An analysis of the reaction kinetics of the hexahaem nitrite reductase of the anaerobic rumen bacterium *Wolinella succinogenes*

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The reduction kinetics of both the resting and redox-cycled forms of the nitrite reductase from the anaerobic rumen bacterium *Wolinella succinogenes* were studied by stopped-flow reaction techniques. Single-turnover reduction of the enzyme by dithionite occurs in two kinetic phases for both forms of the enzyme. When the resting form of the enzyme is subjected to a single-turnover reduction by dithionite, the slower of the two kinetic phases exhibits a hyperbolic dependence of the rate constant on the square root of the reductant concentration, the limiting value of which ($\sim 4 \text{ s}^{-1}$) is assigned to a slow internal electron-transfer process. In contrast, when the redox-cycled form of the enzyme is reduced by dithionite in a single-turnover experiment, both kinetic phases exhibit linear dependences of the rate on the square root of dithionite concentration, with associated rate constants of $150 \text{ M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$ and $6 \text{ M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$. Computer simulations of both the reduction processes shows that no unique set of rate constants can account for the kinetics of both forms, although the kinetics of the redox-cycled species is consistent with a much enhanced rate of internal electron transfer. Under turnover conditions the time course for reduction of the enzyme, in the presence of millimolar levels of nitrite and 100 mM-dithionite, is extremely complex. A working model for the mechanism of the turnover activity of the enzyme is proposed which very closely describes the reaction kinetics over a wide range of substrate concentrations, as shown by computer simulation. The similarity in the action of the nitrite reductase enzyme and mammalian cytochrome *c* oxidase is commented upon.

INTRODUCTION

The anaerobic rumen bacterium *Wolinella succinogenes* is capable of dissimilatory reduction of nitrate to NH_3 (Wolin *et al.*, 1961). This redox process involves two major enzymes, namely the nitrate reductase, which has been shown to reduce nitrate to nitrite, and the nitrite reductase, which carries out the six-electron reduction of nitrite to NH_3 (Liu *et al.*, 1983). The nitrite reductase enzyme has been shown to be a hexahaem protein of M_r 63000. Spectroscopic studies show that, in the isolated protein, the haems are all ferric, with one of the six haems high spin and with the rest of the haems low spin. One of the low-spin haems is weakly exchange-coupled to the high-spin centre (Blackmore *et al.*, 1987); this form is termed 'resting'. When the enzyme is reduced and re-oxidized, the haems in the enzyme are all ferric and low spin, with the exchange-coupled centre now more strongly coupled. This latter form is termed 'redox-cycled'. It has been proposed that the coupled haem pair forms the ligand-binding site (Blackmore *et al.*, 1990b). In the resting form a marked band at 610 nm is seen in the enzyme's absorption spectrum, indicative of high-spin ferric haem, whereas the redox-cycled form is characterized by the presence of a broader ferric Soret band. There is no evidence that corresponding resting and redox-cycled forms of the enzyme exist in the reduced enzyme (Blackmore *et al.*, 1990a).

The enzyme has been found to contain one haem with a very low redox potential, such that it cannot be fully reduced, even in the presence of an excess of dithionite or in the presence of an electron-transfer mediator such as Methyl Viologen. This 'non-reducible' haem can be further reduced, however, in the presence of haem ligands such as CO or cyanide (Blackmore *et al.*, 1990b). The low-redox-potential haem has been proposed to be one of

the exchanged coupled haem pair at which ligand binding triggers full reduction (Blackmore *et al.*, 1990b).

