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INTERACTION OF NITROGENASE FROM *KLEBSIELLA PNEUMONIAE* WITH ATP OR CYANIDE

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SUMMARY

Nitrogenase Mo–Fe protein or Fe protein from *Klebsiella pneumoniae*, horse heart cytochrome *c* or ferredoxin, but not haemoglobin or bovine serum albumin bound [^{14}C]ATP on a Sephadex column equilibrated with [^{14}C]ATP. Fe protein exposed to O_2 did not catalyse acetylene reduction but was still able to bind ATP and also inhibited acetylene reduction by nitrogenase under ATP-limited conditions: the K_m of nitrogenase for ATP was 0.12 mM.

The role of ATP in N_2 fixation remains obscure since both nitrogenase proteins bound ATP.

Mo–Fe protein, Fe protein or bovine serum albumin bound small amounts of [^{14}C]cyanide on a [^{14}C]cyanide-equilibrated Sephadex column and in equilibrium dialysis experiments; CO or acetylene did not affect this binding. Haemoglobin bound greater amounts of [^{14}C]cyanide and CO partially inhibited this binding. The K_m of nitrogenase for cyanide was 0.19 mM. The rate of reduction of cyanide was increased by acetylene whereas acetylene reduction was inhibited in the presence of cyanide.

These results indicate that cyanide can be a misleading model for understanding N_2 reduction and that the binding of [^{14}C]cyanide by nitrogenase protein was a non-specific process probably unconnected with N_2 fixation.

INTRODUCTION

Nitrogenase from a number of bacteria has been separated into two protein components[1–4]. One (Mo–Fe protein) contains iron and molybdenum, the other (Fe protein) contains only iron and both are inactivated by O_2 (ref. 2) though Mo–Fe protein of *Klebsiella pneumoniae*[5] or *Azotobacter chroococcum*[4] is less rapidly inactivated than its corresponding Fe protein. Besides N_2 reduction, nitrogenase catalyses ATP-dependent H_2 evolution[6] or reduction of a variety of compounds including cyanide (ref. 7) and acetylene[8,9]. All these catalytic activities have the same requirements as N_2 reduction, *i.e.* the two proteins, $Na_2S_2O_4$ and an ATP supply (usually as ATP *plus* creatine phosphate *plus* creatine kinase) (ATP: creatine phosphotransferase, EC 2.7.3.2). Considerable use of these substrates has been made for assay of nitrogenase, in particular acetylene[10–12] because the gaseous product is readily detected in small amounts. Though there is some evidence[9,13] that these substrates

bind at the same site as N_2 this has not been proved beyond doubt. HWANG AND BURRIS[14] found that CO, which competitively inhibits N_2 reduction[15], inhibited reduction of some substrates including cyanide or methyl isocyanide[16] in a non-competitive way.

Despite much speculation[17–19], the mechanism of N_2 reduction is not known, nor is the order of reaction of N_2, ATP, $Na_2S_2O_4$ and the two proteins certain. BUI AND MORTENSON[20] described experiments using nitrogenase from *Clostridium pasteurianum* in which each protein was passed down a Sephadex G-50 column equilibrated with either ^{14}C-labelled cyanide or ATP. Radioactivity greater than background level associated with the protein peak was taken to indicate specific binding by that protein. Only Mo–Fe protein gave a radioactivity peak with [^{14}C]cyanide whereas only Fe protein gave a peak with [^{14}C]ATP. They concluded that Mo–Fe protein bound cyanide (and therefore by inference other substrates including N_2) specifically, whereas Fe protein bound ATP specifically.

Since the suggestions of BUI AND MORTENSON[20] are of importance in explaining the mechanism of N_2 reduction and also because their procedure might be developed to give further information, *e.g.* on whether O_2-damaged Fe protein is still able to bind ATP, we have used their procedure with Mo–Fe protein and Fe protein from *K. pneumoniae* and with various other proteins and have endeavoured to extend their observations using equilibrium dialysis. Our results are presented in this paper.

METHODS AND MATERIALS

Nitrogenase of K. pneumoniae

For details of the procedures used for preparation, storage and assay of Mo–Fe protein and Fe protein see ref. 5. Mo–Fe protein and Fe protein were estimated to be at least 90 and 95 % pure, respectively, by disc electrophoresis in polyacrylamide gels[21].

