Biochem. J. (1979) **181**, 569–575
Printed in Great Britain

Characterization of an Oxygen-Stable Nitrogenase Complex Isolated from *Azotobacter chroococcum*

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(*Received* 4 *December* 1978)

In crude cell-free extracts of *Azotobacter chroococcum*, nitrogenase was much less sensitive to irreversible inactivation by O_2 than was the purified enzyme. When nitrogenase was partially purified by anaerobic discontinuous sucrose-density-gradient centrifugation, O_2-tolerance was retained. This preparation was considerably enriched in four polypeptides, three of which were derived from the Mo–Fe (molybdenum–iron) protein and Fe (iron) protein of nitrogenase. The fourth was purified to homogeneity and shown to be an iron–sulphur protein (mol.wt. 14000) probably containing a 2Fe–2S centre. When this protein was added to purified nitrogenase, the enzyme was rendered O_2-tolerant, though stabilization was Mg^{2+}-dependent. The isolated O_2-tolerant nitrogenase was an equimolar stoicheiometric complex between the Mo–Fe, Fe and protective proteins. It is likely that the formation of this complex *in vivo* is the mechanism of 'conformational protection' in this organism.

Nitrogenase is an oxygen-labile enzyme (Bulen & LeComte, 1966; Kelly, 1969*b*), and when purified from *Azotobacter chroococcum* its component proteins, the Mo–Fe (molybdenum–iron) protein and the Fe (iron) protein have half-lives of inactivation by O_2 (dioxygen) of approx. 10 and 0.5 min respectively (Yates & Planqué, 1975). In general, the oxygen-lability is attributable to the irreversible oxidation of the essential non-haem Fe–S (iron–sulphur) groups present in both proteins (Petering *et al.*, 1971; Gomez-Moreno, 1978).

Azotobacter species are obligately aerobic nitrogen-fixing organisms, so for nitrogenase to remain active it must be protected *in vivo* from the effects of O_2. Two protective mechanisms were postulated for *A. chroococcum* (Dalton & Postgate, 1969). It was proposed that when this organism is fixing N_2 its specialized respiratory system has a dual role, not only providing for conservation of energy via oxidative phosphorylation, but also for the removal of excess O_2 at sites of nitrogenase activity: a process termed 'respiratory protection'. When the organism is subjected to an O_2 stress, this protection ceases to be effective, and nitrogenase activity *in vivo* is inhibited. Since nitrogenase is not rapidly irreversibly inactivated under these conditions (Drozd & Postgate, 1970), it was suggested that the enzyme was present in the organism as a relatively O_2-insensitive form termed the 'conformationally protected state' (Hill *et al.*, 1972). However, the mechanism of conformational protection in this organism has not been determined.

In crude extracts prepared from *A. vinelandii* and *A. chroococcum*, nitrogenase is more stable to O_2 than is the purified enzyme, and partially purified preparations may retain this characteristic (Bulen *et al.*, 1965; Kelly, 1969*a*). More recently Haaker & Veeger (1977) demonstrated that, in *A. vinelandii*, a 2Fe–2S protein first isolated by Shethna *et al.* (1968) that was present in an O_2-stable partially purified preparation of nitrogenase (Bulen & LeComte, 1972), could be added back to the O_2-sensitive enzyme, thereby restoring the stability to O_2.

The present paper describes the partial purification of an O_2-stable complex of nitrogenase from *A. chroococcum* and shows that the stability also results from the association of the Mo–Fe and Fe proteins with a low-molecular-weight 2Fe–2S protein.

Abbreviations used: DNAase, deoxyribonuclease; SDS, sodium dodecyl sulphate.

Materials and Methods

Preparative techniques

Azotobacter chroococcum N.C.I.B. 8003 was grown at 30°C on a modified Burk's medium (Newton *et al.*, 1953) containing 2% (w/v) sucrose and no fixed nitrogen source. Precise cultural conditions and methods of harvesting organisms have been described elsewhere (Yates & Planqué, 1975).

