Immunofluorescence Detection of Nitrogenase Proteins in Whole Cells

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SUMMARY

Fluorescent antibodies (FA) prepared against the Mo-Fe and Fe proteins of nitrogenase from *Klebsiella pneumoniae* M5a1 were used to detect these protein components in toluene-treated whole cells that were actively reducing acetylene. The FA were highly specific, staining only nitrogenase component proteins originating from *Klebsiella*. Cross-reactions between the FA and purified nitrogenase proteins from other dinitrogen-fixing micro-organisms did not occur, except in the case of *Bacillus polymyxa*. The tests rapidly and accurately assayed the component proteins in *Klebsiella* mutants and derivatives to which *Klebsiella nif* genes had been transferred either by plasmid or by other means. Cross-reactions also indicated the degree of relatedness between nitrogenase proteins from dinitrogen-fixing micro-organisms of various origins.

INTRODUCTION

Fluorescent antibodies (FA) have been widely used in microbial ecology (Schmidt, 1973) for the identification and enumeration of micro-organisms in various habitats. FA have also been used as 'tracers' to locate and study enzymes in animal cells (Goldman, 1968; Nairn, 1969), but have not been used to study intracellular enzymes in micro-organisms since bacterial membranes are impermeable to antibodies (Kwapinskyi, 1972). Once the wall and membrane have been damaged, however, one would expect intracellular enzymes to be readily stained with their specific, homologous FA.

Nitrogenase, the enzyme which reduces dinitrogen, is a binary protein consisting of non-haem Mo-Fe and Fe proteins, and has been well characterized (Eady & Postgate, 1974). Immunological detection of the two protein components as cross-reacting material (CRM) has involved serial dilutions in Preer tubes (Davis *et al.*, 1972), modifications of the Ouchterlony plate technique (Shah *et al.*, 1973), or polyacrylamide gel electrophoresis (Brill *et al.*, 1974). The immunological detection limit of the FA technique is 1 μg protein ml^{-1} , compared with 0.05 μg ml^{-1} for quantitative agglutination, 0.1 μg ml^{-1} for flocculation, and 0.3 μg ml^{-1} for immunodiffusion (Kwapinskyi, 1972); but once the antigen-antibody complex has been formed, the conjugated dye may be detected at concentrations of 1 pg ml^{-1} and is subjectively easier to observe (Nairn, 1969).

This paper reports the preparation of FA against purified nitrogenase proteins from *Klebsiella pneumoniae* M5a1 and their use in genetic and biochemical studies involving nitrogenase.

METHODS

Antibody induction. The Mo-Fe and Fe proteins of nitrogenase were purified from *Klebsiella pneumoniae* M5a1 (Eady *et al.*, 1972; Smith *et al.*, 1976) and diluted in pyrogen-free, de-gassed 0.85 % (w/v) NaCl (pH 8.0). The solution was divided into lots equivalent to 100 µg nitrogenase; these were frozen as small beads in liquid nitrogen. Antibody formation was induced in New Zealand rabbits by four intramuscular injections at 2-day intervals of 100 µg protein diluted 1:1 (v/v) with Freund's complete adjuvant, followed by two intravenous injections without adjuvant at 2-day intervals. After 4 days rest, the rabbits were test bled through the marginal ear vein to determine antibody titre and then 20 ml blood was removed by cardiac puncture, allowed to coagulate, and the antiserum was removed and frozen.

Antibody purification. All operations were at 4 °C. To 10 ml undiluted antiserum in a centrifuge tube, an equal volume of 3.9 M-(NH₄)₂SO₄ was added slowly, with stirring, and allowed to stand for 16 h. After centrifuging at 10000 *g* for 10 min, the protein precipitate was redissolved in 10 ml saline; the precipitation procedure was repeated three times, but omitting the 16 h settling period. The final precipitate, in 10 ml saline, was dialysed against saline until no sulphate or ammonium could be detected. Gamma globulins (IgG) were further purified with DEAE-cellulose using phosphate-buffered saline [PBS; containing (g l⁻¹): Na₂HPO₄, 11.52; NaH₂PO₄·2H₂O, 4.48; NaCl, 8.50; adjusted to pH 7.2 with 1 M-NaOH] as the eluant (Kwapinskyi, 1972). The protein content of the globulin solution was determined by the biuret procedure (Gornall, Bardawill & David, 1949) and the solution was adjusted to 1.0 % (w/v) protein with saline and stored at -20 °C.

