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Nitrogenase of *Klebsiella pneumoniae*

PURIFICATION AND PROPERTIES OF THE COMPONENT PROTEINS

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1. Nitrogenase from the facultative anaerobe *Klebsiella pneumoniae* was resolved into two protein components resembling those obtained from other nitrogen-fixing bacteria. 2. Both proteins were purified to homogeneity as shown by the criteria of disc electrophoresis and ultracentrifugal analysis. 3. The larger component had a mol.wt. of 218000 and contained one Mo atom, 17 Fe atoms and 17 acid-labile sulphide groups/mol; it contained two types of subunit, present in equal amounts, of mol.wts. 50000 and 60000. All the common amino acids were present, with a predominance of acidic residues. The apparent partial specific volume was 0.73; ultracentrifugal analysis gave $s^0_{20,w} = 11.0$S and $D^0_{20,w} = 4.94 \times 10^{-7}$cm^2/s. The specific activities (nmol of product formed/min per mg of protein) when assayed with the second nitrogenase component were 1500 for H_2 evolution, 380 for N_2 reduction, 1200 for acetylene reduction and 5400 for ATP hydrolysis. The reduced protein showed electron-paramagnetic-resonance signals at $g = 4.3$, 3.7 and 2.015; the Mössbauer spectrum of the reduced protein consisted of at least three doublets. The u.v. spectra of the oxidized and reduced proteins were identical. On oxidation the absorbance increased generally throughout the visible region and a shoulder at 430nm appeared. The circular-dichroism spectra of both the oxidized and reduced proteins were the same, consisting mainly of a negative trough at 220nm. 4. The smaller component had mol.wt. 66800 and contained four Fe atoms and four acid-labile sulphide groups in a molecule comprising two subunits each of mol.wt. 34600. All common amino acids except tryptophan were present, with a predominance of acidic residues. The apparent partial specific volume calculated from the amino acid analysis was 0.732, which was significantly higher than that obtained from density measurements (0.69); ultracentrifugal analysis gave $s^0_{20,w} = 4.8$S and $D^0_{20,w} = 5.55 \times 10^{-7}$cm^2/s. The specific activities (nmol of product formed/min per mg of protein) were 1050 for H_2 evolution, 275 for N_2 reduction, 980 for acetylene reduction and 4350 for ATP hydrolysis. The protein was not cold-labile. The reduced protein showed electron-paramagnetic-resonance signals in the $g = 1.94$ region. The Mössbauer spectrum of the reduced protein consisted of a doublet at 77°K. The u.v. spectra of reduced and O_2-inactivated proteins were identical, and inactivation by O_2 generally increased the absorbance in the visible region and resulted in a shoulder at 460nm. The circular-dichroism spectra exhibited a negative trough at 220nm and inactivation by O_2 decreased the depth of the trough. 5. The reduction of N_2 and acetylene, and H_2 evolution, were maximal at a 1:1 molar ratio of the Fe-containing protein to the Mo–Fe-containing protein; excess of the Mo–Fe-containing protein was inhibitory. All reductions were accompanied by H_2 evolution. The combined proteins had no ATP-independent hydrogenase activity.

Nitrogenase from a variety of organisms has been fractionated into two protein components, an Fe-containing protein and an Fe and Mo-containing protein (see Burris, 1971). Only the component proteins from the nitrogenases of the strict anaerobe *Clostridium pasteurianum* and the strict aerobe *Azotobacter vinelandii* have been examined in detail.

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These are proteins Cp1* (Dalton *et al.*, 1971), Cp2 (Moustafa & Mortenson, 1969), Av1 (Bulen & LeComte, 1966; Burns *et al.*, 1970) and Av2 (Moustafa, 1970). Some of these reports are preliminary in nature, and the characterizations of the proteins are limited. Less information is available about comparable nitrogenase proteins from *Rhizobium japonicum* bacteroids (Bergersen & Turner, 1970; Evans & Russell, 1971).

In the present paper we describe the purification and properties of the component proteins (Kp1 and Kp2) of the nitrogenase of the facultative anaerobe *Klebsiella pneumoniae* and compare their properties with available results for the nitrogenase proteins of *Clostridium pasteurianum* and *Azotobacter vinelandii*. The partial purification of Kp1 protein and Kp2 protein has been reported (Kelly, 1969*b*).

Materials and Methods

Maintenance and growth of the organism

Klebsiella pneumoniae, strain M5a1, a gift from Professor P. W. Wilson, Department of Bacteriology, University of Wisconsin, U.S.A., was maintained at 20°C in air on 2% Oxoid nutrient-agar slopes and was subcultured monthly. For large-scale cultivation the organism was grown at 30°C under N_2 in a 400-litre glass fermenter on the N-free medium of Hino & Wilson (1958), except that the $CaCO_3$ was omitted and the medium was made up with tap water sterilized by filtration. A 5% seed culture, growing in the logarithmic phase under N_2, was used to inoculate the fermenter; bacteria were harvested under N_2 after 24h, by using a Westfalia continuous centrifuge. The paste was frozen and stored in liquid N_2 until required for use.

Assay of nitrogenase activity

Acetylene reduction. Serum bottles (7.5–8ml) capped with Suba-Seal (Griffin and George, Wembley, Middx., U.K.) closures contained, in 1.5ml: 40μmol of 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid (HEPES) buffer, pH7.8, 8μmol of ATP, 20μmol of $MgCl_2$, 15μmol of creatine phosphate, 20μmol of $Na_2S_2O_4$, 0.2mg of creatine kinase, under 10^4 N/m^2 (0.1atm) of acetylene in Ar. In some assays mentioned, creatine phosphate and creatine kinase were omitted and 5.25μmol of ATP was used. The sodium dithionite and acetylene were injected through the Suba-Seal after the bottle had been flushed with Ar. The reaction mixture was shaken through 4cm at 90 strokes/min at 30°C. After 5min the pressure in the bottle was equalized to atmospheric pressure, by momentarily piercing the Suba-Seal with a hypodermic needle, before crude or purified nitrogenase was injected to start the reaction. Except in time-course studies the reaction was stopped after 10min with 0.1ml of 40% (w/v) NaOH. Under these conditions the reaction showed a lag of 20s before the rate became linear and remained so during the rest of the assay time, provided that the amount of ethylene produced did not exceed 1.2μmol. For assays with a Viologen dye as electron donor, a reduced-Viologen-generating system consisting of crude hydrogenase and 1atm (10^2kN/m^2) of H_2 was used instead of dithionite. The hydrogenase was an unfractionated extract of acetone-dried *Desulfovibrio vulgaris* (N.C.I.B. 8303) in 25mM-tris–HCl buffer, pH7.4, which was centrifuged for 10min at 30000*g* to remove debris and was stored under H_2 at −15°C (containing 7.2mg of protein/ml). Approx. 1.5mg of hydrogenase and appropriate amounts (0.1–0.5μmol) of Viologen dye were added to the assay system, without creatine kinase, and, after gassing with H_2 and acetylene, the vessels were left until the colour of the reduced dye had appeared. Creatine kinase was then injected and the reaction was initiated by injection of nitrogenase.

Tests with H_2+Ar mixtures showed that the reaction rate was independent of H_2 partial pressure down to 10^4 N/m^2 (0.1atm) of H_2 and of hydrogenase concentration down to 0.35mg/ml. Incompletely purified *K. pneumoniae* nitrogenase possessed sufficient intrinsic hydrogenase activity to reduce viologen dyes and acetylene in their presence, without added *Desulfovibrio* hydrogenase. The highly purified nitrogenase proteins reported here had been freed of such activity.

Ethylene was determined in a Pye 104 gas–liquid chromatograph fitted with a flame ionization detector. Gas samples (0.5–1ml) were injected into 0.76 or 1.52m (2.5 or 5ft) Porapak R columns (4mm internal diam.) at 65°C with N_2 as a carrier gas flowing at 20–60ml/min. With a fixed sample volume, the peak height was proportional to the amount of ethylene in the sample. The specific activity of the separated nitrogenase proteins was determined by assaying them in the presence of an optimum concentra-

*Abbreviations: the nitrogenase components of the 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 [molybdoferredoxin of Mortenson *et al.* (1967), azofermo of Hardy *et al.* (1971*a*), fraction 1 of various other authors]; and the number 2 indicates the Fe-containing protein [azoferredoxin of Mortenson *et al.* (1967), azofer of Hardy *et al.* (1971*b*) and fraction 2 or Fe-protein of various other authors]. This notation (Postgate, 1971) is less cumbersome than the alternatives, and does not ascribe as yet unsubstantiated roles to the two proteins. Kp is *Klebsiella pneumoniae*, Cp is *Clostridium pasteurianum*, Av is *Azobacter vinelandii*, Ac is *A. chroococcum* and Bp is *Bacillus polymyxa*. Other abbreviations are: e.p.r., electron paramagnetic resonance; c.d., circular dichroism; o.r.d., optical rotatory dispersion.

tion (see below) of the purified complementary protein.

H_2 evolution. This was measured under the conditions described above for acetylene reduction, except that the gas phase was Ar alone and the H_2 formed was determined in a Perkin–Elmer 452 gas–liquid chromatograph fitted with a thermal conductivity detector. Gas samples (1 ml) were injected into a 0.76m (2.5ft) column of a 0.5nm (5Å) molecular sieve, 100–120 mesh (Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, U.K.), at 60°C, with Ar as carrier gas at a setting of $1.03 \times 10^5 N/m^2$ (15lbf/in^2). Peak areas were measured with a Perkin–Elmer type 194B printing integrator.

N_2 reduction. This was measured in similar vessels with the same reaction mixture as for acetylene reduction, except that the gas phase was N_2 and the reaction was stopped with 0.1 ml of 30% (w/v) trichloroacetic acid. Samples (1 ml) of the stopped-reaction mixture were added to 1 ml of a saturated K_2CO_3 solution contained in a serum bottle, which was then quickly stoppered with a rubber bung carrying a glass rod, on which a drop of 0.5M-H_2SO_4 was suspended. The serum bottle was then shaken over 4cm at 130 strokes/min for 2.5h at 20°C. The rod was rinsed in 2ml of water, and 1 ml of this solution was analysed for NH_3 by the indophenol method (Chaney & Marbach, 1962). Recovery of standard amounts of NH_3 in the range 0.1–1 μmol was 98% under these conditions.

ATP hydrolysis. This was measured in assay mixtures from which the ATP-generating system was omitted. Assays were stopped at intervals with 0.1 ml of 30% (w/v) trichloroacetic acid and P_i was measured by the method of Taussky & Shoor (1943).

Specific activity

One unit of activity is defined as the amount of enzyme required to produce 1 nmol of product/min under the conditions given above. In crude extracts, specific activities refer to total protein in the extract; with separated nitrogenase components they are expressed in terms of whichever protein limited the rate of substrate reduction.

Purification procedure

Anaerobic techniques. Throughout the purification, assay and subsequent handling of nitrogenase components, strict anaerobic precautions were taken. All buffers were flushed continuously with O_2-free N_2 and they contained 100mg of sodium dithionite and 100mg of dithiothreitol/l, unless otherwise stated. Column effluents were collected in conical flasks flushed with N_2, and the fractions were manipulated with 50ml syringes fitted with polyvinyl chloride tubing.

Preparation of crude extracts and separation of nitrogenase components. During this work the procedures adopted for the purification of nitrogenase components were changed in the light of experience. Our most successful procedure for purification (now routine) is described below; because the purified components were completely stable at room temperature for at least 30h, all manipulations beyond Step 1 were conducted at room temperature.