Initial kinetic studies were made of the single-turnover reduction process of the resting nitrite reductase enzyme by dithionite. The reduction process was analysed in terms of a three-step mechanism which produced the experimentally observable kinetic steps (Blackmore & Brittain, 1986). The rate of the slower of these steps shows a hyperbolic dithionite-concentration-dependence. The overall rate of reduction of the resting enzyme is controlled by an internal electron-transfer step which represents a rate-limiting process within this form of the protein. The redox-cycled form also exhibits two kinetic steps, but, unlike the resting form, the rate of the slower of these steps shows a linear dithionite-concentration-dependence (Blackmore *et al.*, 1990b). It has also been found that the low rate of dithionite reduction of resting nitrite reductase increased in the presence of cyanide, but the rate limit of this process was unchanged (Blackmore *et al.*, 1990a). This can be accounted for if the reduction of the active-site ligand-binding haems is unfavourable owing to the low redox potential of the exchange-coupled haem pair. Ligand binding to this site would stabilize the reduced state and increase the observed rate of reduction.

In the present paper we re-analyse the single-turnover reduction process for the resting (as isolated) and redox-cycled enzyme using more sophisticated computational algorithms for the simulation of the proposed kinetic model, and compare these results with those obtained experimentally for the dithionite reduction of the resting and redox-cycled enzyme. In addition, we combine the previously obtained mechanistic and structural details in a more general mechanism to simulate the spectral changes observed during nitrite reduction by this enzyme during turnover. The proposed mechanism of this enzyme is then

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compared with that previously proposed mechanism for the turnover of mammalian cytochrome *c* oxidase (Wilson *et al.*, 1981).

MATERIALS AND METHODS

Wolinella succinogenes was grown in 150-litre culture as previously described (Blackmore *et al.*, 1987) and the nitrite reductase enzyme was purified according to the procedures outlined previously (Blackmore *et al.*, 1986). Single-wavelength stopped-flow kinetics were measured with the Gibson–Milnes stopped-flow apparatus (Gibson & Milnes, 1964). Data acquisition and analysis was performed using the procedures described by Blackmore *et al.* (1990b). Rapid-scan measurements were made with a Tracor Northern TN6500 rapid-scan spectrophotometer connected to the Gibson–Milnes stopped-flow apparatus. Redox-cycled enzyme was prepared as described by Blackmore *et al.* (1990b). All working solutions were prepared as outlined by Blackmore & Brittain (1986).

Computer simulations

Simulations were performed using a VAX 8650 computer. Time courses were calculated for a given reaction scheme using the Gear (1971) method of numerical integration, which employs a predictor–corrector approach. The particular form of this method implemented in our studies was based on the NAG FORTRAN routine DO2EBF. It was found to be necessary to employ the Gear (1971) method for our purposes as the Runge–Kutta procedures previously employed in studies of the reduction kinetics of the nitrite reductase (Blackmore & Brittain, 1986) were found to be incapable of coping with the higher degree of ‘stiffness’ inherent in the more complex schemes studied in this work (Clare, 1983). Calculated time courses were usually obtained as a series of 100 equally spaced time points, which were either directly compared with the complex experimental time courses or analysed in terms of the sum of two exponentials, as appropriate, using the non linear least-squares NAG routine EO4FDF, to obtain the apparent rate constants and kinetic proportions.

Analysis of the simple, single-turnover, reduction kinetics of both the resting and redox-cycled forms of the enzyme proceeded according to previously outlined scheme (Blackmore & Brittain, 1986) (Scheme 1) in which *c* refers to the six-co-ordinate, low-spin, rapidly reduced haems, *c** refers to the slowly reducing haems with a high-spin-like difference spectrum, *k*₁ is the rate constant for reduction of the *c* haems by dithionite, *k*₂ is the rate constant for reduction of the *c* haems by dithionite, *k*₃ is the rate constant for internal electron transfer between *c* and *c**, *k*₄ is the rate constant for the reverse process and *k*₅ is the rate constant for re-reduction of the *c* haems by dithionite. It is noteworthy that, in Scheme 1, the rate constants *k*₁ and *k*₅ have units of M⁻¹·s⁻¹. This situation arises from the fact that the active reductant for the nitrite reductase is SO₂⁻ (as is evident by the linear dependence of the faster kinetic phase on the square root of the dithionite concentration; Blackmore & Brittain, 1986). The SO₂⁻ species exists in equilibrium with dithionite according to:

