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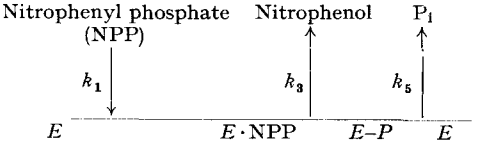
Reaction sequence of the K^+-dependent phosphatase

K^+-dependent phosphatase activity is associated with many partially purified ($Na^+ + K^+$)-dependent ATPase preparations (ATP phosphohydrolase, EC 3.6.1.3)[1], and strong circumstantial evidence links this activity to the terminal K^+-dependent hydrolytic step of the ATPase[2-4]. The reaction sequence of the ATPase involves an initial Na^+-dependent phosphorylation of the enzyme to form a glutamyl phosphate complex[5], but it is quite unlikely that such an intermediate occurs with the K^+-dependent phosphatase since (i) the phosphatase functions in the absence of Na^+, and (ii) its activity is stimulated by agents that themselves phosphorylate the enzyme at that site[4]. It thus seems probable that the phosphatase hydrolytic site, and its enzyme–phosphate group if any, is distinct from (although sensitive to) the glutamyl phosphate complex. Hence, studies on the reaction sequence of the phosphatase, with particular attention to a possible enzyme–phosphate intermediate, would appear pertinent both to the general mechanism of phosphatase enzymes[6] and to the hydrolytic site of the ATPase[4], and experiments were undertaken based on the kinetic analyses of CLELAND[7].

K^+-dependent phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate with a partially purified ($Na^+ + K^+$)-dependent ATPase preparation from rat brain, obtained after treatment with deoxycholate and NaI[8]. The standard incubation medium[3] contained 50 mM Tris–HCl (pH 7.8), 3 mM nitrophenyl phosphate (Tris salt), 3 mM $MgCl_2$, and 10 mM KCl; velocity was calculated, after incubation for 10–20 min at 30°, in terms of the production either of *p*-nitrophenol[3] or of P_i[8]. Velocities are expressed[3] relative to that in the standard medium defined as 1.0, and experimental points are averages of five or more experiments performed in duplicate.

The enzymatic response to the concentration of nitrophenyl phosphate followed Michaelis–Menten kinetics, with a K_m of 3.6 mM (Fig. 1). One product of the reaction, nitrophenol, was a noncompetitive inhibitor (Fig. 1), and the other, P_i, was a competitive inhibitor (Fig. 2). This pattern of inhibition is consistent with an ordered release of products, first nitrophenol and then P_i[6,7], and indicates the existence of an enzyme–phosphate intermediate.

Moreover, certain thermodynamic values may be estimated for the K^+-dependent phosphatase bearing on the reaction sequence. As pointed out by HSU *et al.*[6], for the simple mechanism



$K_{eq} \leqslant (1/K_{ia})\ (K_{ip})\ (K_{iq})$, where K_{ia}, K_{ip}, and K_{iq} are evaluated[6,7] from the slopes and intercepts of the inhibition curves (Figs. 1, 2). This calculated value of K_{eq} is far too low, 2.5 mM, and as with the potato phosphatase[6] implies a more complex mechanism with an isomerization of the enzyme–phosphate intermediate (see

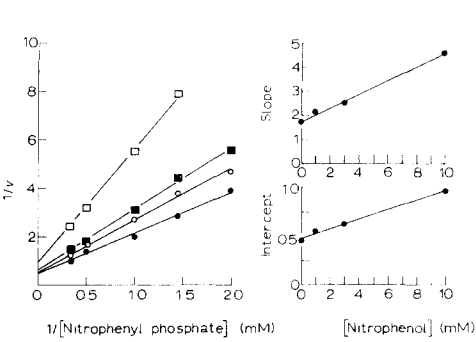


Fig. 1. Inhibition of K^+-dependent *p*-nitrophenyl phosphatase activity by *p*-nitrophenol. Initial velocities were calculated in terms of the production of P_i, after incubation in the standard medium with varying concentrations of nitrophenyl phosphate, in the absence (●) and presence of nitrophenol: ○, 1 mM; ■, 3 mM; and □, 10 mM. Data are presented in the form of a double reciprocal plot in terms of relative velocities, and the slopes (in arbitrary units) and intercepts of these lines are replotted against the concentration of nitrophenol. In the absence of nitrophenol the K_m for nitrophenyl phosphate was 3.5 mM, and, from the replots, K_{ia} was 5.7 mM and K_{ip} was 9.5 mM.