Step 1. Preparation of crude extract. *K. pneumoniae* organisms (425g wet wt.) were suspended in 300ml of 25mM-tris–HCl buffer, pH7.4, and were disrupted by passage through a French pressure cell, previously cooled on ice, at $10^8 N/m^2$ (15000lbf/in^2). The resulting suspension was centrifuged at 5°C for 90min under N_2 at 25000*g* to remove debris and whole organisms. The clear brown supernatant obtained in this way is referred to as 'crude extract'.

Step 2. Chromatography on DEAE-cellulose. The crude extract from Step 1 was mixed with an equal volume of a thick slurry of DEAE-cellulose (Whatman DE52) in 25mM-tris–HCl buffer, pH7.4, and was allowed to equilibrate for 15min. It was then poured on a column (16cm × 6.5cm) of clean anaerobic DEAE-cellulose equilibrated with the same buffer. After the DEAE-cellulose had settled to form a compact column it was washed with 1 bed vol. of buffer. The batch process removed some contaminating protein and made subsequent chromatography faster. The column was developed with stepwise additions of 150ml of 0.15, 0.20, 0.21, 0.22 and 0.23M-NaCl in 25mM-tris–HCl buffer, pH7.4. A dark-yellow protein was eluted first, and then a broad dark-brown band containing protein with Kp1 activity; this peak was collected in fractions of approx. 50ml. Fractions with a specific activity greater than 500nmol of acetylene reduced/min per mg were combined for further purification. The column was then developed with 500ml of 90mM-$MgCl_2$ in 25mM-tris–HCl buffer, pH7.4, which eluted protein with Kp2 activity as a sharp yellowish-brown band, which still had considerable activity when assayed alone, but showed an enhancement of about 4-fold when more Kp1 protein was added.

Step 3. Purification of Kp1 protein. The combined fractions containing Kp1 protein from Step 2 were concentrated to about 30mg of protein/ml by ultrafiltration with a Diaflo UM10 membrane [Amicon N.V., Mechelaastraat, Oosterhout (N.B.), Holland] under $1.72 \times 10^5 N/m^2$ (25lbf/in^2) of Ar. The concentrated material was centrifuged to remove a small amount of precipitate that formed during concentration and then was loaded on an anaerobic column (40cm × 5cm) of Sephadex G-200 in a K50/60 chromatography column [Pharmacia (G.B.) Ltd., London W.5, U.K.). The column was equilibrated with 25mM-tris–HCl buffer, pH8.7, and was developed by using an upward flow at 30ml/h. Kp1 protein was

eluted at 420ml as a discrete brown band. At this stage the purity of the preparation was investigated by using analytical disc-electrophoresis and it was occasionally necessary to repeat this step to remove trace impurities.

Step 4. Purification of Kp2 protein. Fractions containing Kp2 protein activity from Step 2a were diluted fourfold by the addition of 25mM-tris–HCl buffer, pH7.4, and were then adsorbed on a small column (4cm×2.5cm) of DEAE-cellulose equilibrated with this buffer. The protein was then eluted as a light-brown band with 90mM-$MgCl_2$ in the same buffer. Because of the large volume of fractions from Step 2a, this method of concentration was preferred to ultrafiltration at this stage. The concentrated material was loaded on a column (37cm×5cm) of Sephadex G-100 in a K50/60 chromatography column. The column was equilibrated with 25mM-tris–HCl buffer, pH7.4, containing 50mM-$MgCl_2$ and was developed by using an upward flow of 30ml/h. The column resolved the contaminating Kp1 protein (elution volume approx. 400ml) from the Kp2 protein (elution volume 500ml), which was eluted as a discrete brownish-yellow band. Because of the large amount of protein loaded on the column initially, it was usually necessary to repeat this step.

The purification procedure for both components is summarized in Table 1.

Analytical methods

Ultraviolet- and visible-absorption spectra. Samples of reduced Kp1 and Kp2 proteins were prepared by incubating them with 1mM-$Na_2S_2O_4$, and then passing the proteins down an anaerobic Chelex 100 column, to remove non-specifically bound metals, and finally passing them down a Sephadex G-10 column; both columns had been equilibrated with 50μM-$Na_2S_2O_4$. The eluent was 25mM-tris–HCl buffer, pH7.4, containing 0.1mg of dithiothreitol/ml. The sample of buffer that emerged immediately before the protein from the Sephadex G-10 column was used as a blank for spectroscopy. All glassware used after the Chelex treatment of the proteins was previously freed from contaminating metal ions by soaking it for 24h in 10% (v/v) Decon 75 detergent and rinsing successively in deionized water, 5% (v/v) HCl and then twice with deionized water. The Sephadex G-10 was washed with a saturated aqueous solution of EDTA before use.

To prepare oxidized solutions of Kp1 or Kp2 proteins, they were first freed from non-specific

Table 1. *Purification of the components of Klebsiella pneumoniae nitrogenase*

For experimental details see the text.

Step no.	Fraction	Vol. (ml)	10^{-3}×Total activity (units)	Total protein (g)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
		Kp1 protein					
1	Supernatant obtained from crude extract after centrifugation	500	800	17.6	50	1	100
2	DEAE-cellulose effluent	323	1660	1.87	885	17.7	189
3	Sephadex G-200 effluent	42	115	0.093	1247	24.9	135*
		Kp2 protein					
1a	Supernatant obtained from crude extract after centrifugation	610	1190	18.8	63.5	1	100
2a	DEAE-cellulose effluent	375	1310	2.66	350	5.5	110
4	Sephadex G-200 effluent	80	380	0.23	875	13.8	64†
5	Step 4 repeated	100	122	0.128	950	15	20.4

*Overall yield; batches of approx. one-tenth of the protein from Step 2 were chromatographed separately.
† Overall yield; protein from Step 2a was chromatographed in two batches.

metals by passage down a Chelex 100 column, which has been equilibrated with 100mg of $Na_2S_2O_4$/l in 25mM-tris–HCl buffer, pH7.4. The protein and dithionite were then oxidized with potassium ferricyanide or Lauth's Violet (thionine) and the excess of oxidant was removed with an anaerobic Sephadex G-10 column, previously equilibrated with degassed 25mM-tris–HCl buffer, pH7.4, containing 100mg of dithiothreitol/l. Spectra were measured at 25°C with a Unicam SP.1800 spectrophotometer in 1mm or 10mm light-path quartz cuvettes. After the spectral measurements had been made, the acetylene-reducing activity of the protein solutions in the cuvettes was measured and the protein concentration was measured by the Folin–Ciocalteau method as described below.

Circular-dichroism spectra. C.d. spectra were measured on solutions prepared, as for absorption spectroscopy, by using a Jasco U.V./O.R.D. 5 instrument fitted with a c.d. accessory. The machine was calibrated with a solution of 5α-cholestan-3-one in methanol (1mg/ml). The c.d. results are reported as mean residue ellipticities $[\theta]$ in units of degrees·$cm^2 \cdot dmol^{-1}$.

Mössbauer and electron-paramagnetic-resonance spectroscopy. The cuvettes and instrument used for Mössbauer spectroscopy have been described (Kelly & Lang, 1970). Samples for e.p.r. measurements were prepared in 3mm outer diam. quartz tubes fitted with standard tapers. All cuvettes were degassed to less than $0.67 N/m^2$ (5×10^{-3} Torr) and flushed with Ar three times before being filled with protein solutions. E.p.r. spectra were measured by using a Varian E-9 spectrometer fitted with a variable-temperature probe.

Amino acid analysis. Dithionite and dithiothreitol were removed from the protein samples before analysis by chromatography on an anaerobic column of Sephadex G-15, previously equilibrated with 5mM-tris–HCl buffer, pH7.4. The protein concentration of the sample treated in this way was determined by dry-weight measurement (see below). Duplicate samples (approx. 2mg of protein) were hydrolysed with 6M-HCl for 16, 24, 48 and 72h at 110°C in evacuated sealed tubes as described by Moore & Stein (1963) and analysed in a Locarte amino acid analyser. Where there was no obvious trend with time of hydrolysis the means of the 24h, 48h and 72h values were taken; for serine and threonine the values were extrapolated to zero time and for valine and isoleucine to 72h. Half-cystine and methionine were determined as cysteic acid and methionine sulphone respectively in performic acid-oxidized samples prepared by the method of Moore (1963).

Tryptophan. This was determined by the method of Opienska-Blauth *et al.* (1963). Kp2 protein was negative in this test, and up to 3mg added to authentic tryptophan samples did not interfere with the determination.

Cysteine. Dithiothreitol and $Na_2S_2O_4$ were removed from the sample by anaerobic gel-filtration on Sephadex G-15 equilibrated with 25mM-tris–HCl buffer, pH7.4, before analysis by the method of Cavallini *et al.* (1966).

Disc electrophoresis. Analytical disc electrophoresis in 6% (w/v) polyacrylamide gels was done at pH7.9 as described by Hedrick & Smith (1968). Up to 250μg of protein could be run on the 7cm×0.5cm gels. The current was maintained at 2mA/tube. Gels were stained for protein with 1% (v/v) Naphthalene Black 10B (G. T. Gurr Ltd., London S.W.6, U.K.) in 7% (v/v) acetic acid for 1h and were destained by washing overnight with 7% (v/v) acetic acid. Anaerobic disc electrophoresis was done under essentially the same conditions, except that the gel solutions were degassed and flushed with N_2 before use, and the gels were poured under a stream of N_2. The reservoir buffer was degassed before use, and during electrophoresis the apparatus was enclosed in a glass vessel that was continually flushed with N_2.

Preparative disc electrophoresis was done in larger (8cm×1.2cm) gels with a maximum loading of 2.5mg/tube. Good resolution was obtained after 4h at a current of 5mA/tube. Protein was recovered from the gels by cutting the visible bands out of the gel with a scalpel and macerating the gel by anaerobic passage through a syringe. The macerated gels from several tubes were pooled, incubated with an equal volume of 25mM-tris–HCl buffer, pH7.4, under Ar for 1h, and then were centrifuged under Ar to remove the residual gel and were dialysed anaerobically against 25mM-tris–HCl buffer, pH7.4, to remove low-molecular-weight material leached from the gel.

Molecular-weight measurements by disc electrophoresis in polyacrylamide gels were done under two different sets of conditions. Kp1 protein was investigated by using the two-gel system of Hedrick & Smith (1968), except that the gels were prepared and run anaerobically as described above. A plot of log mobility relative to Amido Black (R_m) versus gel concentrations gave a straight line, with a slope that was linearly related to the molecular weight of the protein (see Fig. 3). The subunit structures of Kp1 and Kp2 proteins were investigated by using the single gel sodium dodecyl sulphate system of Weber & Osborn (1969). For these experiments the proteins were incubated for 1h in 10mM-sodium phosphate buffer, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) β-mercaptoethanol. They were then dialysed against 1 litre of the same buffer containing 0.1% (w/v) sodium dodecyl sulphate and 0.1% (w/v) β-mercaptoethanol before electrophoresis at pH7.0 in 7cm×0.5cm gels containing 0.1% sodium dodecyl sulphate. Electrophoresis took 4h at a current of 8mA/tube. Cytochrome *c* was included in all

Kp1 protein samples subjected to electrophoresis and R_m values were measured relative to cytochrome *c*. This procedure was not satisfactory for Kp2 protein, as it interacted with cytochrome *c* under these conditions, so R_m measurements were made relative to Brilliant Cresyl Blue. The gels were stained in Coomassie Brilliant Blue as described by Weber & Osborn (1969).

