LACK OF EVIDENCE FOR COVALENTLY-BOUND CARBOHYDRATES IN ENERGY-TRANSDUCING ATPases FROM MITOCHONDRIA, BACTERIA, AND CHLOROPLASTS

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Received 1 June 1979
Revised version received 19 June 1979

1. Introduction

Recently Muñoz and co-workers proposed that the energy-transducing ATPases from *Micrococcus lysodeikticus* (BF_1) and spinach chloroplasts (CF_1) are glycoproteins [1,2]. Their evidence includes periodic acid-Schiff (PAS) staining of the enzymes' α and β subunits on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE gels), PAS staining of protein bands of the appropriate molecular weight on non-denaturing PAGE gels and sugar analysis of enzyme preparations. In addition, preliminary experiments indicate that the mitochondrial ATPase (F_1) may also contain sugar moieties [3,4]. This would be consistent with a report that mitochondrial F_1 may contain up to 8% non-protein material by weight [5].

Despite the potential appeal of these suggestions, the experiments reported in this communication fail to confirm the presence of covalently-bound carbohydrates in beef-heart mitochondrial F_1, spinach chloroplast CF_1, or *Escherichia coli* BF_1. When SDS is carefully removed from SDS-PAGE gels prior to PAS staining, none of the subunits of the individual ATPases give a positive reaction for sugar. In addition, the ATPases are not retained by concanavalin A-Sepharose columns based on recovery of protein and enzymatic activity.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ATPase, the terminal coupling factors of oxidative phosphorylation in mitochondria (F_1) and bacteria (BF_1) and of photophosphorylation in chloroplasts (CF_1)

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2. Experimental

2.1. *Materials*

Coupling factor, F_1, from beef-heart mitochondria [6] and CF_1 from spinach chloroplasts [7] were prepared as described. BF_1 from two strains of *E. coli* (ML308–225 and K12 (γ)) were kindly provided by Dr. Leon Heppel's laboratory [8,9].

Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals. All other chemicals were of reagent grade quality.

2.2. *SDS-polyacrylamide gel electrophoresis*

10% gels were cast in cylindrical glass tubes (10 × 0.5 cm) and samples were electrophoresed at 6 to 8 mA per tube [10]. Following electrophoresis, the gels were treated to remove SDS as described [11] except that fresh acid-washed charcoal (2 to 3 g per gel) wrapped in miracloth was added at each solvent change. The PAS method was used to stain for glycoproteins and the gels were scanned at 560 nm [11]. The gels were subsequently stained with Coomassie Brilliant Blue G (0.1%) in 25% isopropanol, 10% acetic acid. Gels were destained and scanned at 595 nm. In preliminary experiments, replicate gels

containing CF_1 were stained for protein with or without prior staining for sugars. No significant difference in the intensity or position of the protein bands was noted. Thus gels containing the ATPases were used for both sugar and protein staining in order to increase the accuracy in identifying any possible glycoprotein bands.

2.3. *Concanavalin A affinity chromatography*

Concanavalin A-Sepharose columns were poured in 1 ml tuberculin syringes with a porous polyethylene disc to support a column bed of 1 ml. The columns were equilibrated with 5 ml of 50 mM Tris–SO_4, 2 mM ATP, pH 7.4 (Tris–ATP buffer) before application of sample.

Samples containing between 0.44 and 0.65 mg of each protein were applied in 100 to 250 μl of the Tris–ATP buffer to individual columns. Tris–ATP buffer was added to the column and 2.5 ml of effluent was collected in a graduated conical tube (fraction I). In the case of the bacterial enzymes, effluent was collected in tubes already containing 2.5 ml 50 mM Tris–SO_4 (pH 7.8), 2 mM ATP, 4 mM EDTA, and 20% glycerol to stabilize the enzyme. The columns were then washed with 0.5 ml of buffer, followed by displacement of bound glycoproteins with 2.5 ml of the Tris–ATP buffer containing 200 mM α-methyl mannoside (fraction II). After removing aliquots of fractions I and II for protein analysis and ATP hydrolysis assays, solid ammonium