The Mo–Fe and Fe proteins from *A. chroococcum* were purified as described by Yates & Planqué (1975) to specific activities of 1670 and 1250 units per mg of protein respectively.

The O_2-stable nitrogenase complex was purified by applying 2ml of DNAase-treated cell-free extract (50mg of protein) to a 30ml anaerobic discontinuous sucrose density gradient, which was centrifuged for 22h at 10°C at 91000*g* ($r_{av.}$ 9.1cm) in a 3×35ml swinging-bucket rotor in a Christ Omega II centrifuge. Gradients were prepared by sequential addition of 2ml of 60% followed by 4ml each of 50, 45, 40, 35, 30, 25 and 20% (w/v) deoxygenated sucrose solutions containing 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH7.8, 2mM-$MgCl_2$ and 0.1mg each of sodium dithionite and dithiothreitol/ml. Gradients were fractionated anaerobically after first piercing the bottom of the centrifuge tube with a needle attached to a short section of narrow-bore PVC tubing. The gradient was expelled by applying compressed argon at the top of the gradient and the fractions were collected under a stream of N_2.

The protective protein (AcP) was purified as follows: 500g (wet wt.) of bacteria were thoroughly resuspended in 500ml of 50mM-Hepes buffer, pH7.8, containing 10mg each of deoxyribonuclease and ribonuclease and disrupted by passage through a French pressure cell (Aminco, Silver Springs, MD, U.S.A.) at 151.8MPa (22000lb/in^2). The extract was collected under a stream of N_2 and all procedures up to the gel-filtration step were carried out by using anaerobic techniques described elsewhere (Eady *et al.*, 1972). Crude nitrogenase complex was sedimented by centrifugation [125000*g* ($r_{av.}$ 7cm) for 4h at 5°C] of the supernatant obtained by differential centrifugation of the crude cell extract, first at 21000*g* ($r_{av.}$ 7.5cm) for 40min, and then at 70000*g* ($r_{av.}$ 7cm) for 3h, both at 5°C. The pellets were resuspended in 50ml of 25mM-Hepes buffer, pH7.8, containing 0.1mg each of sodium dithionite and dithiothreitol/ml (buffer A). The slightly turbid brown solution was adsorbed on to a column (5cm×10cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with 3 bed vol. of this buffer and AcP protein was eluted with 2 bed vol. of buffer A containing 0.1M-KCl. The orange-coloured solution obtained was concentrated by ultrafiltration with an Amicon UM-10 membrane and the 10ml concentrate was applied to a column (3.5cm×80cm) of Sephadex G-100 equilibrated in 25mM-Hepes buffer, pH7.8. The protein eluted as a single peak, which was obtained as one 50ml fraction after 345ml had been eluted from the column. The protein was reconcentrated by ultrafiltration and rechromatographed on Sephadex G-100. By this stage the protein was electrophoretically pure. Approx. 50mg was obtained from the original 500g (wet wt.) of organisms.

Analytical techniques

Nitrogenase activity *in vitro* was determined by measuring the rate of acetylene reduction at pH 7.8 and 30°C as previously described (Eady *et al.*, 1972). Specific activities are expressed as nmol of acetylene reduced/min per mg of protein. Specific activities of the pure Mo–Fe and Fe proteins were determined with saturating concentrations of the Fe and Mo–Fe proteins respectively.

Glucose 6-phosphate dehydrogenase was assayed by the method of Senior & Dawes (1971), and NADH and malate oxidase activities were assayed as reported by Ackrell & Jones (1971).

