Klebsiella pneumoniae nitrogenase MoFe protein: chymotryptic proteolysis affects function by limited cleavage of the β -chain and provides high-specific-activity MoFe protein

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Proteinase treatment with chymotrypsin has been used to probe the structure of native *Klebsiella pneumoniae* nitrogenase MoFe protein (Kp1). Reaction with chymotrypsin did not bleach Kp1, suggesting that it did not destroy the metal centres, and the Mo and Fe contents of Kp1 were unchanged. High ratios of chymotrypsin to Kp1 (1:1 by mass) cleaved the β -chain of Kp1 to give 44 and 14 kDa polypeptides, which N-terminal amino acid sequence analysis showed to be derived from cleavage at residue β -Phe¹²⁴. A mutant MoFe protein, Kp1^{Met-124}, in which β -Phe¹²⁴ is replaced by methionine, was not cleaved by chymotrypsin. Under non-denaturing conditions, the 'nicked' β -chain of the wild-type protein remained associated with the α -chain. The α -chain was not cleaved by the proteinase treatment. Fission of the wild-type β -chain was accompanied by loss of enzyme activity, loss of intensity of the $g = 3.7$ e.p.r. signal derived from

dithionite-reduced FeMoco and by changes in the visible spectrum. The e.p.r. spectra of potassium ferricyanide-oxidized native and digested Kp1 show differences in the signals between $g = 1.6$ and 2.0. After prolonged treatment, the final specific activity of Kp1 was about $25 \pm 5\%$ of the initial activity. This corresponded to $25 \pm 5\%$ of the β -chain which was resistant to proteolytic action. Brief treatment of Kp1 with a lower concentration of chymotrypsin (chymotrypsin/Kp1 ratio = 1:10 by mass, for 10 min) preferentially cleaved high-molecular-mass polypeptides that routinely contaminate preparations of Kp1 prepared by standard procedures. Treatment with chymotrypsin followed by gel filtration to remove the proteinase and cleaved protein fragments can therefore be used to increase significantly the specific activity of Kp1 preparations and remove contaminating activities, such as the ATPase activity of myokinase.

INTRODUCTION

Nitrogenase catalyses the eight-electron reduction of dinitrogen to ammonia. It consists of two components, MoFe protein and Fe protein. The substrate-reducing active site is located in the MoFe protein which is involved in an ATP-dependent cyclic interaction with the Fe protein which results in the transfer of a single electron per cycle to the MoFe protein [1]. The MoFe protein of *Klebsiella pneumoniae* (Kp1) is an approximately 227 kDa $\alpha_2\beta_2$ tetramer with α - and β -subunits of 54.1 and 58.4 kDa respectively. Each $\alpha\beta$ unit has two distinct metal-sulphur centres; the P cluster and the iron-molybdenum cofactor, FeMoco. The P cluster is probably involved in acceptance, storage and transfer of electrons, and FeMoco is involved in substrate reduction at the catalytic site [2]. The structural relationships of the metal clusters with the polypeptide chains have been investigated in different ways. The amino acid sequences of the α - and β -chains show significant homology [3]. Three cysteines in the N-terminal segment of the α -chain (α -Cys⁶³, α -Cys⁸⁹, α -Cys¹⁵⁵, using Kp1 sequence numbering, including Met at the first residue) are homologous to cysteines in the β -chain (β -Cys⁶⁹, β -Cys⁹⁴, β -Cys¹⁵²). Mutagenesis, resulting in conversion of these conserved cysteines of the α - and β -chains of *K. pneumoniae* and *Azotobacter vinelandii* MoFe proteins into alanines, has shown that they are essential for activity, for the FeMoco e.p.r. signal and for assembly of the subunits [4–7]. They have been considered to be likely ligands of the P clusters [5,7]. The effects of specific amino acid substitutions on e.p.r. spectra, cofactor binding, electrophoretic mobility and activity have indicated that α -His¹⁹⁷ [8] and α -Cys²⁷⁵ [5,6] interact with the MoFe cofactor. A 0.5 nm resolution X-ray structure of *Clostridium pasteurianum* MoFe protein crystals showed that the

