The Molybdenum–Iron Protein of *Klebsiella pneumoniae* Nitrogenase

EVIDENCE FOR NON-IDENTICAL SUBUNITS FROM PEPTIDE ‘MAPPING’

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The molybdenum- and iron-containing protein components of nitrogenase purified from *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Azotobacter chroococcum* and *Rhizobium japonicum* bacteroids all gave either one or two protein-staining bands after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, depending on the commercial brand of sodium dodecyl sulphate used. The single band obtained with *K. pneumoniae* Mo–Fe protein when some commercial brands of sodium dodecyl sulphate were used in the preparation of the electrode buffer was resolved into two bands by the addition of 0.01 % (v/v) dodecanol to the buffer. Protein extracted from the two bands obtained after electrophoresis of *K. pneumoniae* Mo–Fe protein gave unique and distinct peptide ‘maps’ after tryptic digestion. Undissociated Mo–Fe protein contained both sets of tryptic peptides. These data are consistent with Mo–Fe protein from *K. pneumoniae* being composed of non-identical subunits. Amino acid analyses of the subunit proteins revealed some clear differences in amino acid content, but the two subunits showed close compositional relatedness, with a difference index [Metzer, H., Shapiro, M. B., Mosiman, J. E. & Vinton, J. G. (1968) *Nature (London)* **219**, 1166–1168] of 4.7.

Nitrogenase is the enzyme complex that catalyses the reduction of N_2 to NH_3 . Two distinct proteins have been shown to participate in this reaction, in which electrons are transferred from reduced nitrogenase to N_2 , with concomitant hydrolysis of ATP. Both proteins contain iron–sulphur centres and one of them also contains molybdenum. This Mo–Fe protein, known also as nitrogenase component I or molybdoferredoxin, has been obtained in a highly purified form from six widely different organisms (Eady & Postgate, 1974). In all cases the proteins are very similar and are tetrameric, with a native mol.wt. near 220000.

In most instances, the sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis method (Weber & Osborn, 1969) has been used to investigate the subunit structure of these proteins. Despite the close similarity of their physicochemical properties, both one and two types of subunit have been reported (see Eady & Postgate, 1974; Eady & Smith, 1976), depending on the source of the protein. For the pro-

tein from *Azotobacter vinelandii* there have been conflicting reports from different research groups as to the number of subunit types (Hardy & Burns, 1972; Fleming & Haselkorn, 1973, 1974; Stasny *et al.*, 1974; Kleiner & Chen, 1974; Bulen, 1976).

The number of polypeptide species that comprise nitrogenase Mo–Fe protein is essential information both for elucidating the structure and function of nitrogenase and in determining the number and nature of genes involved in nitrogen fixation. We report here that the commercial brand of sodium dodecyl sulphate used in polyacrylamide-gel electrophoresis influences the number of protein-staining bands observed for nitrogenase Mo–Fe proteins. ‘Mapping’ of peptides derived from presumptive subunits of the Mo–Fe protein from *Klebsiella pneumoniae* (Kpl)† by tryptic digestion provides evidence that, despite their similarity in amino acid composition, the subunits of this protein are of two

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‡ The nitrogenase components of various organisms are denoted by a capital letter indicating the genus, a lower-case letter indicating the species and the number 1 indicating the Mo- and Fe-containing protein (Eady *et al.*, 1972): Kp, *Klebsiella pneumoniae*; Ac, *Azotobacter chroococcum*; Av, *A. vinelandii*; Cp, *Clostridium pasteurianum*; Rj, *Rhizobium japonicum*; Cv, *Chromatium vinosum*.

types. Circumstantial evidence is given that the Mo-Fe proteins from *A. vinelandii* (Avl), *A. chroococcum* (Acl) and bacteroid form of *R. japonicum* (Rjl) are also composed of two non-identical subunits.

Materials and Methods

Growth of organisms and preparation of nitrogenase Mo-Fe proteins

Methods used for maintenance and growth of *K. pneumoniae* strains M5al as well as for preparation and purification of Mo-Fe protein from cell extracts have been described previously (Eady *et al.*, 1972; Smith *et al.*, 1976). Purified Avl and Acl proteins were provided by Dr. M. G. Yates (Unit of Nitrogen Fixation); Rjl protein was from Dr. F. Bergersen (CSIRO, Canberra, Australia).

