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COMPARISONS AND CROSS REACTIONS OF NITROGENASE FROM *KLEBSIELLA PNEUMONIAE, AZOTOBACTER CHROOCOCCUM* AND *BACILLUS POLYMYXA*

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SUMMARY

Crude nitrogenase of *Klebsiella pneumoniae* was less rapidly inactivated by 0.2 atm of O_2 than nitrogenase of *Bacillus polymyxa;* inactivation of *Azotobacter chroococcum* nitrogenase required 1 atm of O_2. Nitrogenase from each organism was separated into two protein components; one was rapidly inactivated by air, the other was only slowly affected. The product of reduction of C_2H_2 in ^2H_2O or $C_2{}^2H_2$ in H_2O by each nitrogenase was *cis*-ethylene, $C_2{}^2H_2H_2$, some *trans*-$C_2{}^2H_2H_2$ was also detected. Each nitrogenase catalysed exchange between $^2H_2$ and H_2O proportional to the partial pressure of $^2H_2$ and not dependent on the presence of N_2. Proteins 1 of each nitrogenase were assayed with their corresponding Protein 2 or those of the other two bacteria for rate of acetylene, azide, cyanide, isocyanide or N_2 reduction; ATP-dependent H_2 evolution and P_i formed from ATP hydrolysis were also determined. Cross reaction, better than 80%, was observed between *Azotobacter chroococcum* and *Klebsiella pneumoniae* and between *Bacillus polymyxa* and *Klebsiella pneumoniae* with acetylene as substrate, but in the crosses *Bacillus polymyxa* Protein 1 + *Azotobacter chroococcum* Protein 2 and its reciprocal only 50 and 12% cross, respectively, were observed. Some differences were found in the degree of cross reaction with the various substrates and the amount of P_i formed did not always correspond with the amount of substrate reduced. These results together with other work are used to support the hypothesis that cyanide or isocyanide are not model substrates, being reduced at more than one site on the nitrogenase complex. The possibility that a two-metal site catalyses N_2 fixation is considered.

INTRODUCTION

The ability to fix N_2 is found in a variety of bacteria, including aerobes such as *Azotobacter vinelandii*[1], facultative anaerobes such as *Bacillus polymyxa*[2] and obligate anaerobes such as *Clostridium pasteurianum*[3]. The nitrogenase from some bacteria, including the three examples above[4–6], has been extracted and fractionated

into two protein components both necessary for the reactions nitrogenase is able to catalyse. In addition to N_2, substrates of nitrogenase include methyl isocyanide[7], cyanide[8] or azide[9]. Acetylene[10,11] is also a substrate being reduced to ethylene and is now widely used since it provides the basis for a rapid and sensitive assay applicable to whole bacteria[12], legume[13] or non-legume[14] nodules or purified nitrogenase[15].

In all nitrogenases examined, one protein contains iron and molybdenum, the other only iron. MORTENSON[16] has termed these proteins molybdo- and azo-ferredoxin respectively, but these names suggest a close similarity with ferredoxin which has not yet been shown, therefore in this paper the proteins will be referred to as Protein 1 and 2, respectively. The role each plays in catalysing N_2 fixation is not understood, though evidence that Protein 1 may bind the substrate whilst Protein 2 reacts with ATP has been published[17]. However Protein 2 has been postulated to contain the N_2-binding site[18] and a N_2-fixation mechanism involving two metals has also been suggested[19].

No significant differences in the nitrogenases from different bacteria have been reported. They have the same substrate specificity and all require an ATP and electron supply. In the crude state the nitrogenase of *A. vinelandii*[20] or *Azotobacter chroococcum*[21] is stable in air whereas other nitrogenases are rapidly inactivated. However, in the purified state all nitrogenases are inactivated by O_2 though Protein 1 of *A. chroococcum* was reported to be stable in air for short periods[18].

DETROY *et al.*[6] prepared the two nitrogenase components from a number of bacteria to determine if Protein 1 from one bacterial nitrogenase could function together with the Protein 2 from another to effect N_2 fixation. They reported that most crosses were positive, *i.e.* the system was able to fix N_2, though apparently at a slower rate than catalysed by components from the same bacteria. Negatives were observed between Protein 2 of *B. polymyxa* and Protein 1 of *A. vinelandii* and between Protein 1 of *C. pasteurianum* and Protein 2 of all other nitrogenases prepared. The authors pointed out that these negatives might have arisen because the optimum ratio of the two components was not used; in their experiments only azide or N_2 reduction was measured and no measurements of ATP-dependent H_2 evolution or P_i formation was reported.

The use of Proteins 1 and 2 from different bacteria, besides giving information on the comparative biochemistry of different nitrogenases, might provide more evidence on the mechanism of N_2 fixation. In this paper the results of a more detailed examination of cross reactions between various nitrogenase components is presented and their significance relative to current theories of N_2 fixation are considered.

METHODS AND MATERIALS

Culture of bacteria

K. pneumoniae strain M5al and *B. polymyxa* were obtained from Professor Wilson. They were grown in 20-l fermentors on a N-free medium[2] at 30° starting with a 5% inoculum and bubbled with N_2 for 36–48 h before harvesting in a continuous centrifuge. No special care was taken to exclude O_2 during harvesting. The cell pastes were stored in liquid N_2 until required though cells could be stored satisfactorily under Ar at −20° for a few days. *C. pasteurianum* was grown under the same condi-

tions using medium containing 3 mg/l of yeast extract. Growth, harvesting and storage of *A. chroococcum* was as described previously[21].

