Oxidation of nitrogenase iron protein by dioxygen without inactivation could contribute to high respiration rates of *Azotobacter* species and facilitate nitrogen fixation in other aerobic environments

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The kinetics of oxidation of the Fe proteins of nitrogenases from *Klebsiella pneumoniae* (Kp2) and *Azotobacter chroococcum* (Ac2) by O_2 and H_2O_2 have been studied by stopped-flow spectrophotometry at 23 °C, pH 7.4. With excess O_2 , one-electron oxidation of Kp2 and Ac2 and their 2 MgATP or 2 MgADP bound forms occurs with rate constants (k) in the range $5.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $1.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. A linear correlation between $\log k$ and the mid-point potentials (E_m) of these protein species indicates that the higher rates of electron transfer from the Ac2 species are due to the differences in E_m of the 4Fe–4S cluster. The reaction of $\text{Ac2}(\text{MgADP})_2$ with O_2 is sufficiently rapid for it to contribute significantly to the high respiration rate of *Azotobacter* under N_2 -fixing conditions and may represent a new respiratory pathway. Excess O_2 rapidly inactivates $\text{Ac2}(\text{MgADP})_2$ and $\text{Kp2}(\text{MgADP})_2$; however, when these protein species are in greater than 4-fold molar excess over the concentration of O_2 , 4 equivalents of protein are oxidized with no loss of activity. The kinetics of this reaction suggest that H_2O_2 is an intermediate in the reduction of O_2 to $2 H_2O$ by nitrogenase Fe proteins and imply a role for catalase or peroxidase in the mechanism of protection of nitrogenase from O_2 -induced inactivation.

INTRODUCTION

The effects of oxygen on biological nitrogen fixation are complex (see reviews by Robson & Postgate, 1980; Bergersen, 1984; Yates, 1988; Hill, 1988). However, two intrinsic properties of nitrogenase, the enzyme that reduces N_2 to $2 NH_3$ with concomitant evolution of H_2 and hydrolysis of 16 MgATP to 16 MgADP + 16 P_i , dominate any discussion. Firstly, the two proteins comprising nitrogenase are rapidly inactivated by O_2 (see Mortensen & Thorneley, 1979; Lowe *et al.*, 1985; Thorneley & Lowe, 1985; Orme-Johnson, 1985; Smith *et al.*, 1987, for reviews of the structure and function of nitrogenase). Secondly, the high ATP requirement for nitrogenase activity is satisfied in aerobes (i.e. *Azotobacter*) largely by aerobic respiration (see review by Hill, 1988). These properties of nitrogenase together with data for the response of N_2 -fixing *Azotobacter chroococcum* cultures to changes in O_2 concentration lead to the concept of 'respiratory protection' (Dalton & Postgate, 1969*a,b*). This is that *Azotobacter* adjusts its respiration rate in response to O_2 in order to maintain a concentration of O_2 in the vicinity of nitrogenase that is compatible with N_2 -fixation. The branched respiratory chain of *Azotobacter* and the O_2 -regulated synthesis of some components of the chain form the basis of the mechanism of respiratory protection (Jones & Redfearn, 1966, 1967; Yates & Jones, 1974; Drozd & Postgate, 1970; Poole *et al.*, 1985; Yates, 1988). All previous studies have assumed that O_2 is removed by reaction at membrane-bound terminal oxidase sites and that any direct interaction between nitrogenase and O_2 in the cytoplasm leads to inactivation. Although previous studies have not been able to show that nitrogenase can be oxidized by O_2 without inactivation, such a reaction could initiate the

process termed 'conformational protection' in which the oxidized components of *Azotobacter* nitrogenase associate with a third Fe–S protein (Shethna protein) to form an oxygen-stable ternary protein complex (Postgate, 1971; Robson, 1979; Scherings *et al.*, 1983).

In the present paper we show that O_2 under certain conditions can react with the nitrogenase Fe protein without inactivating it and that this reaction is sufficiently rapid for it to contribute to the respiration rate of *Azotobacter in vivo* during N_2 -fixation. It therefore represents a potentially new respiratory pathway in *Azotobacter* species and other aerobic nitrogen-fixing organisms.

