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NITROGENASES OF *KLEBSIELLA PNEUMONIAE* AND *AZOTOBACTER CHROOCOCCUM*

COMPLEX FORMATION BETWEEN THE COMPONENT PROTEINS

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Summary

1. Sedimentation velocity analyses of mixtures of highly purified component proteins of *Azotobacter chroococcum* are consistent with the formation of a tight 1 : 1 complex in the absence of $Na_2S_2O_4$. 1 : 1 complex formation between complementary proteins from *A. chroococcum* and *Klebsiella pneumoniae* was also observed. The addition of 5 mM $Na_2S_2O_4$ weakened the interaction between the *A. chroococcum* proteins and also the interaction between complementary proteins of *A. chroococcum* and *K. pneumoniae*.

2. Steady-state kinetic data for acetylene reduction at low protein concentrations have been used to calculate association constants at 30°C for the 1 : 1 protein complexes of nitrogenase proteins from *A. chroococcum*, *K. pneumoniae* and mixtures of complementary proteins from both organisms. Values centred around $3 \cdot 10^7$ M^{-1} were obtained.

3. The temperature dependence of the association constant for the complex formed by the *K. pneumoniae* proteins exhibited a sharp break at 17°C with $\Delta H = 0$ and $\Delta H = 418$ kJ · mol^{-1} above and below 17°C, respectively.

4. The Arrhenius plot for acetylene reduction by the complex formed by the *K. pneumoniae* proteins was linear over the range 12—40°C with $\Delta H^{\ddagger} = 80$ kJ · mol^{-1}.

Introduction

Nitrogenase from all the sources studied to date consists of two non-haem iron proteins, one of which also contains molybdenum. The properties and composition of these proteins from different sources are very similar and often a protein from one organism will cross-react with a component from another organism to form a functional enzyme [1]. Both proteins are essential for all

catalytic activities of nitrogenase: neither protein has been shown to have any enzymic activity alone nor to catalyse any partial reactions involving either ATP or reducible substrates. In addition to dinitrogen a variety of substrate analogues, including acetylene, are reduced by the enzyme [2]. Mossbauer and kinetic EPR studies on nitrogenase from several organisms indicate a role for the Fe protein in the ATP-dependent transfer of electrons from $Na_2S_2O_4$ to the Mo-Fe protein [3].

Spectrophotometric stopped-flow kinetic data [4] showed that the component proteins of *Klebsiella pneumoniae*, Kp1 and Kp2*, must rapidly form a complex ($k > 10^7\ M^{-1} \cdot s^{-1}$) with an association constant, $K_{complex} > 2 \cdot 10^6\ M^{-1}$ at 23°C. Ultracentrifuge studies of mixtures of these proteins have shown tight 1 : 1 complex formation in the absence of $Na_2S_2O_4$ [5]. Other evidence for the existence of a more than transient complex between the two proteins during nitrogenase function is derived from steady-state kinetic studies on the enzymes from *Azotobacter vinelandii* [6] and soybean bacteroids [7]. A dilution effect (disproportionately low activity at low protein concentration) has been observed with nitrogenase isolated from various organisms [8–17]. Although in some cases [16,15], it was observed that this behaviour was consistent with dissociation of an active complex of the nitrogenase proteins no analysis of the data has been reported.

In this paper acetylene reduction data obtained at low protein concentrations have been analysed on the assumption that 'the dilution effect' can be attributed to the dissociation of the active 1 : 1 protein complex. Values of the association constant, ($K_{complex}$) have been calculated for both the *K. pneumoniae* and *Azotobacter chroococcum* proteins and for the heterologous cross-reactions involving a component from each organism, which together form a functional nitrogenase. The temperature dependence of $K_{complex}$ and the variation of the specific activity of the complex with temperature have been investigated for the nitrogenase of *K. pneumoniae*. The data obtained are compared with those available for complexes formed by other iron sulphur proteins.

Evidence for direct formation of homologous and heterologous 1 : 1 complexes between the nitrogenase proteins of *A. chroococcum* and *K. pneumoniae* was obtained from sedimentation studies of protein mixtures in the ultracentrifuge in the absence of ATP, a substrate which is essential for catalytic activity.

Materials and Methods

K. pneumoniae strain M5al was a gift from Dr P.W. Wilson, Department of Bacteriology, University of Wisconsin; *A. chroococcum* was a low-gum-producing variant of strain NCIB 8003 developed in this laboratory. Growth,

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* The nitrogenase components of various organisms are denoted by a capital letter indicating the genus, a lower case letter indicating the species and the number 1 or 2 indicating which of the protein components is referred to. The number 1 indicates the Mo- and Fe-containing protein, the number 2 the Fe-containing protein, Ac is *Azotobacter chroococcum*, Kp is *Klebsiella pneumoniae* and Cp is *Clostridium pasteurianum*.

harvesting and preparation of crude, cell-free extracts from these organisms have been described [18,19].

All biochemicals were purchased from Sigma (London) Chemical Co. Ltd, and all salts from British Drug Houses, Poole, Dorset.

Purification of nitrogenase proteins. Kp1 and Kp2 were purified as described [18,24]. The purification of Ac1 and Ac2 was achieved by similar procedure to that employed for Kp1 and Kp2 [19]. Each protein solution contained 25 mM Tris · HCl buffer, pH 7.4, 10 mM $MgCl_2$ and 0.1 mg/ml each of sodium dithionite and dithiothreitol. All proteins were stored in bead form in liquid nitrogen. The specific activities (expressed as nmol ethylene produced/min per mg of protein) of the proteins used in this work were, for the dilution effect, Kp1, 2100; Kp2, 1000; Ac1, 2000; Ac2, 2150; in ultracentrifuge experiments, Kp1, 1060; Kp2, 940; Ac1, 1800 and Ac2, 2000.

