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THIOPHOSPHORYLATION OF (Na + K$^+$)-ATPase YIELDS AN ADP-SENSITIVE PHOSPHOINTERMEDIATE *

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(1) Treatment of (Na$^+$ + K$^+$)-ATPase from rabbit kidney outer medulla with the γ-^{35}S labeled thio-analogue of ATP in the presence of Na$^+$ + Mg^{2+} and the absence of K$^+$ leads to thiophosphorylation of the enzyme. The K_m value for [γ-S]ATP is 2.2 μM and for Na$^+$ 4.2 mM at 22°C. Thiophosphorylation is a sigmoidal function of the Na$^+$ concentration, yielding a Hill coefficient $n_H = 2.6$. (2) The thio-analogue ($K_m = 35$ μM) can also support overall (Na$^+$ + K$^+$)-ATPase activity, but V_{max} at 37°C is only 1.13 μmol · (mg protein)$^{-1}$ · h^{-1} or 0.09% of the specific activity for ATP ($K_m = 0.43$ mM). (3) The thiophosphoenzyme intermediate, like the natural phosphoenzyme, is sensitive to hydroxylamine, indicating that it also is an acylphosphate. However, the thiophosphoenzyme, unlike the phosphoenzyme, is acid labile at temperatures as low as 0°C. The acid-denatured thiophosphoenzyme has optimal stability at pH 5–6. (4) The thiophosphorylation capacity of the enzyme is equal to its phosphorylation capacity, indicating the same number of sites. Phosphorylation by ATP excludes thiophosphorylation, suggesting that the two substrates compete for the same phosphorylation site. (5) The (apparent) rate constants of thiophosphorylation (0.4 s^{-1} vs. 180 s^{-1}), spontaneous dethiophosphorylation (0.04 s^{-1} vs. 0.5 s^{-1}) and K$^+$-stimulated dethiophosphorylation (0.54 s^{-1} vs. 230 s^{-1}) are much lower than those for the corresponding reactions based on ATP. (6) In contrast to the phosphoenzyme, the thiophosphoenzyme is ADP-sensitive (with an apparent rate constant in ADP-induced dethiophosphorylation of 0.35 s^{-1}, K_m ADP = 48 μM at 0.1 mM ATP) and is relatively K$^+$-insensitve. The K_m for K$^+$ in dethiophosphorylation is 0.9 mM and in dephosphorylation 0.09 mM. The thiophosphoenzyme appears to be for 75–90% in the ADP-sensitive E_1-conformation.

Introduction

The phosphoenzyme of (Na$^+$ + K$^+$)-ATPase can exist in two forms [1]. One form, primarily produced upon phosphorylation by nucleoside triphosphates is a high-energy phosphorylated intermediate, designated $E_1 \sim P$. It is sensitive to decomposition by ADP, but its hydrolysis is not stimulated by K$^+$. The other form is the low-energy phosphorylated intermediate E_2-P, primarily produced upon phosphorylation by inorganic phosphate, which is K$^+$-sensitive but ADP-insensitive. The two forms of the phosphoenzyme can be converted into each other by a conformational change without a chemical shift of the phosphate group.

Under normal conditions phosphorylation with ATP leads predominantly to E_2-P, particularly in the kidney enzyme [2]. Significant quantities of $E_1 \sim P$ are retained only under various abnormal conditions: unphysiologically high Na$^+$ concentra-

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tions [3], replacement of enzyme-bound Mg^{2+} by Ca^{2+} [2,4], treatment with inhibitors like *N*-ethylmaleimide [5], oligomycin [6] or quercetin [7], lipid depletion [8], or limited proteolysis in the presence of Na^+ [9].

For the myosin ATPase system it has been reported that thiophosphorylation by [γ-S]ATP leads to a thiophosphoenzyme, which is resistant to the action of an intrinsic phosphatase activity [10,11]. Although in the phosphoenzyme of myosin ATPase phosphate is linked to serine [12] rather than to an aspartic acid group as in $(Na^+ + K^+)$-ATPase [13], the K^+-stimulated hydrolysis of the latter intermediate can also be seen as an intrinsic phosphatase activity. Hence, we wondered whether thiophosphorylation of $(Na^+ + K^+)$-ATPase would yield a K^+-insensitive, ADP-sensitive thiophosphoenzyme, i.e. whether in this way an $E_1 \sim P$-type phosphoenzyme can be produced without the use of inhibitors or modification of the enzyme protein or its lipid environment. This appears to be the case, as will be set forth here.

