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Nitrogenases from *Klebsiella pneumoniae* and *Clostridium pasteurianum*

KINETIC INVESTIGATIONS OF CROSS-REACTIONS AS A PROBE OF THE ENZYME MECHANISM

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In combination with the Mo–Fe protein of nitrogenase from *Klebsiella pneumoniae*, the Fe protein of nitrogenase from *Clostridium pasteurianum* forms an active enzyme with novel properties different from those of either of the homologous nitrogenases. The steady-state rates of reduction of acetylene and H^+ are 12% of those of the homologous system from *C. pasteurianum*. Acetylene reductase activity exhibited an approx. 10min lag at 30°C before the rate of reduction became linear, consistent with a once-only activation step being necessary for acetylene reduction to occur. No such lag was observed for H_2 evolution. The activity with N_2 as a reducible substrate was very low, implying that acetylene reductase activity is not necessarily an accurate indication of nitrogen-fixing ability. This is of particular relevance to studies on mutant and agronomically important organisms. Stopped-flow spectrophotometric studies showed unimolecular electron transfer from the Fe protein to the Mo–Fe protein to occur at the same rate ($k_2 = 2.5 \times 10^2 s^{-1}$) and with the same dependence on ATP concentration (apparent $K_D = 400 \mu M$) as with the homologous *Klebsiella* nitrogenase. However, an ATP/2e ratio of 50 was obtained for H_2 evolution, indicating that ATP hydrolysis had been uncoupled from electron transfer to substrate. These data indicate that ATP has at least two roles in the mechanism of nitrogenase action. The combination of the Mo–Fe protein of nitrogenase of *C. pasteurianum* and the Fe protein of *K. pneumoniae* were inactive in all the above reactions, except for a weak adenosine triphosphatase activity, 0.5% of that of the homologous *K. pneumoniae* system.

Nitrogenase, the enzyme responsible for nitrogen fixation, has been isolated and purified from a number of micro-organisms, and always consists of two oxygen-sensitive iron–sulphur proteins (see Eady & Postgate, 1974; Zumft & Mortenson, 1975; and references therein). The larger of these proteins (Mo–Fe protein, Protein 1, molybdoferredoxin) has a mol.wt. of about 220000 and contains Mo as well as Fe and acid-labile sulphur. The smaller protein (Fe protein, Protein 2, azoferredoxin) has a mol.wt. between 55000 and 67000, depending on the source, and contains no other metals. Both proteins are essential for enzymic activity together with ATP (which is hydrolysed to $ADP + P_i$), a bivalent metal ion (usually Mg^{2+}), a source of electrons (usually $Na_2S_2O_4$ *in vitro*) and an anaerobic environment. In addition to the reduction of N_2 to NH_3, the enzyme can reduce acetylene (to ethylene), and also a number of other small triple-bonded substrate molecules, which may be regarded as N_2 analogues. In the absence of such substrates it reduces H^+ to H_2; in their presence, this reaction is partially suppressed so that, with acetylene and N_2 at least, the total electron flow through the enzyme remains constant (R. R. Eady, A. Funnell & B. E. Smith, unpublished work).

MgATP binds to the Fe protein (Bui & Mortenson, 1968; Tso & Burris, 1973), causes a conformational change (Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972; Smith *et al.*, 1973; Thorneley & Eady, 1973; G. A. Walker & Mortenson, 1974) and lowers its redox potential from −290mV to −400mV (Zumft *et al.*, 1974; Burris & Orme-Johnson, 1976). The Mo–Fe protein binds the reducible substrate C_2H_2 (Smith *et al.*, 1973). In the presence of MgATP, electrons are transferred from the Fe protein to the Mo–Fe protein to form a 'super-reduced' species (Smith *et al.*, 1972, 1973; Orme-Johnson *et al.*, 1972; Smith & Lang, 1974; M. N. Walker & Mortenson, 1974). Substrate reduction can then occur. The rate-limiting step has not yet been identified, but protein–protein electron transfer occurs at a rate much faster than enzyme turnover (Smith *et al.*, 1973; Thorneley, 1975).

The transfer of two electrons to reducible substrates is accompanied by the hydrolysis of at least four ATP molecules, but this ATP/2e ratio depends on the temperature (Hadfield & Bulen, 1969; Watt *et al.*, 1975), pH (Jeng *et al.*, 1970) and the ratio of the amounts of the nitrogenase components (Ljones & Burris, 1972; Eady & Postgate, 1974). It is not known how ATP hydrolysis is coupled to electron transfer;

however, some results suggest that ATP may be involved in more than one step in the transfer of electrons to reducible substrates (Eady *et al.*, 1975).

There have been a number of reports on the formation of functional nitrogenase enzymes from the Mo–Fe protein isolated from one organism and the Fe protein isolated from another (see Burris, 1971, and references therein; Smith *et al.*, 1971; Biggins *et al.*, 1971; Murphy & Koch, 1971). Most of these studies have used preparations of uncertain purity and often only single and possibly non-optimal concentrations of the two proteins. The nitrogenase components from aerobic and anaerobic organisms generally do not cross-react; the components from facultative anaerobic organisms will usually cross-react with those from either aerobic or anaerobic organisms.

The nitrogenase proteins from *Clostridium pasteurianum* have been reported to generate only one active, heterologous cross-reaction, namely that between Cp1* and Bp2 proteins. The heterologous cross-reaction, with N_2 as the reducible substrate, between nitrogenase proteins from *Klebsiella pneumoniae* and *C. pasteurianum* has been reported as being inactive (Detroy *et al.*, 1968). In the present paper we report the novel properties of the enzyme formed by the highly purified nitrogenase proteins from these two organisms, and demonstrate the potential of cross-reactions of this type for studying the mechanism of nitrogenase action.