Measurement of specific activity. One unit of activity is defined as the amount of enzyme required to produce 1 nmol of product/min under the assay conditions of Eady et al. [18]. Specific activities refer to the concentration of whichever protein limited the rate of substrate reduction.

Homogeneity of nitrogenase components. The nitrogenase proteins of *K. pneumoniae* [18] and *A, chroococcum* [19] were homogeneous when tested by a variety of techniques including ultracentrifugation, disc electrophoresis and electrophoresis in the presence of sodium dodecylsulphate.

Molecular weights. The molecular weights used for Kp1 protein and Kp2 protein were 218 000 and 66 800, respectively [18]. Those of Ac1 protein and Ac2 protein were determined as described in ref. 19 and were 227 000 and 64 000, respectively.

Amounts of protein. Protein concentration was measured by the Folin-Ciocalteau method [20] using bovine serum albumin previously dried for 24 h over P_2O_5 as a standard; dry weight measurements have shown that no correction factor is necessary for the *K. pneumoniae* and *A. chroococcum* nitrogenase proteins [18,19].

Ultracentrifugation. Sedimentation coefficients were determined at 20° C in an An-D rotor, using Schlieren optics by using a Martin Christ 70 000 preparative ultracentrifuge fitted with an analytical attachment. Because of the extreme oxygen sensitivity of the nitrogenase proteins they were purified under anaerobic conditions in buffers containing 1 mM $Na_2S_2O_4$ to scavenge residual O_2. In experiments where $Na_2S_2O_4$-free proteins were used they were prepared by anaerobic gel chromatography on Sephadex G-25 as described by Eady [5]. In experiments where $Na_2S_2O_4$ was added to mixtures of the proteins the contents of the ultracentrifuge cell were tested after ultracentrifugation with methyl viologen to establish the presence of $Na_2S_2O_4$ which decomposes in the presence of nitrogenase proteins [18].

Measurement of nitrogenase activity. Acetylene reduction activity data as a function of protein concentration were obtained by adding an increasing volume (0.4—0.01 ml) of a protein solution containing a 1 : 1 molar ratio of the component proteins to the standard assay system described by Eady et al. [18]. Mg^{2+} and buffer concentrations were maintained constant over the range of protein concentrations. Assays of 10—40 min duration were used, the longer times being for the low temperature experiments. In all cases a linear activity vs

time relationship was observed. Ethylene detected in blank assays from which proteins were omitted was subtracted from readings for assays containing protein.

Calculation of protein complex association constant. Consider the equilibrium

$$A + B \rightleftharpoons AB \tag{1}$$

when A and B are the component nitrogenase proteins and AB the 1 : 1 protein complex. If the total concentration of $A=B=A_0$ then

$$K = x/(A_0 - x)^2 \tag{2}$$

where x is the concentration of protein complex AB and K the apparent association constant. Rearrangement of Eqn 2 gives

$$A_0 = x \pm \sqrt{x/K} \tag{3}$$

If acetylene reduction activity is associated only with the complex and not with the dissociated proteins then

$$A_0 = by \pm \sqrt{by/K} \tag{4}$$

where b is a constant which relates the rate of acetylene reduction y to the concentration of complex x. In order to allow for systematic errors in protein concentration or activity which may result in a non-zero intercept, an additional term, E is included so that

$$A_0 = by \pm \sqrt{(by/K)} + E \tag{5}$$

Since $A_0 > by$, the positive square root is taken. A least squares computer fit using a Newton-Raphson iteration was used to calculate the parameters K, b and E for a series of measured A_0 and y values.

Results

Ultracentrifuge studies

Sedimentation velocity analysis of an equimolar mixture of Ac1 protein and Ac2 protein under rigorously anaerobic conditions in the absence of $Na_2S_2O_4$ showed a single boundary (Fig. 1a). The boundary was asymmetric on the tailing edge (Fig. 1a) with a sedimentation coefficient of 12.6 S, a value significantly greater than 10.5 S obtained for Ac1 protein alone at the same concentration. At higher ratios of Ac2 protein to Ac1 protein, up to a molar ratio of 7 : 1 (Ac2 protein in excess) the sedimentation coefficient of the leading boundary did not change significantly (see Table I) but the boundary became sharper, more symmetrical and increased in area (compare Figs 1a(i) and 1b(i) where the Ac1 protein concentration is the same in both cases). At a molar ratio of 2 : 1 (Ac2 protein in excess) a diffuse tailing boundary was