and thus:

$$[\text{SO}_2^-] \propto [\text{S}_2\text{O}_4^{2-}]^{1/2}$$

Scheme 1. Kinetic scheme for the reduction of nitrite reductase

[For further discussion, see Lambeth & Palmer (1973) and Creutz and Sutin (1973).] The object of computer simulation in the present work was to determine whether the previously reported rate constants provide a unique solution for the proposed mechanism, or if other rate constants would give an equally valid solution. In addition, it was of interest to run the simulation over a wider range of dithionite concentrations than had been possible previously in order to check further the validity of the proposed mechanism, to analyse the effect of the rate constant values on the form of the dithionite-concentration-dependence of the slow phase and to gain a better understanding of the kinetic differences observed between the redox-cycled and resting nitrite reductase.

Fig. 1. Comparison of the simulated and the experimental dithionite-concentration-dependence of the observed slow-phase rate of reduction for resting and redox-cycled nitrite reductase

Experimental points for the resting state (●) and the redox-cycled state (□) were taken from Blackmore *et al.* (1989a). The simulated rate values (■) were obtained using values of *k*₁ = 150 M⁻¹·s⁻¹, *k*₂ = 4 s⁻¹, *k*₃ = 200 s⁻¹ and *k*₄ = 300 M⁻¹·s⁻¹ for the resting form and values of *k*₁ = 150 M⁻¹·s⁻¹, *k*₂ = 500 s⁻¹, *k*₃ = 2.5 × 10⁴ s⁻¹ and *k*₄ = 300 M⁻¹·s⁻¹ for the redox-cycled form.

Fig. 2. Comparison of the simulated (■) and the experimental (●) double-reciprocal plot of the dithionite-concentration-dependence of the slow-phase rate reduction of resting nitrite reductase

Experimental and simulated rates were obtained as described in Fig. 1.

Fig. 3. Time course for the loss of the 610 nm band during the reduction of resting nitrite reductase by dithionite

Dithionite concentrations were: ●, 100 mM; □, 20 mM; and ■, 4 mM. The decrease in absorbance was measured at 610 nm. The nitrite reductase concentration was $5 \mu\text{M}$ in 60 mM-potassium phosphate buffer (pH 7.5)/0.05% (v/v) Triton X-100. The temperature was 20 °C. The lines were drawn by inspection.

RESULTS

When the resting form of the enzyme was rapidly mixed with dithionite under anaerobic conditions and the reaction monitored at 427 nm, the reduction occurred in the form of two exponentials, as described previously (Blackmore & Brittain, 1986; Brittain, 1986). The rate of the faster phase was linearly dependent on the square root of the dithionite concentration, with a rate constant of $150 \text{ M}^{-1/2} \text{ s}^{-1}$. The slower phase was hyperbolically dependent on dithionite concentration (Fig. 1) with a rate limit of $\sim 4 \text{ s}^{-1}$ (Fig. 2), and the ratio of the amplitudes of the two phases showed a non-linear dependence on dithionite concentration, as shown previously (Blackmore & Brittain, 1986). When observation was made in the α/β region, two kinetic phases were again observed, but in this region each kinetic phase contributed equally to the overall absorbance change at all wavelengths. If the reaction with dithionite was observed at 610 nm, then the high-spin ferric haem band was lost with a large period which corresponded in length to the time period required for the rapid reduction process, observed at 427 nm (Fig. 3).

Dithionite reduction of the redox-cycled enzyme in single-turnover experiments proceeded in two kinetic phases. The rate constant associated with the rapid phase was found to be identical with that observed for the resting enzyme. The slower phase, however, showed a linear dependence on dithionite concentration, with an associated rate constant of $6 \text{ M}^{-1/2} \text{ s}^{-1}$. The ratio of the amplitudes of the two kinetic phases in this case was found to be independent of the dithionite concentration employed.

