Differential Effects of Temperature on a Membrane Adenosine Triphosphatase and Associated Phosphatase

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Arrhenius plots of a membrane ($\text{Na}^+ + \text{K}^+$)-dependent ATPase (adenosine triphosphatase) activity showed characteristic discontinuities, whereas those of the associated K^+ -dependent phosphatase activity did not. These findings support the contention that the phosphatase activity does not depend on phospholipid in the same way as does the ATPase activity.

Recently we described experiments that showed differential effects of lipid depletion on a membrane ($\text{Na}^+ + \text{K}^+$)-dependent ATPase* (EC 3.6.1.3) and the associated K^+ -dependent phosphatase (EC 3.6.1.7), from which we concluded that the phosphatase activity does not depend on lipid in the same way that the complete ATPase reaction does (Wheeler & Walker, 1975). We have now extended that work by comparing the responses of the two enzymic activities to temperature variation. Arrhenius plots of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity as a function of temperature show characteristic discontinuities that have been widely interpreted as reflecting the effect of the gel to liquid-crystalline phase transitions of associated phospholipids on the ATPase protein (Gruener & Avi-Dor, 1966; Smith, 1967; Kemp & Smith, 1970; Charnock *et al.*, 1971*a,b*; Taniguchi & Iida, 1972; Priestland & Whittam, 1972; Kimelberg & Papahadjopoulos, 1972, 1974). If that interpretation is correct, it follows that Arrhenius plots of K^+ -dependent phosphatase activity should reveal whether this activity has a similar or different dependence on phospholipids.

Methods

Microsomal fractions were isolated from homogenates of rabbit kidney and treated with 2M-NaI to give 'intact' ATPase preparations. Lipid-depleted samples of the latter were prepared by extraction with Lubrol WX. The details of these procedures, together with the methods by which ATPase and phosphatase activities were measured, have been described previously (Wheeler *et al.*, 1975; Wheeler & Walker, 1975). The incubation medium used for the ATPase assays was prepared as follows. A solution containing approx. 0.24M-NaCl, 0.04M-KCl, 0.006M-ATP (disodium salt), 0.006M-MgSO₄ and 0.06M-Tris was adjusted to pH 7.5 at about 20°C with HCl solution

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetic acid.

and then diluted to give final concentrations of exactly half the above values. The medium used for the phosphatase assays was prepared similarly, but the NaCl was omitted and disodium *p*-nitrophenyl phosphate replaced the ATP disodium salt. In some of the preliminary experiments (see under 'Results and discussion') the pH values were adjusted with the electrodes immersed in the solutions maintained at various different temperatures in the range 6–40°C. Temperatures between 6° and 40°C were maintained by circulating water pumped through the jackets of small vessels from a thermostatically controlled heater/refrigerator unit. The incubation mixtures in the vessels were stirred continuously with small magnetic stirrers and incubation times were varied so that about 10–20% hydrolysis of the substrates was catalysed at each temperature. Variations in the compositions of the incubation media are noted in the Figure legends. All other methods and materials were exactly as detailed by Wheeler *et al.* (1975) and Wheeler & Walker (1975).

The following results provide an indication of the reproducibility of the enzyme assays. Mean values ($\pm$ S.E.M.) of the ATPase activity (μmol of P_i /min per mg of protein) of the 'intact' enzyme preparations were 1.07 ± 0.16 (7) at 38°C and 0.008 ± 0.003 (7) at 6°C. The corresponding values for the K^+ -dependent phosphatase activity (μmol of *p*-nitrophenol/min per mg of protein) were 0.24 ± 0.03 (3) and 0.016 ± 0.003 (3).

Results and discussion

Variation of pH with temperature. Preliminary experiments were carried out to check the importance of variation in pH of the incubation media over the temperature range 6–40°C, because the high temperature coefficient of ionization of the amino group makes the pH of buffers such as Tris particularly sensitive to temperature (see Bull, 1964). Both ($\text{Na}^+ + \text{K}^+$)-dependent ATPase and K^+ -dependent phosphatase

of the various tissue preparations used exhibited rather broad pH optima, between pH 6.8 and 7.6, at 38°C. When the activities were measured over the entire temperature range only small variations were observed between the values obtained when the media used had previously been adjusted to pH 7.5 at the temperature of the experiment, and when the media had been adjusted to pH 7.5 at 20°C (results not shown). Since those small variations that were noted produced no significant changes in the Arrhenius plots, the remaining experiments were conducted with the use of media adjusted to pH 7.5 at 20°C.

