Journal of Bioenergetics and Biomembranes, Vol. 20, No. 5, 1988

Characteristics of 3-*O*-Methylfluorescein Phosphate Hydrolysis by the $(Na^+ + K^+)$-ATPase[1]

Richard L. Davis[2] and Joseph D. Robinson[2,3]

Received February 4, 1988

Abstract

With 3-*O*-methylfluorescein phosphate (3-OMFP) as substrate for the phosphatase reaction catalyzed by the $(Na^+ + K^+)$-ATPase, a number of properties of that reaction differ from those with the common substrate *p*-nitrophenyl phosphate (NPP): the K_m is 2 orders of magnitude less and the V_{max} is two times greater, and dimethyl sulfoxide (Me_2SO) inhibits rather than stimulates. In addition, reducing the incubation pH decreases both the K_m and V_{max} for K^+-activated 3-OMFP hydrolysis as well as the $K_{0.5}$ for K^+ activation. However, reducing the incubation pH increases inhibition by P_i and the V_{max} for 3-OMFP hydrolysis in the absence of K^+. When choline chloride is varied reciprocally with NaCl to maintain the ionic strength constant, NaCl inhibits K^+-activated 3-OMFP hydrolysis modestly with 10 mM KCl, but stimulates (in the range 5–30 mM NaCl) with suboptimal (0.35 mM) KCl. In the absence of K^+, however, NaCl stimulates increasingly over the range 5–100 mM when the ionic strength is held constant. These observations are interpreted in terms of (a) differential effects of the ligands on enzyme conformations; (b) alternative reaction pathways in the absence of Na^+, with a faster, phosphorylating pathway more readily available to 3-OMFP than to NPP; and (c) a $(Na^+ + K^+)$-phosphatase pathway, most apparent at suboptimal K^+ concentrations, that is also more readily available to 3-OMFP.

Key Words: $(Na^+ + K^+)$-ATPase; 3-*O*-methylfluorescein phosphate; nitrophenyl phosphate; dimethyl sulfoxide; P_i; pH.

Introduction

The $(Na^+ + K^+)$-ATPase reaction cycle involves a Na^+-activated phosphorylation of the enzyme to form E_1P, an intermediate that is resistant to

[1] Abbreviations: Et_3N, triethyl amine; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; Me_2SO, dimethyl sulfoxide; NPP, *p*-nitrophenyl phosphate; 3-OMFP, 3-*O*-methylfluorescein phosphate; TNP–ATP, 2′ (or 3′)-*O*-(2,4,6-trinitrophenyl)-ATP.

[2] Department of Pharmacology, SUNY Health Science Center, Syracuse, New York 13210.
[3] To whom correspondence should be addressed.

0145-479X/88/1000-0571$06.00/0

hydrolysis, but is subsequently transformed to a second phosphorylated intermediate, E_2P, that is sensitive to K^+-activated dephosphorylation (Albers, 1967; Robinson and Flashner, 1979; Cantley, 1981; Glynn, 1985):

$$E_1 \xrightarrow[Na^+]{+ATP} E_1P \longrightarrow E_2P \xrightarrow[K^+]{-P_i} E_2 \longrightarrow E_1$$

The enzyme also catalyzes a K^+-activated hydrolysis of several phosphoric acid anhydrides, which appears to reflect the K^+-activated hydrolysis of E_2P (Robinson and Flashner, 1979; Cantley, 1981; Glynn, 1985). NPP is the most commonly studied substrate, and recently a low level of K^+-independent hydrolysis was demonstrated (Robinson, 1985). Another substrate for this reaction, 3-OMFP, is notable for its very low K_m (Huang and Askari, 1975), nearly 2 orders of magnitude less than that for NPP (Davis and Robinson, 1988). Here are described a number of other characteristics of 3-OMFP hydrolysis that contrast sharply with NPP hydrolysis: higher V_{max}, higher activity of K^+-independent hydrolysis compared with K^+-activated hydrolysis, greater stimulation by Na^+, and an opposite response to Me_2SO. These observations are considered both in terms of ligand effects on enzyme conformation and of alternative reaction pathways differentially available to the phosphatase substrates.

While this work was in progress, an article by Nagamune *et al.* (1986) appeared that also describes K^+-independent hydrolysis of 3-OMFP.

Materials and Methods

The enzyme preparation was obtained from frozen canine kidney medullae by the procedure described by Jorgensen (1974). Specific activities for the (Na + K)-ATPase reaction in the standard medium (see below) ranged from 14 to 21 μmol P_i released/min mg of protein.

