Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 552-558

DETERMINATION OF THE BLOCKED N-TERMINAL OF SOYBEAN LEGHEMOGLOBIN *b*

R.G. Whittaker*, B.A. Moss+ and C.A. Appleby#

*School of Biochemistry, University of New South Wales, Kensington, N.S.W., Australia
+CSIRO, Molecular and Cellular Biology Unit, North Ryde, N.S.W., Australia
#CSIRO, Division of Plant Industry, Canberra, A.C.T., Australia

Received June 11,1979

SUMMARY: Soybean leghemoglobins a and b were compared by microscale peptide mapping after heme removal with acid-acetone. Maps generated by trypsin or the combined action of trypsin and thermolysin indicated a large amount of homology between the proteins with the only variations detected being the N-terminal peptides. The N-terminal tryptic peptide of leghemoglobin b was found to be both blocked and to lack the first amino acid of the corresponding leghemoglobin a peptide. Nuclear magnetic resonance and gas chromatography/mass spectroscopy studies showed that the N-terminal of leghemoglobin b was N-acetyl-alanine. It is possible that leghemoglobin b arises from leghemoglobin a by a two-stage modification involving cleavage of the N-terminal valyl residue and subsequent acetylation of the exposed alanyl residue.

INTRODUCTION: Soybean ferric leghemoglobin (1) may be partly separated by anion exchange chromatography (2,3) or completely separated by isoelectric focusing (4) into the major components Lba, Lbc_1, Lbc_2, Lbc_3 and minor components Lbb, Lbd_1, Lbd_2, Lbd_3. The proportions of Lba and the subcomponents of Lbc vary dramatically with nodule age, whereas the ratios of Lba to Lbb, and of each subcomponent of Lbc to the corresponding subcomponent of Lbd, are almost independent of nodule age (4). This led Fuchsman and Appleby (4) to the conclusion that Lba and Lbb might be related biosynthetically, as might the leghemoglobins c and leghemoglobins d. On the other hand, following Lb biosynthesis studies *in vitro*, Verma *et al*. (5) concluded that Lbb was an artifact of protein isolation having heme more tightly bound than in Lba. Because of this disparity, and also because of controversy about the necessity for Lb or its apoprotein to be secreted through a plant cell

Abbreviations: Lb, soybean ferric leghemoglobin; DSS, 2,2-dimethyl-2-silapentane sulfonate; NMR, nuclear magnetic resonance; GC/MS, gas chromatography/mass spectroscopy.

0006-291X/79/140552-07$01.00/0

membrane (5,6) we undertook the characterization of Lbb, in relation to Lba whose amino acid sequence is known (7,8). We considered the possibilities that Lbb might represent a remnant or precursor of Lba containing a signal peptide (9), or acetylated N-terminal (10), or with other modification for facilitation of Lb passage through a plant cell membrane.

MATERIALS AND METHODS: Soybean Lba and Lbb were separated by chromatography on Whatman DE52 cellulose (3). The minor component, Lbb, was further purified by rechromatography on an 86 cm column of DE52 at constant ionic strength (22 mM sodium acetate, pH 5.17). Only the best fractions of Lbb with $A_{403_{nm}}/A_{273_{nm}} > 5.3$ (as obtained for Lba during the first cycle of chromatography) were pooled, concentrated by ultrafiltration over an Amicon UM10 membrane, and used for analysis.

The protoheme was completely removed from both Lba and Lbb by acid-acetone at -20°C (11). It was not necessary to use the harsher silver sulfate procedure at 60°C to remove heme from Lbb, as claimed by Verma et al. (5). The colorless globin precipitates were freeze-dried, dissolved in 1% NH_4HCO_3, pH 8.7, at 1 mg/ml and digested with 10 µg trypsin/ml for 3 hours at 37°C. Thermolytic digestions were for 16 hours, using the same buffer and conditions.

