The effect of haem ligands on the redox states of the hexa-haem nitrite reductase from *Wolinella succinogenes*

Richard S. BLACKMORE,* Paul M. A. GADSBY,† Colin GREENWOOD* and Andrew J. THOMSON††

*School of Biological Sciences and †School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The nitrite reductase of *Wolinella succinogenes* containing six covalently bound haem groups has one haem group that will not reduce fully in the presence of excess $\text{Na}_2\text{S}_2\text{O}_4$. The effect of the extrinsic ligands CO and cyanide on the redox state of this haem was studied by e.p.r. and magnetic c.d. spectroscopy. It was found that both ligands increased the extent of reduction of this haem group, and that in the case of CO binding the level of reduction was correlated with the extent of CO saturation of the enzyme. Stopped-flow studies of the effect of cyanide binding on the rate of dithionite reduction showed that the rate of reduction of the ligand-binding site was increased in the presence of cyanide. This suggests that reduction of the haem groups at the active site is thermodynamically unfavourable in the absence of an extrinsic ligand. The role of the 'non-reducing' haem group and the effect of ligands on this centre and on the rate of reduction are discussed in relation to the reduction of nitrite by this enzyme.

INTRODUCTION

The nitrite reductase of the anaerobic bacterium *Wolinella succinogenes* contains six covalently bound ϵ -type haem groups (Liu *et al.*, 1983). E.p.r. and m.c.d. studies show that five of the haem groups have bis-histidine ligation. Two of the haem groups are magnetically isolated from one another. The other four consist of two pairs, which are exchange-coupled in the oxidized state of the protein (Blackmore *et al.*, 1990b). One pair contains two low-spin ferric haem groups and the other a combination of low-spin and high-spin haem groups. Kinetic and ligand-binding experiments show that the exchange-coupled high-low-spin pair of haem groups are the site at which nitrite reduction takes place when the enzyme is reduced. Blackmore *et al.* (1987) report that treatment of the enzyme with excess $\text{Na}_2\text{S}_2\text{O}_4$ or reduced methyl viologen reduces five of the six haem groups to the low-spin ferrous state. One haem group, called the 'non-reducible' haem group, remained in the low-spin ferric state, however, and could be detected by e.p.r. spectroscopy (with g -values of 3.08, 2.22 and 1.27) and by m.c.d. spectroscopy (Blackmore *et al.*, 1987). The addition of the denaturing detergent SDS at a concentration of 1% (w/v) caused the 'non-reducible' haem group to be reduced by $\text{Na}_2\text{S}_2\text{O}_4$. Therefore we conclude that the integrity of the protein structure is important in maintaining this haem group in the ferric state. The 'non-reducible' haem group may be difficult to reduce either because the protein prevents reductant from approaching the haem group or because it has an unusually low reduction potential.

In the present paper we report the effect of the binding of CO and cyanide to the reduced state of the resting nitrite reductase enzyme. We have studied by stopped flow the effect of cyanide on the rate of dithionite reduction of the resting enzyme, and have examined, by means of low-temperature e.p.r., low-temperature m.c.d. and room-temperature m.c.d., the effect of cyanide and CO on the reduction state of the 'non-reducible' haem group.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Co.

Wolinella succinogenes was grown and nitrite reductase was purified as described previously (Blackmore *et al.*, 1987). E.p.r.,

m.c.d., stopped-flow and absorption spectroscopic studies were performed as described (Blackmore *et al.*, 1987, 1990b). Cyanide solutions were prepared by dissolving NaCN in the chosen buffer solution and titrating to the required pH with HCl. The resulting solutions were standardized by titration into a metmyoglobin solution (Antonini & Brunori, 1971). $\text{Na}_2\text{S}_2\text{O}_4$ and CO solutions were prepared and standardized as previously described (Blackmore & Brittain, 1986). Photolysis at 4.2 K was carried out with white light from a 500 W light source focused on to the sample housed inside the magnet of the m.c.d. apparatus.

Rates and absorbance changes for the stopped-flow traces were calculated by 'peeling' as outlined previously (Blackmore *et al.*, 1990a). Non-linear least-squares fitting was performed by using a Damped Gauss-Newton computer algorithm (Nielsen-Kudsk, 1983) written in Turbo Pascal (Borland International) running on an IBM AT compatible computer.

