BBA 66241

A FLUORIMETRIC ASSAY METHOD FOR THE K^+-PHOSPHATASE ASSOCIATED WITH THE ($Na^+ + K^+$)-ACTIVATED ATPase

B. J. R. PITTS AND A. ASKARI
Department of Pharmacology, Cornell University Medical College, New York, N.Y. (U.S.A.)

(Received September 14th, 1970)

SUMMARY

1. Data are presented which indicate that umbelliferone phosphate is a substrate for the K^+-activated phosphatase which is found in preparations of ($Na^+ + K^+$)-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3).

2. The hydrolysis of umbelliferone phosphate by this enzyme resembles that of *p*-nitrophenyl phosphate in that it requires Mg^{2+} and is activated by K^+ and this activation is inhibited by ouabain.

3. At low concentrations of K^+, it is further activated by ATP in the presence of Na^+ and this extra activation is inhibited by oligomycin.

4. The fluorimetric assay method, which has previously been used in the determination of acid and alkaline phosphatases, is simple, convenient and highly sensitive.

INTRODUCTION

In recent years much interesting information about the mechanism of the ($Na^+ + K^+$)-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been obtained from investigation of the K^+-activated phosphatase activity which appears to be catalysed by the same enzyme[1-15].

It seemed to us that further study of this partial reaction would yield still more interesting information if a more sensitive assay could be found. The hydrolysis of umbelliferone phosphate can readily be followed by measuring the increase in fluorescence due to the production of umbelliferone. This can be continuously monitored on a recorder and permits the direct determination of reaction rates in 1–5 min (depending on the rate) with great accuracy.

In this paper we present data which indicate that umbelliferone phosphate is a substrate for the K^+-activated phosphatase which catalyses the hydrolysis of *p*-nitrophenyl phosphate.

MATERIALS AND METHODS

Umbelliferone phosphate was obtained from Isolabs Inc., Elkhart, Ind. and umbelliferone from K and K laboratories, Plainview, N.Y. ($Na^+ + K^+$)-activated ATPase was prepared from rat brain by the method of SKOU[16].

All fluoresence measurements were made with an Eppendorf photometer model 1101 M fitted with a fluoresence attachment and connected through a recording adaptor to a Honeywell recorder. A Hg 313–366 nm primary filter and a 430–470 nm secondary filter were employed in all measurements.

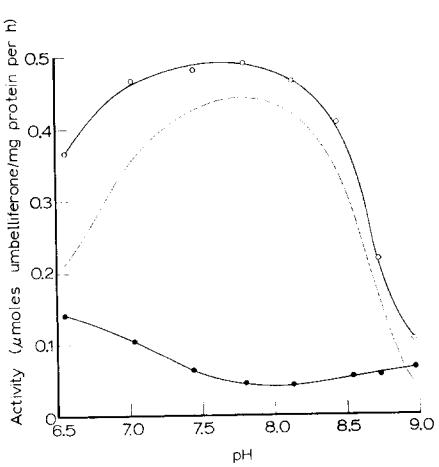
The assay method, which measures the amount of fluoresence due to umbelliferone, has previously been employed in the determination of acid and alkaline phosphatases[17]. Unless otherwise stated, all assays were conducted with an umbelliferone phosphate concentration of 0.2 mM in 0.08 M Tris–HCl (pH 7.8) in a final volume of 2.5 ml at 37°. The fluorimeter was standardised against a solution of umbelliferone ($4 \cdot 10^{-6}$ M) in the same buffer prior to each period of use. When assays were conducted at different pH values it was necessary to standardise with umbelliferone at each pH because the amount of fluoresence produced by umbelliferone is strongly dependent on pH. Mg^{2+}, K^+ and Na^+ were added as their chlorides and umbelliferone phosphate and ATP were converted to their Tris salts by treatment with Dowex 50W-X8 and readjustment to pH 7.8 with Tris base.