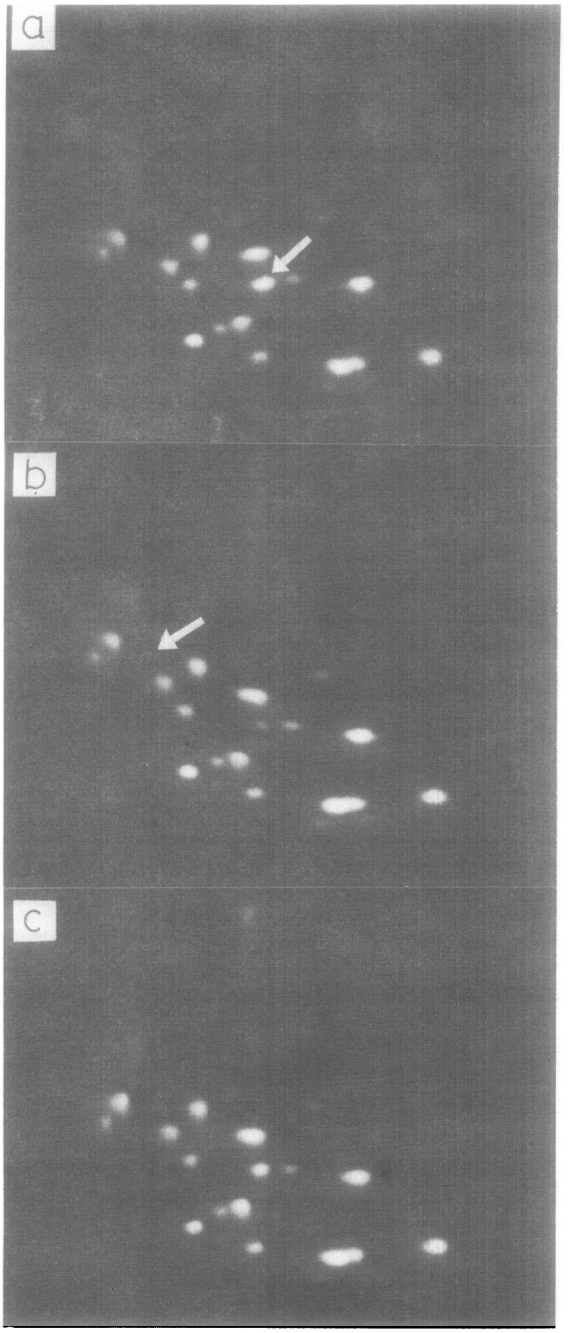
Two dimensional peptide maps (12) were run on cellulose-coated plastic sheets (Macherey-Nagel MN 300). 1 to 2 nanomoles of digested globin were mapped using electrophoresis at pH 3.5 in pyridine:acetic acid:water (1:10:89) as the first dimension and chromatography in butanol:pyridine:acetic acid: water (150:100:3:100) in the second. Plates were washed with this latter solvent prior to use. Peptides were detected by spraying with 0.01 mg/ml fluorescamine (Roche) in acetone (13) and photographed through an orange filter (HOYA O [G]) under ultra-violet illumination.

Peptide zones were scraped from the plates and transferred to glass tubes (2.5 mm x 75 mm) plugged with glass fiber filter paper for elution with 60% pyridine. Samples for analysis were hydrolysed in 6N HCl for 24 hours at 110°C and run on a Beckman 121M analyser. Dansyl determinations of the N-terminal residues (14) used peptides eluted from maps lightly stained with 0.001 mg/ml fluorescamine in acetone (13).