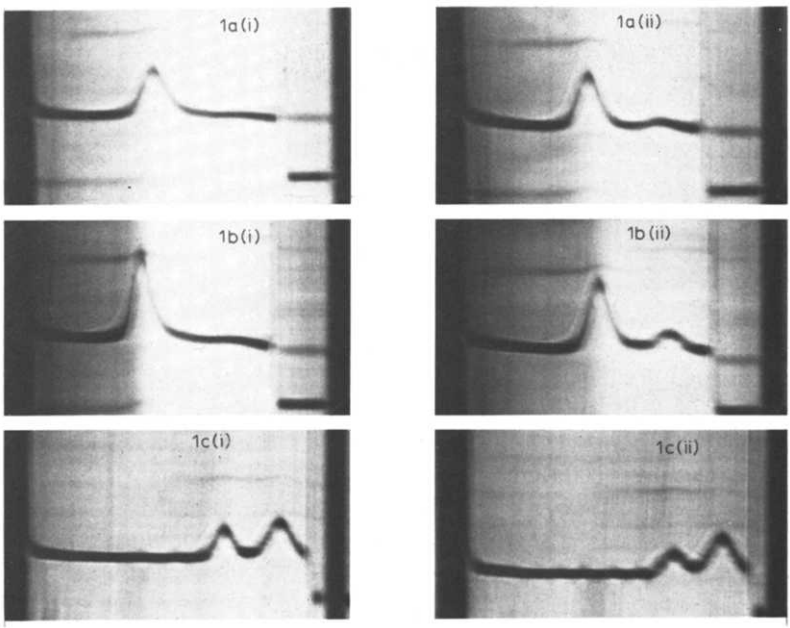

Fig. 1. Ultracentrifuge Schlieren patterns of mixtures of Ac1 protein and Ac2 protein showing the effect of the addition of $Na_2S_2O_4$. The pictures were taken at 32 min after reaching 58 400 rev./min for (a) and (b) and 50 000 rev./min for (c), in a Martin Christ 70 000 preparative ultracentrifuge fitted with an analytical attachment. Sedimentation at 20° C was from left to right under a gas phase of N_2. The proteins were in 25 mM Tris · HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, and, where indicated, 5 mM $Na_2S_2O_4$. The protein concentrations are those of Table I at the corresponding protein ratios. The molar ratios of Ac2 protein to Ac1 protein are (a) 1 : 1, (b) 1 : 2.1, (c) 1 : 7.5. The Schlieren diaphragm angle was 35° C. Mixtures in a(i), b(i) and c(i) contain no $Na_2S_2O_4$ and those in a(ii), b(ii) and c(ii) contain $Na_2S_2O_4$.

TABLE I

SEDIMENTATION COEFFICIENTS OF BOUNDARIES IN MIXTURES OF Ac1 PROTEIN AND Ac2 PROTEIN AND MIXTURES OF *A. CHROOCOCCUM* AND *K. PNEUMONIAE* NITROGENASE PROTEINS

Sedimentation coefficients were determined at 20° C in 25 mM Tris · HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and where indicated 5 mM $Na_2S_2O_4$ under an atmosphere of N_2. The ratio of the Mo-Fe protein and Fe proteins was varied as indicated. The molar ratios were calculated using molecular weights of 220000 for Kp1 protein, 227000 for Ac1 protein, 68000 for Kp2 protein and 64000 for Ac2 protein.

Mo-Fe protein concn (mg of protein/ml)	Fe protein concn (mg of protein/ml)	Molar ratio	Sedimentation coefficient of leading boundary		Sedimentation coefficient of tailing boundary
			Without $Na_2S_2O_4$	With $Na_2S_2O_4$	With $Na_2S_2O_4$
Ac1 protein	Ac2 protein	Ac1 : Ac2			
3.88	1.13	1 : 1	12.6	11.3	—
3.88	2.35	1 : 2.1	12.9	11.5	4.5
2.35	2.85	1 : 4.3	12.8	11.5	4.7
1.5	3.16	1 : 7.5	12.8	12	4.2
Ac1 protein	Kp2 protein	Ac1 : Kp2			
3.88	1.74	1 : 1.5	12.5	11.4	—
Kp1 protein	Ac2 protein	Kp1 : Ac2			
4.9	1.58	1 : 1	11.8	11.1	4.7

apparent, probably corresponding to free Ac2 protein since, at higher ratios where the peak was sharp enough to measure, the tailing peak corresponded in sedimentation coefficient to free Ac2 protein (Table I). These data indicate that, under these conditions, a single 1 : 1 complex predominates over a wide range of ratios of the two nitrogenase proteins.

The addition of 5 mM $Na_2S_2O_4$ to such mixtures of Ac1 protein and Ac2 protein produced pronounced changes in sedimentation behaviour. The area under the leading boundary and its sedimentation coefficient decreased (see Fig. 1 and Table I). At a 1 : 1 molar ratio a tailing boundary appeared (Fig. 1a(ii)) which was too diffuse to be measured accurately, but at the higher ratios the sedimentation coefficient of this boundary corresponded to free Ac2 protein. Although the addition of $Na_2S_2O_4$ to mixtures of Ac1 protein and Ac2 protein caused the sedimentation coefficient of the leading boundary to decrease, it was still larger than that of Ac1 protein alone under the same conditions (see Table I). In addition, comparison of Fig. 1a(ii) and Fig. 1b(ii) (where the Ac1 concentration is the same) shows that the leading boundary is higher and sharper at a molar ratio of 2 : 1 compared with 1 : 1. These data indicate that complex formation occurs in the presence of $Na_2S_2O_4$, but that binding is not so tight as in its absence.

Sedimentation velocity analysis of mixtures of *A. chroococcum* and *K. pneumoniae* at a 1 : 1 molar ratio in the absence of $Na_2S_2O_4$ showed essentially the same behaviour as that described above for mixtures of Ac1 protein and Ac2 proteins. The sedimentation coefficient of the Mo-Fe-containing protein increased in the presence of the Fe protein (see Table I). The increase was most marked in the Ac1-Kp2 combination, when the value obtained for the boundary of 12.5 S was the same as when Ac2 protein was used. When 5 mM $Na_2S_2O_4$ was added to such heterologous protein mixtures the sedimentation coefficient of the leading boundary decreased, but remained larger than that

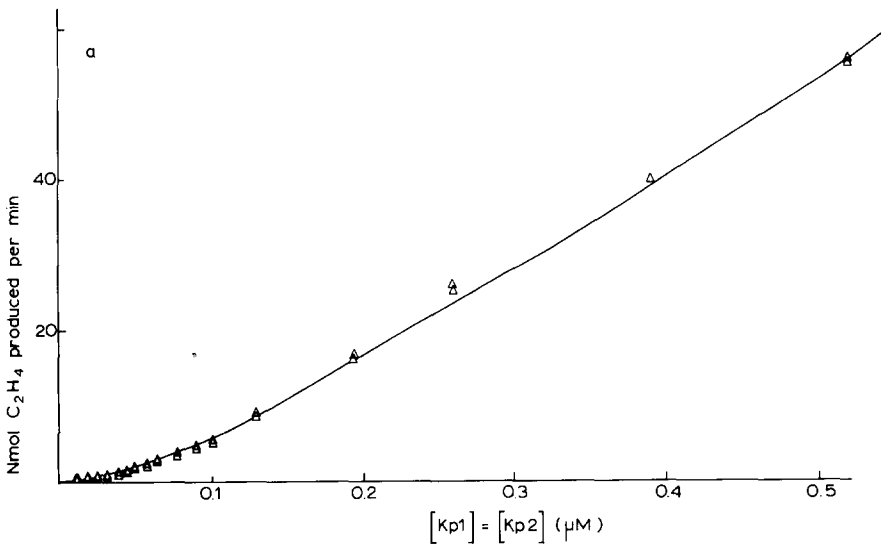

Fig. 2a.