FeMoco in each half molecule is close (1.9 nm) to a P centre and is separated by 7 nm from the other cofactor [9]. Structural models for the P clusters and FeMoco have now been proposed on the basis of crystallographic analysis of *A. vinelandii* nitrogenase MoFe protein at 0.28 nm [10]; the P clusters contain two 4Fe-4S clusters ligated by six cysteine thiol groups, α 63, α 89, α 155, β 69, β 94 and β 152 (using Kp1 numbering corresponding to *A. vinelandii*); α 89 and β 94 bridge the two clusters. FeMoco is ligated to α -Cys²⁷⁵ and α -His⁴⁴².

Proteinases cleave native proteins at flexible loops at the surface or at interdomain links, rather than at rigid core elements of secondary structure [11,12]. We sought to exploit this property to determine the effects on MoFe protein on specific surface or interdomain chain fissions which preserve the binding of the metal clusters. There have been no previous reports of the use of proteolysis of native MoFe protein to probe structure-function relationships. The information yielded by chymotryptic cleavage of Kp1 is very different from that obtained from the study of mutant proteins in that here we start with functional MoFe protein and follow the impact of 'nicking' the polypeptide chain, in this case in a region considered to bind P clusters. This study also provides a purification step of value for biochemical studies of nitrogenase.

MATERIALS AND METHODS

Materials

Proteinases, including α -chymotrypsin (type II from bovine pancreas) and endoprotease Glu-C (V8 proteinase) (*Staphylococcus aureus* strain V8, type XVII-B) (EC 3.4.21.19), and all other biochemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Salts were from BDH chemicals. Cylinder

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Ar and N₂ were purchased from Air Products, Walton-on-Thames, Surrey, U.K. Sephadex G-100 was from Pharmacia, Milton Keynes, Bucks., U.K. Acetylene was prepared by the action of water on calcium carbide.

K. pneumoniae nitrogenase component proteins, MoFe protein (Kp1) and Fe protein (Kp2), were purified from *K. pneumoniae* (oxytoca) N.C.I.B. 12204 as described by Thorneley and Lowe [13]. Kp1 and Kp2 had specific activities at 30 °C of 1300 and 1600 nmol of ethylene produced/min per mg of protein respectively. The Mo content of the Kp1 was 1.4 ± 0.1 g-atom of Mo/mol of protein. Protein concentrations were determined by the method of Lowry et al. [14].

Kp1^{Met-124} was isolated from *K. pneumoniae* UNF2392 (*nifH*2231::Tn7, *RecA*56, *sr1300*::Tn10, *hsdR1*) [15], containing the mutated *nifK* on the pWF23 low-copy-number plasmid which contains a 6.4 kb *EcoRI* fragment spanning *nifHDK* [16,17]. The codon for Phe¹²⁴ was changed to Met by oligonucleotide-directed mutagenesis of a single-stranded DNA template derived from *nifK* gene fragments ligated into M13mp19 as described by Kent et al. [5].

Proteinase digestion of Kp1

Solid proteinase, in the various quantities described in the Results section, was flushed with N₂ in serum bottles fitted with rubber 'Suba-seal' closures and thermostatically maintained at 25 °C in a shaker bath. Digests were initiated by adding Kp1 (1 ml at about 10 mg/ml) in a solution containing 25 mM Hepes/NaOH, pH 7.4, 10 mM MgCl₂ and 10 mM Na₂S₂O₄ by means of a gas-tight syringe. The high concentrations of proteinase necessitated steps in addition to rapid heating to 100 °C with sample buffer to arrest proteolysis before electrophoresis. Initially two methods were used: (1) addition of a soyabean trypsin-chymotrypsin inhibitor (0.3 mg/mg of chymotrypsin); (2) separation of chymotrypsin from Kp1 by gel filtration chromatography on Sephadex G-100 equilibrated with anaerobic 50 mM Tris/HCl, pH 8.7, 50 mM MgCl₂, containing 1 mM Na₂S₂O₄. The latter was found to be sufficient alone, and was subsequently used.

