BBA 63404

Some properties of two phosphatases attached to insoluble cellulose matrices

Membrane-bound enzymes are involved in a variety of physiological processes, yet studies of such enzymes *in vitro* are usually made either with fragmented membranes or with isolated and purified material, so that properties associated with the original intact membrane structure may be lost. An alternative approach is to attach the isolated enzyme to an artificial membrane of well-defined chemical composition and then examine its properties[1]. A particularly interesting enzyme for such studies is the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3), because of the directional movements of Na^+ and K^+ associated with its activity *in situ*. It seemed possible that the behaviour of this enzyme attached to an artificial membrane might provide some insight into the relationship between enzyme activity and transport processes. This aim has not been achieved but the information acquired during our investigation may be of value to others working in this field.

The complex nature of the 'transport' enzyme, or system of enzymes, as it might be, made it necessary to start with a simpler system, to develop the techniques and to gain experience with them. We selected the potato ATPase, apyrase (ATP diphosphohydrolase, EC 3.6.1.5), as a suitable enzyme for this purpose, since it is readily obtainable, stable, easy to assay, and cation activated[2,3].

The apyrase was obtained as described previously[3] and for preliminary experiments it was attached to powdered forms of CM-cellulose azide or dichloro-*s*-triazinyl cellulose, using methods developed for other enzymes[4–7]. Protein concentrations were measured by the method of LOWRY *et al.*[8] and the amount of bound enzyme was estimated as the difference between that originally present in the reaction mixture and that remaining in the supernatant and washing solutions after attachment. Enzymic activity was assayed by measuring the initial rate of P_i release from ATP during incubation with the enzyme preparation at 37°, usually in a medium containing 2.5 mM ATP, 4 mM $CaCl_2$, 100 mM NaCl and 50 mM succinate buffer (pH 6.5). Trichloroacetic acid was added to stop the reactions and the concentration of P_i in the acid extracts was measured by the method of FISKE AND SUBBAROW[9].

The cellulose-bound apyrase preparations showed specific activities which were only 18–33% of that of the original enzyme. In most other respects the bound enzyme exhibited properties similar to those of the starting material. For example, neither form of the enzyme showed much activity in the absence of added Ca^{2+}, and both forms required about 0.2 mM $CaCl_2$ for half-maximal activation and 2–3 mM for complete activation. Similarly, there was no significant difference in the response of free and bound apyrase to variation in pH (contrast ref. 10). Both forms of the enzyme also possessed the peculiar property of behaving as both an ATPase and an ADPase at 37°, but only as an ATPase at 0°. The kinetic behaviour of the free and bound enzyme, however, was different. Fig. 1 shows a typical plot of v against $v/[ATP]$ for both forms of the enzyme: clearly, coupling the enzyme decreased the v_{max} and increased the K_m. Mean values from three experiments were 5.6 and 1.9 (mmoles P_i per g protein per min) for v_{max} of the free and bound enzyme, respectively, and 0.07 and 0.21 mM ATP for K_m.

The method[7] used for attaching apyrase to dichloro-*s*-triazinyl cellulose powder

proved to be suitable for the preparation of intact membranes containing apyrase, simply by replacing the cellulose powder with filter paper or dialysis tubing. The feasibility of using such preparations for permeability studies was checked by measuring the flux of Ca^{2+} through membranes of dialysis tubing to which apyrase had been linked. A small Perspex diffusion cell was constructed for this purpose and Ca^{2+} concentrations were measured by atomic absorption spectrophotometry. Although the techniques were developed satisfactorily, there was no difference in Ca^{2+} flux through the membranes when the bound apyrase was active (*i.e.* hydrolysing ATP) compared with the inactive state (*i.e.* in the absence of ATP).

On the basis of these preliminary studies with apyrase, attempts have been made to produce similar systems with a $(Na^+ + K^+)$-ATPase. 'Soluble' ATPase preparations, containing up to 75% ouabain-sensitive activity, were obtained from rabbit kidney by deoxycholate treatment, essentially as described by SKOU[11]. ATPase activity was assayed in the same way as apyrase activity, except that the incubation medium contained 120 mM NaCl, 20 mM KCl, 3 mM $MgSO_4$, 3 mM ATP and 20 mM Tris–HCl (pH 7.8). Ouabain-sensitive ATPase activity was determined as the difference in activity during incubation in the presence and absence of ouabain (0.1 mM). In spite of many attempts with a variety of experimental conditions, however, the techniques described above proved unsuitable with the kidney ATPase preparation. No coupling was achieved with dichloro-*s*-triazinyl cellulose prepared from dialysis tubing. Active preparations were obtained with this compound prepared from cellulose powder and from filter paper, but they proved to be unstable, losing all activity after a few consecutive incubations.

