An immunological comparison of nitrogenase proteins of fast and slow growing rhizobia

T. Bisseling, R.C. van den Bos, L. Moen, J.G.J. Hontelez and A. van Kammen

Laboratory of Molecular Biology, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands

Received 24 June 1982

Radioimmunoassay *Nitrogenase* *Component I* Rhizobium Klebsiella Azotobacter

1. INTRODUCTION

The enzyme complex nitrogenase, which catalyzes nitrogen reduction consists of two component proteins [1,2]: component I, a molybdenum and iron containing protein comprising two types of subunits and component II, a dimer iron–sulfur protein with identical subunits. The actual nitrogen reduction takes place on component I and this component is called dinitrogenase; component II which supplies component I with electrons is called dinitrogenase reductase [3]. The nitrogenase complex shows a remarkable structural similarity [2] among widely divergent organisms. The genus *Rhizobium* can be divided into two groups, *viz.* slow and fast-growing species [4,5]. These two groups differ in several characteristics. The fast-growing rhizobia have a generation time of 2–4 h, are acid producers, have laterally arranged flagella and do not reduce nitrogen in the free-living form [4,5]. The slow-growing rhizobia have a generation time of 6–8 h, are alkali producers, have polar or subpolar flagella and reduce nitrogen in free-living and symbiotic form. Furthermore there are differences in the metabolic pathways and DNA base ratios. The results of Ruvkun and Ausubel [6] on the sequence homology between the *nif* genes of a fast and a slow-growing *Rhizobium* pointed to a rather restricted homology. This result implies that a substantial difference may exist between nitrogenase proteins of fast and slow-growing rhizobia. In this paper we report on the immunological comparison of component I proteins of fast and slow-growing rhizobia by the use of radioimmunoassays. We have used this technique because comparative immunological analyses of proteins from different organisms have been useful in asserting taxonomic relationships [7,8] and only small amounts of proteins are required in this procedure.

2. MATERIALS AND METHODS

2.1. *Growth conditions*

The following combinations of fast-growing rhizobia with legumes were cultured: *Rhizobium leguminosarum* (PRE) – *Pisum sativum* (var. Rondo); *R. meliloti* (KA145) – *Medicago sativa* (var. du Puits); *R. phaseoli* (KA47) – *Phaseolus vulgaris* (var. Walcherse witte); *R. lupini* (GK82) – *Ornithopus sativus*. The combinations of slow-growing rhizobia with legumes were: *R. japonicum* (SM), *Rhizobium spp.* (32H1) or *Rhizobium spp.* (CB756) with *Vigna unguiculata* Walp (var. Blackeye Early Ramshorn); *R. lupini* (RCR3211) – *Ornithopus sativus*. *P. sativum* was grown as described before [9] at 18°C. The other legumes were cultivated and nodulated essentially under the same conditions, but at 24°C. Bacteroids of the different rhizobia were isolated from root nodules as described before [9]. *Klebsiella pneumoniae* was cultured as described by Eady et al. [10].

2.2. *Nitrogenase purification*

Bacteroids and *K. pneumoniae* were lysed anaerobically with a French press. Component I of nitrogenase was purified by chromatography on DEAE cellulose (DE 52, Whatman) and Ultrogel ACA 34 (LKB) as described before [11]. Nitrogenase component I of *Azotobacter vinelandii* was a generous gift from G. Scherings [1]. For the com-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

ponent I proteins of the different organisms the following abbrevations are used: Rle1, Rp1, Rm1, Rlu1, Rj1, Rspp1, Kp1 and Av1 for the component I proteins from respectively *R. leguminosarum, R. phaseoli, R. meliloti, R. lupini, R. japonicum, R.* spp., *K. pneumoniae* and *A. vinelandii*. Kp1 and Rle1 were electrophorectically pure. The nitrogenase component I proteins of the other rhizobia were analysed by polyacrylamide gel electrophoresis and were 70–90% pure, as determined by scanning the gels after staining with Coomassie Brilliant Blue [9].