Preparation of nitrogenase

Crude nitrogenase was obtained from all four bacteria either by passage of a thick cell suspension through the French press at a pressure of 12 000 lb/inch^2 or by use of an ultrasonic disintegrator. However, except for *A. chroococcum*, great care was necessary to keep the system anaerobic by flushing with high purity N_2. The French press was used for large scale preparations and was flushed with N_2 before filling with a suspension containing 50–80 mg dry wt./ml of bacteria in 25 mM Tris–HCl buffer (pH 7.4) to which a small amount of $Na_2S_2O_4$ (approx. 10 mg/100 ml) was added to scavenge O_2. The effluent from the press was collected in a N_2-flushed flask cooled in ice. Whole cells and large fragments were removed by centrifuging in sealed tubes under N_2 at 40 000 $\times$ *g* for 45 min. The clear dark brown supernatant from this stage containing 40–50 mg protein/ml was decanted into a N_2 flushed flask. Such extracts were stored for short periods at 5° but for longer periods were frozen in liquid N_2 and stored at −190°. Material stored at this temperature showed no loss of activity at the end of 18 months.

Crude nitrogenase of *A. chroococcum* was prepared in essentially the same way though no precautions to exclude air were taken nor was $Na_2S_2O_4$ added at that stage.

Fractionation of nitrogenase into Protein 1 and Protein 2

During purifications nitrogenase was assayed with acetylene as substrate since this assay is more sensitive and rapid than N_2-fixation assays. Partial purification and separation of the nitrogenase proteins of each bacteria was achieved using the procedures described for *A. chroococcum*[18] though a preliminary precipitation of nitrogenase with protamine sulphate was not used with extracts of *K. pneumoniae* or *B. polymyxa*. A single chromatographic step on DEAE-cellulose of crude nitrogenase gave two protein components but, though Protein 1 was devoid of any reducing activity, Protein 2 had, in each case, some residual activity. Further chromatography of each Protein 1 was carried out using conditions described for *A. chroococcum*[18] to remove inactive material. Further purification of each Protein 2 was obtained by chromatography on DEAE-cellulose at pH 5.5. Material was dialysed anaerobically against 25 mM Tris–maleate buffer (pH 5.5), then loaded onto a column equilibrated with the same buffer and washed with 3 bedvol. of this buffer followed by 2 bedvol. of this buffer containing 250 mM NaCl which eluted a light brown band. This eluted material was identified as Protein 1 by assaying for acetylene reduction with added Protein 2. The remaining material (Protein 2) was eluted in a small volume from the column by washing with 90 mM $MgCl_2$ in 25 mM Tris–HCl buffer (pH 7.4), diluted with an equal volume of the same buffer containing approx. 1 mg/100 ml of $Na_2S_2O_4$ and concentrated by ultrafiltration anaerobically[18]. This dilution and concentration procedure was repeated several times to bring the pH up to 7.4 and lower the salt concentration below 30 mM. A similar procedure was used for each Protein 1. The Protein 2 of each bacterium was assayed alone to determine the range over which a linear relationship between protein concentration and rate of reduction occurred. This was particularly important since below a Protein 2 concentration of about

0.1 mg/1.5 ml, reduction was not linear in absence of Protein 1, consequently assays using lower concentrations produced a misleading impression of the purity. The residual activity of each Protein 2 was very low; addition of the corresponding Protein 1 gave enhancement of about 250-fold for *K. penumoniae;* 300-fold for *B. polymyxa*, and 100-fold for *A. chroococcum* with acetylene as substrate. Using the same assay conditions, each Protein 2 alone had no N_2-fixing activity and the system therefore had an absolute requirement for its corresponding Protein 1. This apparent discrepancy is because acetylene reduction is a more sensitive assay system than N_2 fixation and therefore detects much lower levels of residual activity.

For convenience the nitrogenase proteins of *A. chroococcum* are referred to in the rest of this paper as A_1 and A_2; those of *K. pneumoniae* as K_1 and K_2, and *B. polymyxa* as B_1 and B_2.

Assay of nitrogenase

The procedures used for assay of reduction of acetylene, cyanide, methyl isocyanide and N_2, for ATP-dependent H_2 evolution and P_i formation have been described (refs. 17, 21). Reduction of azide was determined using conditions reported by KELLY *et al.*[15].

Infrared spectroscopy

Measurement of the gaseous products of acetylene reduction (C_2H_2 in ^2H_2O or $C_2{}^2H_2$ in H_2O) were made with a Unicam S.P. 1200 instrument using a 100-ml cell with NaBr plates and a 10-cm pathlength. The $C_2{}^2H_2$ was prepared by addition of commercial grade CaC_2 to 99.7% ^2H_2O. Each assay was carried out in a 25-ml double side-armed Warburg flask attached to the spectrophotometer cell. The whole system was flushed with Ar before closing off the flask from the infrared cell and introducing acetylene, $Na_2S_2O_4$ and nitrogenase into the flask. At the end of the reaction the connection between flask and infrared cell, which had been partly evacuated, was opened and gas displaced into the cell by injecting water into the flask. For approximate time-course experiments, the infrared cell was not closed off from the flask and a 50-ml syringe with hypodermic needle inserted into the flask through a serum cap, was used as a pump to circulate gas between the infrared cell and the Warburg flask.

