625

Doc n° 942.

Vol. 15, No. 3, September 1987 BIOCHEMISTRY INTERNATIONAL

Pages 509-516

Commission Scientifique 4
"Sciences du Monde Végétal"

pas vu dls H B

THE AMINO ACID SEQUENCE OF LEGHEMOGLOBIN II FROM SESBANIA ROSTRATA STEM NODULES

Alexander A. Kortt*, Philip M. Strike*, Didier Bogusz+ and Cyril A. Appleby++

*CSIRO, Division of Protein Chemistry, Parkville (Melbourne), 3052, Australia,

+ORSTOM Dept. F, 75480 Paris, France, and

++CSIRO, Division of Plant Industry, Canberra, 2601, Australia.

Received June 11, 1987

SUMMARY: The complete amino acid sequence of leghemoglobin II from the stem nodules of Sesbania rostrata has been established. The hemoglobin consists of 147 amino acids and has a M_r of 16,283 including the heme group. The homology between Sesbania leghemoglobin and the leghemoglobins from lupin and soybean is 59% and 62% respectively, and the prediction of secondary structure indicates a high content of α-helix (79%). The major structural features of this stem nodule leghemoglobin are very similar to those of the root nodule leghemoglobins from other symbioses.

The tropical legume Sesbania rostrata is unusual insofar as it has nitrogen-fixing stem nodules as well as the normal root nodules (1). These stem nodules contain chloroplasts (1) with consequent potential for oxygen evolution adjacent to the Rhizobium domain (2). While it might have been expected that Sesbania stem nodule symbiosis would operate at higher than usual oxygen concentration and have unique leghemoglobin (Lb) components, we purified Sesbania Lb and showed that stem and root nodules contained the same seven species (2). The major component from stem or root nodules, Sesbania LbII, had the highest oxygen affinity ($K_{diss} \sim 30$ nM) yet recorded for a symbiotic hemoglobin (3). Also, we demonstrated (4) that the isolated Rhizobium bacteroids from Sesbania stem or root nodules, in the presence of Sesbania LbO_2, were capable of respiration-coupled nitrogenase activity at a free oxygen concentration less than 10 nM, which is extremely low.

Hence, it appears that both Sesbania stem nodule and root nodule symbioses operate at lower than usual (4), rather than higher than usual, Lb-buffered free oxygen concentration. This paper reports our determination of Sesbania stem LbII amino acid sequence to discern whether unusual substitutions in or around the heme pocket could account for its extraordinarily high oxygen affinity (3).

Fonds Documentaire IRD
Cote : B*24826 Ex : unique

Fonds Documentaire IRD
010024826

0158-5231/87/090509-08$01.00/0

Copyright © 1987 by Academic Press Australia.
All rights of reproduction in any form reserved.

MATERIALS AND METHODS

Total ferric S. rostrata Lb was isolated from stem nodules and separated into seven components by chromatography on DEAE-Sephacel as described previously (2). The major component, Sesbania LbII, was judged to be homogeneous by analytical gel electrophoresis after rechromatography on DEAE-Sephacel as the nicotinate complex (2) and was used for the sequence determination.

Separate aliquots of Sesbania LbII (40 nmoles) were dissolved in 70% (v/v) formic acid and after 2h at 37°C were dried under vacuum at 50°C. This treatment enhanced the subsequent enzymatic digestion of the protein. The protein aliquots were dissolved in 0.5 ml 0.1M ammonium bicarbonate, adjusted to pH 8.0, and digested with trypsin (Worthington), α-chymotrypsin (Worthington) or Staphylococcus aureus V8 protease (Pierce) at 37°C for 4h at an enzyme:substrate ratio of 1:50 (w/w). The digests were dried under vacuum at 50°C, dissolved in 0.25 ml 0.1% (v/v) trifluoroacetic acid and the soluble peptides separated by RP-HPLC on a Vydac 218 TP54 column (4.6 x 250 mm) using a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Peptides which co-eluted or were poorly resolved were purified by rechromatography under optimized conditions.

The amino-terminal sequence of the intact protein and the sequences of several longer peptides were determined with a gas-phase sequencer (Applied Biosystems, U.S.A.). Most of the peptides isolated from the enzymatic digests were sequenced manually by a modified (5) Edman technique (6). PTH-amino acids were identified after separation by RP-HPLC as described (7). The carboxy-terminal residues of the intact protein were determined by analysis after digestion with carboxypeptidase Y (1% (w/w)) in 0.1M pyridine-acetate buffer, pH 5.6, at 37°C for 1h and 2h.