RESULTS

Cyanide binding

The effect of the binding of cyanide on the level of reduction of the enzyme brought about by dithionite was monitored by absorption (Fig. 1), e.p.r. and m.c.d. (Fig. 2) spectroscopies. These results show that the single low-spin ferric haem group that remains oxidized in the reduced enzyme in the absence of cyanide is induced to reduce to the low-spin ferrous state by its addition. The e.p.r. spectrum of the cyanide-bound form has lost the low-spin ferric haem signals at $g = 3.08$, 2.22 and 1.27, associated with the non-reducible haem group. The Soret band develops a shoulder on the long-wavelength edge (Fig. 1), showing that binding of cyanide to a haem group takes place. The m.c.d. spectra, recorded at 4.2 K (Fig. 2), also give evidence that the signals associated with the low-spin ferric haem group have been lost. Fig. 2 provides a comparison between forms of the reduced enzyme with and without cyanide added. The absence of temperature-dependent signals in the Soret-band region shows that all the haem in the enzyme is low-spin ferrous in nature. This contrasts with the intense temperature-dependent signals in the reduced enzyme (Fig. 2b). The Soret-region m.c.d. of the cyanide-treated enzyme is unusual, having a band between 410 and

Abbreviation used: m.c.d., magnetic circular dichroism.

† To whom correspondence should be addressed.

Fig. 1. Soret-region absorption spectra of dithionite-reduced nitrite reductase (—) and the cyanide derivative of dithionite-reduced nitrite reductase (---)

Nitrite reductase concentration was $1 \mu\text{M}$ in 60 mM-potassium phosphate buffer, pH 7.5, containing 0.05% (v/v) Triton X-100. The cyanide concentration was 100 mM and the dithionite concentration was 10 mM.

480 nm, apparently corresponding to the shoulder in the absorption spectrum (Fig. 1). Photolysis of the cyanide-inhibited sample at 4.2 K with white light for 20 min gives rise to a new high-spin ferrous haem group in the m.c.d. spectrum. After warming the sample to 80 K, in the dark, and re-cooling to 4.2 K, the spectrum of the unphotolysed sample is regained. Photolytic displacement of ligands bound to ferrous haem is not abnormal.

Stopped-flow studies of cyanide binding to dithionite-reduced nitrite reductase

Previous stopped-flow studies of the reduction of nitrite reductase by dithionite have shown that the reaction time course is biphasic (Blackmore & Brittain, 1986). If cyanide is added to the dithionite solution that is rapidly mixed with resting ferric nitrite reductase in the stopped-flow apparatus, a triphasic reaction time course is observed. The two faster phases can be associated with reduction of the enzyme by dithionite, and the third and slowest phase can be associated with the binding of cyanide to the reduced enzyme. The rate of and absorption change associated with the first, fastest, phase is unaffected by the presence of cyanide, as is the absorption change associated with the second phase. The dithionite-concentration-dependence of the pseudo-first-order rate constant of this second phase is, however, different in the presence and in the absence of cyanide (Fig. 3). In the presence of 100 mM-cyanide the shape of the dithionite-concentration-dependence of the observed rate constant of the second phase has clearly altered. An equation of the form $k_{\text{obs}} = p_1[S]/(p_2 + [S])$ can be fitted to these data by using a Damped-Gauss-Newton non-linear least-squares algorithm. p_1 represents the rate limit of reduction, and p_2 defines how quickly the curve approaches the rate limit. In the absence of cyanide p_1 is 4.42 s^{-1} with a standard deviation of 0.36 s^{-1} , and p_2 is $-0.433 \text{ M}^{\frac{1}{2}}$ with a standard deviation of $8.9 \times 10^{-5} \text{ M}^{\frac{1}{2}}$. In the presence of 100 mM-cyanide p_1 is 4.0 s^{-1} with a standard deviation of 0.065 s^{-1} and p_2 is $0.167 \text{ M}^{\frac{1}{2}}$ with a standard deviation of

Fig. 2. Low-temperature m.c.d. spectra of (a) the cyanide derivative of dithionite-reduced nitrite reductase, (b) dithionite-reduced nitrite reductase and (c) the CO derivative of dithionite-reduced nitrite reductase