MATERIALS AND METHODS

The nitrogenase component proteins from *Klebsiella pneumoniae* (oxytoca) N.C.I.M.B. 12204 and *Azotobacter chroococcum* N.C.I.M.B. 8003 (non-gummy variant) were purified, characterized and assayed as previously described (Thorneley & Lowe, 1983; Yates & Planque, 1975). Kp1, Kp2, Ac1 and Ac2 had specific activities of 1800, 1150–1500, 1700 and 1450 nmol of ethylene produced $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ respectively. $Na_2S_2O_4$ was removed from solutions of reduced Kp2 and Ac2 proteins by anaerobic gel filtration using Bio-Gel P-6DG (Bio-Rad) (17 cm $\times$ 1 cm column) equilibrated with deoxygenated buffer (25 mM-Hepes/10 mM-MgCl $_2$, pH 7.4). Samples of protein (0.7 ml) were completely resolved from $Na_2S_2O_4$ (1 mM).

Solutions containing defined concentrations of O_2 were prepared by dilution of either air-saturated (250 μM at 23 °C) or O_2 -saturated (1.25 mM) solutions with deoxygenated buffer (N_2 -saturated) using syringes and avoid-

ing any gas space or bubbles (N_2) with which the dissolved O_2 might equilibrate.

Solutions of O_2 -oxidized proteins for e.p.r. spectroscopy or acetylene reduction activity determinations were prepared using two all-glass syringes (2 ml volume), one containing protein, the other O_2 dissolved in buffer. After filling, the syringes were joined together by a double female Luer connector. The syringe with the protein solution (0.75 ml) also contained a small glass bead (4 mm diameter) to ensure complete mixing of the solutions which was initiated by depressing the plunger of the syringe containing the O_2 solution (0.75 ml). Two or three rapid inversions of the syringes which moved the glass bead through the reacting solution ensured mixing was complete within 2–3 s. After various reaction times, samples (0.2 ml) were frozen for e.p.r. spectroscopy or syringed into 10 mM- $Na_2S_2O_4$ (0.8 ml) for acetylene reduction assays. A minimum reaction time after mixing of 10 s was obtained, most of this time being required to replace the Luer connector by a hypodermic needle prior to syringing aliquots into the quenching agent. E.p.r. spectra were obtained as previously described (Ashby & Thorneley, 1987). Strict anoxia was ensured by preparing all protein solutions and performing all reactions inside a glove box through which N_2 gas containing less than 1 p.p.m. of O_2 was continuously circulated (Thorneley & Lowe, 1983).

Stopped-flow spectrophotometry was performed at 23 °C with a Hi-Tech SF-51 instrument (Hi-Tech Scientific, Salisbury, Wilts., U.K.) installed with a Techne-C-400 thermostat [Techne (Cambridge), Duxford, Cambridge, U.K.] inside the glove box. Problems associated with the loss of dissolved O_2 by diffusion through the walls of the Teflon tubing connecting the drive syringes to the mixing chamber were overcome by using 0.3 ml of solution from each syringe. This volume ensures that the solution observed in the mixing chamber has only passed transiently through the Teflon tubing and between shots has resided in the drive syringes. Kinetic data acquisition, analysis and curve fitting were done as previously described (Ashby & Thorneley, 1987).

H_2O_2 solutions were standardized by titration with potassium permanganate. Large dilutions of H_2O_2 (30 % by volume) with deoxygenated buffer ensured that solutions were essentially free of dissolved O_2 at the concentrations of H_2O_2 used in these studies.

Bovine liver catalase (twice recrystallized) and all biochemicals were obtained from Sigma Chemical Co., and salts were from BDH Chemicals.

The mid-point potentials (E_m) of Ac2 and Ac2(Mg-ATP)₂ were determined spectrophotometrically following equilibration with diquat cation [E_m – 350 mV (NHE)] as previously described (Thorneley & Deistung, 1988).