Gel electrophoresis

The two discontinuous gel systems used for analytical work were either the method of Laemmli (1970) or Ortec (Instruction Manual, Ortec 4200 electrophoresis system, Ortec, Oak Ridge, TN, U.S.A.). Gels prepared by the Laemmli method were composed of 8 or 10% (w/v) acrylamide and 0.15% (w/v) bisacrylamide buffered with 0.37M-Tris/HCl buffer, pH8.8, in the main gel and with 0.125M-Tris/HCl buffer, pH6.8, in the single stacking gel layer. The electrode buffer contained 0.025M-Tris/glycine buffer, pH8.3, and 0.1% (w/v) sodium dodecyl sulphate. Ortec gels were made with 0.36M-Tris/H₂SO₄ buffer, pH9.0, in both gel layers. The stacking gel layer contained 5.8% (w/v) acrylamide and 0.16% (w/v) bisacrylamide; the main gel had 10% (w/v) acrylamide and 0.85% (w/v) bisacrylamide. Ortec electrode buffer was 0.065M-Tris/borate buffer, pH9.0, containing 0.1% (w/v) sodium dodecyl sulphate. In all cases, Serva-brand sodium dodecyl sulphate was used in the preparation of the protein samples and the polyacrylamide gel. Different brands of sodium dodecyl sulphate were used only in the preparation of the electrode buffer at 0.1% (w/v). Proteins in sample buffer were incubated for 2min in a boiling-water bath. Normally 5–20 µg of protein was applied to each 8mm sample well of the gel in 5–50 µl of buffer. Gels were cast between glass plates as 1.5mm-thick slabs. Electrophoresis was at an applied current not exceeding 40mA. After electrophoresis, gels were stained with freshly prepared Coomassie Blue stain as described by Weber & Osborn (1969) and destained with 6% (v/v) acetic acid and 25% (v/v) methanol in water. When sodium dodecyl sulphate from Koch-Light was used in the electrode buffer, we found it necessary to stain and destain twice to obtain maximum colour development; the second staining was unnecessary when Serva or Schwartz sodium dodecyl sulphate was used.

Acrylamide and bisacrylamide were purchased from Eastman Kodak Co., Rochester, NY, U.S.A.; sodium dodecyl sulphate was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. (lot no. 40143), or Serva Fine Biochemicals, Heidelberg, Germany (lot no. 20760), or Schwartz, Orangeburg, NY, U.S.A. Glycine and Trizma base were from Sigma (London) Chemical Co., London S.W.6, U.K. Proteins used for molecular-weight calibration of gels were purchased from Boehringer Corp. (London), London W5 2TZ, U.K.

Preparation of putative subunits

Putative subunits of Kpl protein were isolated from 4mm-thick and 13.5mm-wide preparative Laemmli slab gels by using Koch-Light-brand sodium dodecyl sulphate in the electrode buffer. The sample well was continuous across the top of the pH6.8 stacking gel layer. To each gel 2mg of purified Kpl protein was applied in a volume of 0.5–1 ml. Larger quantities of Kpl protein did not separate into two discrete bands on preparative gels.

After electrophoresis, gels were immersed in Coomassie Blue stain solution for 5min, after which time the surface protein was sufficiently stained to be visible as two bands. The bands were excised by cutting the gel with a scalpel and the gel strips containing protein were macerated by forcing the material through a 5ml syringe. The protein was extracted by mixing the macerated gel with 5vol. of 5mM-NH₄HCO₃+0.1% (w/v) sodium dodecyl sulphate (Serva brand) in a 250ml flask. The pH of the mixture was adjusted to 8 with aq. NH₃ and the flask shaken vigorously at 30°C for 24h. The protein solution was then filtered twice through Whatman no. 1 filter paper to remove gel material. Protein was precipitated in the cold by addition of sufficient 100% (w/v) trichloroacetic acid to give a final concentration of 20%. After 30min on ice, the solution was warmed to 37°C for 30min to allow the sodium dodecyl sulphate, which precipitates under these conditions, to redissolve. Precipitated protein was recovered by centrifugation at 8000g for 12min. The pellet was washed first with acetone containing 0.1M-HCl and finally with acetone.