Chromatography of proteins on Sephadex

This procedure was carried out using similar conditions to those described by BUI AND MORTENSON[20]. Columns (2 cm × 19 cm) containing 6 g of coarse Sephadex G-50 (Pharmica, Uppsala, Sweden) were run anaerobically at 15°. After equilibrating the column by passing approximately 2 l of N_2-sparged 25 mM Tris–HCl buffer (pH 7.4), the same buffer containing ^{14}C-labelled ATP or cyanide was introduced onto the top of the column with fine tubing attached to a 50-ml syringe. In experiments with ATP the buffer contained $MgCl_2$ at 4 times the final concentration of ATP. The ATP or cyanide solution was prepared by adding the appropriate amount of concentrated labelled material to N_2-sparged buffer in a flask; unlabelled ATP or cyanide was also added at this time if required. With cyanide the flask was then closed and samples withdrawn through a tap without further bubbling with N_2 to avoid the considerable loss of HCN to the gas phase which otherwise occurred at pH 7.4. At least 4 bed volumes of buffer containing ^{14}C-labelled compound were passed through the column and collected before introducing a 1-ml sample containing 20 mg protein also in 25 mM buffer but containing no radioactivity. The sample was allowed to just enter the column before syringing on further buffer containing [^{14}C]ATP or cyanide. Samples were normally collected aerobically but anaerobically

if required for enzyme assay, in 1-ml volumes during protein elution, otherwise in 5- or 10-ml volumes. In experiments with [^{14}C]cyanide, 5 M NaOH was added during collection to give a final concentration of 0.5 M and sample bottles were then stoppered. These precautions were necessary to minimise loss of cyanide as HCN before counting.

Equilibrium dialysis

Samples of protein were placed anaerobically in 8/32-inch dialysis tubing and then immersed in 25 mM Tris–HCl buffer kept sparged with either Ar, CO or acetylene. The flask was tightly stoppered with a suba-seal rubber closure and [^{14}C]-ATP or cyanide injected. The system was then shaken at 12° in a cooled water bath for up to 48 h before samples were removed. In experiments with cyanide a sample of the dialysing liquid was removed through the seal with syringe and needle before the contents of each dialysis sac were drawn into syringes and injected into bottles containing 5 M NaOH to give a final concentration of 0.5 M. In some cases a separate sample, untreated with NaOH, was retained for enzyme assay. No NaOH was added to samples of [^{14}C]ATP.

Counting of radioactivity

An I.D.L. instrument (Nucleonic Accessories Ltd., Knowle Road, Mirfield, York, England) was used. 0.2-ml samples were counted for 10 sec in the scintillation fluid described by BUI AND MORTENSON[20]. In addition, 0.1-ml amounts were dried onto stainless steel dimple planchettes (Nuclear Enterprise, Bath Road, Beenham, Nr. Reading, England) and counted for 10 or 60 sec using an end window counter. Some variation in the effects of different proteins on the scintillation count was observed. Blanks were carried out with each protein added to the standard amount of [^{14}C]cyanide to correct for this. Though less sensitive, planchette counting was prefered since comparison of the two methods showed that the scintillation procedure required more blanks. All the peaks observed for [^{14}C]cyanide with planchette counting were also confirmed by scintillation counting. Samples from [^{14}C]ATP-equilibrated columns gave greater than 800 counts/min using the planchette method. A self-absorption correction was found unnecessary. Samples from [^{14}C]cyanide-equilibrated columns gave greater than 1000 counts/min with the planchette method and greater than 8000 counts/min with the scintillation technique.

Radiochemicals

Sodium [^{14}C]cyanide and [8-^{14}C]ATP were obtained from the Radiochemical Centre, Amersham, England, and contained 52.8 mC/mmole or 38 mC/mmole, respectively. Concentrated solutions, prepared by addition of a small volume of buffer, were stored at −20° and diluted as required.

Chemicals

Biochemicals used in this work were obtained from Sigma (London) Chemical Co., London, S.W.6. The source of other chemicals and details of various procedures used in this work have been published previously[5].

RESULTS

Co-chromatography of ATP with Mo–Fe protein or Fe protein

BUI AND MORTENSON[20] observed that Fe protein of *C. pasteurianum*, but not Mo–Fe protein, bound ATP. The results of repeating their experiments using Fe protein or Mo–Fe protein from *K. pneumoniae* are shown in Figs. 1a and 1b. Both proteins were eluted in the void volume. A radioactivity peak was observed about 22% above the background level corresponding to the Fe protein peak; this Fe protein was still active when assayed for acetylene reduction with Mo–Fe protein[5].