Materials and Methods

Enzyme preparation. $(Na^+ + K^+)$-ATPase is purified from rabbit kidney outer medulla as described by Jørgensen [14], followed by removal of contaminating ATP, washing and storage of the preparation according to the procedure of Schoot et al. [15]. Protein is determined by the Lowry method, following trichloroacetic acid precipitation and using bovine serum albumin as standard [14].

Phosphorylation and thiophosphorylation. Phosphorylation by ATP at 22°C is carried out as previously described by us [16]. In all experiments, except in the determination of the Na^+-dependence of the phosphorylation process, the Na^+ concentration is 100 mM. Batches of [γ-^{32}P]ATP (Amersham International Ltd., U.K., code PB 168) of high specific radioactivity ($3 \cdot 10^6$ Ci/mol) contained radioactive contamination and gave 40% lower apparent specific enzymatic hydrolysis in the radioactive $(Na^+ + K^+)$-ATPase assay as compared to the non-radioactive assay [15]. Batches containing appreciably more carrier (Amersham International Ltd., U.K., code PB 170, spec. radioact. 500–3000 Ci/mol; New England Nuclear, Dreieich, F.R.G., Cat. No. NEG-002, spec. radioact. 2000–10000 Ci/mol) give comparable results when tested by the two assay methods and have been used in this study.

For thiophosphorylation non-radioactive [γ-S]ATP (tetralithium salt, Boehringer Mannheim GmbH, Biochemica, F.R.G.) is converted to its imidazole salt by passage over a Dowex 50W-X4 cation exchange resin in the imidazole form [17] and then mixed with [γ-^{35}S]ATP (Amersham International Ltd., U.K.) to give a specific radioactivity of 32–420 Ci/mol. Dithioerythritol (10 mM) is added to prevent disulfide formation. Thiophosphorylation by [γ-S]ATP follows the same procedure as for phosphorylation by ATP, but 1 mM dithioerythritol is added to assay medium and stopping solution. Samples are filtered, washed and transferred to the non-aqueous cumene-based Aqualuma Plus counting solution (Lumac/3M, Schaesberg, The Netherlands) within 10 min after stopping thiophosphorylation in order to minimize loss of radiolabel from the acid-labile thiophosphorylated enzyme. All data are the average of assays performed in duplicate or triplicate.

Simultaneous dual phosphorylation with [γ-^{32}P]ATP (20 μM) and [γ-^{35}S]ATP (20 μM) is carried out according to the same procedure described for thiophosphorylation by [γ-S]ATP alone, but the liquid scintillation counter is now programmed for double label analysis to determine enzyme-incorporated ^{32}P and ^{35}S. The content of each isotope is calculated by means of the external standard ratio method. The time elapsing between addition of nucleotide and completion of homogenization by stirring is called mixing time. The kinetic semilogarithmic plots reveal a mixing time (no reaction) of 1.8 ± 0.18 s (see Results section). Kinetic data have been corrected for this mixing time.

Assay of acid stability and effect of pH on stability of the phosphoenzymes. The acid stability of the phosphoenzymes EP and EPS is assayed by (thio)phosphorylating the enzyme with [γ-^{32}P]ATP for 10 s or with [γ-^{35}S]ATP for 15 s at 22°C and stopping the reaction by addition of an ice-cold solution of 5% (w/v) trichloroacetic acid + 0.1 M H_3PO_4, without or with 1 mM dithioerythritol, respectively. The suspension is kept at 0°C and samples containing 25 μg protein are removed and

filtered 0, 5, 15, 30 and 60 min after stopping the reaction. The filters are immediately washed and transferred to counting vials.

The pH effect on the stability of EP and EPS is determined by (thio)phosphorylating the enzyme as described in the preceding paragraph. The reaction is terminated by adding an equal volume of ice-cold 50% (w/v) trichloroacetic acid. Subsequently, 0.2 ml aliquots of the acid denatured enzyme suspension, each containing 25 μg protein, are added to 2 ml of a buffer medium containing 5% (w/v) trichloroacetic acid, 0.1 M H_3PO_4, 0.1 M sodium acetate, 0.1 M imidazole and 1 mM dithioerythritol. The pH of the buffer medium had been adjusted to one of twelve values in the range of pH 0.9– 9.3 by addition of solid NaOH. After 2 h at 0°C the samples are filtered, washed three times on the filter with their incubation buffer and counted for ^{32}P or ^{35}S in the liquid scintillation analyzer. As 100% controls serve samples, which immediately after acid denaturation are filtered, washed with 5% (w/v) trichloroacetic acid + 0.1 M H_3PO_4 + 1 mM dithioerythritol and counted.