PAGE and N-terminal amino acid sequence determination

Proteolysis was monitored by SDS/PAGE in 10% (w/v) acrylamide vertical slab gels stained with Coomassie Brilliant Blue R-250 [18]. Samples, freed from proteinase as described above, were diluted to 2 mg/ml, added to an equal volume of electrophoresis sample buffer and boiled for 5 min before electrophoresis. Densitometry of the stained gels was carried out with a Molecular Dynamics model 300A computing densitometer. The α - and β -subunits of Kp1 do not stain equally with Coomassie Blue [19]. Proteolytic fragments for N-terminal sequence determination were separated by SDS/PAGE on gels pre-run at 80 V for 2 h with 50 μ M glutathione in the upper running buffer and with 0.1 mM thioglycollate in the running buffer [20]. The protein sample (0.8 ml of Kp1 at 1.4 mg/ml) was loaded across the entire width of the gel (16 cm). After electrophoresis, protein bands were transferred to a polyvinylidene difluoride membrane [Immobilon, from Millipore (U.K.) Ltd., Harrow, Middx, HA1 2YH, U.K.] using a semi-dry electroblotting procedure [21]. The blotted proteins were stained with 0.2% Coomassie Blue R-250 in 50% methanol/7.5% acetic acid for 5 min and then destained in 50% methanol/10% acetic acid for 10 min. The membrane was finally rinsed in distilled water and air-dried before the protein bands were cut out. N-Terminal amino acid sequence determination was carried out using standard procedures on an ABI pulsed liquid analyser.

Assay for nitrogenase activity

Acetylene reduction assays were performed at 30 °C and a 20:1 molar ratio of Kp2/Kp1 as described by Eady et al. [19], except that the reaction volume was 1.0 ml and dithionite concentration 30 mM. Assays were of 5 min duration and terminated by addition of 0.1 ml of 30% (w/v) trichloroacetic acid. H₂ evolution and NH₃ production were measured after incubation in an atmosphere of Ar or N₂ respectively. Gas samples were assayed for ethylene on a Pye Series 204 gas chromatograph operated at 100 °C and fitted with a 1 m $\times$ 6 mm column of Poropak N (Phase Separations) (80–100 mesh) with N₂ (20 ml/min) as carrier gas.

Metal determinations

Fe and Mo were determined colorimetrically; acid-labile Fe was determined with 4,7-diphenyl-1,10-phenanthroline [22] and Mo with toluene-3,4-dithiol by the method of Clarke and Axley [23] as described by Bulen and LeComte [24].

F.p.l.c.

Gel-filtration f.p.l.c. was carried out with a Pharmacia f.p.l.c. system (Pharmacia, Uppsala, Sweden) using a Superose 12 column equilibrated and eluted with 50 mM Tris/HCl, pH 8.7, 10 mM MgCl₂. Samples (20 mg) applied to the column were eluted at a flow rate of 0.5 ml/min. Anion-exchange f.p.l.c. was carried out on a Mono Q HR5/5 column (Pharmacia Biosystems) with a 750 ml 100–500 mM NaCl gradient in 50 mM Tris/HCl, pH 8.7, and a flow rate of 8 ml/min.

E.p.r.

Protein samples (0.3 ml of Kp1 at 10 mg/ml) were transferred by syringe to pre-N₂-flushed quartz e.p.r. tubes (3 mm internal diameter) inside a 'Schlenk tube' fitted with a 'Suba-seal' closure and attached via a side arm to a vacuum-N₂ manifold. Samples were stored in liquid nitrogen. E.p.r. spectra were recorded on a Bruker ER200D spectrometer. The amplitudes of the $g = 3.7$ spectral feature were measured, corrected for differences in tube diameter, and used as a measure of the e.p.r. signal intensity. The intensities were expressed as a percentage of the zero-time intensity.