(a) Low-temperature m.c.d. spectrum of the cyanide derivative of dithionite-reduced nitrite reductase. Spectra in the Soret and visible regions are shown before (—) and after (---) 20 min photolysis. The cyanide concentration was 100 mM. (b) Low-temperature m.c.d. spectrum of dithionite-reduced nitrite reductase. The spectrum in the visible and Soret regions is shown. (c) Low-temperature m.c.d. spectrum of the CO derivative of dithionite-reduced nitrite reductase. The spectra in the Soret and visible regions are shown before (—) and after (---) 20 min photolysis. CO concentration was 1 mM. Measurement and sample conditions for (a), (b) and (c) were as follows: temperature, 4.2 K; path length, 1.5 mm; magnetic field, 5 T. The nitrite reductase concentration was $5 \mu\text{M}$ in 50 mM-Hepes buffer, pH 7.5, containing 50% (v/v) glycerol and 0.05% (v/v) Triton X-100. The dithionite concentration was 10 mM.

Fig. 3. Dithionite-concentration-dependence of the observed rate constant of the second phase in the dithionite reduction of nitrite reductase

The reduction was performed both in the absence ($\square$) and in the presence (+) of cyanide. The inset shows the time course of dithionite reduction of nitrite reductase in the presence (upper trace) and in the absence (lower trace) of cyanide. The nitrite reductase concentration was $0.5 \mu\text{M}$ in 60 mM-potassium phosphate buffer, pH 7.5, containing 0.05 % (v/v) Triton X-100. The cyanide concentration was 100 mM, and the experiments were performed at 20 °C. The continuous lines show best fit calculated by non-linear least-squares analysis (Nielsen-Kudsk, 1983).

Fig. 4. Dithionite-concentration-dependence of the observed rate constant of the slowest phase in the dithionite reduction of nitrite reductase in the presence of cyanide

The nitrite reductase concentration was $0.5 \mu\text{M}$ in 60 mM-potassium phosphate buffer, pH 7.5, containing 0.05 % (v/v) Triton X-100. The cyanide concentration was 100 mM and the experiments were performed at 20 °C.

Fig. 5. Cyanide-concentration-dependence of the phase observed on cyanide binding to the dithionite-reduced nitrite reductase

The nitrite reductase concentration was $0.5 \mu\text{M}$ in 60 mM-potassium phosphate buffer, pH 7.5, containing 0.05 % (v/v) Triton X-100. The dithionite concentration was 20 mM and the experiments were performed at 20 °C.

Fig. 6. Dependence on CO concentration of the intensity of the m.c.d. signal of the 'non-reducible' haem group

The signal of the 'non-reducible' haem group was measured at 400 nm ($\blacksquare$) and is compared with the hyperbolic best-fit line (—) for the CO titration of nitrite reductase (from Blackmore *et al.*, 1986). The experiment was performed at 4.2 K with a path length of 1.5 mm and at a magnetic field of 5 T. The nitrite reductase concentration was $5 \mu\text{M}$ in 50 mM-Hepes buffer, pH 7.5, containing 50 % (v/v) glycerol and 0.05 % (v/v) Triton X-100. The dithionite concentration was 10 mM.

third, slow, phase also shows a rate-limited dithionite-concentration-dependence (Fig. 4). The observed rate constants of the three phases in the dithionite reduction of the enzyme were not dependent on cyanide concentration above 2 mM-cyanide. At cyanide concentrations below 1 mM the dithionite reduction reaction was approximated by a biphasic reaction.

The dependence on cyanide concentration of the rate of the reaction with the ferrous nitrite reductase is shown in Fig. 5. The reaction is monophasic and independent of cyanide concentration above 2 mM.

CO binding

In the presence of 1 mM-CO (Fig. 6) the level of ferric haem signal in the 4.2 K m.c.d. spectrum decreased by 60 % from that normally expected in the dithionite-reduced enzyme. The decrease in the ferric haem signal was monitored at 400 nm, because the m.c.d. signal at this wavelength is principally due to low-spin ferric haem. A CO titration is shown in Fig. 6. There is a clear

$5.7 \times 10^{-6} \text{ M}^{\frac{1}{2}}$. Clearly the rate limits (p_1) are not significantly different. However, in the presence of cyanide the reduction approaches the rate limit at a much lower dithionite concentration. The observed pseudo-first-order rate constant of the

correlation between the intensity of the low-spin ferric haem signal and the amount of CO bound. This correlation was also observed by absorption spectroscopy in the Soret region (Blackmore *et al.*, 1986).