$^2H_2/H_2O$ exchange and mass spectroscopy

Analysis for H_2, $^2H_2$ and H^2H were made in a MS 3 instrument (A.E.I. Ltd., Barton Dock Road, Urmston, Manchester); some details of the procedures used have been described previously[22]. Warburg flasks were attached to mass spectrometer tubes of about 7 ml volume. These tubes could be closed by means of wide bore high vacuum taps at either end. For each assay, after flushing the whole system with gas, the upper tube tap and the sidearm tap of each Warburg flask were closed. The Warburg flask containing the assay components was closed off from the mass spectrometer tube after a further 15-min delay to ensure adequate diffusion of gas throughout the system. The nitrogenase-catalysed reaction was started by tipping in Protein 1 and 2 from the side arm, and stopped by the addition of 30% (w/v) of trichloroacetic acid. The contents of the mass spectrometer tube were analysed for H_2, H^2H and $^2H_2$ to give the initial levels of these gases. The tubes were then evacuated, still attached to the mass spectrometer, and a sample from the Warburg flask introduced into the

evacuated tube by opening the lower tap with the upper one closed off. This sample was then analysed for H_2, H^2H and $^2H_2$ giving the level of these gases at the end of the reaction. The mass spectrometer was calibrated with gas samples containing known amounts of H_2, H^2H and $^2H_2$. Samples of liquid were removed from Warburg flasks for determination of NH_3 produced by N_2 fixation.

For further details of the procedures used in this work including protein determinations, handling of Protein 1 and 2, gas chromatography and source of chemicals see refs. 15, 18, 21.

RESULTS

Assay of nitrogenase from C. pasteurianum

Crude nitrogenase from *C. pasteurianum* gave rates of acetylene reduction only about 4% of those observed with nitrogenase from *K. pneumoniae* using $Na_2S_2O_4$ and an ATP-generating system (creatine kinase *plus* creatine phosphate). This low rate of activity was observed whether extracts were obtained using a French press, a sonic disintegrator or by extraction from dried cells[23]. However, when pyruvate was added to such extracts to provide both ATP and electrons[24,25] the rate of acetylene reduction increased considerably to levels comparable with those observed with nitrogenase from *K. pneumoniae*. Acetylene reduction catalysed by nitrogenase from *K. pneumoniae* was not inhibited by addition of nitrogenase from *C. pasteurianum* indicating that the low acetylene-reducing activity of the *C. pasteurianum*/$Na_2S_2O_4$ + ATP system was not simply due to presence of inhibitors, *e.g.*, blocking the ADP *plus* creatine phosphate → ATP reaction.

Repeated fractionations of *C. pasteurianum* nitrogenase on DEAE-cellulose, in attempts to obtain its Protein 1 and 2, gave material with low acetylene reducing activity and addition of Protein 1 from *B. polymyxa* produced enhancemen tof activity up to 30-fold using the $Na_2S_2O_4$/ATP system. Addition of material thought to be Protein 1 of *C. pasteurianum* produced only slight enhancement. Only a small enhancement (2–3-fold) was observed if B_1 was added to crude nitrogenase of *C. pasteurianum*.

These observations suggest that $Na_2S_2O_4$ reacts rapidly with Protein 1 of most nitrogenases but not that of *C. pasteurianum* in crude or partially purified state. B_1 functions with Protein 2 of *C. pasteurianum* but in the crude nitrogenase interaction between the two is not possible hence B_1 cannot give enhancement. In the physiological system, ferredoxin transfers electrons effectively from the phosphoroclastic reaction to the nitrogenase.

Titration of Protein 2 with Protein 1

The ratio of the two proteins which gave maximum rate of acetylene reduction was determined by assaying a fixed level of Protein 2 with a varying amount of Protein 1 for nitrogenase of *A. chroococcum, K. pneumoniae* and *B. polymyxa*. Since methyl isocyanide required a different ratio of the two proteins for maximum rate of reduction in *A. chroococcum*[18] a second titration was carried out with isocyanide as substrate; ATPase determinations were also made. The results for $B_1 + B_2$ are given in Fig. 1 and similar results were obtained for $A_1 + A_2$ or $K_1 + K_2$. The ratio of B_1 to B_2 for maximum rate of acetylene reduction was about 2:1 (mg of each

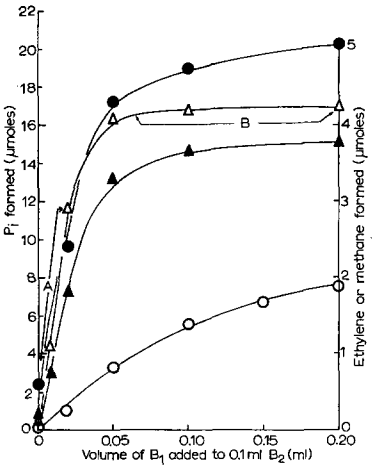

Fig. 1. Titration of B_2 with B_1. Activity regained for reduction of acetylene to ethylene (△—△) or methyl isocyanide to methane (○—○) upon addition of B_1 to B_2. ▲—▲ and ●—●, corresponding ATPase activities. All assays contained 0.95 mg B_2 and indicated volume of B_1 (20.0 mg/ml).

protein) compared to 6:1 for methyl isocyanide reduction, also the amount of ATP hydrolysed was higher during methyl isocyanide reduction than during acetylene reduction. This observation is in agreement with work reported previously for $A_1 + A_2$ (ref. 18).

These titration results were used to determine levels of each component for different assays. Thus for assay of Protein 1 a level within the region marked A on Fig. 1 was used, conversely in assays of Protein 2 levels of Protein 1 in range marked B were used.