Materials and Methods

Growth of organisms and purification of nitrogenase proteins

C. pasteurianum W5 and *K. pneumoniae* M5al were grown, and the nitrogenase proteins from these organisms purified by methods previously described (Eady *et al.*, 1972; Zumft & Mortenson, 1973; Smith *et al.*, 1976). These methods yielded nitrogenase proteins with the following specific activities in the acetylene-reduction assay at optimal pH: Cpl, 1100; Cp2, 1030; Kp1, 1700; and Kp2, 1130 (nmol of acetylene produced/min per mg of protein).

The mol.wts. used were 220000 for Kp1 and Cp1 proteins, 55000 for Cp2 protein and 67000 for Kp2 protein (Huang *et al.*, 1973; Eady *et al.*, 1972; Nakos & Mortenson, 1971).

* Abbreviations: The nitrogenase proteins from various organisms are denoted by a capital letter indicating genus, a lower case letter indicating the species and either the number '1' indicating Mo–Fe protein or '2' indicating Fe protein. Kp is *Klebsiella pneumoniae*, Cp is *Clostridium pasteurianum*, Bp is *Bacillus polymyxa*, Av is *Azotobacter vinelandii*, Ac is *Azotobacter chroococcum*.

Assay procedures

Production of ethylene from acetylene, H_2 from H^+ and NH_3 from N_2 by nitrogenase were measured by established techniques (see Eady *et al.*, 1972). The published assay systems for the nitrogenases from *C. pasteurianum* (Dalton *et al.*, 1971) and *K. pneumoniae* (Eady *et al.*, 1972) contain the same components, but in different concentrations, and also differ in pH. For the cross-reactions described in the present paper, the *K. pneumoniae* assay system was used.

To improve the sensitivity of the H_2 evolution assay, 2ml in place of 7.8ml assay bottles were used.

The ATPase (adenosine triphosphatase) activity of 0.1mg of Cp2 protein + 0.4mg of Kp1 protein was measured in a 1ml assay system containing 10mM-Mg^{2+} and 10mM-ATP in 25mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]/NaOH buffer at pH7.8, without an ATP-regenerating system, to avoid the problems involved in assaying P_i in the presence of creatine phosphate. Reactions were stopped by addition of 0.1ml of trichloroacetic acid (30%, w/v), the reagents cooled in an ice bath and all subsequent operations conducted at 0°C to minimize further ATP hydrolysis. H_2 in the gas phase was determined on a 0.5ml gas sample which was withdrawn from the assay bottle after addition of 0.5ml of ice-cold water. P_i was measured on 1ml of the final solution by a modification of the method of Taussky & Shoor (1954). At 0°C a 30min incubation was required for complete development of the blue colour of reduced phosphomolybdic acid, which was then estimated by measuring the E_{815} with a Perkin–Elmer 55 spectrophotometer. Before measurements were made, the solutions were clarified by centrifugation at 5000*g* for 3min. Control assays in the presence of MgATP were carried out on the individual proteins, alone and with the homologous complementary proteins, on Cp2 and Kp1 proteins added after the addition of trichloroacetic acid and on MgATP alone to obtain suitable blanks.

$Na_2S_2O_4$ oxidation assays

The rate of $Na_2S_2O_4$ oxidation was measured at 30°C in a stopped-flow spectrophotometer by following the decrease in E_{350}nm. An experimentally determined value of 1200litre·mol^{-1}·cm^{-1} for the molar extinction coefficient of $S_2O_4^{2-}$ at 350nm was used. The assay contained Kp1 protein and Cp2 protein (both 44μM), 5mM-ATP, 10mM-$MgCl_2$, 20mM-creatine phosphate, creatine kinase [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH U.K.; 0.1mg/ml] and 0.6mM-$Na_2S_2O_4$ in N_2-saturated 25mM-Hepes/NaOH buffer at pH7.4.

Stopped-flow spectrophotometric studies

These were carried out in a commercial stopped-flow apparatus (American Instrument Co., Silver Spring, MD, U.S.A.) modified as described by Thorneley (1974). The buffer used throughout was 25mM-Hepes/NaOH, pH7.4.

E.p.r. (electron-paramagnetic-resonance) studies

Samples were prepared in 25mM-Hepes/NaOH buffer, pH7.4. The reaction, at 21°C, was initiated anaerobically by addition of MgATP and quenched by freezing the contents of the e.p.r. tube by immersion of the tube in an isopentane bath cooled to approx. −140°C. The procedure for measuring e.p.r. spectra has been described by Smith *et al.* (1973).

Results

Cross-reactivity of Cp2 protein with Kp1 protein

Time-course of substrate reduction. The enzyme formed by Cp2 and Kp1 proteins at a 1:1 molar ratio showed a pronounced time-lag of 8–10min at 30°C before the rate of acetylene reduction became linear with a specific activity of 51nmol of ethylene produced/min per mg of Cp2 protein (see Fig. 1). By contrast, the rate of acetylene reduction by an equivalent nitrogenase from either *K. pneumoniae* or *C. pasteurianum* was linear after initiation of the reaction by the addition of protein to the assay system. Owing to the difficulty of starting and stopping assays of this type at such short time-intervals, these data for the homologous proteins cannot exclude a 10–15s lag for acetylene reduction.

