}} K_{\text{ND}}$ shows that the redox state of the T1Cu center affects the binding of $\text{SO}_2^-$ at the T2Cu site.

**Fig. 6**

It is interesting to note that $1/K_{\text{ND}}$, which corresponds to the Michaelis constant for dithionite, is $1.79 \times 10^{-6} \text{ M}$ for the fully oxidized (T1Cu(II)T2Cu(II)) and $\leq 3.82 \times 10^{-6} \text{ M}$ for the partially reduced (T1Cu(I)T2Cu(II)) state of the enzyme. Both of these values are significantly lower than the Michaelis constant $3.6 \times 10^{-5} \text{ M}$ reported for $\text{NO}_2^-$ under turnover conditions. Thus, the tight binding of $\text{SO}_2^-$ to the T2Cu center compared to that of $\text{NO}_2^-$ under turnover conditions, is not a function of the redox state of the enzyme. Both spectroscopic data and the crystal structures of AcNiR and AxNiR indicate $\text{NO}_2^-$ only binds to the oxidized type-2 Cu centers. Thus, $\text{SO}_2^-$ would be expected to behave as a competitive inhibitor. However, a marginal excess (or even a moderately less amount) of $\text{NO}_2^-$ over dithionite will preclude any effective inhibition by $\text{SO}_2^-$ due to the very small value of $K_{\text{DT}}$ of $1.6 \times 10^{-9} \text{ M}$ (10 mM dithionite solution contains only 4 µM $\text{SO}_2^-$). Under these conditions electrons will enter NiR via the type-1 Cu centers. This is clearly the most effective route, since mutations of the ligands to the type 1 Cu ion which result in either Zn replacing Cu, (15) or an increase in redox potential of the type 1 center to $\sim 750 \text{ mV}$ thereby preventing intramolecular electron transfer (16) have markedly lower activity than the native enzyme.
As discussed above, this analysis is unable to ascertain whether $k_2$ follows first order ($T_{1\text{red}}k_1$) or second order kinetics ($k_2$). The two types of kinetic behaviors of $k_2$ depend on the value of the y-intercept of Fig. 6. If this value is equal to zero then Eq. VIIIc of the Supplementary Data applies, and $k_2$ becomes a second order rate constant. Otherwise, Eq. VIIIe of the Supplementary Data applies, and $k_2$ changes to $T_{1\text{red}}k_1$ and follows first order kinetics. In other words, smaller is the value of the y-intercept, the more difficulty is encountered in differentiating between the two possibilities. The value of the y-intercept of this plot is not only sensitive to small errors in the extreme values of the plot, but the upper limit of this value is as small as 0.2. Thus it is very difficult to assign unequivocally whether $k_2$ follows first or second order kinetics. However, as the plot conforms to Eq. VIIIe of the Supplementary Data, and as slope of the straight line gives a reasonable value for $T_{1\text{red}}K_{\text{ND}}$, it is likely that $k_2$ follows first order kinetics and like the fully oxidized $T_1\text{Cu(II)}T_2\text{Cu(II)}$ state of the enzyme, the semi-reduced $T_1\text{Cu(I)}T_2\text{Cu(II)}$ state also reacts through the adduct formation with $\text{SO}_2^{-}$. 

**Intramolecular Electron Transfer Between the two Cu sites of AxNiR**: Electron transfer between the two types of Cu centers present in Cu-containing NiR’s has been studied by pulse radiolysis of both the blue and green enzymes (10-14). In these studies the type 1 center is rapidly reduced but does not become fully re-oxidized, because of the redox equilibrium with the type 2 center. The first-order rate constant for electron transfer $k_{\text{ET}}$, is equal to the sum of the forward $k_{1,2}$ and backward $k_{2,1}$ rates. Only in the presence of nitrite does the equilibrium lie to the right due to the reduction of $\text{NO}_2^{-}$ to NO at the type 2 Cu center. In the presence of
nitrite, $k_{\text{ET}}$ is decreased 3 to 4-fold, an effect attributed to the change in redox potential of the type 2 site. It has recently been shown for AxNiR (14) that amino acid residues not directly ligated to either Cu center also control $k_{\text{ET}}$. Directed mutagenesis of these residues His255 and Asp92 has shown them to be essential for activity, and the crystal structure shown them to be involved in hydrogen bonding to the water molecule bound to the oxidized type 2 Cu ion. Thus the binding of nitrite, or in the present case $\text{SO}_2^-$ to the type 2 Cu ion could disrupt the H-bonding network to perturb both $k_{\text{ET}}$ and the equilibrium between the two centers.