By this method, at least 50% of the total protein applied to gels was recovered.

Oxidation and trypsin digestion

Native Kpl protein and purified subunit proteins were oxidized with performic acid as described by Hirs (1967). A 2mg portion of protein was dissolved in 0.5ml of formic acid. Freshly prepared performic acid (0.125ml) was added and the mixture kept at –5°C for 120min. Water was added to a final volume of 20ml, and the protein was immediately freeze-dried.

Protein was dissolved in 2ml of 10mM-NH₃. The

excess of NH_3 was removed by bubbling air through the solution until the pH was approx. 7.5. Trypsin (Sigma; diphenylcarbamoyl chloride-treated, no. T-1005) was added to a final concentration of 0.002% (w/v). The mixture was then incubated for 18–36 h at 37°C. The resulting peptide solution was freeze-dried and stored at -20°C.

Tryptic-peptide analysis

Two-dimensional tryptic-peptide analyses were performed as described by Bennett (1967). Approx. 1 mg of peptide dissolved in 50 μl of water was applied to Whatman 3MM paper. Chromatography was carried out for 20 h by using the upper organic phase of a mixture of butanol/acetic acid/water (4:1:5, by vol.) as a solvent. The lower phase was added to the bottom of the chromatography tank for equilibration. Phenol Red was applied as a marker dye.

After chromatography, high-voltage paper electrophoresis was performed in pyridine/acetate buffer, pH 3.5, for 1 h 15 min at 2000 V in a Gilson model D Electrophorator. The peptides and amino acids were then located by using cadmium/ninhydrin reagent (Dreyer & Bynum, 1967). Amino acid markers were added along the edge of the paper at the origin, just before electrophoresis.

Amino acid analysis

A portion (1–5 nmol) of the protein to be analysed was dissolved in 0.5 ml of constant-boiling 6M-HCl containing 0.1% (w/v) phenol and 10 μl of butanedithiol. The hydrolysis tubes were evacuated, sealed and incubated at 110°C for 24 h. The tubes were then opened and the contents dried in a desiccator containing pellets of NaOH. Then 50–100 μl of 0.2M-sodium citrate buffer, pH 2.2, was added to dissolve the residue, and 10–20 μl of this was used for each dialysis. Samples were analysed with a Durrum D500 amino acid analyser.

Iron analysis

Iron was determined by atomic-absorption spectrophotometry with an A1750 atomic-absorption spectrophotometer (Southern Analytical Ltd., Camberley, Surrey, U.K.). Before analysis Kpl protein was treated with 1% (v/v) β -mercaptoethanol and 1% (w/v) Koch-Light sodium dodecyl sulphate under the conditions of Laemmli (1970). Samples (10 ml) were then dialysed for 48 h against 2 litres of electrode buffer containing 0.1% (w/v) Koch-Light sodium dodecyl sulphate. The dialysis was done under aerobic conditions and the buffer changed twice during the dialysis time.

Results

Effect of commercial brand of sodium dodecyl sulphate on number of Mo-Fe-protein bands observed after electrophoresis

The brand of sodium dodecyl sulphate used to prepare the electrode buffer for polyacrylamide-gel electrophoresis influenced the number of protein-staining bands observed with the Mo-Fe protein of nitrogenase isolated from several different organisms. This discovery was made by systematically checking component reagents after occasional failure to obtain two bands from Kpl protein (as observed by Eady *et al.*, 1972).