O_2-inactivation experiments were performed by using 1ml plastic hypodermic syringes fitted with 17-gauge needles and containing a stainless-steel ball to ensure rapid mixing within the syringe. Protein solution (0.5ml) containing 500 units of nitrogenase activity was drawn into the argon-flushed syringe, and at zero time O_2-saturated 50mM-Hepes buffer, pH7.8, was drawn into the syringe until the base of the plunger registered 1ml. After the syringe had been inverted several times, 0.1ml was discarded, and at timed intervals thereafter further 0.1ml samples were expelled into capped serum bottles containing nitrogenase assay mix (including sodium dithionite, but excluding ATP) under an atmosphere of acetylene/argon) (3:47). Nitrogenase assays were initiated by adding deoxygenated ATP. Controls were provided by mixing test samples with argon rather than O_2-sparged buffer. Samples that contained sodium dithionite and dithiothreitol were desalted by using a column (1cm×25cm) of Sephadex G-25, which was equilibrated with deoxygenated 25mM-Hepes buffer, pH7.8, containing 2mM-$MgCl_2$.

Protein in crude extracts and partially purified preparations was determined by Folin–Ciocalteau reagent (Lowry *et al.*, 1951) or by the biuret reagent (Gornall *et al.*, 1949) for purified proteins, with crystalline bovine serum albumin as a standard.

AcP protein was analysed for Fe (Doeg & Ziegler, 1962) and acid-labile S (King & Morris, 1967). Analytical centrifugation was performed with an MSE Centriscan 75 instrument at 20±0.5°C and 55000 rev./min.

Generally, SDS/polyacrylamide-gel electrophoresis was performed under the conditions described by Laemmli (1974), though analytical SDS/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969) by using the slab-gel equipment described by Reid & Bielski (1968). Gels were fixed in ethanol/acetic acid/water (2:1:7, by vol.), for 2h, stained with Coomassie Brilliant Blue R250, (0.1%, w/v) in ethanol/acetic acid/water (10:1:10, by vol.) for 1h and then destained in ethanol/acetic acid/water (1:1:8. by vol.) for 2h and then 5% (v/v) acetic acid until the background was clear.

Isoelectric focusing was carried out by using 7.5% (w/v) acrylamide tube gels (0.5cm×10cm) containing 2% (w/v) Ampholine (pH range 3–10). Protein

samples in 15% (w/v) sucrose were layered on to the gel and overlayed with 10% (w/v) sucrose. The upper buffer reservoir (anode) contained 70mM-H_2SO_4, and the lower, 20mM-NaOH. Gels were focused for 18h at 3°C and 1mA/tube. pH profiles were determined by cutting the gels into 0.5cm transverse sections, which were incubated for 2h in 0.25ml of 10mM-NaCl before the pH of the solution was measured with a miniature combined pH electrode. Gels were stained as described by Otavsky & Drysdale (1975).

U.v. and visible spectra were recorded with a Pye–Unicam SP. 1800 spectrophotometer. Polyacrylamide gels were scanned at 600nm with a Pye–Unicam microdensitometer attachment for the SP.1800 instrument.

Materials

All biochemicals and Coomassie Brilliant Blue R250 were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. SDS was obtained from Serva, Heidelberg, Germany (Kennedy *et al.*, 1976). Ampholines were obtained from LKB Instruments Ltd., South Croydon, Surrey, U.K., and proteins used in the calibration of analytical SDS/polyacrylamide-gel electrophoresis were obtained from Boehringer, Lewes, East Sussex, U.K. All other chemicals used were obtained from either BDH, Poole, Dorset, U.K., or Hopkin and Williams, Chadwell Heath, Essex, U.K. Sephadex G-100 and G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-cellulose was purchased from Whatman, Maidstone, Kent, U.K. Chelex 100 was obtained from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.

Results

To determine whether a specific factor or factors stabilized nitrogenase against O_2 inactivation in crude extracts, purification of the protected enzyme was attempted so that such factors might be more easily identified. Factors that possibly could have been involved ranged from association of nitrogenase with one or more proteins, with membrane material, with metabolites or cations or with any combination of these cell components. Yates (1972) reported that O_2 stability in crude cell-free extracts was considerably lowered after anaerobic dialysis in the presence of EDTA but that addition of Mg^{2+}, Ca^{2+} or Mn^{2+} to the dialysed extract restored stability. Since the addition of Mg^{2+} to purified nitrogenase does not exert a marked stabilizing effect, it is apparent that whatever factors are required, the presence of Mg^{2+} or other bivalent cations was necessary for the stabilization to be expressed .Therefore buffers used throughout the present study generally contained Mg^{2+}.