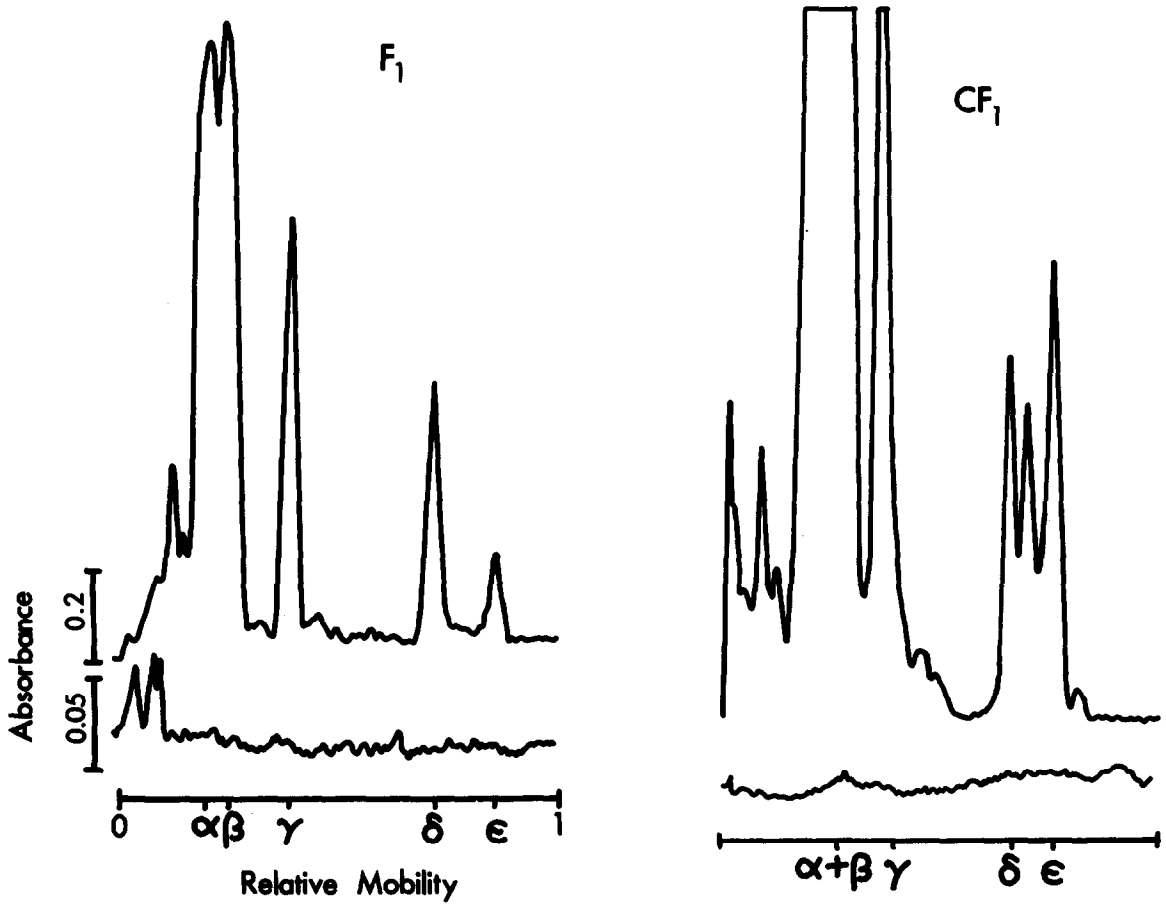

Fig. 1. Sugar and protein staining of F_1 and CF_1 subunits. Samples containing 150 μg F_1 (left panel) and 220 μg CF_1 (right panel) were run on SDS-PAGE gels, stained for sugar and protein, and scanned as described under Experimental. The upper scan shows the protein stain and the lower scan shows the sugar stain. The absorbance scales and the five accepted subunits of each ATPase are indicated.

sulfate was added to the remaining effluent to give 50% saturation. The precipitated protein was collected by centrifugation and characterized by SDS-PAGE.