Assay of hydroxylamine sensitivity of the phosphoenzymes. After (thio)phosphorylation and termination of the reaction as described in the second paragraph of the previous section (stopping with 10% (w/v) trichloroacetic acid instead), 0.2 ml aliquots are filtered and washed with 5% (w/v) trichloroacetic acid + 0.1 M H_3PO_4 + 1 mM dithioerythritol. They are then incubated on the filter at 0–4°C for up to 10 min with ice-cold buffer solution, containing 50 mM imidazole-HCl (pH 7.5) in the presence or absence of hydroxylamine-HCl (0.2 M, pH 7.5) and 1 mM dithioerythritol. About half (5 ml) of the supernatant solution is immediately used to impregnate protein and filter by suction, while the remainder is removed by suction following the proper incubation time. The filters are washed once more with trichloroacetic acid-H_3PO_4-dithioerythritol solution and then counted. The 100% control values are determined as indicated in the previous section.

Assay of sensitivity of the phosphoenzymes to ADP to K^+. Assay of ADP-sensitive dephosphorylation at 22°C follows prior phosphorylation at 22°C with [γ-^{32}P]ATP (20 μM) for 10 s or thiophosphorylation with [γ-^{35}S]ATP (20 μM) for 15 s, both in the presence of 100 mM Na^+. To 100 μl of the (thio)phosphorylation mixture we add 25 μl of the medium without tracer and enzyme but containing excess non-radioactive ATP (final concentration 1 mM) or ADP (final concentration 5 mM) in order to stop incorporation of radioactive (thio)phosphate. The spontaneous and the ADP-dependent dephosphorylation are then followed for time intervals up to 60 s, whereupon the reaction is stopped by addition of trichloroacetic acid/H_3PO_4/dithioerythritol. The K_m value for ADP is determined in similar fashion, except that incorporation of thiophosphate is stopped by addition of 0.1 mM ATP in the presence of 20–1000 μM ADP, followed by acid denaturation after 4 s. The apparent rate constant k is calculated with the equation $\log EPS_o/EPS_t = k \cdot t/2.3$, where EPS_o is the steady-state thiophosphoenzyme level prior to the chase and EPS_t the amount of radioactive thiophosphoenzyme following the chase with non-radioactive ATP and ADP. A mixing time correction is determined from the intercept of the linear semi-logarithmic plot of EPS vs. time (3–15 s) with the EPS_o level in spontaneous dethiophosphorylation (chasing with ATP only).

Assay of K^+-sensitive dephosphorylation of EP and EPS follows the same procedure, except that incorporation of radioactive (thio)phosphate is interrupted by addition of 1 mM non-radioactive ATP in the presence of increasing concentrations of K^+ (up to 10 mM) and dephosphorylation is stopped after 5 s by acid denaturation. As 100% controls serve samples that are acid denatured after 10 s phosphorylation or 15 s thiophosphorylation.

Results

Overall hydrolysis of [γ-S]ATP

Under standard assay conditions [15] and in the presence of 10 mM dithioerythritol, [γ-S]ATP (1 mM) is hydrolyzed by ($Na^+ + K^+$)-ATPase at a specific rate of 1.13 $\mu mol \cdot (mg\ protein)^{-1} \cdot h^{-1}$ (S.E. 0.07, $n = 6$), which is 0.09% of the maximal rate of ATP hydrolysis by the same enzyme preparations. However, the K_m for [γ-S]ATP under these conditions (mean ± S.E. 35.4 ± 2.8 μM, n = 4) is only 8.2% of that for ATP (0.43 ± 0.04 mM, $n = 7$, Ref. 18). This reduced K_m may be due to a

general reduction in the rate constants of the partial reactions, as will be explained in the Discussion.

Kinetics and capacity of thiophosphorylation

The course of thiophosphorylation at saturating levels of [γ-S]ATP (50 μM) and Na^+ (100 mM) is shown in Fig. 1A. The semilogarithmic plot of the reciprocal of the free thiophosphorylation capacity (total number of sites minus occupied sites divided by total number of sites) versus time shows a straight line up to 8 s or 90% of the steady-state thiophosphorylation level (Fig. 1B). This indicates that spontaneous dephosphorylation must be negligible. The rate constant of thiophosphorylation (0.56 s^{-1} in Fig. 1; mean ± S.E. 0.4 ± 0.1 s^{-1}, $n = 3$) is very much lower than that for phosphorylation by ATP at the same temperature (180 s^{-1}, Ref. 19). Fig. 1B also displays a mixing time of 2.5 s with a mean ± S.E. for eight experiments of 1.8 ± 0.18 s^{-1}.