Kpl protein gave two protein-staining bands with Koch-Light or Schwartz sodium dodecyl sulphate, and only a single band when Serva brand was used (see Plate 1). Avl and Acl proteins gave two well-separated bands with sodium dodecyl sulphate from Serva, two poorly separated bands with that from Schwartz and a single band with that from Koch-Light. Rjl protein separated into two bands with sodium dodecyl sulphate from either Koch-Light or Serva, but yielded two poorly separated bands when that from Schwartz was used. These differences are seen in the photograph of representative slab gels in Plate 1. Protein standards of known molecular weights were included to estimate molecular weights of the Mo-Fe-protein bands observed. Because of the variation in migration observed for both the standard proteins and the Mo-Fe proteins with different brands of sodium dodecyl sulphate, we are somewhat uncertain whether such estimates are meaningful. However, a plot of mol.wts. from Koch-Light sodium dodecyl sulphate/polyacrylamide-gel data gave 56500 and 61000 for the two bands of Kpl protein, in fair agreement with the previously determined values of 51300 and 59600 (Eady *et al.*, 1972). The single band of Kpl protein on the Serva sodium dodecyl sulphate/polyacrylamide gel ran in a position corresponding to the lower molecular-weight protein band of the Koch-Light sodium dodecyl sulphate/polyacrylamide gels. Rjl protein, previously reported to run as a single band of mol.wt. 50000 (Whiting & Dilworth, 1974), ran as two bands of estimated mol.wts. of 58000 and 61000 on gels containing Koch-Light sodium dodecyl sulphate and 50700 and 54300 in the presence of Serva sodium dodecyl sulphate.

The differences in banding patterns and therefore molecular-weight estimates among the various Mo-Fe proteins may be due to different amounts of impurities in the sodium dodecyl sulphate preparations. The addition of 0.01% (v/v) dodecanol, a plausible contaminant, to electrode buffer containing Serva sodium dodecyl sulphate resulted in Kpl protein migrating as two bands. The buffer system used during electrophoresis did not affect the behaviour

of Kpl protein since the latter migrated as a single band with Serva sodium dodecyl sulphate and as two bands with Koch-Light sodium dodecyl sulphate in either of the buffer systems used in this work.

The single band of Kpl protein seen on gels run with Serva sodium dodecyl sulphate contained material which separated into two distinct bands on gels run with Koch-Light sodium dodecyl sulphate. This was established by running a 'two-dimensional' gel; two strips were cut from a gel on which Kpl protein had been electrophoresed with electrode buffer containing Serva sodium dodecyl sulphate, giving a single band. One strip was stained to identify the position of the band; the other strip was trimmed of all gel except that carrying the band, then soaked in 1 M-Tris/HCl buffer, pH 6.8, for 2 h. The strip was then incorporated into the stacking layer of a second gel with the band oriented lengthwise with respect to the direction of current flow, and was electrophoresed in electrode buffer containing Koch-Light sodium dodecyl sulphate. The previously obtained single band yielded two distinct well-separated bands when re-run on gels with Koch-Light sodium dodecyl sulphate (Plate 2, sample *a*), these two bands had the same mobilities as those from native Kpl protein applied in a sample well in the middle of the gel (Plate 2, sample *b*). Sample *c* shows a comparable experiment in which Kpl protein was electrophoresed with Koch-Light sodium dodecyl sulphate, the two bands cut out and reoriented on the second gel as before, and re-run with Koch-Light sodium dodecyl sul-

phate. No new bands appeared, demonstrating that the primary bands were not artifacts of interaction of Kpl protein with the brand of sodium dodecyl sulphate.

Iron content of sodium dodecyl sulphate-treated protein

To determine if either of the protein-staining bands observed was associated with bound iron, Kpl protein was subjected to electrophoresis by using Koch-Light sodium dodecyl sulphate, and the gel was subsequently stained for iron with bathophenanthroline. Two red bands were observed, a sharp reddish-brown band with a mobility relative to the dye Bromophenol Blue (R_m) of 0.93 and a diffuse pink band 3 mm wide (R_m 0.89). Both these iron-containing zones were well separated from the two protein bands (R_m 0.71 and 0.77), which were stained with Coomassie Blue. In a similar experiment in which Serva sodium dodecyl sulphate was used in the electrode buffer, iron was not associated with the single protein band. Protein that had been treated with Koch-Light sodium dodecyl sulphate and β -mercaptoethanol under the conditions of Laemmli (1970) and subsequently dialysed against electrode buffer containing Koch-Light sodium dodecyl sulphate had lost all iron.