Several attempts were also made to trap this ATPase in porous membranes with

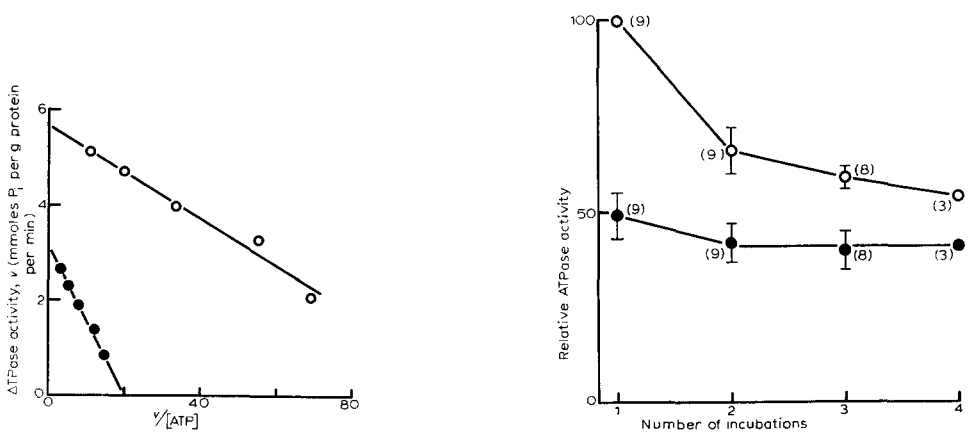

Fig. 1. Increase in K_m and decrease in v_{max} with bound apyrase. Experimental conditions were as described in the text, except that the ATP concentration was varied from 0.2 to 3.2 mM. ○, free apyrase; ●, bound apyrase.

Fig. 2. Loss of ouabain-sensitive ATPase activity after adsorption to a porous membrane. Adsorption of the kidney ATPase to Millipore filters is described in the text. The activity of the adsorbed material was determined by incubating the membranes in the standard medium, either with (●) or without (○) ouabain, for 30 min and measuring the amount of P_i liberated. The membranes were then transferred to fresh samples of the medium for each subsequent incubation. The results have been normalized by setting the initial total activity equal to 100. Mean values, with S.E., are given from the number of experiments noted in parentheses.

the use of bisdiazobenzidine or bisdiazobenzidine disulphonic acid as cross-linking reagents[12]. A variety of membranes were tried, including collodion membrane, filter paper and 'Millipore' filters. Again, these methods had worked with apyrase, but none of the attempts was successful with the kidney ATPase. Subsequent investigation of the effect of the diazo compounds on the ATPase showed that they completely inhibited the ouabain-sensitive activity when present at a concentration of 0.25 μM.

In the course of these experiments we found that substantial quantities of the ATPase were adsorbed to 'Millipore' and 'Sartorius' membrane filters when the ATPase preparation was forced through them under pressure. The 'Millipore' filters with average pore size of 220 mμ were examined in most detail. After thorough washing with cold 150 mM NaCl the adsorbed material consisted of approximately equal proportions of ouabain-sensitive and ouabain-insensitive ATPase. However, when these membranes were repeatedly incubated at 37°, the ouabain-sensitive activity gradually declined until practically none was detectable after three or four thirty-minute incubations. In contrast, the ouabain-insensitive activity remained almost constant (Fig. 2).

So far our findings illustrate the difficulties involved in trying to attach the ouabain-sensitive ATPase to cellulose membranes with the use of various techniques which were successful with apyrase. Although it is possible that the 'transport' ATPase merely requires further purification, the most promising approach seems to be simple adsorption to 'Millipore' filter membranes. This technique might possibly enable some orientation of the enzyme molecules within the membrane pores to be achieved, for example, by application of an electrical potantial difference across the membranes. It is first necessary, however, to find some means of preventing the preferential loss of the ouabain-sensitive activity after attachment to the artifical membrane. Recent observations suggest that this loss of activity could be caused by a gradual elution of an essential phospholipid component from the enzyme complex.

We are greatly indebted to the Science Research Council for financial support.

Department of Physiology,
University of Leicester,
Leicester (Great Britain)

K. P. WHEELER
B. A. EDWARDS
R. WHITTAM

1 H. I. SILMAN AND E. KATCHALSKI, *Ann. Rev. Biochem.*, 35 (1966) 873.
2 K. H. LEE AND J. J. EILER, *Science*, 114 (1951) 393.
3 R. WHITTAM, W. BARTLEY AND G. WEBER, *Biochem. J.*, 59 (1955) 590.
4 F. MICHEEL AND J. EWERS, *Makromol. Chem.*, 3 (1949) 200.
5 M. A. MITZ AND L. J. SUMMARIA, *Nature*, 189 (1961) 576.
6 W. E. HORNBY, M. D. LILLY AND E. M. CROOK, *Biochem. J.*, 98 (1966) 420.
7 G. KAY AND E. M. CROOK, *Nature*, 216 (1967) 514.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
10 H. D. BROWN, A. PATEL AND S. K. CHATTOPADHYAY, *Plant Physiol.*, 41 Suppl. (1966) IXVI.
11 J. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
12 R. GOLDMAN, O. KEDEM, I. H. SILMAN, S. R. CAPLAN AND E. KATCHALSKI, *Biochemistry*, 7 (1968) 486.

Received June 20th, 1969