$$\begin{align*}
    \text{T1Cu(II)T2Cu(I)} & \rightleftharpoons \frac{K_4}{K_s} \text{T1Cu(I)T2Cu(II)} \\
    K_{\text{NR}} &= \frac{[\text{T1Cu(I)T2Cu(II)}]}{[\text{T1Cu(II)T2Cu(I)}]}
\end{align*}$$

In the pulse-radiolysis studies, the extent of the partial re-oxidation of the type 1 center is used to calculate the equilibrium constant $K_{\text{NR}}$ and redox potentials of the centers. In these calculations it is assumed that during the initial pulse only the type-1 Cu is reduced while type-2 Cu remains oxidized. The pulse radiolysis studies give a value for $K_{\text{NR}} = k_4/k_s = 1.45$ while the present kinetic work estimates this constant to be 0.0902. This difference may arise from two different species involved in the two studies. The redox states of NiR studied in the pulse radiolysis work are appropriately designated as $\text{T1Cu(I)T2Cu(II)}$ and $\text{T1Cu(II)T2Cu(I)}$. The nature of the corresponding species involved in this work is less certain, but is expected to be $\text{T1Cu(I)T2Cu(II)}\cdot\text{SO}_2$ and $\text{T1Cu(II)T2Cu(I)}\cdot\text{SO}_2$ respectively. The
existence of the T1Cu(I)T2Cu(II).SO₂ species is fully supported by the transient nature and the relatively large value of its association constant 2.62 - 5.58 x 10^5 M⁻¹ discussed above. In the case of AcNiR, it has been reported that the value of K₉ decreases by 4-fold (i.e. changes from 0.25 to 0.0625) when NO₃⁻ is added into a pulse radiolysis mixture. The SO₂⁻ may have similar effects that cause K₉ to change from 1.43 to 0.0902. The relatively large drop in the value of K₉ may reflect the analogy of SO₂⁻ with the substrate NO₂⁻. It is for these reasons that whenever needed we have used the present value of K₉=0.0902 rather than the reported value of 1.45.

Mathematical Analysis
As we were unable to find a mathematical analysis of Scheme-1 or that of any related kinetic scheme in the literature, we developed our own mathematical model. Details of the model are provided as Supplementary Data. The analysis is of a general nature and can be applied to any system that contains two intercommunicating sites but only one site can be monitored kinetically.

CONCLUSIONS
Our stopped-flow kinetic study of the reduction of AxNiR by dithionite shows unexpected biphasic kinetics. The reductant for both phases is the SO₂⁻ radical, formed by dissociation of the parent ion S₂O₄²⁻. The unexpected kinetics are explained by assigning the biphasic reduction of the T1Cu center to a direct reaction with SO₂⁻ and indirect reduction by intramolecular electron transfer from the T2Cu center. Reduction of the T2Cu center follows saturation kinetics.
consistent with a rapid pre-equilibration reaction to form a T1Cu(II)T2Cu(II)-SO$_2^-$
adduct of NiR, followed by transfer of electron from SO$_2^-$ to T2Cu(II). The $K_m$ for
binding SO$_2^-$ is some 20-fold tighter than that for NO$_2^-$. A mathematical analysis of
these kinetics is presented, which is of general application to systems containing
two redox centers in redox equilibrium but only one center is kinetically monitored.
The equilibrium constant for the intra-molecular electron transfer equilibrium was
calculated from the amplitudes of the fast and slow phases of the reaction. The
value 0.0902 is significantly lower than the reported value of 1.43, calculated from
pulse radiolysis studies of the enzyme, possibly due to perturbation of the redox
potential of the type 2 Cu center by the binding of SO$_2^-$ to the center.
REFERENCES


Footnotes

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§ The on-line version of this article (available at http://www.jbc.org) contains Mathematical Analysis of Scheme-1.