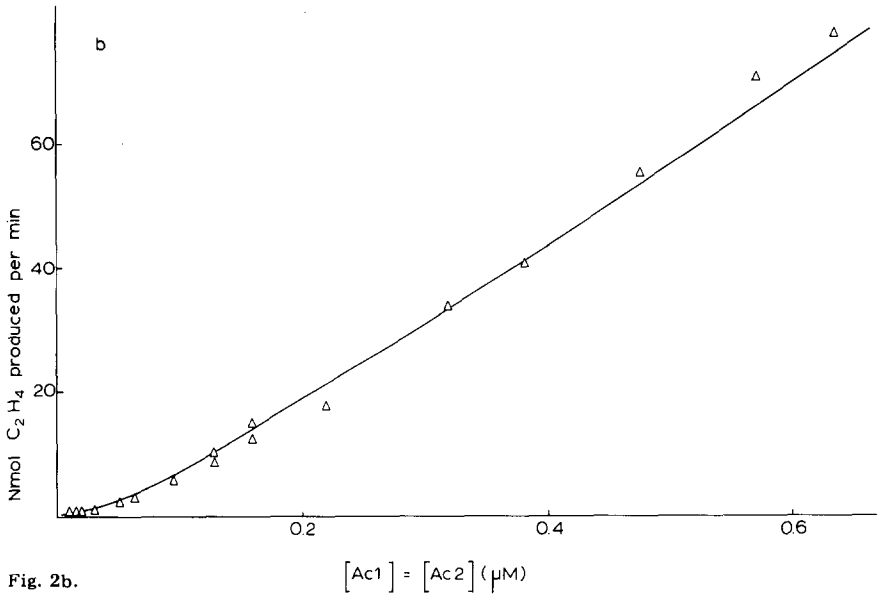

Fig. 2b.

obtained with the Mo-Fe protein when centrifuged alone. A slower sedimenting boundary, probably corresponding to free Fe protein, appeared in each case.

The dilution effect

1 ; 1 molar ratio of component proteins. Typical acetylene reduction activity data as a function of protein concentration for a 1 : 1 molar ratio of nitrogenase components of *K. pneumoniae* and *A. chroococcum* are shown in Fig. 2, together with data obtained with complementary proteins from these

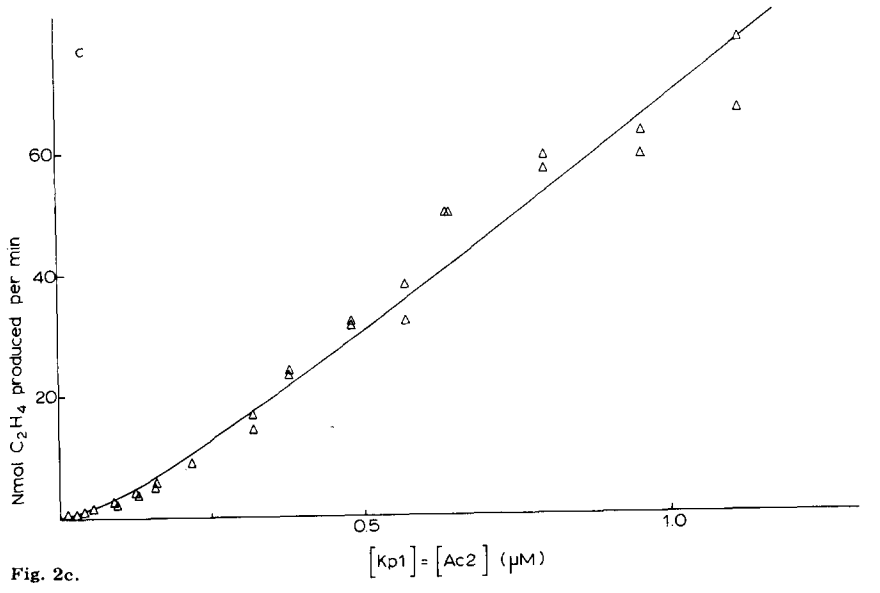

Fig. 2c.

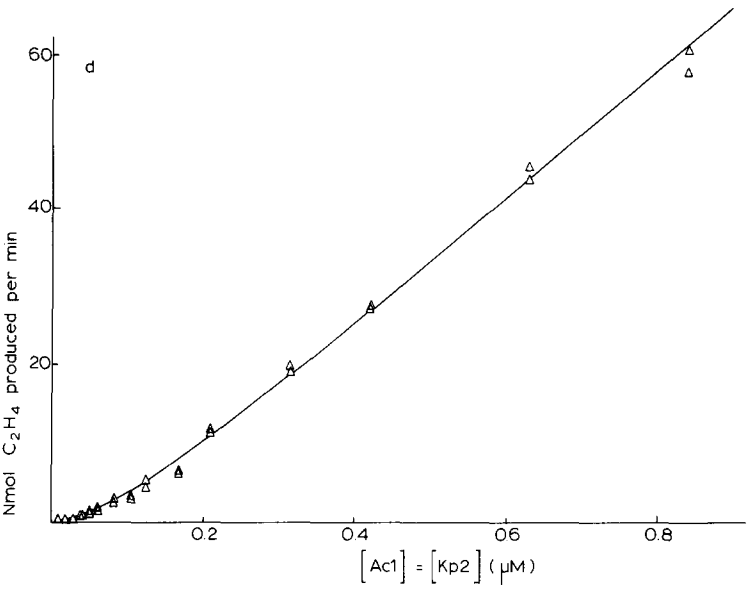

Fig. 2. Dilution effect curves obtained by plotting the rate of ethylene formation against protein concentration at 30°C for various combinations of the component proteins from the nitrogenases of *K. pneumoniae* and *A. chroococcum*. Lines are a computer fit to the experimental points. Assay conditions are described in the text. a, Kp1-Kp2; b, Ac1-Ac2; c, Kp1-Ac2; d, Ac1-Kp2.