Sensitivity of crude nitrogenases, Proteins 1 and Proteins 2 to O_2

A comparison of the O_2 sensitivity of crude nitrogenase from *A. chroococcum*, *K. pneumoniae*, *B. polymyxa* and *C. pasteurianum* was made in the following way. The protein content of each nitrogenase extract was adjusted to 20 mg/ml by addition of 25 mM Tris–HCl buffer, pH 7.4. The buffer was sparged with Ar before use to remove dissolved air. The same volume of each extract was magnetically stirred at the same rate under air. Controls of each nitrogenase were incubated under the same conditions except that the vessels were flushed with Ar and sealed. At intervals aliquots were removed from each sample and the rate of acetylene reduction determined. The nitrogenase of *A. chroococcum* showed no loss of activity after 60 min under air at 20° compared with its control, which retained complete activity. Nitrogenase of *K. pneumoniae* showed 20% loss, that of *B. polymyxa* 70% and of *C. pasteurianum* 75% after only 10 min. The corresponding losses after 30 min were 60, 100 and 100% respectively. The same rate of loss of activity was observed for *B. polymyxa* nitrogenase under air or Ar–O_2 (80:20, by vol.) or Ar–acetylene–O_2 (75:5:20, by vol.) indicating that the substrates of nitrogenase (acetylene or N_2) were not able to protect against O_2 inactivation. Slower rates of inactivation were observed at lower temperatures or if the partial pressure of O_2 was decreased. Thus nitrogenase

of *B. polymyxa* was only 20% inactivated after 10 min at 0° or under 5% O_2 at 20°. The thiol compounds cysteine, glutathione and dithiothreitol did not protect against O_2 inactivation nor have any attempts to reactivate damaged nitrogenase been successful. A protective effect obtained with $Na_2S_2O_4$ in unstirred nitrogenase preparations under air was apparently due to its efficiency at scavenging O_2 before this could inactivate the nitrogenase.

Results for the O_2 sensitivity of *C. pasteurianum* nitrogenase were essentially the same using either the pyruvate or $Na_2S_2O_4$/ATP-generating assay systems.

Though no inactivation of *A. chroococcum* nitrogenase was detected at 20% O_2, exposure of such extracts to 100% O_2 at 20° for 30 min produced 75% inactivation. These results indicate that a gradation in O_2 sensitivity exists ranging from very O_2 sensitive nitrogenases from some anaerobic bacteria through less sensitive ones like that of *K. pneumoniae* to the relatively stable nitrogenase of *A. chroococcum*. These differences in O_2 sensitivity may be due to contaminating protein having a protective effect, present in crude nitrogenase of *A. chroococcum* but absent from nitrogenase of *C. pasteurianum* or *B. polymyxa*. Possibly some of the other aerobic N_2-fixing bacteria, *e.g. Derxia gummosa* or *Beijerinckia indica*, will have nitrogenases that occupy an intermediate position in O_2 sensitivity, though nitrogenase of *Mycobacterium flavum* which, on N_2-free medium, fixes N_2 best under 5% O_2 is very sensitive to O_2 (ref. 26).

The effect of O_2 on Protein 1 and Protein 2 of each nitrogenase was determined using similar conditions to those described above for crude nitrogenases. The activity of each Protein 1 was determined by assaying it for acetylene-reducing activity in the presence of excess of its corresponding untreated Protein 2 so that the rate of reduction was determined by the amount of active Protein 1. Similarly, the activity of treated Protein 2 was determined with untreated Protein 1. Each protein was

TABLE I

EFFECT OF TEMPERATURE ON O_2 INACTIVATION OF K_1

K_1 containing 13 mg protein per ml was exposed to air and 0.02 ml was assayed with excess untreated K_2 for acetylene reduction. Results are expressed as percent of control (untreated K_1 + K_2).

Temperature of storage	*Time of exposure*	*Activity remaining*	
		C_2H_2 reduction	*ATPase*
20°	30 min	100	100
	60 min	58	75
	120 min	22	38
	24 h	1.5	10
10°	24 h	31	45
	48 h	1.5	5
	48 h (under Ar)	100	95
5°	24 h	25	45
	48 h	3	8
−20°	24 h	100	100
	2 weeks	100	100

also assayed on its own; controls of untreated 1 *plus* untreated 2 were assayed and in all cases ATPase activity was determined.

A_2, B_2 or K_2 protein was completely inactivated by exposure to air for 10 min at 20°, being unable to catalyse acetylene reduction, ATP-dependent H_2 evolution or ATP hydrolysis. By contrast A_1, B_1 or K_1 showed no loss of any of these activities after exposure to air for 30 min at 20°. The effect of exposing K_1 to air at different temperatures for varying times was determined with the results shown in Table I. Some loss of acetylene-reducing activity occurred after 1 h and there was an approximately corresponding loss of ATPase activity. After 24 h the material which partially bleached from dark brown to pale orange-brown retained only slight activity. K_1 stored at 10° also slowly bleached and retained 31% activity after 24 h but at −20° no colour change or loss of activity was detected after 2 weeks. Bleached K_1 (12°, 48 h under air) was dialysed to remove iron and molybdenum not firmly bound to protein. There was no change in the amount of iron and molybdenum in this K_1 compared with a control stored under Ar at 10° for 48 h (which did not bleach) and also dialysed. The amount of iron was about 0.5% (mg iron per mg protein) and of molybdenum about 0.035%.