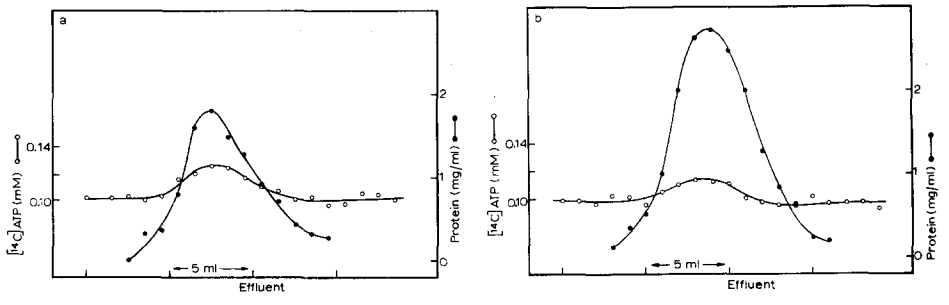

Fig. 1. a. Co-chromatography of Fe protein with 0.1 mM ATP. For details of the Sephadex column technique see METHODS AND MATERIALS. Buffer (25 mM Tris–HCl (pH 7.4)) contained 0.1 mM ATP (in which [^{14}C]ATP was 1.25 μM) and 0.4 mM $MgCl_2$. The radioactivity scale for Figs. 1a, 1b and 2a–2d is the same; the ATP concentration scale is changed. b. Co-chromatography of Mo–Fe protein with 0.1 mM ATP. Conditions as for a.

Since the final protein concentration in this experiment was only about half of that obtained by BUI AND MORTENSON[20], who observed a peak about 45% above background, the percent increase in activity per mg protein was about the same for Fe proteins from the two nitrogenases. However, as Fig. 1b shows, Mo–Fe protein of *K. pneumoniae* gave a very similar peak which suggested that the binding might be a non-specific property of both proteins. Before considering the effect of other proteins and of ATP concentration on the ATP–protein chromatography, the K_m of nitrogenase for ATP was determined for comparison with the published figure of 0.3 mM obtained using nitrogenase from *Azotobacter vinelandii*[22].

Apparent K_m of nitrogenase for ATP

Reciprocals of the rates of acetylene reduction by *K. pneumoniae* nitrogenase using an ATP-generating system of creatine phosphate *plus* creatine kinase and ATP varied from 0.033 to 6.6 mM were plotted to determine the apparent K_m for ATP. A straight line was obtained from which a K_m value of about 0.12 mM was calculated[23]. BUI AND MORTENSON[20] quoted a figure of 2.0 mM for optimum rate of reduction.

Co-chromatography of ATP at low concentration with various proteins

Diluting the ^{14}C-labelled ATP with unlabelled material, produced a final concentration of ATP (0.1 mM) comparable to the K_m but reduced the sensitivity of the chromatographic procedure. Chromatography was carried out using conditions as previously described but with the ATP concentration only 1.25 μM. No unlabelled

ATP was added to the [^{14}C]ATP, the $MgCl_2$ concentration was lowered to maintain the Mg^{2+} to ATP ratio of 4:1. Figs. 2a and 2b show that both Fe protein and Mo–Fe protein gave a considerably increased radioactivity peak associated with protein, about 150–170 % above the background compared to about 22 % observed with the high level of ATP (Figs. 1a and 1b). No increase in activity associated with bovine serum albumin was detected (Fig. 2c). Haemoglobin chromatographed under these conditions also gave a negative result but horse heart cytochrome *c* or ferredoxin from *C. pasteurianum* (prepared according to the procedure of MORTENSON[24]) gave a small peak associated with protein. Because of their relatively low molecular weight these proteins spread considerably on Sephadex G-50 and were retarded whereas bovine serum albumin and haemoglobin passed through in the void volume. The increase in activity per mg protein was 40 % for cytochrome and 50 % for ferredoxin compared with 75 % for Fe protein.

Fig. 2a also shows the volume of [^{14}C]ATP buffer needed to equilibrate the column completely. The rate of change of radioactivity in the effluent was low as equilibrium approached but 120 ml were needed before the radioactivity of the effluent equalled that applied to the top of the column. The background level of ATP subtracted from that eluted with the protein peak gave an amount of [^{14}C]ATP associated with Fe protein (Fig. 2a) equivalent to 12 ml of equilibrating buffer. Obviously the column was no longer at equilibrium and the counts immediately after protein elution were slightly less than the plateau level.