2.4. *Other methods*

Specific activities for ATP hydrolysis by F_1 and BF_1 were determined at 30 and 37°C respectively using an NADH-linked, ATP-regenerating assay system [12]. CF_1 was heat-activated [7], and the Mg^{2+}-dependent ATP hydrolysis activity was measured at 37°C using the same spectral assay containing in addition 75 mM sodium bicarbonate, pH 8.0 [13]. $MgCl_2$ was present at a concentration of 1 mM and ATP at 500 μM.

Protein was determined using a modified Lowry procedure [14]. Fraction II contains α-methyl mannoside which interferes with this assay. In addition, small amounts of a protein of 25 000 daltons (concanavalin A based on molecular weight) is eluted from the columns. To correct for these effects, aliquots of fractions I and II from a control column with no added protein were used as blanks in the protein assays. In some experiments, protein was precipitated [15] prior to protein determination.

3. Results

3.1. *PAS staining of SDS-polyacrylamide gels*

Staining for sugars by the PAS method after

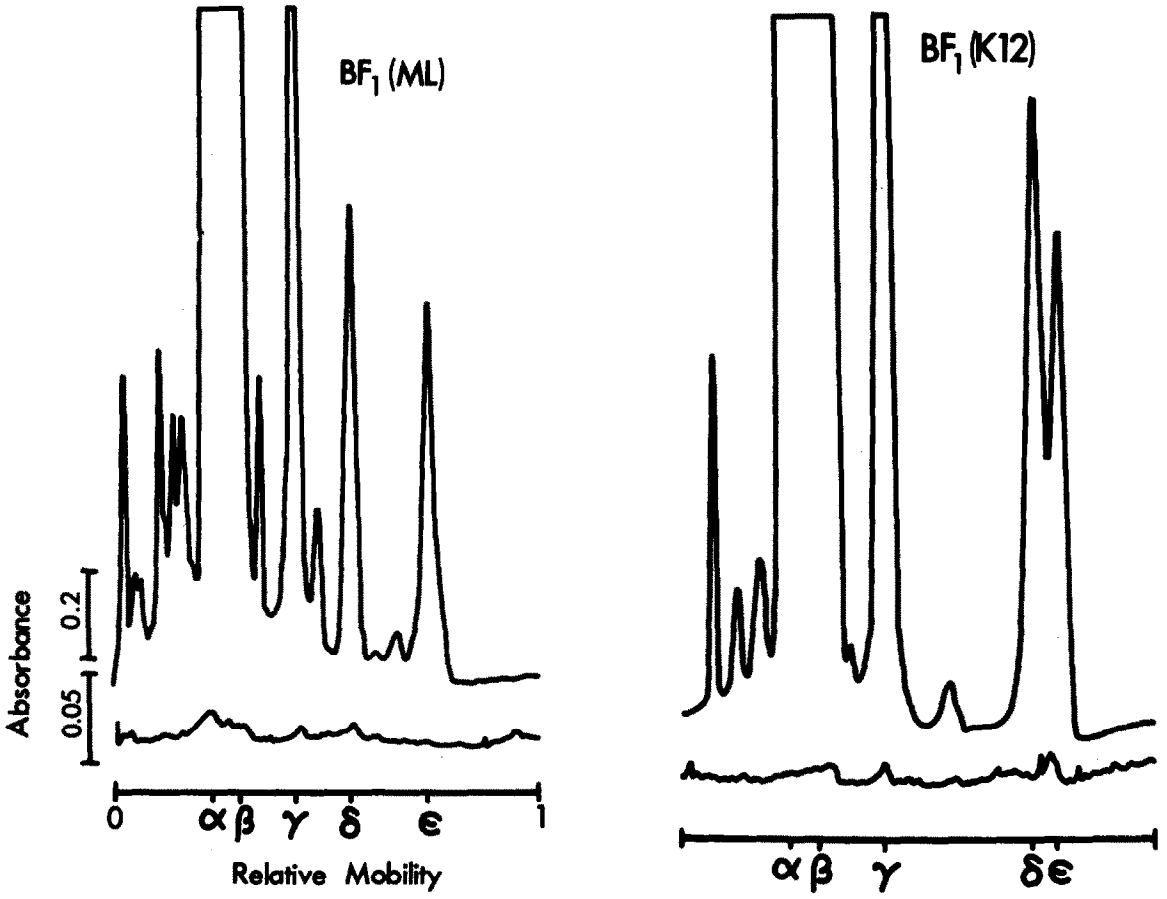

Fig.2. Sugar and protein staining of BF_1 subunits. Samples containing 150 μg of *E. coli* BF_1 from ML308–225 (left panel) and K12 (λ) (right panel) were run on SDS-PAGE gels, stained for sugar and protein, and scanned as described under Experimental. The upper scan shows the protein stain and the lower scan shows the sugar stain.