Gel isoelectric focusing. Analytical gel isoelectric focusing in 7.5% polyacrylamide gels with LKB Ampholine carrier ampholytes (pH range 3–10) was done as described by Wrigley (1968). The gel solutions were degassed and flushed with N_2 before use, and the gels were poured under a stream of N_2. The electrode-compartment solutions were degassed before use, and the entire apparatus was contained in a vessel that was flushed with N_2 during electrofocusing. Up to 150 μg of protein was run on the 7 cm × 0.5 cm gels. The current was maintained at 0.5 mA/tube for 45 min before protein samples were loaded and was maintained at this value for a further 3 h. Proteins were located on the gels by precipitation with 2.5% (w/v) sulphosalicylic acid. Nitrogenase components were visible before this treatment as brown bands on the gels. Approximate values for the isoelectric points of visible bands were obtained by cutting out these bands from several gels and eluting the protein into 1 ml of water.

Analytical centrifugation. This was done in a Beckman–Spinco model E ultracentrifuge by using an An-D rotor at 20±0.2°C and schlieren optics. Sedimentation and diffusion coefficients were determined over a range of concentrations and $s^0_{20,w}$ and $D^0_{20,w}$ values were determined by extrapolation to infinite dilution. In concentration-dependence studies the coefficients were evaluated at initial solute concentrations and the best fit to the result was determined by the method of least mean squares. The coefficients were corrected to standard conditions of 20°C and water as solvent, by using standard viscosity tables and results from Svedberg & Pedersen (1940*b*) on the effect of salts on viscosity. The analytical cell was flushed with N_2 for 10 min after it had been assembled and experiments were done under N_2 as gas phase.

Density measurements. These were made in a water bath at 20±0.02°C by using 5 ml and 10 ml dilatometers fitted with stoppers. Protein solutions were prepared by dialysis against degassed 5 mM-tris–HCl buffer, pH 7.4, containing 50 μM-$Na_2S_2O_4$. The protein concentration was determined by dry-weight measurements on known weights of the protein solution dried to constant weight (see below). The dilatometers were calibrated with double-distilled water.

Amount of protein. This was measured colorimetrically by the biuret method (Gornall *et al.*, 1949) and by the Folin–Ciocalteau method (Lowry *et al.*, 1951), or by dry-weight determination. For colorimetric analyses bovine serum albumin, previously dried in a desiccator for 24 h over P_2O_5, was used as a standard. Dry-weight measurements indicated that no correction factor was necessary for Kp1 and Kp2 proteins. For dry-weight determinations approx. 10 mg of protein was dialysed for at least 25 h against two changes of 3 litres of 5 mM-tris–HCl buffer, pH 7.4, before being evaporated to dryness in a hot-air oven at 95°C. At intervals the containers were removed from the oven, and were allowed to cool in a desiccator before being weighed and returned to the oven. This procedure was repeated until a constant weight was obtained (approx. 24 h). The dry weight of an equal volume of dialysate was subtracted from that of the sample to give the dry weight of protein.

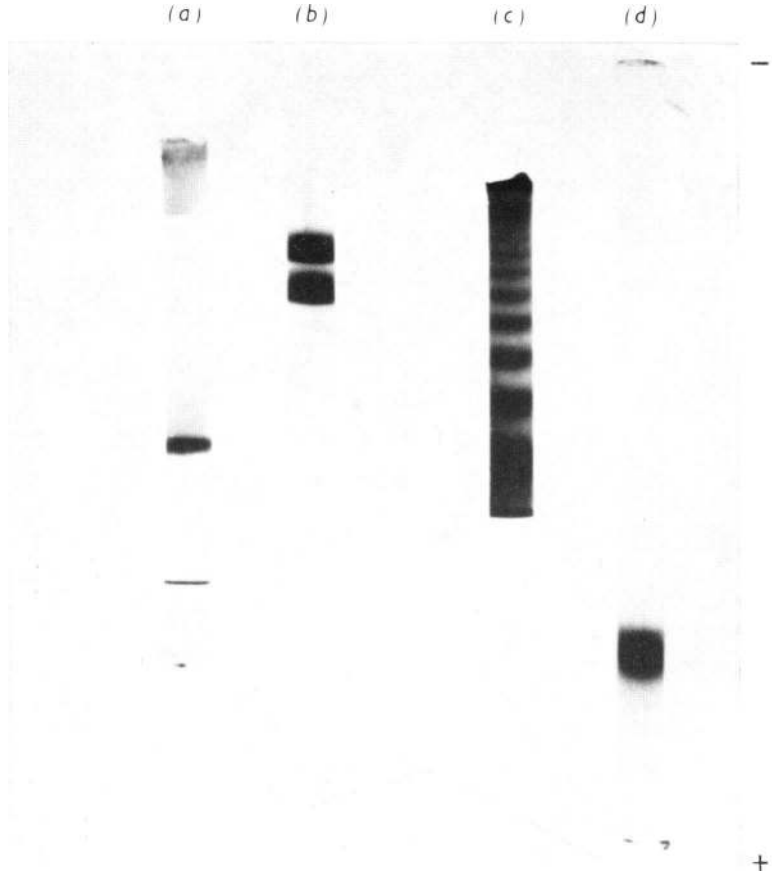
Metal analyses. Before metal analyses, samples were treated with Chelex 100 (Bio-Rad Laboratories Ltd., Porter's Wood, St. Albans, Herts., U.K.) to remove non-specifically bound metals. The samples to be analysed were passed down an anaerobic column (42 cm × 0.5 cm) of resin, previously equilibrated with 25 mM-tris–HCl buffer, pH 7.4, at a flow rate of 0.5 ml/min. Under these conditions neither nitrogenase component lost activity. Fe, Zn, Mo, Mn, Mg, Co, Cu, Cd and Ca were determined by atomic-absorption spectrometry with an A1750 atomic-absorption spectrophotometer (Southern Analytical Ltd., Camberley, Surrey, U.K.). Fe and Mo were also determined colorimetrically; acid-labile Fe was determined with 4,7-diphenyl-1,10-phenanthroline (Doeg & Ziegler, 1962) and Mo with toluene-3,4-dithiol by the method of Clarke & Axley (1955) as described by Bulen & LeComte (1966).

Amount of acid-labile sulphide. Dithionite and dithiothreitol were removed from the sample by anaerobic gel filtration on Sephadex G-15, previously equilibrated with 25 mM-tris–HCl buffer, pH 7.4, before analysis by the method of King & Morris (1967) with *NN'*-dimethyl-*p*-phenylenediamine.

Amount of N. Total N was measured in a Coleman Nitrogen Analyser (Baird and Tatlock Ltd., Chadwell Heath, Essex, U.K.) with carefully dried specimens.

Protein standards

Peptide molecular weights are those from Weber & Osborn (1969); bovine serum albumin (68000), catalase (60000), glutamate dehydrogenase (53000), ovalbumin (43000), creatine kinase (40000), carboxypeptidase A (34000), trypsin (23300), lysozyme (14300) and cytochrome *c* (11700). The molecular weights used for native proteins were: urease (483000), xanthine oxidase (275000), catalase (240000), lactate dehydrogenase (130000), β-amylase (215000), hexokinase (99000), bovine serum albumin (68000), ovalbumin (47000), chymotrypsin (22500) and myoglobin (17800).



EXPLANATION OF PLATE 1

Polyacrylamide electrophoresis of native and sodium dodecyl sulphate-treated nitrogenase components

(*a*) Electrophoresis of 90μg of native Kp1 protein, at pH 7.9 under anaerobic conditions; (*b*) electrophoresis of Kp1 protein, after treatment with 1% (w/v) sodium dodecyl sulphate and 1% (v/v) β-mercaptoethanol, in sodium dodecyl sulphate–polyacrylamide gel at pH 7.0; (*c*) electrophoresis of 120μg of native Kp2 protein at pH 7.9 under anaerobic conditions; (*d*) electrophoresis of Kp2 protein treated as in (*b*).

Chemicals

Creatine phosphate, ATP, ITP, UTP, GTP, bovine serum albumin, fraction V, and creatine phosphokinase (EC 2.7.3.2) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Dithiothreitol was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and sodium dithionite (99% pure) was a gift from Albright and Wilson, Stratford, London E.15, U.K. Methyl Viologen, Benzyl Viologen and Lauth's Violet were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Mo–Fe protein (Kp1) of Klebsiella nitrogenase

As indicated below, the purification procedure described in the Materials and Methods section gave a preparation of Kp1 protein that behaved as a single molecular species at normal pH values in electrophoresis, sedimentation behaviour and during isoelectric focusing. It was free of Kp2 protein and conventional hydrogenase activity. It was therefore considered to be homogeneous, although attempts to crystallize it by the method used by Burns *et al.* (1970) for Av1 protein were not successful.

Specific activity. A sample (5mg) of Kp1 protein assayed alone was completely inactive. Kp1 protein reduced acetylene with an optimum concentration of Kp2 protein (see below) 30 times faster than the crude extract. The specific activities are given in Table 3.

Disc electrophoresis. When 90μg of Kp1 protein was subjected to electrophoresis anaerobically it ran as a single band with R_m 0.67 (see Plate 1). If anaerobic precautions were not taken before and during disc electrophoresis multiple bands were produced. In 6% acrylamide gels at pH7.9, run anaerobically by the method of Hedrick & Smith (1968), one major brown band and two minor brown bands were formed. Isolation and assay of these bands from preparative disc-electrophoresis gels, prepared anaerobically as described in the Materials and Methods section, showed all three of them to have protein containing Kp1 activity. The splitting occurred also in the pH range 7.9–9.5 and in the single-gel system of Hjertén *et al.* (1965), in which the ammonium persulphate is removed from the gels before electrophoresis.

Isoelectric point. A sample (100μg) of Kp1 protein, when analysed by isoelectric focusing under anaerobic conditions in 5% polyacrylamide gels containing 0.1% (v/v) Ampholines, ran as two bands with isoelectric points at pH5.0 and 5.5. Both of these bands were brown and contained Fe as judged from their colour reaction with bathophenanthroline after treatment of the gels with 2.5% (w/v) sulphosalicylic acid. Oxidation of Kp1 protein with Lauth's Violet as described in the Materials and Methods section and subsequent isoelectric focusing produced a single band with a pI of 5.

Subunits. Electrophoresis of Kp1 protein, after treatment with 1% (v/v) β-mercaptoethanol and 1% (w/v) sodium dodecyl sulphate, in gels containing sodium dodecyl sulphate [under the conditions of Weber & Osborn (1969)] produced two bands of equal intensities (see Plate 1). By comparison of the rates of migration of these bands with standard curves for proteins of known molecular weight (see Fig. 1), the values in Table 3 were obtained for the molecular

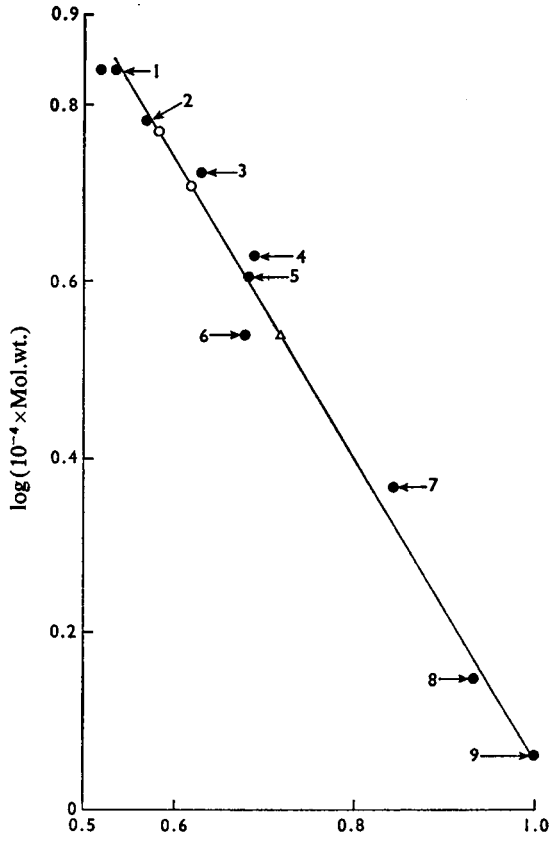


Fig. 1. *Molecular-weight determination of subunits of Kp1 and Kp2 proteins by disc electrophoresis*

The conditions were those of Weber & Osborn (1969). Proteins used for molecular-weight calibration (●): 1, bovine serum albumin; 2, catalase; 3, glutamic dehydrogenase; 4, ovalbumin; 5, creatine kinase; 6, carboxypeptidase; 7, trypsin; 8, lysozyme; 9, cytochrome *c*. (For molecular weights see the Materials and Methods section.) Subunits of Kp1 protein (○); subunit of Kp2 protein (△).

