Kinetic studies on the nitrite reductase of *Wolinella succinogenes*

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The six haem groups of the nitrite reductase enzyme isolated from *Wolinella succinogenes* are rapidly reduced by the addition of dithionite ($S_2O_4^{2-}$). The reduction, however, is not homogeneous. Two of the haem groups, namely those that show spectral characteristics typical of five-co-ordinated haem groups, are reduced in a dithionite-concentration-dependent fashion with a rate limit of 1.5 s^{-1} . The other four haem groups, which show spectral characteristics very similar to those of normal six-co-ordinate *c*-haem groups, reduce in a linear dithionite-concentration-dependent manner with a second-order rate constant of $150\text{ M}^{-1/2}\cdot\text{s}^{-1}$. The ratio of the amplitudes of the two reduction phases observed in stopped-flow studies is found to be dependent on the concentration of dithionite used. A model is proposed to account for these observations, and computer simulations show that the model represents a good fit to the experimental data. The two haem groups with five-co-ordinate spectral characteristics bind CO. Flash photolysis of the CO complex exhibits one major recombination process with a linear dependence in rate on CO concentration with a second-order rate constant of $2 \times 10^6\text{ M}^{-1}\cdot\text{s}^{-1}$. By contrast, stopped-flow mixing of the reduced protein with CO shows a very complex pattern of combination, with most of the observed absorbance change associated with a concentration-independent step. These findings are rationalized in terms of structural changes in the protein consequent to ligand binding.

INTRODUCTION

Although the existence of a nitrite reductase in *Wolinella succinogenes* has been suspected since the discovery of the bacterium by Wolin *et al.* (1961), it is only recently that the enzyme has been purified. The first report of a purification procedure was by Liu *et al.* (1983), and this preparation allowed a preliminary characterization of the protein associated with nitrite reductase activity to be made.

The nitrite reductase of *Wolinella succinogenes* has been found to have an M_r of 63000 and this single polypeptide contains six covalently bound *c*-type haem groups [Liu *et al.*, 1983; Blackmore *et al.*, 1986 (the preceding paper)]. The enzyme plays an important role in the dissimilatory metabolism of nitrate by this bacterium. The enzyme is induced by nitrite during the anaerobic growth of the bacterium on nitrate as a substrate and the enzyme takes part in the direct six-electron reduction of nitrite to ammonia.

In the preceding paper we reported a more complete study on the molecular characterization of this enzyme and some of its equilibrium properties (Blackmore *et al.*, 1986). In the present study we have extended our static findings to investigate the kinetic properties of the enzyme and in particular to investigate any differences that might exist between the haem groups present within the protein.

MATERIALS AND METHODS

Nitrite reductase was isolated from the bacterium *Wolinella succinogenes* and purified by the methods described in the preceding paper (Blackmore *et al.*, 1986). The high- M_r form of the enzyme (Blackmore *et al.*, 1986)

was used in all the experiments described here. Stopped-flow and flash-photolysis experiments were performed by using the techniques and apparatus previously described (Brittain, 1980, 1984). Computer simulations were performed by using a fourth-order Runge–Kutta numerical-integration procedure to solve the simultaneous differential equations derived from the kinetic scheme (Barber *et al.*, 1978).

Dithionite solutions were prepared by adding anaerobically a preweighed amount of sodium dithionite to previously thoroughly deoxygenated buffer. The resulting solution was then calibrated in terms of the active concentration of dithionite present by anaerobic titration against a solution of deoxygenated cytochrome *c* by using the previously published absorption coefficient for ferrous cytochrome *c* (Lemberg & Barrett, 1973).

CO solutions were prepared by dilution of a thoroughly deoxygenated buffered solution equilibrated with 100 kPa (1 atm) of CO with deoxygenated buffer by using standard syringe techniques.