The amino acid composition of Sesbania LbII was determined after hydrolysis in 6M HCl (24h, 48h and 72h) at 110°C or in 4M methane sulfonic acid - 0.2% (w/v) tryptamine (24h) at 115°C under vacuum. Peptides were hydrolysed under vacuum in 0.2 ml of 6M HCl (24h) at 110°C. Analyses were performed on a Waters amino acid analyser.

RESULTS AND DISCUSSION

The amino-terminal sequence of intact Sesbania LbII (residues 1-27) was determined using the gas-phase sequencer. The complete amino acid sequence of Sesbania LbII was deduced after manual sequencing of the peptides derived from tryptic, chymotryptic and staphylococcal protease digests as illustrated in Fig. 1. The tryptic peptides, isolated by RP-HPLC, accounted for the complete structure of Sesbania LbII. Alignment of these tryptic peptides was established with overlapping chymotryptic and staphylococcal protease peptides as shown in Fig. 1. Although C11 provided only a single residue overlap between T7 and T8, the placement of these two peptides is consistent with the homology comparisons with other leghemoglobins (Fig. 2). Serine was identified as the carboxy-terminal residue from sequence data and amino acid composition of peptides T13, C19 and S9.

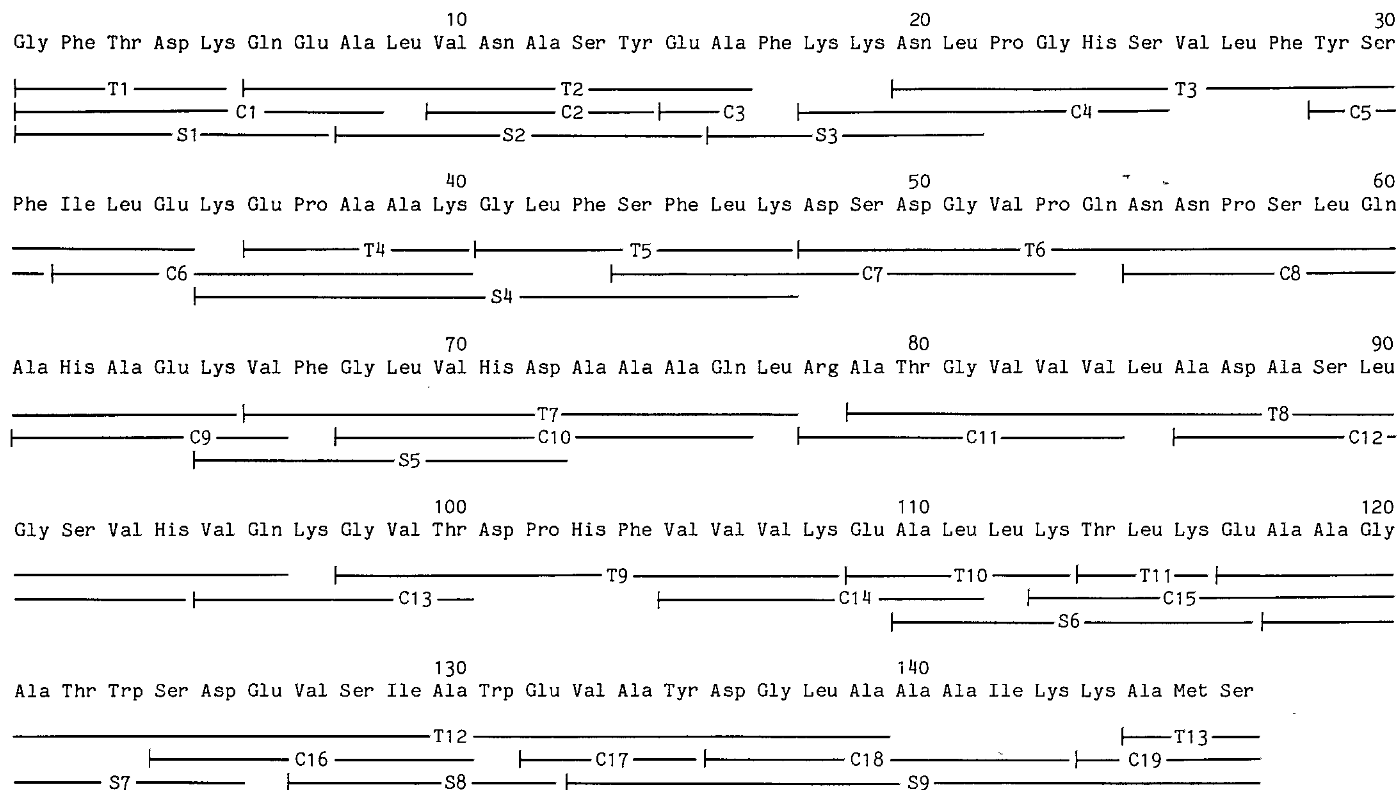

Figure 1. Amino acid sequence of Sesbania LbII. The vertical bar indicates the amino-terminus of the peptide and the residues sequenced are indicated by the solid line. Peptides are numbered from the N-terminus of the protein where T,C and S signify tryptic, chymotryptic and staphylococcal protease peptides, respectively.

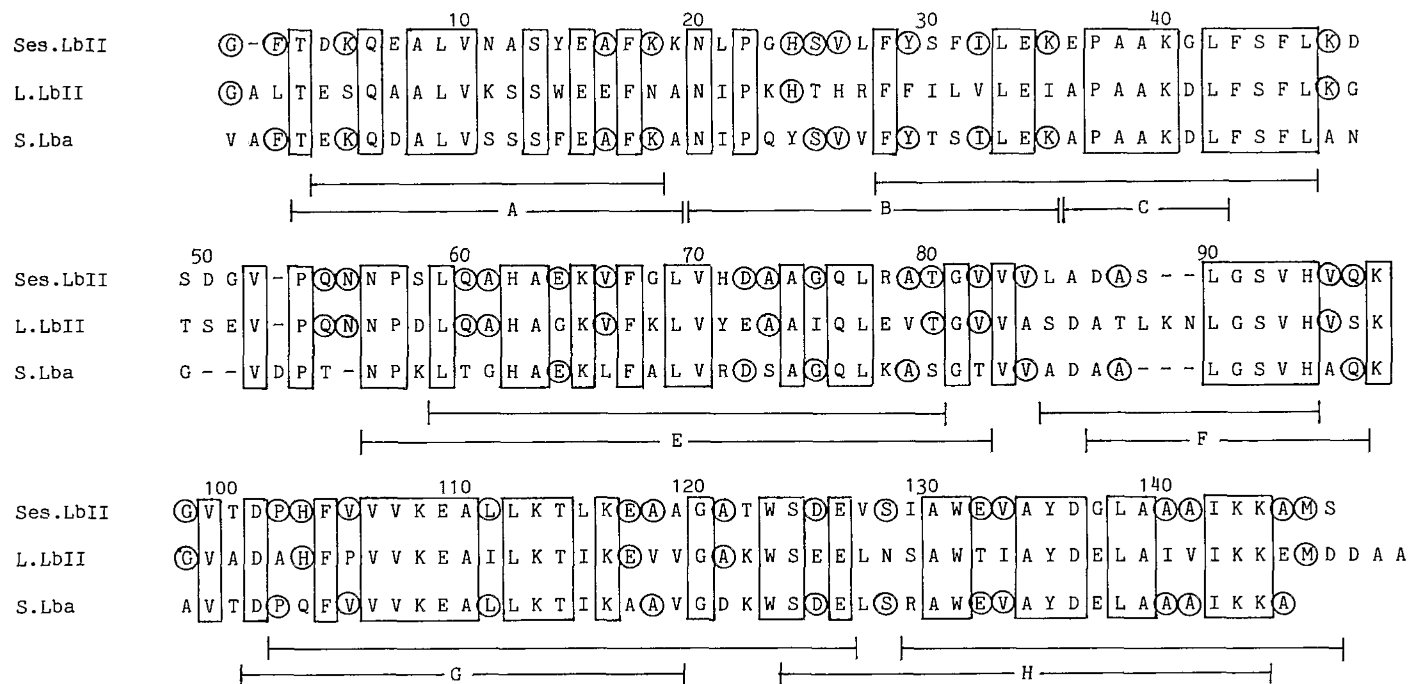

Figure 2. Comparison of the amino acid sequences of Sesbania LbII, lupin LbII (8) and soybean Lba (9) to illustrate the extent of homology. Residues identical in the three proteins are boxed and residues identical in Sesbania LbII and one of the other leghemoglobins are ringed. (----) deletions introduced to maximize homology. (├──┤) upper designates the predicted helical regions in Sesbania LbII and (├──┤) lower designates the known helices in soybean and lupin Lb from X-ray crystallographic studies (17,18).

