GLYCOPROTEIN NATURE OF ENERGY-TRANSDUCING ATPases

Chemical characterization of glycopeptides isolated from bacterial and chloroplast coupling factors

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1. Introduction

Evidence to support the notion that the ATPases or F_1 coupling factors from bacteria and organelles are glycoproteins has been presented [1,2]. The significance of this finding with regard to the peripheral location of these membrane proteins as well as to their role as phosphorylating coupling factors have been discussed [2]. A further characterization of the glycoprotein nature of these proteins has been attempted by extensive digestion with pronase and isolated glycopeptides by Sephadex filtration. This report describes the quantitative and qualitative characterization of these glycopeptides from *Micrococcus lysodeikticus* and chloroplast ATPase. The results further support to the idea that these coupling factors are glycoproteins.

2. Materials and methods

2.1. *ATPase preparations*

The ATPase (form B) from *Micrococcus lysodeikticus* (BF_1) was isolated and purified by polyacrylamide gel electrophoresis as in [3]. Crude coupling factor from spinach chloroplasts (CF_1) was prepared as in [4] and purified by preparative gel electrophoresis. Purified BF_1 and CF_1 were highly homogenous (> 98%) as judged by analytical gel electrophoresis (fig.1 and [3]). Purified CF_1 had no detectable tryptophan fluorescence and had an ATPase activity of 35 μmol substrate transformed/min/mg protein if assayed as in [4]. Protein was estimated by as in [5] or by ultraviolet absorption from the molar extinction coefficients [6,7].

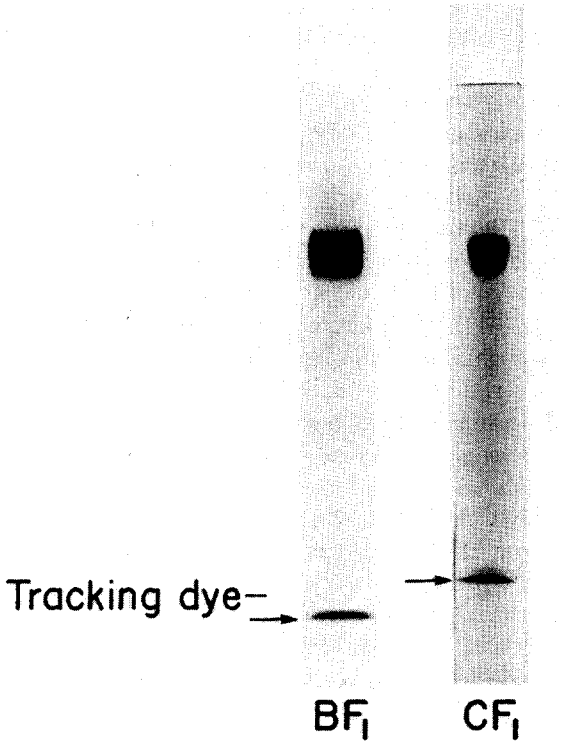

Fig.1. Polyacrylamide gel electrophoresis of purified *M. lysodeikticus* and chloroplast ATPase (60 μg and 40 μg) in Tris–glycine (pH 8.4 ± 0.2). For details see [1,3].

2.2. *Digestion with pronase of bacterial and chloroplast ATPases*

Purified BF_1 (10 mg protein/ml) and CF_1 (2 mg protein/ml) were incubated in 100 mM Tris–HCl/2 mM $CaCl_2$ (pH 8.2) for 48 h at 37°C with two additions (separated by 24 h) of pronase (Koch-Light; weight ratio pronase/ATPase 1:70). The incubations were performed under toluene atmosphere [8]. Blanks without ATPase were incubated under identical conditions and run subsequently through the chromatographic systems and in each analytical procedure to subtract their values.

2.3. *Amino acid analysis*

The analysis was carried out by ion exchange chromatography in a Durrum D500 autoanalyzer following hydrolysis of approx. 10 μg protein samples in 100 μl 6 M HCl for 22 h at 110°C. Norleucine was added as internal standard before hydrolysis. Free tryptophan and tyrosine were estimated by fluorescence relative to standards in a Fica 55 MK II spectrofluorimeter.