RESULTS AND DISCUSSION

Kinetics of the oxidation of Ac2, Kp2 and their MgATP- or MgADP-bound forms by excess O_2

These reactions were studied by stopped-flow spectrophotometry, monitoring at 430 nm, as described in the Materials and methods section. All protein solutions were dithionite-ion-free with the concentrations given in the legends to Figs. 1, 2 and 3. The second syringe of the stopped-flow apparatus contained various concentrations of dissolved O_2 (125 μ M–1.25 mM) and when necessary MgATP (10 mM) or MgADP (10 mM). Pre-

Fig. 1. Oxidation of Kp2(MgADP)₂ by excess O_2 at 23 °C, pH 7.4

Dithionite-ion-free Kp2 (30 μ M) with ADP (5 mM) in buffer [25 mM-Hepes (pH 7.4)/10 mM- $MgCl_2$] was mixed in a stopped-flow spectrophotometer with air-saturated buffer (250 μ M- O_2 , 5 mM-ADP) and the absorbance changes at 430 nm monitored. The one-electron oxidation of the [4Fe-4S]⁺ centre in Kp2(MgADP)₂ is complete after approx. 2 s (trace a) and is followed by a slow decrease in absorbance (trace b). Parallel experiments in which reacting solution was quenched with $Na_2S_2O_4$ (10 mM) showed that > 75 % of the activity of Kp2 was lost after 10 s (first time point).

incubation of the proteins with either MgATP or MgADP did not affect the kinetics of oxidation, confirming that the binding of these nucleotides to Kp2 or Ac2 is rapid compared with the rate of oxidation (Thorneley & Cornish-Bowden, 1977). Fig. 1 shows typical traces, which in this example are for the reaction of Kp2(MgADP)₂ with excess O_2 . Fig. 1(a) shows a single exponential increase in absorbance ($k_{obs} = 2.6 s^{-1}$) with an amplitude corresponding to $\Delta\epsilon = 3.8 mM^{-1} \cdot cm^{-1}$. This amplitude is consistent with a one-electron oxidation of the [4Fe-4S] centre of Kp2, since Ashby & Thorneley (1987) obtained a value of $\Delta\epsilon_{430} = 4.0 \pm 0.5 mM^{-1} \cdot cm^{-1}$ when Kp2 was oxidized without loss of activity by indigocarmine dye or by Kp1 in the presence of MgATP. Fig. 1(b) shows the subsequent decrease in absorbance that occurs over a period of 1400 s. Although we have not analysed this slow phase in detail, inspection gives some insight into the nature of the inactivation process that occurs when Kp2(MgADP)₂ is oxidized with excess O_2 . After the initial rapid increase in absorbance, no

significant decrease has occurred by 10 s, yet by this time Kp2(MgADP)₂ has lost 75 % of its activity [shown by the quenching of reacting solutions of Kp2(MgADP)₂ + O₂ with Na₂S₄O₄ at times > 10 s followed by acetylene reduction assays for Kp2 activity as described in the Materials and methods section]. Thus inactivation does not involve a change of oxidation level of the [4Fe-4S]²⁺ cluster as judged by the absorbance change at 430 nm, i.e. we only see the change expected for conversion of [4Fe-4S]⁺ to [4Fe-4S]²⁺. It is therefore more likely that inactivation is due to a reaction of O₂, O₂⁻, HO₂⁻ or OH[•] with a group other than the 4Fe-4S cluster. When Kp2(MgADP)₂ was in a greater than 4-fold molar excess over O₂, no loss in activity was observed after oxidation (see below). This suggests that O₂⁻, generated in the oxidation reaction, is responsible for inactivation. The reduction of O₂⁻ to H₂O₂ by the excess Kp2(MgADP)₂ presumably prevents inactivation under conditions of excess protein. The involvement of O₂⁻ has previously been suspected since addition of superoxide dismutase decreased the extent of inactivation when the Fe-protein from *Clostridium pasteurianum* was oxidized by O₂ (Mortenson *et al.*, 1976). Fig. 1(b) also shows that after 1400 s the absorbance at 430 nm has returned to the initial value that is characteristic of a reduced [4Fe-4S]⁺ cluster although this protein has lost more than 90 % of its activity. However, the assignment of this oxidation level to the cluster is inconsistent (a) with the Mössbauer spectrum of O₂-inactivated Kp2 protein, which resembled that of an oxidized ferredoxin, and (b) with the e.p.r.-silent nature of this protein (Smith & Lang, 1974). We do not wish to speculate further on the nature of the 4Fe-4S cluster in these species.

Figs. 2 and 3 show the dependence of $k_{\text{obs.}}$ on [O₂]

Fig. 2. Kinetics of one-electron oxidation of Ac2, Ac2(MgATP)₂ and Ac2(MgADP)₂ by excess O₂ at 23 °C, pH 7.4

First-order rate constants ($k_{\text{obs.}}$) obtained by fitting traces such as that shown in Fig. 1(a) to a single exponential function are plotted as a function of O₂ concentration. Reaction conditions are as described for Fig. 1 with Ac2 protein alone, with added ATP or ADP (5 mM after mixing). The slopes of the lines give the second-order rate constants indicated (see also Table 1).