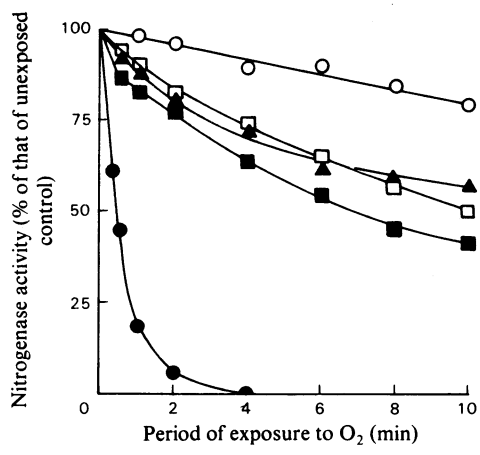

Fig. 1. *Inactivation of nitrogenase by O_2*
Exposure of various preparations to O_2 was carried out as described in the Materials and Methods section. All syringes contained 500 units of nitrogenase before inactivation. The various preparations tested are denoted as follows: ○, crude extract; □, 125000*g* pellet; ▲, sucrose-density-gradient preparation; ■, mixture of purified proteins containing 0.8mg of Mo–Fe protein, 0.2mg of Fe protein and 0.2mg of protective protein; ●, mixture of purified proteins as above but minus protective protein.

By using differential centrifugation, 80% of the nitrogenase activity in the crude extract was sedimented by centrifugation at 125000*g* ($r_{av.}$ 7cm) for 3h. The bulk of the cytoplasmic membrane (with NADH oxidase and malate oxidase activities as markers) was collected at lower gravitational forces [70000$g_{max.}$ ($r_{av.}$ 7cm) for 2h]. Fig. 1. shows the O_2 sensitivity of the various fractions compared with that of purified nitrogenase. It indicated that although the sedimented nitrogenase was not as stable as that in the crude extract, it was nevertheless more stable than the purified enzyme. Since the 125000*g* pellet was not free of NADH oxidase or malate oxidase activities, it was not clear at this stage whether the cytoplasmic membrane was involved in stabilization.

Crude extracts were subjected to anaerobic discontinuous density-gradient centrifugation. Fig. 2 shows the distribution of cytoplasmic membrane markers, glucose 6-phosphate dehydrogenase (a 'soluble enzyme' marker) and nitrogenase after 22h centrifugation. Although NADH oxidase and malate oxidase were distributed as a broad band towards the bottom of the gradient and glucose 6-phosphate dehydrogenase just entered the top of the gradient, nitrogenase collected as a sharp band at the zone corresponding to the original 30/25% (w/v) sucrose