2.4. *Sugar analysis*

Hydrolyses of the ATPases and their fractions after digestion with pronase were carried out in 1 N H_2SO_4 (approx. 2 μg sugar/20 μl) for 4 h at 100°C. Hydrolysates were neutralized with $BaCO_3$ and reduction and acetylation were performed essentially as in [9], keeping the volumes as small as possible. In some instances, samples were chromatographed twice through Dowex 50 × 8 (H^+) to eliminate the residual Tris that could interfere in the chromatograms. Inositol, xylose and galactose were used as internal standards for all sugars, pentoses and hexoses, respectively. The alditol acetates were analyzed by gas–liquid chromatography on ECNSS-M (Applied Science Laboratories) at 180°C or 200°C in a Varian 1440/10 gas chromatograph with a Hewlett Packard 3380 A integrator. Peak assignments were made on the basis of sample coincidence with the relative retention times (± 0.01) of standards at the two column temperatures. Sialic acid was assayed with the thiobarbituric acid in [10]. The small amounts of hexosamines present were estimated from their well-separated peaks in the amino acid chromatograms with appropriate corrections for loss by hydrolysis [11].

To rule out that the sugars present in the ATPases and their fractions were contaminants from the procedures of protein purification, buffers were run in parallel through all the separation and concentration steps as controls. These controls were analyzed for their sugar composition and their values, if any, subtracted from the samples.

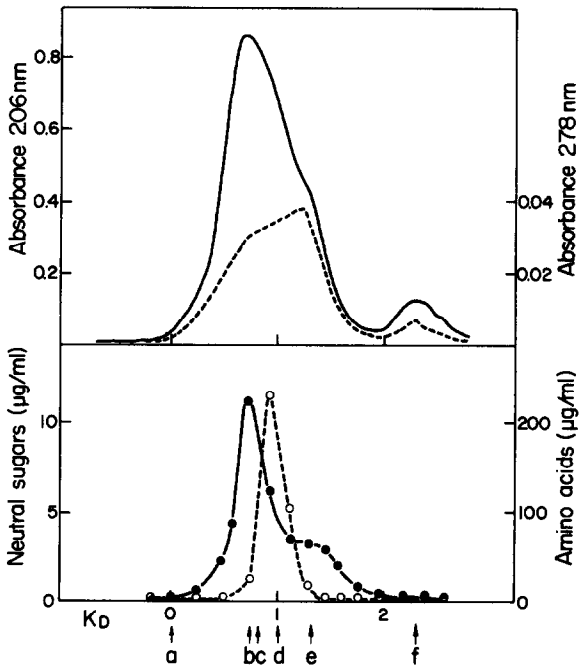

Fig.2. Sephadex G-25 chromatography of *M. lysodeikticus* ATPase or BF_1 digested with pronase. The protein sample (200 μg) was applied to a 0.9 × 25 cm column with 50 mM NaH_4PO_4–Na_2HPO_4 (pH 7.0) as elution buffer. The flow rate was kept constant at 40 ml/h with the help of a SAGE 375 A peristaltic pump and the effluent was monitored with a double beam 2 channel Uvicord III (LKB) equipped with 206 nm and 278 nm filters and connected to a Honeywell FB90 recorded in the 10 V and 1 V scales, respectively. Aliquots (50 μl) of each fraction were used for a micro-scale orcinol reaction [1] to detect sugars and 0.3 ml aliquots were used for ninhydrin reaction to reveal amino compounds. The upper part shows the monitoring of eluate A_{206} (——) and A_{278} (– – –) and the lower part the sugar (•——•) and amino acid (○——○) concentrations in the effluent. Arrows at the bottom indicate the elution position of markers: (a) dextran blue; (b) ATP; (c) hexoses; (d) Cl^-; (e) tyrosine; (f) tryptophan.

3. Results

3.1. *Analysis and preparative isolation by gel chromatography of ATPases digested with pronase*

Figure 2 shows the elution profile on Sephadex G-25 of the products resulting from digestion with pronase of BF_1 from *M. lysodeikticus*. No high molecular weight material eluting at the void volume was observed but a minimum of 4 components could be detected although they were poorly resolved. A glycopeptide material ($K_d \simeq 0.75$) with a high sugar content eluted first, followed by a peak with a $K_d \simeq 1$, theoretically corresponding to free amino acids. Finally, 2 other peaks showed a high retention on the gel (K_d values, 1.3 and 2.3, respectively) and possessed high absorption at 278 nm, suggesting they were compounds enriched in aromatic amino acids. The analysis of the products of CF_1 digestion with pronase gave a similar pattern of elution with absence of the last peak.

A better resolution of these peaks could be achieved when the filtration of the ATPase digest was carried out in a two column system of Sephadex G-25 and G-10. Five components from BF_1 were now resolved (fig.3) which gave single peaks after rechromatography on the same system. Component 5 was absent in the digestion product of CF_1. When electrophoresed at neutral pH, components 1, 3, 4 and 5 gave single ninhydrin-positive spots but component 1 revealed a certain heterogeneity by electrophoresis at pH 3.7. Therefore, this component cannot be considered as pure glycopeptide but as a peptide family representing a sugar-enriched fraction. Component 2 also showed marked heterogeneity.

