Biochimica et Biophysica Acta, 686 (1982) 7–12
Elsevier Biomedical Press

BBA 71077

PURIFICATION AND CHARACTERIZATION OF AN $(Na^+ + K^+)$-ATPase PROTEOLIPID LABELED WITH A PHOTOAFFINITY DERIVATIVE OF OUABAIN

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(Received July 27th, 1981)
(Revised manuscipt received October 29th, 1981)

Key words: Proteolipid; $(Na^+ + K^+)$-ATPase; Ouabain binding site; Photoaffinity labeling; Amino acid composition

Highly purified lamb kidney $(Na^+ + K^+)$-ATPase was photoaffinity labeled with the tritiated 2-nitro-5-azidobenzoyl derivative of ouabain (NAB-ouabain). The labeled $(Na^+ + K^+)$-ATPase was mixed with unlabeled carrier enzyme. Two proteolipid ($\gamma 1$ and $\gamma 2$) fractions were then isolated by chromatography on columns of Sepharose CL-6B and Sephadex LH-60. The two fractions were interchangeable when rechromatographed on the LH-60 column, suggesting that $\gamma 1$ is an aggregated form of $\gamma 2$. The total yield was 0.8–1.5 mol of γ component per mol of catalytic subunit recovered. This indicates that the γ component is present in stoichiometric amounts in the $(Na^+ + K^+)$-ATPase. The proteolipids that were labeled with NAB-ouabain copurified with the unlabeled proteolipids.

Introduction

Ouabain and other cardiac glycosides are specific inhibitors of $(Na^+ + K^+)$-ATPase, the enzyme which is responsible for the active transport of Na^+ and K^+ across cell membranes [1,2]. Highly purified $(Na^+ + K^+)$-ATPases from various sources contain two major proteins: the α, or catalytic subunit ($M_r \sim 100000$) and the β, or glycoprotein subunit ($M_r \sim 50000$). In 1972, Rivas, et al. [3] obtained evidence suggesting that ouabain may bind to a small proteolipid associated with the $(Na^+ + K^+)$-ATPase. (Proteolipids are very hydrophobic peptides that are soluble in organic solvents, such as chloroform-methanol mixtures. They do not necessarily contain covalently bound lipids.) Later, Racker [4] suggested that the $(Na^+ + K^+)$-ATPase contains a proteolipid of $M_r \sim 10000$ that may form an ion channel through the cell membrane. More definitive evidence for the existence of a proteolipid component in $(Na^+ + K^+)$-ATPase was recently provided by Forbush [5], who found that NAB-ouabain labels the cardiac glycoside binding site of pig kidney $(Na^+ + K^+)$-ATPase, with about half the label being bound to the α subunit and half to a proteolipid of $M_r \sim 12000$. These results have been confirmed and extended in other studies [6–8]. These studies suggest that there may be two regions on the cardiac glycoside binding site: a primary region, located on α, which recognizes the lactone ring and steroid portions of the glycoside molecule, and a secondary region, formed by α, perhaps β, and the proteolipid, that binds to the carbohydrate por-

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Abbreviations: NAB-ouabain, 2-nitro-5-azidobenzoyl derivative of ouabain; SDS, sodium dodecyl sulfate; $(Na^+ + K^+)$-ATPase, (sodium plus potassium)-stimulated adenosine triphosphatase (EC 3.6.1.3).

tion. Recently, Reeves et al. [9] reported a procedure for the preparative-scale isolation of a small protein from lamb kidney $(Na^+ + K^+)$-ATPase which was designated the γ component of $(Na^+ + K^+)$-ATPase. In this report, we present evidence that the γ component is identical (except for possible species-specific differences) to the proteolipid labeled with NAB-ouabain by Forbush et al. [5].