Not infrequently it was found that there was a considerable rate of apparently spontaneous hydrolysis. This could be eliminated if the deionised water to be used in the assay was boiled for 30 min.

RESULTS

pH optimum

Umbelliferone phosphatase activity was measured at different pH values in the presence of Mg^{2+} with and without K^+. These results are shown in Fig. 1 together with the K^+-stimulated activity, obtained by subtraction, which has a maximum at pH 7.8. The same pH optimum has been reported with *p*-nitrophenyl phosphate, carbamyl phosphate or acetyl phosphate as the substrate[3,6,8,11].



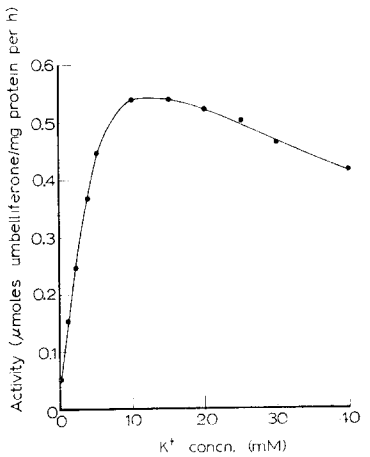


Fig. 1. Optimum pH for umbelliferyl phosphatase activity. All reaction mixtures contained 0.2 mM umbelliferone phosphate and 8 mM Mg^{2+}. ●, without K^+; ○, with 30 mM K^+. The dotted line indicates the K^+-stimulated activity obtained by subtraction.

Fig. 2. Effect of K^+ concentration on umbelliferone phosphatase activity .All reaction mixtures contained 0.2 mM umbelliferone phosphate and 8 mM Mg^{2+}.

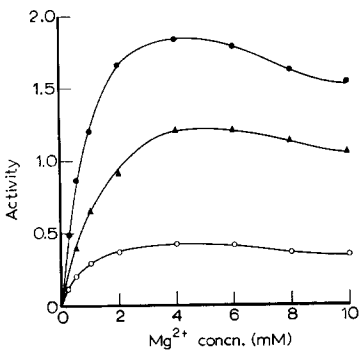
Effect of monovalent cations

The umbelliferone phosphatase activity was strongly dependent on the K^+ concentration. Maximal activation (10-fold) was obtained at a K^+ concentration of 10 mM as shown in Fig. 2. Very similar results have been reported for other substrates[4].

Activation by K^+ did not occur in the absence of Mg^{2+} and neither was any activation observed when Na^+ was added in the absence of K^+. Na^+ inhibited the activation by K^+ as previously observed with other substrates[6,10].

Optimal Mg^{2+} concentration

The effect of Mg^{2+} was tested at three widely different substrate concentrations with the same concentration of K^+ (10 mM). The optimal concentration of Mg^{2+} was found to be about 4 mM in each case (Fig. 3). Since the highest substrate concentration tested was 400 times greater than the lowest it is obvious that with this sub-



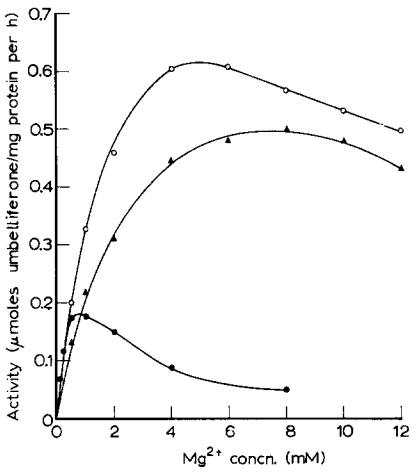


Fig. 3. Effect of Mg^{2+} at three widely different substrate concentrations. Different units of activity apply to each curve and are given in parentheses below. ○, 0.01 mM umbelliferone phosphate (μmoles/10 mg protein per h); ▲, 0.2 mM umbelliferone phosphate (μmoles/2 mg protein per h); ●, 4 mM umbelliferone phosphate (μmoles/mg protein per h).