3.2. *Comparison of the chemical composition of* M. lysodeikticus *BF_1 and CF_1 and the main glycopeptide fractions isolated after their digestion with pronase*

Table 1 compares the amino acid and sugar composition of whole ATPase from *M. lysodeikticus* with the main glycopeptide fraction isolated after its digestion with pronase. All the mannose, glucose and glucosamine of the whole protein were virtually recovered in component 1. This component also had a high serine content. Component 3 (results not shown) contained all the ribose present, smaller

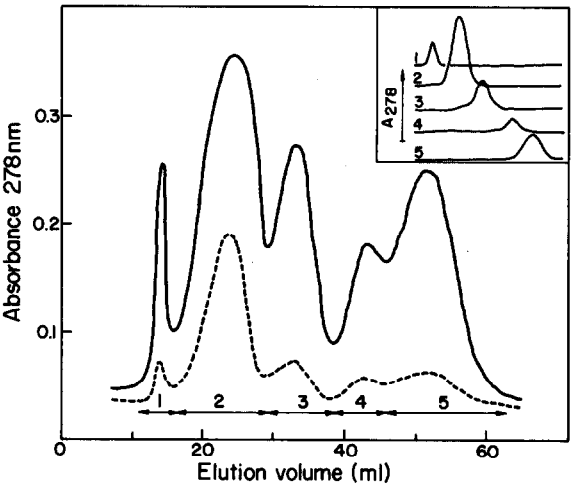

Fig.3. Separation of the components of BF_1 (8 mg) after its digestion with pronase by Sephadex G-25. G-10 chromatography in 0.2 M pyridine–acetic acid, pH 6.5. The broken line is an off-scale indication of the effluent A_{206}. Numbers 1–5 indicate the collected fractions. The inset shows the rechromatography of each separated in the same system. Fractions 1, 2, 3 correspond to the components of K_d 0.75, 1.0, 1.3 of fig.2, respectively, as checked by analysis of their chromatographic behavior in this Sephadex G-25–G-10 system. High-voltage paper electrophoresis of these fractions was performed in a flat bed Shandon HVE apparatus at 150–300 V. h/cm on Whatmann 3 MM washed paper using as buffers either 1 M pyridine–acetic acid (pH 6.5) or pyridine acetic acid water (1:10:289, v/v/v) at pH 3.7 [8]. The yields of the 5 fractions from BF_1, were in % weight 1.6, 67.0, 1.9, 0.2 and 0.1 whereas those of the 4 fractions from CF_1 were 3.1, 47.1, 4.4 and 0.6, respectively.

percentages of glucose and glucosamine as well as most of the tyrosine and phenylalanine of the protein. These aromatic amino acids were probably free since most of the dinitrophenyl derivatives of component 3 were extractable with ether under acidic conditions. This was confirmed by the ultraviolet and fluorescence spectra of the fraction which had the same characteristics as free tyrosine. However it is worth noting that the sugars present in this fraction had an abnormal chromatographic retention and were therefore not free sacharides. Component 2 had a similar composition to that of whole BF_1 and consisted of free amino acids. Component 5 seemed to consist of free tryptophan.

The sugar composition of CF_1 recorded in table 1 showed certain similarities with that of BF_1 although

Table 1
Sugar and amino acid composition of *M. lysodeikticus* and chloroplast ATPase and the glycopeptide fractions isolated after their digestion with pronase

Residues	Fractions (mol/100 mol amino acid)			
	BF_1	BF_1^{-1}	CF_1	CF_1^{-1}
Monosaccharide				
Rhamnose			0.2	7.5
Fucose			0.2	2.5
Ribose	0.2		0.7	35.8
Arabinose				
Xilose				
Mannose	1.7	51.6		
Galactose			0.9	34.6
Glucose	1.7	12.2	0.5	13.4
Glucosamine	0.2[a]	3.5	0.1	3.1
Galactosamine			0.3	3.3
Sialic acid				
Total	3.8	67.3	3.0	101.9
Amino acid				
Lys	2.1	5.0	4.4	3.7
His	0.9	3.7	0.4	2.9
Arg	4.1	2.4	4.8	3.4
Asp	9.5	7.6	8.0	11.2
Thr	6.9	4.4	6.3	5.3
Ser	5.6	18.8	5.4	16.6
Glu	12.7	14.0	12.5	15.0
Pro	4.6			
Gly	11.2	13.2	10.0	9.6
Ala	12.4	14.6	10.0	9.6
Val	9.1	5.2	8.0	4.4
Met	1.8	0.4	2.8	0.7
Ile	5.7	2.1	7.4	3.5
Leu	8.8	3.6	10.9	6.6
Tyr	1.5	2.5	1.7	3.6
Phe	2.7	2.1	3.0	3.0
Trp	0.4[b]	$\lesssim$ 0.4[c]		$\lesssim$ 0.4[c]
Hydrophobic/ Hydrophilic	0.69	0.26	0.78	0.33