Exposure to O_2 with gentle stirring for 10 min made Fe protein completely inactive in assays with Mo–Fe protein for acetylene reduction. However, such O_2-damaged material gave essentially the same protein to [^{14}C]ATP ratio in Sephadex chromatography (Fig. 2d) as undamaged Fe protein. In a NADH oxidase system from *Escherichia coli*, O_2 inactivation is reported to have no effect on affinity of the enzyme for NADH[25].

Effect of O_2-damaged Fe protein on acetylene reduction

Though O_2-damaged Fe protein did not catalyse acetylene reduction it gave a protein to ATP peak in chromatography experiments (Fig. 2d) like undamaged Fe protein (Fig. 2a). If this peak represented binding of ATP to the protein, O_2-damaged Fe protein should partially inhibit acetylene reduction in assays with limiting ATP by binding some of the ATP.

Acetylene reduction with 800 μg Mo–Fe protein *plus* 400 μg Fe protein to which 1600 μg O_2-damaged Fe protein was added was compared with controls containing no O_2-damaged Fe protein with various amounts of ATP in a final assay volume of 1.5 ml. There was no inhibition with 5 μmoles ATP but about 20 % inhibition at 0.10 μmole (67 μM). During determination of the K_m for ATP we observed that the rate of acetylene reduction was directly proportional to ATP below 0.15 μmole, so we can calculate that the activity observed above with O_2-damaged Fe protein with 0.10 μmole ATP corresponded to an apparent ATP level of 0.08 μmole, *i.e.* 1600 μg of O_2-damaged Fe protein bound about 0.02 μmole of ATP. This compares with a figure of about 0.001 μmole/1600 μg calculated from column experiments where the ATP concentration was only 1.25 μM (Fig. 2a) and 0.02 μmole/1600 μg protein when the ATP concentration was 100 μM (Fig. 1a).