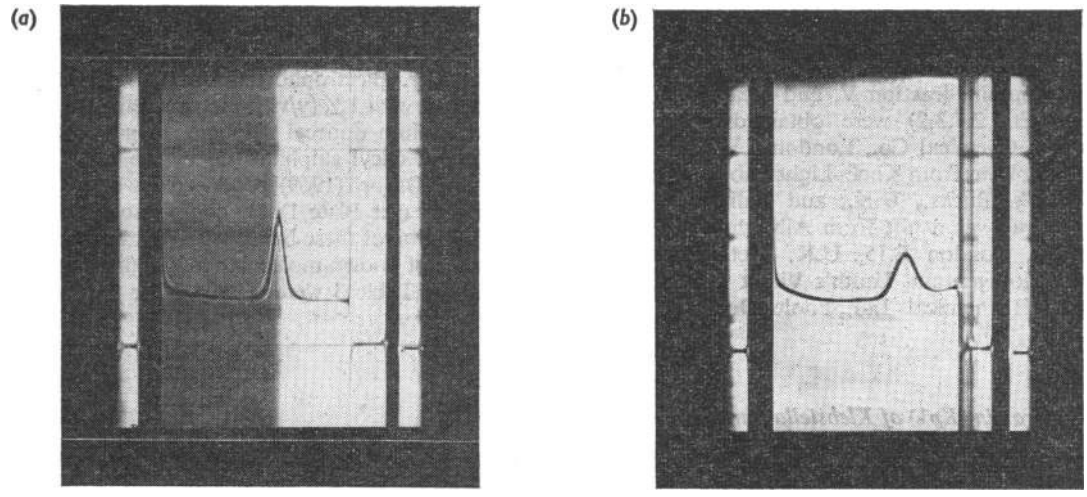


Fig. 2. *Ultracentrifuge schlieren patterns of Kp1 and Kp2 proteins*

The pictures were taken at 32 and 56min after reaching a speed of 60200rev./min. Sedimentation at 20°C was from right to left under a gas phase of N_2. (*a*) Kp1 protein, 5.3mg of protein/ml in 25mM-tris–HCl buffer, pH8.7, containing 0.1mg of dithiothreitol/ml and 0.1mg of $Na_2S_2O_4$/ml; (*b*) Kp2 protein, 4.8mg of protein/ml in 25mM-tris–HCl buffer, pH7.9, containing 50mM-$MgCl_2$, 0.1mg of dithiothreitol/ml and 0.1mg of $Na_2S_2O_4$/ml.

weights of the subunits of Kp1 protein. Integration of the peak areas of densitometer traces of these bands showed a difference of 11%. We suggest that this difference is due to differential dye-binding by the two subunits and that native Kp1 protein comprises two subunits of each type. From these results a minimum mol.wt. of 110900 was calculated.

Sedimentation coefficient. Kp1 protein sedimented as a single symmetrical peak in the ultracentrifuge in 25mM-tris–HCl buffer, pH8.7 (see Fig. 2), and analysis of the boundary shape during sedimentation (Schachman, 1957) indicated that the material was homogeneous. Sedimentation coefficients were determined at concentrations of Kp1 protein from 1.5 to 5.3mg/ml and extrapolated to zero concentration (Table 3).

Diffusion coefficient. The diffusion coefficient (see Table 3) of Kp1 protein in 25mM-tris–HCl buffer, pH7.4, was measured in the ultracentrifuge. Since the apparent diffusion coefficients were concentration-dependent within the range 1.5–5.3mg of Kp1 protein/ml, the values were extrapolated to infinite dilution. The frictional ratio (see Table 3), calculated from the diffusion coefficient and the mol.wt. of 218000 (see below), was typical of a globular protein.

Partial specific volume ($\bar{v}$). The apparent specific volume, ϕ, of Kp1 protein was obtained by use of the relationship:

$$\phi = \frac{1}{n}\left[\frac{1}{\rho_t} - \frac{(1-n)}{\rho_{o,t}}\right]$$

where n is the weight fraction of solute, ρ_t is the density of the solution at temperature t, and $\rho_{o,t}$ is the density of the solvent at the same temperature. At 7mg/ml the density of Kp1 protein gave $\bar{v} = 0.73 \pm 0.01$ ml/g in good agreement with $\bar{v} = 0.732$ ml/g calculated from the amino acid composition.

Amino acid composition. The amino acid composition of Kp1 protein is shown in Table 2. There are many more acidic than basic amino acids, an observation which agrees with its high affinity for DEAE-cellulose and its relatively low pI. From the results in Table 2, a minimum mol.wt. of 114560 was calculated. The apparent partial specific volume was calculated from the equation of Cohn & Edsall (1943). The values of residue partial specific volumes used in the calculation were those of Cohn & Edsall (1943), except for cysteine, where the revised value of McMeekin & Marshall (1952) was used.

The calculated N content from the amino acid composition is 15.75%. This does not agree with the value of 14.4% found on analysis of a sample of Kp1 protein, as described in the Materials and Methods section. We have no clear explanation for this discrepancy. Kp1 protein contains no carbohydrate when analysed by the method of Trevelyan & Harrison (1952).

Molecular weight. The molecular weight calculated from the equation of Svedberg & Pedersen (1940*a*), by using a value for $\bar{v}$ of 0.73, was 200400.

The molecular weight of Kp1 protein, determined

by disc electrophoresis by the method of Hedrick & Smith (1968) and by using the calibration curve in Fig. 3, was 217000. The molecular weight determined by thin-layer gel filtration on Sephadex G-200 (superfine grade), by using the calibration curve in Fig. 4, was 220000. These molecular weights are in reasonable agreement and compare with the value of 229000, calculated as a multiple of the minimal molecular weight of 114560 based on the amino acid composition, and 221800 as two of each of the subunits reported above.

The mean of the molecular-weight values of Kp1 protein summarized in Table 3 is 218000, and this value will be used subsequently in the present paper.

Acid-labile sulphide content. Kp1 protein contained 33.6ng-atoms of sulphide/mg, which corresponds to 16.7±1g-atoms of sulphide/mol and is equivalent to the Fe content.

Cysteine content. The amino acid analysis of performic acid-oxidized samples gave 36 cysteic acid residues/mol. Colorimetric analysis for cysteine under conditions that did not reduce disulphide bonds gave 9.5μg of cysteine/mg of Kp1 protein, which is equivalent to 17±1 cysteine residues in a molecule.

Metal content. Treatment with Chelex 100 as described in the Materials and Methods section did not affect the activity of Kp1 protein, but it removed 1.2μg of Fe/mg of protein. Table 3 shows the relative abundance of the metals analysed. The atomic ratio of Mo to Fe is 1:17/molecule. There is good evidence on nutritional grounds for the involvement of Mo and Fe in nitrogen fixation (Wilson, 1958), but the significance of the presence of approx. 2g-atoms of Mg/mol and 1g-atom of Zn, Cu and Ca/mol in Kp1 protein is difficult to assess. Zn has been reported in a nitrogenase component from *A. vinelandii* (Kajiyama *et al.*, 1969), and Cp1 protein contains 1.7g-atoms of Ca/mol, and significant amounts of Mg (Dalton *et al.*, 1971).

Stability. Purified Kp1 protein was completely stable at room temperature for up to 50h under Ar or N_2 in 25mM-tris–HCl buffer, pH7.8. On exposure to O_2 [0.2atm (2×10^4N/m^2] of O_2 in Ar) at 30°C in the absence of $Na_2S_2O_4$, activity was lost rapidly (Fig. 5). Over 60% of the activity was destroyed in 10min and attempts to restore activity by treatment with Na_2S, β-mercaptoethanol and $FeSO_4$ were unsuccessful.

The protein had a fairly wide pH-stability range. At 0°C activity was unchanged over 4h at pH values between 4.5 and 9.2. Below pH4 precipitation and loss of activity occurred.

Ultraviolet- and visible-absorption spectra. The u.v.

Table 2. *Amino acid composition of Kp1 protein*

The results, which are in nmol, are the average of two duplicate hydrolyses. In determining the best value for serine and threonine the results were extrapolated to zero time; those for valine and isoleucine are the 72h recoveries. Cysteine was determined as cysteic acid in a performic acid-oxidized sample and tryptophan by colorimetric analysis. The best ratio was derived as described by Thornber & Olson (1968).

Hydrolysis time (h) ...	16	24	48	72	Oxidized protein 48	Best value	Best ratio	Nearest integer
Asx	34.7	34.9	35.6	35.8	33.5	35.4	105.4	105
Thr	17.1	17.0	16.4	16.1	15.8	17.4	51.8	52
Ser	16.1	17.0	15.3	13.7	18.4	18.4	54.8	55
Glx	35.2	32.9	35.0	33.9	33.9	34.4	102.5	103
Pro	16.1	15.5	15.8	14.9	16.1	15.4	45.9	46
Gly	25.1	25.4	24.8	26.1	25.8	25.4	76	76
Ala	26.6	26.4	26.5	26.7	28.5	26.5	78.9	79
Val	17.1	19.4	20.2	20.8	20.3	20.8	62	62
Met	13.1	13.0	12.4	12.5	—	12.6	37.5	38
Ile	14.1	15.0	15.8	16.7	15.1	16.7	49.7	50
Leu	30	30.4	30.5	30.3	29.7	30.4	90.6	91
Tyr	11.6	11.5	11.3	11.3	—	11.3	33.7	34
Phe	15.1	16.5	15.8	16.1	13.8	16.1	48	48
His	8.0	8.0	7.9	7.7	7.8	7.9	23.5	24
Lys	17.1	17.0	16.9	17.3	15.9	17.0	50.6	51
Arg	17.1	17.0	16.9	17.3	15.8	17.0	50.6	51
Cys(O_3H)	—	—	—	—	6.2	6.2	18.5	19
Trp	—	—	—	—	—	9.3	27.7	28

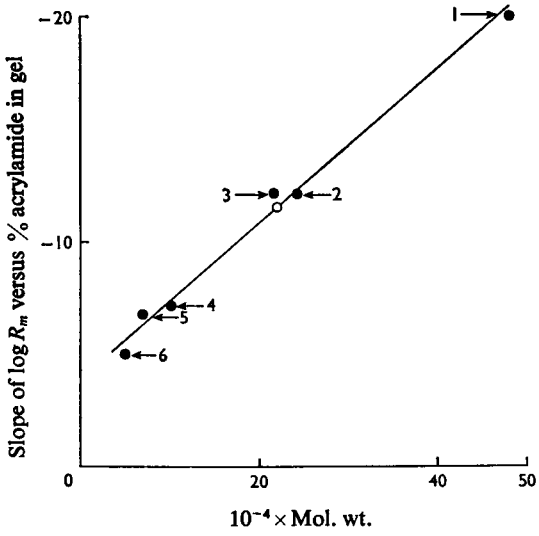


Fig. 3. *Molecular-weight determination of Kp1 protein by electrophoresis in polyacrylamide gels*

The conditions used were those of Hedrick & Smith (1968), except that the gels were prepared and run anaerobically. Proteins used for molecular-weight calibration (●): 1, urease; 2, catalase; 3, β-amylase; 4, hexokinase; 5, bovine serum albumin; 6, ovalbumin. (For molecular weights see the Materials and Methods section.) Kp1 protein (○).