Effect of temperature on 'intact' enzyme preparations. The changes with temperature of both ATPase and phosphatase activities of the 'intact' enzyme preparations (i.e. microsomal fractions treated with NaI) are illustrated in Fig. 1. Ouabain-sensitive activities are shown by lines A and B, whereas lines C and D give

Fig. 1. Effects of temperature on activity of 'intact' enzyme

Enzyme activities were measured as described in the text. Line A (▲), ouabain-sensitive ATPase activity, difference measured with and without 0.1 mM-ouabain (mean values from three to seven experiments). Line B (●), ouabain-sensitive phosphatase activity, difference measured with and without 0.2 mM-ouabain (mean values from three experiments). Line C (Δ), (Na⁺+K⁺)-dependent ATPase activity, difference measured in the presence and absence of K⁺ (mean values from two experiments). Line D (○), K⁺-dependent phosphatase activity, difference measured in the presence and absence of K⁺ (mean values from three experiments).

the differences in activities measured in the presence and absence of K⁺. Clearly, ouabain-sensitive and (Na⁺+K⁺)-dependent ATPase activities (lines A and C) were almost identical with each other over the entire temperature range and the biphasic nature of these Arrhenius plots are similar to those reported in the literature (see references listed in the introduction). These results are consistent with the common interpretation that the activation energy of the (Na⁺+K⁺)-dependent ATPase is influenced by the physical state of the phospholipids associated with the enzyme protein, the discontinuity in the Arrhenius plot reflecting the thermotropic gel to liquid-crystalline phase transition of the lipids.

In contrast, ouabain-sensitive and K⁺-dependent phosphatase activities were not always identical with each other. Under the conditions noted in Fig. 1, ouabain did not completely inhibit the K⁺-dependent phosphatase activity at any temperature. Moreover, this discrepancy between ouabain-sensitive and K⁺-dependent activities varied with temperature, becoming much more pronounced as the incubation temperature was lowered. The net result was that, although the Arrhenius plot of K⁺-dependent phosphatase activity produced a single straight line (Fig. 1, line D), the corresponding plot of ouabain-sensitive activity (Fig. 1, line B) gave a biphasic curve, which was similar to that exhibited by the ATPase activity (Fig. 1, line A).

It is well established that the sensitivity of this membrane ATPase system to inhibition by ouabain is markedly influenced by the experimental conditions used; in particular, the concentrations of Mg²⁺, Na⁺, K⁺, ATP and P_i are very important. In general, the conditions required for measuring ATPase activity (namely Mg²⁺, ATP, Na⁺ plus K⁺) favour interaction with and inhibition by ouabain, whereas those normally used for assaying the phosphatase activity (i.e. Mg²⁺ plus K⁺) do not. However, if Na⁺ and a low concentration of ATP are added to the incubation mixture used for measuring the phosphatase activity, so as to mimic the conditions of the ATPase assay, then the sensitivity of the phosphatase to inhibition by ouabain is increased, so that there is little difference between the concentrations of ouabain required to inhibit the ATPase and the phosphatase (see Whittam & Wheeler, 1970; Dahl & Hokin, 1974; Skou, 1975). The effect of temperature on the ouabain-sensitive phosphatase activity of the 'intact' enzyme was therefore checked again, this time with the use of 2 mM-ouabain, instead of 0.2 mM, and with the further addition of 0.1 mM-ATP plus 40 mM-NaCl. As shown in Fig. 2 (line A), the Arrhenius plot of ouabain-sensitive phosphatase activity then gave a single straight line, like that for K⁺-dependent phosphatase activity (Fig. 1, line D).