electrophoresis revealed no significant stain in the five subunits of F_1 (fig.1, left panel), CF_1 (fig.1, right panel), or BF_1 (fig.2). In contrast, ovalbumin, used as a glycoprotein standard, shows strong staining (fig.3).

When large amounts of the mitochondrial coupling factor are run on gels (i.e., 150 μg F_1 in fig.1), two minor bands are observed which were consistently stained. These proteins migrate more slowly than the α subunit of F_1 and have molecular weights of approximately 65 000 and 75 000 daltons. The ratios of sugar stain to protein stain for the two bands are similar to those of ovalbumin, suggesting that these contaminating proteins are glycosylated.

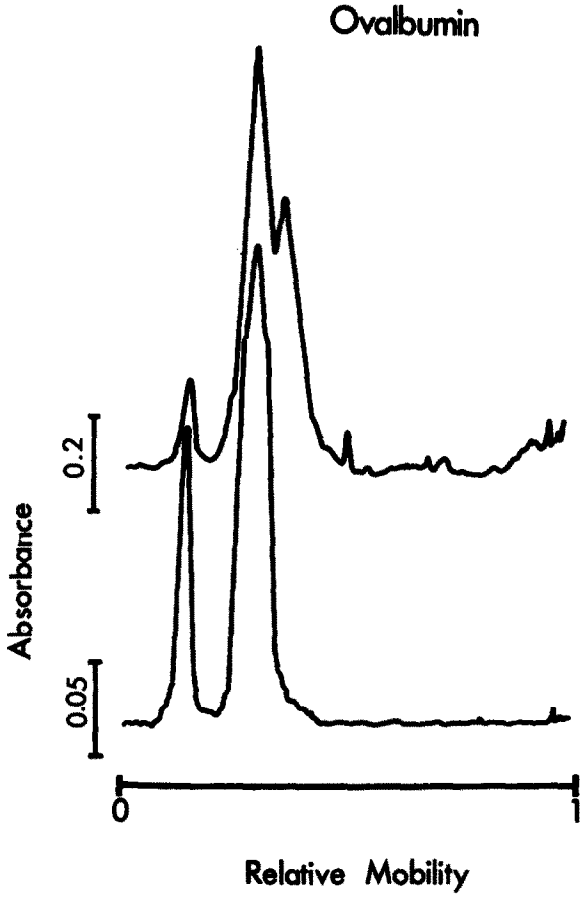

Fig.3. Sugar and protein staining of ovalbumin. A sample containing 50 μg ovalbumin was run on SDS-PAGE gels, stained for sugar and protein, and scanned as described under Experimental. The upper scan shows the protein stain and the lower scan shows the sugar stain.

3.2. *Affinity chromatography of F_1, CF_1, and BF_1 on concanavalin A-Sepharose*

The ATPases and standard proteins were applied to concanavalin A-Sepharose columns. Based on protein recovery, the ATPases as well as the non-glycoprotein standard, bovine serum albumin, eluted exclusively in the first fraction. In contrast, 85% of the ovalbumin sample was recovered in the second fraction by elution with buffer containing α-methyl mannoside.