Unlike acetylene reduction, H_2 evolution by the Cp2/Kp1 protein mixture did not show a lag (Fig. 2). At a 1:1 molar ratio of proteins, a specific activity of 53nmol of H_2 evolved/min per mg of Cp2 protein was observed in assays containing an ATP-regenerating system, in good agreement with the specific activity of 58nmol of $S_2O_4^{2-}$ oxidized/min per mg of Cp2 protein obtained in the stopped-flow apparatus, and the final linear rate of acetylene reduction. These specific activities are approx. 12% of those obtained for Cp2 protein in combination with Cp1 protein at a 1:1 molar ratio at the same pH.

Dependence of activity on protein concentration. When a 1:1 molar ratio of a mixture of Cp2 and Kp1 proteins was added in decreasing amounts to a series of assays, the acetylene reduction activity remained linear with protein concentration down to 8μg of protein/ml, corresponding to [Cp2] = [Kp1] = 29nM. Below this concentration the activity was disproportionately low.

Activity titration of Kp1 protein with Cp2 protein. When increasing amounts of Cp2 protein were added to assays containing a fixed amount of Kp1 protein, the ATP-dependent hydrogenase activity increased rapidly from zero to a plateau at an approximate molar ratio (Cp2/Kp1) of 2:1. The activity with N_2 as a reducible substrate was very low. With 37min assays, a specific activity of about 1.4nmol of NH_3 produced/min per mg of Cp2 protein was obtained; this is close to the limits of the sensitive indophenol method (Chaney & Marbach, 1962) used.

Activity titration of Cp2 protein with Kp1 protein. When increasing amounts of Kp1 protein were added to assays containing a fixed amount of Cp2 protein, the ATP-dependent hydrogenase activity increased rapidly from zero to a plateau at an approximate molar ratio (Cp2/Kp1) of 2:1. In contrast with the homologous *Klebsiella* nitrogenase (Eady *et al.*, 1972) no inhibition by excess of Kp1 protein was observed even at a 6:1 molar ratio. Again the activity with N_2 as the reducible substrate was so low as to be at the limit of the reliability of the analytical methods used.

Electron-paramagnetic-resonance studies. When Kp1 and Cp2 proteins (both 31μM) were made to react with 5mM-ATP in N_2-saturated buffer containing 10mM-$MgCl_2$, an approximately 50% loss of intensity of the $g = 3.7$ line from the Kp1-protein

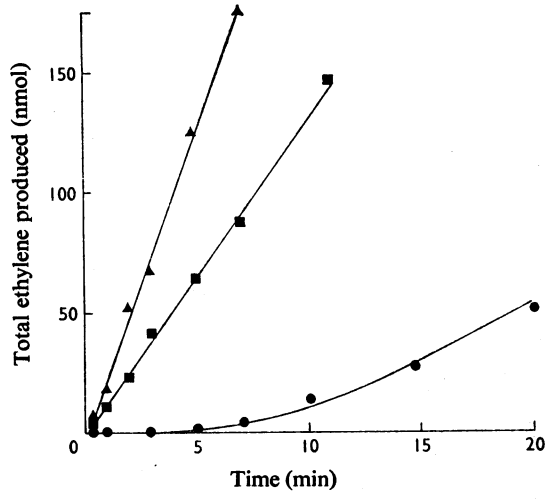


Fig. 1. *Time-course of acetylene reduction by homologous and heterologous nitrogenase systems*

Acetylene reduction was measured at pH7.8 as described in the Materials and Methods section. Individual assays were stopped at the times indicated and the C_2H_4 formed was measured by g.l.c. Homologous nitrogenase systems contained: ▲, 65μg of Kp1 protein plus 20μg of Kp2 protein; ■, 55μg of Cp1 protein plus 24μg of Cp2 protein; the heterologous nitrogenase system, ●, contained 475μg of Kp1 protein plus 120μg of Cp2 protein in a 1ml assay.

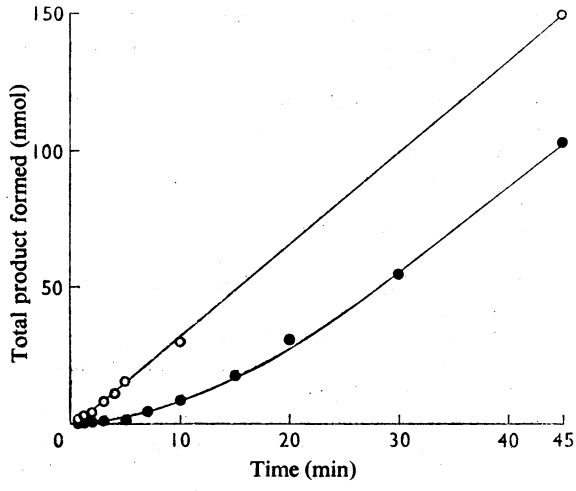


Fig. 2. *Time-course of H_2 evolution compared with acetylene reduction by the Cp2/Kp1 enzyme*

H_2 evolution (○) was measured, at pH7.8, with assays containing a creatine kinase/creatine phosphate ATP-regenerating system as described in the Materials and Methods section. Individual assays were stopped at the times indicated and the H_2 formed was measured by g.l.c. Each assay contained 97μg of Cp2 + 345μg of Kp1 proteins. ●, Acetylene reduction, as in Fig. 1; note that at long times the rates of acetylene reduction and H_2 evolution become similar.

e.p.r. spectrum was observed after 90s reaction time. This demonstrated MgATP-induced electron transfer from Cp2 to Kp1 protein similar to that observed with the homologous nitrogenases (Smith *et al.*, 1972, 1973; Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972). An extensive investigation into the time-course and the extent of the loss of intensity of the Kp1 protein e.p.r. spectrum, and its dependence on reducible substrates, will be necessary to determine whether the H^+- and acetylene-reducing forms of the enzyme can be defined in terms of their e.p.r. parameters.