Photolysis of the CO-bound enzyme at 4.2 K produced a high-spin ferrous haem m.c.d. spectrum (Fig. 2) that is broadly similar to that produced by photolysis of the cyanide-bound enzyme (Fig. 2). This high-spin ferrous haem spectrum was lost on warming to 80 K. In the absence of cyanide and CO, no spectral changes on photolysis of the dithionite-reduced enzyme were observed.

DISCUSSION

The loss of the e.p.r. signal of the 'non-reducible' haem group in the dithionite-reduced enzyme in the presence of cyanide and the decreased intensity of the low-spin ferric m.c.d. signal in the presence of CO show that ligand binding to a single haem group leads to further reduction of the enzyme. Whether the difficulty of reducing this haem group in the absence of a ligand is due to protein restriction of access of reductant to the haem or to the presence of a haem group with an unusually low redox potential is a question that can be answered by reference to the results of the m.c.d. CO titration. In the former case ligand binding would open the structure to allow reductase access and reduction would be irreversible. The reaction would go to completion even at low ligand concentrations, in a mechanism similar to Scheme 1, in which $c^{III}c^{II}$ represents the resting reduced state of the enzyme, which contains the 'non-reducible' haem group, and $c^{II}c^{II}$ represents the fully reduced state of the enzyme. The essentially irreversible character of the dithionite reduction step arises from the low redox potential of dithionite and ensures that at equilibrium the enzyme will be fully reduced. Clearly this was not observed. Alternatively, if the haem group cannot be reduced because it has an unusually low redox potential, and the potential is reversibly raised by the binding of ligand to the enzyme, then it would be expected that the level of reduction would reflect the extent of ligand binding in a mechanism similar to Scheme 2. In this case the reaction of the protein with dithionite is a redox equilibrium, unlike that in Scheme 1, where the reaction with the dithionite is essentially irreversible. From the CO titration data of Fig. 6 it is clear that Scheme 2 represents the most likely scheme.

Mayhew (1978) has shown that a redox equilibrium can be formed between dithionite and (bi)sulphite ions and low-potential electron carriers such as flavodoxin hydroquinone or methyl viologen semiquinone. The redox potential of dithionite is pH-dependent (midpoint potential -660 mV at pH 7.0): the lower the pH the higher the potential. Therefore at low pH the level of reduction of nitrite reductase should be lower. This therefore gives further evidence for a redox equilibrium between the 'non-reducible' haem group and $\text{Na}_2\text{S}_2\text{O}_4$. It is also possible that pH-dependent structural changes in the protein might change the absorption coefficient of one or more of the haem groups without changing the redox potential. Mayhew (1978) also noted that the redox potential of dithionite solution increased as the bisulphite concentration was increased. No effect on the level of reduction of nitrite reductase was observed when 100 mM-bisulphite was

Scheme 1.

Scheme 2.

added to nitrite reductase that had been reduced with a minimal amount of dithionite, nor was there any evidence that the level of reduction decreased with increasing dithionite concentration, as might have been expected. (The solution potential should increase by 29 mV per decade as a result of the $\text{S}_2\text{O}_4^{2-} \rightleftharpoons 2\text{SO}_3^{\cdot-}$ equilibrium.) There is, however, evidence from low-temperature m.c.d. studies that bisulphite or another component of dithionite solutions binds to the enzyme (R. S. Blackmore & P. M. A. Gadsby, unpublished work). This may raise the redox potential of the 'non-reducible' haem group in the same way as does cyanide and CO binding.

A haem group with such a low redox potential is very unusual. Histidine-histidine-ligated haem groups typically have redox potentials in the range -200 to -500 mV (Moore & Williams, 1977). That of this haem group is lower by up to -150 mV. In previous work two exchange-coupled haem pairs were identified in this enzyme (Blackmore *et al.*, 1987). Further studies of this centre (Blackmore *et al.*, 1990a) lead to the proposal that one pair is bridged by a common histidine ligand with two nitrogen atoms providing the sixth iron ligands to each haem. The redox potentials of the haem groups in such a system will not be independent. A second electron would be more difficult to add to the pair. If the 'non-reducible' haem group were part of this pair this would account for the second low redox potential. Any process that disrupted the interaction between the two haem groups such as a ligand binding to one of the haem groups, should raise the redox potential of the ferric haem and allow it to be reduced. This process would be reversible on loss of ligand, however, so that at equilibrium the extent of saturation of the nitrite reductase with ligand would control the level of reduction. A process that removed the ligand, such as flash photolysis, would leave the haem group free of ligand, but more reduced than normal for the unbound form. In this state the ligand would re-bind more rapidly and also have a different dissociation constant. Flash-photolysis results reported previously show this to be the case. The dissociation constant estimated from a CO titration was $415 \mu\text{M}$ (Blackmore *et al.*, 1986) and that obtained from flash photolysis was $93 \mu\text{M}$ (Blackmore & Brittain, 1986).

