PHOTOAFFINITY LABELLING OF A SMALL PROTEIN COMPONENT OF A PURIFIED (Na^+-K^+)ATPase

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1. Introduction

The Na^+ pump, or the (Na^+-K^+)ATPase, is a well-known receptor for ouabain and the transmembrane ion-transport system of this enzyme is inhibited as a result of this interaction (reviewed [1]). We have recently synthesized and characterized two photoaffinity labels useful for the labelling of the ouabain-binding site of the (Na^+-K^+)ATPase system, either in a purified or membrane-bound form [2]. It was found that the large molecular weight subunit ($M_r \simeq$ 100 000) was exclusively labelled after photolysis of a highly-purified (Na^+-K^+)ATPase from *Electrophorus electricus* in the presence of either photoaffinity label.

After our experiments were completed and sent for publication [2], a very similar photoaffinity-labelling study reported [3] the radiolabelling of a small proteolipid ($M_r \simeq 12\,000$) in addition to the large chain of the (Na^+-K^+)ATPase purified from pig kidney. A small radiolabelled protein of this size would not have been detected in our experiments [2] with the *Electrophorus electricus* (Na^+-K^+)ATPase because the acrylamide concentrations in the SDS–gel electrophoresis used to analyze the proteins that were photolabelled, were too low to separate such a small protein from the tracking dye front.

We have initiated further studies on the photoaffinity labelling of the (Na^+-K^+)ATPase from the electric organ of *Electrophorus electricus* for two reasons:

(i) To see if different photoaffinity labels of the ouabain binding site, in addition to the one reported in [3] are capable of labelling a small protein component;

(ii) To know if there is a small protein component, in addition to the major protein chains with molecular weights in the regions of 100 000 and 50 000, which is present in other purified (Na^+-K^+)ATPase preparations besides the pig kidney preparation used in [3].

2. Materials and methods

A solubilized purified (Na^+-K^+)ATPase from *Electrophorus electricus* was prepared according to [4]. The specific activity of this preparation was 8.9 μmol P_i/min/mg protein at 25°C using the enzyme assay in [2]. The photoaffinity labels used in these studies, [^{3}H]NAP-ouabain and [^{3}H]NAP-strophanthidin were prepared according to [2]. Their structures are shown in fig.1.

In the photoaffinity labelling experiments, 200 μg protein of the (Na^+-K^+)ATPase was incubated with 0.8 μM [^{3}H]NAP-ouabain (5 Ci/mmol) or [^{3}H]NAP-strophanthidin (2 Ci/mmol) in 250 μl 50 mM triethanolamine, 120 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP at pH 7.5 for 30 min at ambient temperature in the dark. After incubation, the unbound photolabel was removed by diluting each

Abbreviations: SDS, sodium dodecylsulfate; NAP-ouabain, *N*-(ouabain)-*N'*-(2-nitro-4-azidophenyl)ethylenediamine; NAP-strophanthidin, *N*-(strophanthidin)-*N'*-(2-nitro-4-azidophenyl)ethylenediamine

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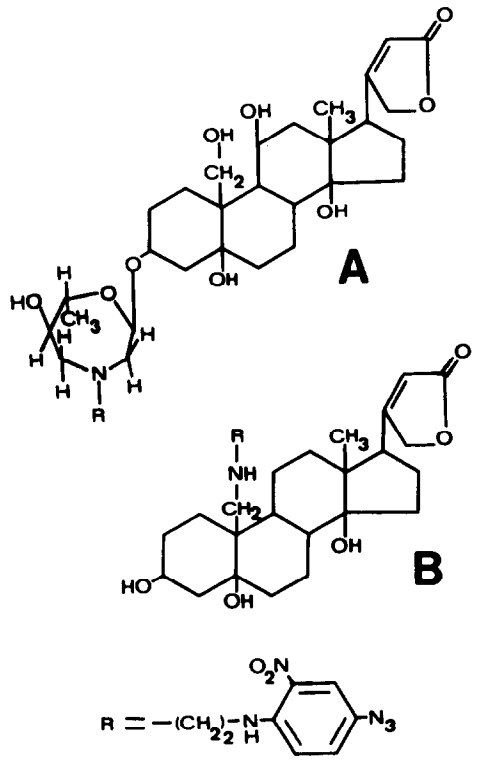