Stopped-flow spectrophotometric studies on the Cp2/Kp1 enzyme. When Cp2 and Kp1 proteins (both 14μM) were mixed in the stopped-flow apparatus with 10mM-MgATP at 23°C, a rapid increase in E_{420} was observed, with a time-constant $\tau = 7.7 \pm 0.3$ms and an amplitude of 0.011 ± 0.001 in a single exponential process. In Fig. 3 we compare this experiment with those carried out on the homologous *K. pneumoniae* ($\tau = 5.6 \pm 0.3$ms; $\Delta E = 0.011$) and *C. pasteurianum* ($\tau = 14 \pm 1$ms; $\Delta E = 0.012$) nitrogenases under comparable conditions. Increasing the Cp2/Kp1 molar protein ratio to 4:1 ([Cp2 protein] = 60μM) did not affect τ, but increased the amplitude about threefold, to 0.030 ± 0.002.

The dependence of τ on MgATP concentration for a 1:1 molar mixture of Cp2 and Kp1 proteins (both 14μM) is shown in Fig. 4. The observed linear dependence of τ on $[MgATP]^{-1}$ is virtually identical with that found with *K. pneumoniae* nitrogenase (Thorneley, 1975), which was consistent with Scheme 1. Analysis of the data for the Cp2/Kp1 enzyme on the basis of Scheme 1 yielded $k_{+2} = 2.5 \times 10^2 s^{-1}$ ($2 \times 10^2 s^{-1}$) and $K_{D(MgATP)} = 400\mu M$ (400μM), where the values in parentheses refer to the corresponding constants for the homologous Kp1/Kp2 nitrogenase.

ATPase activity and the ATP/2e ratio. C. pasteurianum nitrogenase is normally assayed at pH7.0 (Dalton *et al.*, 1971) but at pH7.8, a 1:1 molar mixture of Cp1 and Cp2 proteins showed a decreased specific activity for both reduction of acetylene and H^+, with values of about 440nmol of product formed/min per mg of Cp2 protein for both substrates. However, the ATPase activity, at 5300nmol of PO_4^{3-} released/min per mg, was approximately that expected at pH7.0 (Ljones & Burris, 1972). Thus at pH7.8 the ATP/2e ratio for a 1:1 molar ratio mixture of Cp1 and Cp2 proteins was 12.

To determine the ATP/2e ratio for the Cp2/Kp1 nitrogenase, the ATPase activity and the rate of concomitant H_2 evolution was measured. In assays of up to 5min duration containing 10mM-Mg^{2+} and 10mM-ATP, but no ATP-regenerating system, a 1:1 molar mixture of Cp2 and Kp1 proteins showed a linear ATPase activity of 2100nmol of PO_4^{3-} produced/min per mg of Cp2 protein with H_2 evolution at 85–90% of that observed in assays with an ATP-regenerating system. The ATPase activity was about 40% of that observed with the homologous *C. pasteurianum* nitrogenase under comparable conditions and gave an ATP/2e ratio of about 50. Neither Cp2 nor Kp1 protein had any ATPase activity when assayed alone.

Cross-reactivity of Kp2 protein with Cp1 protein

Combination of Kp2 protein with Cp1 protein did not result in enzymic activity as determined by C_2H_2 reduction or H_2 evolution. The use of a relatively long (35min) incubation period, and high protein concentrations, would have permitted the detection of an activity of less than 0.01% that of the potential activity of these proteins when assayed with their homologous proteins. In addition, no changes in E_{420} were observed when the potentially active system was monitored in the stopped-flow apparatus (Fig. 3). However, a 1:1 molar mixture of Kp2 and Cp1 proteins in assays containing 10mM-ATP and 10mM-Mg^{2+} did exhibit weak ATPase activity with a specific activity of 30nmol of PO_4^{3-} released/min per mg of Kp2 protein, about 0.5% of the activity in a 1:1 molar Kp2/Kp1 mixture. Neither Kp2 nor Cp1