RESULTS

Dithionite reduction

Anaerobic rapid mixing of the nitrite reductase enzyme with dithionite in the stopped-flow apparatus produced biphasic reaction curves at 427.5 nm. An analysis of the dithionite-concentration-dependence of the two exponential components of the reduction time courses indicates the presence of two distinct processes. When the observed rate constant for the faster process is plotted against the square root of the dithionite concentration, a straight line is obtained (Fig. 1). The slope of this line yields an observed second-order rate constant, in terms of dithionite concentration, of $150\text{ M}^{-1/2}\cdot\text{s}^{-1}$. The square-

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Fig. 1. Concentration-dependence of the fast phase observed in the reduction of nitrite reductase by dithionite

Deoxygenated nitrite reductase ($1 \mu\text{M}$) was allowed to react anaerobically with dithionite in the stopped-flow apparatus in 60 mM-potassium phosphate buffer containing 0.05% Triton X-100 at pH 7.6 supplemented with Na_2SO_4 to I 0.3

root-dependence indicates that the active reductant in this process is $\text{SO}_2^{\cdot-}$ (Lambeth & Palmer, 1973). In order to obtain the true second-order rate constant for this process it is necessary to take into account the dissociation reaction of dithionite which leads to the formation of $\text{SO}_2^{\cdot-}$. Lambeth & Palmer (1973) have shown that, under the conditions employed in this study, the observed rate constant ($k_{\text{obs.}}$) is related to the true second-order rate constant (k) by the relationship:

$$k_{\text{obs.}} = kK^{1/2}[\text{S}_2\text{O}_4^{2-}]^{1/2}$$

in which K is the equilibrium dissociation constant for the reaction:

using the value of $K = 8.5 \times 10^{-8} \text{ M}$ (Lambeth & Palmer, 1973), we obtain a true second-order rate constant for the reduction process, in terms of $\text{SO}_2^{\cdot-}$ concentration, of $1.63 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The slower process observed presents a more complex hyperbolic dependence of rate on dithionite concentration (Fig. 2). The hyperbolic character of this process was unchanged up to ionic strengths of 0.3, and as such must indicate a true rate-limiting case rather than an ionic-strength effect. The contribution of each phase to the total absorbance change was found to be wavelength-dependent. This finding, together with the significant difference in rates between the two phases, allowed the separation of the kinetic difference spectrum arising from each kinetic phase (Fig. 3). The sum of the two phases at each wavelength measured was equal to the expected total absorbance change predicted from static measurements, and hence shows that no process is being overlooked in the dead time of the apparatus. Furthermore, the relative contribution of each process to the total absorbance change was found to be concentration-dependent at all wavelengths investigated (Fig. 4).

CO binding

Flash photolysis of the dithionite-reduced CO complex of the nitrite reductase produced recombination reaction profiles consisting of one major (> 95%) and one minor

Fig. 2. Concentration-dependence of the slow phase observed in the reduction of nitrite reductase by dithionite

The reaction was performed under the conditions given in Fig. 1.

Fig. 3. Kinetic difference spectra for the dithionite reduction of nitrite reductase

The contribution of the fast (Δ) and slow ($\circ$) kinetic phases to the total absorbance change ($\square$) observed when $1 \mu\text{M}$ -nitrite reductase was reduced by 1.85 mM -dithionite is shown. The reaction was carried out in 60 mM-potassium phosphate buffer containing 0.05% Triton X-100 at pH 7.6.

(< 5%) phase (Fig. 5). Both phases showed a linear dependence of rate on CO concentration, although the major phase was associated with a much higher rate constant ($2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ compared with

Fig. 4. Concentration-dependence of the ratio of the two kinetic phases observed in the reduction of nitrite reductase

The ratio of the contributions of the fast phase to that of the slow phase observed by stopped-flow (○) is shown compared with that simulated by the computer model (—) given in the text. Reaction conditions were as in Fig. 3.