Carboxypeptidase Y digestion of the intact protein did not release a major residue; however, a comparison of 1h and 2h digests showed an increase in the yields of Ser, Met and Ala released consistent with the C-terminal sequence deduced from the peptide data.

Separation of the tryptic peptides by RP-HPLC yielded a number of peptides with the same amino-terminal sequence. Incomplete cleavage of the Lys(5)-Gln(6) and Lys(18)-Lys(19) bonds yielded 3 well resolved peptides from the amino-terminal region of the protein. In addition several unusual cleavages were noted with trypsin: a major cleavage site occurred at Phe(31)-Ile(32) and a minor site at Phe(43)-Ser(44). The Lys(35)-Glu(36) bond was only partially cleaved and thus the Phe(31)-Ile(32) cleavage produced a major peptide overlap for T3 and T4. Some unexpected cleavage sites were also found in the chymotryptic digests of Sesbania LbII. Cleavages were obtained at Gln(54)-Asn(55) and His(94)-Val(95) with minor cleavages at Gln(60)-Ala(61) and Lys(143)-Lys(144). No hydrolysis was observed at several chymotrypsin specific residues e.g. Phe(2)-Thr(3), Tyr(29)-Ser(30) and Leu(59)-Gln(60). These cleavages were confirmed by the composition of the isolated peptides.

The sequence determination showed that Sesbania LbII contains 147 amino acid residues which together with the heme group corresponds to a M_r of 16283 in close agreement with estimates of M_r from gel electrophoresis and chromatography (2). Also, the sequence composition is in close agreement with the amino acid composition of the protein (Table 1). In Fig. 2 the sequence of Sesbania LbII is compared with the sequence of lupin LbII (8) and soybean Lba (9). Sesbania LbII contains 4 more residues than soybean Lba and 4 fewer residues than lupin LbII. Maximum homology was obtained with an alignment containing 4 deletions in Sesbania LbII, 1 deletion in lupin LbII and 6 deletions in soybean Lba (Fig. 2). In this alignment the three Lb's show 46% identity and when compared individually the homology between Sesbania LbII and lupin LbII or soybean Lba is 59% and 62%, respectively.

Sesbania LbII has an amino-terminal glycine which is a common feature of soybean Lb components C_1, C_2, C_3 (9), broad bean Lb (10), green pea LbI (11) and kidney bean LbI (12). Six of the seven Sesbania Lb components (2) contain the amino-terminal sequence, Gly-Phe-Thr, with component IV having the sequence Gly-Ala-Phe as found for kidney bean Lb (11). In contrast soybean Lba (9) and Vigna sesquipedalis LbI (13) have an amino-terminal valine while in the non-legume plant hemoglobins,

Table 1. Amino Acid Composition of Sesbania LbII

Amino acid	Analysis	Sequence
Lys	12.9	13
His	4.9	5
Arg	0.9	1
Asx	11.6	12
Thr	4.8	5
Ser	10.2	11
Glx	14.9	14
Pro	5.2	5
Gly	9.4	10
Ala	22.3	23
Cys($\frac{1}{2}$)	0.0	0
Val	14.9	16
Met	0.9	1
Ile	3.0	3
Leu	14.2	15
Tyr	2.9	3
Phe	7.9	8
Trp	1.6	2

Data are expressed as residues/molecule

Casuarina hemoglobin (14) has lost the N-terminal glycine residue and the Parasponia hemoglobins (15) have an additional six residue at the amino terminus. Four of the five histidine residues in Sesbania LbII are also present in homologous positions in lupin LbII (8) and Casuarina hemoglobin (10); the single methionine residue in the C-terminal region of the Sesbania LbII sequence is homologous to the single methionine found in lupin LbII. Broad bean leghemoglobin contains two methionine residues but they are located in the middle of the molecule with Met(69) (10) corresponding to a methionine found in this position in the hemoglobin from the nodules of Parasponia andersonii, a non-legume (15), which contains five methionine residues.