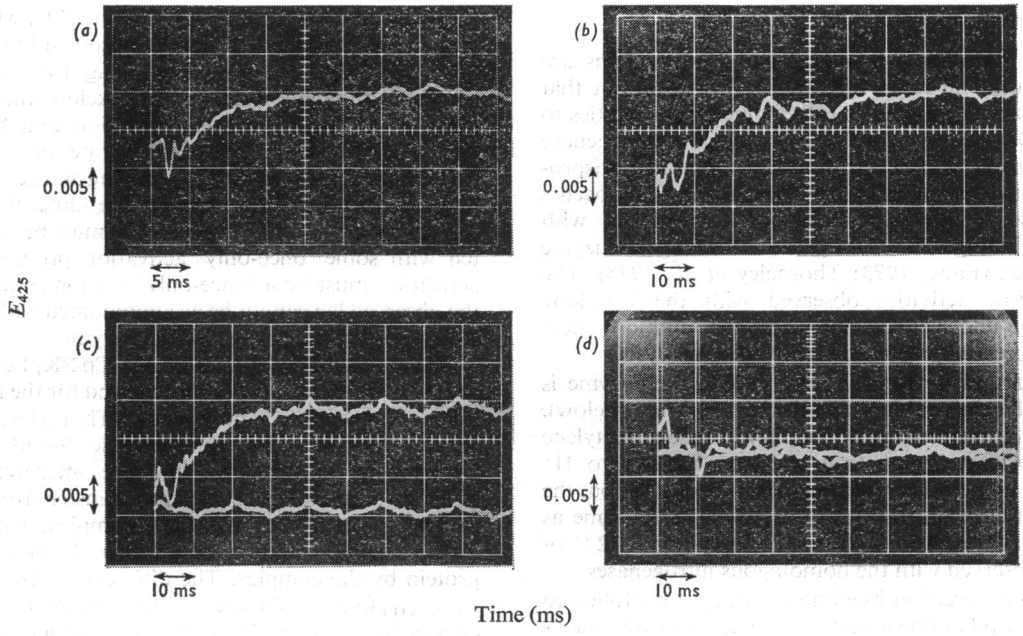


Fig. 3. *Stopped-flow oscillographs for the MgATP-induced electron transfer at 23°C from (a) Kp2 to Kp1 protein, (b) Cp2 to Cp1 protein, (c) Cp2 to Kp1 protein, (d) Kp2 to Cp1 protein (no reaction)*

Concentrations after mixing in all cases were: both component proteins, 7 μM; ATP, 5 mM; $MgCl_2$, 10 mM; $Na_2S_2O_4$, 10 mM; Hepes/NaOH buffer, 25 mM, pH 7.4. The reaction was monitored at 425 nm with a 1 cm path-length cell.

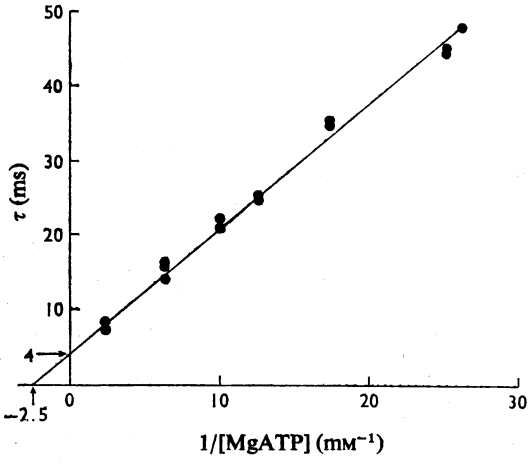


Fig. 4. *Dependence of τ on the reciprocal of the MgATP concentration at 23°C*

[Kp1] = [Cp2] = 7 μM, [$MgCl_2$] = 10 mM, [Hepes/NaOH] = 25 mM, pH 7.4, [$Na_2S_2O_4$] = 10 mM. The reaction was monitored at 425 nm with a 1 cm path-length cell. MgATP concentrations were calculated from the equilibrium data of Frey *et al.* (1972). For (*a*), τ = 5.6 ms, for (*b*) τ = 14 ms and for (*c*), τ = 8 ms.

$$\text{MgATP} + (\text{MoFe})\cdot(\text{Fe}) \underset{\text{(Fast)}}{\overset{k_{+3}}{\underset{k_{-3}}{\rightleftharpoons}}} (\text{MoFe})\cdot(\text{Fe})\cdot\text{MgATP} \quad (1)$$

$$(\text{MoFe})\cdot(\text{Fe})\cdot\text{MgATP} \xrightarrow[\text{(Slow)}]{k_{+2}} (\text{MoFe})_{\text{red.}}\,(\text{Fe})_{\text{ox.}}\begin{pmatrix}\text{MgATP}\\ \text{MgADP}\end{pmatrix} \quad (2)$$

where (MoFe) = Mo–Fe protein and (Fe) = Fe protein. Then with $k_{-3}/k_{+3} = K_{D\,(MgATP)}$,

$$\tau = \frac{K_{D\,(MgATP)}}{k_{+2}\cdot[\text{MgATP}]} + \frac{1}{k_{+2}}$$

Scheme 1. *MgATP-induced electron transfer from Fe protein to Mo–Fe protein*

protein exhibited ATPase activity when assayed separately.

Thus with the Kp2/Cp1 potential nitrogenase, both of the ATP-dependent steps discussed below are largely inhibited, resulting in complete inhibition of electron transfer from Kp2 to Cp1 protein and thus to substrates.

Discussion

Our data show that Cp2 and Kp1 proteins are compatible in terms of complex-formation but that the enzyme formed has rather different properties to those formed by combining homologous nitrogenase proteins. The concentration range in which disproportionately low acetylene-reduction activity occurs (below [Cp2] = [Kp1] = 29 nM) is consistent with tight binding between the proteins forming the enzyme complex (Eady, 1973; Thorneley *et al.*, 1975). The very low activities observed with the Cp2/Kp1 enzyme under conditions where the 'dilution' effect was observed precluded any detailed analysis.

As found by Detroy *et al.* (1968), the enzyme is virtually incapable of reducing N_2 (see below). However, after a lag period, it will reduce acetylene to ethylene. By contrast, electron transfer to H^+ shows no demonstrable lag. After the lag period, the rate of electron transfer to acetylene is the same as that to H^+ and in both cases is only about 12% of that observed with the homologous nitrogenases.

We interpret the observation of a lag period followed by a period of linear acetylene reduction with time as evidence for the build-up of a steady-state concentration of an acetylene-reducing enzyme. This is clearly different from the H^+-reducing enzyme, since no lag is observed in either H^+ reduction or $S_2O_4^{2-}$ oxidation, i.e. acetylene is reduced not simply at a different site but by a different form of the enzyme from that which reduces H^+. It seems probable that N_2 is reduced by yet another form of the enzyme, the formation of which is interrupted by H^+ reduction and thus the steady-state concentration of the N_2-reducing form of the enzyme is very low. We are unable, from our current data, to determine whether there is a lag in the formation of this N_2-reducing form of the enzyme.