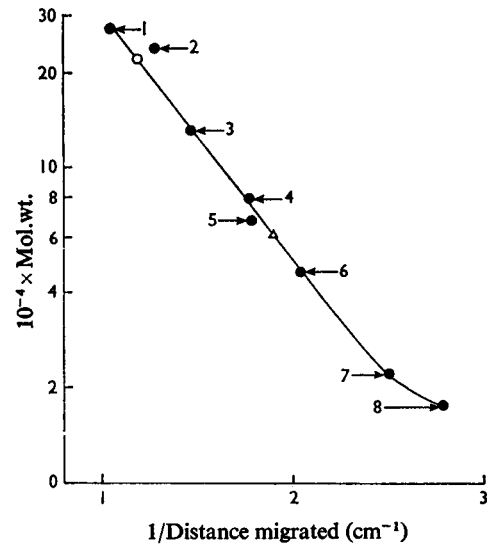


Fig. 4. *Molecular-weight determination of Kp1 and Kp2 proteins by thin-layer gel chromatography in Sephadex G-200*

The gel used was Sephadex G-200 (superfine), 1mm thick, previously equilibrated with 25mM-tris–HCl buffer, pH7.4, containing 50mM-$MgCl_2$, 0.1mg of dithiothreitol/ml and 0.1mg of $Na_2S_2O_4$/ml. Development was for 28h at an angle of 10° under N_2. Proteins used for molecular weight calibration (●): 1, xanthine oxidase; 2, catalase; 3, lactate dehydrogenase; 4, creatine kinase; 5, bovine serum albumin; 6, ovalbumin; 7, α-chymotrypsin; 8, myoglobin. (For molecular weights see the Materials and Methods section.) Kp1 protein (○); Kp2 protein (△).

and visible absorption spectra of reduced and oxidized Kp1 protein are shown in Fig. 6. Extinction coefficients are given in Table 3. Both species were characterized by identical strong absorption spectra in the u.v. region with a peak at 277.5nm and shoulders at 258.5, 269, 282 and 289nm. Reduced Kp1 protein exhibited lower absorbance than oxidized Kp1 throughout the visible region, the largest difference occurring at about 430nm, where oxidized Kp1 exhibited a shoulder. The absorption spectra of both species extended well into the near-infrared region. These results are more comprehensive than, but broadly similar to, those obtained for Cp1 protein (Dalton *et al.*, 1971) and Av1 protein (Burns *et al.*, 1970). We encountered some difficulty in obtaining reproducible extinction coefficients for reduced Kp1 protein between 290 and 360nm in the presence of $Na_2S_2O_4$. This was because the dithionite decomposed slightly faster in the presence than in the absence of Kp1 protein. As the difference spectrum between reduced and oxidized Kp1 protein was always identical with the absorption spectrum of $Na_2S_2O_4$ in this region, we concluded that the extinction coefficients for the two species were identical between 290 and 360nm.

Circular-dichroism spectra. The c.d. spectra of reduced and oxidized Kp1 protein from 210 to 250nm were identical and are shown in Fig. 7. The results below 210nm were very erratic, presumably because of the high background absorption caused by $Na_2S_2O_4$ and dithiothreitol, and are not reported. At wavelengths longer than 250nm, reduced Kp1 protein exhibited a very weak positive band (not shown), which began in the visible region and increased slowly until the sharp decrease to the trough at 220nm. As the positive band began well outside the region of aromatic absorption and there were no apparent changes in the latter region, this band may be associated with the metal chromophores. Most iron–sulphur proteins, e.g. the ferredoxins and xanthine oxidase, exhibit considerable dissymmetry of the metal chromophores as shown by their c.d. and o.r.d. spectra in the visible region (Tsibris & Woody,

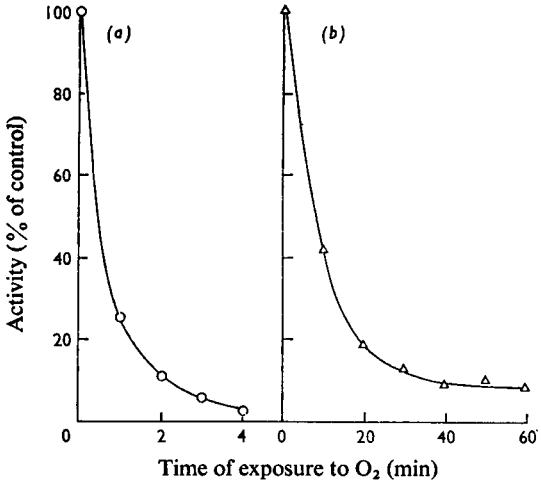


Fig. 5. *Effect of exposure to oxygen on the activity of Kp1 and Kp2 proteins*

The protein samples were incubated under 20kN/m² (0.2atm) of O_2 in Ar at 30°C in serum bottles capped with Suba-Seals, which were shaken over 7.5cm at a rate of 120 strokes/min. Samples (0.05ml) were removed and assayed with acetylene as substrate at the time-intervals indicated. The activity was compared with a control incubated under Ar alone. (*a*) ○, Kp2 protein, 0.9mg of protein/ml in 2ml of 25mM-tris–HCl buffer, pH7.9, containing 50mM-$MgCl_2$; (*b*) △, Kp1 protein, 1.45mg of protein/ml in 0.75ml of 25mM-tris–HCl buffer, pH8.7.

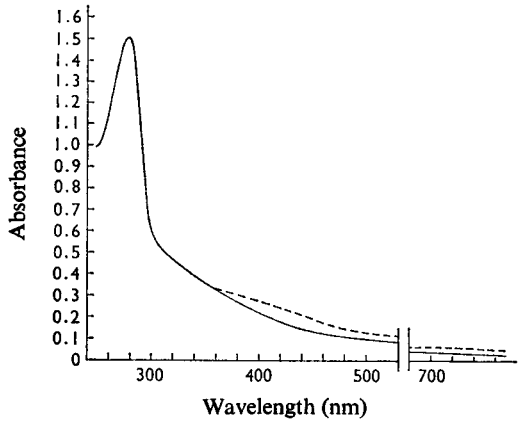


Fig. 6. *U.v.- and visible-absorption spectra of oxidized and reduced Kp1 protein*

The spectra are plotted as absorbance ($mg^{-1} \cdot ml$) of protein and are the average of several experiments on Chelex 100-treated protein solutions with concentrations from 0.5 to 2.0mg/ml. Solutions of both reduced (——) and oxidized (– – – –) Kp1 protein contained 25mM-tris–HCl buffer, pH7.4, and 0.1mg of dithiothreitol/ml. The reduced Kp1 protein solution also contained 50mM-$Na_2S_2O_4$. Preparation of the protein solutions and blanks is described in the Materials and Methods section.

1970). Both Kp1 and Kp2 proteins (see below) seem to be exceptional in this respect.

Between 210 and 250nm the c.d. spectra (Fig. 7) consisted of a single negative trough at 220nm with the suggestion of another below 210nm. The negative trough at 220nm has been associated with an α-helical structure, and by comparison with polyglutamic acid (Timasheff *et al.*, 1967), Kp1 protein, with the reduced mean residue ellipticity given in Table 3, was 38% α-helical.

Electron-paramagnetic-resonance spectra. The e.p.r. spectrum of reduced Kp1 protein exhibited g values shown in Table 3. After inactivation by O_2, the spectrum showed resonances at $g = 4.3$ and 2.0. Similar spectra have been reported for Av1 (Davis *et al.*, 1972; Hardy *et al.*, 1971) and Cp1 (Dalton *et al.*, 1971) proteins.

In addition, careful oxidation of reduced Kp1 protein resulted in a complex series of reversible changes in the e.p.r. spectrum, with the disappearance of the resonance at $g = 3.7$ and the appearance of new resonances in the region $g = 2.0$–1.6 (B. E. Smith, D. Lowe & R. C. Bray, unpublished work).

Mössbauer spectra. Kelly & Lang (1970) described Mössbauer spectra of Kp1 preparations with about one-quarter the specific activity of the present preparations. Their findings have been substantiated in general but with certain differences that are summarized below.

The Mössbauer spectrum of reduced Kp1 protein consisted of at least three doublets (see Table 3). That with $\delta = 0.35$mm/s and $\Delta E = 0.7$mm/s at 77°K broadened at 4.2°K indicating half-integral spin.

On brief exposure to O_2 a broad paramagnetic component appeared in the spectrum at 4.2°K. On further exposure to O_2 and loss of activity this component disappeared, and was replaced by one doublet with $\delta = 0.35$mm/s and $\Delta E = 0.9$mm/s (B. E. Smith & G. Lang, unpublished work).

The minor differences between the above observations and those reported by Kelly & Lang (1970) probably arise from the use of purer Kp1 protein and more rigid control of its oxidation state. The properties of Kp1 protein are summarized in Table 3.

Iron protein of Klebsiella nitrogenase, Kp2

The purification procedure described in the Materials and Methods section gave a preparation

of Kp2 protein, which behaved as a single molecular species in the ultracentrifuge, and, after treatment with sodium mersalyl or sodium dodecyl sulphate and β-mercaptoethanol, migrated as a single band on disc electrophoresis. It was free of Kp1 protein and conventional hydrogenase activity. Because of the extreme O_2 sensitivity of this protein (see Fig. 5), it was considered desirable to establish that the purification procedure would separate O_2-inactivated Kp2 protein from the native protein. When such a mixture was subjected to Step 4 of the purification procedure, complete resolution of Kp2 protein from O_2-damaged Kp2 protein was obtained, based on specific activity measurements before and after this treatment.

Specific activity. When up to 2mg of Kp2 protein was assayed alone it was completely inactive. Kp2 protein reduced acetylene in the presence of an optimum concentration of Kp1 protein (see below) 15 times faster than the crude extract. The specific activities of Kp2 protein are given in Table 3.

Disc electrophoresis. Electrophoresis in 6% acrylamide gels at pH7.9 gave the multiple band pattern as shown in Plate 1. Up to eight bands were observed with R_m values of 0.93, 0.71, 0.58, 0.46, 0.36, 0.28, 0.23, 0.13. This appearance was typical of gels prepared and run anaerobically as well as of gels prepared and run aerobically. It also occurred in gels run at pH9.7 and in the single gel system of Hjertén *et al.* (1965) when the ammonium persulphate is removed from the gels before electrophoresis. Analysis of the rate of migration of these bands by the method of Hedrick & Smith (1968), to determine if this unusual pattern was due to aggregation, was not successful, as both molecular size and charge isomers were apparently present. Pretreatment of Kp2 protein with 1mM-sodium mersalyl before electrophoresis changed the pattern to that of a single band with R_m of 0.2.

Isoelectric point. A portion (120μg) of Kp2 protein, when subjected to anaerobic isoelectric focusing as described for Kp1 protein, gave two major bands with pI of about 4 and a minor band with pI of 5.9. All these bands were brown and contained Fe. The pattern was the same when O_2-inactivated Kp2 protein was used, suggesting that the reduced material was becoming oxidized during isoelectric focusing.

Subunits. Treatment of Kp2 protein with sodium dodecyl sulphate as described for Kp1 protein gave a single band on electrophoresis (see Plate 1), and the rate of migration corresponded to a subunit mol.wt. of 34600±200 (see Fig. 1).