organisms. The non-linearity of activity at low concentrations of nitrogenase proteins is evident in all cases. The lines drawn through the experimental points were those calculated according to Eqn 5. The computed values of the association constants for complex formation between the component proteins, ($K_{complex}$) are given in Table II. The error term E, Eqn 5, showed no systematic trend and was in the range $\pm 1 \cdot 10^{-8}$ M. The specific activities for the various 1 : 1 molar complexes, calculated from the computed values of b, Eqn 4, are given in Table II.

Excess of either component protein. Data for acetylene reduction for a 1 : 1 molar ratio of Kp1 : Kp2 proteins as a function of protein concentration are shown in Fig. 3, curve b, and may be compared with those obtained for a 1 : 12 molar ratio of Kp1 : Kp2 proteins (Fig. 3, curve a). At a given Kp1

TABLE II

ASSOCIATION CONSTANTS AND SPECIFIC ACTIVITIES OF THE NITROGENASE COMPLEX

Calculated association constants for complex formation between the component proteins of *K. pneumoniae* and *A. chroococcum* nitrogenases at 30°C, pH 7.8, and specific activities of the complex for acetylene reduction determined from the parameter b in Eqn 5.

Protein combination	$K_{complex}$ (M^{-1})	Specific activity of complex (nmol C_2H_4 produced/ min per mg of Ac2 or Kp2 protein)
Kp1 + Kp2	$2.0 \pm 0.6 \cdot 10^7$	2000 ± 100
Ac1 + Ac2	$5.5 \pm 1.5 \cdot 10^7$	2200 ± 100
Kp1 + Ac2	$2.8 \pm 0.5 \cdot 10^7$	1500 ± 200
Ac1 + Kp2	$1.4 \cdot 10^7$	1500

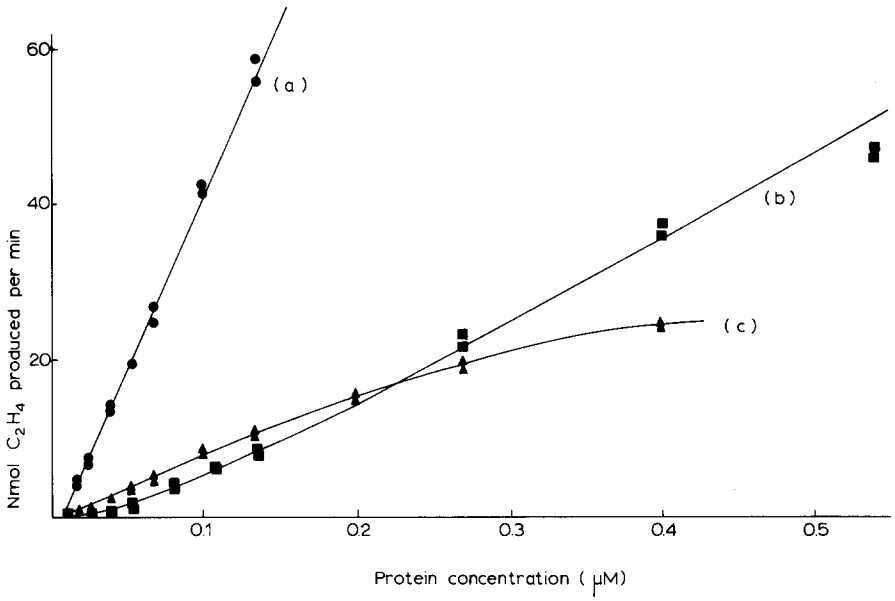

Fig. 3. Effect of increasing the molar ratio of the nitrogenase component proteins from *K. pneumoniae* on the rate of ethylene formation. a, molar ratio of Kp1 : Kp2 = 1 : 12, abscissa refers to concentration of Kp1 protein; b, molar ratio Kp1 : Kp2 = 1 : 1; c, molar ratio Kp1 : Kp2 = 8 : 1, abscissa refers to concentration of Kp2 protein. All data were obtained at 30°C, assay conditions are described in the text.

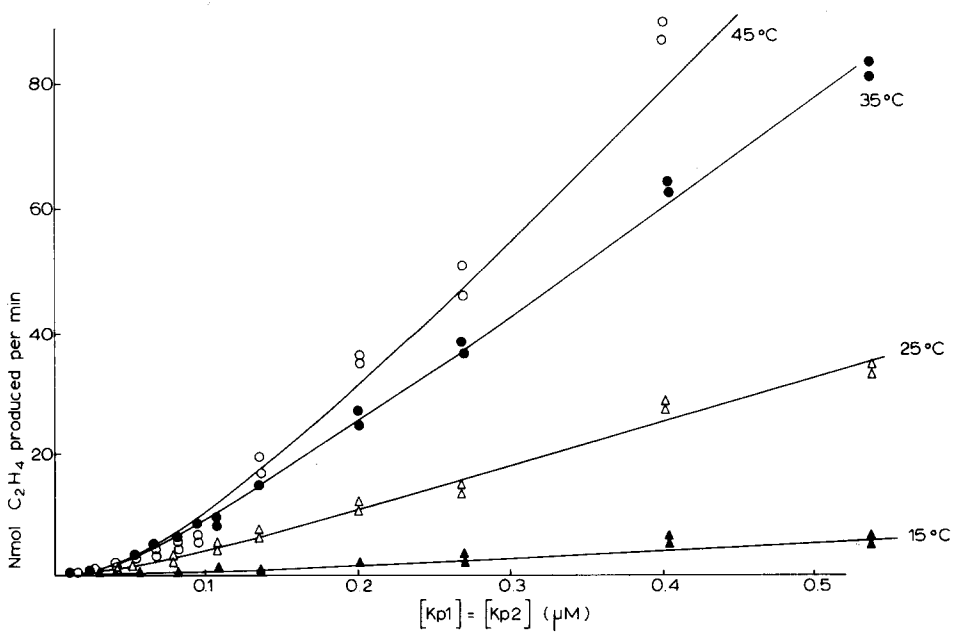

Fig. 4. Dilution effect curves at various temperatures obtained by plotting rate of ethylene production against *K. pneumoniae* protein concentration. Lines are a computer fit to experimental points. Assay conditions are described in the text.