Fig.1. The structures of the photoaffinity labels. The following compounds have been used to label the (Na^+-K^+)-ATPase: (A) NAP-ouabain; (B) NAP-strophanthidin. See [2] for details.

solution 10-fold with 50 mM triethanolamine, 100 mM KCl at 0°C and concentrating the enzyme by centrifugation (50 000 × *g*, 15 min). The pellet was resuspended in 200 μl 50 mM triethanolamine, 100 mM KCl at 0°C and irradiated as in [2]. After photolysis, the enzyme was reconcentrated by centrifugation and dissolved in SDS. Protein (50 μg) was applied to each gel track for analysis. The addition of K^+ to the buffer during the washing and photolysis steps is useful in decreasing the rate of dissociation of the (Na^+-K^+)ATPase–cardiac glycoside complex [5].

A discontinuous SDS–gel electrophoresis system was used according to [6] except that *N,N*-diallyltartaramide was used to crosslink the acrylamide to impart a periodate solubility to the gels. The gels were cast in a slab-gel apparatus as in [2] using a gel gradient from 10% acrylamide (w/v), 0.3% *N,N*-diallyltartaramide (w/v) to 30% acrylamide, 0.9% *N,N*-diallyltartaramide. In most cases the gels were stained before slicing into 2 mm segments and counting for radioactivity. These methods are described in [2].

3. Results and discussion

The results of the photoaffinity labelling experiments with [^{3}H]NAP-oubain and the purified (Na^+-K^+)ATPase are shown in fig.2A. It is clear that there are two bands of radioactivity on the gels. The molecular weights of these two bands correspond to 93 300 and 12 800 when compared with molecular weight standards. The large peak contains 58% of the

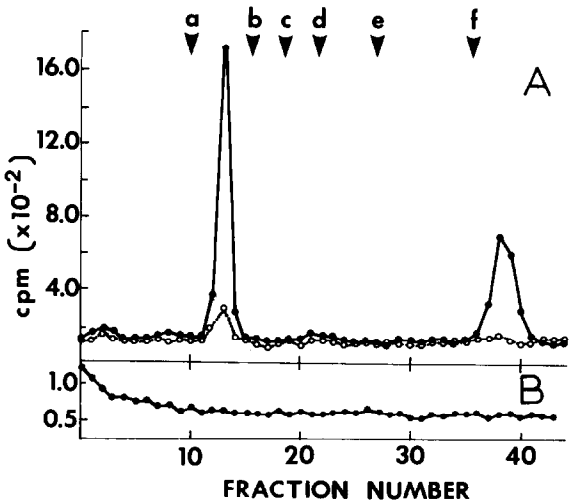

Fig.2. Photoaffinity labelling of the (Na^+-K^+)ATPase using [^{3}H]NAP-ouabain. Panel A shows the pattern of radiolabelling after photolysis when the proteins are separated by SDS–gel electrophoresis with an acrylamide gradient of 10–30% (w/v). The experimental details are in the text. The enzyme was photolyzed after incubation with 0.8 μM [^{3}H]NAP-ouabain (•–•) or with 0.8 μM [^{3}H]NAP-ouabain plus 0.1 mM ouabain (○–○). The gels were calibrated using molecular weight standards prepared according to [7]. The labelled arrows indicate the mobility of the following molecular weights: (a) 132 000, (b) 66 000; (c) 57 200; (d) 42 900; (e) 28 600; (f) 14 300. Panel B shows the radioactivity pattern for photolyzed [^{3}H]NAP-ouabain when it is applied directly to the gel.