Methods and Materials

$(Na^+ + K^+)$-ATPase was purified from lamb kidney outer medulla as described previously [12]. All the experiments described in this report were carried out on a single large batch of enzyme. A small amount of this (0.4 mg) was labeled with NAB-[^{3}H]ouabain as described previously [5]; after binding NAB-[^{3}H]ouabain to the enzyme in the presence of Mg^{2+} and phosphate, the enzyme was pelleted in a Beckman airfuge, washed twice, resuspended in 1 ml of binding medium lacking NAB-[^{3}H]ouabain and photolyzed (0°C, 15 min, long wave hand held lamp UVSL, Ultra-Violet Products). Prior to solubilization with SDS [9], 0.04 mg of the labeled $(Na^+ + K^+)$-ATPase was mixed with 20 mg of unlabeled enzyme. The solubilized $(Na^+ + K^+)$-ATPase was dialyzed overnight vs. column buffer (see Fig. 2), and applied (in a volume of 9–13 ml, containing 28000 cpm/mg protein) to a 1.5×195 cm column of Sepharose CL-6B. The three fractons (α, β and γ) from this column were lyophilized. The γ fraction was then dissolved in 2 ml of 88% formic acid and applied to a 2×195 cm column of Sephadex LH-60 run in a 3 : 1 (v/v) mixture of 95% ethanol and 88% formic acid.

Amino acid analyses were carried out as described previously [11]. All protein concentrations were determined by amino acid analysis. Gradient gel electrophoresis of labeled $(Na^+ + K^+)$-ATPase, including slicing and radioisotope counting, was done as described previously [5]. SDS-urea gel electrophoresis of isolated proteolipids was done according to the method of Swank and Munkres [13]. For N-terminal amino acid determination, proteolipids were first oxidized with performic acid [14], then reacted with dansyl chloride in the presence of 1% SDS and hydrolyzed as described by Gray [15]. The dansylated amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets [15].

Results and Discussion

The results of SDS-gel electrophoresis of the lamb kidney $(Na^+ + K^+)$-ATPase labeled with NAB-[^{3}H]ouabain are shown in Fig. 1. As was the case with pig kidney enzyme [5], about half of the covalently bound NAB-ouabain migrated with the α subunit and half with the γ component. It should be noted, however, that only about half as much total label was covalently incorporated into the lamb kidney $(Na^+ + K^+)$-ATPase preparation as compared to that from the pig kidney (Ref. 5, and unpublished results with samples run in parallel). The non-covalently bound NAB-ouabain (about 80% of the total label) moved with the tracking dye. The separation of the protein subunits of a mixture of labeled (0.04 mg) and unlabeled (20 mg) $(Na^+ + K^+)$-ATPase by Sepharose CL-6B chromatography is shown in Fig. 2. This separation pattern has been obtained with three different enzyme samples. When the labeled enzyme was omitted from the sample, the profile of absorbance at 280 nm was unchanged. The recovery of protein and counts applied to the Sepharose column was 90–95%. Although the $(Na^+ + K^+)$-ATPase was

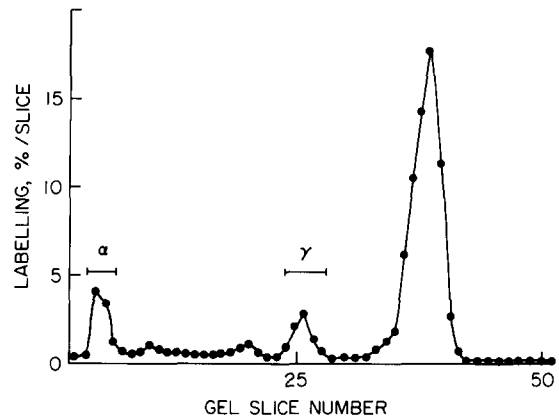

Fig. 1. Gradient gel electrophoresis of lamb kidney $(Na^+ + K^+)$-ATPase covalently labeled with NAB-[^{3}H]ouabain. Labeling per slice is plotted as the percent of total NAB-[^{3}H]ouabain in the sample. Direction of migration is from left to right. The large radioactive peak that follows γ comigrates with the tracking dye.