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Figures Legends

Fig:1: Reduction kinetics of AxNiR by dithionite.
Reduction of the NiR was recorded at 594nm as a change in the blue color of the Type 1 Cu center of the enzyme. The reaction was monitored at 25 °C in a stopped flow spectrophotometer placed in a glove box with oxygen level ≤ 1 ppm. Both NiR (11µM in syringe A) and dithionite (0.56 mM in syringe B) were prepared in HEPES buffer pH 7.0 and ionic strength adjusted to 0.1M NaCl.

Fig:2 Dependence of the observed rate constants $k_{obs1}$ and $k_{obs2}$ on dithionite concentrations.
The observed rate constants $k_{obs1}$ (○, ●) and $k_{obs2}$ (◆, ◊) were computed by fitting exponential functions to the kinetic traces using Kinetasyst 3. Both constants show a non-linear dependence on the dithionite concentrations used. The observed data points (○, ◊) and (●, ◆) were recorded on two different days. Lines drawn through the points are not theoretical fits to the observed data; they are drawn to shown the general non-linear trend of the data.

Fig:3 The dependence of $k_{obs1}$ and $k_{obs2}$ on the square root of the concentration of $[S_2O_4^{2-}]$ used to ascertain the nature of the effective reducing agent.
The plot of $k_{obs2}$ versus $[S_2O_4^{2-}]^{1/2}$ is linear, indicating that the $SO_2^{•-}$ radical is the effective reducing agent. The slope of the line was used to calculate the value of the second order rate constant $k_2 = 3.33 \times 10^7$ M$^{-1}$ s$^{-1}$, see text for further details. Values of [ SO$_2^{•-}$] were calculated from [ SO$_2^{•-}$] = $K_{DT}^{1/2}[S_2O_4^{2-}]$ where $K_{DT}$=1.6 $\times 10^{-9}$. 
Similar plot of $k_{\text{obs}1}$ is non-linear, see the inset, indicating a more complex mechanism.

**Fig: 4 The observed rate constant $k_{\text{obs}1}$ follows saturation kinetics.**

A plot of the reciprocal of $k_{\text{obs}1}$ versus the reciprocal of the square root of $S_2O_4^{2-}$ is linear indicating that $k_{\text{obs}1}$ follows saturating kinetics as outlined in Eqs.s. 3 - 4. The y-intercept of the line gave the value $T^{1ox}k_1 = 122 \text{ s}^{-1}$ while the slope was used to calculate $T^{1ox}K_{ND} = 5.58 \times 10^5 \text{ M}^{-1}$. As a similar plot for $S_2O_4^{2-}$ (rather the square root of $S_2O_4^{2-}$) is non-linear, see the inset, it indicates that the effective reducing agent is the $SO_2\cdot^- \text{ radical rather than the dimeric species } S_2O_4^{2-}$.

**Fig: 5 Reduction of the type 2 depleted (T2D) NiR.**

The reduction kinetics of the T2DNiR were recorded under the same conditions as the T2 loaded protein. The mono-phasic traces were fitted to a single exponential function and the observed rate constant $T^{2D}k_{\text{obs}}$ was linearly dependent on the square root of $[S_2O_4^{2-}]$, see the main plot above, indicating the $SO_2\cdot^- \text{ radical as the reducing agent. Slope of the plot was used to calculate the second order rate constant } T^{2D}k = 3.06 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Plot of $T^{2D}k_{\text{obs}}$ versus was $[S_2O_4^{2-}]$ was non-linear, see the inset.
Fig: 6 Assessment of the rate constant $k_2$ of the partially-reduced $\text{T1Cu(I)T2Cu(II)}$ state of the enzyme as a first or second order constant.