'Mapping' of the peptides generated by tryptic digestion of Kpl protein and its constituent subunits

The putative subunits prepared as described in the Materials and Methods section were not contamin-

Table 1. *Amino acid composition of Kpl protein and the separated subunits*

Results are expressed as (a) mol of amino acid/100 mol of residues and (b) residues per polypeptide chain, by using mol.wts. of 54 000 and 57 000 for the faster- and slower-migrating subunit bands respectively and 111 000 for the heteromeric dimer of Kpl protein, which contains one subunit of each type. No correction was made for destruction during hydrolysis; cysteine and tryptophan were not determined.

	Faster subunit band		Slower subunit band		Native Kpl protein (dimer)		Ratio of residues in subunits (a) (faster/slower)	Recovery of residues (b) heteromeric dimer (slower+faster subunits)
	(a)	(b)	(a)	(b)	(a)	(b)		
Asx	11.0	54	11.0	59	10.8	111	1.00	0.980
Thr	5.13	25	7.13	38	5.72	58	0.718	0.920
Ser	4.37	22	4.22	22	4.15	43	1.04	0.975
Glx	12.2	60	13.0	69	11.7	119	0.938	0.920
Pro	4.93	24	5.23	28	5.41	55	0.942	1.06
Gly	10.0	49	8.23	44	8.95	91	1.21	0.980
Ala	7.82	38	7.93	42	8.44	86	0.985	1.07
Val	5.88	29	5.76	31	6.33	64	1.02	1.06
Met	3.46	17	3.44	18	3.21	33	1.01	0.940
Ile	5.50	27	3.39	18	4.99	51	1.62	1.13
Leu	8.51	42	10.6	57	9.68	99	0.803	1.00
Tyr	3.78	19	3.00	16	3.38	34	1.26	0.970
Phe	4.32	21	5.16	27	4.33	44	0.837	0.910
His	2.40	12	2.54	13	2.40	24	0.941	0.965
Lys	5.10	25	4.99	26	5.10	52	1.02	1.02
Arg	5.55	27	4.28	23	5.30	54	1.29	1.08

EXPLANATION OF PLATE I

Banding pattern of various nitrogenase Mo-Fe proteins on gels containing three different brands of sodium dodecyl sulphate

Purified protein (10–20 μ g) in sample buffer were applied to sample wells; 10% polyacrylamide gels were prepared by the method of Laemmli (1970). Gels and sample buffer were prepared with Serva-brand sodium dodecyl sulphate. The electrode buffer contained sodium dodecyl sulphate from (a) Schwartz, (b) Serva or (c) Koch–Light. Electrophoresis was as described in the Materials and Methods section. Proteins marked M, used for mol.wt. calibration, were: (1) bovine serum albumin (67000); (2) catalase (57500); (3) hen's-egg albumin (45000); (4) aldolase (40000).

EXPLANATION OF PLATE 2

Two-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of Kp1 protein

Gel strips (2 cm wide) were cut from 8% polyacrylamide gels on which Kp1 protein had been electrophoresed. These protein-containing strips were incorporated into the stacking layer of a second gel and a middle sample well was formed in which Kp1 protein was applied. This gel was electrophoresed by using Koch–Light sodium dodecyl sulphate in the electrode buffer and is shown in the photograph. For further details see the text. (a) Electrophoresis of a strip cut from gel run with Serva-brand sodium dodecyl sulphate in the electrode buffer; (b) Kp1 protein (not previously electrophoresed); (c) the first strip was cut from gel run with Koch–Light sodium dodecyl sulphate in the electrode buffer and carried two bands (see the text).

EXPLANATION OF PLATE 3

Peptide 'maps' of putative subunits and undissociated Kp1 protein

(a) Undissociated Kp1 protein; (b) faster-migrating subunit; (c) slower-migrating subunit. Proteins were digested with trypsin and peptide 'maps' prepared as described in the Materials and Methods section. Samples of each digest were applied at the origin. After chromatography, but before electrophoresis, the markers alanine, arginine and aspartic acid were applied to both sides of the paper at D.

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ated with each other as judged by their behaviour on electrophoresis in the presence of sodium dodecyl sulphate. Samples of the isolated subunits and also the unresolved Kp1 protein were oxidized with performic acid and digested with trypsin, and peptide 'maps' of the resulting peptides were prepared as described in the Materials and Methods section.