The maximal thiophosphorylation level of 1.6 nmol · (mg protein)$^{-1}$ in Fig. 1A is rather low. From the previously established relation between the phosphorylation capacity (y, in nmol · (mg protein^{-1}) and the specific ($Na^+ + K^+$)-ATPase activity (x, in μmol · (mg protein)$^{-1}$ · h^{-1}) of $y = 0.00174x + 0.22$ [20] we would expect a capacity of 2.4 nmol · (mg protein)$^{-1}$ for the preparation used in Fig. 1A with specific activity 1250 μmol · (mg protein)$^{-1}$ · h^{-1}. Phosphorylation by ATP initially

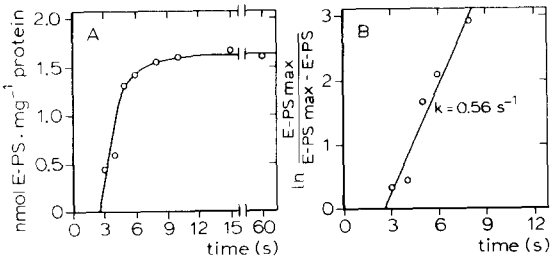


Fig. 1. Kinetics of thiophosphorylation of ($Na^+ + K^+$)-ATPase. (A) Amount of thiophosphoenzyme plotted vs. time. (B) Semilog plot of reciprocal relative free thiophosphorylation capacity vs. time, derived from (A). Thiophosphorylation takes place in the presence of 50 μM [γ-S]ATP and 100 mM Na^+. The line through the experimental points in (B) is calculated by linear-regression analysis. The slope yields a rate constant of thiophosphorylation of 0.56 s^{-1}, while a mixing time of 2.5 s is obtained from the abscissa intercept. This 'silent' mixing time has been applied in the plot shown in (A).

also gave about 40% too low steady-state levels, which is due to a radioactive contamination in the [γ-^{32}P]ATP (see Materials and Methods). However, the low thiophosphorylation level is actually due to the lability of the acid-denatured thiophosphoenzyme, as will be shown in the next section.

Acid stability of the thiophosphoenzyme

In contrast to the phosphoenzyme EP (2% decomposition in 1 h, rate constant 0.018 h^{-1}), the thiophosphorylated intermediate displays a rather high acid lability (> 50% decomposition, rate constant 0.74 h^{-1}) at 0 °C (Fig. 2A). The thiophosphoenzyme EPS is most stable in the pH range of 5–6 (Fig. 2B) near the isoelectric point of 5 of the enzyme [21], whereas the phosphoenzyme EP is stable up to pH 7.0. We do not know whether the low stability of EPS below pH 5 is due to the release of H_2S or thiophosphate, since we do not have available [γ-^{32}PS]ATP for use together with [γ-^{35}S]ATP in double label experiments.

The substrate [γ-^{35}S]ATP is totally resistant to loss of label in the trichloroacetic acid/H_3PO_4/dithioerythritol medium for periods of up to 4 h at 20 °C. However, as soon as it is hydrolyzed by the enzyme, label escapes from the incubation medium, probably as the result of hydrolysis of

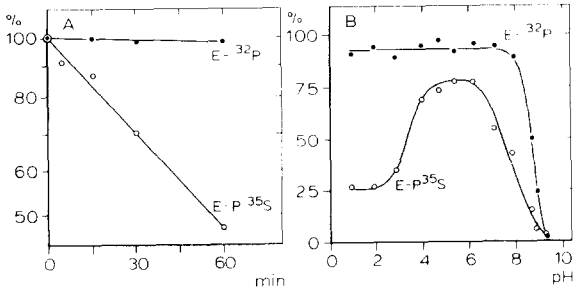


Fig. 2. Acid lability of the phosphoenzymes. (A) Loss of thiophosphoenzyme and phosphoenzyme in 5% (w/v) trichloroacetic acid + 0.1 M H_3PO_4 with or without 1 mM dithioerythritol, respectively, at 0 °C. The logarithm of the percent residual intermediate is plotted against time. The lines through the experimental points have been calculated by linear-regression analysis. The slopes give acid hydrolysis rate constants of 0.74 h^{-1} for the thiophosphoenzyme and of 0.018 h^{-1} for the phosphoenzyme. (B) Percent residual intermediate following acid denaturation and subsequent incubation for 2 h at 0 °C at the pH indicated on the abscissa.

thiophosphate to P_i and $H_2^{35}S$. This suggests that the low stability of EPS below pH 5 could be due to the release of H_2S. In all further experiments we have kept the time between acid-denaturation of EPS and filtration below 10 min, which reduces decomposition to less than 10% (Fig. 2A).