2.3. *Radioimmunoassays*

Specific antisera against Kp1 and Rle1 were raised in rabbits [11] and radioimmunoassays for these two proteins were performed as described previously [11]. Iodinated Rle1 or Kp1 (20 μg) and 1 μl of Rle1 or Kp1 antiserum was used in each assay. The final volume was made up to 1 ml with RIA buffer: 0.05 M phosphate (pH 7.5), 0.85% (w/v) NaCl, 0.2% (w/v) bovine serum albumin, 0.01% (w/v) thimerosal, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate. Homologous or heterologous CI was added and the assay mixture was incubated overnight at 4°C. Component I–antibody complexes were separated from unbound component I by precipitation with protein A Sepharose CL-4B (Pharmacia). The pellet after centrifugation (1 min, 10 000 × *g*) was washed 3 times with RIA buffer. The pellet was suspended in RIA buffer and radioactivity was quantified with Hydroluma (Lumac, Basel) in a Packard 2450 liquid scintillation spectrometer. The abilities of the component I proteins of the different rhizobia to inhibit binding of [^{125}I]Rle1 or [^{125}I]Kp1 to their homologous antibodies was determined and considered to represent the degree of homology between that component I and Rle1 or Kp1. Protein was assayed by the Lowry method [12] with bovine serum albumin as standard.

3. RESULTS

The immunological relationship between nitrogenase component I proteins of different *Rhizobium* species was studied with a radioimmunoassay specific for Rle1. The ability of component I proteins isolated from fast and slow-growing rhizobia, to inhibit the binding of [^{125}I]Rle1 are

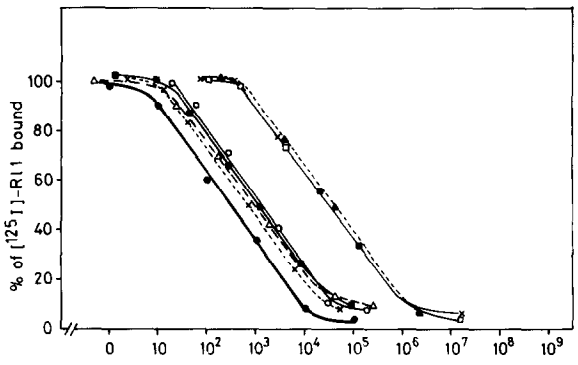

Fig.1. Inhibition of [^{125}I]Rle1 binding by anti-Rle1-serum as a function of amount of non-radioactive CI added. (•—•) Rle1, (△—△) Rp1, (○—○) Rm1, (×---×) Rlu1 (GK82), (▪—▪) Rlu1 (RCR 3211), (▲—▲) Rj1, (×—×) *R.* spp. (CB 756), (□—□) *R.* spp. 1 (32H1).

shown in fig.1. The component I proteins of the fast (Rm1, Rp1, Rlu1 (GK82)) as well as the slow growing rhizobia (Rj1, Rspp1, Rlu1 (RCR 3211)) tested, all were able to inhibit binding of [^{125}I]Rle1 completely. This indicates that these component I proteins are able to compete with Rle1 for all the binding sites on the anti-Rle1. However, there is a large difference in the binding affinity of the different proteins. Component I proteins of all fast-growing rhizobia show an affinity for anti-Rle1 which is only slightly lower than that of Rle1; only about twice as much component I protein of fast-growing rhizobia was required to cause an equal decline in the percentage of [^{125}I]Rle1 bound as with Rle1. In contrast about 200-fold larger quantities of component I proteins from slow-growing rhizobia were necessary to effect the same degree of competition. Of the slow-growing rhizobia only Rlu1 (RCR 3211) was exceptional in that it has a competitive ability against Rle1 similar to that found for fast-growing rhizobia.