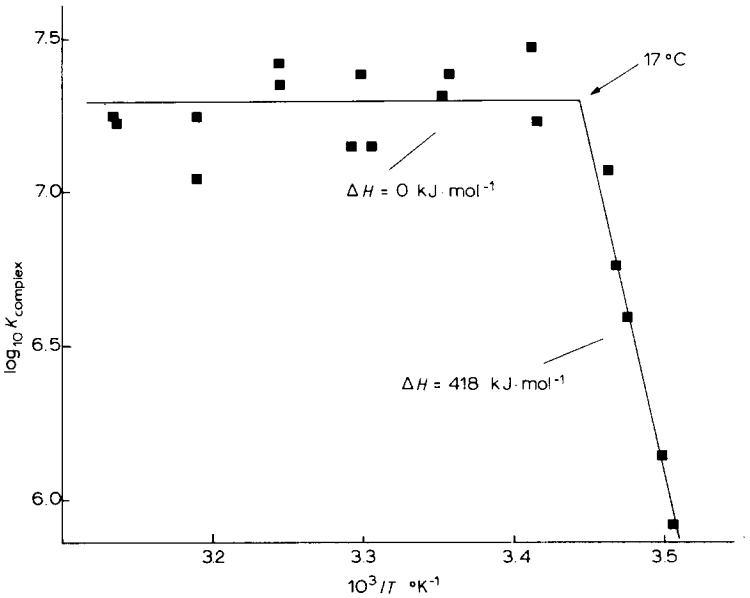

Fig. 5. Temperature dependence of the association constant $K_{complex}$ for the 1 : 1 Kp1-Kp2 protein complex. Values of $K_{complex}$ obtained by computer fit according to Eqn 5 at the various temperatures.

protein concentration, the excess of Kp2 protein caused about a 7-fold increase in acetylene reduction activity compared with that obtained with the 1 : 1 molar ratio of Kp1 : Kp2 protein. No curvature was discerned in the plot of the 1 : 12 (Kp1 : Kp2) protein data at the lower concentrations, i.e. excess Kp2

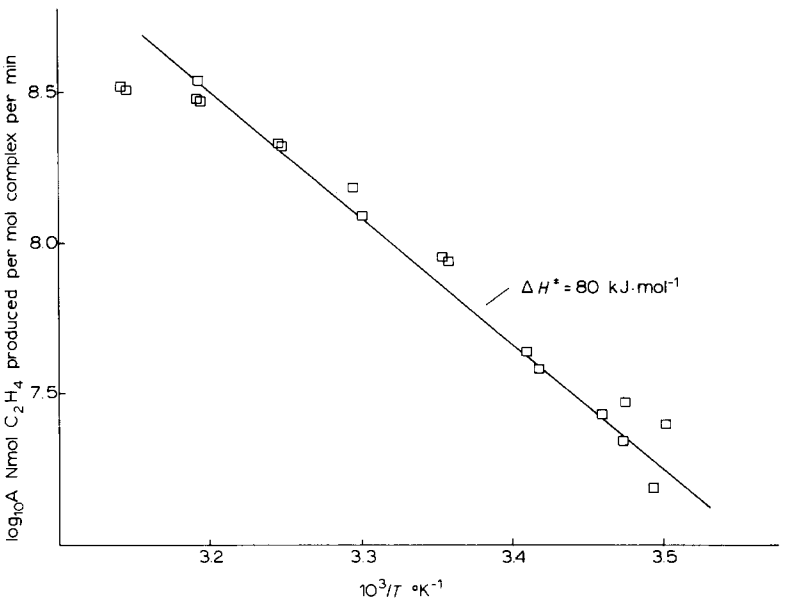

Fig. 6. Arrhenius plot for acetylene reduction activity for the Kp1-Kp2 1 : 1 protein complex. Activity of the complex at each temperature was obtained from the calculated value of b in Eqn 5.

overcame the dilution effect. Data for an 8 : 1 molar ratio of Kp1 : Kp2 proteins are given in Fig. 3, curve c. At the lower concentrations the enhancement of the acetylene reduction rate was only sufficient to overcome the dilution effect. At the higher concentrations the acetylene reduction rate was inhibited relative to the corresponding concentration for the 1 : 1 molar ratio of Kp1 : Kp2 proteins.

Effect of temperature

The temperature dependence of $K_{complex}$ for the Kp1 : Kp2 complex was determined from data such as that in Fig. 4. A plot of $\log_{10} K$ against $1/T(^\circ K)$, Fig. 5, showed that over the range 45—17°C, $K_{complex} = 2 \pm 0.6 \cdot 10^7$ M^{-1} with $\Delta H = 0$ kJ · mol^{-1} and $\Delta S = 140$ J · K^{-1} · mol^{-1}. Below 17°C, $K_{complex}$ was strongly temperature dependent, falling to $8.3 \cdot 10^5$ M^{-1} at 12°C with $\Delta H = 418$ kJ · mol^{-1} and $\Delta S = 1600$ J · K^{-1} · mol^{-1}. The precision of the data did not allow a distinction to be made between a sharp break in the Arrhenius plot at 17°C and a tight curve over the range 20—16°C. In contrast to this behaviour, the temperature dependence of the specific activity of the complex calculated from the values of b (Eqn 5) gave a linear Arrhenius plot with $\Delta H = 80$ kJ · mol^{-1} complex over the range 12—40°C, Fig. 6.