Using the figure of 0.02 μmole ATP per 1600 μg Fe protein and assuming a

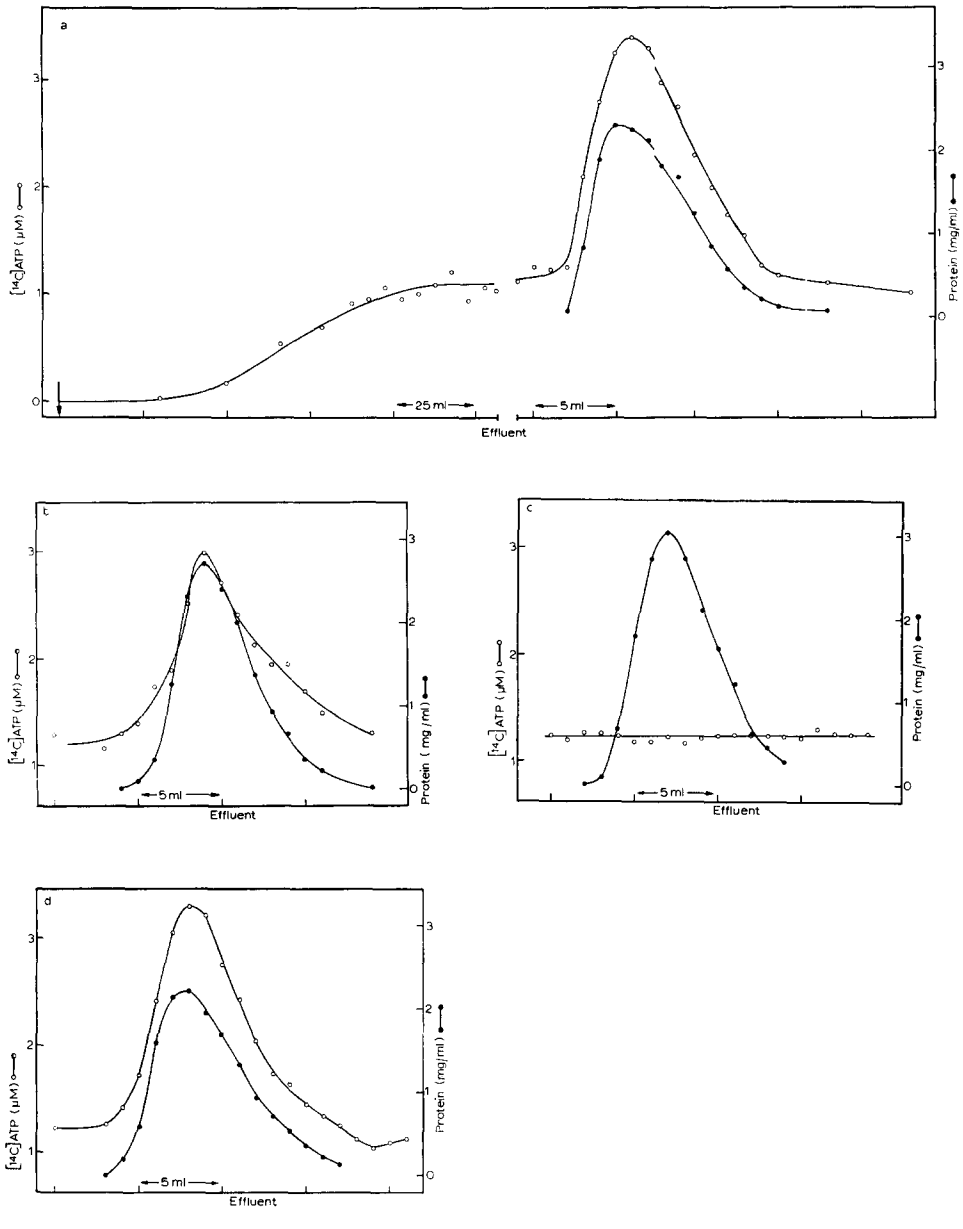

Fig. 2. a. Co-chromatography of Fe protein with 1.25 μM ATP. For details of the Sephadex column technique see METHODS AND MATERIALS. Buffer (25 mM Tris–HCl (pH 7.4)) contained 1.25 μM [^{14}C]ATP and 5 μM $MgCl_2$. The amount of ATP-containing buffer necessary to achieve a plateau is indicated; application of this buffer was begun at the arrow. Note the change in scale on the effluent axis. The radioactivity scale for Figs. 1a, 1b and 2a–2d is the same; the ATP concentration scale is changed. b. Co-chromatography of Mo–Fe protein with 1.25 μM ATP. Conditions as for a. c. Co-chromatography of bovine serum albumin with 1.25 μM ATP. Conditions as for a. d. Co-chromatography of O_2-damaged Fe protein with 1.25 μM ATP. Conditions as for a. Fe protein was exposed to O_2 with gentle stirring for 10 min, rendering it completely inactive in assays with Mo–Fe protein for acetylene reduction.

molecular weight of 50000, the number of ATP molecules per molecule of Fe protein was about 0.6. The K_m for ATP was 120 μM, therefore the maximum amount of ATP Fe protein can bind probably approaches 2. O_2-damaged Fe protein would also inhibit acetylene reduction if it bound ADP since this would not then be available for regeneration to ATP by the creatine phosphate–creatine kinase system.

Equilibrium dialysis with [^{14}C]ATP

Attempts to show binding of [^{14}C]ATP to Mo–Fe protein or Fe protein by equilibrium dialysis were unsuccessful. The amount of radioactivity associated with each protein inside the dialysis tubing was about the same or slightly less than that detected in the outside buffer after 12, 24 or 48 h dialysis. To ensure that the equilibrium was reached as rapidly as possible, an experiment was carried out in which an equal volume of protein and ATP in buffer were mixed before transferring to tubing for dialysis against ATP diluted with buffer to make the ATP concentration inside and outside the dialysis tube the same at the beginning of the experiment. Radioactivity associated with Mo–Fe protein and Fe–protein did not increase after 14, 24 or 48 h. When Mo–Fe protein and Fe protein were dialysed together the level of associated radioactivity was only about half the background level after 24 h, presumably because ATP was hydrolysed to ADP which then diffused out of the dialysis tube. Mo–Fe protein and Fe protein separately also had slight ATPase activity tending to reduce the level of [^{14}C]ATP inside the tubing so that no significant conclusions could be made from these results.

Co-chromatography of [^{14}C]cyanide with Mo–Fe protein

Cyanide at the concentration used by BUI AND MORTENSON[20] was equilibrated with a Sephadex column at pH 7.4. An increase in radioactivity of about 25% was detected associated with Mo–Fe protein (Table I). Cyanide-equilibrated columns were run by BUI AND MORTENSON[20] at pH 8.4 but in this work pH 7.4, which was

TABLE I

CO-CHROMATOGRAPHY OF [^{14}-C]CYANIDE WITH VARIOUS PROTEINS

Proteins were chromatographed on Sephadex G-50 columns as described in METHODS AND MATERIALS, with 25 mM Tris–HCl buffer (pH 7.4) containing cyanide at the concentration indicated.