According to the mathematical analysis, plot of $\frac{1}{k_{\text{obs}2}} - \{(1/1+K_{\text{NR}})k_3K_{\text{DT}}^{1/2}[\text{S}_2\text{O}_4^{2-}]^{1/2}\}$ versus $[\text{S}_2\text{O}_4^{2-}]^{1/2}$ gives a straight line if $k_2$ follows saturation kinetics. Although the plot is linear the line is very sensitive to the extreme values of the plot and the precise value of the y-intercept cannot be ascertained. Hence, the y-intercept is unable to determine whether $k_2$ follows first order kinetics through adduct formation or it follows normal second order kinetics. Values of the y-intercept were not used to calculate any kinetic parameters, see text for further details.
Table 1

Kinetic and equilibria parameters of the dithionite reduction reaction of the copper containing NiR isolated from Alcaligenes xylosoxidans.

A Hi-Tech SF-61 stopped-flow rapid-scan spectrophotometer was used to monitor the reactions at 25 °C using the single wavelength mode of the machine. The mixing and reaction chambers of the stopped-flow spectrometer were installed in an anaerobic glove box with oxygen level less than 1 ppm. The observed traces were fitted to exponential functions using KinetAsyst.3.

<table>
<thead>
<tr>
<th>Rate Constants</th>
<th>Values</th>
<th>Reactions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{10K_1}$</td>
<td>112 S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} + SO_2^- \rightarrow T_{1Cu(II)T2Cu(II)} + SO_2$</td>
<td>present work</td>
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<tr>
<td>$T_{10K_1}$</td>
<td>52.6 S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} + SO_2^- \rightarrow T_{1Cu(II)T2Cu(II)} + SO_2$</td>
<td>present work</td>
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<tr>
<td>$k_2$</td>
<td>$3.33 \times 10^7$ M$^{-1}$ S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} + SO_2^- \rightarrow T_{1Cu(II)T2Cu(II)} + SO_2$</td>
<td>present work</td>
</tr>
<tr>
<td>$T_{20K_1}$</td>
<td>3.06 $\times 10^6$ M$^{-1}$ S$^{-1}$</td>
<td>$T_{1Cu(II)} + SO_2^- \rightarrow T_{1Cu(II)} + SO_2$</td>
<td>present work</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$T_{20K_1} = 3.06 \times 10^5$ M$^{-1}$ S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} + SO_2^- \rightarrow T_{1Cu(II)T2Cu(II)} + SO_2$</td>
<td>present work</td>
</tr>
<tr>
<td>$k_4$</td>
<td>265 ± 18 S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} \rightarrow T_{1Cu(II)T2Cu(II)}$</td>
<td>13</td>
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<tr>
<td>$k_5$</td>
<td>185 ± 12 S$^{-1}$</td>
<td>$T_{1Cu(II)T2Cu(II)} \rightarrow T_{1Cu(II)T2Cu(II)}$</td>
<td>13</td>
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<table>
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<th>Equilibria Constants</th>
<th>Values</th>
<th>Equilibria</th>
<th>References</th>
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<tr>
<td>$K_{DT}$</td>
<td>1.6 $\times 10^9$ M</td>
<td>[SO$_2^-]^2$/[S$_2$O$_4^{2-}$]</td>
<td>24</td>
</tr>
<tr>
<td>$T_{10K_{ND}}$</td>
<td>5.58 $\times 10^5$ M$^{-1}$</td>
<td>$[T_{1Cu(II)}T2Cu(II),SO_2][T_{1Cu(II)}T2Cu(II)][SO_2^-]$</td>
<td>present work</td>
</tr>
<tr>
<td>$T_{10K_{ND}}$</td>
<td>2.62 $\times 10^5$ M$^{-1}$</td>
<td>$[T_{1Cu(II)}T2Cu(II),SO_2][T_{1Cu(II)}T2Cu(II)][SO_2^-]$</td>
<td>present work</td>
</tr>
<tr>
<td>$K_{NR}$</td>
<td>0.0892</td>
<td>$k_4/k_5 = [T_{1Cu(II)}T2Cu(II)]/[T_{1Cu(II)}T2Cu(II)]$</td>
<td>present work</td>
</tr>
<tr>
<td>$K_{NR}$</td>
<td>1.43</td>
<td>$k_4/k_5 = [T_{1Cu(II)}T2Cu(II)]/[T_{1Cu(II)}T2Cu(II)]$</td>
<td>13</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 6