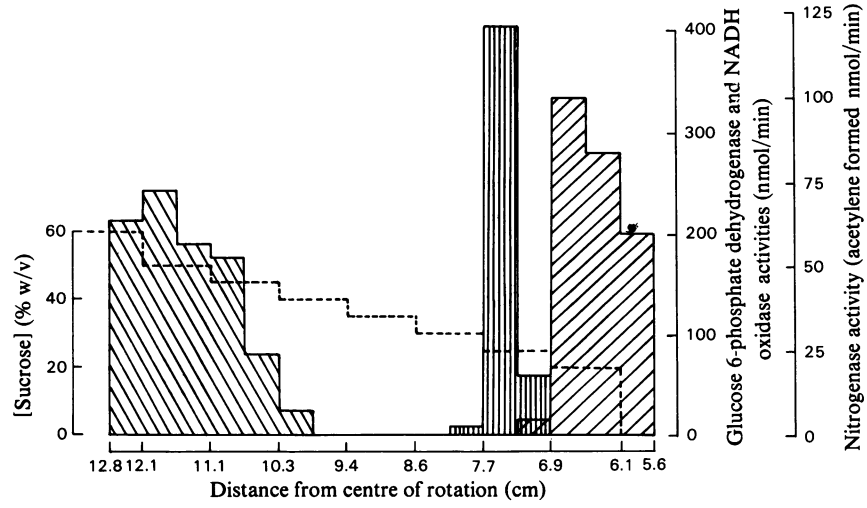

Fig. 2. *Distribution of enzyme activities after anaerobic discontinuous-sucrose-density-gradient centrifugation*
For details of gradient preparation, centrifugation and fractionation, see the Materials and Methods section. A portion (2ml) of DNAase-treated cell-free extract (50mg of protein) was applied to the gradient (----). Samples (0.1 ml) from gradient fractions (2ml) were assayed for NADH oxidase (■) and malate oxidase, glucose 6-phosphate dehydrogenase (■) and nitrogenase (▥). Activities refer to a 0.1 ml sample. Data for malate oxidase were essentially similar to those for NADH oxidase and were omitted for clarity.

interface. The specific activity of the nitrogenase removed from the gradient was on average 400 as compared with 50units/mg for the crude extract.

The inactivation by O_2 of the crude nitrogenase preparation isolated on sucrose density gradients is shown in Fig. 1 and resembles that of nitrogenase collected by differential centrifugation. Thus the separation of nitrogenase from cytoplasmic membrane markers and also from more soluble cytoplasmic constituents did not markedly increase the O_2-sensitivity of the enzyme. Analysis by SDS/polyacrylamide-gel electrophoresis of this nitrogenase-containing fraction (Fig. 3) revealed that compared with the crude extract, this preparation was not only markedly enriched in the Mo–Fe and Fe proteins, but also in a polypeptide of mol.wt. approx. 14000.

To test the possibility that this polypeptide was a stabilizing factor, it was first separated from the nitrogenase by the following procedure. Several nitrogenase-containing fractions prepared by sucrose-density-gradient centrifugation were pooled, and sucrose was removed by anaerobic dialysis in buffer A containing 2mM-$MgCl_2$, for 18h at 20°C. This partially purified nitrogenase preparation, which retained its O_2 stability at this stage, was adsorbed on to an anaerobic DEAE-cellulose column equilibrated with buffer A. The column was developed by washing first with 3 bed vol. of buffer A, followed by 2 bed vol. of buffer A containing 0.1M-KCl, which eluted an orange-coloured fraction. Finally, washing with 2 bed vol. of buffer A containing 0.09M-$MgCl_2$ eluted nitrogenase as a brown band.

SDS/polyacrylamide-gel electrophoresis indicated that the 0.1M-KCl wash contained the 14000-mol.wt. polypeptide free of the nitrogenase polypeptides. The nitrogenase-bearing fraction (0.09M-$MgCl_2$ eluate) contained no detectable amount of the 14000-mol.wt. polypeptide, but was now as sensitive to O_2 inactivation as was the highly purified enzyme.

To determine whether the low-molecular-weight protein alone was the stabilizing factor, it was purified to homogeneity as described in the Materials and Methods section. Fig. 3 shows that the protein gave a single band on SDS/polyacrylamide-gel electrophoresis and one band was also observed after isoelectric focusing (p*I* 6). Fig. 4 shows that addition of increasing amounts of the protein to a constant amount of purified nitrogenase (as an equimolar mixture of the Mo–Fe and Fe proteins) rendered the enzyme increasingly more stable to O_2 inactivation, until saturation was reached. The rate of inactivation of the most highly stabilized mixture was comparable with that observed in the partially purified, O_2-stable nitrogenase complex (Fig. 1). In the absence of Mg^{2+}, very little stabilization could be demonstrated even at high concentrations of the protective protein. These findings confirm previous reports that Mg^{2+} is essential for protection (Yates, 1972).