Identity of the thiophosphorylation and phosphorylation sites

The difference in acid stability between EP and EPS raises the question whether the thiophosphorylation site is identical with the phosphorylation site. From the aspartylphosphate formed upon phosphorylation a hydroxamic acid can be derived by treatment with hydroxylamine under simultaneous release of P_i. The findings shown in Fig. 3 indicate that EPS is hydroxylamine-sensitive (Fig. 3A), although hydroxamate formation is faster than that for EP (Fig. 3B). This indicates that both phosphoenzymes are acylphosphates.

The thiophosphorylation capacity of the enzyme, determined at saturating [γ-S]ATP level ($K_m = 2.2$ μM, see Fig. 5) without ATP, is the same as the phosphorylation capacity at saturating ATP level ($K_m = 0.1$–0.2 μM). The maximal phosphorylation level shown in Fig. 4 is determined in the presence of equimolar [γ-S]ATP, which is equal to that in the absence of [γ-S]ATP. The near absence of thiophosphorylation in the initial 20 s in the presence of ATP against maximal thiophosphorylation reached after 15 s in the absence of ATP, coupled with the time-course of the $E\text{-}^{32}P$ level obtained with labeled ATP, indicates

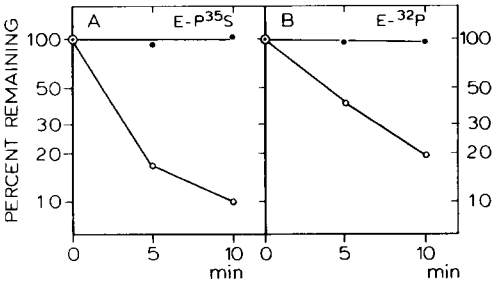


Fig. 3. Hydroxylamine sensitivity of thiophosphoenzyme (A) and phosphoenzyme (B). Following acid denaturation the phosphoenzymes are treated with 0.2 M hydroxylamine at pH 7.5 and at 0–4 °C for up to 10 min (○). The controls (●) are treated similarly but without hydroxylamine. The ordinate gives the percent residual intermediate on a log scale.

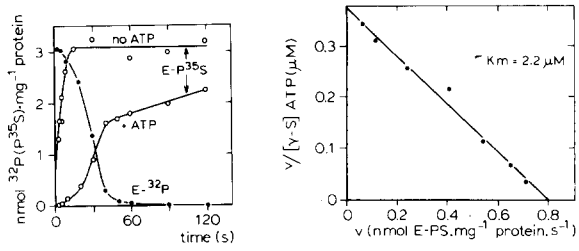


Fig. 4. Exclusion of thiophosphorylation by phosphorylation. The amounts of $E\text{-}^{32}P$ and $E\text{-}P^{35}S$, following simultaneous addition of equimolar (20 μM) ATP and [γ-S]ATP to the enzyme, are plotted against time. The time-course of thiophosphorylation in the absence of ATP is included for comparison. The maximal phosphorylation in the absence of ATP is included for comparison. The maximal phosphorylation levels in the presence or absence of [γ-S]ATP are the same.

Fig. 5. Scatchard plots of the initial rate of thiophosphorylation. The incorporation of thiophosphate after 3 s is taken, correction being made for a mixing time of 2 s. Thiophosphorylation occurs in the presence of 0.2–20 μM [γ-S]ATP and 100 mM Na^+. The [γ-S]ATP concentration used in calculating the ordinate value is corrected for its utilization during formation of the thiophosphoenzyme. The line through the experimental points is determined by linear-regression analysis. Its slope yields a K_m value for the substrate of 2.2 μM.

that phosphorylation excludes thiophosphorylation (Fig. 4). The slower rise of the thiophosphorylation level after 40 s in the presence of ATP is probably due to the accumulation of ADP (ATP being nearly exhausted at this time), which either inhibits [γ-S]ATP binding or promotes the back-reaction or both.