![Graph showing the relationship between \(1/k_{\text{obs2}}\), \((1+(1+K_{NR})k_3K_{DT})^{1/2}\), and \([S_2O_4^{2-}]^{1/2}\) (M\(^{-1/2}\)).]
Supplementary Data

Mathematical analysis:

Consider a hypothetical molecule AB where both A and B are redox active. If AB is reduced by dithionite according to the following scheme and the reaction is monitored at wavelength where only A absorb (i.e. $\Delta_{OD}$ (change in optical density) of B at that wavelength is equal to zero), then a two exponential decay of A will be observed provided $k_1$ is slower than the equilibration reaction ($k_4 + k_5$) but faster than $k_3$.

$$
\mathrm{A_{ox}B_{ox}} \xrightarrow{k_1[S_2O_4^{2-}]} \mathrm{A_{ox}B_{red}} \xrightarrow{k_4} \mathrm{A_{red}B_{ox}} \xrightarrow{k_2[S_2O_4^{2-}]} \mathrm{A_{red}B_{red}}
$$

$$
K_{NR} = k_4/k_5 = A_{redB_{ox}}/A_{oxB_{red}} \quad (I)
$$

**Fast phase**

Under such conditions the rate at which A disappears is given by the following Eq. (IIa)

$$
-d[A_{oxB_{ox}}]/dt = k_1[A_{oxB_{ox}}] \quad (IIa)
$$
\[ [A_{oxB_{ox}}] = [AB_T]e^{-k_1t} \]  

Since the total optical density of \( A_{ox} \) at any time \( t \) is equal to the sum of the optical density of \( A_{oxB_{ox}} \) and \( A_{oxB_{red}} \), therefore, \( A_{oxB_{red}} \) has to be considered. According to law of mass action

\[ [AB_T] = [A_{oxB_{ox}}] + [A_{oxB_{red}}] + [A_{redB_{ox}}] + [A_{redB_{red}}] \]  

(IIIa)

As \( [A_{redB_{red}}] \) is negligible during the initial phase of the reaction, comparison of Eqs. (I) and (IIIa) followed by rearrangement, gives

\[ [AB_T] = [A_{oxB_{ox}}] + [A_{oxB_{red}}] + K_{NR}[A_{oxB_{red}}] \]  

(IIIb)

\[ [A_{oxB_{red}}] = \frac{[AB_T] - [A_{oxB_{ox}}]}{1 + K_{NR}} \]

\[ [A_{oxB_{red}}] = \frac{[AB_T] - [AB_T]e^{-k_1t}}{1 + K_{NR}} \]

\[ [A_{oxB_{red}}] = \frac{k_5[AB_T] \{1 - e^{-k_1t}\}}{k_5 + k_4} \]

Thus the decay of \( A \) during the fast phase of the reaction is given as

\[ [A_{oxB_{ox}}] + [A_{oxB_{red}}] = [AB_T]e^{-k_1t} + \frac{k_5[AB_T] \{1 - e^{-k_1t}\}}{k_5 + k_4} \]  

(IVa)

\[ [A_{oxB_{ox}}] + [A_{oxB_{red}}] = [AB_T] \{ \frac{k_5 + k_4 e^{-k_1t}}{k_5 + k_4} \} \]  

(IVb)
The exponential term of Eq. (IVb), defines the observed rate constant \( k_{\text{obs}_1} \) of the fast phase of the reaction and Eq. (IVc) applies.