Unlike CO, cyanide binds to the enzyme rapidly and with high affinity. Dithionite reduction of nitrite reductase in the presence of cyanide was triphasic. The first two phases were due to dithionite reduction and were also observed in the absence of cyanide. The last, slowest, phase was found only in the presence of cyanide ion. The most interesting feature of the reaction was the increase in the rate of the observed intermediate phase in the presence of cyanide. The reduction mechanism developed previously (Blackmore & Brittain, 1986) is shown in Scheme 3, where c refers to the low-spin haems and c^* refers to the exchange-coupled haem pair at the active site. If cyanide were able to bind to species 4, no effect on the intermediate rate would be expected. However, if cyanide were to bind to intermediate 3, the mixed-valence form, as well, then the effect would be to stabilize the reduced reaction forms of the active-site haem group(s). This can be seen as a decrease in the value of k_{-2} with no change in k_{+2} .

k_{-2} can be calculated from the values of p_2 , p_1 and k_{+3} (Yandell *et al.*, 1973). The value of k_{+3} was obtained from Blackmore & Brittain (1986). In the absence of cyanide k_{-2} is -130 s^{-1} , and in the presence of cyanide k_{-2} is -50 s^{-1} .

The rate of reduction of the intermediate phase should be dependent on the concentration of cyanide until the reductive

Scheme 3.

rate limit of 4 s^{-1} is reached. This was, however, not observed. The binding of cyanide to intermediate 3 must be rate-limited, presumably by protein conformational changes.

The final phase observed in the reduction of nitrite reductase in the presence of cyanide therefore does not arise directly from cyanide binding, but arises from the reduction of the 'non-reducible' haem group facilitated by cyanide binding. This is consistent with the observation that the rate of this slowest phase is dependent on dithionite concentration but is independent of cyanide concentration, the binding of cyanide occurring earlier in the reaction and causing no significant spectral changes.

The reaction of cyanide with dithionite-reduced nitrite reductase was monophasic and the observed rate showed a hyperbolic dependence on cyanide concentration. If cyanide binding were to cause significant spectral changes directly, the overall reaction would be biphasic, since cyanide binding is evidently faster than the reduction of the 'non-reducible' haem group. The observation of a monophasic reaction is therefore consistent with cyanide binding causing no significant spectral changes other than those associated with the reduction of the 'non-reducible' haem group. The hyperbolic dependence of the rate on cyanide concentration is also consistent with this phase arising from reduction of the 'non-reducible' haem group. At low concentrations cyanide binds slowly to the enzyme, and this limits the rate of reduction of the 'non-reducible' haem group. At higher concentrations of cyanide other enzyme-dependent factors come into play to rate-limit the rate of electron transfer to the 'non-reducible' haem group.

Cyanide binding to the nitrite reductase thus has two effects: first, to alter the redox equilibrium between the initial electron-acceptor haem groups and the active site, to allow faster active-site reduction, and, secondly, to alter the interaction between the two active-site haem groups to allow the 'non-reducible' haem group to accept an electron. The effect of ferrous ligands on the rate of reduction of the active site of nitrite reductase has interesting implications for the enzyme in the reduction of its natural substrate, nitrite. It is now possible to see how fast turnover of substrate can be achieved with an apparently low rate of active-site reduction (Blackmore & Brittain, 1986). In the

absence of substrate the reduction of the active site will be slow because of the unfavourable redox equilibrium that exists even in the redox-cycled form (Blackmore *et al.*, 1990b). Substrate will cause the rapid reduction of the active site in a similar way to cyanide, by binding to the mixed-valence form, stabilizing intermediate 3 (Scheme 3) and, in effect, decreasing k_{-2} to a small value. It is tempting to speculate that the existence of the 'non-reducible' haem group in the nitrite reductase acts to link substrate binding and electron transfer, as it is clear from the above data that the redox state of the nitrite reductase enzyme is dependent on the presence of extrinsic haem ligands.

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