(Na + K)-ATPase activity, assayed in terms of P_i release (Robinson, 1967), was determined at 37° C in a standard medium containing 20 mM HEPES/Et_3N (pH 7.5), 0.1 mM EGTA, 3 mM $MgCl_2$, 90 mM NaCl, 10 mM KCl, and 3 mM ATP (Et_3N salt).

Phosphatase activity at pH 7.5 and 37° C was assayed in a similar standard medium, but lacking ATP and NaCl. Phosphatase activity at pH 6.0 was assayed in the same medium, except that 20 mM MES/Et_3N (pH 6.0) was substituted for 20 mM HEPES/Et_3N (pH 7.5). 3-OMFP hydrolysis was routinely assayed continuously in a stirred cuvette in terms of 3-*O*-methylfluorescein release, measured spectrophotofluorometrically, with excitation at 475 nm and emission at 515 nm (Davis and Robinson, 1988); the standard medium contained 20 μM 3-OMFP. The unit of phosphatase activity

is the difference between the activity present in the standard media at pH 7.5 with 10 mM KCl and the activity present in the same media but in the absence of KCl and the presence of 0.3 mM ouabain, with that activity defined as 1.0. NPP hydrolysis was in this instance assayed in terms of P_i release (Robinson, 1967).

Data are presented as the average of three or more experiments, each performed in duplicate or triplicate. Standard errors of the mean are presented where pertinent.

Frozen kidneys were obtained from Pel-Freeze, TNP–ATP from Molecular Probes, and ouabain, oligomycin, ATP, and 3-OMFP from Sigma.

Results

Substrate Kinetics

With 3-OMFP as substrate, the phosphatase reaction catalyzed by the (Na + K)-ATPase differs in a number of characteristics from that with the more commonly used substrate NPP. In the presence of 10 mM KCl and saturating 3-OMFP concentrations, the K-phosphatase activity is twice that with saturating NPP concentrations, approaching the (Na + K)-ATPase activity (Table I). Moreover, the K_m for 3-OMFP, 50 μM, is nearly 2 orders of magnitude less than that for NPP (Fig. 1, and Tables I and II), as previously reported (Davis and Robinson, 1988).

The enzyme also catalyzes the hydrolysis of 3-OMFP in the absence of KCl (the only monovalent cations present being H^+ and Et_3NH^+), at a maximal velocity that is 7% of the maximal velocity in the presence of 10 mM KCl (Table II). By contrast, the maximal velocity with NPP in the absence

Table I. Kinetic Parameters for Hydrolytic Activities[a]

Activity	Relative V_{max}	K_m (mM)
$(Na^+ + K^+)$-ATPase	1.0	0.4
K^+-3-OMFPase	0.6	0.05
K^+-NPPase	0.3	3[b]

[a] $(Na^+ + K^+)$-ATPase activity was measured in the standard media but with ATP varied ($MgCl_2$ was kept 0.5 mM greater than the ATP concentration); for this Table, the maximal velocity was defined as 1.0; the K_m reported is for the low-affinity substrate sites. K^+-3-OMFPase activity was similarly measured by varying the 3-OMFP concentration in the standard medium for 3-OMFP hydrolysis, but for evaluating the relative V_{max} the velocity was measured in terms of P_i release, and expressed relative to that of the ATPase reaction. Relative K^+-NPPase activity was also measured for this purpose in terms of P_i release, in incubations in which NPP was substituted for 3-OMFP.

[b] From Robinson *et al.* (1986).