\[
k_{\text{obs}_1} = k_1[S_{2O_4}^{2-}] \quad \text{(IVc)}
\]

The non-exponential term of Eq. (IVb) defines the amplitude of the un-reacted \( A_{\text{ox}} \) which under the two extreme conditions of \( t = 0 \) and \( t = \infty \) acquires the following two forms.

at \( t = 0 \)

\[
[A_{\text{ox}}B_{\text{ox}}] + [A_{\text{ox}}B_{\text{red}}] = [AB_T] \quad \text{(IVd)}
\]

at \( t = \infty \)

\[
[A_{\text{ox}}B_{\text{ox}}] + [A_{\text{ox}}B_{\text{red}}] = \frac{k_5 [AB_T]}{k_5 + k_4} \quad \text{(IVe)}
\]

However, when \( t = \infty \), \( [A_{\text{ox}}B_{\text{ox}}] = 0 \), see Eq. (IIb), thus

\[
at t = \infty \quad [A_{\text{ox}}B_{\text{red}}] = \frac{k_5 [AB_T]}{k_5 + k_4} \quad \text{(IVf)}
\]

This is an important equation that will be referred to later.

**Slow phase**

The slow phase of the reaction can be analyzed by considering the rate of formation of \( A_{\text{red}}B_{\text{red}} \)
\[
\frac{d[A_{\text{redB}}]}{dt} = k_3[A_{\text{oxB}}] + k_2[A_{\text{redB}}]
\]  

(Va)

Comparing Eqs. (I) and (Va) followed by rearrangement gives

\[
\frac{d[A_{\text{redB}}]}{dt} = \left\{k_3 + k_2 K_{NR}\right\}[A_{\text{oxB}}]
\]  

(Vb)

Since, \([A_{\text{oxB}}]\) is negligible during the slow phase of the reaction, Eq. (IIIa) by comparison with Eq. (I) can be rearranged to Eq. (VIa)

\[
[A_{\text{oxB}}] = \frac{[AB_T] - [A_{\text{redB}}]}{1 + K_{NR}}
\]  

(VIa)

Putting this value in Eq. (Vb)

\[
\frac{d[A_{\text{redB}}]}{dt} = \left\{k_3 + k_2 K_{NR}\right\} \frac{[AB_T] - [A_{\text{redB}}]}{1 + K_{NR}}
\]  

(VIb)

If \((k_3 + k_2 K_{NR})/(1 + K_{NR}) = y\) 

(VIc)

\[
\frac{d[A_{\text{redB}}]}{dt} = y [AB_T] - y[A_{\text{redB}}]
\]  

(VIIa)

Rearrangement and Integration of Eq. (VIIa) yields

\[
\int \frac{d[A_{\text{redB}}]}{y [AB_T] - y[A_{\text{redB}}]} = \int dt
\]
\[
\frac{\ln[y[AB_T] - y[A_{redB_{red}}]]}{-y} = t + c \quad (VIIb)
\]

Introducing the condition \( t = 0, [A_{redB_{red}}] = 0 \), followed by rearrangement, Eq. (VIIb) is converted to Eq. (VIIc) which gives the value of \([A_{redB_{red}}]\) as a function of time \(t\).

\[
[A_{redB_{red}}] = [AB_T](1-e^{-yt}) \quad (VIIc)
\]

According to Eq. (VIa)

\[
[A_{redB_{red}}] = [AB_T] - \{(1+K_{NR})[A_{oxB_{red}}]\}
\]

Putting this value in Eq. (VIIc)

\[
[AB_T] - \{(1+K_{NR})[A_{oxB_{red}}]\} = [AB_T](1-e^{-yt})
\]

\[
[A_{oxB_{red}}] = \frac{[AB_T]e^{-yt}}{1 + K_{NR}} \quad (VIIId)
\]

It is the exponential term of Eq. (VIIId) that defines the observed rate constant \((k_{obs2})\) of the slow phase of the reaction.

\[
k_{obs2} = y[S_2O_4^{2-}]
\]

