IMMUNOCHEMISTRY OF NITROGENASE AS A PROBE FOR THE ENZYME MECHANISM

Evidence for multiple enzyme forms and an $MgATP^{2-}$ binding site on the MoFe protein

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1. Introduction

Immunochemistry has been shown to be a powerful tool in the characterization of several enzyme systems including the elucidation of the structure of the catalytic site, the determination of the role of the prosthetic group and the study of allostery in enzymes [1].

The interaction of an enzyme with its specific, homologous antibody generally leads to a reduction in enzyme activity, although stimulation may occur. The extent of inhibition is frequently related to the size of the substrate [1] since steric hindrance is an important means of inhibition. Inhibition may also occur due to aggregation of enzyme–antibody clusters since this will also contribute to steric hindrance by interfering with access of the substrate to the catalytic site.

Nitrogenase, the enzyme catalyzing the reduction of dinitrogen to ammonia, consists of two, non-haem iron proteins the MoFe and Fe proteins (reviewed [2,3]). The enzyme will also reduce acetylene to ethylene and protons to hydrogen although this latter reaction is largely inhibited in the presence of other reducible substrates. All substrate reductions are accompanied by the hydrolysis of ATP to ADP and PO_4^{3-}. At its most efficient the enzyme hydrolyses about 4 ATP molecules for every electron pair transferred to substrates.

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Antibodies, K1 and K2, induced against the component proteins, Kp1 (MoFe protein) and Kp2 (Fe protein) of nitrogenase from *Klebsiella pneumoniae* M5al have been used successfully, by employing immunofluorescence, to detect nitrogenase in whole cells of dinitrogen-fixing organisms and their derivatives [4]. This paper reports initial observations on the effect of these antibodies on the major nitrogenase activities in vitro.

2. Methods and materials

2.1. *Nitrogenase*

The Kp1 and Kp2 proteins of nitrogenase purified [5,6] from *Klebsiella pneumoniae* M5al had molecular weights [5] of 218 000 for Kp1 and 66 700 for Kp2 and spec. act. 1200–1500 and 1000–1200 nmol C_2H_2 reduced/min/mg protein, respectively.

2.2. *Antibody induction and purification*

Kp1 and Kp2 proteins were used as antigens to induce specific antibody formation in New Zealand rabbits [4]. These antibody preparations, and one from a control rabbit which had not been injected with nitrogenase proteins, were purified by precipitation with 3.9 M $(NH_4)_2SO_4$ [4]. The preparations were equilibrated with 25 mM Tris–HCl, pH 7.4–1 mM $Na_2S_2O_4$–0.1 mg/ml dithiothreitol (DTT), by dialysis or by passage through a Sephadex G-25 column, and frozen as small beads in liquid nitrogen until required. Antibody molecular weights were taken to be 155 000 since they consisted mostly of IgG [7].

2.3. *Assays*

The nitrogenase proteins were assayed under optimum conditions, i.e., Kp2 protein was assayed with equimolar Kp1 protein and Kp1 protein with a 20-fold molar excess of Kp2 protein [5]. Assays for the reduction of acetylene, protons and dinitrogen at 30°C, pH 7.8, were performed according to [5]. In some activity assays a creatine phosphate/creatine kinase ATP-regenerating system was used [5]. When this was omitted the ATP concentration was increased from 5–10 mM with 11 mM Mg^{2+}. Phosphate was assayed by the method in [8] as modified [9]. Protein concentrations were measured by the Folin-Ciocalteau method [10]. Specific techniques involving interactions with antibodies are described in section 3.

3. Results and discussion

Preliminary work established the protein:antibody ratio and interaction time required to observe substantial inhibition of the acetylene-reducing activity of nitrogenase after prior incubation of either Kp1 or Kp2 protein with its respective antibody. These conditions were then used to investigate whether there were any differential effects on the various nitrogenase activities. The degree of inhibition obtained with the same antibody in different incubations varied by about 10%. With 90% inhibition this could result in a 2-fold variation in the residual activity of the inhibited protein, thus it was necessary to make comparisons with antibody/protein mixtures from the same incubation.

3.1. *The Kp2 protein–K2 antibody interaction*

A 20-fold molar excess of a K2 antibody preparation was added to Kp2 protein and the resultant solution incubated anaerobically under argon for 15 min. Aliquots were then removed for assay with Kp1 protein for acetylene, proton or dinitrogen reduction activities in reaction mixtures without an ATP-regenerating system. The assays were stopped by addition of 0.1 ml trichloroacetic acid (30%) and kept on ice until the gas phase had been assayed for hydrogen and ethylene (if required). The liquid phase was then centrifuged to remove precipitated protein, 0.2 ml supernatant removed for phosphate estimation and, if required, 1.0 ml for ammonia estimation. The results were compared with those obtained from assays on comparable incubations in which the antibody preparation was replaced by an equal volume of 25 mM Tris, pH 7.4–1 mM $Na_2S_2O_4$ –0.1 mg/ml DTT. In the assays containing antibody acetylene, proton and dinitrogen reduction and ATP hydrolysis in the presence of these substrates were all inhibited, by one preparation of K2 antibody, to 25–30% of the control activities. In similar experiments with another antibody preparation, but using an ATP-regenerating system in the assays, the substrate reductions were again all inhibited equally.

An IgG preparation from a rabbit which had not been injected with nitrogenase proteins was not inhibitory under similar conditions.

Unequivocal interpretation of these data is not yet possible but among possible reasons for inhibition by K2 antibody are perturbation of complex formation between Kp1 and Kp2 proteins, or inhibition of $MgATP^{2-}$ binding to Kp2 protein.