Cyanide concn. (μM)	*Protein*	*Peak increase in radioactivity above background (%)*	*Peak protein concn. (mg/ml)*	*% Increase in radioactivity per mg protein*
*[^{14}C]Cyanide diluted with unlabelled cyanide**				
100	Mo–Fe protein	25	2.67	9.4
*Undiluted [^{14}C]cyanide***				
4.7	Mo–Fe protein	42	3.64	11.5
4.5	Fe protein	17	2.12	8.0
2.9	bovine serum albumin	51	3.33	15.3
4.7	haemoglobin	338	2.14	158.0
*Undiluted [^{14}C]cyanide**; acetylene-saturated column****				
4.7	Mo–Fe protein	41	3.46	11.9

* 100 μM cyanide contained 21.1 μM Na^{14}CN, remainder of cyanide unlabelled.
** All cyanide was Na^{14}CN.
*** Column run with acetylene-saturated buffer and under acetylene gas.

optimum for cyanide reduction, was prefered. The rate of cyanide reduction at pH 8.4 was only about 20 % of the maximum.

Determination of K_m for cyanide reduction

The published figure for the K_m for cyanide reduction to methane (4 mM) was obtained with nitrogenase from *A. vinelandii*[7]; for comparison a value was determined for nitrogenase of *K. pneumoniae*. Assays were carried out as described previously[26]; cyanide, prepared immediately before use, was injected into the assay bottles after these had been flushed with Ar and sealed. A reciprocal plot of the cyanide concentration against the reciprocal of the methane formed is shown in Fig. 3 and a value for the K_m of 0.2 mM was calculated. During these assays very small amounts of acetylene were detected. This compound was formed from cyanide in the absence of nitrogenase or $Na_2S_2O_4$; isocyanide can also break down to yield acetylene[4].

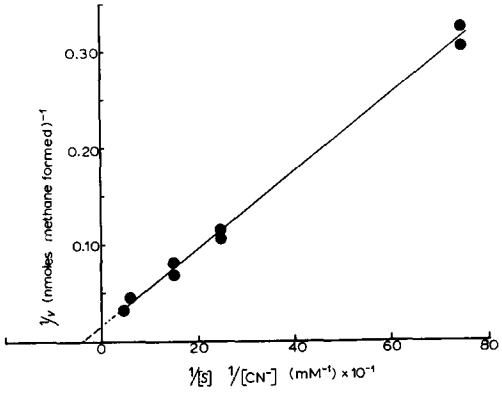

Fig. 3. Cyanide reduction by nitrogenase. Lineweaver–Burk plot of the rate of methane production *vs.* cyanide concentration. Intercept on *x*-axis gives K_m = 0.2 mM.

Samples of gas and liquid in the assay system (which had a gas to liquid ratio of 4:1) *minus* nitrogenase but containing [^{14}C]cyanide were counted in the scintillation counter. About 14 % of the radioactivity was in the gas phase at pH 7.4; inclusion of Mo–Fe protein and Fe protein reduced the amount in the gas phase to about 7 %. This observation suggests that the K_m for cyanide given above was slightly too high since no allowance was made for HCN in the gas phase; when this correction was made the value for the K_m was 0.19 mM.

Co-chromatography of cyanide at low concentrations with various proteins

Experiments were carried out using [^{14}C]cyanide undiluted with unlabelled cyanide to determine the maximum labelling that could be obtained. In addition to Mo–Fe protein (Fig. 4), columns were eluted with Fe protein, haemoglobin or bovine serum albumin. As Table I shows there was some increase in radioactivity over the background level which was greatest for haemoglobin and much lower for Mo–Fe protein or Fe protein or bovine serum albumin. Analysis of the bovine serum albumin by atomic absorption spectroscopy indicated less than 0.01 % iron.

If the radioactivity associated with Mo–Fe protein (Fig. 4 and Table I) was

due to cyanide bound at the N_2-reducing site of nitrogenase then other substrates should prevent this binding. However, no change was observed in the radioactivity associated with Mo–Fe protein when a column was run with acetylene-saturated buffer and under acetylene gas (Table I). Measurements of the acetylene in the eluate were made by gas chromatography to confirm that samples were saturated with this substrate.