Discussion

A theoretical treatment of interacting macromolecules undergoing mass transport as in sedimentation velocity experiments [21] indicates that the properties of the faster sedimenting boundary give only a qualitative idea of the properties of the complex formed by the interacting species. The boundary is a reaction boundary at which the equilibrium between the Mo-Fe protein and the Fe protein is continually readjusting as a consequence of the differential transport of the complexed and individual proteins. The area of the leading peak is not necessarily a good measure of the concentration of the complex, and the sedimentation coefficient is intermediate between those of the heavier component and the complex, were the latter to exist in solution alone. However, the combining ratio between nitrogenase components can readily be determined from sedimentation velocity experiments because of the large difference in sedimentation coefficient between the two proteins. When complex formation between Ac1 protein $S^0_{20,w} = 9.9$ S [19] and Ac2 protein ($S^0_{20,w} = 4.3$ S) [19] occurred, the smaller protein was well resolved from the peak corresponding to the complex, and the changes in sedimentation coefficient of Ac1 protein which occurred on complex formation could easily be measured. Experiments of this type, with nitrogenase proteins from *K. pneumoniae* showed [5] tight binding in the absence of $Na_2S_2O_4$ to form a 1 : 1 complex. The addition of 2 mM $Na_2S_2O_4$ to mixtures of the *Klebsiella* proteins suppressed complex formation and the leading boundary corresponded in sedimentation coefficient to free Kp1 protein [5]. The data of Fig. 1 and Table I are consistent with a similar 1 : 1 molar complex being formed between *A. chroococcum* nitrogenase proteins. $Na_2S_2O_4$ changed the sedimentation coefficient of the leading boundary from 12.65 to 11.3 S, a value higher than the 10.5 S obtained with Ac1 protein alone at the same concentration. These data are

consistent with the interaction of the *Azotobacter* proteins still occurring but being weaker in the presence of $Na_2S_2O_4$, in contrast to the *Klebsiella* proteins where $Na_2S_2O_4$ suppresses such interaction. Mixtures of *A. chroococcum* and *K. pneumoniae* proteins interacted both in the presence and absence of $Na_2S_2O_4$. In the presence of $Na_2S_2O_4$, complex formation was tightest in the Ac1 protein-Kp2 protein mixture.

Because complex formation is weaker in the presence of $Na_2S_2O_4$, a normal component of the nitrogenase assay system the interactions between the nitrogenase proteins observed in the ultracentrifuge may not be relevant to those formed under assay conditions. However, 1 : 1 complexes of *K. pneumoniae* [18] and *A. chroococcum* (Yates, M.G., unpublished) proteins have been postulated on the basis of activity titration data, since optimum enzymic activity for the Fe-containing protein is obtained at a 1 : 1 molar ratio of the two proteins. The 'dilution effect' data reported here are also consistent with a 1 : 1 molar complex being formed. Since the binding of Mg^{2+}-ATP produces a conformational change in the Fe protein (see ref. 1) the effect of $Na_2S_2O_4$ of weakening or preventing complex formation may not occur if the Fe protein has the Mg^{2+}-ATP conformation state. Satisfactory analysis of the sedimentation behaviour of both proteins, Mg^{2+}-ATP and a reductant is not practicable because this system reduces H^+ to H_2 and produces ADP, an inhibitor of nitrogenase activity. The steady-state data for acetylene reduction as a function of protein concentration have been analysed assuming a single 1 : 1 equilibrium between the component proteins. Good agreement between the calculated and observed data points was obtained and the calculated association constant for the Kp1-Kp2 protein complex, $2.0 \pm 0.6 \cdot 10^7\ M^{-1}$ is consistent with the lower limit of $K_{complex} > 2.0 \cdot 10^6\ M^{-1}$ obtained in pre-steady-state stopped-flow studies of absorbance changes in Kp2 protein which occur during enzyme turnover [4]. However, the lower limit refers to the equilibrium between reduced Kp2 protein (in the Mg^{2+}-ATP-induced conformation) and Kp1 protein in the half-reduced (EPR active) state, while the apparent equilibrium constant obtained from steady-state data in this study is presumably for the equilibrium between oxidised Kp2 protein and wholly reduced (EPR inactive) Kp1 protein (EPR studies have shown that these oxidation states dominate the steady state [22]).

The association constants for the complexes Kp1-Kp2, Ac1-Ac2, Ac1-Kp2 and Kp1-Ac2 are in the range $1 \cdot 10^7$—$5 \cdot 10^7\ M^{-1}$ at 30°C and no large loss of stability is apparent for the heterologous protein complexes. These values are higher than that of $1 \cdot 10^4\ M^{-1}$ obtained for unresolved *A. vinelandii* nitrogenase from analysis of the dependence of H_2 evolution at various nitrogenase concentrations [6]. The difference may be due to species variation, although the similarity of the *K. pneumoniae* and *A. chroococcum* values makes this improbable. It is more likely a consequence of the fact that unresolved *A. vinelandii* preparations contain a large excess of Av1 over Av2 protein since the value of 5 obtained for the ATP/2*e* ratio is characteristic of this situation [1,23].