When the reductions of both the resting and redox-cycled forms of the enzyme were performed in the presence of 1 mM-CO, the kinetics of CO binding were indistinguishable from the triphasic kinetics previously described for the binding of CO to the pre-reduced enzyme (Blackmore & Brittain, 1986).

In turnover experiments where the nitrite reductase was rapidly mixed with an excess of nitrite in the presence of a large excess of dithionite, extremely complex reaction kinetics were observed at 427 nm. Each time course, although very complex, exhibited the same major features (Fig. 4). Rapid reduction occurred at the same rate as that observed in the rapid phase of single-turnover-reduction experiments outlined above. This process was followed

Fig. 4. Time course for the reduction of nitrite reductase by dithionite in the presence of nitrite

The time course was observed at 427 nm (—); ---- shows the maximum absorbance obtained. The nitrite concentration was 4 mM, the dithionite concentration was 100 mM and the temperature was 20 °C. The nitrite reductase concentration was $0.5 \mu\text{M}$ in 60 mM-potassium phosphate buffer (pH 7.5)/0.05% (v/v) Triton X-100. ---- shows the computer-simulated time course.

Fig. 5. Comparison of computer-simulated (—) and experimentally observed (----) time courses for the reduction of resting nitrite reductase in the presence of nitrite

Nitrite concentrations were 0.1, 0.2, 0.5, 1.0, 2 and 4 mM (from left to right). The reaction was observed at 427 nm and the temperature was 20 °C. The nitrite reductase concentration was $0.5 \mu\text{M}$ in 60 mM-potassium phosphate buffer (pH 7.5)/0.05% (v/v) Triton X-100. All reductions were performed in the presence of 100 mM-dithionite. The rate constants used in the simulation are as shown in Table 1.

by a slower increase in absorbance at 427 nm, which lasted several seconds and lead to a steady-state phase. The steady state continued whilst nitrite was reduced, and the enzyme finally reached the 'fully reduced' form when nitrite was exhausted. This fully reduced form had e.p.r. and absorption spectra identical with the form produced by dithionite reduction of ferric nitrite reductase in the absence of nitrite. The level of reduction during the steady state and the length of the steady-state period (Fig. 5) were proportional to the amount of nitrite used in the experiments, at a fixed dithionite concentration of 100 mM.

DISCUSSION

Previous spectroscopic and kinetic studies (Blackmore *et al.*, 1990b) clearly identified major structural and functional differences between the ferric resting and ferric redox-cycled

Scheme 2. Kinetic scheme for the redox reactions of nitrite reductase in turnover conditions

forms of the protein. It has been shown that the dithionite reduction kinetics of the resting and redox-cycled forms of the enzyme show the same general pattern, with reduction occurring in two phases (Blackmore & Brittain, 1986; Blackmore *et al.*, 1990b). Although the internal electron-transfer process appears to have become rate-limiting at high dithionite concentrations, in reduction studies of the resting form of the enzyme when the redox-cycled form is reduced, no rate limit is evident up to the highest reductant concentrations employed. Some new insights into these processes have, however, been gained from the present studies. Observations at 610 nm show that this absorption band is not altered until after the rapid phase of reduction is completed, and it is lost during the slow reduction process. This is suggestive of a significant structural change in the enzyme accompanying, or rapidly following, the initial electron-transfer process. In addition, the kinetic data obtained for the dithionite reduction of both forms of the protein in the presence of CO suggest that, once having been reduced, the resting and redox-cycled forms of the enzyme become functionally equivalent.

It would thus appear that Scheme 1 should be able to account for the single-turnover reduction of both the resting and redox-cycled forms of the protein by dithionite if the internal electron-transfer rate is significantly different in the two structural forms of the protein.