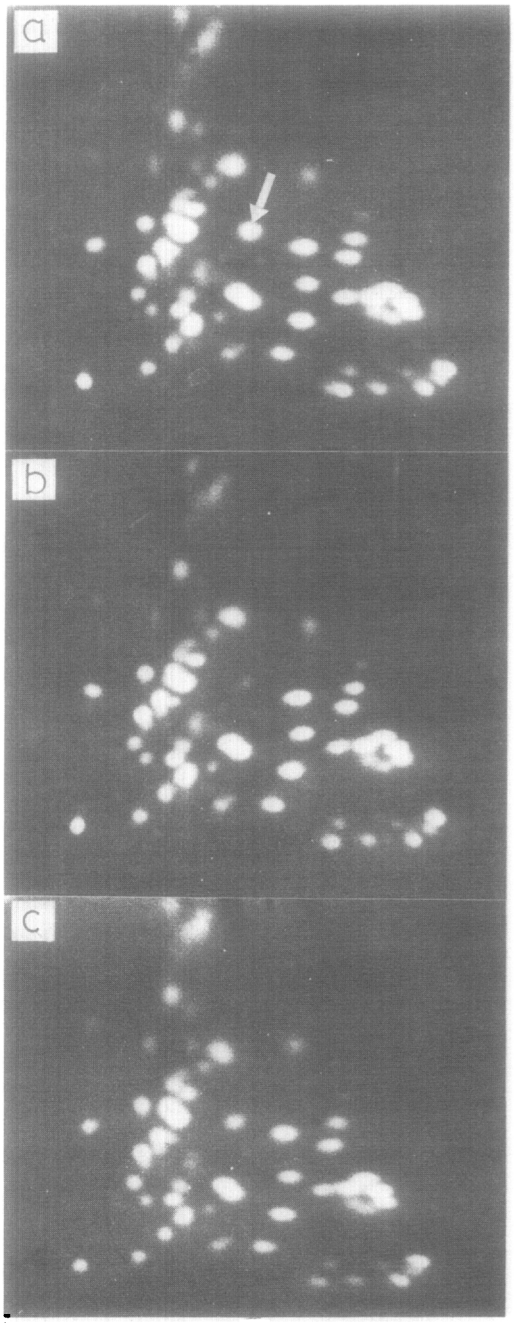
Samples for analysis of the blocked N-terminal were prepared by fractionation of a thermolytic digest of 2mg of Lbb. After freeze-drying, the digest was dissolved in water, loaded, and eluted with water from a 1 ml column of sulfonated polystyrene resin (Bio-Rad AG 50W-X2, 200-300 mesh, in the acid form).

Proton NMR spectra of the non-binding fraction were recorded on a JEOL 99.6 MHz JNM-FX100 spectrometer in the Fourier Transform mode using 1.7 mm O.D. tubes in a JEOL dual $^{1}H/^{13}C$ mini-probe; pulse width was 36 µs (90°) with a pulse repetition of 5 seconds. Chemical shifts are reported downfield of the sodium salt of DSS.

Chemical ionisation mass spectra on samples methylated by treatment with ethereal diazomethane were recorded with a Finnigan Model 3200 GC/MS system interfaced to a Finnigan Model 6115 Data System. GC separations were accom-



(1)



(2)

FIGURE 1: Micro-peptide maps of the tryptic peptides of (a) Lb$\underline{a}$, (b) Lb$\underline{b}$ and (c) equal amounts of Lb$\underline{a}$ and Lb$\underline{b}$. Electrophoresis is in the horizontal direction and ascending chromatography in the vertical. Arrows indicate the variant peptides.

FIGURE 2: Micro-peptide maps of the peptides generated by combined tryptic and thermolytic digestion. (a) Lb$\underline{a}$, (b) Lb$\underline{b}$ and (c) equal amounts of Lb$\underline{a}$ and Lb$\underline{b}$. The arrow indicates the N-terminal dipeptide (Val-Ala) of Lb$\underline{a}$.

TABLE 1

AMINO ACID ANALYSES OF VARIANT TRYPTIC PEPTIDES FROM Lba and Lbb

AMINO ACID	Lba	Lbb
Lysine	1.0(1)	1.0(1)
Aspartic Acid	0.2	-
Threonine	1.0(1)	1.0(1)
Serine	0.1	0.1
Glutamic Acid	1.1(1)	1.1(1)
Glycine	0.3	0.1
Alanine	1.0(1)	1.0(1)
Valine	1.0(1)	-
Phenylalanine	0.9(1)	1.0(1)

Samples were hydrolysed in 6N HCl for 24 hrs at 110°C. Results are expressed as moles per mole of peptide.

plished using Tabsorb (0.65% EGA on Chromosorb W AW, 80/100 mesh - Supelco) in glass columns (2mm I.D. x 1.5 m).

RESULTS: Micro-peptide maps of tryptic digests of Lba and Lbb gave similar patterns with only one peptide variation being noted (Figure 1). The map of Lbb contained only trace amounts of one strongly staining Lba peptide (which is attributed to cross contamination of the Lbb preparation by a small amount of Lba). In addition the Lbb map contains an extra peptide of weak intensity (arrowed); this peptide showed more clearly when the fluorescamine-stained map was oversprayed with ninhydrin (0.2% in 95% ethanol). The Lba peptide has a composition consistent with the reported sequence of the first six residues of Lba (7), (Table 1). The unique Lbb peptide gave a similar analysis with the exception that it lacked the reported N-terminal valyl residue. Valine was confirmed as the N-terminal residue of the Lba peptide by dansylation (14).