Fig. 3. Kinetics of one-electron oxidation of Kp2, Kp2(MgATP)₂ and Kp2(MgADP)₂ by excess O₂ at 23 °C, pH 7.4

Reaction conditions and procedures were as indicated in the legends to Figs. 1 and 2.

Table 1. Second-order rate constants for the oxidation of nitrogenase Fe proteins by excess O₂ and the mid-point potentials of the nucleotide-bound forms of the proteins at 23 °C, pH 7.4

Protein species	k (M ⁻¹ ·s ⁻¹)	E_m (mV, NHE)
Kp2	5.3×10^3	-200*
Kp2(MgATP) ₂	4.2×10^4	-320*
Kp2(MgADP) ₂	2.1×10^4	-350*
Ac2	7.8×10^3	-230
Ac2(MgATP) ₂	3.5×10^4	-350
Ac2(MgADP) ₂	1.6×10^5	-463†

* Thorneley & Deistung (1988).

† Bergström *et al.* (1988).

for the oxidation of Ac2 and Kp2 and their nucleotide-bound forms ($k_{\text{obs.}}$ was calculated from a single exponential best fit to curves such as that shown in Fig. 1a). For each species of Fe protein, the kinetics were first order in [O₂] except in the cases of Kp2(MgATP)₂ and Ac2(MgATP)₂ when some deviation from linearity occurred at the two highest concentrations of O₂. The second-order rate constants (k) were calculated from the slopes of the lines in Figs. 2 and 3 and are given in Table 1 together with the mid-point potentials (E_m) of the various forms of Fe protein. Fig. 4 shows the dependence of $\log k$ on E_m and the slope of this line gives a value of $\log k / \log K = 0.4$ (where K is the equilibrium constant for the reaction of O₂ with each form of Fe protein and is readily calculated from the E_m values in Table 1). The linear dependence of $\log k$ on E_m and the value of $\log k / \log K = 0.4$ suggest that the electron transfer from the [4Fe-4S]⁺ cluster to O₂ can be described by Marcus and related theories (see Marcus & Sutin, 1985, for a review). Fig. 4 also shows that the relative rates of reaction of O₂ with the various forms of Fe protein are determined by the free energy change of the reaction (i.e. E_m of the Fe protein species). Thus the lower E_m values for the Ac2

Fig. 4. Correlation between the rates of oxidation of Fe protein species by excess O_2 and their mid-point potentials at 23 °C, pH 7.4

The logarithm of the second-order rate constants (k) obtained from the data of Figs. 2 and 3 and given in Table 1 are plotted as a function of the mid-point potential of each protein species (E_m , NHE). The linear free energy relationship indicates that the relative rates of electron transfer to O_2 are determined by the free energy change on reaction.

species relative to the corresponding Kp2 species causes them to react faster with O_2 . The difference in E_m values for Kp2 and Ac2 species does not seem to be related to the role of these proteins as constituents of nitrogenase inasmuch as they will substitute for each other in combination with Kp1 and Ac1 to form fully active nitrogenases (Detroy *et al.*, 1968; Kelly, 1969). We therefore suggest that the lower potentials of the Ac2 species, in particular that of $Ac2(MgADP)_2$, are required to facilitate the reaction with O_2 *in vivo* and that these reactions contribute to the high respiration rates of *Azotobacter* species under N_2 -fixing conditions. We discuss this further below after considering the kinetics and stoichiometry of reaction of O_2 with an excess of $Kp2(MgADP)_2$ and $Ac2(MgADP)_2$ and introduce the concept of 'auto-protection' from oxygen-induced inactivation of nitrogenase in *Azotobacter*.