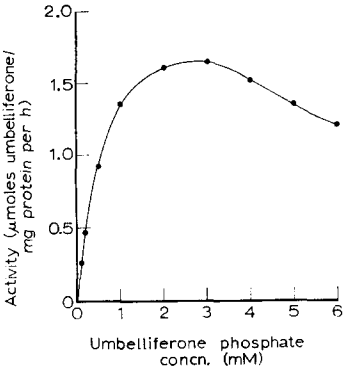
Fig. 4. Effect of Mg^{2+} at three different K^+ concentrations. All reaction mixtures contained 0.2 mM umbelliferone phosphate. ●, 1 mM K^+; ○, 10 mM K^+; ▲, 30 mM K^+.

strate the optimal Mg^{2+} concentration is not in any way related to the substrate concentration.

When, however, the effect of Mg^{2+} was tested at three different K^+ concentrations with the same umbelliferone phosphate concentration (0.2 mM), it was found that the optimal concentration increased as the K^+ concentration was increased (Fig. 4).

Effect of substrate concentration

The effect of umbelliferone phosphate was tested over a concentration range from 0.1 to 6 mM keeping the concentration of Mg^{2+} at 4 mM and K^+ at 10 mM



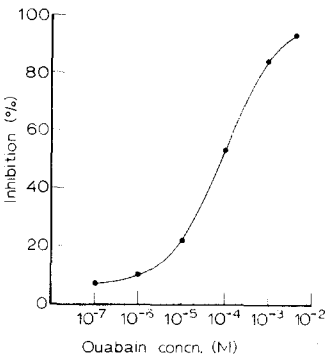


Fig. 5. Effect of substrate concentration. All reaction mixtures contained 4 mM Mg^{2+} and 10 mM K^+.

Fig. 6. Inhibition by ouabain. All reaction mixtures contained 0.2 mM umbelliferone phosphate, 4 mM Mg^{2+} and 10 mM K^+.

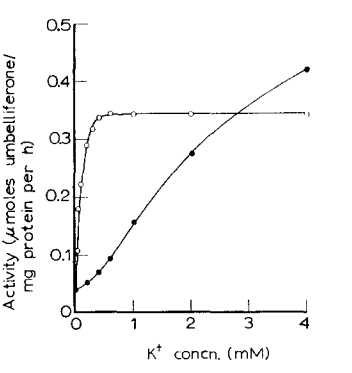
(Fig. 5). Maximal activity was observed at 3 mM and a K_m value of about 0.9 mM was obtained in good agreement with results previously reported for other substrates[4].

Inhibition by ouabain

The effect of ouabain on the K^+-stimulated umbelliferone phosphatase activity was tested over a range of concentrations from $1 \cdot 10^{-7}$ to $4 \cdot 10^{-3}$ M and the results obtained (Fig. 6) are very similar to those previously reported in which acetyl phosphate or *p*-nitrophenyl phosphate was the substrate[6,10]. Half maximal inhibition was obtained at about $8 \cdot 10^{-5}$ M ouabain.

Effect of ATP and Na^+ at low concentrations of K^+

Either ATP or Na^+ alone inhibited the K^+-stimulated umbelliferone phospha-



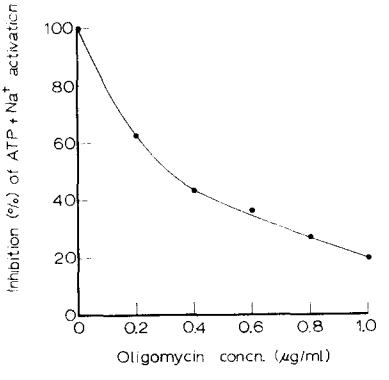


Fig. 7. Effect of K^+ on the activation by ATP + Na^+. All reaction mixtures contained 0.2 mM umbelliferone phosphate and 1 mM Mg^{2+}. ●, control; ○, with 12 μM ATP and 10 mM Na^+.