Conjugation. Globulin solution (10 ml) was mixed with 4.0 ml 0.15 M-sodium phosphate buffer pH 9.0, added to a solution of 4.0 ml 0.10 M-sodium phosphate buffer pH 8.0 and FITC Isomer I [fluorescein isothiocyanate, Sigma; 0.02 mg FITC (mg protein)⁻¹], the volume was made up to 20 ml with saline and the pH was adjusted to 9.0 with 0.1 M-NaOH. The mixture was stirred for 6 h at 25 °C after which conjugated and unconjugated FITC were separated on a column of Sephadex G-25 using PBS as the eluant. The fluorescent antibody was dispensed in 1.0 ml quantities into sterile bijoux and stored frozen at -20 °C until required.

Bacteria. Bacteria were transferred from slants to nutrient broth and grown for 24 h, and a 1 % inoculum was added to the appropriate medium: NFDm (Cannon, Dixon & Postgate, 1974a) for *Klebsiella* and *Salmonella* Nif⁺ strains; NFDm with 100 µg aspartate ml⁻¹ for *Agrobacterium*, *K. aerogenes* and *Klebsiella* Nif⁻ strains; Burk's sucrose (Newton, Wilson & Burris, 1953) for *Azotobacter* and *Beijerinckia*; Hino and Wilson's medium (Hino & Wilson, 1957) for *Clostridium* and *Bacillus*; Kalininskaya's medium, with sodium lactate in place of ethanol, for *Rhodospirillum* and *Spirillum* (Biggins & Postgate, 1969); and NFDm supplemented with 100 µg Casamino acids ml⁻¹ (for *Escherichia coli* C603) or 25 µg tryptophan ml⁻¹ (for *E. coli* JC5466). Bacteria were grown in 25 ml Erlenmeyer flasks, sealed with a Suba-Seal and shaken on a rotary shaker at 30 °C, and were sampled at mid-exponential phase (10⁶ to 10⁸ cells ml⁻¹) and assayed for acetylene-reducing activity. Samples (0.01 ml) were placed within a 1 × 1 cm marked area on a sterile glass slide, 0.05 ml toluene was added, and the solution was evaporated to a smear at 34 °C and heat fixed by slowly passing the slide through a flame three times.

Enzymes. The oxygen-sensitive nitrogenase enzymes were maintained under argon. Samples (0.01 ml; 1 mg protein ml⁻¹) were transferred under anaerobic conditions on to sterile glass slides in a BBL Gas Pack container which was sealed under an atmosphere of argon, hydrogen and CO₂. The container was maintained at 34 °C until the solution had

Table 1. *Immunological specificity of fluorescent antibodies (FA) prepared against the Mo-Fe and Fe proteins of nitrogenase from Klebsiella pneumoniae M5a1*

Test	Objective	Antibody stain*
'Normal' serum FA used to stain <i>Klebsiella pneumoniae</i> nitrogenase	Antigen should be non-reactive, i.e. negative to non-specific staining	—
Homologous, unconjugated (unlabelled) serum, followed by homologous conjugated serum (FA)	Blocking of specific sites with unlabelled antibody should prevent specific staining	—
Stain with homologous FA that has been absorbed with antigen	Absorption should remove all or most of the staining reaction	—
Stain Kp1 with K1†	Test cross-reactions between protein fractions	+4
Stain Kp1 with K2		—
Stain Kp2 with K1	Test cross-reactions between protein fractions	—
Stain Kp2 with K2		+4

* —, Negative; ±, trace; +1 feeble to +4 maximal.

† Kp1 and Kp2 are Mo-Fe and Fe proteins of nitrogenase respectively; K1 and K2 are fluorescent antibodies prepared against proteins 1 and 2 respectively.

evaporated to a smear. The smear was then heat fixed by slowly passing the slide through a flame three times.