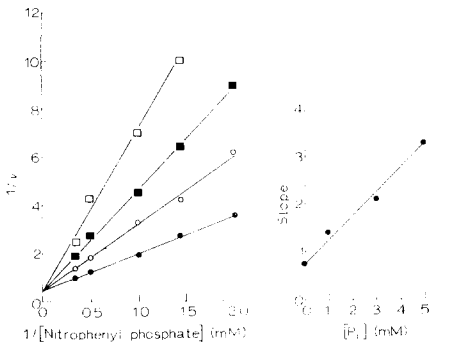
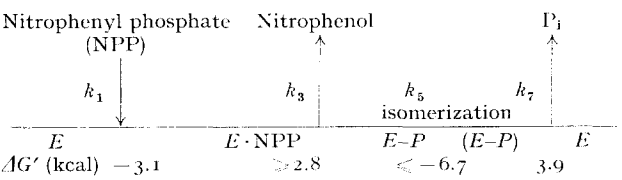


Fig. 2. Inhibition of K^+-dependent *p*-nitrophenyl phosphatase activity by P_i. Initial velocities were calculated in terms of the production of nitrophenol, after incubation in the standard medium with varying concentrations of nitrophenyl phosphate, in the absence (●) and presence of P_i: ○, 1 mM; ■, 3 mM; and □, 5 mM. Data are presented as in Fig. 1; the value of K_{iq} from the replot of the slopes was 1.5 mM.

below), such that $K_{eq} \leqslant [(K_{ip}\,K_{iq})/K_{ia}]\,[(k_5/k_6) + 1]$. Assuming 3 kcal as the minimal value for the free energy of hydrolysis of nitrophenyl phosphate[6], limiting values of the free energy changes ($\Delta G'$) may then be assigned to the reaction sequence of the K^+-dependent phosphatase, in close agreement with those for the cation-independent potato phosphatase[6]:



Nucleotides in order to activate the K^+-dependent phosphatase apparently must phosphorylate the enzyme to form the glutamyl phosphate complex[4]. Clearly, then, if the nucleotide-activated enzyme is also phosphorylated by nitrophenyl phosphate during catalysis, this phosphorylation must occur at a second site distinct from that of the activating phosphorylation. In the presence of CTP and Na^+, with resultant activation of the phosphatase (in terms of increased sensitivity to low K^+ concentrations[3,9]), the K_m for nitrophenyl phosphate was increased to 5.7 mM (Fig. 3), presumably due to competition for the hydrolytic site, and P_i was a competitive inhibitor (Fig. 3). (Unfortunately, it is not technically feasible to study nitrophenol inhibition in the presence of CTP.) These data are consistent with the formation of an enzyme–phosphate intermediate at the hydrolytic site (from nitrophenyl phosphate) while there coexists an activating glutamyl phosphate complex (from CTP) on the enzyme[4].

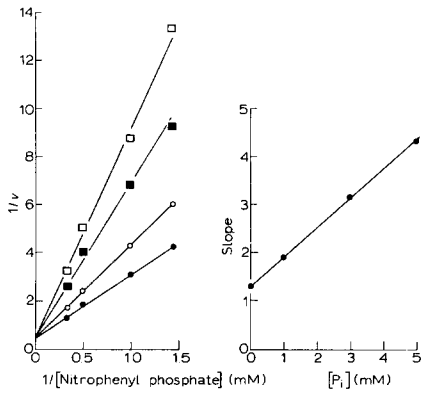


Fig. 3. Inhibition of K^+-dependent *p*-nitrophenyl phosphatase activity by P_i, in the presence of CTP *plus* Na^+. Experiments were performed and the data are presented as in Fig. 2, except that the incubation media contained 50 mM Tris–HCl (pH 7.8), 3 mM $MgCl_2$, 1.0 mM KCl, 20 mM NaCl, 0.3 mM CTP (Tris salt) and the concentration of nitrophenyl phosphate indicated, in the absence (●), and presence of P_i: ○, 1 mM; ■, 3 mM; and □, 5 mM. The K_m for nitrophenyl phosphate in the absence of P_i was 5.7 mM, and the value of K_{iq} from the replot of the slopes was 2.2 mM.

For the ATPase it would seem that initially ATP phosphorylates the glutamyl residue, activating the enzyme; this phosphate group is then transferred in a K^+-dependent step to the distinct hydrolytic site (perhaps as a succeeding phosphate is donated by ATP to the glutamyl residue). This hydrolytic site would thus be identical to the hydrolytic site of the phosphatase with its enzyme–phosphate intermediate.

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