Cross reactions between nitrogenase components

Products of acetylene reduction

DILWORTH[10] reported that reduction of acetylene by crude nitrogenase of *C. pasteurianum* in ^2H_2O produced *cis*-$C_2{}^2H_2H_2$ which was identified by its infrared spectrum, in particular a strong absorption band at 843 cm^{-1} (ref. 27). Other workers obtained similar results using nitrogenase of *A. vinelandii*[12]. In this work the product of acetylene reduction in ^2H_2O was determined with freeze-dried $A_1 + A_2$. The infrared spectrum of gaseous products shown in Fig. 2 revealed a strong absorption at 843 cm^{-1} and a smaller one at 988 cm^{-1} which was ascribed to *trans*-$C_2{}^2H_2H_2$[27,28]. Absorption was also noted at 677 cm^{-1} and was ascribed to C_2H^2H (ref. 29) probably formed by non-enzymic exchange between C_2H_2 and ^2H_2O. $C_2H_3{}^2H$, reported to have strong absorption at 943 cm^{-1} (ref. 29), was also detected.

The reduction of $C_2{}^2H_2$ was next examined because it offered two advantages: firstly, the necessity either to freeze-dry Proteins 1 and 2 (when loss of activity, particularly of Protein 2, occurred) or to subject them to prolonged dialysis against several changes of ^2H_2O, was avoided. Secondly, C_2H_2 has a strong absorption centred at 729 cm^{-1} and this shifts to about 537 cm^{-1} in $C_2{}^2H_2$ making interpretation of results easier. Preliminary experiments showed that $C_2{}^2H_2$ was reduced at about the same rate as C_2H_2. An infrared spectrum of products of $C_2{}^2H_2$ reduction by $K_1 + K_2$ is shown in Fig. 3a. Again the major product was *cis*-$C_2{}^2H_2H_2$ though some *trans*-$C_2{}^2H_2H_2$ was also formed and a trace of $C_2{}^2HH_3$ produced from $C_2{}^2HH$, itself probably formed by non-enzymic exchange between $C_2{}^2H_2$ and H_2O and also detected in the spectrum. Asymmetric $C_2{}^2H_2H_2$ (H_2C–C^2H_2) which absorbs at 944.752 and 676 cm^{-1} (ref. 28) was not detected. By adding CaC_2 to a mixture of about 66% ^2H_2O *plus* 34% H_2O (v/v) C_2H_2, $C_2{}^2HH$ and $C_2{}^2H_2$ were obtained and reduction of these by nitrogenase gave a mixture of the various products (see Fig. 3b) confirming the identification of the absorption bands, in particular distinguishing C_2H_4 from $C_2H^2H_3$.

Reduction of $C_2{}^2H_2$ by $B_1 + B_2$, $A_1 + A_2$, $B_1 + A_2$, $A_1 + K_2$ or crude extracts of *C. pasteurianum plus* pyruvate gave essentially the same results and as Fig. 3c

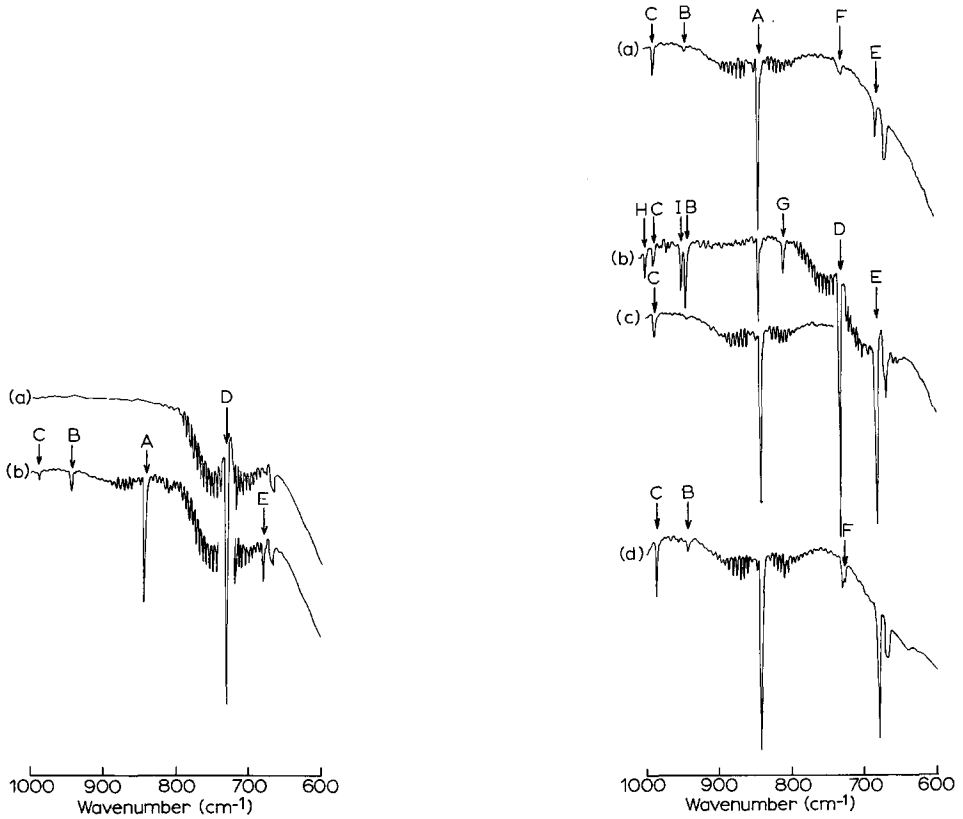

Fig. 2. Infrared spectrum of products of acetylene reduction by $A_1 + A_2$. (a) Acetylene control; (b) gaseous products of acetylene reduction; mixture contained about 25 mg freeze-dried A_1; about 20 mg freeze-dried A_2; 10 times the usual amount of ATP-generating system and $Na_2S_2O_4$ (see ref. 18) all in ^2H_2O. 5 ml acetylene injected into system and gas samples examined in infrared after 60 min. Identification of absorption bands: A, *cis*-$C_2{}^2H_2H_2$ (843 cm^{-1}); B, $C_2{}^2HH_3$ (943 cm^{-1}); C, *trans*-$C_2{}^2H_2H_2$ (988 cm^{-1}); D, C_2H_2 (729 cm^{-1}); E, $C_2{}^2HH$ (677 cm^{-1}).