Staining. The appropriate FA (FA prepared against the Mo-Fe and Fe proteins of *K. pneumoniae* M5a1 are designated K1 and K2 respectively) was thawed slowly, diluted 1:4 (v/v) with PBS, filter sterilized using a Sweeney filter and 0.45 µm Millipore filter, and kept cold until required. Smears of bacteria and pure enzymes were stained with 0.5 ml rhodamine gelatin solution to control autofluorescence and non-specific staining (Bohloul & Schmidt, 1968) and dried at 50 to 60 °C. K1 or K2 (0.5 ml) was added, and the slides were incubated in a moist chamber for 15 min and then washed with PBS for 30 min. Slides were dried, mounted with Difco U.V. Free Mounting Media-Aqueous and covered with a 19 mm coverslip.

Microscopy. Slides were viewed on a Leitz Orthoplan fluorescence microscope equipped with a Wotan HBO 200 W super pressure mercury lamp and a tungsten white lamp, both in transmitted mode. Excitation of the FITC at 490 nm was by 2 mm KG1 heat filter, 3 mm BG38, 3 mm BG12, 1.5 mm BG12 and KP500 filters in conjunction with a K510 barrier filter. Plano 10/25/40 objectives were used in conjunction with phase 5, bright or dark field fluorescence microscopy. The fields viewed showed green fluorescing organisms and fluorescing debris from toluene-lysed cells. Fluorescence brightness of staining was determined qualitatively.

RESULTS AND DISCUSSION

The immunological specificities of the FA prepared against the Mo-Fe and Fe proteins of nitrogenase were evaluated according to the criteria of Schmidt (1973). The FA (Table 1) were highly specific, exhibiting no non-specific staining or cross-reactions, and absorption effectively blocked the staining as expected.

Reactions with proteins

Cross-reactions between K1 and K2 and nitrogenase proteins isolated from other dinitrogen-fixing bacteria as well as some related enzymes are shown in Table 2. K1 and K2 stained

Table 2. *Cross-reactions of fluorescent antibodies against Klebsiella pneumoniae M5a1 nitrogenase with purified nitrogenase from other dinitrogen-fixing micro-organisms and with other molybdenum-containing proteins*

Enzyme	Origin	Source	Antibody stain†	
			K1	K2
Nitrogenase protein				
Kp1* }	<i>K. pneumoniae</i> M5a1	R. R. Eady	{ +4 —	— +4
Kp2 }				
Ac1 }	<i>Azotobacter chroococcum</i>	M. G. Yates	{ — ±	— ±
Ac2 }				
Cp1 }	<i>Clostridium pasteurianum</i>	L. E. Mortenson	{ — —	— —
Cp2 }				
Bp1 }	<i>Bacillus polymyxa</i>	B. E. Smith	{ +3 —	— +3
Bp2 }				
Rj1	<i>Rhizobium japonicum</i> bacteroids	F. Bergersen	—	—
Glutamine synthetase				
	Adenylated <i>K. aerogenes</i> MK53	S. L. Streicher	—	—
	Deadenylated <i>K. aerogenes</i> MK53		—	—
	Deadenylated <i>E. coli</i>		—	—
Xanthine oxidase	<i>E. coli</i>	R. C. Bray	—	—
Nitrate reductase	<i>E. coli</i>	R. C. Bray	—	—
Formate dehydrogenase	<i>E. coli</i>	R. C. Bray	—	—
Bovine serum albumin		Sigma	—	—

* Proteins 1 and 2 are Mo-Fe and Fe proteins of nitrogenase respectively.

† —, Negative; ±, trace; +1 feeble to +4 maximal.

Table 3. *Staining of toluene-treated micro-organisms actively reducing acetylene with fluorescent antibodies against nitrogenase from Klebsiella pneumoniae M5a1*