Ferricyanide-oxidized Kp1 was prepared after the removal of sodium dithionite with a Bio-Gel P-6DG column (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., U.K.) equilibrated with 25 mM Hepes/NaOH, pH 8.0, 200 mM NaCl, the high salt concentration being necessary to prevent precipitation on the column. Dithionite-free Kp1 was allowed to react with K₃Fe(CN)₆ [1 mol of Kp1/10 mol of K₃Fe(CN)₆] for 10 min. E.p.r. samples were prepared inside an anaerobic glove box [13].

RESULTS

Proteinase digestion of Kp1

Several preliminary experiments were carried out using proteinases of different specificities at various proteinase/Kp1 ratios, pHs, temperatures and incubation times to determine which proteinase (chymotrypsin, carboxypeptidase, papain, pepsin, proteinase K, trypsin, V8 proteinase, subtilisin) would not bleach Kp1. Only V8 proteinase and chymotrypsin did not decolorize the protein. SDS/PAGE and acetylene reduction assays revealed that Kp1 was fully resistant to the action of V8 proteinase (protease/Kp1 ratio of 0.5:1 by mass, 48 h incubation at pH 8.7, 25 °C).

Figure 1 Chymotryptic proteolysis of *K. pneumoniae* nitrogenase MoFe protein (Kp1) analysed by SDS/PAGE

Samples of the reaction mixtures were digested under conditions described in the Materials and methods section and chromatographed on Sephadex G-100 to remove chymotrypsin. The gels were stained with Coomassie Brilliant Blue R250 and the lanes containing the stained proteins scanned with a scanning densitometer. The gels were run from left to right on the horizontal axis. (a) Kp1 incubated for 6 h in the absence of chymotrypsin; (b) after 6 h digestion with chymotrypsin at a proteinase/Kp1 ratio of 1:1 by mass; (c) after 6 h digestion of Kp1^{Met-124}, in which β -Phe¹²⁴ of the wild-type protein is replaced by methionine.

No digestion was observed when Kp1 was incubated with chymotrypsin for 10 min at 25 °C at a proteinase/Kp1 ratio = 1:10 by mass. Treatment of Kp1 with a higher concentration of chymotrypsin (chymotrypsin/Kp1 ratio = 1:1 by mass, 25 °C) resulted in the appearance of fragments of approx. 44 and 14 kDa (Figure 1). The appearance of these fragments was accompanied by diminution of the intensity of the band for the β -chain. However, proteolysis never resulted in complete disappearance of this band. After prolonged treatment (72 h), the remaining protein was calculated to be $25 \pm 5\%$ of its initial

Figure 2 Sequence alignment of nitrogenase α - and β -chains between the cysteines considered to bind P clusters

Cysteines at positions 69, 94 and 152 in the β -chain of *K. pneumoniae* MoFe protein are numbered, as well as the conserved phenylalanine residue (124) in the β -chain, the C-terminal peptide bond of which is cleaved by chymotrypsin. Sequences of the α - and β -chains of *K. pneumoniae* nitrogenase (KpNifK, KpNifD) are placed at the top of the alignments. The remaining sequences are from α - and β -chains (NifD and NifK respectively) of molybdenum nitrogenases, and of *Azotobacter* vanadium nitrogenase (VnNifD and VnNifK) and nitrogenase-3 (AnNifD and AnNifK) [25,26]. Av = *A. vinelandii*; Ac = *A. chroococcum*; Rh = *Rhizobium* sp.; Bj = *Bradyrhizobium japonicum*; An = *Anabaena* sp.; Tf = *Thiobacillus ferrooxidans*; Mt = *Methanococcus thermolithotrophicus*; Cp = *Clostridium pasteurianum*. The PIR database accession code numbers for the sequences are: *K. pneumoniae*, S06987, S06987; *A. vinelandii* nitrogenase-3, B32057, D32057; *A. chroococcum* vanadium nitrogenase, S04113 and S04115; *A. vinelandii* Mo nitrogenase, NIAVMA, NIAVMB; *Anabaena* sp., NIAIMA, NIAIMB; *Clostridium pasteurianum*, N1CLMA, N1CLMB; *Rhizobium* sp., A23874, B23874; *Methanococcus thermolithotrophicus* α -chain S06987; *Thiobacillus ferrooxidans*, N1BCAT, N1BCBT. The sequences were aligned by the program PILEUP in the GCG sequence analysis package [27].