Fig. 3. Infrared spectra of products of acetylene reduction by nitrogenase. Each assay contained about 20 mg Protein 1 and 10 mg of Protein 2, amounts of other reactants as described for Fig. 2 though reactions carried out in H_2O. (a) $C_2{}^2H_2$ substrate under Ar; $K_1 + K_2$. Identification of absorption peaks: A, B, C and E as Fig. 2b F, possibly *trans*-$C_2{}^2H_2H_2$ (727 and 730 cm^{-1}). (b) Mixture C_2H_2, C_2H^2H and $C_2{}^2H_2$ as substrate under Ar; $K_1 + K_2$. Identification of absorption peaks: B, G: $C_2{}^2HH_3$ (943 and 809 cm^{-1}); H, I: C_2H_4 (949 and 995 cm^{-1}), rest as Fig. 2a. (c) As (a) but with $A_1 + K_2$. Identification of A, B *etc.* as Fig. 2(b). (d) As (a) but reaction carried out under H_2. Identification of absorption peaks as Fig. 2a.

shows for the $A_1 + K_2$ cross there was no significant change in the ratio of *trans*- to *cis*-$C_2{}^2H_2H_2$. Incubation of $K_1 + K_2$ with $C_2{}^2H_2H_2$ in the absence of acetylene did not produce isomerisation of *cis*-$C_2{}^2H_2H_2$ to *trans*-$C_2{}^2H_2H_2$. Variation in temperature between 10 and 40° and the ratio of Protein 1 to Protein 2 had no effect on the relative amounts of *cis* and *trans* products nor was any change in the ratio observed during time-course experiments. Nevertheless, although quantitative determinations of the amounts of *trans*-$C_2{}^2H_2H_2$ to *cis*-$C_2{}^2H_2H_2$ were not made, this ratio appeared to be slightly greater in the $C_2{}^2H_2/H_2O$ system than in the $C_2H_2/{}^2H_2O$ system. Also if the $C_2{}^2H_2$ reduction was carried out under H_2 or $^2H_2$, although no significant

inhibition of reduction was observed, the amount of *trans*-$C_2{}^2H_2H_2$ apparently increased (Fig. 3d).

$^2H_2/H_2O$ exchange catalysed by nitrogenase

Early reports that exchange between $^2H_2$ and H_2O to form H^2H catalysed by nodules of soybean, was greater under N_2 than under Ar[30,31] was re-examined by using nitrogenase from *A. vinelandii*[32,33]. Exchange required nitrogenase, $Na_2S_2O_4$, ATP supply and N_2, supporting the earlier suggestions that an enzyme-bound intermediate of N_2 reduction (possibly di-imide) catalysed the exchange. KELLY[22] observed that $H_2/^2H_2O$ or $^2H_2/H_2O$ exchange, using a variety of nitrogenase preparations, was inhibited by CO or acetylene and depended on $Na_2S_2O_4$/ATP supply but not N_2. The discrepancy between results would be explained if two types of exchange can be catalysed by nitrogenase. Type 1, not requiring N_2, would occur at maximum rate with a high partial pressure of $^2H_2$ (or H_2) as a result of interaction of this gas with the substrate-binding site of nitrogenase. All substrates including N_2 would compete for the site and therefore cause inhibition. Metal hydrides complexes are known which catalyse such exchange[34]. Type 2, N_2-dependent exchange, would occur by interaction of $^2H_2$ at low partial pressure with enzyme bound di-imide. Exchange actually observed would then be the sum of both types and the contribution each made might be determined by experiments with widely varying partial pressure of $^2H_2$ with or without N_2 present. At high partial pressure of $^2H_2$, Type 1 exchange would predominate and N_2 fixation be partially inhibited, but Type 2 exchange should be most obvious when maximum fixation was occurring *i.e.*, low partial pressure of $^2H_2$ *plus* N_2.

Measurements of exchange were made by procedures described in METHODS AND MATERIALS. The purity of Ar, N_2 and $^2H_2$ was checked by gas chromatography; only traces of contaminating gases were detected in particular the level of CO was

TABLE II

EFFECT OF PARTIAL PRESSURE OF $^2H_2$ AND OF N_2 ON EXCHANGE REACTION

Assays contained 0.1 ml Protein 1; 0.1 ml Protein 2. Protein concentration: A_1, 1.8; A_2, 0.8; B_1, 2.0; B_2, 0.95; K_1, 1.9; K_2, 1.1 mg/0.1 ml. Reactions stopped after 30 min and H_2, H^2H and NH_3 analysis carried out as described in text.