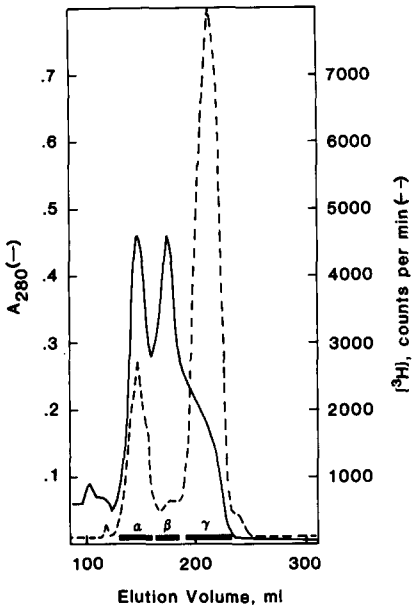

Fig. 2. Separation of the subunits of sheep kidney ($Na^+ + K^+$)-ATPase which has been partially labeled with NAB-[^{3}H]ouabain. Column: Sepharose CL-6B, 1.5×195 cm. Solvent: 5 mM sodium phosphate, pH 8.0, containing 0.1% SDS, 1 mM 2-mercaptoethanol and 0.01% sodium azide. Fractions of 3 ml each were collected at a flow rate of 14 ml/h. Horizontal bars indicate fractions pooled.

solubilized and dialyzed extensively before applying to the column, a large amount of noncovalently bound NAB-ouabain remained in the sample and was eluted in the γ fraction of the Sepharose column, along with phospholipids, excess SDS and other low molecular weight material. The α fraction contained 15–20% of the total counts recovered, and the β fraction about one-third that amount.

In our previous report on the proteolipids of ($Na^+ + K^+$)-ATPase [9], we extracted the lyophilized γ fraction ilustrated in Fig. 2 with chloroform/methanol/water (46 : 46 : 8, v/v) containing 60 mM NH_4HCO_3, pH 7.5, and 1 mM 2-mercaptoethanol. This solvent extracts only 40–60% of the total γ protein. Subsequent chromatography on Sephadex LH-60 in this solvent yielded two similar fractions: γ1, which emerged at the void volume of the column, and γ2. The total protein recovery from the column was variable and often quite low (50% or less). Furthermore, we subsequently found that rechromatography of either γ1 or γ2 on the same column gave extremely poor yields (less than 10%). These low yields were unexpected, since a proteolipid by definition should be completely soluble in chloroform-methanol mixtures. This poor solubility in the LH-60 column solvent may be due to denaturaton of the γ component by our preparative procedures, since this protein can be completely extracted from the intact ($Na^+ + K^+$)-ATPase with acidic chloroform-methanol [5]. We experimented with several different LH-60 solvents in an effort to improve these yields. The most satisfactory procedure found so far is based on that used by Gerber et al. [10] for purification of peptides derived from the intrinsic membrane protein bacteriorhodopsin. The γ fraction is completely solubilized with 88% formic acid and then chromatographed on a Sephadex LH-60 column run in a 3 : 1 (v/v) mixture of 95% ethanol and 88% formic acid. This procedure yields a total of 0.8–1.5 mol of γ component per mol of α subunit recovered, assuming molecular weights of 12000 and 100000, respectively. This indicates that the γ component is present in stoichiometric amounts in the ($Na^+ + K^+$)-ATPase, and thus may play a vital role in enzyme function. Two fractions, γ1 and γ2, are again obtained. The weight ratio of γ1 to γ2 recovered ranges from 1 to 4, with an average value of 2.0. We have also considerably improved the resolution of γ1 and γ2 by increasing the length of the

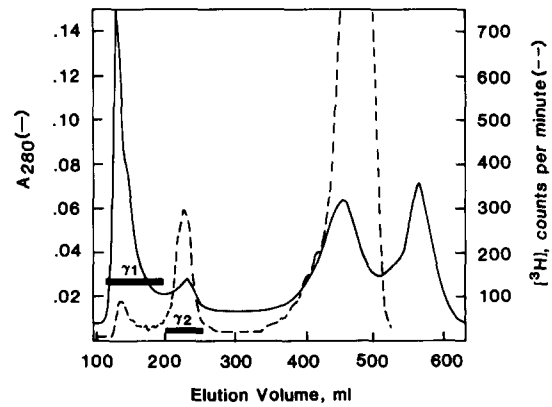

Fig. 3. Chromatography of the γ fraction from Fig. 2. on a 2×195 cm column of Sephadex LH-60. Solvent: a 3 : 1 (v/v) mixture of 95% ethanol and 88% formic acid. Fractions of 3 ml were collected at a flow rate of 7 ml/h. Horizontal bars indicate fractions pooled.