Putting the value of \(y\) from Eq. (VIc),
\[
K_{\text{obs}2} = \frac{k_3 + k_2 K_{\text{NR}}}{1 + K_{\text{NR}}} [S_2O_4^{2-}] \]  \quad (\text{VIIe})

If \( SO_2^- \) rather than the \( S_2O_4^{2-} \) is the reducing agent then Eq. (VIIe) is modified into Eq. (VIIf)

\[
K_{\text{obs}2} = \frac{k_3 + k_2 K_{\text{NR}}}{1 + K_{\text{NR}}} K_{\text{DT}}^{\frac{1}{2}} [S_2O_4^{2-}]^{\frac{1}{2}} \]  \quad (\text{VIIf})

The non-exponential term of Eq. (VIIId) defines the amplitude of the un-reacted \( A_{\text{ox}}B_{\text{red}} \) species during the slow phase of the reaction. Under the two extreme conditions of \( t = 0 \) and \( t = \infty \), it acquires the following two values.

at \( t = 0 \) \[ A_{\text{ox}}B_{\text{red}} = \frac{k_6 [AB_T]}{k_5 + k_4} \]  \quad (\text{VIIg})

At \( t = \infty \) \[ A_{\text{ox}}B_{\text{red}} = 0 \]

It is important to note that if the analysis is valid, then \( A_{\text{ox}}B_{\text{red}} \) present at the start of the slow phase should be the same as that at the end of the fast phase. Eqs. (IVf) and (VIIg) clearly verify that condition and validate the analysis.

**Special case**

As a special case, if \( A_{\text{ox}}B_{\text{red}} \) reacts with the monomeric \( SO_2^- \) radical in a bimolecular fashion while reduction of \( A_{\text{red}}B_{\text{ox}} \) follows the mechanism of adduct formation, see Eqs. 6 - 7, then Eq. (VIIe) is modified as follow.

\[
K_{\text{obs}2} = \frac{k_3 + k_2 K_{\text{NR}}}{1 + K_{\text{NR}}} [SO_2^-] \]  \quad (\text{VIIla})
Since \([\text{SO}_2^-] = K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}\), see Eq. 3

\[
K_{\text{obs}2} = \frac{k_3 + k_2 K_{NR}}{1 + K_{NR}} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}
\]  

(VIIIb)

\[
K_{\text{obs}2} = x k_3 K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2} + x k_2 K_{NR} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}
\]

where \(x = 1/(1 + K_{NR})\)

\[
K_{\text{obs}2} - x k_3 K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2} = x k_2 K_{NR} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}
\]  

(VIIIc)

If \(k_2\) follows the mechanism outlined in Eqs. 6-7, then Eq. 8 applies and \(k_2\) is replaced by the rate constant \(T_{1\text{red}}k_1\) (first-order rate constant for the reduction of \(T_2\text{Cu}\) when the \(T_1\text{Cu}\) is in the reduced state), Eq. (VIIIc) can be written as

\[
K_{\text{obs}2} - x k_3 K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2} = \frac{x T_{1\text{red}}k_1 K_{NR} K_{ND} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}}{1 + K_{ND} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}}
\]  

(VIIIId)

Taking a reciprocal of Eq. (VIIIId)

\[
\frac{1}{K_{\text{obs}2} - x k_3 K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}} = \frac{1}{x T_{1\text{red}}k_1 K_{NR} K_{ND} K_{DT}^{1/2} [\text{S}_2\text{O}_4^{2-}]^{1/2}} + \frac{1}{x T_{1\text{red}}k_1 K_{NR}}
\]  

(VIIId)
This is straight-line equation that was used to calculate the values of $^{T_{\text{red}}}_1 k_1$ and $K_{ND}$ for the special case considered above.
Dithionite reduction kinetics of the dissimilatory Cu-containing nitrite reductase of Alcaligenes xylosoxidans; the SO2^- radical binds to the substrate binding type 2 Cu site before the type 2 Cu is reduced
Faridoon K. Yousafzai and Robert R. Eady

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