This interpretation is in contrast with that of Hwang *et al.* (1973), who studied the mutual inhibition of a number of nitrogenase-catalysed reductions and concluded that H^+, acetylene and N_2 were all reduced at different sites on the enzyme. Our interpretation also differs from that of Silverstein & Bulen (1970), who found that, at low ATP concentrations, nitrogenase channelled relatively more electrons into H_2 evolution than into N_2 reduction and interpreted their results in terms of three forms of the nitrogenase enzyme, each generated by successive interactions with 2 mol of ATP and involved in the transfer of two, four and six electrons to substrates respectively. Although not stated explicitly, on this model, presumably both H^+ and acetylene would be reduced by the 'two-electron' form.

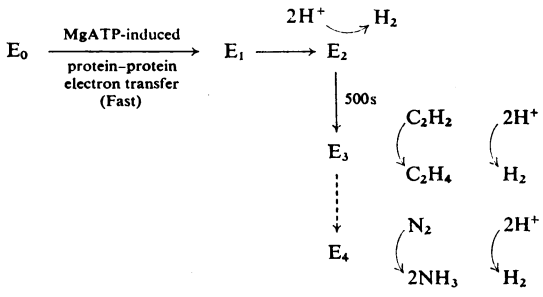
Our data show that the steady-state specific activity of both the H^+- and acetylene-reducing forms of the enzyme is about 53 nmol of electron pairs transferred/min per mg of Cp2 protein. This gives a turnover time for the enzyme of about 20 s, which is far too short to account for the observed lag in the formation of the acetylene-reducing form of the enzyme, although our data do not exclude the possibility of a 20 s lag for H_2 evolution and $S_2O_4^{2-}$ oxidation. Since the steady-state rate of electron transfer through both the H^+-reducing and acetylene-reducing forms of the enzyme is the same, then the difference between these two species must be associated with some 'once-only' activation process. The activation must be a 'once-only' phenomenon since the observed lag cannot be accommodated within the steady-state turnover time of the enzyme.

The stopped-flow studies on the Cp2/Kp1 enzyme are virtually identical with those found for the homologous *K. pneumoniae* nitrogenase (Thorneley, 1975) with τ intermediate between the values found for the homologous nitrogenases, and are interpreted in terms of a unimolecular electron transfer from Cp2 to Kp1 protein within a tight complex, with the further rapid oxidation of another 2 mol of Cp2 protein by the complex. The difference between the $K_{D(MgATP)}$ found in the present study for the Cp2/Kp1 enzyme (400 μM) and that of 20 μM determined by Tso & Burris (1973) for isolated Cp2 protein may be due to a modification of the MgATP-binding site as a consequence of protein–protein interaction.

However, we cannot exclude the possibility that the $K_{D(MgATP)} = 400\,\mu M$ for electron transfer between the two proteins is associated with a site on the enzyme completely different from that associated with the $K_{D(MgATP)} = 20\,\mu M$ for MgATP binding to Cp2 protein alone (Tso & Burris 1973). This 'alternative-site' hypothesis is supported by the observation (Walker *et al.*, 1976) that the release of Fe from reduced Cp2 protein is dependent on MgATP concentration and has a $K_{D(MgATP)}$ of about 400 μM.

The amplitude of the optical change contains information on the number of electrons transferred from Cp2 to Kp1 proteins in this rapid reaction. The change in the optical spectrum of Cp2 protein on physiological oxidation has been measured by Walker & Mortenson (1973) and Ljones (1973) as $\Delta\varepsilon_{mM}^{420} = 4.55$ and 3.9 cm^{-1} respectively. Walker & Mortenson (1973) equated this change in the spectrum with that obtained by removing two electrons/mol of protein with an oxidizing dye.

By using the above data we calculate that the amplitude of the optical change observed with the 1:1 and 4:1 molar ratios of Cp2 and Kp1 proteins corresponds to the transfer of 0.69–0.81 electron and 1.9–2.2 electrons respectively to a molecule of Kp1 protein. Two possible interpretations of these data are as follows: (*a*) three or more molecules of Cp2 protein transfer two electrons to Kp1 protein. This implies a redox equilibrium between the two proteins in which an excess of Cp2 protein is needed to drive the electron transfer to completion. This is difficult to



Scheme 2.

For details and an explanation of the symbols see the text.

reconcile with the essentially irreversible unimolecular electron-transfer process observed with these proteins (see below) and with the observation of Thorneley (1975) that excess of Kp1 protein did not affect the measured amplitude with the homologous Kp2/Kp1 nitrogenase. (*b*) Each Cp2 molecule transfers one electron to Kp1 protein. The observed values are lower than this, either because of the presence of some inactive Cp2 protein or because there is a compensating decrease in the E_{420} of Kp1 protein on reduction. The transfer of one electron/molecule of Cp2 protein would be consistent with its containing an Fe_4S_4 cluster, as has been suggested for Kp2 protein from Mössbauer spectroscopy by Smith & Lang (1974), and from cluster-extrusion experiments from Cp2 protein by Orme-Johnson (W. H. Orme-Johnson, unpublished work) and Gillum and co-workers (G. Gillum, L. E. Mortenson & R. H. Holm, unpublished work). In addition, the e.p.r. signal of Ac2 protein has been shown to be fully formed after a one-electron reduction of the phenazine methosulphate-oxidized protein with $Na_2S_2O_4$ (Thorneley *et al.*, 1976).