$2.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$; Figs. 6a and 6b). As the enzyme used in these studies is known to contain about 5% of contaminating proteins (Blackmore *et al.*, 1986), it is possible that the smaller phase seen in CO recombination experiments arises from CO binding to this contaminant. In rapid-mixing experiments the combination reaction profiles showed a complex pattern of phases. Most of the absorbance change was associated with a very slow concentration-independent phase that required many minutes to reach the final absorbance value. It should be noted that, owing to the low affinity of the protein for CO, only small absorbance changes were associated with these reactions and therefore it was possible to obtain data only in the region 200–500 μM . As a consequence of these restrictions, only approximate values could be obtained for the rate constants associated with the CO-concentration-dependent phase seen in rapid-mixing studies. The values obtained were approx. $2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the binding rate constant and approx. 1 s^{-1} for the dissociation rate constant.

Computer simulations of dithionite reduction kinetics

Both kinetic and spectral measurements indicate that two types of haem groups are present in this enzyme. These will be referred to as 'c' for the haem groups presenting c-type kinetic difference spectra, and 'c*' for those haem groups presenting typically five-co-ordinate kinetic difference spectra. The concentration-dependence for each of these types of haem groups shows that the c-haems

Fig. 5. Kinetic difference spectra for CO recombination with nitrite reductase

The contribution of the fast (○) and slow (□) phases observed in the recombination of the nitrite reductase (1 μM) with CO (500 μM) after photolysis of the CO complex is shown. The reaction was performed at pH 7.6 in 60 mM-potassium phosphate buffer containing 0.05% Triton X-100.

are reduced in essentially a simple second-order reaction, whereas the c* haem groups show a rate-limiting concentration-dependence. The rate-limiting nature of the slower phase can arise in a number of ways; two commonly observed cases seen in biochemical systems being either due to (a) complex-formation (Strickland *et al.*, 1975) or else (b) the existence of an equilibrium between an active and an inactive form of the protein (Yandell *et al.*, 1973). The concentration-dependence of the amount of each phase associated with the two types of haem groups indicates that the two types are not reduced independently. This information led us to investigate the properties of a scheme analogous to that previously treated by Halford (1971) and Adams & Berman (1980), extended to include a second second-order step, namely:

Scheme 1.

In this Scheme the rapid second-order phase arises essentially from the reduction of $c^{III}c^{*III}$ with a rate constant k_{+1} , whereas the second phase arises from the

Fig. 6. Concentration-dependence of the kinetic phases observed when CO recombines with nitrite reductase

The concentration-dependence of the fast (a) and slow (b) phases observed on recombination of nitrite reductase ($1 \mu\text{M}$) with CO after photolysis of the CO complex is shown.

rate-limiting internal electron transfer followed by rapid dithionite reduction. The value of k_{+1} was obtained from the slope of the graph of the concentration-dependence of the fast phase and was initially set equal to k_{+3} . This was necessary, since it was not possible to obtain a value for k_{+3} directly by using the stoichiometrically reduced

Table 1. Rate constants used in the computer simulation of Scheme 1

Rate constant	Value
k_{+1}^*	$150 \text{ M}^{-1/2} \cdot \text{s}^{-1}$
$k_{+2}^\dagger$	1.5 s^{-1}
$k_{-2}^\ddagger$	23 s^{-1}
$k_{+3}^\ddagger$	$300 \text{ M}^{-1/2} \cdot \text{s}^{-1}$

* Obtained from the slope of Fig. 1.

† Obtained from the double-reciprocal plot of the data of Fig. 2.

‡ Obtained iteratively by best fit simulation of experimental data.