The purified protective protein was orange in colour and exhibited a u.v. and visible absorption

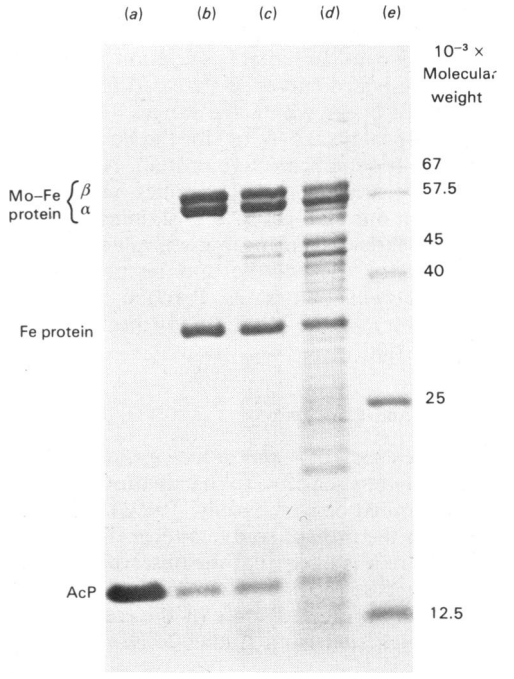

Fig. 3. *Analysis by SDS/polyacrylamide-gel electrophoresis of polypeptides present in various preparations*
Slots contained the following samples: (*a*) purified protective protein (AcP) (5 μg); (*b*) 'O_2-stable' nitrogenase isolated by discontinouus-sucrose-density-gradient centrifugation (20 μg); (*c*) 'O_2-stable' nitrogenase prepared by differential centrifugation (20 μg); (*d*) crude cell-free extract (20 μg); (*e*) molecular-weight standards provided by bovine serum albumin, catalase, ovalbumin, aldolase, chymotrypsinogen A and cytochrome *c*.

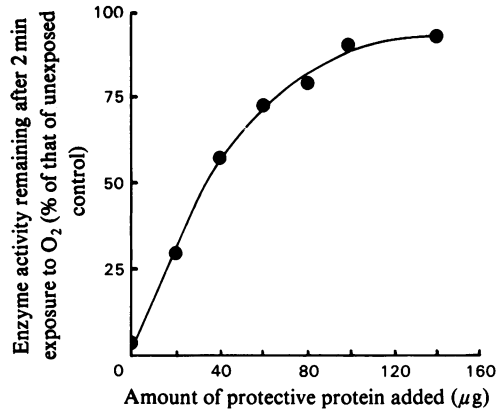

Fig. 4. *Effects of protective protein on stability of nitrogenase in the presence of* O_2
Syringes were first filled with an anaerobic but $Na_2S_2O_4$-free solution containing 0.8 mg of Mo–Fe protein, 0.2 mg of Fe protein and varying amounts of protective protein. The proteins were mixed with O_2-saturated buffer and 2 min later were injected into anaerobic nitrogenase assay mix containing $Na_2S_2O_4$. For details see the Materials and Methods section.

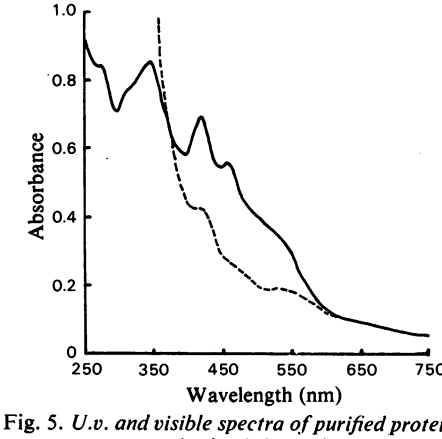

Fig. 5. *U.v. and visible spectra of purified protein*
The spectra were obtained by using an aqueous solution containing 1.2 mg of protein/ml in 25 mM-Hepes buffer, pH 7.8. The protein was isolated as the oxidized form (——) and the fully reduced form (----) was obtained in the presence of 5 mM-$Na_2S_2O_4$ under argon.