Micro-organism	Phenotype	Antibody stain*	
		K1	K2
<i>K. pneumoniae</i> M5a1	Nif ⁺	+4	+4
<i>K. pneumoniae</i> M5a1 (NH ₄ ⁺ -repressed)	Nif [—]	—	—
<i>K. pneumoniae</i> 107	Nif [—]	—	—
<i>Klebsiella</i> serotype 54	Nif ⁺	+4	+4
<i>Klebsiella</i> serotype 54(1281-70)	Nif [—]	—	—
<i>K. aerogenes</i> 418	Nif [—]	—	—
<i>K. aerogenes</i> MK54	Nif [—]	—	—
<i>Azotobacter vinelandii</i> OP	Nif ⁺	±	±
<i>Az. chroococcum</i> 8003	Nif ⁺	±	±
<i>Beijerinckia indica</i> 8597	Nif ⁺	±	±
<i>Bacillus polymyxa</i> 8599	Nif ⁺	+3	+3
<i>Clostridium pasteurianum</i> 9486	Nif ⁺	±	±
<i>Rhodospirillum rubrum</i> 8740	Nif ⁺	±	±
<i>Spirillum lipoferum</i> 7	Nif ⁺	±	±
<i>Sp. lipoferum</i> Br10	Nif ⁺	±	±
<i>Sp. lipoferum</i> M81	Nif ⁺	±	±
<i>Sp. lipoferum</i> 51e	Nif ⁺	+2	+2
<i>Sp. lipoferum</i> RG5b	Nif ⁺	+2	+2
<i>Sp. lipoferum</i> USA5C	Nif ⁺	±	±

* —, Negative; ±, trace; +1 feeble to +4 maximal.

homologous nitrogenase proteins from *K. pneumoniae* M5a1 at maximal fluorescence. No cross-reaction was observed with nitrogenase from *Rhizobium japonicum*, *Clostridium pasteurianum* or *Azotobacter chroococcum* except for traces of staining of the Fe protein of *Az. chroococcum*. An interesting observation was the good cross-reaction with analogous nitrogenase from *Bacillus polymyxa*, which suggested close structural homology at least of the antigenic sites of the *B. polymyxa* and *K. pneumoniae* protein. Glutamine synthetase was studied because it is involved in the regulation of nitrogenase synthesis (Streicher *et al.*, 1974; Tubb, 1974); nitrate reductase, xanthine oxidase and formate dehydrogenase were tested because, like nitrogenase, they are molybdoenzymes; serum albumin was a blank control; none was stained by K1 or K2.

Reactions with natural dinitrogen-fixing bacteria

Table 3 records tests of K1 and K2 with toluene-treated bacteria, grown as described in Methods. The parent strain stained well; ammonium repressed the synthesis of stainable material. A Nif⁺ *Klebsiella* sp. of serotype 54 stained well; a comparable Nif⁻ species did not. Nif⁻ *K. aerogenes* strains did not stain at all but, as expected from the behaviour of its nitrogenase proteins, *B. polymyxa* stained well. Trace cross-reactions were seen with some aerobic and anaerobic dinitrogen-fixing bacteria but the only substantial cross-reactions were seen with two strains of *Spirillum lipoferum* designated 'small' by Dr J. Dobereiner (personal communication). These, like *B. polymyxa*, probably have a nitrogenase which has some structural homology to *Klebsiella* nitrogenase.

Tests with mutants and strains carrying nif plasmids

Table 4 records tests with well-characterized mutants of *K. pneumoniae* M5a1 and organisms carrying the *nif* plasmids RP41 (Dixon, Cannon & Kondorosi, 1976; Cannon, Dixon & Postgate, 1976; Cannon & Postgate, 1976), FN68 (Cannon *et al.*, 1976) and uncharacterized plasmids (Dixon & Postgate, 1972). *Klebsiella pneumoniae* 107 is a mutant of strain M5a1 deleted throughout the *nif* region and showed no cross-reaction at all. Mutant UN318, a point Nif⁻ mutant (St John *et al.*, 1975), had lost both immunological and biochemical Mo-Fe protein activity; mutant 279 showed the reverse pattern – absence of activity and antigen for the Fe protein. Mutant 285 lacked the Fe protein activity but retained an antigenic polypeptide. *Escherichia coli* JC5466 with the plasmids RP41 or FN68 added (Cannon *et al.*, 1976) reduced acetylene and had both component proteins. Similarly, *E. coli* C603 with non-characterized plasmids added (Dixon & Postgate, 1972) reduced acetylene and had both component proteins irrespective of whether the *nif* genes remained on the plasmid, as was the case for C-M9 and C-L4 (Cannon *et al.*, 1974b), or were integrated into the chromosome, as for C-M7 (Cannon *et al.*, 1974a). The strains of *Az. vinelandii* (Shah *et al.*, 1973; Brill, 1976) were the mutants used by Cannon & Postgate (1976) to correct structural *nif* mutations with *K. pneumoniae* *nif* genes on RP41. Mutants FC10, FC91 and FC100 showed cross-reaction towards the nitrogenase protein in which they were originally defective (as UW10, UW91 and UW100) but, surprisingly, no cross-reaction with FA to the *Klebsiella* nitrogenase protein they did not need. Selective regulation of chromosomal and plasmid *nif* would seem to be the only interpretation. Strains of VKS1 and the parent LT7-1201 of *S. typhimurium* showed the expected pattern of cross-reaction but 602 was unsatisfactory: both VKS1 and 602 were strongly Nif⁺ (Cannon *et al.*, 1976; Postgate & Krishnapillai, unpublished). *Agrobacterium tumefaciens*, though phenotypically Nif⁻, carried RP41 (Dixon *et al.*, 1976) and formed antigens to serum prepared against