Sedimentation coefficient. The preparation sedimented as a single symmetrical peak in the ultracentrifuge at a concentration of 4.8mg of protein/ml in 25mM-tris–HCl buffer, pH7.4, containing 50mM-

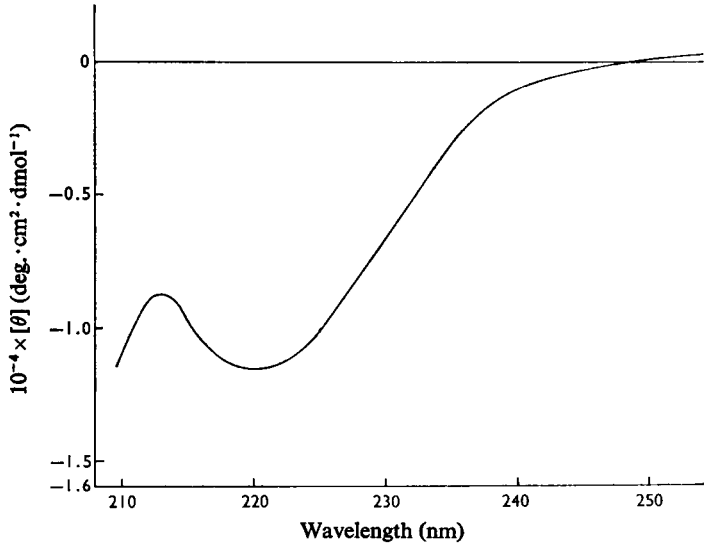


Fig. 7. *Circular-dichroism spectrum of Kp1 protein*

The reduced and oxidized species had identical spectra. Solutions were those used for u.v. and visible spectroscopy. The spectrum of each solution was measured at least three times. The spectra shown are the average of the spectra of three different solution concentrations.

$MgCl_2$ (see Fig. 2). Under these conditions, analysis of the boundary shape (Schachman, 1957) indicated that the material was homogeneous. Sedimentation coefficients determined at concentrations of Kp2 protein from 1 to 5.5 mg/ml were identical (see Table 3).

Diffusion coefficient. The diffusion coefficient of Kp2 protein (Table 3) was determined as described for Kp1 protein, except that 50 mM-$MgCl_2$ was included in the buffer. Assuming a mol.wt. of 66800 (see below), a value of 1.45 was calculated for the frictional ratio. This rather high value indicates a considerable degree of asymmetry.

Partial specific volume. The partial specific volume of Kp2 protein was calculated, as for Kp1 protein, from both density measurements and the amino acid composition. These two methods gave $\bar{v} = 0.69 \pm 0.01$ and 0.732 ml/g respectively. Differences of this order between calculated and measured partial specific volume have been reported for spinach ferredoxin, where the measured $\bar{v}$ is also 0.69 ml/g (Petering *et al.*, 1971).

Amino acid composition. The amino acid composition of Kp2 protein is shown in Table 4; a minimal mol.wt. of 11294 was calculated from these results. Acidic amino acids predominated markedly over basic ones, and tryptophan was completely absent. The calculated N content from the amino acid composition (15.4%, w/w) is in reasonable agreement with the value found by analysis (14.8%, w/w). Kp2 protein contains no carbohydrate when analysed by the method of Trevelyan & Harrison (1952).

Molecular weight. The molecular weight, calculated from the equation of Svedberg & Pedersen (1940*a*), by using the values of $\bar{v} = 0.69$ ml/g, $s^0_{20,w} = 4.8$ S and $D^0_{20,w} = 5.55 \times 10^{-7}$ cm^2/s (see Table 3), was 68200.

Because extensive aggregation occurred on electrophoresis under the conditions of Hedrick & Smith (1968) (see Plate 1), it was not possible to obtain a value for the molecular weight of Kp2 protein by this method. The molecular weight measured by thin-layer gel filtration through Sephadex G-200 (superfine grade) was 62000 (see Fig. 4).

These molecular weights are in reasonable agreement and compare with the value of 67800 calculated as a multiple of the minimal mol.wt. of 11294 based on the amino acid composition (see above) and 69200 as two of the subunits above.

The mean of the molecular-weight values of Kp2 protein summarized in Table 3 is 66800 and this value will be used subsequently in the present paper.

Acid-labile sulphide content. Kp2 protein contained 56.5 ng-atoms of sulphide/mg, which corresponds to 3.85 g-atoms of sulphide/molecule, and is approximately equivalent to the Fe content (see below).

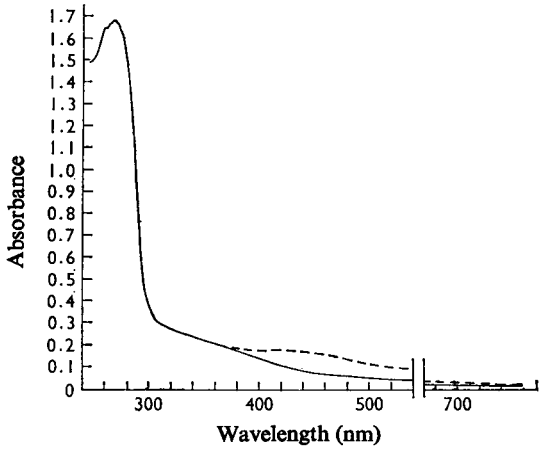


Fig. 8. *U.v.- and visible-absorption spectra of reduced and oxygen-inactivated Kp2 protein*

The spectra are plotted as absorbance ($mg^{-1} \cdot ml$) of protein and are the average of at least two experiments on Chelex 100-treated protein solutions with concentrations from 0.5 to 1.5 mg/ml. Solutions of reduced Kp2 protein (——) contained 25 mM-tris–HCl buffer, pH 7.4, 0.1 mg of dithiothreitol/ml and 50 μM-$Na_2S_2O_4$. Preparation of these solutions and blanks is described in the Materials and Methods section. The solutions of O_2-damaged Kp2 protein (----) were obtained by exposing the reduced Kp2 protein solutions and blanks to air for 15 min.

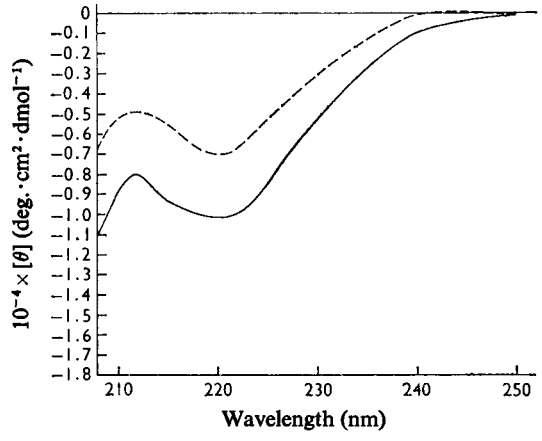


Fig. 9. *Circular-dichroism spectra of reduced and oxygen-damaged Kp2 protein*

Solutions of reduced (——) and O_2-damaged (----) Kp2 protein were those used for u.v. and visible spectroscopy. The spectrum of each solution was measured at least three times and the results from two different Kp2 protein concentrations were averaged to obtain the spectra shown.

Cysteine content. The amino acid analysis of performic acid-oxidized samples gave 18 cysteic acid residues/mol. Colorimetric analysis for cysteine under conditions that did not reduce disulphide bonds gave 0.158 μmol of cysteine/mg of Kp2 protein, equivalent to 10.6 cysteine residues in a molecule.

Metal content. Table 3 shows the relative abundances of the metals analysed. Only Fe was present in amounts greater than 0.4 g-atom/mol, at 3.3 μg of Fe/mg. This Fe content corresponds to 4.0 Fe atoms in a molecule.

Stability. Purified Kp2 protein (1.5 mg/ml) was completely stable at room temperature for up to 30 h under N_2 in 25 mM-tris–HCl buffer, pH 7.4, containing 50 mM-$MgCl_2$. Under comparable conditions no activity was lost on storage for up to 15 days at 1°C. This behaviour contrasts with that of Cp2 and Av2 proteins, which are reported to be cold-labile (Moustafa & Mortenson, 1969; Moustafa, 1970).

On exposure to O_2 (0.2 atm of O_2 in Ar) at 30°C, after removal of $Na_2S_2O_4$, activity was lost very rapidly; over 70% of the activity was destroyed after 1 min (see Fig. 5).

Ultraviolet- and visible-absorption spectra. The u.v.- and visible-absorption spectra of reduced Kp2

Table 3. *Summary of the physicochemical properties of the nitrogenase components of Klebsiella pneumoniae*

For details see the text. N.D. is not detected.

	Kp1 protein	Kp2 protein
Sedimentation coefficient ($s^0_{20,w}$, S)	11	4.8
Diffusion coefficient ($D^0_{20,w}$, cm²/s)	4.95×10^{-7}	5.55×10^{-7}
Frictional ratio (f/f_0)	1.08	1.45
Apparent partial specific volume ($\bar{v}$, ml/g)	0.73	0.69
Molecular weight		
From amino acid composition	229000	67800
From diffusion and sedimentation coefficients	200400	68200
From gel chromatography on Sephadex G-200	220000	62000
From disc electrophoresis	217000	—
From subunit composition	221800	69200
Subunit composition	Two types of subunit, mol.wts. 51300±1700 and 59600±1900	One type of subunit, mol.wt. 34600±2000
Acid-labile sulphide content (g-atoms/mol)	*16.7±1	†3.85
Metal content (g-atoms/mol)		
Molybdenum	*1.04±0.1	†<0.2
Iron	17.5±0.7	4
Copper	1.4	<0.1
Magnesium	1.8	<0.1
Calcium	1.2	<0.4
Zinc	0.8	<0.05
Cadmium	N.D.	—
Manganese	N.D.	N.D.
Cobalt	N.D.	N.D.
Specific activity		
Substrates		
Acetylene (nmol of substrate reduced/min per mg of protein)	1200	980
H^+ (nmol of substrate reduced/min per mg of protein)	1500	1050
Nitrogen (nmol of substrate reduced/min per mg of protein)	380	275
ATP (nmol of phosphate/min per mg of protein)	5400	4350
Oxygen sensitivity	$t_{\frac{1}{2}}$ = 10 min	$t_{\frac{1}{2}}$ = 45 s

Table 3—*continued*

	Millimolar extinction coefficients ($l \cdot mmol^{-1} \cdot cm^{-1}$)			Millimolar extinction coefficients ($l \cdot mmol^{-1} \cdot cm^{-1}$)	
	Wavelength (nm)	Reduced protein	Oxidized protein	Wavelength (nm)	Reduced protein	O_2-inactivated protein
U.v. and visible spectra	258.5 (shoulder)	260	260	258 (shoulder)	110	110
	269 (shoulder)	300	300	268	112	112
	277.5	330	330	460 (shoulder)	4.5	10
	282 (shoulder)	320	320			
	289 (shoulder)	250	250			
	430 (shoulder)	35	50			
C.d. spectra	Reduced and oxidized protein $[\theta]_{220} = -11\,600$ deg. $\cdot cm^2 \cdot dmol^{-1}$			Reduced protein $[\theta]_{220} = -10\,100$ deg. $\cdot cm^2 \cdot dmol^{-1}$	O_2-inactivated protein $[\theta]_{220} = -7000$ deg. $\cdot cm^2 \cdot dmol^{-1}$	
E.p.r. spectra	Reduced protein‡ $g = 4.3, 3.7, 2.015$	O_2-inactivated protein $g = 4.3, 2.0$		Reduced protein $g_1 = 2.05; g_2 = 1.94; g_3 = 1.86$		
Mössbauer spectra	Reduced protein $\delta = 0.65$ mm/s; $\Delta E = 3.05$ mm/s at 4.2°K $\delta = 0.6$ mm/s; $\Delta E = 0.8$ mm/s at 4.2°K $\delta = 0.35$ mm/s; $\Delta E = 0.7$ mm/s at 77°K	O_2-inactivated protein $\delta = 0.35$ mm/s; $\Delta E = 0.9$ mm/s at 4.2°K		Reduced protein $\delta = 0.45$ mm/s; $\Delta E = 1.1$ mm/s at 77°K		

* g-atoms/mol based on an average mol.wt. of 218000.
† g-atoms/mol based on an average mol.wt. of 66800.
‡ Spectral data on protein as normally prepared; addition of $Na_2S_2O_4$ to 20mM final concentration resulted in the appearance of an additional relatively weak peak at an approx. *g* value of 1.94, as observed by Hardy *et al.* (1971*b*).

protein and Kp2 protein inactivated by O_2 are shown in Fig. 8. Extinction coefficients are given in Table 3. We were unable to oxidize Kp2 protein by any means without concomitant inactivation. The spectra were identical in the u.v. region, with a peak at 268nm and a shoulder at 258nm. In the visible region the absorbance of reduced Kp2 protein was generally lower than that of O_2-inactivated Kp2 protein; this difference was greatest at about 460nm, where the O_2-inactivated species exhibited a shoulder. The spectra converged at longer wavelengths and extended into the near-infrared region. These results are similar to, but more comprehensive than, those obtained with Cp2 protein (Moustafa & Mortenson, 1969).