TABLE I

AMINO ACID COMPOSITIONS OF $(Na^+ + K^+)$-ATPASE PROTEOLIPIDS

The γ1 and γ2 fractions were obtained by LH-60 chromatography of the whole γ fraction (see Fig. 2) or by rechromatography of the γ1 and γ2 fractoins shown in Fig. 3. Results expressed as residues per hundred residues. Cys not determined.

	from γ		from γ1		from γ2	
	γ1	γ2	γ1	γ2	γ1	γ2
Residues						
Asp	7.48	9.66	7.97	6.67	8.82	10.9
Thr	5.37	3.99	5.28	5.77	5.19	3.80
Ser	7.00	5.15	6.11	7.19	8.17	5.26
Glu	11.2	11.6	9.92	11.7	10.8	12.3
Pro	5.51	5.67	4.20	4.01	4.33	5.20
Gly	8.55	9.39	8.50	8.74	11.6	10.6
Ala	9.02	8.08	8.95	8.42	9.40	6.79
Val	5.92	4.66	7.03	7.46	9.44	4.58
Met	2.38	1.83	2.25	1.38	2.05	1.67
Ile	5.58	4.94	5.68	6.75	4.16	3.62
Leu	10.7	8.63	11.3	11.5	8.23	7.94
Tyr	3.69	5.06	3.54	3.18	2.90	4.68
Phe	5.98	5.46	6.14	6.46	3.60	4.50
Lys	4.91	6.92	5.77	3.46	4.71	7.94
His	2.18	1.77	2.13	1.75	3.17	3.13
Arg	4.62	7.04	5.16	5.28	4.01	7.23
Yield of protein, %	65	25	27	3	32	23
^3H cpm/μg protein	5.60	40.50	6.20	5.50	38.00	44.00

LH-60 column from 95 to 195 cm.

The results of LH-60 chromatography of the γ fraction from Fig. 2 are shown in Fig. 3. The γ1 and γ2 peaks are well-resolved, with 80% of the labeled protein recovered as γ2. Most of the counts (85–90%) recovered from the column are located in a large peak which contains phospholipids, SDS, and other low molecular weight material; this peak contains no protein, peptides or amino acids, as judged by amino acid analysis. The amino acid compositions of γ1 and γ2 (see Table I) are very similar to those of the fractions we isolated previously [9] using the chloroform-methanol solvent. The content of hydrophobic amino acids is not particularly high, and in fact the amino acid composition of the γ component is rather similar to that of the α subunit [12]. Our experience with more than thirty LH-60 columns has revealed that there is no correlation between the absorbance at 280 nm and the amount of protein in the γ1 peak. The absorbance at 280 nm of γ1 is usually about 20 times that of γ2, but the amount of protein in γ1 is only 2- to 4-times that in γ2. Most of the absorbance in γ1 is therefore due to the presence of some unidentified, non-protein contaminant, or possibly to light-scattering by large aggregates of protein.

The γ2 peak of Fig. 3 contains 7.2-times as many ^3H cpm/mg of protein as the γ1 peak. This would suggest that differences exist between γ1 and γ2 which lead to the preferential labeling of γ2. However, when γ1 and γ2 were individually rechromatographed on the same Sephadex LH-60 column, they appeared to be interchangeable, with the ^3H cpm/mg of protein recovered remaining fairly constant (see Table I for data). It may be that the labeled γ component in the initial sample has less tendency to aggregate than the unlabeled γ component.