Also the immunological relationship between Rle1 on one hand and Kp1 and Av1 on the other was determined. Figure 2 shows that Kp1 has the ability to inhibit the binding of Rle1 completely, while Av1 is only able to inhibit 80% of this binding. Therefore Av1 is the only component I protein tested which is not capable of blocking out all the binding sites on Rle1. Figure 2 shows that 200

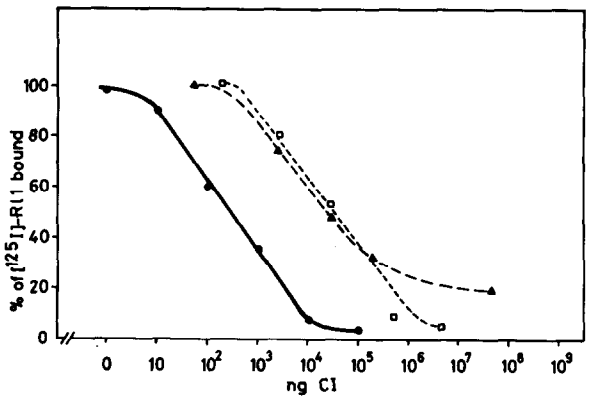

Fig.2. Inhibition of [^{125}I]Rle1 binding by anti-Rle1-serum as a function of amount of non-radioactive Cl added. (•—•) Rle1, (□—□) Kp1, (▲—▲) Av1.

times more Kp1 or Av1 than Rle1 is necessary to effect the same inhibition of binding of [^{125}I]Rle1 to anti-Rle1. This means that the component I proteins of slow-growing rhizobia differ as much from Rle1 as Kp1 and Av1 do. Therefore we determined whether component I of slow-growing rhizobia is more related to Kp1 than to Rle1. The ability of component I from different rhizobia to compete with [^{125}I]Kp1 for binding to anti-Kp1 was determined. Component I proteins of fast- as well as slow-growing rhizobia had a similar low affinity for anti-Kp1. In all cases (result not shown) about 200 times more *Rhizobium* component I was required than Kp1 to effect an equal inhibition of binding of labeled [^{125}I]Kp1 to anti-Kp1.

4. DISCUSSION

The present results show that component I proteins from four fast-growing rhizobia, Rle1, Rm1, Rp1 and Rlu1 (GK 82) are very similar to each other. This is in contrast to the component I proteins from slow growers, which have a much lower affinity for anti-Rle1, with only Rlu1 (RCR 3211) as an exception. *R. lupini* (RCR 3211) is a slow-growing *Rhizobium*, but its component I protein falls within the range of the component I proteins of fast growers, in the radioimmunoassay shown in fig.2. Our results indicate that within the genus *Rhizobium* two different types of nitrogenase component I occur. One type is present in all fast-growers investigated; another is mainly present in slow-growing strains (with the exception of Rlu1 in strain RCR 3211, a slow-growing strain having the 'fast-growing' type component I). We cannot easily explain this exception at present. One might conjecture that *nif*-genes being located on large indigenous plasmids [13–15] can be transferred by conjugation [16] from one *Rhizobium* strain to another and that strain RCR 3211 has acquired *nif* genes from a fast-growing strain in this way. These results confirm that rhizobia of the fast and slow-growing groups are taxonomically not closely related. Our results are also in agreement with the work of Ruvkun and Ausubel [6], who showed that plasmid pRmR1 containing cloned *nif* sequences from *R. meliloti* hybridizes strongly with DNA, from other fast-growing rhizobia (*R. leguminosarum, R. trifolii, R. phaseoli*) but only weakly with DNA from *R. japonicum* and *R.* spp (32H1) (slow growers).