Since the amplitude of the optical change is unaffected by decreasing the MgATP concentration to 60μM, we can draw some conclusions about the irreversibility of reaction (2) in Scheme 1. For the amplitude to remain constant down to 60μM-MgATP, then

$$60 \gg \frac{K_{D(MgATP)}}{K_{redox}}$$

where K_{redox} is the equilibrium constant for the electron transfer from the Fe protein to the Mo–Fe protein in the complex (Gutfreund, 1972). With $K_{D(MgATP)} = 400\mu M$, and equating the inequality sign with a factor of 10 (from the minimum detectable change in amplitude) then

$$K_{redox} > 60$$

This value may be converted into a redox potential difference between the two proteins by using

$$\Delta G = -RT\ln K_{redox} = -nFE$$

where F = Faraday constant, n = the number of electrons involved in the redox couple and E = the potential difference between the two proteins. A value of E = 107mV is calculated for a one-electron process with $K_{redox} = 60$. This value, although a minimum, is remarkably close to the change in redox potential of 105mV observed (−295mV to −400mV) when MgATP binds to Cp2 protein (Zumft *et al.*, 1974).

Our results are consistent with Scheme 2. Cp2 and Kp1 proteins in the presence of MgATP form a tight 1:1 complex, E_0. One electron is transferred from Cp2 to Kp1 protein within the complex to form E_1. We have been able to kinetically distinguish the formation of one form of the enzyme, E_2, which can only reduce H^+, from another form, E_3, which can reduce acetylene but only after a lag phase.

Enzyme E_3 is initially formed from E_2 by a once-only activation process in a time of approx. 500s at 30°C. After activation, enzyme turnover for acetylene reduction is too fast to involve the conversion of enzyme E_2 into E_3, thus in subsequent catalytic cycles this modification must be maintained.

Experiments with ATP analogues (Eady *et al.*, 1975) have shown that acetylene reduction is more readily inhibited by these analogues than is H^+ reduction, and in fact H^+ reduction can be enhanced at the expense of acetylene reduction by their use. These data indicate that ATP may be involved in the formation of E_3 from E_2, for example by phosphorylation or adenylation, although this may be by a stoicheiometric rather than a catalytic process. If this is so, then presumably enzyme E_3 is formed from E_2 even in the absence of acetylene and is capable of reducing protons. An alternative hypothesis is that acetylene induces the formation of enzyme E_3 from E_2.

The reduction of N_2 by the Cp2/Kp1 enzyme is very slow. On scheme 2 we have shown this as being catalysed by yet another form of the enzyme, E_4, although our evidence for this is tenuous. We have tentatively assumed that enzyme E_3 is not the N_2-reducing form, since N_2 reduction is inefficient, whereas acetylene reduction is as efficient as H^+ reduction, but we stress the speculative nature of this assumption.

It is noteworthy that acetylene-reduction activity is commonly used in genetical work for screening micro-organisms and in field-work as an index of potential N_2-fixing ability of soil micro-organisms. The uncoupling of acetylene-reduction activity and N_2-fixation that we have reported here indicates that, particularly with mutated proteins or with nitrogenase proteins transferred to exotic environments,

the assumed relationship may not hold, and ^{15}N-enrichment techniques must be used for confirmation.

Another conclusion may be drawn from the MgATP-dependence of the optical change observed in the present paper and by Thorneley (1975). Even at the lowest MgATP concentration used (60 μM) the optical change was completed in a time (40 ms) much shorter than the turnover time of the enzyme (about 0.5 s/two electrons for a homologous nitrogenase). However at this low concentration of MgATP the activity of the enzyme is well below its maximum, since the apparent K_m of MgATP for substrate reduction is 400 μM (Thorneley & Willison, 1974). This implies that MgATP is also involved in a stage in the enzymic reaction other than electron transfer between the two proteins.

We have found that the overall ATP hydrolysis reactivity of the Cp2/Kp1 enzyme is lowered to 40%, but the electron transfer to substrate to 12% of that of the homologous enzyme; thus ATP hydrolysis and electron transfer are uncoupled. Kelly (1969) made a similar observation, without comment, in an earlier study on nitrogenase protein cross-reactions. However, in our studies the rate of the ATP-induced electron transfer from Cp2 protein to Kp1 protein and its ATP concentration-dependence is apparently unaffected. This is yet another indication of an additional role for ATP in nitrogenase action, with one ATP site unaffected in the heterologous cross-reaction but another being considerably perturbed and virtually uncoupled from the enzyme reaction.

Hadfield & Bulen (1969) discussed the temperature-dependence of the ATP/2e ratio for *A. vinelandii* nitrogenase, which varied from 5.8 at 40°C to 4.3 at 20°C, in terms of two ATP-hydrolysis reactions having different activation energies and with only one of the reactions being coupled to electron transfer. Our data suggest that the 'uncoupled' reaction of Hadfield & Bulen (1969) is in fact an inefficiently coupled step in the nitrogenase enzymic reaction. Possibly this step is also that which is affected by pH and by high Mo–Fe/Fe protein molar ratios (Ljones, 1973; Eady & Postgate, 1974). It may also be associated with the 'reductant-independent' ATPase activity of nitrogenase (Jeng *et al.*, 1970).

The present data have indicated a number of roles for MgATP in the enzymic reaction, namely: (*a*) the induction of electron transfer from the Fe protein to the Mo–Fe protein with an apparent K_D of 400 μM; (*b*) an additional role, during enzyme turnover subsequent to (*a*), since the coupling of ATP hydrolysis to electron transfer is largely disrupted: (*c*) the activation of enzyme E_2 to E_3; this process may be stoicheiometric with enzyme concentration, and thus not reflected in the ATP/2e ratios derived from steady-state experiments. The relationship between the above roles and the reported binding constants for MgATP to the Fe-protein alone has yet to be determined.