When γ1 was rechromatographed, about 10% of the recovered protein was eluted in the γ2 fraction. The γ1 and γ2 from this rechromatography had

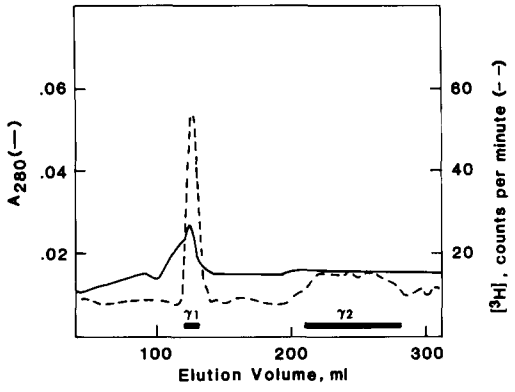

Fig. 4. Rechromatography of the γ2 fraction on Sephadex LH-60. Chromatographic conditions same as in Fig. 3. Horizontal bars indicate fractions pooled.

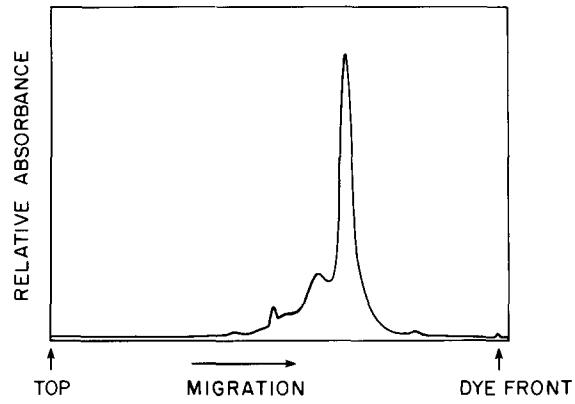

Fig. 5. Densitometric scan of SDS-urea gel electrophoresis of the γ2 fraction of Fig. 4.

similar amino acid compositions (see Table I). When γ2 was rechromatographed (see Fig. 4), the majority of the protein recovered was eluted at the void volume in the γ1 position. It thus appears as though γ1 is an aggregated form of γ2, and that there may in fact be only one type of γ component in $(Na^+ + K^+)$-ATPase. The copurification of labeled and unlabeled material provides evidence that the γ component of Reeves et al. [9] is identical to the proteolipid of Forbush et al. [5]. The γ2 shown in Fig. 4 is especially similar in amino acid composition to the original γ2, while the γ1 illustrated in Fig. 4 is about equally similar to all of the fractions listed in Table I.

The small differences in amino acid composition between γ1 and γ2, and the slight changes that occur after rechromatography, indicate that one or more impurities are present in the proteolipid fractions. We usually observe a single, diffuse band of $M_r \sim 12000$ after electrophoresis of γ1 and γ2 on SDS-urea polyacrylamide gels [9]. Occasionally, however, better resolution is obtained, as illustrated in Fig. 5. In these cases, we observe a major band (which accounts for about 70% of the Coomassie blue staining dye bound) of $M_r \sim 12000$ and several minor bands with mobilities corresponding to M_r 14000–18000. Similar patterns have been observed for both γ1 and γ2. The minor bands would seem to represent impurities, although it is possible that they may be aggregates of the major band. It should be also pointed out that the true molecular weight of the γ component may be less than 12000, since its mobility on gel electrophoresis may be due to comigration with SDS micelles. N-terminal analysis by the dansyl method was carried out on two different γ preparations. In both cases, arginine was found to be the only N-terminal amino acid, although this result needs to be confirmed by another method. This suggests that the proteolipid may be homogeneous, although the question will not be settled unambiguously until further structural analysis has been carried out. It will be of particular interest to identify the amino acid residues which have been labeled by NAB-ouabain, since these may be involved in forming a cardiac glycoside binding site on the $(Na^+ + K^+)$-ATPase. It will also be of interest to compare the structure of the γ component to those of similar proteins which we have recently isolated from both cardiac [11] and skeletal (unpublished observations) muscle sarcoplasmic reticulum.

Acknowledgements

We thank Ms. Grace Jones for excellent technical assistance and Ms. Pamela Smith for the expert preparation of the manuscript. This work was supported by a grant from the Muscular Dystrophy Association and by NIH grants R01-AM-20875, R01-GM-27920, K04-HL-00555, P01-HL-22619 and 5-T32-HL-07382.

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