Photographs of their peptide 'maps' are seen in Plate 3. The tryptic peptides from the slower- and faster-migrating protein bands are clearly different from one another; those of the unresolved Kp1 protein are a composite of both subunit sets of peptides. A few peptides seen on the 'map' of the trypsin blank were too faint to interfere with comparisons of Kp1 protein and subunit peptide 'maps'.

After close inspection of the original stained peptide 'maps' of the unresolved protein, we identified at least 66 distinct ninhydrin-positive spots, some of which are indistinct in the photograph. The 'map' of the faster-migrating subunit had 50 ninhydrin-positive spots and that of the slower-migrating subunit protein had 36. Only 11 spots were in similar positions on both subunit 'maps'. Each subunit had a few characteristically spaced peptides which stained orange or yellow with the cadmium/ninhydrin reagent (among a background of pink spots); the unresolved protein gave orange- and yellow-staining peptides which were a composite of the two subunit sets.

In all cases the amount of staining material remaining in the area where the peptide hydrolysate was applied was slight (Plate 3), suggesting that little undigested protein was left at the origin.

Amino acid composition of the subunits of Kp1 protein

The amino acid compositions of the putative subunits of Kp1 protein prepared from gels containing Koch-Light sodium dodecyl sulphate are shown in Table 1. The overall compositions are similar and when they are compared by a method in which all amino acid residues are treated equally (Metzer *et al.*, 1968) a difference index of 4.7 was obtained. For two proteins of identical amino acid composition this method gives a value of zero for the difference index, and when the compositions of pairs of 25 proteins were compared a mode difference index of 26 was obtained (Metzer *et al.*, 1968). The low value obtained for putative subunits of Kp1 protein indicates that they have a close degree of compositional relatedness. Of those residues that are stable under the conditions of hydrolysis, the biggest differences were in the content of arginine, phenylalanine, tyrosine, leucine, isoleucine and glycine. The composition of unresolved Kp1 protein is in good agreement with the sum of the subunit compositions (Table 1).

Discussion

Since its introduction (Shapiro *et al.*, 1967) the sodium dodecyl sulphate/polyacrylamide-gel-electro-

phoresis technique has found wide application in studies on the subunit structure of multimeric proteins. All highly purified preparations of the Mo-Fe protein of nitrogenase obtained from different organisms have been investigated by this method, and the following data reported. Those from the free-living N_2 -fixing organisms *K. pneumoniae* (Eady *et al.*, 1972) and *C. pasteurianum* (Nakos & Mortenson, 1971; Huang *et al.*, 1973; Tso, 1974) have two types of subunit, of mol.wts. about 50000 and 60000, present in equal numbers in a native tetrameric molecule of mol.wt. near 220000. Cp1 protein has been shown to have alanine and leucine as C-terminal amino acids (Chen *et al.*, 1973). Cv1 protein also has two subunits (Evans *et al.*, 1973), but the molecular weights were not reported. Av1 protein has been reported to have both one (Stasny *et al.*, 1974; Kleiner & Chen, 1974) or two (Fleming & Haselkorn, 1973, 1974) types of subunit, whereas Ac1 protein has given either one or two depending on the pretreatment of the protein and the gel system used during electrophoresis (Yates & Planqué, 1975). The proteins from the symbiotic N_2 -fixing organisms *R. japonicum* (Israel *et al.*, 1974) and *Rhizobium lupini* (Whiting & Dilworth, 1974) are reported to have only one type of subunit of mol.wt. near 50000.

For Av1 protein it has been suggested that the variability in the number of bands observed is due to incomplete removal of iron from the protein (Bulen, 1976), and that one of the bands represents an iron-free species of the subunit polypeptide. This explanation cannot account for the appearance of two bands with the Kp1 protein, since pretreatment with sodium dodecyl sulphate and β -mercaptoethanol released all of the iron from the protein, in agreement with results obtained with Cp1 protein (Huang *et al.*, 1973). Also, in Kp1 protein, the iron, as determined by complex formation with bathophenanthroline, was well separated from the protein bands after electrophoresis in the presence of sodium dodecyl sulphate.