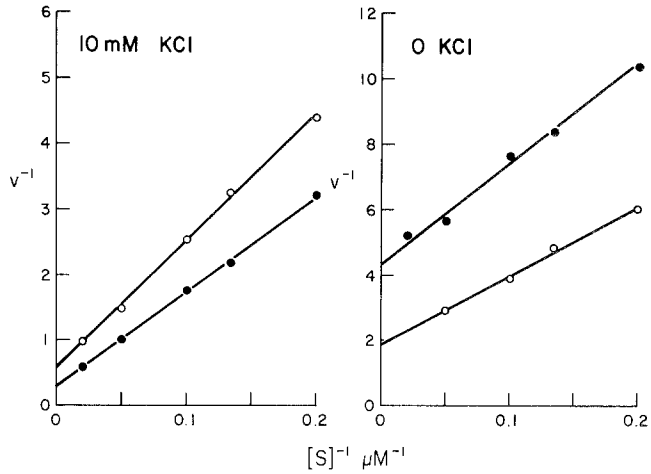

Fig. 1. Effect of 3-OMFP concentration on phosphatase activity. Activity was measured in media containing either 20 mM HEPES/Et_3N (pH 7.5) (●) or 20 mM MES/Et_3N (pH 6.0) (○), 3 mM $MgCl_2$, 0.1 mM EGTA, the concentrations of 3-OMFP shown, and either 10 mM KCl (left panel) or no KCl (right panel). Velocities are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both with 20 μM 3-OMFP) defined as 1.0. Data are presented in double-reciprocal form and represent the means of three or more experiments, each performed in duplicate or triplicate.

of KCl is 3–4% of the maximal velocity with 10 mM KCl (Robinson and Davis, 1987), half that seen with 3-OMFP as substrate. In the absence of KCl, the K_m for 3-OMFP is markedly reduced, to 7 μM (Fig. 1 and Table II); with a suboptimal KCl concentration (1 mM), the observed K_m is 37 μM (not shown), intermediate between these values.

Reducing the pH of the incubation medium from pH 7.5 to 6.0 (changing the buffer anion from HEPES to MES, the ionic strength remaining

Table II. Substrate Kinetics[a]

	pH 7.5			pH 6.0		
	K_m (μM)	V_{max}	Ratio of V_{max}	K_m (μM)	V_{max}	Ratio of V_{max}
No KCl	7	0.24	0.07	12	0.54	0.3
10 mM KCl	50	3.6		35	1.8	

[a] Activity was measured in media containing either 20 mM HEPES/Et_3N (pH 7.5) or 20 mM MES/Et_3N (pH 6.0), 3 mM $MgCl_2$, 0.1 mM EGTA, varying concentrations of 3-OMFP, and either 10 mM KCl or no KCl. Velocities are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both with 20 μM 3-OMFP) defined as 1.0. Results are averages of three or more experiments, each performed in duplicate or triplicate.

Table III. Effects of Inhibitors[a]

	K_i for ATP (μM)	K_i for TNP–ATP (μM)	K_i for P_i (μM)	
	At pH 7.5	At pH 7.5	At pH 7.5	At pH 6.0
No KCl	0.5	0.01	45	130
1 mM KCl	–	0.35	700	–
10 mM KCl	300	1.4	1000	350

[a] Activity was measured in media containing either 20 mM HEPES/Et_3N (pH 7.5) or 20 mM MES/Et_3N (pH 6.0), 3 mM $MgCl_2$, 0.1 mM EGTA, various concentrations of 3-OMFP and of the inhibitors listed, and either 10 mM KCl, 1 mM KCl, or no KCl. Results are averages of three or more experiments, each performed in duplicate or triplicate; K_i values were obtained from Dixon plots.

essentially the same) reduces the maximal velocity of 3-OMFP hydrolysis with 10 mM KCl twofold (Table II). 3-OMFP hydrolysis in the absence of KCl, however, doubles with that reduction in pH (Table II), so that the ratio of activities without KCl to that with KCl is then 0.3. The K_m for 3-OMFP, on the other hand, changes relatively little with pH (Table II).

ATP acts as a competitor to the phosphatase substrates (Robinson, 1976; Davis and Robinson, 1988) with a K_i in the presence of 10 mM KCl (Table III) near the K_m for ATP at its low-affinity substrate sites (Robinson, 1976). In the absence of KCl, the K_i for ATP is reduced nearly 3 orders of magnitude (Table III). TNP–ATP, an ATP analog not hydrolyzed by this enzyme, has a far higher apparent affinity, but the K_i is 2 orders of magnitude less in the absence of KCl than in its presence (Table III).

P_i is also a competitor to the phosphatase substrates (Robinson, 1970b), and the K_i in the presence of KCl also decreases sharply as the pH is reduced (Table III). At pH 6.0, the K_i with 10 mM KCl is decreased to a third of that at pH 7.5, whereas the K_i in the absence of KCl, although still lower than that with KCl, is greater than at pH 7.5 (Table III).

Effects of Cations

Divalent cations are required for phosphatase activity, and Mg^{2+} is the optimal cation (Robinson, 1985). For 3-OMFP hydrolysis in the presence of 10 mM KCl, the K_m for $MgCl_2$ is 0.5 mM at pH 7.5, but increases fourfold at pH 6.0 (Fig. 2). In the absence of KCl, the K_m at pH 7.5 is far less, 0.1 mM, but increases at pH 6.0 to almost the same value as with 10 mM KCl (Fig. 2).