Table 4. Application of immunofluorescence to genetic analysis of nitrogen-fixing derivatives of *Klebsiella*, *Azotobacter*, *Salmonella*, *Agrobacterium* and *Escherichia*

Micro-organism	Known 'Nif' phenotype		Plasmid introduced	Altered 'Nif' phenotype			Antibody stain†	
	Biochemical	CRM*		Code	Presumed CRM	C ₂ H ₂ reduction	K1	K2
<i>K. pneumoniae</i>								
M5a1	I ^k II ^k	I ^k II ^k	.	.	.	+	+4	+4
107	I-II ⁻	I-II ⁻	.	.	.	-	-	-
UN318	I-II ^k	I-II ^k	.	.	.	-	-	+4
279	.	.	.	.	I ^k II ⁻	-	+2	-
285	.	.	.	.	I ^k II ⁻	-	+4	+2
<i>E. coli</i>								
JC5466	.	.	.	.	I-II ⁻	-	-	-
JC5466	.	.	FN68	JC5466(FN68)	I ^k II ^k	+	+4	+4
JC5466	.	.	RP41	JC5466(RP41)	I ^k II ^k	+	+4	+4
C603	.	.	.	.	I-II ⁻	-	-	-
C603	.	.	NC‡	C-M7	I ^k II ^k	+	+4	+4
C603	.	.	NC	C-M9	I ^k II ^k	+	+4	+4
C603	.	.	NC	C-L4	I ^k II ^k	+	+4	+4
<i>S. typhimurium</i>								
LT7-1201	.	.	.	.	I-II ⁻	-	-	-
LT7-1201	.	.	RP41	VKS1	I ^k II ^k	+	+2	+4
LT7-1201	.	.	FN68	602	I ^k II ^k	+	±	+2
<i>A. tumefaciens</i>								
544	.	.	.	.	I-II ⁻	-	-	-
544	.	.	RP41	544	I ^k II ^k	+	+2	+2
<i>Az. vinelandii</i>								
OP	I ^a II ^a	I ^a II ^a	.	.	.	+	±	±
UW10	I-II ^a	I ^a II ^a	RP41	FC10	I ^{ak} II ^{ak} §	+	+4	±
UW91	I ^a II ⁻	I ^a II ^a	RP41	FC91	I ^{ak} II ^{ak}	+	±	+4
UW100	I-II ^a	I ^a II ^a	RP41	FC100	I ^{ak} II ^{ak}	+	+4	±

* Cross-reacting material determined immunologically: k designates *Klebsiella*; a, *Azotobacter* origin.

† -, Negative; ±, trace; +1 feeble to +4 maximal.

‡ NC, Not sufficiently characterized to be named.

§ Dominance of chromosomal over plasmid DNA unknown.

K. pneumoniae proteins. The lower degree of staining may indicate that this nitrogenase was present in lower concentration or was conformationally distorted.

The use of specific, homologous fluorescent antibodies against the Mo-Fe and Fe proteins of *Klebsiella* nitrogenase permitted the rapid and precise detection of these components in mutants and derivatives carrying *Klebsiella nif* genes. Such tests could be a useful tool for characterization of mutants and derivatives which synthesize inactive nitrogenase polypeptides. Differing degrees of cross-reactions, as noted with *B. polymyxa* and two *Sp. lipoferum* strains, may be used to study the relatedness of nitrogenase from various origins.

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