As with Kp1 protein, we had difficulty obtaining reproducible results with reduced Kp2 protein in the region of dithionite absorption between 290 and 360nm, as Kp2 protein slightly enhanced the rate of decomposition of dithionite. The difference spectrum between reduced and O_2-damaged Kp2 protein was always identical with the absorption spectrum of $Na_2S_2O_4$ in this region and we therefore concluded that oxidation produced no change in the spectrum

Table 4. *Amino acid composition of Kp2 protein*

For details see Table 2 and the text.

Hydrolysis time (h) ...	16	24	48	72	Oxidized protein 36	Best value	Best ratio	Nearest integer
Asx	43.8	45.7	42.8	44.0	42.5	44.1	10	10
Thr	24.7	23.3	22.6	20.9	16.0	25.1	5.7	6
Ser	14.7	14.4	13.5	11.8	13.7	15.4	3.5	4
Glx	64.2	63.1	60.7	63.5	52.2	62.9	14.3	14
Pro	14.7	13.2	13.2	13.0	9.7	13.5	3.1	3
Gly	42.9	43.4	43.0	43.7	37.5	43.3	9.9	10
Ala	44.5	45.0	44.9	44.9	40.7	44.8	10.2	10
Val	24.8	27.7	31.6	31.2	28.6	31.2	7.1	7
Met	24.8	24.1	24.4	25.0	—	24.6	5.6	6
Ile	26.5	29.4	32.1	33.7	29.4	33.7	7.7	8
Leu	31.9	32.7	32.3	32.8	28.2	32.4	7.4	7
Tyr	13.0	12.5	13.0	12.8	6.9	12.8	2.9	3
Phe	9.5	9.1	9.7	9.6	4.1	9.5	2.2	2
His	3.4	3.7	3.5	3.2	2.9	3.5	0.8	1
Lys	24.7	24.9	24.5	24.7	27.4	24.7	5.6	6
Arg	19.5	20.0	18.8	19.6	11.6	19.5	4.4	4
Cys (O_3H)	—	—	—	—	11.6	12.9	2.9	3
Trp	—	—	—	—	—	0.0	0.0	0

of Kp2 protein between 290 and 360nm.

Circular dichroism spectra. The c.d. spectra between 200 and 250nm of reduced Kp2 protein and Kp2 protein inactivated by O_2 are shown in Fig. 9. Neither species exhibited any c.d. in the visible region. As noted for Kp1 protein, this is a major difference between these nitrogenase proteins and other iron–sulphur proteins. The c.d. spectra consist of a single negative trough at 220nm with the suggestion of a further trough below 210nm. The spectra below 210nm were very erratic, owing to the large background absorption by sodium dithionite and dithiothreitol.

Inactivation by O_2 resulted in a smaller amount of α-helix in the protein. The reduced mean residue ellipticity (see Table 3) for reduced Kp2 protein corresponded to 33% of α-helix (Timasheff *et al.*, 1967), whereas for Kp2 inactivated by O_2 it corresponded to 23% of α-helix.

Electron-paramagnetic-resonance spectra. Kp2 protein reduced by $Na_2S_2O_4$ exhibited e.p.r. signals in the $g = 1.94$ region similar to those of spinach ferredoxin (Hall *et al.*, 1966). The g values are shown in Table 3. Integration of these signals was variable and always less than 1 electron/mol; they disappeared on inactivation with O_2 (B. E. Smith, D. Lowe & R. C. Bray, unpublished work).

Mössbauer spectra. Purification of Kp2 protein led to considerable changes in the Mössbauer spectra compared with those reported by Kelly & Lang (1970). Kp2 protein of the purity reported in the present paper exhibited a single doublet at 77°K (see Table 3). This broadened into a multiplet at 4.2°K, indicating unpaired spin.

The doublet with $\delta = 1.4$mm/s and $\Delta E = 2.8$mm/s at 77°K, which Kelly & Lang (1970) associated with substrate interaction, was found only in reduced, O_2-inactivated Kp2 protein (B. E. Smith & G. Lang, unpublished work). The properties of Kp2 protein are summarized in Table 3.

Activity of the purified components of Klebsiella pneumoniae nitrogenase

Purified Kp1 and Kp2 proteins, when added together to the standard assay system, reduced acetylene, HCN, N_2, H^+, N_3^- or methylisocyanide, giving reaction products typical of other nitrogenase preparations (see Hardy & Burns, 1968). In common with nitrogenases from other sources, the recombined components of *Klebsiella pneumoniae* nitrogenase required the absence of air, a reductant (Bulen *et al.*, 1965), ATP and Mg^{2+} (Mortenson, 194; D'Eustachio & Hardy, 1964; Bulen *et al.*, 1964). Particular conditions relevant to the *Klebsiella* proteins are described below.

ATP dependence. In these experiments ATP or other nucleotides at 3.5mM replaced the ATP-generating system of the standard assay when a 2min incubation time was used. During this time the reaction rate was linear. The reaction required ATP specifically; UTP, GTP and ITP were inactive. An

apparent K_m of 0.1mM for ATP was determined from a Lineweaver & Burk (1934) plot at a Kp1/Kp2 molar ratio of 1:1. No inhibition of acetylene reduction was observed at ATP concentrations of up to 25mM.

Bivalent cation requirement. In the assay conditions described above for ATP dependence, Mg^{2+} was the most effective bivalent cation tested, with an apparent K_m of 15mM. At 5mM, Mg^{2+} could be replaced by the following ions: Mn^{2+} (33%), Fe^{2+} (22%) and Co^{2+} (25%). The percentage values are the rates of acetylene reduction relative to Mg^{2+}.

Reductant. Sodium dithionite was the usual reductant used. Reduced Benzyl or Methyl Viologen functioned as electron donors for acetylene reduction by the highly purified *Klebsiella* nitrogenase. With partially purified preparations the specific activities were 50% and 30% of those obtained with $Na_2S_2O_4$. The optimum dye concentrations, with both partially and wholly purified preparations of *Klebsiella pneumoniae* nitrogenase, depended on the protein concentration and were similar at about 250μmol of dye/mg of nitrogenase proteins (Kp1/Kp2 molar ratio of 1:2). Both above and below this concentration the specific activity decreased sharply. Activity of Viologens with crude nitrogenase has been reported with *Clostridium pasteurianum* (D'Eustachio & Hardy, 1964), *Azotobacter chroococcum* (Yates & Daniel, 1970), *Rhizobium japonicum* bacteroids (Klucas & Evans, 1968) and in extracts of *Mycobacterium flavum* (Biggins & Postgate, 1971), but not with pure nitrogenase.

pH optimum. Nitrogenase activity in crude extracts showed a broad pH optimum around pH7.4. The purified nitrogenase activity showed a somewhat sharper pH optimum around pH7.8, and this value was adopted for the standard assay system. No inhibition by buffers up to 25mM was observed, and no significant difference was noted among tris–HCl, piperazine-*NN'*-bis-(2-ethanesulphonic acid), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid and 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid buffers at 25mM.

Titration of Kp2 protein with Kp1 protein. Addition of increasing amounts of Kp1 protein to a fixed amount of Kp2 protein resulted in a rapid increase in nitrogenase activity to a maximum, followed by a region where activity was inhibited. The shape of this curve was the same for N_2 and acetylene as substrates and for H_2 evolution (see Fig. 10), but was different from that for KCN (R. R. Eady & M. Kelly, unpublished work). Comparable inhibition at high concentrations of the Mo–Fe-containing protein has been observed with nitrogenase preparations from other organisms, namely Cp1 (Vandecasteele & Burris, 1970) and Ac1 (Kelly, 1969*a*) proteins. Comparable differences in substrate titration curves were reported for partially purified Ac1 and Ac2 (Kelly, 1969*a*) and Bp1 and Bp2 (Kelly, 1969*b*) proteins. The specific activities of Kp2 protein towards the first group of substrates were listed in Table 3, and were determined at the optimum Kp1/Kp2 ratio for activity (42μg of Kp2 protein/177μg of Kp1 protein), which, taking mol.wts. of 66800 and 218000 respectively, corresponds to a Kp1/Kp2 molar ratio of 1:1.2. Plots at this ratio gave apparent K_m values for ATP, 0.1mM, N_2, 0.12mM, acetylene, 0.18mM.

Titration of Kp1 protein with Kp2 protein. The effect of adding increasing amounts of Kp2 protein to a fixed amount of Kp1 protein on nitrogenase activity is shown in Fig. 11. Curves of this type for H_2 evolution and reduction of N_2 or acetylene showed a sigmoidal region at low concentrations of Kp2 protein and then a gradual rise to a maximum. Specific activities of Kp1 protein towards these substrates were determined at a ratio of 33μg of Kp1 protein/1.3mg of Kp2 protein. Assuming mol.wts. of 218000 and 66800 respectively this corresponds to a Kp1/Kp2 molar ratio of 1:80.

Effect of protein concentration on specific activity. Nitrogenase activity of partially purified preparations is not proportional to enzyme concentration below about 1mg of protein/ml (Burns & Bulen, 1965;

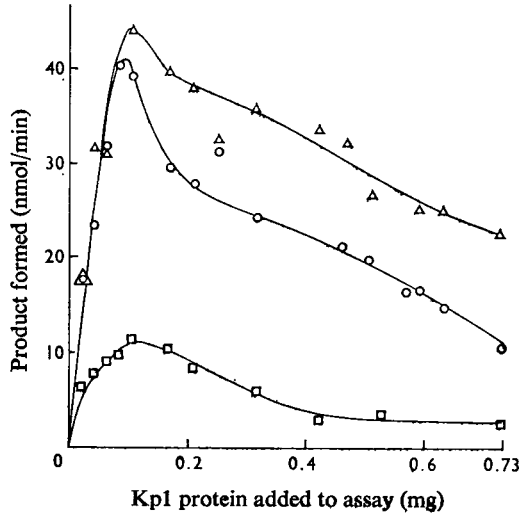


Fig. 10. *Substrate-reduction-activity curves for the titration of Kp2 protein with Kp1 protein*

The activity measurements of H_2 evolution and acetylene and N_2 reduction were made under the conditions described in the Materials and Methods section. Each assay contained 42μg of Kp2 protein (0.42μM, assuming a mol.wt. of 66800) and various amounts of Kp1 protein as indicated. △, H_2 evolution; ○, acetylene reduction; □, N_2 reduction.