Evaluating the effects of KCl on 3-OMFP hydrolysis is complicated by the significant activity in the absence of KCl. When the increment in 3-OMFP hydrolysis due to KCl, Δv, is plotted against KCl concentration in double-reciprocal form, then the lines curve concavely upward (Fig. 3) and the slopes of the Hill plots are near 2 (not shown). However, expressing velocity

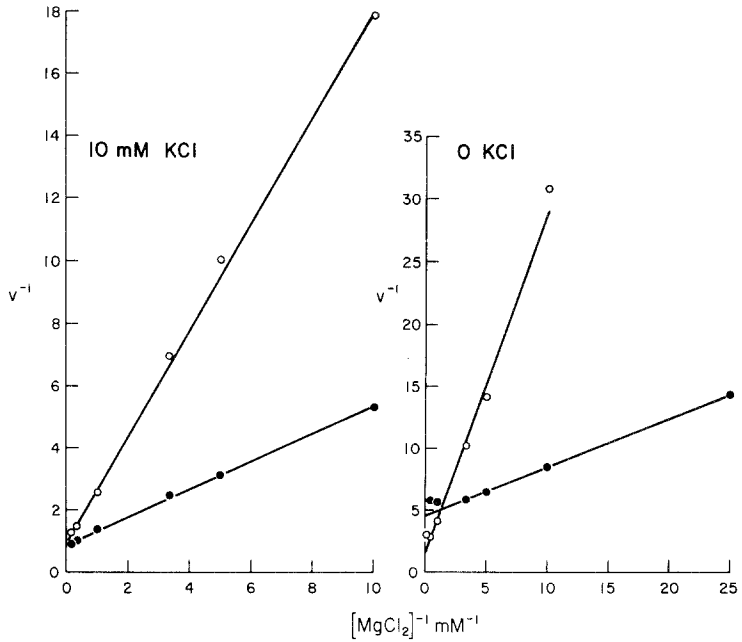

Fig. 2. Effect of $MgCl_2$ on phosphatase activity. Activity was measured in media containing either 20 mM HEPES/Et_3N (pH 7.5) (●) or 20 mM MES/Et_3N (pH 6.0) (○), 0.1 mM EGTA, 20 μM 3-OMFP, the concentrations of $MgCl_2$ shown, and either 10 mM KCl (left panel) or no KCl (right panel). Velocities are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both with 3 mM $MgCl_2$) defined as 1.0. Data are presented in double-reciprocal form and represent the means of three or more experiments, each performed in duplicate or triplicate.

as the difference between that in the presence and absence of KCl is somewhat inaccurate, since as KCl is increased the fraction of the total enzyme catalyzing K-independent 3-OMFP hydrolysis undoubtedly declines, and consequently the higher the KCl concentration the more Δv underrepresents K^+-phosphatase activity [to a maximum of 7% at pH 7.5 and 40% at pH 6.0 (Table II)], thereby increasing the curvature of the double-reciprocal plots. [A more complex but slightly better approximation would be to increase the plotted values of velocity from Δv toward v at successively higher concentrations of KCl. This change would then reflect the increasing fraction of the total enzyme that is catalyzing K^+-activated hydrolysis as the KCl concentration is raised. However, such modification of the plots (not shown) affects the estimated $K_{0.5}$ values scarcely at all: the only change is in the $K_{0.5}$ for K^+ with 50 μM 3-OMFP, which then decreases from 0.7 mM to 0.6 mM.] In any case, several aspects of the response to KCl are apparent. Raising the 3-OMFP concentration from 20 μM to 50 μM increases this estimated $K_{0.5}$ somewhat,

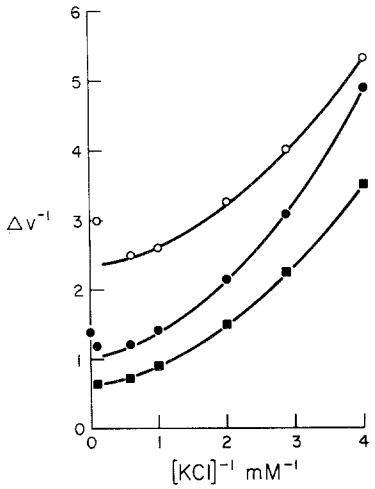

Fig. 3. Effect of KCl concentration on phosphatase activity. Activity was measured in media containing either 20 mM HEPES/Et_3N (pH 7.5) (● and ■) or 20 mM MES/Et_3N (pH 6.0) (○), 3 mM $MgCl_2$, 0.1 mM EGTA, either 20 μM (● and ○) or 50 μM (■) 3-OMFP, and the concentrations of KCl shown. Δv is defined as the increment in activity produced by a given concentration of KCl over the activity observed in the absence of both KCl and ouabain. The magnitudes of the plotted values of Δv are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both at pH 7.5 and with 20 μM 3-OMFP) defined as 1.0. Data are presented in double-reciprocal form and represent the means of three or more experiments, each performed in duplicate or triplicate.