[a]Not calculated in this work – Estimated from [1] where the hexosamine value should read 0.003 g/g peptide
[b]Not done – calculated from [1]
[c]Estimated from the fluorescence spectra (see section 2)

differences were also evident. The main difference stem from the presence in CF_1 of arabinose, 6-deoxy-hexoses, galactosamine besides glucosamine and galactose instead of mannose. Component 1 of CF_1 also contained most of the sugar of the whole protein and a relatively high percentage of serine. Some galactose and glucose were now found in component 4 (results not shown) representing a minor proportion of the whole protein (fig.3). Sialic acid was not detected in either BF_1 or CF_1.

4. Discussion

The aim of the present work was to extend our findings on the glycoprotein nature of the energy-transducing ATPases or F_1 factors from various origins [1,2]. The isolation of glycopeptide fractions from two ATPases represents new and strong support to the notion that these energy-transducing proteins are in fact glycoproteins. To carry out this work we have taken advantage of the use of microscale analytical and preparative procedures which increased the sensitivity of the methods. The possibility that the sugars present in the F_1 factors arose from a contamination of the purification procedures was ruled out by running appropriate controls (see section 2). Moreover, the differences in sugar composition of ATPases and fractions isolated by the same procedures also argue against that possibility. Most of the sugar components of bacterial and chloroplast F_1 were found in glycopeptide fractions which represented a small proportion of the total protein weight and resembled to some extent the whole proteins in the amino acid composition although they became poorer in hydrophobic amino acids and were enriched in serine. It was, therefore, tempting to speculate that their glycan portion might be linked to the peptide moiety through *O*-glycosidic bonds between the hydroxy-amino acids and sugar residues. However, we were unable to detect significant β-elimination of serine residues as it occurred already with the whole proteins [2]. The hydrophilic character of the main glycopeptide fractions suggested that as in all glycoproteins, the carbohydrate moiety of F_1 ATPases appears to be part of a hydrophilic domain which may in turn be oriented towards the exterior of the protein or less likely represent a hydrophilic pore in the protein interior [2].

Preliminary evidence on the glycoprotein nature of mitochondrial F_1 is in [2]. However, the confirmation and a detailed analysis of this property are still

lacking. The report [12] estimates that 8% of mitochondrial F_1 may not be protein. The glycoprotein nature as a general property of F_1 coupling factors may raise questions with regard to their topography and assembly as well as to the analysis of mutants which may be affected in that component of the energy-transducing machinery.

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References

[1] Andreu, J. M., Carreira, J. and Muñoz, E. (1976) FEBS Lett. 65, 198–203.
[2] Andreu, J. M., Larrage, V. and Muñoz, E. (1977) in: Bioenergetics of Membranes (Packer, L., Popageorgiou, G. C. and Trebst, A. eds) pp. 529–538, Elsevier/North Holland Biomedical Press, Amsterdam.
[3] Carreira, J., Andreu, J. M., Nieto, M. and Muñoz, E. (1976) Mol. Cell. Biochem. 10, 67–76.
[4] Lien, S. and Racker, E. (1971) in: Meth. Enzymol. (San Pietro, A. ed) Vol. XXIII, part A, pp. 547–555, Academic Press, New York, London.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[6] Nieto, M., Muñoz, E., Carreira, J. and Andreu, J. M. (1975) Biochim. Biophys. Acta 413, 394–414.
[7] Farron, F. (1970) Biochemistry 9, 3823–3828.
[8] Glick, M. C. (1974) in: Methods in Membrane Biology (Korn, E. D. ed) Vol. 2, pp. 157–204, Plenum Press, New York, London.
[9] Laine, R. A., Esselman, W. J. and Sweeley, C. C. (1972) in: Meth. Enzymol. (Ginsburg, V. ed) Vol. XXVII, part B, pp. 159–167, Academic Press, New York, London.
[10] Warren, L. (1959) J. Biol. Chem. 234, 1971–1975.
[11] Allen, A. K. and Neuberger, A. (1975) FEBS Lett. 6, 76–80.
[12] Muller, J. L. M., Rosing, J. and Slater, E. C. (1977) Biochim. Biophys. Acta, in press.