These findings, together with the ADP-sensitivity of EPS (see later section), strongly suggest that thiophosphorylation and phosphorylation occur at the same site.

Affinities for substrate and Na^+ in thiophosphorylation

Fig. 5 shows a Scatchard plot of the initial rate of thiophosphorylation as a function of the concentration of [γ-S]ATP. A straight line is obtained, which yields a K_m value of 2.2 μM for [γ-S]ATP.

Subsequently, we have studied the Na^+-dependence of thiophosphorylation. Fig. 6A shows a sigmoidal curve for thiophosphorylation as a function of the Na^+ concentration. The Hill plot de-

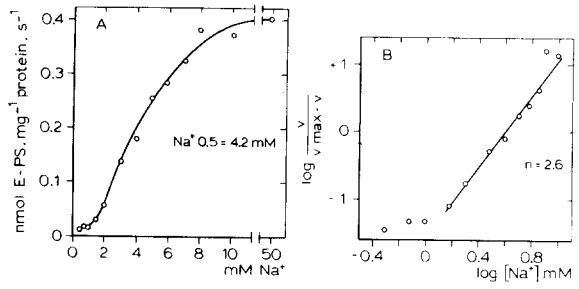


Fig. 6. Thiophosphorylation as a function of the Na^+ concentration. The initial rate of thiophosphorylation has been determined as indicated in the legend to Fig. 5, except that a constant (20 μM) concentration of [γ-S]ATP and a variable Na^+ concentration is used. (A) Amount of thiophosphoenzyme formed vs. Na^+ concentration. (B) Hill plot derived from the data in (A). The straight line for the range of 1.5–10 mM Na^+ yields a K_m value for Na^+ of 4.2 mM and a Hill coefficient $n_H = 2.6$.

rived from these data (Fig. 6B) yields an apparent Hill-coefficient of 2.6 in the Na^+ concentration range of 1.5–10 mM and a K_m value for Na^+ of 4.2 mM. This resembles the ($Na^+ + Mg^{2+}$)-dependent hydrolysis of 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*), where phosphorylation appears to be rate-limiting and which shows for Na^+ an apparent Hill-coefficient of 2.4 and a K_m value of 5–9 mM [22]. On the other hand, for phosphorylation by ATP (3 s levels) we find an apparent Hill-coefficient of 1.4 and a K_m value for Na^+ of 1.1 mM. The affinity for Na^+ appears to be directly related to the substrate affinity [23]: K_m ATP 0.1–0.2 μM, K_m [γ-S]ATP 2.2 μM, K_m Ado*PP*[NH]*P* = 17 μM [22].

Sensitivity of the thiophosphoenzyme to ADP

Fig. 7A shows that the thiophosphoenzyme is highly sensitive to ADP, inasmuch as the initial dethiophosphorylation rate in the presence of ADP is much higher than in its absence ($k = 0.35\ s^{-1}$ vs. $0.05\ s^{-1}$). This is in contrast to the dephosphorylation of the natural phosphoenzyme, which is not stimulated by ADP (Fig. 7B).

For the affinity of EPS for ADP, determined as the K_m for ADP in the presence of 0.1 mM ATP, a value of 48 μM is found. A corresponding value for EP from this enzyme preparation cannot be obtained, since EP is largely in the E_2-P conforma-