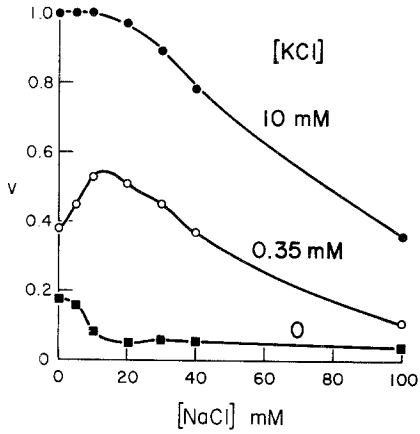

Fig. 4. Effect of NaCl concentration on phosphatase activity. Activity was measured in media containing 20 mM HEPES/Et_3N (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EGTA, 20 μM 3-OMFP, the concentrations of NaCl shown, and either 10 mM KCl (●), 0.35 mM KCl (○), or no KCl (■). Velocities are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 ouabain (both with no NaCl) defined as 1.0. Data presented are means of three or more experiments, each performed in duplicate or triplicate.

from 0.5 mM to 0.7 mM (or 0.6 mM) at pH 7.5 (Fig. 3). Reducing the pH from 7.5 to 6.0 decreases the estimated $K_{0.5}$, with 20 μM 3-OMFP, to 0.3 mM (Fig. 3). Finally, inhibition at higher KCl concentrations is apparent with 20 μM 3-OMFP, attributable at least in part to the effect of KCl on the K_m for 3-OMFP (Fig. 1).

Adding NaCl reduces 3-OMFP hydrolysis in both the absence of KCl and the presence of 10 mM KCl, but 5–30 mM NaCl stimulates in the presence of a suboptimal KCl concentration, 0.35 mM (Fig. 4). Inhibition by NaCl could result not only from competition with K^+ for K^+ sites (when KCl is present), but also from effects of ionic strength (Robinson and Davis, 1987). To assess this latter possibility, the effects of choline chloride were also examined (Fig. 5). Choline chloride inhibits in both the absence and presence of 10 mM KCl, but with quite different sensitivities: 60 mM choline chloride is required for 50% inhibition in the presence of 10 mM KCl, but only 15 mM in the absence of KCl. Moreover, when NaCl and choline chloride are varied reciprocally, to maintain a constant total concentration of 100 mM and thus a constant ionic strength, then the percentage decline in K^+-phosphatase activity due to NaCl is far less (Fig. 6) than when NaCl is increased without compensating for the changes in ionic strength (Fig. 4). Even more striking is the response to NaCl of 3-OMFP hydrolysis in the absence of KCl (Fig. 6): with compensating choline chloride, Na^+ then stimulates over the entire concentration range. [With 0.35 mM KCl, adding chlorine chloride only

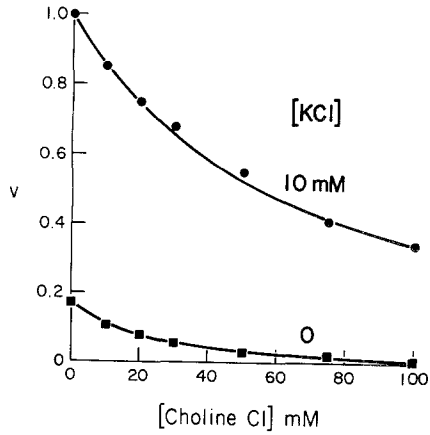

Fig. 5. Effect of choline chloride on phosphatase activity. Activity was measured in media containing 20 mM HEPES/Et_3N (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EGTA, 20 μM 3-OMFP, the concentrations of choline chloride shown, and either 10 mM KCl (●) or no KCl (■). Velocities are expressed relative to the difference between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both with no choline chloride) defined as 1.0. Data presented are means of three or more experiments, each performed in duplicate or triplicate.