To test this hypothesis, we have simulated time courses for Scheme 1 under a variety of conditions, firstly in order to identify whether a unique set of rate constants is capable of describing these systems, and secondly to identify whether a change in the relative magnitudes of k_2 and k_3 is capable of representing the differences in kinetics observed between the resting and redox-cycled forms of the enzyme.

In terms of Scheme 1, for the resting enzyme, k_1 and k_2 were set to their experimentally determined values and then, in two sets of simulations, either k_3 or k_4 were systematically altered whilst the other was kept constant. k_3 was varied from 6 s^{-1} to 72 s^{-1} and k_4 varied from 100 s^{-1} to 900 s^{-1} . The results were then compared with the experimental data. It was evident from these simulations that, for values of $k_3 > 24 \text{ s}^{-1}$, a particular value of k_4 exists which gives reaction rates and a simulated dithionite-concentration-dependence which is the same as that observed experimentally (Fig. 1). Thus, although this mechanism can adequately describe the observed single-turnover reduction of nitrite reductase by dithionite, it is only possible to set lower limits to the values of k_3 and k_4 . They cannot be below 24 s^{-1} and

$300 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. For the redox-cycled enzyme, in which a hyperbolic dependence on dithionite concentration is not observed, k_2 and k_3 were systematically altered whilst k_4 was set to $300 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Blackmore & Brittain, 1986) and the other rate constants and absorption coefficients were taken from the previous simulation. In this case k_2 was varied from 200 s^{-1} up to 1000 s^{-1} . In this situation, good agreement between simulation and experiment was obtained at all values of k_2 explored, as long as the ratio of k_3 and k_2 used for this simulation was maintained at 50:1. Thus from these data it is evident that no unique set of values of k_2 and k_3 exist to describe best the single-turnover reduction of the redox-cycled form of the enzyme; however, there does exist a unique ratio of k_2 and k_3 which best fits the experimental data. The simulations show that, in going from the resting form to the redox-cycled form, the ratio of k_2 and k_3 remains unchanged; that is, reduction of the active-site haems is thermodynamically unfavourable as compared with the electron-donor haems. However, the increase in the absolute values of the rate constants k_2 and k_3 in the redox-cycled state allows for a much faster electron exchange between the donor haems and the exchange-coupled active-site haem pair. It has previously been shown that the reduced form of the active-site haems could be stabilized by the binding of ligands (Blackmore *et al.*, 1990b). Thus, in the presence of substrate, it would be predicted that electron transfer to the active site would be much more rapid than might be expected from the single-turnover dithionite-reduction experiments.

Building on these findings we next simulated the kinetic processes observed in turnover experiments where the enzyme is reduced by a vast excess of dithionite in the presence of an excess of nitrite. It is clear that the enzyme exists in two ferric forms and, as outlined above, certain rate-constant values and their ratios can be assigned for the reduction process. In the ferrous state there is no evidence for more than one form of the enzyme. It has also been demonstrated that ligands will bind to the mixed-valence, partially reduced, forms of the enzyme as well as to the fully reduced form (Blackmore *et al.*, 1990b). Recently it has been found that the previously-referred-to reduced or ferrous form of the enzyme does in fact contain only five reducing equivalents and will only take up the sixth and last equivalent subsequent to ligand binding (Blackmore *et al.*, 1989a). It might therefore be assumed that once nitrite has bound to the enzyme it will induce total reduction of the enzyme and only then will the six-electron reduction of nitrite to NH_3 occur. On this basis we

Table 1. Rate constants used in computer simulation of enzyme turnover

Rate constants (k) were experimentally determined; the remainder were obtained from computer simulation. k_{13} was assigned a value of $1 \times 10^{-6} \text{ s}^{-1}$ from experimental observation of this process (Blackmore *et al.*, 1989a). k_{12} was assigned a value of 10^3 s^{-1} , as all attempts to measure this rate using stopped-flow techniques have indicated the reaction is complete within the dead-time of the apparatus, i.e. $t_{1/2} < 2 \text{ ms}$ (Blackmore, 1988).