The data presented here show that the commercial brands of sodium dodecyl sulphate used in the electrode buffer influenced the number and separation of the protein-staining bands obtained from Kp1, Ac1, Av1 and Rj1 proteins. The 'two-dimensional' gels of Kp1 protein shown in Plate 2 illustrate that a single band is the consequence of a failure to resolve material that will separate when the appropriate brand of sodium dodecyl sulphate is used. The commercial source of sodium dodecyl sulphate has also been shown to influence the banding pattern of foot-and-mouth-disease-virus proteins (Swaney *et al.*, 1974), but these authors were unable to identify the causative agent.

These results emphasize the unreliability of the sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis method as applied to the determination of

the subunit structure of the Mo-Fe proteins. They also indicate that the unequivocal identification of this protein on sodium dodecyl sulphate-containing gels of material prepared from whole organisms can be rather difficult. However, this technique has been successfully used to study the regulation of nitrogenase in the N_2 -fixing alga *Nostoc muscorum*, where extracts of heterocysts have been shown to contain two proteins of mol.wt. 56000 and 62000 that are thought to represent subunits of the Mo-Fe protein of this organism (Fleming & Haselkorn, 1973, 1974).

The small differences in amino acid composition between the putative subunit proteins of Kp1 protein (difference index of 4.7) is consistent with that observed between the subunits of Cp1 protein, which have a difference index of 5.6 (Chen *et al.*, 1973). These values are at the lower end of the range of 4.6–11.5 obtained when the compositions of the Mo-Fe proteins as a group were compared (Eady & Smith, 1976) and are almost at the limit of reproducibility of the analysis of different preparations of Acl protein (Yates & Planqué, 1975). For this reason it is very difficult to distinguish confidently between subunits on the basis of amino acid composition alone. However, the distinctly different 'fingerprint' patterns enable this to be done for Kp1 protein.

The differences between the peptide 'maps' of protein isolated from the two bands obtained with Kp1 protein after electrophoresis with Koch-Light sodium dodecyl sulphate, provide good evidence for this protein being composed of two types of subunit. Although analysis of peptide 'maps' composed of large numbers of peptides can be difficult, the peptide 'maps' of the two subunit proteins are clearly distinct from each other, and the undissociated protein contains both sets of peptides (see Plate 3). A maximum of 11 peptides may be common to the two subunits, as that number of ninhydrin-positive spots appear in similar positions on each map. However, some of these almost certainly represent free amino acids, and, provided that tryptic digestion was complete, one may reasonably conclude that at least 80% of the subunit polypeptides are composed of unlike amino acid sequences, despite their similarity in amino acid composition.

Since trypsin cleaves proteins at lysine and arginine residues, one can predict the number of expected tryptic-peptide spots generated from native Mo-Fe protein, depending on whether there are like or unlike subunits. For example, native Kp1 protein contains about 200 lysine plus arginine residues (Eady *et al.*, 1972; and the present paper). If the four subunits were identical they would each contain 50 lysine plus arginine residues, and the peptide 'map' of trypsin-digested protein would have a maximum of 51 peptides (assuming that digestion is complete and that no more than a single free lysine or arginine residue is released by trypsin).

Experimentally, the situation can be more complex, and the number of ninhydrin-positive spots is not necessarily simply related to the amino acid composition in the way described above (Harris & Hindley, 1965). Nevertheless, the peptide 'map' of the unresolved Kp1 protein itself is consistent with there being more than one type of subunit since it has 66 ninhydrin-positive spots. The number of trypsin-digestion products is almost certainly greater than this, since the region corresponding to free arginine is intensely stained, suggesting that multiple free arginine residues have been generated; in addition there are stained areas common to both subunit 'maps'.

The peptide 'maps' of unresolved Kp1 and Acl proteins are very similar (C. Kennedy, R. R. Eady & D. K. Rekosh, unpublished work). It is probable therefore that the *Azotobacter* protein is also composed of two types of subunit, although a definite assignment must await peptide 'mapping' of these separated subunits of this protein. Since under appropriate conditions of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the Mo-Fe proteins from *K. pneumoniae*, *A. chroococcum*, *A. vinelandii* and *R. japonicum* bacteroids resolve into two bands, which for Kp1 protein represent distinct polypeptides, these data provide circumstantial evidence that all these proteins, which have very similar physico-chemical properties, are composed of two types of subunit.

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