To ensure that the lectin did not selectively remove small amounts of essential subunits from the coupling factors, samples were taken from each fraction and assayed for ATP hydrolysis activity. Table 1 shows that the specific activities of F_1, CF_1, and BF_1 remained essentailly unchanged following chromatography. No detectable ATP hydrolysis was observed in fractions II. In control experiments, α-methyl mannoside had no effect on the activity of the ATPases at the level introduced during assay of fractions II.

SDS-PAGE of material precipitated by ammonium sulfate from fractions I showed protein bands corresponding to α and β subunits for each ATPase and no such bands in fractions II (data not shown). Examination of these gels for elution of γ, δ, and ϵ subunits was precluded by the presence of small amounts of protein which leached from the columns (see Experimental). In contrast, over 90% of the material in the ovalbumin sample which is stained on gels by the PAS method was recovered in fraction II (data not shown).

4. Discussion

It is difficult to accommodate our results with several published reports [1–4]. The sugar compositions provided in table 1 of ref. 3 allow the calculation of the percent by weight of carbohydrates to be approximately 3% in CF_1, 11% in form A, and 6% in form B of BF_1 from *M. lysodeikticus*. These authors also report that purified mitochondrial F_1 is 3% carbohydrate by weight [3]. Considering the ease with which the sugar moieties were detected in ovalbumin (fig.3) which contains 3% carbohydrate by weight [16], we would have had a clear indication by

Table 1
Recovery of ATP hydrolysis activity from concanavalin A-Sepharose columns

	Specific activity	Recovery of specific activity	
	Prior to affinity chromatography ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	Fraction I (buffer) (%)	Fraction II (+ α-methyl mannoside) (%)
F_1	55.4	97	< 0.05
CF_1	3.9	90	< 0.15
BF_1 (ML308–225)	28.9	103	< 0.05
BF_1 (K12, λ)	34.9	108	< 0.05

Column chromatography was performed as described under Experimental. Aliquots containing 0.5 μg of F_1 and BF_1 and 1.4 μg of CF_1 from enzyme stock solutions and from fractions I were assayed for ATP hydrolysis activity as described. A volume of fraction II, 80 times that used to assay fraction I, was also assayed

the PAS method if these levels were present in our preparations of the ATPases. The estimated lower limits of detection for the percentage carbohydrate by weight in the subunits of F_1 (fig.1) are 0.1% for α and β, 0.5% for γ, 1.0% for δ and 3% for ε. In addition, the lack of retention of the ATPases on concanavalin A affinity columns indicates the absence of α-D-mannosyl terminal groups.

We believe it unlikely that our procedures for isolating the ATPases labilized glyco-peptide linkages, particularly since we used the same procedure as Muñoz and co-workers to obtain CF_1 from spinach chloroplasts [7]. We also believe that the procedure used to remove SDS from gels prior to PAS staining would not account for the complete absence of stainable material. With the exception of the use of charcoal to facilitate removal of SDS, our washing procedure was identical to that previously developed for the detection of glycoproteins on SDS-PAGE gels [11]. In addition, the PAS staining of ovalbumin was not significantly affected by the duration of the wash steps.

The PAS staining procedure [17] referenced by Muñoz and co-workers [2] was not designed for use on SDS gels. The short 30-min wash prior to PAS staining prescribed by this method may not have been sufficient to remove SDS. SDS is known to give a false positive stain for carbohydrate by the PAS method [11], and while the detergent can be easily removed from the gel matrix, it binds tenaciously to proteins [18]. The PAS staining of protein bands on non-denaturing polyacrylamide gels and the sugar analysis of their enzymes [1–3] may be related to non-covalently bound carbohydrates or to glycoprotein contaminants such as those observed in our F_1 preparation (fig.1).

Our results are in accord with those of Penefsky and Warner, who failed to obtain evidence that beef-heart mitochondrial F_1 contains hexosamines [19].

Acknowledgements

We wish to thank Thomas Barra for excellent technical assistance and John Power for helpful advice on removal of SDS from polyacrylamide gels. This work was supported by Grant GM 23152 of the Institute of General Medical Science and Grant HD 09046, United States Public Health Service.

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