concentration. The 44 and 14 kDa fragments were not produced when a mutant protein (Kp1^{Met-124}), in which β -Phe¹²⁴ was replaced by methionine, was subjected to proteolysis (Figure 1).

A portion of the proteolysis mixture after 6 h reaction was analysed by gel-filtration f.p.l.c. This showed two peaks, one at the position expected for Kp1, the other at the position for chymotrypsin (results not shown). Analysis by SDS/PAGE of the protein eluted at the position expected for Kp1 showed that this contained the α -chain, the β -chain with diminished intensity, as well as the 44 and 14 kDa fragments. The last two fragments are derived from proteolytic cleavage of the β -subunit (see below). Anion-exchange f.p.l.c. of the proteolysis mixture also showed a single Kp1 peak which contained the α - and β -chains as well as the 44 and 14 kDa fragments.

The N-terminal amino acid sequence of the 44 kDa fragment was determined and found to be GGNNNMNLGLQ-NASALYKPE (one letter amino acid code). This sequence occurs in the β -chain of Kp1 at residues 125–144 [based on the sequence deduced from *K. pneumoniae* nifK (Figure 2)] [17]. The N-terminal amino acid sequence of the α -subunit after chymotryptic digestion was found to be DNATGEXRLALIQ-

(D/E)VLEVF, which corresponds to the N-terminal sequence of the native α -subunit.

Mo and Fe contents of chymotrypsin-digested Kp1

A portion of chymotrypsin-treated Kp1, with the proteinase removed, was analysed for Fe and Mo content. Digested Kp1 contained 1.4 g-atom of Mo/mol and therefore had not lost any Mo during proteinase treatment. It was also shown to retain its full complement of Fe after digestion (25 g-atom of Fe/mol).

Effect of chymotrypsin on Kp1 activity

Chymotryptic action resulted in a decrease in the activity of Kp1. This was similar when N_2 , H^+ or acetylene were reduced (only the data for acetylene reduction are shown; Figure 3a). During acetylene reduction, Kp1 activity was lost in an approximately exponential form with a half-life of about 2 h. However, activity was not totally lost and, even after prolonged proteolysis (72 h, 25 °C), $25 \pm 5\%$ of the initial Kp1 activity was retained. After 6 h digestion, the proteinase was removed by gel filtration and fresh proteinase added at the initial concentration. The residual activity was not decreased further. Also, addition of 5 mM ATP or ADP together with the fresh chymotrypsin had no effect on the fragments and did not decrease the remaining activity (not shown).

Visible spectrum changes

U.v.-visible scanning of Kp1 (2 mg/ml) before and after chymotrypsin digestion showed an increase in absorption of 15–20% over the entire spectrum after digestion. This is characteristic of an oxidation of the protein [19].

E.p.r.

The dithionite-reduced cofactor FeMoco exhibits a characteristic $S = 3/2$ e.p.r. spectrum with features at $g = 4.3$, 3.7 and 2.01. There was an approximately exponential decrease in the height of the $g = 3.7$ e.p.r. signal during the digestion of Kp1, with a similar half-life to that observed for the decrease in Kp1 activity (Figure 3b). However, after 6 h digestion, 50% of the original e.p.r. signal intensity still remained, and the specific activity was $25 \pm 5\%$ of that before the start of proteolysis. There was no

Figure 3 Time courses of the effect of chymotryptic digestion on the activity and $g = 3.7$ e.p.r. feature of Kp1

E.p.r. spectra were measured at 10 K using 100 mW of microwave power at 9.47 GHz with 2.1 mT field modulation at 100 kHz. (a) Specific activities of Kp1 in the absence of chymotrypsin ($\square$) and after digestion with 10 mg of chymotrypsin/10 mg of Kp1/ml ($\circ$). (b) Intensity of the $g = 3.7$ e.p.r. of Kp1 after chymotryptic digestion.

further decrease after 50 h digestion. Figure 4 shows the effect of digestion on the e.p.r. signals, due to P centres, that are elicited when the protein is oxidized by ten equivalents of ferricyanide.