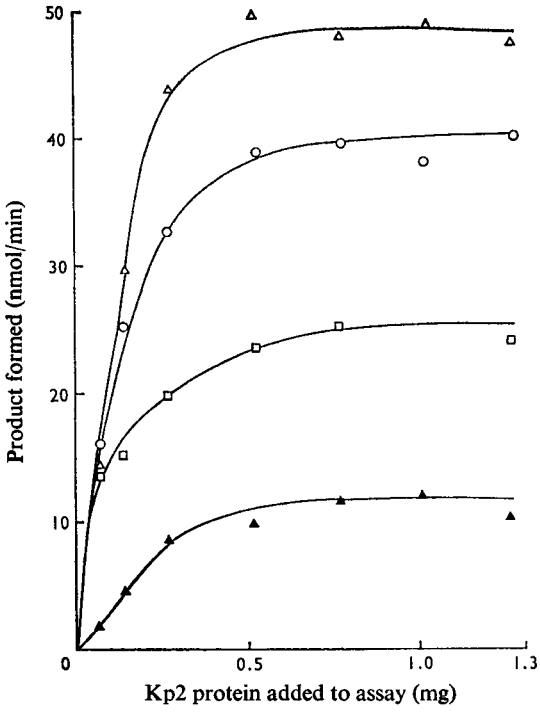


Fig. 11. *Substrate-reduction activity curves for the titration of Kp1 protein with Kp2 protein*

The activity measurements of H_2 evolution and acetylene and N_2 reduction were made under the conditions described in the Materials and Methods section. Each assay contained 33 μg of Kp1 protein (0.1 μM, assuming a mol.wt. of 218000) and various amounts of Kp2 protein as indicated. △, H_2 evolution; ○, acetylene reduction; □, N_2 reduction; ▲, H_2 evolution concomitant with acetylene reduction.

Yates, 1970; Sorger, 1971). Non-linearity was evident with purified *K. pneumoniae* nitrogenase components below 15 μg of protein/ml. This concentration was much lower than those usually used to measure activity. In titration curves of the type described above, Kp2 protein could be diluted to 10 μg/ml and Kp1 protein to 15 μg/ml without effect on the measured specific activity.

Discussion

The purification procedures described in this paper allowed the complete separation of the nitrogenase components of *K. pneumoniae* in good yield. Kp1 protein prepared by these methods was homogeneous by the analytical techniques of disc electrophoresis and ultracentrifugation. It resolved into two bands on isoelectric focusing in polyacrylamide gels, but this we attribute to the separation of the protein in different oxidation states, because after oxidation with Lauth's Violet it ran as a single band. Haemoglobin separates on isoelectric focusing into four bands, depending on the oxidation state of the haem groups (Bunn & Drysdale, 1971). Kp2 protein, because of its extreme O_2 sensitivity, was difficult to handle in the absence of $Na_2S_2O_4$. It is probable that, under our conditions of isoelectric focusing and disc electrophoresis, Kp2 protein existed in a mixture of oxidation states that gave rise to multiple bands. Only after the protein had been denatured with sodium dodecyl sulphate or inactivated with sodium mersalyl did it migrate as a single band. In conditions under which it could be maintained in its reduced form, as during ultracentrifugation, it behaved as if it was homogeneous.

The nitrogenase proteins of *K. pneumoniae* were similar to the corresponding proteins of *C. pasteurianum* (Moustafa & Mortenson, 1969; Nakos & Mortenson, 1971*a*,*b*; Dalton *et al.*, 1971; Vandecasteele & Burris, 1970) and *A. vinelandii* (Bulen & LeComte, 1966; Burns *et al.*, 1970; Moustafa, 1970) in their general properties, and in the conditions they require for activity.

The properties of Kp1, Av1 and Cp1 proteins are summarized in Table 5. They differ considerably in their molecular weights, but are of comparable specific activity. Both Cp1 and Kp1 proteins show an equivalence of acid-labile sulphide and Fe content, but Av1 protein does not. The Mo and Fe content/mol of Kp1 and Cp1 proteins is in good agreement, 1 Mo and 14–17 Fe atoms/molecule. Burns *et al.* (1970) report 2 Mo and 36 Fe atoms in a molecule of Av1 protein, and a minimum mol.wt. of 150000 based on 1 g-atom of Mo/mol. Recalculation of the results in Table 2 of Burns *et al.* (1970) gives an average Mo/Fe ratio of 1:16.6, and assuming 1 g-atom of Mo/mol, a minimum mol.wt. of 135000. This latter value is in good agreement with the value of 270000 determined in the ultracentrifuge for the molecular weight of Av1 protein (Burns *et al.*, 1970), assuming that it exists as a dimer in solution. The conditions of low ionic strength under which Av1 protein crystallizes do not result in crystallization of Cp1 (H. Dalton, personal communication) or Kp1 proteins. This difference may be due to dimer formation by Av1 protein in solution.

Because these proteins differ in size, it might be expected that their specific activities would be in proportion to their molecular weights. Available results suggest that this is not the case (see Table 5). Calculation of the nitrogen-fixing activity/Mo atom gives values of 83.5 for Kp1, 58.5 for Cp1 and 49 for Av1 proteins (mol of NH_3 produced/min per Mo atom). Assuming a comparable degree of purity for these proteins, this would indicate that Mo is not

Table 5. *Comparison of the properties of Kp*1, *Cp*1 *and Av*1 *proteins*

Data for Kp1 protein (the present work); Cp1 protein (Dalton *et al.*, 1971; Nakos & Mortenson, 1971*a*; Vandecasteele & Burris, 1970); Av1 protein (Burns *et al.*, 1970; Hardy *et al.*, 1971*b*);— signifies no published values available.

	Kp1 protein	Cp1 protein	Av1 protein
Molecular weight			
From ultracentrifuge data	200400	168000	270000
From gel filtration	220000	170000	
Subunit composition	Four subunits, two of each type, mol.wts. 51300, 59600	Three subunits, two of mol.wt. 59500, one of mol.wt. 50700	Two types of subunit, mol.wts. approx. 40000
Partial specific volume	0.73	0.72	—
Metal content (g-atoms/mol)			
Iron	17.5	14–15	33
Molybdenum	1.04	1	2
Calcium	1.2	1.7	—
Acid-labile sulphide (g-atoms/mol)	16.7	16	25.6
Cysteine content	17	23	37
Specific activity (nmol of substrate reduced/min per mg of protein)			
Substrates			
Nitrogen	380	345	362
Hydrogen	1500	—	1488
Acetylene	1200	1200	—
E.p.r. spectra			
Native protein	—	$g = 4.3, 2.01$	$g = 4.3, 3.67, 2.01$
Protein reduced with $Na_2S_2O_4$	$g = 4.3, 3.7, 2.015$	—	$g = 4.3, 3.67, 2.01, 1.94$
Oxygen-damaged protein	$g = 4.3, 2.0$	$g = 2.01$	$g = 4.3, 2.01$

Table 6. *Comparison of the properties of Kp*2 *and Cp*2 *proteins*

Data for Kp2 protein (the present work) and Cp2 protein (Moustafa & Mortenson, 1969; Jeng *et al.*, 1969; Vandecasteele & Burris, 1970; Nakos & Mortenson, 1971*b*).

	Kp2 protein	Cp2 protein
Molecular weight		
From ultracentrifuge	68200	40000
From gel filtration	62000	55000
Subunit composition	One type, mol.wt. 34600±2000	One type, mol.wt. 27500±1350
Tryptophan content	Absent	Absent
Iron content (g-atoms/mol of dimer)	4	4.05
Acid-labile sulphide content (g-atoms/molecule)	3.85	4.0
Cysteine content	10.6	11
Specific activity (nmol of substrate reduced/min per mg of protein)		
Substrates		
Nitrogen	275	460
Acetylene	980	2708
Stability	Not cold-labile; O_2-sensitive; $t_{\frac{1}{2}} = 45$ s	Cold-labile; O_2-sensitive; all activity lost after 5 min

involved in the rate-determining step of N_2 reduction.

When treated with β-mercaptoethanol and sodium dodecyl sulphate and then analysed by gel electrophoresis in gels containing sodium dodecyl sulphate, all these proteins are apparently composed of two types of subunits see (Table 5).

Microdensitometer traces of Cp1 protein subjected to electrophoresis in sodium dodecyl sulphate-containing gels showed that the higher-molecular-weight band predominated in the ratio of 2:1 (Nakos & Mortenson, 1971*a*). These authors conclude that native Cp1 protein consisted of three subunits, two of mol.wt. approx. 60000 and one of mol.wt. 50000. Kp1 protein appears to be different, as the ratio of the two bands was equal, compatible with a native mol.wt. of 220000 comprising four subunits, two of mol.wt. 50000 and two of mol.wt. 60000.

The properties of Kp2 and Cp2 proteins are given in Table 6. Av2 protein is not included, as it is not well characterized; it has a reported specific activity of 532nmol of N_2 reduced/min per mg (Moustafa, 1970). Kp2 protein is bigger than Cp2 protein. Both consist of two subunits and contain Fe and acid-labile sulphide in equivalent amounts (see Table 6). Tryptophan is absent from both Kp2 (the present work) and Cp2 (Jeng *et al.*, 1969) proteins. Despite these close similarities their apparent specific activities differ. That of Cp2 protein has been reported as 2708nmol of ethylene produced/min per mg (Moustafa & Mortenson, 1969), and Av2 protein as 2480 (Moustafa, 1970). Both these values are much higher than that of 1380 reported by Vandecasteele & Burris (1970) for Cp2 protein. The high values for both Av2 and Cp2 proteins were obtained in an assay system in which a cold-inactivated crude bacterial extract was a source of the complementary protein, so the assay system is not comparable with that of Vandecasteele & Burris (1970), nor with that used in the present work. The reports that ferredoxin and other non-specific proteins can stimulate nitrogenase activity appreciably in partially-purified nitrogenase preparations from *A. chroococcum* (Yates, 1970), and that *Chloropseudomonas ethylicum* ferredoxin stimulates nitrogenase activity three- or four-fold in crude extracts of that organism (Evans & Smith, 1971), mean that results obtained in assays involving crude extracts must be interpreted with caution. Despite these reservations about the high values obtained for Cp2 and Av2 proteins, the specific activity of the Kp2 preparation described here is about 70% that reported for Cp2 protein in a comparable assay. When allowance is made for the ratio of the molecular weights of these proteins the difference becomes 15% and may not be significant. However, in a titration curve of the type shown in Fig. 10, used to determine the specific activity of Kp2 protein, it is clear that the maximum activity depends on the relationship between the equilibrium constant for the formation of the active complex and that for the formation of the inhibited species apparently present at high concentrations of Kp1 protein. A similar inhibition is observed with Ac1 protein and Kp2 protein (M. G. Yates, personal communication), and in titrations of Cp2 protein with Cp1 protein (Vandecasteele & Burris, 1970) the latter was attributed to the presence of contaminating hydrogenase in the Cp1 preparation. As our Kp1 preparations were free of conventional hydrogenase activity, and the H_2-evolution reaction as well as N_2 and acetylene reduction shows the inhibition, we prefer to attribute the inhibition to the formation of an inhibited complex between Kp1 and Kp2 proteins. The relationship between the equilibrium constants mentioned above might well be species-dependent, and thus the specific activity of the Fe proteins determined from titration curves of this type would not be a valid indication of the relative degree of purity of these proteins from different species.

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