Maps of peptides from combined tryptic - thermolytic digestion showed only one difference (Figure 2). The unique Lba peptide contained only valine

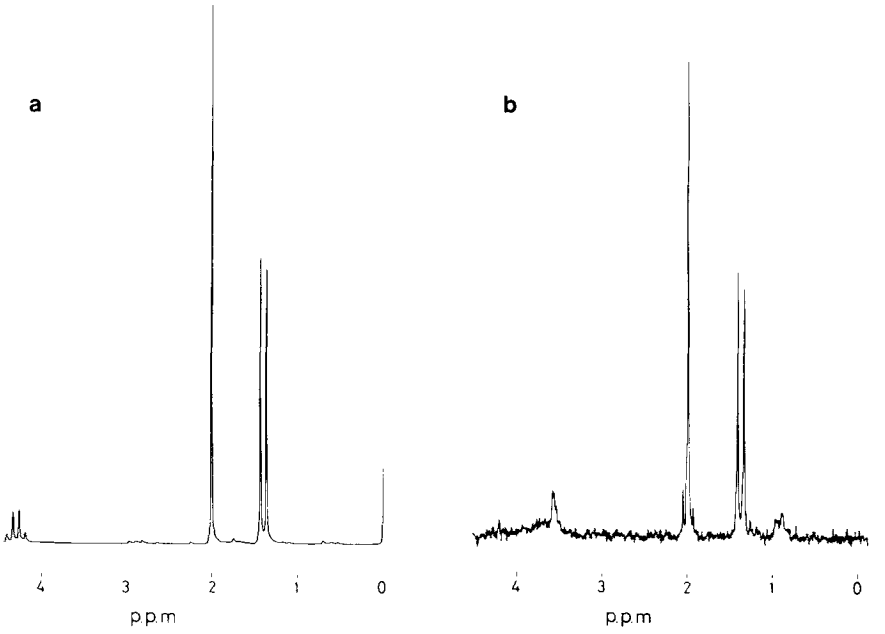


FIGURE 3: 99.6 MHz proton nuclear magnetic resonance spectra of (a) N-acetyl-alanine (20 mg/ml in ^2H_2O, containing DSS, 0.1 mg/ml; and (b) blocked N-terminal of leghemoglobin b (approximately 10 µg in 20 µl of ^2H_2O).

and alanine, corresponding to the known N-terminal sequence of Lba (7). Failure to detect a variant spot in the Lbb map suggested that the N-terminal peptide of Lbb was blocked by chemical modification.

A thermolytic digest of Lbb was fractioned on sulfonated polystyrene and the non-binding fraction analysed by NMR and GC/MS. Both procedures (Figures 3 and 4 respectively) showed the presence of N-acetyl-alanine.

DISCUSSION: Combined tryptic and thermolytic digestion, which gives rise to tri- or tetrapeptides on average, resulted in complete solubilization of Lba and Lbb. Amino acid differences in small peptides are readily detectable by our mapping procedure, especially when a map spotted with equal amounts of both digests is used to check for small mobility changes. In practice, synthetic tripeptides varying by one amino acid can be easily resolved, and in

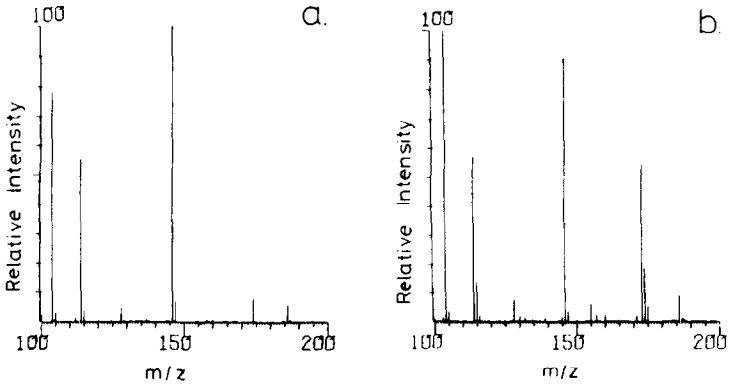


FIGURE 4: Chemical ionisation mass spectra of (a) N-acetyl-alanine and (b) the blocked N-terminal of Lbb.

some cases it is possible to distinguish between peptides of similar composition but different sequence (12).