The reduction of mixed substrates by nitrogenase

The failure of acetylene to inhibit apparent binding of [^{14}C]cyanide suggests that the two substrates are bound to different sites on the nitrogenase. These might be the same sites as those occupied during reduction, in which case assays of simultaneous cyanide and acetylene reduction would be expected not to show mutually competitive inhibition. Assays were made of ethylene and methane production in the presence of acetylene and various concentrations of cyanide, and compared with the rate of reduction of each substrate alone. Fig. 5 shows that acetylene reduction was

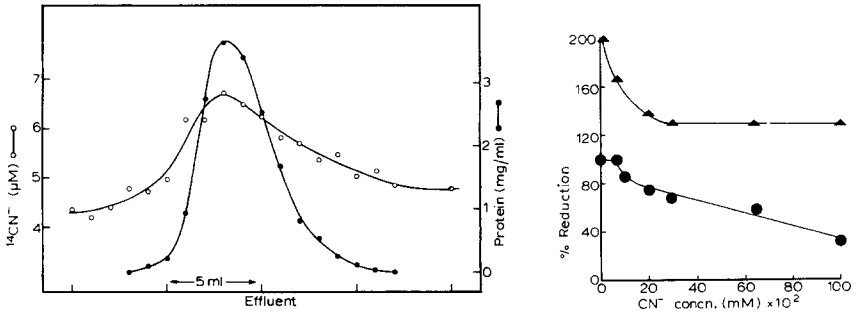

Fig. 4. Co-chromatography of Mo–Fe protein with 4.7 μM cyanide. For details of the Sephadex column technique see METHODS AND MATERIALS. Buffer (25 μM Tris–HCl (pH 7.4)) contained 4.7 μM Na^{14}CN.

Fig. 5. Mixed substrate reduction by nitrogenase. ●—●, reduction of acetylene to ethylene in the presence of cyanide expressed as a percentage of the acetylene reduced in the absence of cyanide; ▲—▲, reduction of cyanide to methane in the presence of acetylene expressed as a percentage of the cyanide reduced in the absence of acetylene.

inhibited by cyanide but reduction of cyanide to methane was actually enhanced by acetylene. For example 1.0 mM cyanide inhibited acetylene reduction 70 % whereas methane production was increased 30 % over the control without acetylene. The K_m of nitrogenase for cyanide was not altered by acetylene but v_{max} increased. The increase in methane formed from cyanide was not equal to the decrease in ethylene, assuming two electrons for reduction of acetylene to ethylene and six for reduction of cyanide to methane and ammonia.

100 % CO completely inhibited both cyanide or acetylene reduction when these substrates were assayed singly or together.

Equilibrium dialysis with [^{14}C]cyanide

Mo–Fe protein and Fe protein, haemoglobin and bovine serum albumin were dialysed under the conditions shown in Table II. (The activity of various samples was checked at the end of dialysis by assay for acetylene reduction: Mo–Fe protein

retained full activity, Fe protein or Mo–Fe protein *plus* Fe protein retained about 40 % of their original activity.) Neither acetylene nor CO had a significant effect on the small amount of radioactivity associated with Mo–Fe protein or Fe protein but CO caused about 50 % inhibition of [^{14}C]cyanide binding to haemoglobin which was much greater. The change in pH from 7.4 to 8.4 caused some increase in amounts of radioactivity associated with Mo–Fe protein or Fe protein or Mo–Fe protein *plus* Fe protein. Dialysis of Mo–Fe protein and Fe protein together did not produce any increase, nor did the addition of an ATP-generating system in absence of $Na_2S_2O_4$. In the presence of $Na_2S_2O_4$ the cyanide was reduced.

TABLE II

EQUILIBRIUM DIALYSIS OF VARIOUS PROTEINS WITH [^{14}C]CYANIDE

Figures are percent increase in radioactivity above background per mg protein. Samples containing 20 mg protein per ml were dialysed against 20 μM [^{14}C]cyanide before determining the level of radioactivity as described in METHODS AND MATERIALS. —, not determined.

Protein	*Ar*		*CO*	*Acetylene*
	pH 7.4	*pH 8.4*	*pH 7.4*	*pH 7.4*
Mo–Fe protein	2.2	4.2	1.8	2.2
Fe protein	3.9	7.7	4.3	5.3
Mo–Fe protein and Fe protein	3.6	7.4	3.4	4.5
Bovine serum albumin	1.2	—	—	—
Haemoglobin	109	—	53	105

DISCUSSION

BUI AND MORTENSON[20] explained their observations that only Mo–Fe protein of *C. pasteurianum* gave a cyanide peak and that only Fe protein gave an ATP peak by postulating that Mo–Fe protein contained the substrate-binding site and Fe protein the ATP-binding site. In their work, no results with other proteins were presented nor were alternative experimental approaches such as equilibrium dialysis used. Nevertheless, if their interpretations were correct all nitrogenases would be expected to show these selective binding properties. The data presented here for nitrogenase of *K. pneumoniae* do not completely support this conclusion.