Fig. 8. Inhibition of ATP + Na^+ activation by oligomycin. The scale indicates the % inhibition of the difference between the activity with ATP + Na^+ and that with K^+ alone. All reaction mixtures contained 0.2 mM umbelliferone phosphate, 1 mM Mg^{2+}, 0.5 mM K^+, 10 mM Na^+ and 20 μM ATP.

tase activity, but when added together activation was observed at low K^+ concentrations as shown in Fig. 7. These results are remarkably similar to those previously obtained with *p*-nitrophenyl phosphate as the substrate[13].

The concentration of ATP employed ($1.2 \cdot 10^{-5}$ M) was lower than has previously been used in such studies and, even at this concentration, was definitely higher than optimal. If, however, a concentration of ATP lower than $1 \cdot 10^{-5}$ M was added the reaction rate did not remain linear for a sufficient period of time for an accurate estimation of the activity to be made. This was presumably due to hydrolysis of the ATP by the ATPase preparation which could be expected to occur when K^+ and Na^+ are both present.

Effect of oligomycin

Oligomycin caused little or no inhibition of the K^+-stimulated umbelliferone phosphatase activity but did inhibit the activation by ATP and Na^+ at low concentrations of K^+ as shown in Fig. 8. Similar results have been obtained with *p*-nitrophenyl phosphate as the substrate by ASKARI AND KOYAL[12].

DISCUSSION

The data presented in this paper indicate that the conditions under which umbelliferone phosphate and *p*-nitrophenyl phosphate are hydrolysed by $(Na^+ + K^+)$-activated ATPase are alike in that:

(1) Each has an optimum pH at 7.8.

(2) Each requires Mg^{2+} and is activated by K^+.

(3) Ouabain inhibits the K^+-stimulated activity.

(4) ATP and Na^+ activate at low K^+ concentrations.

(5) Oligomycin inhibits the activation by ATP and Na^+ but not the K^+-stimulated activity.

We feel that the results in this paper are sufficient evidence that umbelliferone phosphate is a substrate for the K^+-activated phosphatase which is associated with $(Na^+ + K^+)$ activated ATPase.

The method of assay used in the determination of umbelliferone phosphatase activity is much more sensitive than those previously employed in the determination of K^+-stimulated phosphatase with other substrates allowing assays to be conducted with substrate concentrations as low as $1 \cdot 10^{-6}$ M. Furthermore the method is continuous which has obvious advantages over a sampling method.

Another very useful feature of this assay method is that, with a suitable choice of substrate and enzyme concentrations, less than 1% of the substrate is hydrolysed in the period of time required to obtain an accurate estimate of the activity (3–5 min). Since the rate remains constant over a considerably longer period of time it is possible to determine the effect of increasing the concentration of a particular substance by sequential additions of μl quantities of concentrated solutions to the same reaction mixture. This technique eliminates inaccuracies due to variation between duplicate reaction mixtures.

In most investigations an umbelliferone phosphate concentration of 0.2 mM was employed. This concentration was chosen chiefly because, for most purposes, it gave a convenient reaction rate with 0.05 ml of the enzyme preparation (35 μg pro-

tein) and because the reaction rate remained linear for a considerable period of time. With a substrate concentration of 4 mM, the concentration normally used with *p*-nitrophenyl phosphate as the substrate, the reaction rate was linear for a much shorter period of time probably due to inhibition by products. Another disadvantage of high substrate concentrations was that the fluorescence due to umbelliferone phosphate was high enough to be inconvenient and after the substrate solution had been stored for some time at 2° the slow spontaneous hydrolysis caused the initial fluorescence of the reaction mixture to be so high as to be off the scale of the fluorimeter. This could only be avoided if fresh substrate solutions were prepared every few days while the same solution could be used for several weeks if assays were conducted with 0.2 mM umbelliferone phosphate.