Reactions of O_2 and H_2O_2 with excess $Kp2(MgADP)_2$ and $Ac2(MgADP)_2$

Fig. 5 shows the absorbance changes occurring at 430 nm when $Ac2(MgADP)_2$ (80 μM) reacts with O_2 (12.5 μM). The reaction is biphasic with the first phase essentially complete after 1 s (Fig. 5b) and the second complete after 100 s (Fig. 5a). The total amplitude of the two phases ($\Delta A = 0.193$) corresponds to the oxidation of 48 μM - $Ac2(MgADP)_2$ (assuming $\Delta\epsilon_{430} = 4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Ashby & Thorneley, 1987). Since $[O_2] = 12.5 \mu M$, these data show that O_2 oxidizes four equivalents of $Ac2(MgADP)_2$ which suggests that the O_2 is being reduced to $2H_2O$. Assuming that the first phase corresponded to the reduction of O_2 to H_2O_2 with an amplitude of 0.097 (lower of the two dotted lines in Fig. 5b), this phase was fitted to a single exponential over 75% of the reaction with $k_{obs.} = 5.1 \text{ s}^{-1}$. This corresponded to a second-order rate constant of $6.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction of

Fig. 5. Oxidation of four equivalents of $Ac2(MgADP)_2$ by O_2 without loss of enzyme activity

Dithionite-free $Ac2(MgADP)_2$ (160 μM) was mixed in the stopped-flow apparatus with an equal volume of buffer containing O_2 (25 μM) and the oxidation of the protein monitored at 430 nm. Both syringes contained ADP (5 mM) and $MgCl_2$ (10 mM). The reaction is biphasic with the amplitude of each phase corresponding to the oxidation of approx. two equivalents of protein (see also Table 2). The $Ac2_{ox}(MgADP)_2$ product was fully active in Ac1-complemented acetylene reduction assays. Trace (a) shows the complete reaction and trace (b) the first phase in which H_2O_2 is thought to be formed with the oxidation of two equivalents of $Ac2(MgADP)_2$.

excess $Ac2(MgADP)_2$ with O_2 . This compares with the value of $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction of $Ac2(MgADP)_2$ with excess O_2 (Table 1). The factor of approx. 2 difference between these values may be associated with the heterogeneous nature of the Ac2 protein (about 40% active with respect to MgATP-dependent electron transfer to MoFe proteins; see Ashby & Thorneley, 1987, for a discussion of this problem) or to the kinetic complexity of a system involving sequential reactions possibly with free-radical intermediates. We have not attempted a detailed kinetic analysis at this stage but stress the biphasic nature of the reaction and the amplitudes corresponding to two 2-electron processes (Table 2) which are consistent with H_2O_2 being an intermediate. However in the case of $Kp2(MgADP)_2$ the corresponding second-order rate constants are in much closer agreement with $k = 2.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (with excess O_2) and $1.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (with excess protein).

Further evidence to support this conclusion is shown in Figs. 6 and 7. Replacement of O_2 (12.5 μM) by H_2O_2

Table 2. Stoichiometries for the reactions of excess Kp2(MgADP)₂ and Ac2(MgADP)₂ with O₂ and H₂O₂ at 23 °C, pH 7.4, and the specific activities of the oxidized protein

Specific activity (nmol of C₂H₄ produced · min⁻¹ · mg of Fe protein⁻¹) was determined by titration with Kpl or Acl under the standard assay conditions of Eady *et al.* (1972) at 30 °C. N.D., not determined.

Reaction	Products	Equivalents of protein oxidized	Specific activity	
			Before	After
Kp2(MgADP) ₂ (187 μM) + O ₂ (25 μM)	Kp2 _{ox.} (MgADP) ₂ (97 ± 5 μM) + 2H ₂ O	3.9 ± 0.2	1130 ± 50	1070 ± 40
Kp2(MgADP) ₂ (187 μM) + H ₂ O ₂ (25 μM)	Kp2 _{ox.} (MgADP) ₂ (42 ± 3 μM) + 2H ₂ O	1.7 ± 0.1	1130 ± 50	N.D.
Ac2(MgADP) ₂ (80 μM) + O ₂ (12.5 μM)	Ac2 _{ox.} (MgADP) ₂ (48 ± 2 μM) + 2H ₂ O	3.8 ± 0.2	1500 ± 60	1460 ± 50
Ac2(MgADP) ₂ (80 μM) + H ₂ O ₂ (12.5 μM)	Ac2 _{ox.} (MgADP) ₂ (19 ± 2 μM) + 2H ₂ O	1.6 ± 0.2	1500 ± 60	N.D.