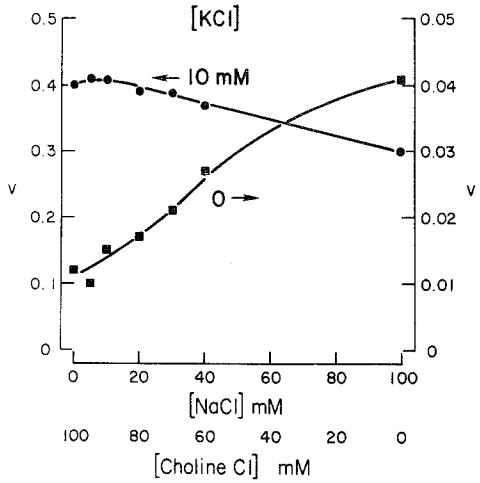

Fig. 6. Effect of varying NaCl and choline chloride concentrations reciprocally on the phosphatase activity. Activity was measured in media containing 20 mM HEPES/Et_3N (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EGTA, 20 μM 3-OMFP, the concentrations of NaCl and choline chloride shown (so that the sum of the two concentrations equaled 100 mM), and either 10 mM KCl (●) (left ordinate) or no KCl (■) (right ordinate). Velocities are expressed relative to the differences between the activity with 10 mM KCl and that in the absence of KCl and the presence of 0.3 mM ouabain (both with no NaCl or choline chloride) defined as 1.0. Data presented are means of three or more experiments, each performed in duplicate or triplicate.

inhibits, and substituting 10 mM NaCl plus 90 mM choline chloride for 100 mM choline chloride stimulates 3-OMFP hydrolysis as 10 mM NaCl does (Fig. 4) in the absence of choline chloride.]

Effects of Inhibitors

Oligomycin inhibits 3-OMFP hydrolysis in the absence and presence of 10 mM KCl (Table IV), and inhibits more with suboptimal (0.2 mM) than optimal (10 mM) KCl. However, with 0.2 mM KCl plus 10 mM NaCl, oligomycin does not inhibit. By contrast, with NPP as substrate, oligomycin inhibits negligibly with 10 mM KCl, appreciably with suboptimal KCl concentrations, and stimulates with suboptimal KCl plus NaCl (Robinson *et al.*, 1984).

Me_2SO inhibits 3-OMFP hydrolysis under all conditions (Table IV). Again, these results contrast with those measuring NPP hydrolysis: Me_2SO stimulates NPP hydrolysis in the absence and presence of KCl, decreases the $K_{0.5}$ for KCl as activator, and markedly stimulates NPP hydrolysis with suboptimal KCl plus NaCl (Robinson *et al.*, 1984; Robinson, 1985). However, the response of 3-OMFP hydrolysis to Me_2SO resembles that of NPP hydrolysis in one regard: Me_2SO decreases the K_m for 3-OMFP (at pH 7.5

Table IV. Inhibition of Phosphatase Activity[a]

Addition	Percent Inhibition by	
	Me_2SO (10% V/V)	Oligomycin (10 μg/ml)
None	65 ± 4	37 ± 5
0.2 mM KCl	51 ± 5	39 ± 4
10 mM KCl	40 ± 1	12 ± 1
10 mM choline chloride	61 ± 4	39 ± 1
0.2 mM KCl plus 10 mm choline chloride	45 ± 6	30 ± 3
10 mM NaCl	63 ± 2	12 ± 4
0.2 mM KCl plus 10 mM NaCl	43 ± 7	2 ± 2

[a]Activity was measured in media containing 20 mM HEPES/Et_3N (pH 7.5), 3 mM $MgCl_2$, 0.1 EGTA, 20 mM 3-OMFP, the salts indicates, and either Me_2SO or oligomycin. The percent inhibition (plus or minus the standard error of the mean) is calculated relative to the corresponding control, treated identically except for the absence of inhibitor. Results are averages of four experiments, each performed in duplicate.

with 10 mM KCl) to 35 μM (not shown) just as Me_2SO decreases the K_m for NPP (Robinson, 1972).