Figure 4 Effect of chymotryptic digestion on the e.p.r. spectrum of 10 electron-oxidized Kp1

A, Spectrum of undigested Kp1; B, spectrum of Kp1 after 6 h of digestion. E.p.r. spectra were measured at 10 K using 100 mW of microwave power at 9.43 GHz with 3.1 mT field modulation at 100 kHz.

Figure 5 Effect of brief chymotryptic proteolysis of Kp1

Samples of the reaction mixtures were separated by SDS/PAGE and analysed as in Figure 1. (a) Kp1 without chymotryptic treatment; (b) Kp1 after treatment with chymotrypsin (Kp1/chymotrypsin ratio = 10:1 by mass) for 10 min. The gels were overloaded for Kp1 to show the effect of chymotryptic treatment on contaminating high-molecular-mass bands. The Kp1 preparation also contained lower-molecular-mass contaminating proteins. The gels were run from left to right on the horizontal axis.

The shape of this complex e.p.r. spectrum is clearly affected by digestion.

Purification of Kp1 from contaminating proteins

SDS/PAGE analysis of samples taken during proteolysis showed that the high-molecular-mass contaminating proteins that are often copurified with Kp1 using conventional chromatographic techniques were rapidly lost. Subsequent digestions with reduced levels of chymotrypsin (chymotrypsin/Kp1 = 1:10 by mass) for short times (10 min) showed no indications of attack on the β -subunit and no change in total Kp1 activity, but the contaminating proteins were still preferentially degraded (Figure 5). The digestion products of the contaminating proteins and chymotrypsin were easily removed by gel-filtration chromatography on Sephadex G-100. Removal of the contaminating proteins, and possibly damaged Kp1, resulted in an increased specific activity, which was most pronounced when low-specific-activity Kp1 was treated with chymotrypsin. Thus the Kp1 specific activity for acetylene reduction could be increased from 1200 to 2200 nmol of ethylene produced/min per mg of protein. The latter value is close to the estimated limiting specific activity of 2400 for Kp1 containing 2 Mo atoms per molecule [28].

V8 proteinase was also investigated as an alternative to chymotrypsin for Kp1 purification. It has the advantage that it does not cleave native Kp1 but does cleave contaminating proteins. However, no increase in Kp1 specific activity was obtained, presumably because the digestion products were not separated from Kp1 by gel filtration.

DISCUSSION

Endoproteinase Glu-C from *S. aureus* (V8 proteinase) cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues [29]. The inability of V8 proteinase to cleave Kp1 indicates the absence of accessible peptide bonds adjacent to glutamate and aspartate residues in Kp1. Two aspartate residues on each chain (α -Asp^{162,163}, β -Asp^{159,160}), with the adjoining Cys, form part of a motif present in all nitrogenases [30].

Chymotrypsin cleaves peptide bonds on the C-terminal side of aromatic residues, giving 47 potential cleavage sites in the β -chain of Kp1. The N-terminal sequence of the 44 kDa fragment formed by chymotryptic digestion of Kp1 indicated that it is formed by cleavage at Phe¹²⁴. The sizes of this and the 14 kDa fragment are those expected if Kp1 were cleaved once at Phe¹²⁴. The 44 and 14 kDa fragments remained associated with the α -chain in non-denaturing conditions. As expected, the mutant protein Kp1^{Met-124}, which has methionine in place of β -Phe¹²⁴, does not produce the 44 and 14 kDa fragments.