Fig. 6. Oxidation of Ac2(MgADP)₂ by H₂O₂, 23 °C, pH 7.4

Dithionite-free Ac2(MgADP)₂ (160 μM) was mixed in the stopped-flow apparatus with an equal volume of buffer containing H₂O₂ (25 μM) and the oxidation of the protein was monitored at 430 nm. Both syringes contained ADP (5 mM) and MgCl₂ (10 mM). Trace (a) shows a monophasic absorbance change occurring at a similar rate to that of the second, slow phase for the reaction of Ac2(MgADP)₂ with O₂ (cf. Fig. 5, trace a). Trace (b) shows that no rapid phase is observed when H₂O₂ is the oxidant (cf. Fig. 5, trace b, when O₂ is the oxidant).

(12.5 μM) caused the rapid phase to disappear (Fig. 6b) with retention of only the slow phase (Fig. 6a). The amplitude of the slow phase (0.078) corresponded to the oxidation of 1.6 equivalents of Ac2(MgADP)₂ by H₂O₂ with a first-order rate constant of $k_{\text{obs.}} = 0.03 \text{ s}^{-1}$. This compares with $k_{\text{obs.}} = 0.05 \text{ s}^{-1}$ for the second phase of the reaction with oxygen. Considering the likely involvement of OH[•] as an intermediate and the attendant kinetic

Fig. 7. Effect of catalase on the oxidation of Ac2(MgADP)₂ by O₂ at 23 °C, pH 7.4

Dithionite-free Ac2(MgADP)₂ (140 μM) was mixed in the stopped-flow apparatus with an equal volume of buffer containing O₂ (25 μM) and the oxidation of the protein was monitored at 430 nm. Both syringes contained ADP (5 mM) and MgCl₂ (10 mM). Trace (b) shows a biphasic reaction similar to that shown in Fig. 5. Trace (a) shows that the presence of catalase (0.5 μM after mixing) essentially eliminates the slow phase by removing the H₂O₂ that is found as an intermediate in the reduction of O₂.

complexity we consider that these data provide further evidence for H₂O₂ being an intermediate in the reduction of O₂ to H₂O by Ac2(MgADP)₂ and Kp2(MgADP)₂. The data for both proteins are summarized in Table 2 together with the specific activities of the proteins after oxidation. Under these conditions of excess Fe protein over O₂ no inactivation occurred.

Fig. 7 shows the effect of catalase on the reaction of excess Ac2(MgADP)₂ with O₂. The amplitude of the slow phase is significantly decreased presumably because the H₂O₂ generated in the first phase is rapidly converted by the catalase to H₂O + ½O₂. The initial and final absorbances are increased by approx. 0.05 due to the absorbance of the catalase at 430 nm.

Two pieces of evidence show that the O₂-oxidized form of Kp2(MgADP)₂ and Ac2(MgADP)₂ which are fully active (Table 2) are the same as the oxidized forms generated by reaction with either indigocarmine dye or with MoFe protein in the presence of MgATP (see Ashby & Thorneley, 1987, for experimental details). Firstly, the e.p.r. feature at $g = 1.95$ characteristic of native nitrogenase Fe proteins was quantitatively

bleached after reaction with O_2 and more than 80% recovered when the O_2 -oxidized proteins were subsequently reduced by $Na_2S_2O_4$. Secondly the absorbance change at 430 nm and the rates of reduction of these oxidized proteins by SO_2^{2-} (formed by the predissociation of $S_2O_4^{2-}$) were identical to those of the dye and MoFe protein/MgATP-oxidized proteins ($\Delta\epsilon = 4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, $k = 3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for both $Ac2_{ox}(\text{MgADP})_2$ and $Kp2_{ox}(\text{MgADP})_2$; Ashby & Thorneley, 1987).