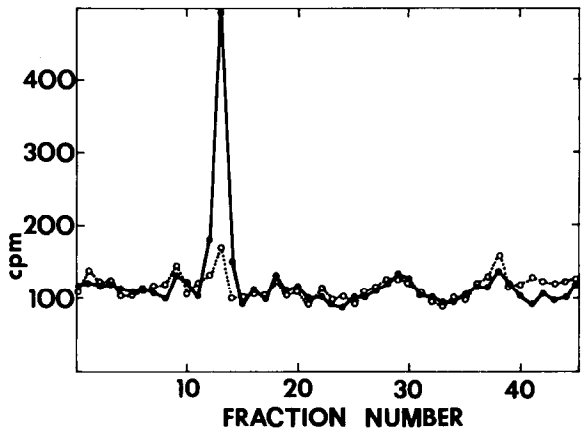

Fig.3. Photoaffinity labelling of the (Na^+-K^+)ATPase using [^{3}H]NAP-strophanthidin. The enzyme was photolyzed after preincubation with 0.8 μM [^{3}H]NAP-strophantidin (●–●) or 0.8 μM [^{3}H]NAP-strophanthidin plus 0.1 μM ouabain (○–○). The same SDS–gel system was used here as in fig.2.

total radioactivity and the smaller band contains 42%, a ratio of labelling that is in agreement with the results in [3] with an enzyme from kidney. In the absence of light no label is incorporated. The labelling in the low molecular weight region of the gel cannot be due to free photolyzed [^{3}H]NAP-ouabain as shown in fig.2B. Most of this radioactivity has been washed from the gels during staining.

In the photolabelling experiments where [^{3}H]NAP-strophanthidin was used, the protein band at 93 300 mol. wt was radiolabelled exclusively. As shown in fig.3 there is no labelling in the 12 000 mol. wt region. In the results shown here the gels were stained before slicing, but the same results were obtained if the gels were sliced immediately after electrophoresis. This was an important control since it was reported [3] that the labelling observed at 12 000 mol. wt was significantly decreased during the staining procedure.

In conclusion, affinity labelling experiments with a highly-purified (Na^+-K^+)ATPase and NAP-ouabain indicate that there is a specific labelling of a band on SDS–gels with an apparent molecular weight in the region of 12 000. In addition to the 100 000 mol. wt chain, we have also observed the labelling of a protein 12 500 mol. wt in cardiac plasma membranes using [^{3}H]NAP-ouabain (in preparation). These results are completely consistent with [3] where 2-nitro-5-azidobenzoyl ouabain was used as a photoaffinity label. In all of these studies with purified (Na^+-K^+)ATPase preparations a small protein, which is a minor component of the preparation and barely visible on SDS–gels after staining, is labelled to a very significant extent (in fact, ~50%). In addition, the β-chain glycoprotein (M_r = 50 000) is always a major component of the purified (Na^+-K^+)ATPase and is not labelled significantly. It would appear that these photoaffinity labels are capable of labelling a small protein which is a component of the (Na^+-K^+)ATPase enzyme system rather than an insignificant minor contaminant.

The functional role of such a small protein component remains unclear, as does the precise function of the major protein components, the α- and β-chains, as well. It is interesting to note that a photeolipid of similar molecular weight has been found in the Ca^{2+}-ATPase of sarcoplasmic reticulum and that this protein may form an ionophore in this transport system [8,9].

A second observation was that the position of the photoreactive side chain on the affinity label has important effects on the pattern of photolabelling. Apparently the photoreactive side chain, the place where covalent attachment occurs, must be located on the sugar portion of the ouabain molecule in order to label the small protein. This was observed here for NAP-ouabain and in [3] where the nitrophenylazide moiety was incorporated into the sugar sidechain as well. However, NAP-strophanthidin has the nitrophenylazide group attached to the steroid moiety (fig.1) and it was clear that the small protein was not labelled to any extent under similar conditions. It appears that the small protein is associated more closely with the sugar-binding site than the steroid-binding site of the ouabain-binding center.

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