spectrum (Fig. 5) similar to that of a 2Fe–2S protein isolated from *Azotobacter vinelandii* (Shethna *et al.*, 1968). The protein was partially bleached on reduction by sodium dithionite, the most notable change being the loss of an absorption peak at 460 nm. The reduced protein was autoxidizable, having a $t_{\frac{1}{2}}$ for oxidation at 20°C of 4.5 min in air-saturated buffer (25 mM-Hepes, pH 7.5). The protein was stable in the presence of O_2. The molecular weight was determined by sedimentation equilibrium by using the meniscus-depletion method of Yphantis (1964). Fig. 6 shows that a plot of ln [protein] against (radius)^2 was linear, with a slope of 2.4691, corresponding to a mol.wt. of 13950, assuming a value for $\bar{v}$ of 0.74, in close agreement to the value 14000 determined by analytical electrophoresis (Fig. 7). The protein was analysed for Fe and labile-S content after passage down a Chelex 100 column to remove non-specifically bound cations, particularly transition metals, and average values obtained were 1.95 and 1.78 g-atoms/mol respectively, indicating that the protein probably contained a 2Fe–2S centre.

As the protective protein co-sedimented with the Mo–Fe and Fe proteins, it is likely that the three proteins form a complex that is important for O_2 protection. The stoicheiometry of the three proteins

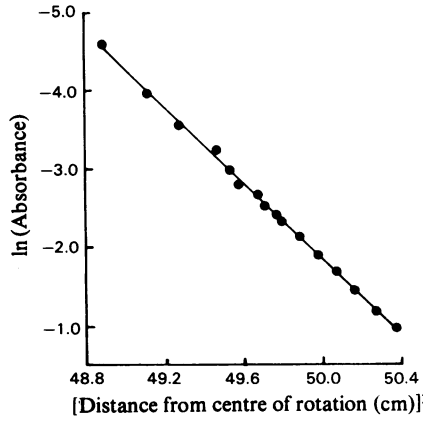

Fig. 6. *Determination of the molecular weight of the protective protein by equilibrium sedimentation using the meniscus depletion method*

Distribution of protein throughout the cell before and after the attainment of equilibrium was determined by measurement of absorbance with a wavelength filter having a band pass of approx. 555±15nm. The initial protein concentration was 0.15mg/ml. Other details are described in the Materials and Methods section.

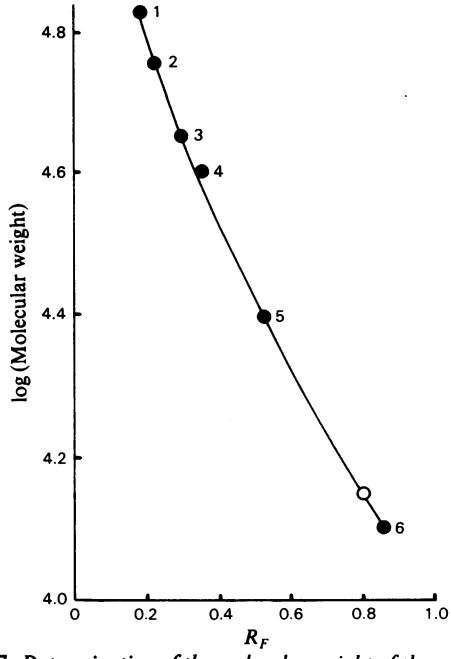

Fig. 7. *Determination of the molecular weight of the protective protein polypeptide by analytical SDS/polyacrylamide-gel electrophoresis*

The relative mobility in a 12.5% (w/v) acrylamide gel of the protective protein (○) was compared with those of polypeptides of purified protein standards (●) (1, bovine serum albumin; 2, catalase; 3, ovalbumin; 4, aldolase; 5, chymotrypsinogen A; 6, cytochrome *c*).

in the complex isolated by sucrose-density-gradient centrifugation was determined by densitometry of stained polypeptides after separation by electrophoresis. Standard curves constructed for each pure protein were linear within the ranges 1.2–7.2μg for the Mo–Fe protein, 0.5–3.3μg for the Fe protein, and 0.15–0.9μg for the protective protein. All standards and the O_2-stable-complex samples were electrophoresed on one slab gel so that staining conditions would be identical. By using mol.wt. values previously determined for the Mo–Fe and Fe proteins from *A. chroococcum* (Yates & Planqué, 1975), the stoicheiometry for the Mo–Fe/Fe/protective proteins was 1:1.08:1.06.