Rate constant	Value	Rate constant	Value
k_1	$150 \text{ M}^{-1/2} \cdot \text{s}^{-1}$	k_7	$2.5 \times 10^4 \text{ s}^{-1}$
k_2	4 s^{-1}	k_8	$300 \text{ M}^{-1/2} \cdot \text{s}^{-1}$
k_3	200 s^{-1}	k_9	$1 \times 10^5 \text{ M}^{-1/2} \cdot \text{s}^{-1}$
k_4	$300 \text{ M}^{-1/2} \cdot \text{s}^{-1}$	k_{10}	$300 \text{ M}^{-1/2} \cdot \text{s}^{-1}$
k_5	$7 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$	k_{11}	1000 s^{-1}
k_6	500 s^{-1}		

have constructed Scheme 2 as a model for the action of the nitrite reductase enzyme in turnover. In this scheme the rate constants k_1 , k_2 , k_3 and k_4 were determined experimentally. The underlined species represent the redox cycled forms and

represents the six-electron-reduced form of the enzyme accessible only after nitrite has bound to the enzyme. 'Dith' is dithionite. To test the validity of Scheme 1, we simulated time courses using rate-constant values shown in Table 1.

In Scheme 2 we have assumed that the oxidation process is very rapid and can be represented, for the purposes of the simulation, as a simple one-step process. We do this simply on the basis that all attempts to measure the rate of oxidation of this enzyme have yielded evidence that this process is complete in the dead time of our stopped-flow apparatus. It should also be pointed out that, in this Scheme, the process of conversion of the fully oxidized redox-cycled form into the resting form has been included simply for completeness, although the rate constant for this process (k_{13}) is set at 10^{-6} s^{-1} on the basis that this conversion, as followed spectroscopically, occurs on the time scale of days.

It should also be emphasized that, as in the case of Scheme 1, each of the reaction steps in Scheme 2 does not represent a single electron-redox process, but rather the change in redox state of either the 'cytochrome-*c*-type' haems or the 'myoglobin-type haems' (as defined in Blackmore & Brittain, 1986). Thus, if defined rigorously, many of the steps in Scheme 2 would be associated with a number of elementary steps. In a scheme such as Scheme 2 clearly many of the rate constants cannot be independently determined experimentally and must be set at values we consider reasonable. As such the simulated time courses are not rigorously justifiable, but we present them as simply representations of a working model which does not conflict with any experimental data so far obtained for this system. Nevertheless, it is clear that, using the parameter set of Table 1, this Scheme not only predicts the very complex overall shape of any particular time course, but also predicts the observed

level of steady-state reduction of the system and also the length of the turnover, for a wide range of substrate concentrations, without recourse to any alteration to the parameter set.

On the basis of the assumed appropriateness of Scheme 2, the similarity of operation of the nitrite reductase and mammalian cytochrome *c* oxidase is striking.

In the case of cytochrome oxidase, both resting and pulsed forms (equivalent to the redox-cycled state in nitrite reductase) have been identified (Antonini *et al.*, 1977), which interconvert during turnover (Brunori *et al.*, 1983; Wilson *et al.*, 1981). Moreover, the pulsed form of the oxidase exhibits a much-enhanced internal electron-transfer rate as compared with the resting form, and it has been shown that ligand binding to partially reduced forms of the enzyme can lead to an increase in turnover rate (Bickar *et al.*, 1986). Cytochrome *c* oxidase thus appears to be almost identical with nitrite reductase in the molecular mechanisms it uses to obtain its enzymic activity and control. At the structural level it would also appear that both enzymes use magnetically coupled metal centres to produce an active site for reduction (Powers *et al.*, 1981; Palmer *et al.*, 1976; Blackmore *et al.*, 1987). Both enzymes prevent the formation of potentially toxic partial reduction products by the use of the same complex structural changes, employing complex multi-metal-centre proteins in an example of convergent mechanistic evolution.

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