ON THE NATURE OF ANAEROBIC OXIDATIVE DAMAGE TO THE MO–FE PROTEIN OF *KLEBSIELLA PNEUMONIAE* NITROGENASE

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1. Introduction

Nitrogenase [1], the enzyme responsible for dinitrogen reduction to NH_3 consists of two oxygen-sensitive metallo-proteins, the Mo–Fe protein and the Fe protein. Both proteins, MgATP, a reductant ($Na_2S_2O_4$ in vitro) and the exclusion of oxygen are required for activity. MgATP is hydrolysed to MgADP plus P_i during enzyme turnover. At its most efficient the enzyme hydrolyses 2 ATP molecules/electron transferred to substrate.

A generally accepted scheme for electron transfer by nitrogenase has been deduced from EPR and Mössbauer spectroscopy and stopped-flow spectrophotometric studies. In this scheme the MgATP-activated reduced Fe protein transfers electrons to the Mo–Fe protein which then reduces the substrate.

The most active preparations of Kp1 protein contained 2.01 Mo atoms and 32.5 ± 3 Fe atoms in a molecule of 218 000 M_r consisting of 2 × 60 000 and 2 × 50 000 M_r subunits [2]. The Mo atoms are in an extractable cofactor, the Fe–Mo-cofactor, which has Mo/Fe in the ratio 1:6–8 [3]. The exact arrangement of the other 14–20 Fe atoms into clusters is the subject of some controversy but there is evidence from cluster extrusion experiments [4] for up to four 4 Fe–4 S clusters/molecule.

The enzyme catalyses the reduction of a number of small, triple-bonded substrates in addition to N_2. The most important of these is acetylene which is reduced to ethylene. The enzyme also catalyses the formation of H_2 from water. This reaction is not completely suppressed by other substrates.

Studies [5] on the mutual inhibition patterns of a number of nitrogenase substrates and inhibitors have indicated the presence of up to 5 sites or modifications of sites for their binding and reduction. Other data have indicated that different substrates might be reduced by different forms [6,7], in particular by different redox states [8], of the enzyme.

There is circumstantial evidence indicating that the Fe–Mo-cofactor centres are involved in N_2 reduction [9]. But it has been shown that synthetic 4 Fe–4 S clusters can reduce C_2H_2 to C_2H_4 [10] and the enzyme hydrogenase which reduces H^+ to H_2 contains 4 Fe–4 S clusters [11,12] and thus such centres in the Mo–Fe protein might be involved in these substrate reductions.

In our work on the redox properties of the Mo–Fe protein [13–15], aimed at an understanding of the nature and function of its metal centres, we discovered that Kp1 protein is oxidatively damaged with regard to C_2H_2 reduction when incubated at potentials above +200 mV relative to the normal hydrogen electrode (NHE). It seemed probable that specific centres were being damaged and therefore that the substrate reduction activities of the enzyme might be differentially affected. Therefore we now report further investigation of anaerobic oxidative damage of Kp1 protein with respect to the reducible substrates N_2, C_2H_2 and H^+ and also in terms of ATP hydrolysis by the enzyme.

2. Materials and methods

The chemicals and biochemicals used were as described elsewhere [15]. *Klebsiella pneumoniae* M5al

Abbreviations: Kp1 protein, Mo–Fe protein from *Klebsiella pneumoniae;* Kp2 protein, Fe protein from *Klebsiella pneumoniae*

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was grown and the nitrogenase proteins purified as in [2,16].

2.1. *Redox measurements and activity assays*

The redox cell and the anaerobic potentiometric titration procedure were as in [13]. During the potentiometric titration the Kp1 protein and dye mediator mixture were allowed to equilibrate for 10 min at each potential before sampling anaerobically into assay vials containing $Na_2S_2O_4$ and other assay chemicals. Assays at 30°C were then started by addition of Kp2 protein and stopped after 10 min by addition of 0.1 ml trichloroacetic acid C_2H_2 reduction, N_2 reduction and H_2 evolution were assayed as in [16] P_i production from ATP hydrolysis was measured as in [17] in assay mixtures from which the ATP-regenerating system had been omitted.