Possible physiological significance

Nitrogenase can constitute up to 40% of the cytoplasmic protein of bacteria fixing N_2 (Jouanneau *et al.*, 1985) although for *Azotobacter* species estimates of about 10% are more realistic (Dingler *et al.*, 1988). A concentration of $Ac2(\text{MgADP})_2$ of approx. 200 μM , reacting with O_2 at $1.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Table 1), would reduce O_2 at a rate approaching the reported Q_{O_2} of 2700 μl of O_2/h per mg dry wt. (Drozd & Postgate, 1970). This calculation assumes that $Ac2(\text{MgADP})_2$ comprises 10% of the total protein present. Clearly we are not suggesting that this reaction accounts *in vivo* for the entire Q_{O_2} but only that any model for respiration in *Azotobacter* under N_2 -fixing conditions should consider that oxygen that is not reduced by the membrane-bound terminal oxidase complexes will be reduced by nitrogenase Fe protein in the cytoplasm. The proportion of O_2 reduced by nitrogenase Fe protein *in vivo* is at this stage impossible to estimate since it depends on several factors including the absolute and relative concentrations of reduced Fe protein, flavodoxin hydroquinone and O_2 and the relative rates of electron transfer between these species. In addition the 'energy charge' will determine the proportion of Fe protein with MgATP or MgADP bound which in turn determines not only the E_m and electron transfer rates for these species to O_2 (Table 1) but also the electron flux to the nitrogenase MoFe protein which may also reduce O_2 (see below). For the present we wish to state that, provided the flux of O_2 into the cytoplasm is such that the concentration of $Ac2(\text{MgADP})_2$ is in at least approx. 4-fold excess of the O_2 , the nitrogenase Fe protein will not be inactivated. Although we have shown that $Ac2(\text{MgADP})_2$ will slowly reduce H_2O_2 to $2H_2O$ *in vitro*, it is more likely that *in vivo* catalase or peroxidase will rapidly remove H_2O_2 . Thus nitrogen fixation may be occurring in the cytoplasm of *Azotobacter* with the dissolved O_2 concentration kept low by the ability of the reduced Fe protein to transfer electrons not only to the MoFe protein for N_2 reduction but also to O_2 without itself being inactivated (Scheme 1). Such a mechanism would explain the approx. 3-fold increase in respiration rate observed by Drozd (1978), and previously attributed to an increase in the maintenance oxygen requirement, when N_2 -fixing cultures of *A. vinelandii* were switched from oxygen-limited to presumably iron-limited oxygen-sufficient conditions.

The ability of nitrogenase Fe proteins to reduce O_2 without being inactivated therefore provides the basis for a new mechanism for protecting nitrogenase from O_2 -induced inactivation which we suggest be called 'auto-protection' by analogy with the term 'respiratory protection'. It may be necessary to maintain a low O_2 concentration in the vicinity of functioning nitrogenase not only to prevent inactivation of Fe protein by excess O_2 but also to minimize the effects of O_2 at the N_2 -binding and reduction site on the MoFe protein. Whether

Scheme 1. Mechanism of 'auto-protection' of nitrogenase from O_2 -induced inactivation in which nitrogenase Fe protein not only donates electrons in an MgATP-dependent reaction to the nitrogenase MoFe protein for N_2 reduction but also reduces O_2 to H_2O_2 and $2H_2O$ probably with the involvement of catalase or peroxidases (not shown)

O_2 binds competitively with respect to N_2 and can be reduced to H_2O_2 or $2H_2O$ on the MoFe protein is not known. Wong & Burris (1972) showed that O_2 was an uncompetitive inhibitor of several substrates of nitrogenase, including N_2 , by using the photochemical electron-donating system of Benemann *et al.* (1969). However, since this system involved electron transfer from ascorbate to *Azotobacter vinelandii* nitrogenase via dichlorophenolindophenol, heat-treated spinach chloroplasts and flavodoxin, the site of interaction of O_2 remains uncertain, i.e. oxidation of any component of the electron transport chain would have yielded an uncompetitive inhibition pattern. It should now be possible, using a large excess of Fe protein in the absence of $Na_2S_2O_4$, together with MoFe protein and MgATP, to investigate the reduction of substrates at the MoFe protein in the presence of O_2 . The known rates of oxidation of Fe protein will enable conditions to be selected that should allow several turnovers of the MoFe protein in the presence of reducible substrates and O_2 .

Although *Klebsiella pneumoniae* is usually considered to be a facultative anaerobe when grown diazotrophically, Hill *et al.* (1984) showed that it can fix N_2 under microaerobic conditions. Thus the data for the reactions of Kp2 and its nucleotide-bound forms with O_2 have a physiological relevance in terms of the mechanism of regulation of nitrogenase activity by O_2 in this organism. Similarly, 'auto-protection' may be important in allowing nitrogen fixation to occur in new aerobic environments such as the plant mitochondrion, chloroplast or root plastid following transformation of the plant cell nucleus with bacterial *nif* genes.

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