The secondary structure predicted from the amino acid sequence (16) indicated that the Sesbania LbII contained considerable α-helix (79%). The predicted helical regions correlated well with the known helical regions of soybean (17) and lupin (18) Lb's determined by X-ray crystallographic studies. However, the method (16) did not predict part of the B-helix for Sesbania LbII as found previously (18) in the prediction of the soybean Lba and lupin LbII secondary structures.

Furthermore, residues involved in the interactions with the heme group and between helices have been conserved indicating that the major structural features of Sesbania stem nodule LbII are very similar to those of the root nodule leghemoglobins of other symbioses. Hence, very subtle structural changes must be responsible for the higher oxygen affinity of Sesbania Lb as compared with other symbiotic leghemoglobins (3).

ACKNOWLEDGEMENTS

We thank J.E. Burns for technical assistance, and L. Whittaker for amino acid analyses and the HPLC analyses of PTH amino acids.

REFERENCES

1. Dreyfus, B. and Dommergues, Y.R. (1981) FEMS Microbiol. Lett. 10, 313-317.
2. Bogusz, D., Kortt, A.A. and Appleby, C.A. (1987) Arch. Biochem. Biophys. 254, 263-271.
3. Wittenberg, J.B., Wittenberg, B.A., Trinick, M.J., Gibson, Q.H., Fleming, A.I., Bogusz, D. and Appleby, C.A. (1985) In Nitrogen Fixation Research Progress (H.J. Evans, P.J. Bottomley and W.E. Newton, eds.), p.354, Martinus Nijhoff. Dordrecht.
4. Bergersen, F.J., Turner, G.L., Bogusz, D., Wu, Y.-Q. and Appleby, C.A. (1986) J. Gen. Microbiol. 132, 3325-3336.
5. McKern, N.M., O'Donnell, I.J., Inglis, A.S., Stewart, D.J. and Clarke, B.L. (1983) FEBS Lett. 164, 149-153.
6. Petersen, J.D., Nehrlich, S.D., Oyer, P.E. and Steiner, D.F. (1972) J. Biol. Chem. 247, 4866-4871.
7. Woods, E.F. and Inglis, A.S. (1984) Int. J. Biol. Macromol. 6, 277-283.
8. Egorov, T.A., Kazakor, V.K., Shakhparonov, M.I. and Feigina, M.Y. (1980) Bioorg. Chem. 6, 666-683.
9. Hyldig-Nielsen, J.J., Jensen, E.O., Paludan, K., Wilborg, O., Garret, R., Jorgensen, P. and Marker. (1982) Nucleic Acids Res. 10, 689-701.
10. Richardson, M., Dilworth, M.J. and Scawen, M.D. (1975) FEBS Lett. 51, 33-37.
11. Lehtovaara, P., Lappalainen, A. and Ellfolk, N. (1980) Biochim. Biophys. Acta 623, 98-106.
12. Lehtovaara, P. and Ellfolk, N. (1975) Eur. J. Biochem. 54, 577-584.
13. Dilworth, M.J. and Appleby, C.A. (1979) In A Treatise on Dinitrogen Fixation. (R.W.F. Hardy, F. Bottomley and R.C. Burns, eds.), Section I and II, pp.691-764. Wiley, New York.
14. Fleming, A.I., Appleby, C.A., Dudman, W.F., Kortt, A.A., Wittenberg, B.A. and Wittenberg, J.B. (1986) in AIAS Occasional Publ. No. 25. The Eighth Australian Nitrogen Fixation Conference (W. Wallace and S.E. Smith, eds.) pp.85-86, Aust. Inst. Agric. Sci., Sydney.
15. Landsmann, J., Dennis, E.S., Higgins, T.J., Appleby, C.A., Kortt, A.A. and Peacock, W.J. (1986) Nature 324, 166-168.

16. Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. 120, 97-120.
17. Ollis, D.L., Appleby, C.A., Colman, P.M., Cutten, A.E., Gus, J.M., Venkatappa, M.P. and Freeman, H.C. (1983) Aust. J. Chem. 36, 451-468.
18. Haratyunyan, E.G., Kuranova, I.P., Vainshtein, B.K. and Steigemann, W. (1980) Kristallografya 25, 80-103.
19. Kortt, A.A., Burns, J.E., Trinick, M.J. and Appleby, C.A. (1985) FEBS Lett. 180, 55-60.