In the competition assay with ^{125}I-labeled Rle1 the component I proteins of *K. pneumoniae* and *A. vinelandii* also show a low degree of competition; this implies that their antigenic structures differ as much from Rle1 as component I proteins of slow-growing rhizobia do. The reciprocal assay in which competition against [^{125}I]Kp1 for anti-Kp1 was tested, proved that all the component I proteins of the rhizobia we have tested only have a low binding affinity for anti-Kp1. Ruvkun and Ausubel [6] showed that a part of *K. pneumoniae nif* DNA hybridized with *nif* DNA sequences of *R. meliloti*. The homologous region is restricted to genes D (component II) and H (subunit of component I) or a part thereof. The extent of divergence between the hybridizing part of *R. meliloti* and *K. pneumoniae nif* DNA is 8–20%. However the other *nif* genes of *K. pneumoniae*, including gene K (subunit of component I) do not hybridize at all with *R. meliloti nif* DNA. This means that there exists a large divergence between the K genes and consequently between the nitrogenase component I proteins of *R. meliloti* and *K. pneumoniae*.

ACKNOWLEDGEMENTS

The authors thank Mr. Houwers for culturing nodulated plants, Mr. Hoogeveen for making the illustrations and Mrs. M.J. van Neerven for typing

the manuscript. We thank the Curator of the Rothamstead Collection of *Rhizobium* for providing strain RCR 3211. This investigation was partly supported by the Netherlands Foundation for Biological Research (BION) and by the Niels Stensen Stichting.

REFERENCES

[1] Scherings, G., Haaker, H. and Veeger, C. (1977) FEBS Lett. 77, 621–630.
[2] Eady, R.R. and Smith, B.E. (1979) in: 'A treatise on dinitrogen fixation' (Hardy, R.W.F., Bottomley, F. and Burns R.C. eds) pp. 401–409, John Wiley, New York.
[3] Burris, R.H., Arp, D.J., Benson, D.R., Emerich, D.W., Hageman, R.V., Ljones, T., Ludden, P.W. and Sweet, W.J. (1980) in: Nitrogen fixation, Proc. Phytochem. Soc. Eur. Symp. (Stewart, W.D.P. and Gallon, J.R. eds) pp. 37–54, Academic Press, London.
[4] Paau, A.S. (1978) J. Theor. Biol. 74, 139–142.
[5] Vincent, J.M. (1974) in: The Biology of Nitrogen Fixation (Quispel, A., ed) pp. 266–341, Elsevier Biomedical, Amsterdam, New York.
[6] Ruvkun, G.R. and Ausubel, F.M. (1980) Proc. Natl. Acad. Sci. USA 77, 191–195.
[7] Hurrell, J.G., Thulborn, K.R., Broughton, W.J., Dilworth, M.J. and Leach, S.J. (1977) FEBS Lett. 84, 244–246.
[8] Metzger, H., Shapiro, M.B., Mosimann, J.E. and Vinton, J.E. (1968) Nature 219, 1166–1168.
[9] Bisseling, T., van den Bos, R.C. and van Kammen, A. (1978) Biochim. Biophys. Acta 539, 1–11.
[10] Eady, R.R., Issack, R., Kennedy, C., Postgate, J.R. and Ratcliffe, H. (1978) J. Gen. Microbiol. 104, 277–285.
[11] Bisseling, T., Moen, A.A., van den Bos, R.C. and van Kammen, A. (1980) J. Gen. Microbiol. 118, 377–381.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Hombrecher, G., Brewin, N.J. and Johnston, A.W.B. (1981) Mol. Gen. Genet. 182, 133–136.
[14] Krol, A.J.M., Hontelez, J.G.J., van den Bos, R.C. and van Kammen, A. (1980) Nucleic Acids Res. 8, 4337–4347.
[15] Rosenberg, C., Boistard, P., Dénarié, J. and Casse-Delbart, F. (1981) Mol. Gen. Genet. 184, 326–333.
[16] Hooykaas, P.J.J., van Brussel, A.A.N., den Dulk-Ras, H., van Slogteren, G.M.S. and Schilperoort, R.A. (1981) Nature 291, 351–353.