The homologous cysteine pairs α -Cys⁶³/ β -Cys⁶⁹, α -Cys⁸⁹/ β -Cys⁹⁴ and α -Cys¹⁵⁵/ β -Cys¹⁵² are involved in ligation of the P clusters [10]. α -Cys⁶³/ β -Cys⁶⁹ and α -Cys⁸⁹/ β -Cys⁹⁴ are separated by 26/25 amino acids, which could accommodate at least two secondary-structure units. A segment of approximately twice this length (66/58 amino acids) intervenes between α -Cys⁸⁹/ β -Cys⁹⁴ and α -Cys¹⁵⁵/ β -Cys¹⁵². β -Phe¹²⁴ lies in a short segment which is highly conserved in both α - and β -chains, and midway between the homologous α -Cys⁸⁹/ β -Cys⁹⁴ and α -Cys¹⁵⁴/ β -Cys¹⁵² pairs (Figure 2). The residue homologous to *K. pneumoniae* β -Phe¹²⁴ is only substituted, by tyrosine, in the α -chain of nitrogenase from the archaebacterium *Methanococcus thermolithotrophicus* [31]. The absence of cleavage in the α -chain indicates that, despite the high conservation of this segment in both chains, they are not equally accessible to chymotrypsin. The absence of strict equivalence between the α - and β -chains has been indicated by differences in

mutations of homologous residues in the two chains. Thus mutation of α -Cys¹⁵⁵ to Ser produces an inactive MoFe protein, whereas the protein produced by changing β -Cys¹⁵² to Ser shows 25% activity [5]. Again, chemical cross-linking of *A. vinelandii* Fe and MoFe proteins revealed a cross-linking site (Lys³⁹⁹) only in the β -subunit [32]. Examination of the aligned sequences in the region surrounding β -Phe¹²⁴ shows clear differences between the α - and β -chains. A seven-residue gap has to be introduced into the segment preceding β -Phe¹²⁴ in order to align the chains (Figure 2). This may underlie the topological difference between the two chains at Phe¹²⁴ which results in the differential action of chymotrypsin.

Chain fission clearly affected the e.p.r. spectrum of the semi-reduced cofactor, reducing the intensity of the $g = 3.7$ peak by 50%. The absence of bleaching and unchanged Mo and Fe contents indicated that chymotryptic cleavage does not result in release of the metal clusters. The consequences of the chain fission are, however, complex, and are not easily interpretable with our current understanding of the structure-function relationships of nitrogenase. First, all attempts to completely cleave the β -subunit were unsuccessful. The final activity was always $25 \pm 5\%$ of the initial activity, corresponding to the amount of uncleaved β -chain. It therefore appears that a proportion of the active enzyme is in a conformation in which β -Phe¹²⁴ is inaccessible to chymotrypsin. ATP and ADP did not affect the susceptibility of the proteinase-resistant fraction. Cleavage of those molecules in which β -Phe¹²⁴ is susceptible to chymotryptic cleavage resulted in loss of enzyme activity. The u.v.-visible spectrum of the cleaved protein suggested that cleavage was accompanied by oxidation of the metal centres; this was consistent with the parallel decrease in intensity in the $g = 3.7$ e.p.r. signal. The e.p.r. signals observed between $g = 1.6$ and $g = 2.0$ from ferricyanide-oxidized Kp1 were changed on proteolysis. Although these signals, which are believed to be due to P clusters, have not been fully characterized, similar changes have been observed previously with a decrease in pH [33]. The changes observed after proteolysis may be due to some distortion of the protein ligands to these clusters. While the dithionite-reduced state of the cofactor is e.p.r.-active in the wild-type MoFe protein, all other redox states are e.p.r.-silent.

An unexpected outcome of this study is a simple procedure for producing high-specific-activity MoFe protein. It is particularly useful for removing myokinase activity which contaminates most preparations of MoFe protein (R. W. Miller and R. R. Eady, personal communication).

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