Nitrogenase	*Partial pressure*			*Product (μmoles)*		
	$^2H_2$	N_2	*Ar*	H_2	H^2H	NH_3
$B_1 + B_2$	1.0	—	—	11.3	2.8	—
	0.48	—	0.52	11.8	1.6	—
	0.51	0.49	—	7.2	1.3	3.1
	0.12	—	0.88	11.4	0.4	—
	0.11	0.50	0.39	4.2	0.4	4.8
$K_1 + K_2$	0.48	—	0.52	9.0	2.0	—
	0.54	0.46	—	6.4	1.4	2.3
	0.09	—	0.91	9.1	0.5	—
	0.10	0.51	0.39	2.2	0.6	4.3
$A_1 + A_2$	1.0	—	—	9.2	1.8	—
$B_1 + A_2$	1.0	—	—	4.5	1.1	—
$A_1 + B_2$	1.0	—	—	2.8	0.6	—

not greater than 20 ppm. The data in Table II show that exchange in *B. polymyxa* occurred in the absence of N_2 and was proportional to partial pressure of $^2H_2$; 50% N_2 caused some inhibition of exchange. At low partial pressure of $^2H_2$ only a small amount of exchange was detected and this was scarcely increased by presence of N_2 though N_2 fixation was detected. In the $B_1 + B_2$ system the amount of exchange observed with 1 atm of $^2H_2$ was about 25% of the H_2 evolved. The two cross reactions, $B_1 + A_2$ and $A_1 + B_2$ also showed some exchange though only about 60 and 16%, respectively, of the exchange observed with $A_1 + A_2$ and $B_1 + B_2$.

Relative rates of nitrogenase activity with different substrates

The rate of acetylene reduction was determined with A_1, B_1 or K_1 crossed with B_2, K_2 or A_2. The ratios of Protein 1 to Protein 2 used were those found to be optimum for the homologous crosses ($A_1 + A_2$, $B_1 + B_2$ etc.). Taking the appropriate homologous cross as 100, *e.g.* for $A_1 + B_2$ this would be $B_1 + B_2$, the degree of cross reaction varied from 100 for $A_1 + K_2$ or $B_1 + K_2$ to only 13 for $A_1 + B_2$. The varying degrees of cross reaction might have been due to insufficient amounts of the appropriate Protein 1 therefore a titration of each Protein 2 was carried out with various proteins as described for each homologous cross (Fig. 1). The results in Fig. 4

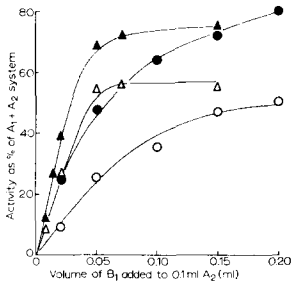

Fig. 4. Titration of A_2 with B_1. Activity regained for reduction of acetylene to ethylene (△—△) or methyl isocyanide to methane (○—○) upon addition of B_1 to A_2. ▲—▲ and ●—●, corresponding ATPase activities. All assays contained 0.8 mg A_2 and indicated volume of B_1 (20.0 mg/ml).

for $B_1 + A_2$ show that approximately the same amount of B_1 effected maximum rate of acetylene reduction when added to a fixed amount of A_2 as in the $B_1 + B_2$ assays (see Fig. 1); addition of further B_1 had no effect on the rate of acetylene reduction. Also included in Fig. 4 are the results with isocyanide as substrate and again the ratio of B_1 to A_2 necessary for maximum rate of isocyanide reduction was higher than that necessary for maximum rate of acetylene reduction. Similar results were obtained for other crosses.

Since B_1A_2 might not have the same K_m for acetylene as the B_1B_2 complex, assays were carried out with various levels of acetylene. No significant differences in K_m values were observed between the two systems. The results of a more detailed examination of cross reactions using a variety of substrates are presented in Table III. There was considerable variation in the degree of cross reaction both between crosses and within a particular cross for the different substrates, *e.g.* acetylene reduction was 100 in $B_1 + K_2$ but only 13 in $A_1 + B_2$ and in the $K_1 + B_2$ cross the figure for

TABLE III

CROSS REACTION OF A_1 WITH B_2 OR K_2; B_1 WITH A_2 OR K_2; K_1 WITH A_2 OR B_2

Each assay marked * contained 0.1 ml Protein 1 + 0.1 ml Protein 2, others contained 0.2 ml Protein 1 + 0.1 ml Protein 2; for protein concentrations see Table II. Reactions were carried out at 30°, stopped after 10 min by addition of 0.1 ml 40% KOH (w/v) and analysed. Figures in parentheses denote ATPase activity.

Assay	*Cross (% of the homologous cross)*					
	$A_1 + K_2$	$A_1 + B_2$	$K_1 + A_2$	$K_1 + B_2$	$B_1 + A_2$	$B_1 + K_2$
*Acetylene reduction	98	13	100	92	55	100
	(95)	(56)	(95)	(105)	(75)	(95)
Methyl isocyanide reduction	100	16	95	37	50	130
	(105)	(55)	(95)	(95)	(80)	(105)
Potassium cyanide reduction	95	9	90	38	47	120
	(110)	(65)	(105)	(92)	(74)	(96)
*Sodium azide reduction	75	16	120	40	44	85
	(97)	(55)	(110)	(110)	(90)	(107)
*N_2 fixation	62	8	80	40	38	70
	(106)	(60)	(105)	(95)	(73)	(98)
*ATP-dependent H_2 evolution	100	25	95	94	48	95
	(105)	(57)	(110)	(90)	(68)	(108)