References

Biggins, D. R., Kelly, M. & Postgate, J. R. (1971) *Eur. J. Biochem.* **20**, 140–143

Bui, P. T. & Mortenson, L. E. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 1021–1027

Burris, R. H. (1971) in *Chemistry and Biochemistry of Nitrogen Fixation* (Postgate, J. R., ed.), 105–160, Plenum Press, London

Burris, R. H. & Orme-Johnson, W. H. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st, 1974*, vol. 1, pp. 208–233, Washington State University Press

Chaney, A. L. & Marbach, E. P. (1962) *Clin. Chem.* **8**, 130–136

Dalton, H., Morris, J. A., Ward, M. A. & Mortenson, L. E. (1971) *Biochemistry* **10**, 2066–2071

Detroy, R. W., Witz, D. F., Parejko, R. A. & Wilson, P. W. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 537–541

Eady, R. R. (1973) *Biochem. J.* **135**, 531–535

Eady, R. R. & Postgate, J. R. (1974) *Nature (London)* **249**, 805–810

Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) *Biochem. J.* **128**, 655–675

Eady, R. R., Kennedy, C., Smith, B. E., Thorneley, R. N. F., Yates, M. G. & Postgate, J. R. (1975) *Biochem. Soc. Trans.* **3**, 488–492

Frey, C. M., Banyasz, J. L. & Stuer, J. E. (1972) *J. Am. Chem. Soc.* **94**, 9198–9204

Gutfreund, H. (1972) *Enzymes: Physical Principles*, p. 205, Wiley-Interscience, London

Hadfield, K. L. & Bulen, W. A. (1969) *Biochemistry* **8**, 5103–5108

Huang, T. C., Zumft, W. G. & Mortenson, L. E. (1973) *J. Bacteriol.* **113**, 884–890

Hwang, J. C., Chen, C. H. & Burris, R. H. (1973) *Biochim. Biophys. Acta* **292**, 256–270

Jeng, D. Y., Morris, J. A. & Mortenson, L. E. (1970) *J. Biol. Chem.* **245**, 2809–2813

Kelly, M. (1969) *Biochim. Biophys. Acta* **191**, 527–540

Ljones, T. (1973) *Biochim. Biophys. Acta* **321**, 103–113

Ljones, T. & Burris, R. H. (1972) *Biochim. Biophys. Acta* **275**, 93–101

Murphy, P. M. & Koch, B. L. (1971) *Biochim. Biophys. Acta* **253**, 295–297

Nakos, G. & Mortenson, L. E. (1971) *Biochemistry* **10**, 455–458

Orme-Johnson, W. H., Hamilton, W. D., Ljones, T., Tso, M-Y., Burris, R. H., Shah, V. K. & Brill, W. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3142–3145

Silverstein, R. & Bulen, W. A. (1970) *Biochemistry* **9**, 3809–3815

Smith, B. E. & Lang, G. (1974) *Biochem. J.* **137**, 169–180

Smith, B. E., Lowe, D. J. & Bray, R. C. (1972) *Biochem. J.* **130**, 641–643

Smith, B. E., Lowe, D. J. & Bray, R. C. (1973) *Biochem. J.* **135**, 331–341

Smith, B. E., Thorneley, R. N. F., Yates, M. G., Eady, R. R. & Postgate, J. R. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st, 1974*, vol. 1, pp. 150–176, Washington State University Press

Smith, R. V., Telfer, A. & Evans, M. C. W. (1971) *J. Bacteriol.* **107**, 574–575

Taussky, H. H. & Shoor, E. (1954) *J. Biol. Chem.* **202**, 675–682
Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta* **333**, 487–496
Thorneley, R. N. F. (1975) *Biochem. J.* **145**, 391–396
Thorneley, R. N. F. & Eady, R. R. (1973) *Biochem. J.* **133**, 405–408
Thorneley, R. N. F. & Willison, K. R. (1974) *Biochem. J.* **139**, 211–214
Thorneley, R. N. F., Eady, R. R. & Yates, M. G. (1975) *Biochim. Biophys. Acta* **403**, 269–284
Thorneley, R. N. F., Yates, M. G. & Lowe, D. J. (1976) *Biochem. J.* **155**, 137–144
Tso, M.-Y. W. & Burris, R. H. (1973) *Biochim. Biophys. Acta* **309**, 263–270
Walker, G. A. & Mortenson, L. E. (1974) *Biochemistry* **13**, 2382–2388
Walker, G. A., Walker, M. N. & Mortenson, L. E. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st, 1974*, vol. 1, pp. 117–149, Washington State University Press
Walker, M. N. & Mortenson, L. E. (1973) *Biochem. Biophys. Res. Commun.* **54**, 669–676
Walker, M. N. & Mortenson, L. E. (1974) *J. Biol. Chem.* **249**, 6356–6358
Watt, G. D., Bulen, W. A., Burns, A. & Hadfield, K. L. (1975) *Biochemistry* **14**, 4266–4272
Zumft, W. G. & Mortenson, L. E. (1973) *Eur. J. Biochem.* **35**, 401–409
Zumft, W. G. & Mortenson, L. E. (1975) *Biochim. Biophys. Acta* **416**, 1–52
Zumft, W. G., Cretney, W. C., Huang, T. C., Mortenson, L. E. & Palmer, G. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1525–1532
Zumft, W. G., Mortenson, L. E. & Palmer, G. (1974) *Eur. J. Biochem.* **46**, 525–535