The investigations reported here were concerned chiefly with demonstrating that umbelliferone phosphate is a substrate for the K^+-activated phosphatase. However, the results shown in Fig. 3 which indicate that the optimal Mg^{2+} concentration is independent of the substrate concentration are worthy of further consideration. In contrast, ROBINSON[14] has reported that the optimal Mg^{2+} concentration was dependent on the substrate concentration with *p*-nitrophenyl phosphate as the substrate for the K^+-activated phosphatase. While the different results could be due to the different substrates employed, the data on which he drew his conclusions appear to be inadequate and since he also reported that only one-fifth of the *p*-nitrophenyl phosphate existed as the magnesium complex under the conditions employed more extensive data would seem to be required.

The $(Na^+ + K^+)$-activated ATPase has also been found to have an optimal Mg–ATP ratio[18–20] but this can readily be explained if it is assumed that a Mg–ATP complex is the substrate for the ATPase reaction while no comparable complex is involved in the phosphatase reaction.

The results shown in Fig. 4 indicate that the optimal Mg^{2+} concentration is dependent on the K^+ concentration. This interdependence of Mg^{2+} and K^+ has previously been noted with *p*-nitrophenyl phosphate as the substrate[3,9]. The mechanism of this interdependence is not clear and the kinetics are complex. It may well be that the binding of Mg^{2+} to the enzyme results in a conformational change which alters its capacity to bind K^+.

ACKNOWLEDGEMENT

This investigation was supported by U.S. Public Health Service Grant HE-10884 from the National Heart Institute and by a grant-in-aid from the New York Heart Association.

REFERENCES

1 J. D. JUDAH, K. AHMED AND A. E. M. MCLEAN, *Biochim. Biophys. Acta*, 65 (1962) 472.
2 K. AHMED AND J. D. JUDAH, *Biochim. Biophys. Acta*, 93 (1964) 603.
3 K. NAGAI, F. IZUMI AND H. YOSHIDA, *J. Biochem. Tokyo*, 59 (1966) 295.
4 F. IZUMI, K. NAGAI AND H. YOSHIDA, *J. Biochem. Tokyo*, 60 (1966) 533.
5 H. YOSHIDA, F. IZUMI AND K. NAGAI, *Biochim. Biophys. Acta*, 120 (1966) 183.
6 M. FUJITA, T. NAKAO, Y. TASHIMA, N. MIZUNO, K. NAGANO AND M. NAKAO, *Biochim. Biophys. Acta*, 117 (1966) 42.
7 K. NAGAI AND H. YOSHIDA, *Biochim. Biophys. Acta*, 128 (1966) 410.
8 H. BADER AND A. K. SEN, *Biochim. Biophys. Acta*, 118 (1966) 116.

9 R. W. ALBERS AND G. J. KOVAL, *J. Biol. Chem.*, 241 (1966) 1896.
10 Y. ISRAEL AND E. TITUS, *Biochim. Biophys. Acta*, 139 (1967) 450.
11 G. SACHS, J. D. ROSE AND B. I. HIRSCHOWITZ, *Arch. Biochem. Biophys.*, 119 (1967) 277.
12 A. ASKARI AND D. KOYAL, *Biochem. Biophys. Res. Commun.*, 32 (1968) 227.
13 H. YOSHIDA, K. NAGAI, T. OHASHI AND Y. NAKAGAWA, *Boichim. Biophys. Acta*, 171 (1969) 178.
14 J. D. ROBINSON, *Biochemistry*, 8 (1969) 3348.
15 A. ASKARI, *Biochim. Biophys. Acta*, 191 (1969) 198.
16 J. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
17 G. G. GUILBAULT, S. H. SADER, R. GLAZER AND J. HAYNES, *Anal. Letters*, 1 (1968) 333.
18 J. C. SKOU, *Biochim. Biophys. Acta*, 42 (1960) 6.
19 K. P. WHEELER AND R. WHITTAM, *Biochem. J.*, 85 (1962) 495.
20 W. SCHONER, C. VON ILBERG, R. KRAMER AND W. SEUBERT, *European J. Biochem.*, 1 (1967) 334.