It has been suggested (5) that Lbb is an artifact, derived from Lba by binding of a strong ligand to the heme. We disagree, because protoheme is easily removed from Lbb by the standard acid-acetone treatment (cf. MATERIALS AND METHODS) and because Lbb and Lba have similar spectral properties and affinities for ligands such as nicotinic acid (3). Our evidence suggests that Lba and Lbb vary only in N-terminal sequence and, while the two proteins could be separate gene products, it would seem more probable that Lba gives rise to Lbb by post-transcriptional modification, involving cleavage of the N-terminal valyl residue of Lba followed by acetylation of the new N-terminal alanyl residue. We can detect no other difference between Lba and Lbb, and R.A. Davey and W.F. Dudman (personal communication), using procedures which gave positive results with small samples of seed proteins (15), were unable to obtain any evidence of Lba or Lbb glycosylation. It is most unlikely that Lbb is a precursor of Lba. Verma et al (5) show that, during the in vitro translation of Lb mRNA, the nascent peptide of apo-Lba contains methionine as initiator followed by valine. They assume that methionine is cleaved from the nascent chain, leaving an N-terminal valyl residue on apo-Lba.

The loss of the original N-terminal residue followed by acetylation is an unusual event and we do not yet understand its significance, particularly as the parent molecule retains a free α-amino group and remains the major species. A somewhat similar pattern is found with fetal hemoglobins where the minor component F_1 is identical to the major F_0 component except for acetylation of the gamma globin chains (16), this modification, however does not involve prior cleavage of the N-terminal residue.

Preliminary results of mappings of digests of the other Lb major and minor components; Lbc (comprising Lbc_1, Lbc_2, Lbc_3) and Lbd (comprising Lbd_1, Lbd_2, Lbd_3), are also consistent with Lbd's arising from Lbc's by N-terminal modification. It will be of interest to compare the isolated individual components, as during isoelectric focusing (4) the Lbd components show a similar relationship to the Lbc components as Lbb does to Lba.

ACKNOWLEDGEMENTS: The authors wish to thank Ms. J. Meers for expert technical assistance and Professor E.O.P. Thompson for helpful discussion and support. We are indebted to Mr. G. Grossman and Dr. K. Barrow for NMR spectroscopy, Dr. A. Duffield for GC/MS analysis and Drs. R.A. Davey and W.F. Dudman for carbohydrate analyses. R.G. Whittaker is supported by a grant from CSIRO.

REFERENCES:

1. Appleby, C.A. (1974) in The Biology of Nitrogen Fixation, Quispel, A. Ed., pp. 521-554, North Holland Publ. Co. Amsterdam.
2. Ellfolk, N. (1960) Acta. Chem. Scand. 14, 609-616.
3. Appleby, C.A., Nicola, N.A., Hurrell, J.G.R., and Leach, S.J. (1975) Biochemistry 14, 4444-4450.
4. Fuchsman, W.H., and Appleby, C.A. (1979) Biochim. Biophys. Acta, in press
5. Verma, D.P.S., Ball, S., Guérin, C., and Wanamaker, L. (1979) Biochemistry, 18, 476-483.
6. Goodchild, D.J.G. (1977) in Intl. Review of Cytology Supplement 6, Studies in Ultrastructure, Bourne, G.H. et al., Eds., pp. 235-288, Academic Press, New York.
7. Ellfolk, N., and Sievers, G. (1971) Acta. Chem. Scand. 25, 3532-3534.
8. Ellfolk, N., and Sievers, G. (1974) Acta Chem. Scand. B. 28, 1245-1246.
9. Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67, 852-862.
10. Palmiter, R.D., Gagnon, J., and Walsh, K.A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 94-98.
11. Anson, M.L., and Mirsky, A.E. (1930) J. Gen. Physiol. 13, 469-476.
12. Whittaker, R.G., and Moss, B.A. (1979) In preparation.
13. Vandekerckhove, J., and Van Montagu, M. (1974) Europ. J. Biochem. 44, 279-288.
14. Gray, W.R. (1967) in Methods of Enzymology, Hirs, C.H.N., Ed., Vol. 11, pp. 469-475, Academic Press, New York.
15. Davey, R.A., and Dudman, W.F. (1979) Aust. J. Plant Physiol., in press.
16. Stegink, L.D., Meyer, P.D. and Brummel, M.C. (1971) J. Biol. Chem., 246, 3001-3007.