The principle of the Sephadex column is important since it may contribute to the difference between the two sets of results. A Sephadex G-50 column equilibrated with respect to a small molecule such as ATP or cyanide contains these molecules both in the void and exclusion volume. In addition, some ATP appears (from the gradual rise to a plateau, Fig. 2a) to be bound. Only ATP or cyanide in the void volume is directly accessible to a protein of molecular weight greater than about 50000 whereas a protein with lower molecular weight would have access to these compounds in both void and exclusion volumes. If a protein of molecular weight greater than 50000 binds ATP, the equilibrium between ATP in the void and exclusion volumes is disturbed and ATP passes out in the void volume, but meanwhile the protein moves on down the column. Re-equilibration does occur until after the protein has passed (Figs. 2a, 2d). If the column is completely equilibrated there

may be enough ATP in the void volume, *plus* that which will diffuse out of the exclusion volume rapidly enough, to satisfy the binding capacity of the protein. However, if the column is not completely equilibrated protein of molecular weight less than 50000 has an advantage since it has direct access to all the ATP on the column. BUI AND MORTENSON[20] quote molecular weights of 160000 and 40000, respectively, for Mo–Fe protein and Fe protein of *C. pasteurianum*. Thus in their experiments Fe protein but not Mo–Fe protein had access to both void and exclusion volume and this may explain why an ATP peak was observed only with Fe protein. Since both proteins from *K. pneumoniae* were eluted in the void volume, neither had access to the exclusion volume. A small degree of apparent binding was observed for ferredoxin or cytochrome *c*, both of which had access to the exclusion volume but negative results were obtained for haemoglobin or bovine serum albumin (Fig. 2c) both of which passed through the column in the void volume. Other workers have shown binding of ATP to human haemoglobin[27]. The observation that O_2-damaged Fe protein caused inhibition of acetylene reduction in ATP-limited assays support the inference that the radioactivity peaks associated with Mo–Fe protein and Fe protein result from actual binding of ATP. However, further work is necessary before the relevance of this binding to the mechanism of N_2 reduction can be determined.

The considerations above on the principle of the Sephadex column cannot explain the difference between our results on binding of cyanide to Mo–Fe protein and Fe protein and those of BUI and MORTENSON[20] since they observed binding to Mo–Fe protein (mol. wt. 160000) but not to Fe protein (mol. wt. 40000). However, variations between the two nitrogenases may account for the apparently different affinities for cyanide. The binding of cyanide to Mo–Fe protein does not, in any case, appear to be specific for the following reasons: (1) Fe protein or bovine serum albumin showed similar amounts of binding. Non-specific binding of cyanide to proteins is known[28]. (2) CO, which completely inhibited cyanide reduction, did not affect binding of cyanide to Mo–Fe protein in equilibrium dialysis experiments whereas the binding to haemoglobin (Tables I and II), which is known to react strongly with cyanide[29], was much greater and in equilibrium dialysis was inhibited 50% by CO. The small change in peak size observed on changing the cyanide concentration on Sephadex columns with Mo–Fe protein (25 *cf.* 42%, Table I) may indicate that the associated radioactivity was not due to cyanide but to an impurity or breakdown product of the [^{14}C]cyanide which is known to decompose to a variety of compounds[30] including formate and acetylene.

The failure of acetylene to inhibit binding of cyanide to Mo–Fe protein in either column or equilibrium dialysis experiments is not evidence for the non-specificity of cyanide binding since in mixed substrate assays acetylene did not inhibit, but actually enhanced, reduction of cyanide to methane (Fig. 5), whilst cyanide caused considerable inhibition of acetylene reduction. A complete explanation of this observation requires further studies of cyanide reduction including estimation of all the products (ammonia, methane, methylamine[7]) and possibly non-enzymic by-products[30] and the other activities occurring simultaneously (ATP hydrolysis and H_2 evolution). Our results suggest that cyanide is not a model substrate and therefore considerably caution is necessary in applying results with cyanide to speculation on the mechanism of N_2 fixation.

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