Discussion and Conclusions

In crude extracts of *A. chroococcum*, nitrogenase was considerably less sensitive to inactivation by O_2 than was the purified enzyme (Kelly, 1969*a*). The results obtained in the present study confirm those findings and therefore it is likely that the observed stability in crude extracts is the expression *in vitro* of the 'conformationally protected' state of the enzyme, which has been suggested from studies *in vivo* (Hill *et al.*, 1972).

The O_2 sensitivity of nitrogenase can be altered by associated molecules; Yates (1972) showed that ATP enhanced its O_2-lability. The stability observed in crude extracts may result from association of nitrogenase with one or more extrinsic stabilizing factors. Indeed crude preparations containing cytochromes (Kelly, 1969*a*) or NADH dehydrogenase (Yates, 1970) from *A. chroococcum* stabilized nitrogenase in this organism, and Mg^{2+} or other bivalent cations are important (Yates, 1972).

In the present study, nitrogenase was purified 8-fold by sucrose-density-gradient centrifugation in a form that still exhibited considerable stability to O_2. The separation of nitrogenase from other enzyme markers indicated that stability was not mediated by association of the enzyme with membrane or any readily dissociable factor.

The 14000-mol.wt. protein alone conferred stability on purified nitrogenase to an extent comparable with that of partially purified preparations, an effect dependent on Mg^{2+}, though it is probable that other bivalent cations would be effective. That this is a specific effect of this protein can be judged by the failure of other purified proteins such as flavodoxin, ferredoxin, cytochrome *c*, or NADH dehydrogenase from *A. chroococcum* to stabilize the pure enzyme (Yates, 1970).

Studies of the stoicheiometry of the proteins in the partially purified stable nitrogenase preparation indicated that the conformationally protected form of the enzyme as isolated was a tight equimolar complex. Complete stability of nitrogenase was not

observed (even for crude extracts) under the assay conditions described. Though the partially purified and reconstituted preparations were less stable than the crude extracts, they were considerably more stable than the purified enzyme, which has a $t_{\frac{1}{2}}$ of 0.5 min for inactivation by O_2, a value similar to that shown the pure Fe protein alone (Yates & Planqué, 1975). Therefore it is evident that the principal effect of the protective protein is the stabilization of the Fe protein, and it is not clear whether the Mo–Fe protein is protected. However, the evidence that all three proteins form a tight complex indicates that the mechanism of protection might depend on the relative orientation of the proteins within the complex.

From the general point of view of protection of nitrogenase from O_2 in obligate aerobes, the mechanism of conformational protection in *A. chroococcum* is strikingly similar to that found by Haaker & Veeger (1977) in *A. vinelandii* (see the introduction). The only point of difference is the molecular weight of the protective proteins, which for the *A. vinelandii* protein is 23000 (Shethna *et al.*, 1968). I suggest that, for reference purposes, the naming of these proteins should follow the widely used nomenclature of nitrogenase proteins (Av1, Av2, etc., Eady *et al.*, 1972), but with the suffix P, the two proteins under discussion being AvP and AcP. These symbols do not imply that protection is necessarily the sole function of these proteins. Scherings *et al.* (1977) suggested that AvP protein regulates nitrogenase activity, and both AvP and AcP proteins may have other functions not necessarily related to nitrogenase or nitrogen fixation.

I wish to thank Mr. K. Baker and Mrs. J. Strudwick for growing bacteria, Mr. M. Wingfield for skilled technical assistance, and Professor J. R. Postgate for constructive criticism of the manuscript.

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