The specific activity for the Fe proteins in the 1 : 1, Ac1-Ac2, Ac1-Kp2 and Kp1-Ac2 complexes obtained from the values of *b* (Eqn 5) are in good agreement (Table II) with the experimental values obtained in activity titration

curves [19,24]. However, the value calculated for Kp2 protein in the Kp1-Kp2 complex is about double that experimentally observed at higher concentrations [18]. At the higher concentrations inhibition by excess Kp1 has been observed [18] and additional inhibitory equilibria involving Kp1 may prevent the expression of the potential activity of the 1 : 1 Kp2 protein complex. The data at a 8 : 1 Kp1 : Kp2 protein ratio also support this explanation, since inhibition, relative to the 1 : 1 Kp1 : Kp2 protein ratio data, occurred at [Kp2] $> 2 \cdot 10^{-7}$ M. The data (Fig. 3) also show that, at the lower concentrations, the dilution effect can be overcome by increasing the concentration of Kp1 protein. This is presumably a mass action effect on the reversible equilibrium binding of Kp1 to Kp2 protein. The analysis applied to the data above must be a simplification since addition of excess Fe protein produced an increase in activity greater than that predicted from the displacement of the 1 : 1 Kp1 : Kp2 protein equilibrium (Fig. 3). At higher protein concentrations, when complex formation must be essentially complete, maximal activity for acetylene reduction for Kp1 and Ac1 proteins was not obtained until at least a 10-fold excess of Kp2 or Ac2 protein was present [18,19]. Thorneley [4] has discussed the possibility of electron transfer by excess Fe protein to a complex of Fe and Fe-Mo protein and suggested that the high molar ratio of Kp2 : Kp1 required may be a reflection of the slow rate of reduction by $Na_2S_2O_4$ of oxidised Fe protein during nitrogenase turnover, as is indicated by the loss of EPR signal from Kp2 protein in the steady state [22]. However, titration of Ac2 or Kp2 protein with increasing Ac1 or Kp1 protein, does give maximal activity at a 1 : 1 protein ratio [18,24]. Further complications arise since excess Mo-Fe protein inhibits substrate reduction and there is some evidence that Ac1 (but not Kp1) protein dissociates at concentrations less than 3 mg/ml in the ultracentrifuge [18,19]. The distribution of electrons between the two substrates available in this study (H^+ and C_2H_2) also depends on the concentration of Mg^{2+}-ATP [25] and the component:protein ratio [18]. At the low concentrations of protein necessary in order to observe the 'dilution effect', the sensitivity of the detection equipment was not sufficient to enable H_2 production concomitant with C_2H_4 to be monitored.

The break in the temperature dependence of the association constant for the Kp1-Kp2 complex implies a conformation change in one of the proteins, occurring close to 17°C, which leads to a rapid loss of complex stability below this temperature. This conformation change may be in the Kp1 protein since Dr W.H. Orme-Johnson (personal communication) has recently observed a sharp increase in the rate of reaction of CO with Cp1 protein above 15°C: little or no reaction was observed below this temperature. Burns [26] has reported a break in the Arrhenius plot for acetylene reduction by *A. vinelandii* nitrogenase at 21°C with apparent activation energies of $\Delta H^{\ddagger} = 63$ kJ · mol^{-1}, $\Delta H^{\ddagger} = 150$ kJ · mol^{-1}, above and below 21°C, respectively. Since ΔH^0 for Kp1-Kp2 complex formation is zero above 17°C, the activation energy, $\Delta H^{\ddagger} = 80$ kJ · mol^{-1} for acetylene reduction by the complex obtained in this study can be compared with the $\Delta H = 63$ kJ · mol^{-1} obtained by Burns [26]. Below 21°C, Burns's value is probably a composite of ΔH^0 for complex formation and $\Delta H^{\ddagger}$ for acetylene reduction.

A comparison is made in Table III of thermodynamic and kinetic data for

TABLE III

COMPARISON OF DATA FOR 1 : 1 COMPLEX FORMATION BETWEEN IRON-SULPHUR ELECTRON TRANSFER PROTEINS

Source	Proteins	Association constant K (M^{-1})	ΔH ($kJ \cdot mol^{-1}$)	ΔS ($J \cdot K^{-1} \cdot mol^{-1}$)	Rate of formation ($M^{-1} \cdot s^{-1}$)	Comments	Reference
K. pneumoniae	Nitrogenase component proteins	$2.0 \pm 0.6 \cdot 10^7$	0	+ 140	$>10^7$	For temperature range 20—40°C	This work and ref. 4
A. chroococcum	Nitrogenase component proteins	$5.5 \pm 1.5 \cdot 10^7$	—	—	—	At 30°C	This work
Spinach	Ferredoxin-ferredoxin-NADP reductase	$2.0 \cdot 10^7$	≈0	≈+ 130	$>10^8$	Temperature range 5—30°C Extrapolated to zero ionic strength	Ref. 27
Pseudomonas oleovorans	Rubredoxin-rubredoxin-NAD reductase	$4.8 \cdot 10^6$	—	—	—	At 20°C	Ref. 28

complex formation involving iron-sulphur proteins. The similarity between the parameters for the *K. pneumoniae* nitrogenase proteins and the spinach ferredoxin-NADP reductase system is quite remarkable. The rates of complex formation must be very close to or at the diffusion controlled limit. Foust et al. [27] for the ferredoxin-NADP reductase system have argued that the thermodynamic parameters, together with a pH and ionic strength dependence of $K_{complex}$, favour an electrostatic interaction between the proteins. Consistent with this view, an inhibition of acetylene reduction and suppression of complex formation at high ionic strength (50 mM $MgCl_2$) has been reported for *K. pneumoniae* nitrogenase [29].

The rapid loss of stability of the protein complex with decreasing temperature below 17°C, which we have interpreted as a consequence of a conformation change in one of the component proteins of *K. pneumoniae* nitrogenase occurring close to 17°C, may be significant with regard to the recent isolation in this laboratory, with an unexpectedly high frequency, of cold sensitive mutants of *K. pneumoniae* over the temperature range 16–22°C (acetylene reduction test) (Krishnapillai, V., personal communication). It is quite conceivable that a single amino acid change could impose a conformation of one of the proteins which could destabilise the active nitrogenase complex sufficiently to essentially prevent complex formation at the protein concentrations in vivo.

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