acetylene was 90 but only 40 for N_2. Figures for ATPase are only approximate (± 5% at best) because P_i determinations were made in the presence of acid-labile creatine phosphate. Nevertheless the degree of cross for ATPase was noticeably higher in most cases than that observed for other activities, *e.g.* in $A_1 + B_2$ the figure for ATPase was 80 but for N_2 fixation 8. Anomalous results were observed in the $B_1 + K_2$ and $K_1 + B_2$ crosses with cyanide or isocyanide as substrate. However for $B_1 + B_2$, cyanide or isocyanide was reduced at a greater rate relative to acetylene than in other systems. If this specificity difference was taken into account by expressing the $B_1 + K_2$ result as a percent of the $B_1 + B_2$ rather than $K_1 + K_2$, the figures become 70 and 78 respectively for cyanide and isocyanide. The corresponding figures for the $K_1 + K_2$ cross become 80 and 85 respectively. A similar calculation does not affect the acetylene figures.

Some differences in the ratio of byproducts ethane and ethylene formed from isocyanide were observed between the nitrogenases. Thus for $K_1 + K_2$ the ratio of ethylene to ethane was about 2.2, for $A_1 + A_2$ about 4, and for $B_1 + B_2$ 0.5. In cross reactions Protein 1 apparently determined the ratio so that for $B_1 + A_2$ a ratio of 0.3 was observed whereas in the $A_1 + B_2$ the ratio was about 4.

DISCUSSION

The nitrogenases examined in this work had very similar properties. Each could be separated into two proteins, one more rapidly inactivated by O_2 than the other and both essential, together with $Na_2S_2O_4$ and an ATP supply, for reduction of a variety of substrates. The relative rates of reduction of these substrates were generally about the same for each nitrogenase although some differences were observed in the relative rates of isocyanide or cyanide to acetylene reduction between

B. polymyxa and *K. pneumoniae*. In assays with components from different bacteria (Table III) some degree of cross reaction was observed in all cases though with considerable variations between different crosses. The reported failure of Protein 1 of *C. pasteurianum* to cross react with any other nitrogenase Protein 2 (ref. 6) may be due to its apparently slow reaction with $Na_2S_2O_4$.

If each substrate was bound and reduced at the same site which was entirely on one protein, small differences in structure around this region would explain small specificity differences. In heterologous reactions the rate of reduction of each substrate might then be approximately the same percent of the appropriate homologous control. Alternatively, in heterologous crosses the affinity of the binding site for various substrates could change giving different degrees of cross reaction.

The data of Table III show greater variations in percent of cross reaction with different substrates than was explicable by experimental error and differences in sensitivity of various assays. Whereas N_2 reduction gave lowest percent cross, the ATPase activity was higher in most cases than the rate of H_2 evolution or substrate reduction. Possibly ATP hydrolysis can be partially uncoupled from substrate reduction; in such cases the system would have a very low efficiency *i.e.*, high ratio of ATP: electrons used for reduction. Earlier work supports this possibility (ref. 18).

The difference in the optimum ratios of Protein 1 to 2 for maximum rate of reduction of isocyanide (or cyanide) compared with acetylene (Figs. 1 and 4) was observed previously[18]. One explanation for these observations is that isocyanide may bind and be reduced at two sites, one site on Protein 1 and the other on Protein 2.

An extension of this hypothesis which may explain the data presented in this paper is as follows: Protein 2 *plus* ATP interacts with $Na_2S_2O_4$-reduced Protein 1 to produce a metal hydride on Protein 2. Cyanide or isocyanide may complex either with Protein 1 or Protein 2 and are reduced by the Protein 2 hydride. Acetylene is bound and reduced stereospecifically only by the Protein 2 site. N_2 binding and reduction is postulated to have similarities with both that of cyanide or acetylene. The N_2 binds end on to Protein 1; the free end interacts with the metal hydride on Protein 2, and the N_2 bridge complex formed is rapidly reduced to NH_3. A two-metal site for N_2 reduction has been suggested before and complexes containing N_2 between two metals are known[35].

The low degree of cross reactions with N_2 as substrate (Table III) could be explained if the binding of this substrate involved both proteins since small differences in the stereospecific match of Protein 1 and 2 from different bacteria would then have most effect on N_2, least on cyanide, isocyanide or acetylene.

There is no direct evidence for a hydride site on Protein 2 and a metal in low reduction state could also explain the various observations. However $^2H_2/H_2O$ exchange and stereospecific reduction of acetylene are known to occur in chemical systems involving metal hydride[36], though as Fig. 3d shows the reduction of $C_2{}^2H_2$ is not completely stereospecific giving significant amounts of *trans*-$C_2{}^2H_2H_2$. CO, which inhibits substrate reduction but not H_2 evolution[20], might bind at the site on Protein 1 and Protein 2 or form a bridge complex[37] between the two metals as postulated for N_2.

The mechanism of N_2 fixation suggested above implies that the nitrogenase is a type of homogeneous hydrogenation catalyst with a specialised region enabling the system to complex and then reduce N_2. For this two metals are involved, conse-

quently other compounds are reduced, but the mechanism of their reduction differs from that for N_2. If this hypothesis is correct the various N_2 complexes now known[38] are unlikely to be reduced by any mechanism comparable with that of the biological system but two-metal complexes into which hydride can be introduced might catalyse N_2 fixation. For the biochemist the important conclusion may be that cyanide, isocyanide or acetylene are all valid assay procedures for determining the amount of active nitrogenase present but the data obtained using these substrates, may not always be directly applicable in explaining the mechanism of N_2 fixation.

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