Taken together, these observations show quite clearly that the K⁺-dependent phosphatase was not

Fig. 2. Effects of temperature on phosphatase activity

Phosphatase activity was measured as described in the text. Line A (■), ouabain-sensitive activity of 'intact' enzyme, measured as difference with and without 2 mM-ouabain in the presence of 0.1 mM-ATP and 40 mM-NaCl. Lines B (○) and C (●), K^+ -dependent and ouabain-sensitive phosphatase activity, respectively, of lipid-depleted enzyme, measured as described in Fig. 1.

affected by temperature in the manner expected if it were dependent on phospholipid in the same way as the ATPase, in agreement with our previous conclusion (Wheeler & Walker, 1975). On the other hand, the effect of temperature on the extent of inhibition of the phosphatase by ouabain indicates that the interaction of ouabain with the enzyme was somehow affected by the physical state of the phospholipid present.

When Gruener & Avi-Dor (1966) examined the effects of temperature on ATPase preparations from rat brain, they concluded that the 'temperature effect in activation and inhibition of the phosphatase is much less striking than that for ATPase', and also noted that 0.1 mM-ouabain was ineffective as an inhibitor of the phosphatase below 16°C. Although our studies were not identical with those by Gruener & Avi-Dor (1966), and had a more specific aim, our findings are qualitatively in agreement with theirs. Since the present work was completed we have discovered that Barnett & Palazzotto (1974) have also reported that Arrhenius plots of the phosphatase

activity of ATPase preparations gave single straight lines, with no discontinuities. Direct comparison of results is impossible because those authors apparently measured only K^+ -dependent, and not ouabain-sensitive, phosphatase activity, but in general their findings and conclusions obviously are consistent with both our previous findings (Wheeler & Walker, 1975) and with those presented here.

Effect of temperature on lipid-depleted enzyme preparations. As described previously (Wheeler & Walker, 1975), ATPase preparations that have been depleted of about 90% of their endogenous phospholipids by extraction with Lubrol retain, on average, about 50% of the phosphatase activity of the 'intact' enzyme. Repetition of the experiments outlined above with such Lubrol-extracted preparations gave the results shown in Fig. 2, lines B and C. This time, K^+ -dependent phosphatase activity was only slightly greater than the ouabain-sensitive activity at most temperatures and Arrhenius plots of both gave virtually identical single straight lines, again similar to line D in Fig. 1. These results therefore support the conclusion drawn above that endogenous phospholipids somehow modify the interaction of ouabain with the enzyme under certain experimental conditions, because removal of most of those lipids removed the discontinuity from the Arrhenius plot of ouabain-sensitive activity, and almost abolished the difference between ouabain-sensitive and K^+ -dependent activities.

The residual ATPase activity after lipid depletion was far too low to permit accurate measurement over the temperature range below 38°C unless the enzyme was reactivated with exogenous phospholipid. We did check that the temperature-dependence of an enzyme reactivated with added phosphatidylserine was similar to that of the 'intact' enzyme, but such results are well documented in the literature and do not warrant further elaboration.

Activation energies. The activation energies for the ATPase and phosphatase were calculated from the slopes of the lines in Figs. 1 and 2. The values for the ATPase, both above the transition temperature (63–67 kJ/mol) and below (159 kJ/mol), were similar to those reported in the references listed in the introduction. Two features of those for the phosphatase are notable. First, the values for the 'intact' enzyme (63 kJ/mol from Fig. 1, line D; 46 kJ/mol from Fig. 2, line A) were about the same as those for the lipid-depleted preparations (54 kJ/mol, from lines B and C, Fig. 2). Secondly, these values were also similar to those for the ATPase at temperatures above the transition point, and therefore much lower than the activation energy of the ATPase below the transition temperature. Hence it seems clear that the presence of the phospholipids in the less fluid state below the transition temperature somehow increased the activation energy of the ATPase reaction but not of

the phosphatase. This suggests that some molecular motion, such as a conformation change, might be involved in the ATPase reaction but not in the phosphatase reaction. The complex relationships among the various ionic ligands, enzyme protein, lipids and extent of inhibition by ouabain merit further study.

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