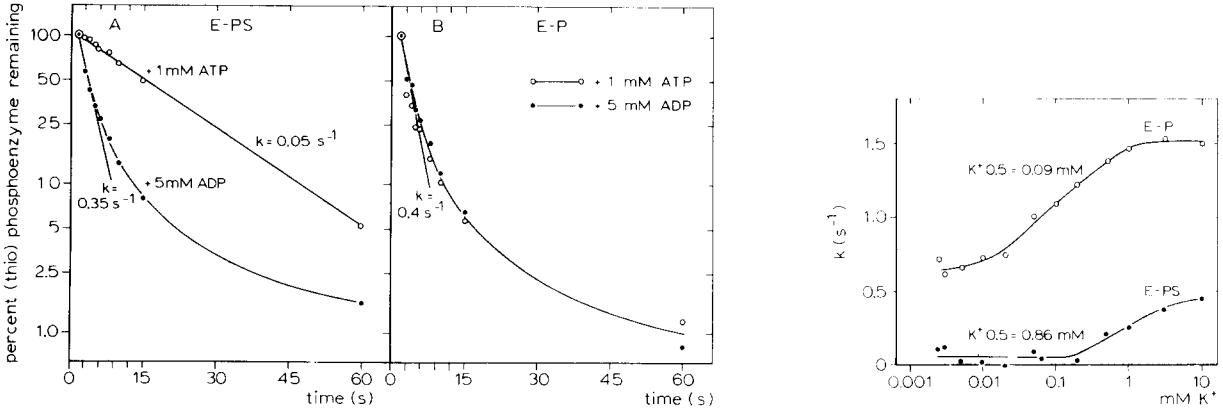


Fig. 7. Spontaneous and ADP-induced dephosphorylation of the thiophosphoenzyme (A) and the phosphoenzyme (B). The logarithm of the amount of enzyme-bound label, following a chase with excess non-radioactive ATP (spontaneous dephosphorylation) or non-radioactive ADP (ADP-induced dephosphorylation), is plotted against time. The apparent rate constants are determined from the tangents to the plots through zero-time corrected for 1.7 s mixing time. The mixing time correction is determined from the intercept of the linear-regression line for spontaneous dephosphorylation in (A) with the 100% level. The apparent rate constants thus obtained are: 0.05 s^{-1} for spontaneous dephosphorylation of EPS, 0.35 s^{-1} for ADP-induced dephosphorylation of EPS, and 0.4 s^{-1} for both spontaneous and ADP-induced dephosphorylation of EP.

Fig. 8. Kinetics of K^+-stimulated hydrolysis of the phosphoenzymes. Apparent rate constants (k in s^{-1}) are calculated from the equation $\log(EP_0/EP_t) = k \cdot t/2.3$, where EP_0 is the steady-state phosphoenzyme (EPS or EP) level prior to the chase and EP_t the amount of radioactive phosphoenzyme following the chase with excess non-radioactive ATP at the indicate K^+ concentrations. The chase is for 5 s, including 2 s mixing time, hence $t = 3$ s. The k_m for K^+ in dephosphorylation of EP is 0.09 mM and of EPS 0.86 mM.

tion. However, the enzyme in bovine brain and electric eel electroplax yields phosphoenzymes with higher proportions of the $E_1 \sim P$ conformation, for which K_m values of 50 μM [24] and 100 μM [25], respectively, have been reported. Hence the ADP affinities of $E_1 \sim PS$ and $E_1 \sim P$ do not greatly differ.

A minor point is that in Fig. 7B the data points for the dephosphorylation in the presence of ADP are not only not below those for spontaneous dephosphorylation (presence of 1 mM ATP), but actually slightly higher. This may be because ADP increases the K_m for ATP (from 0.14 to 350 μM at 5 mM ADP) and thus reduces the residual incorporation of ^{32}P less drastically than non-radioactive ATP does by carrier dilution. The small ADP contamination (approx. 0.7 mol%) present in the commercial ATP preparation cannot be responsible, since we find that a 10-fold reduction of the ATP concentration hardly lowers the rate constant for spontaneous dethiophosphorylation (0.03 s^{-1} vs. 0.05 s^{-1}) and since the concentration of the contaminating ADP (7 μM) is far less than the K_m value of ADP (50–100 μM) for $E_1 \sim P$ dephosphorylation. This phenomenon does not vitiate our method for assaying the ADP-sensitivity of the phosphoenzyme, since this method has given sizeable accelerations in dephosphorylation of phosphoenzymes with high $E_1 \sim P$ content (brain: 38–45% $E_1 \sim P$ at 100–150 mM Na^+, Refs. 24 and 26).

The very low ADP-sensitivity of EP from the kidney enzyme, shown in Fig. 7B and indicating a low $E_1 \sim P$ content in EP, is confirmed by findings for the effect of Na^+ on the ADP-dependent dephosphorylation. Increasing the Na^+ concentration from 16 to 160 mM does not lead to a significant acceleration by ADP of dephosphorylation [2,5,27], in contrast to the findings for the phosphoenzyme from brain and electroplax, which at high Na^+ concentrations have a sizeable $E_1 \sim P$ content [3,4,7,24,26].