3.2. *The K1 antibody–Kp1 protein interaction*

Much more interesting results were obtained with this interaction. K1 antibody in 20-fold molar excess was added to Kp1 protein and the resultant solution incubated anaerobically under argon for 1 h. The Kp1–K1 solution was then assayed with an excess of Kp2 protein for nitrogenase activity and the results compared with those obtained with Kp1 from control incubations containing buffer or a non-specific IgG preparation in place of K1 antibody. The non-specific IgG preparation was not inhibitory.

Table 1 shows the results of duplicate 30 min assays in reaction mixtures containing an ATP-regenerating system. Twice as much of each incubation was used in the dinitrogen-reduction assays as with the other substrates since the ammonia estimation technique was relatively less sensitive. The data show that K1 antibody inhibited the reduction of each substrate equally. Proton reduction in the presence of acetylene or dinitrogen was inhibited to the same extent as the other reductions.

The amount of phosphate produced by hydrolysis of ATP was determined in similar experiments but without an ATP-regenerating system in the assays. These assays were carried out for 15 min only to avoid excessive build up of the inhibitor ADP, but

Table 1
Inhibition by K1 antibody of Kp1 protein substrate reduction activities

Conditions	Products (nmol) H_2 under argon	$C_2H_4 + H_2$ under C_2H_2	= Total e^- pairs under C_2H_2	$1.5\ NH_3 + H_2$ under N_2	= Total e^- pairs under N_2
Uninhibited	148	152 + 11	= 163	157 + 135	= 292
	138	149 + 9.5	= 158.5	151 + 130	= 281
Inhibited	15	14.6 + 1.3	= 15.9	10.6 + 14.8	= 24.4
	16	13.8 + 1.3	= 15.1	14.2 + 16.2	= 30.4
% Activity remaining (average)	10.8	9.6		9.6	

the substrate-reducing activities were nevertheless only about 90% of those obtained with an ATP-regenerating system. The control assays showed the expected ATP:$2e^-$ ratio of about 4. The substrate reductions were all inhibited by K1 antibody to similar extents: about 16% activity remained. Table 2 shows that ATP hydrolysis under acetylene was inhibited by the same amount as substrate reduction but with the proton and dinitrogen reductions more ATP was hydrolysed than would be expected for the amount of substrate reduced. The result is most obvious with dinitrogen as a substrate, where approximately twice as much ATP as expected was hydrolysed. This result was reproducible on three occasions. The results with protons as the only reducible substrate showed greater variability.

The above data show that results obtained with an artificial, heterologous nitrogenase consisting of Cp2 protein and Kp1 protein, are a direct reflection of the substrate reduction mechanism by the homologous *K. pneumoniae* enzyme. Experiments with the heterologous nitrogenase showed that protons, acetylene and dinitrogen were all reduced by different forms of the enzyme [11,12]. Similar results for proton and acetylene reduction have been observed with the homologous *K. pneumoniae* enzyme [13]. The results reported here show that acetylene and dinitrogen are reduced by different forms of the homologous enzyme. Since binding K1 antibody to Kp1 protein inhibited ATP hydrolysis less when dinitrogen was being reduced than when acetylene was the reducible substrate, it follows, that the two substrates were reduced by different conformations of the enzyme. In the acetylene-reducing conformation the K1 antibody did not disrupt the normal relationship between the ATP hydrolysis and electron

Table 2
Inhibition by K1 antibody of Kp1 protein ATP hydrolysis activity

Reducible substrate	PO_4^{3-} (nmol) produced by hydrolysis of ATP		% Activity remaining (average ± range)
	Uninhibited	Inhibited	
$C_2H_2 + H^+$	276	45	15.2 ± 1.1
		39	
H^+ under argon	281	53	21.9 ± 3.4
	283	69	
$N_2 + H^+$	559	167	28.65 ± 1.03
	587	161	

transfer to substrates whereas in the dinitrogen-reducing conformation ATP hydrolysis became partially uncoupled from electron transfer.

Our results also have a bearing on the possible involvement of $MgATP^{2-}$ in substrate reduction. $MgATP^{2-}$ is known to be involved in electron transfer through interaction with Fe protein (reviewed [2,3]) but indirect evidence for a role for ATP in substrate reduction, arises from 4 lines of investigation:

1. Heterologous crosses of Cp2 and Kp1 gave very high ATP:$2e^-$ ratios [11] although the ATP dependence of protein–protein electron transfer was the same as with the homologous enzyme.

2. Azotobacter nitrogenase showed preferential channelling of electrons into H_2 evolution [17] or acetylene reduction [18] rather than dinitrogen reduction at low ATP concentrations.

3. The kinetics of $MgADP^-$ inhibition of $MgATP^{2-}$-activated electron transfer within nitrogenase were different from those of proton reduction [19].

4. The ATP analogues ATP-γS and AMPPNP preferentially channelled electrons into proton rather than acetylene reduction [20].

Thus there may be a second $MgATP^{2-}$ binding and hydrolysing site on the enzyme which is only generated during turnover. Our results provide an additional correlation between substrate reduction and $MgATP^{2-}$ hydrolysis and suggest that since $MgATP^{2-}$ hydrolysis can be affected by the binding of the antibody molecule to the MoFe protein then the proposed additional site for $MgATP^{2-}$ binding may be on the MoFe protein. If this site is only generated during turnover it would explain why $MgATP^{2-}$ has not been observed to bind to the isolated MoFe protein [2,3].

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