Discussion

Two sets of issues are raised by these experiments: the particular properties of 3-OMFP hydrolysis and the mechanistic implications both of these properties and of their differences from those of NPP hydrolysis. Because of the relationships between these sets of issues, however, they will be discussed in parallel. Interpretations are based on the following considerations: (a) phosphatase activity is catalyzed by E_2 enzyme conformations (Robinson *et al.*, 1983; Berberian and Beauge, 1985); (b) K^+ favors E_2 conformations and also promotes substrate hydrolysis (Glynn 1985); (c) H^+ favors E_2 conformations (Skou and Esmann, 1980), although probably a different subset, and may inhibit substrate hydrolysis; (d) P_i can phosphorylate the enzyme to form E_2P (Post *et al.*, 1975), but K^+ favors hydrolysis of E_2P whereas H^+ stabilizes E_2P; and (e) Me_2SO favors E_2 conformations also (Robinson *et al.*, 1984) as well as E_2P formation from P_i (Goncalves de Moraes and de Meis, 1987), whereas oligomycin favors E_1 conformations (Robinson *et al.*, 1984).

The far lower K_m for 3-OMFP, compared with NPP and other phosphatase substrates, is readily attributable to the fluorescein ring structure of 3-OMFP occupying the adenine-binding region of the enzyme substrate site,

as previously proposed (Davis and Robinson, 1988). Although 3-OMFP may bind preferentially to the high-affinity substrate sites of E_1 (Nagamune *et al.*, 1986), as also argued for NPP (Berberian and Beauge, 1985), it seems unlikely that such occupancy is required. With the FITC-modified enzyme, which is unable to bind ATP at the high-affinity sites of E_1 because of the fluorescein ring system of FITC occupying those cites (Carilli *et al.*, 1982), the V_{max} for K^+-activated hydrolysis of both 3-OMFP and NPP is unaffected: this is attributable to the FITC, which is tethered through its isothiocyanate chain to Lys 501 of the enzyme, being pulled from the adenine pocket with the E_1 to E_2 transition (Davis and Robinson, 1988). 3-OMFP hydrolysis in the absence of K^+ is also not blocked by FITC treatment (unpublished observations). Thus, the active site of E_1, which by these arguments is unavailable on the FITC-treated enzyme, cannot be essential.

The decrease in K_m for 3-OMFP when the pH is reduced from 7.5 to 6.0 is consistent with lower pH's favoring the E_2 conformations catalyzing the phosphatase reaction. However, K^+ also favors E_2 conformations, but raising the K^+ concentration increases the K_m: obviously, H^+ and K^+ favor different subconformations of the E_2 family, and act separately. Nagamune *et al.* (1986) argued that the phosphatase substrates add to E_1 conformations, but that catalysis is effected by an E_2 conformation; although this proposal accounts for K^+ effects on the K_m for 3-OMFP, it does not account for the H^+ effects.

These considerations also bear on the K^+-independent phosphatase activity: although K^+-phosphatase activity decreases when the pH is reduced to 6.0, the apparent $K_{0.5}$ for K^+ is not increased and thus no competition between H^+ and K^+ for the K^+ sites is apparent: H^+ seems not to activate as a K^+ analog at K^+ sites. Thus, K^+ has two actions, to favor (certain) E_2 conformations and to promote substrate hydrolysis, whereas H^+ favors (certain) E_2 conformations but inhibits hydrolysis; accordingly, even at pH 6.0, hydrolytic activity with K^+ is greater than in its absence (Table II).

Another, contrasting, mechanism by which H^+ could act is as a substitute for Na^+ (Blostein, 1983; Hara and Nakao, 1986): activity at low pH in the absence of K^+ would then be comparable to Na^+-activated hydrolysis. If this were so, H^+ would seem to be a better activator than Na^+ for, with the H^+ concentration of pH 6.0, the K^+-independent activity is greater than the activity with 100 mM NaCl at pH 7.5, also in the absence of KCl (Table I and Fig. 6). A further suggestion that H^+ might act like Na^+ is the increased sensitivity to K^+ seen at pH 6.0 (Fig. 3): this could reflect the increased sensitivity to low K^+ concentrations seen with Na^+ in (Na^+ + K^+)-activated NPP hydrolysis (Robinson, 1970a). Neither argument seems compelling in light of evidence that Na^+ favors E_1 conformations whereas H^+ favors E_2 (Skou and Esmann, 1980) and actions through E_2 seem plausible here.

Mg^{2+} activation is also sensitive to pH, the K_m rising with a reduction in pH in both the absence and presence of K^+ (Fig. 2). This observation also indicates that Mg^{2+} is not substituting for K^+ in activating 3-OMFP hydrolysis: reducing the pH does not favor activation by Mg^{2+}.