3. Results and discussion

3.1. *The effect of increasing potential on Kp1 protein substrate-reduction activity*

Samples of Kp1 protein equilibrated at different potentials were transferred to assay mixtures and the specific activities for C_2H_2, N_2 and H^+ reduction measured as section 2. The assay mixtures contained sodium dithionite to reduce the Kp1 protein and stop oxidative damage and the assays were started by addition of excess Kp2 protein. The data obtained are shown in fig.1. Fig.1b shows the total electron flow (i.e., substrate reduction + hydrogen evolution) through the enzyme during reduction of each of these three substrates. All reductions showed identical behaviour with full retention of activity at incubation potentials up to +200 mV followed by a decrease in substrate reduction activity at potentials above +200 mV. Hydrogen evolution concomitant with substrate reduction showed the same behaviour (fig.1a).

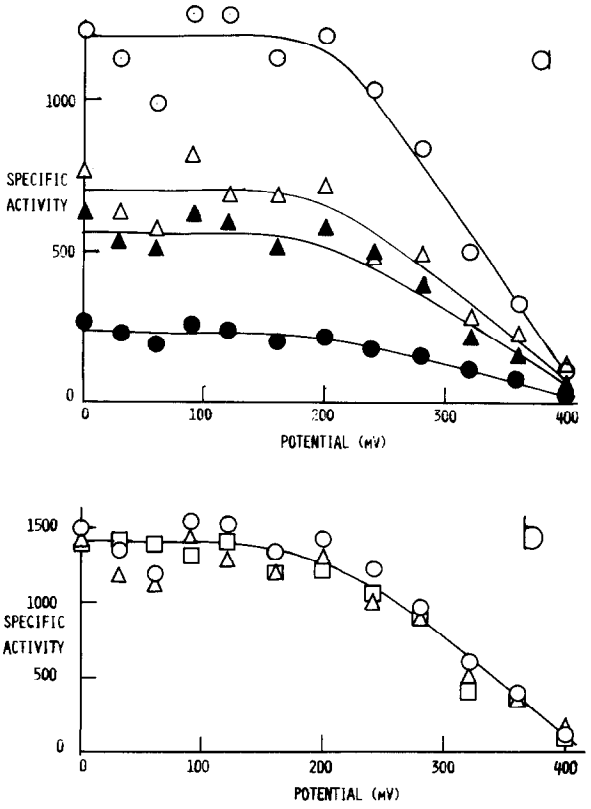

Fig.1. Effect of Kp1 protein preincubation potential on substrate reduction activity. The effect of preincubation potential in 50 mM Hepes: NaOH (pH 8) on Kp1 protein specific activity (nmol electron pairs to substrate . min^{-1} . mg^{-1}); (a) for (○) C_2H_2 reduction and (●) concomitant H_2 evolution and (△) N_2 reduction and (▲) concomitant H_2 evolution; (b) as measured by total electron flow to (○) $C_2H_2 + H^+$, (△) H^+ under Ar, (□) $N_2 + H^+$.

3.2. *ATP hydrolysis*

Fig.2 shows the P_i production during enzyme turnover as a function of the potential to which the Kp1 protein had been subjected. The number of electron pairs transferred to acetylene per ATP molecule hydrolysed is also shown (the ATP/2 e^- ratio). The pattern of P_i production is very similar to that of substrate reduction (cf. fig.1). With protein samples whose incubation potential was <+320 mV the ATP/2 e^- ratio apparently remained constant at 5.6 but with those whose incubation potential exceeded +320 mV the ATP/2 e^- ratio increased as the ability to reduce substrate was lost, i.e., ATP hydrolysis became uncoupled from substrate reduction. Under the prevailing assay conditions reductant-independent ATPase activity by nitrogenase is ~5% of the reductant-dependent ATPase activity with fully active proteins [18]. The observed increased ATP/2 e^- ratio can be explained in terms of the reductant-independent ATPase activity being affected less by oxidative damage than the reductant-dependent activity. Even the reductant-independent ATPase activity was affected eventually.