Fig. 7. Comparison of simulated and observed time courses for the dithionite reduction of nitrite reductase

A comparison of the simulated (—) and observed ($\circ, \square$) time courses for the reduction of nitrite reductase by dithionite ($\circ$, 26.5 mM; and $\square$, 1.85 mM) is shown.

mixed-valence form ($c^{\text{III}} c^{\text{II}}$), owing to the instability of this form of the enzyme on the time scale for the preparation for stopped-flow measurements. Values for k_{+2} and k_{-2} were obtained from the slope and intercept of a double-reciprocal plot of the data for the rate-limiting phase (Table 1). The rate constants and absorption coefficients for each species, estimated from kinetic difference spectra, were then included in the Runge-Kutta computer simulation of the reaction scheme. By using these values it was found that the model accounted quite well for the observed time courses. However, it was found that this set of parameters only poorly accounted for the observed concentration-dependence of the ratio of the extents of the two phases. On this basis the value of k_{+3} was changed in an iterative fashion to optimize the fit of the model to the concentration-dependence of the ratio of the two phases. A final value of $300 \text{ M}^{1/2} \cdot \text{s}^{-1}$ for k_{+3} was found to give an optimum fit to both the time courses and the concentration-dependence of the ratio of phases. Fig. 7 shows a comparison of the observed data at 427.5 nm with those predicted by the model. The model was found

to fit the observed data to the same degree of accuracy at all wavelengths investigated.

DISCUSSION

Kinetic difference spectra obtained from rapid-mixing experiments with dithionite show that the nitrite reductase enzyme from *Wolinella succinogenes* contains two types of haem centres. One type (*c*) has spectral characteristics typical of six-co-ordinate *c*-type haem groups, whereas the other type (*c**) has spectral characteristics similar to those of five-co-ordinate high-spin centres such as those seen in cytochromes of the *c'* class (Kamen & Bartsch, 1961). A comparison of the absorption coefficients for each group, obtained from both reduction and CO-binding studies, with those of known absorption coefficients for five- and six-co-ordinate haem groups (Antonini & Brunori, 1971; Cusanovich & Gibson, 1973; Lemberg & Barrett, 1973) leads to the conclusion that two of the six haem groups present in the molecule are of the *c**-type and four are of the *c*-type.

A model has been proposed for the reduction of the nitrite reductase enzyme by dithionite (Scheme 1). The quality of the fit of the proposed model to the reduction kinetics supports the view that initial reaction occurs via electron transfer from dithionite to the enzyme at the *c*-type sites. This is then followed by a relatively slow internal electron transfer to the *c**-sites and a subsequent rapid re-reduction of the *c*-sites by excess dithionite. There appears to be no evidence, either kinetic or spectral, for any heterogeneity within the two haem subgroups. That the enzyme should reduce in this manner is physiologically reasonable, since this mechanism would minimize the formation of any toxic partially reduced nitrogen intermediates. The formation of a high steady-state level of the enzyme containing only reduced *c** haem groups (the nitrite-binding site) is prevented by the unfavourable internal electron-transfer equilibrium and the rapid reduction of the remaining *c*-type haem in this species. In this way the nitrite-reducing site is essentially only present in the active form (*c**^{II}) when the enzyme is fully reduced. In common with some *c'*-cytochromes, the nitrite reductase enzyme exhibits CO rebinding kinetics subsequent to flash photolysis, which show much higher rates than, and indeed a different character from, those seen in rapid-mixing experiments. In the case of this nitrite reductase, however, the clear

concentration-dependence of the rate constant for CO recombination after photolysis rules out any 'protein cage' effects such as those which lead to geminate recombination seen in some other systems (Gibson & Kamen, 1966; Cusanovich & Gibson, 1973). The very slow and complex pattern of CO binding to the enzyme, seen in rapid-mixing experiments, shows that the reaction occurs via a number of intermediates. The experimental restrictions imposed on the stopped-flow studies by the very low affinity of the enzyme for CO has unfortunately prevented us from carrying out an extensive rapid-mixing study on the CO-binding reaction. Nevertheless, the limited experimental data obtained from stopped-flow studies are consistent with the existence in solution of a large steady-state level of unreactive reduced protein. This species once having undergone the binding process exists in an altered, more reactive form, as evidenced by the rapid recombination rate observed subsequent to photolysis of the liganded enzyme.

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