Inhibition by P_i may bear on these issues as well, for P_i readily phosphorylates the enzyme to form E_2P. Consequently, reducing the pH, to favor that subset of E_2 conformations, would be expected to promote phosphorylation by P_i and thus inhibition of 3-OMFP hydrolysis (Table III); whereas increasing the K^+ concentration, to promote E_2P hydrolysis, would be expected to lessen inhibition by P_i (Table III).

Although hydrolysis of 3-OMFP in the absence of K^+ may be attributable to that fraction of the enzyme in the E_2 conformation effecting hydrolysis without benefit of K^+ at its activating sites, the observation that such K^+-independent activity is greater with 3-OMFP than with NPP remains unexplained. An obvious possibility is that alternative catalytic pathways are differently available to the two substrates. Shaffer *et al.* (1978) concluded from ^{18}O exchange studies that NPP hydrolysis did not involve enzyme phosphorylation, whereas acetyl phosphate hydrolysis did, and acetyl phosphate hydrolysis is also quite rapid (Bader and Sen, 1966). Consequently, if 3-OMFP phosphorylates the enzyme more readily than does NPP, and if this E_2P formation is favored by the absence of K^+ yet is also hydrolyzed appreciably in the absence of K^+ (as is the E_2P intermediate of the Na^+-ATPase reaction catalyzed by this enzyme), then these observations seem reasonable.

Another indication that 3-OMFP may phosphorylate the enzyme, although in this case to form first E_1P, is the stimulation seen with Na^+ when the K^+ concentration is suboptimal (Fig. 4). This (Na^+ + K^+)-phosphatase activity seems analogous to (Na^+ + K^+)-ATPase activity, involving Na^+-activated formation of E_1P with subsequent K^+-activated hydrolysis of E_2P, and is appreciably greater with 3-OMFP as substrate (Fig. 4) than with NPP as substrate (Robinson *et al.*, 1984), and approaches the stimulation of NPP hydrolysis measured with Na^+, suboptimal K^+, and a phosphorylating nucleotide (Robinson, 1969). This latter phenomenon is explainable as the phosphorylated enzyme, E_2P, having a higher affinity for the K^+ present at suboptimal concentrations (Robinson and Flashner, 1979; Cantley, 1981).

The stimulation of 3-OMFP hydrolysis by NaCl in the absence of KCl has a very high apparent $K_{0.5}$ (Fig. 6) and may be due to Na^+ activating hydrolysis at K^+ sites, just as in the Na^+-ATPase reaction high concentrations of extracellular Na^+ may activate E_2P hydrolysis in lieu of K^+ (Blostein, 1983). However, low concentrations of Na^+ could also activate through a pathway akin to the Na^+-ATPase reaction.

A third suggestion that 3-OMFP hydrolysis proceeds differently from NPP hydrolysis is their opposite response to Me_2SO. Me_2SO stimulates NPP hydrolysis, particularly at low KCl concentrations (Robinson, 1972), whereas Me_2So inhibits 3-OMFP hydrolysis (Table IV) just as it does ATP and acetyl phosphate hydrolysis (Robinson, 1972; Albers and Koval, 1972). Since Me_2SO favors enzyme phosphorylation from P_i and thus stabilizes E_2P (Goncalves de Moraes and de Meis, 1987), then, if 3-OMFP hydrolysis does involve E_2P formation (as do ATP and acetyl phosphate hydrolysis) whereas NPP hydrolysis does not, Me_2SO should inhibit phosphatase activity with 3-OMFP but not with NPP. This is the case (Table IV). That oligomycin, favoring E_1 conformations, inhibits 3-OMFP hydrolysis seems straightforward; that it can stimulate NPP hydrolysis (Robinson *et al.*, 1984) remains puzzling.

Differences between 3-OMFP and NPP hydrolysis may thus be largely explained by 3-OMFP following a phosphorylating pathway to form E_2P, as suggested for acetyl phosphate but not NPP (Shaffer *et al.*, 1978). But why 3-OMFP and NPP traverse alternative pathways is unclear. It obviously is not a simple matter of standard free energies of hydrolysis, for P_i readily phosphorylates the enzyme to form the acyl phosphate E_2P, whereas NPP, 3-OMFP, and acetyl phosphate are all acid anhydrides with large negative free energies of hydrolysis.

Acknowledgments

We thank Catherine Leach and Eva Nemeth for careful technical assistance, and Drs. Marcia Steinberg and Richard Cross for criticisms and suggestions. This work was supported by a Javits Neuroscience Investigator Award from the National Institutes of Health, NINCDS.

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