Reductant-dependent ATP hydrolysis by nitrogen-

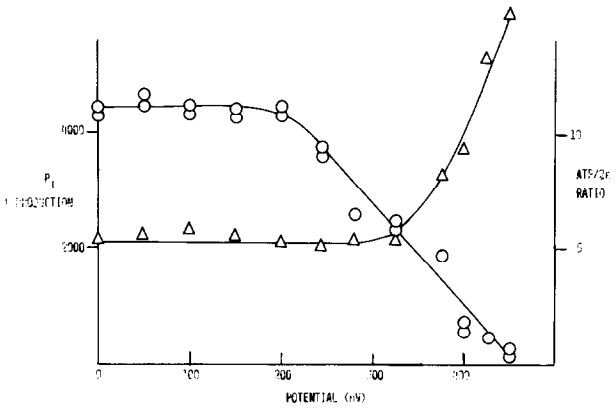

Fig.2. Effect of Kp1 protein preincubation potential on ATP hydrolysis during C_2H_2 reduction. Preincubations were in 50 mM Hepes:NaOH (pH 8). Assays were as in section 2: (○) denotes P_i production (nmol P_i . min^{-1} . mg^{-1}); (△) denotes the ATP/2 e^- ratio.

ase accompanies electron transfer from the Fe to the Mo–Fe protein [19] and is in our experiments affected identically with substrate reduction activity by oxidative damage. Reductant-independent ATP hydrolysis requires both proteins [18] and therefore presumably a complex between them and is less affected by oxidative damage than other activities. We therefore infer that the loss of substrate reduction activity is not primarily due to damage to the ability of the Mo–Fe protein to form a complex with the Fe protein.

If a particular site on the Mo–Fe protein on the electron transfer path from the Fe protein to the enzyme active site/Fe–Mo-cofactor centres were the site of oxidative damage, all substrate reductions would be affected equally as observed. Possible sites of such damage are:

(a) The site which accepts electrons from the Fe protein;
(b) A site between site (a) and the substrate-reducing site;
(c) The substrate-binding and -reducing site.

Which of these possibilities is operative can be deduced by considering another condition in which electron transfer is uncoupled from ATP hydrolysis. If the Mo–Fe/Fe protein ratio is raised considerably above 1:1 then the rate of ATP hydrolysis remains constant at the level observed at the 1:1 ratio but electron transfer to substrates is inhibited [20,21]. This has been rationalised [22] with the assumptions that only one electron is transferred at a time from the Fe to the Mo–Fe protein but that at least two electrons are required for substrate reduction. With a large excess of Mo–Fe over Fe protein the Mo–Fe protein will be largely singly reduced and the Fe protein largely oxidised. Under these conditions the singly reduced Mo–Fe protein can re-reduce the oxidised Fe protein which can then again reduce the Mo–Fe protein with the concomitant hydrolysis of ATP. Thus a futile electron transfer cycle accompanied by ATP hydrolysis can occur resulting in an enhanced ATP/2 e^- ratio for the reductant-dependant ATPase activity. Similar observations would be expected in any situation, e.g., possibilities (b) or (c) above in which Kp1 protein was able to accept electrons from Kp2 protein but unable to pass them on to substrate.

This situation clearly does not occur with oxidatively damaged Kp1 protein, indicating that the electron transfer from Kp2 protein cannot occur. Since our evidence on reductant-independant ATPase activity indicates that complex formation between Kp1 and Kp2 proteins still occurs we conclude that the oxidative damage to Kp1 protein is at that site which accepts electrons from Kp2 protein.

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