A final remark on the method for determining the $E_1 \sim P$ content in the thiophosphoenzyme is in order. In earlier work on the ADP-sensitive phosphointermediate, performed at a lower temperature (0–4°C [3,7,9]) or at the same temperature (21°C) but with enzyme from a different source (bovine brain cortex, Ref. 28), the ADP-stimulated dephosphorylation occurred biphasically. The fraction of $E_1 \sim P$ was determined by linear extrapolation of the second (slow) phase to zero-time. This is not possible in the present experiments, where ADP-stimulated dethiophosphorylation is curvilinear (Fig. 7A). However, the fact that the experimental points fit the tangent to the curve from zero reaction time (after 1.7 s mixing time) down to a point representing 25% residual EPS suggests that a relative amount of 75% $E_1 \sim PS$ is initially present, and in another experiment with a different enzyme preparation a value of 90% is obtained. This high $E_1 \sim PS$ content is in contrast to the natural phosphoenzyme EP, which is nearly ADP-insensitive and thus appears to be predominantly in the E_2-P form.

Sensitivity of the thiophosphoenzyme to K^+

The difference in ADP-sensitivity of the phosphoenzymes EPS and EP is reflected in a much lower K^+-sensitivity if the thiophosphoenzyme (Fig. 8). Although the apparent rate constant of K^+-stimulated hydrolysis of EP is greatly underestimated, due to the fact that a large part of the kinetics belongs to the slow stages of dephosphorylation (1–11% EP remaining after 5 s, cf. Fig. 7B), it is clear that EP has a relatively high K^+-sensitivity. The K_m value of about 0.1 mM (Fig. 8) is the same as the K_d for the dephosphorylation sites (0.08–0.20 mM), determined by Robinson [29] by means of a different technique (K^+-accelerated inactivation of the enzyme by F^-). Dethiophosphorylation is K^+-insensitive up to 0.2 mM K^+ and then the rate constant starts to increase to 0.45–0.63 s^{-1} at 10 mM K^+ with a K_m value for K^+ of 0.9 mM. This means that the thiophosphoenzyme is 10-fold less sensitive to K^+ than the natural phosphoenzyme.

Discussion

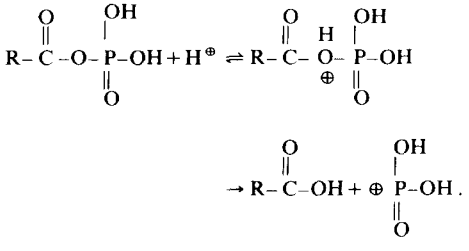
Characteristics of the thiophosphoenzyme

The major conclusion from this study is that reaction of $(Na^+ + K^+)$-ATPase with [γ-S]ATP leads predominantly to an ADP-sensitive, K^+-insensitive thiophosphointermediate of the $E_1 \sim P$ type. This contrasts with the phosphointermediate produced by ATP, which is relatively ADP-insensitive and 10-fold more sensitive to K^+, and thus

appears to be predominantly of the E_2-P type.

Yet, there are four arguments, which together strongly suggest that thiophosphorylation occurs at the same site as phosphorylation: (1) the exclusion of thiophosphorylation by phosphorylation from ATP, (2) the similar sensitivity (K_m) of EPS and $E_1 \sim P$ for ADP, (3) the equal capacities for phosphorylation and thiophosphorylation and (4) the acylphosphate nature of both phosphoenzymes indicated by their sensitivity to hydroxylamine.

The difference in acid stability of the two phosphoenzymes may be due to the different conformational state of $E_1 \sim PS$ as compared to that of E_2-P or, more likely, to the different chemical nature of the thiophosphate group in $E_1 \sim PS$. The chemical identity of $E_1 \sim P$ and E_2-P has already been well documented [27]. It is likely that hydrolysis occurs via phosphoryl-oxygen fission:



Substitution of one of the –OH groups surrounding the P atom by the less electronegative –SH group will lead to less electron withdrawal from the acyl-oxygen link and thus to a higher probability of proton attack. A direct exchange of –SH for –OH from water cannot be excluded, but it should be noted that under similar conditions the substrate [γ-S]ATP is resistant to hydrolysis at acid pH.

Rate constants of formation and hydrolysis of the thiophosphoenzyme

Replacing the –OH group by the more bulky, less electronegative –SH group has other consequences than the introduction of acid lability of the phosphorylated intermediate. A major consequence is the reduction of the rate constants of some of the partial reactions. For example, the apparent rate constants for the Na^+-dependent thiophosphorylation (0.3–0.6 s^{-1}) and the K^+-stimulated dethiophosphorylation (0.45–0.63 s^{-1}) are only a few tenth of a percent of those for phosphorylation (180 s^{-1}) and dephosphorylation (230 s^{-1}) at 21°C [19]. However, the rate constant of spontaneous dethiophosphorylation (0.03–0.05 s^{-1}) is much less reduced relative to that for spontaneous dephosphorylation (0.4–0.6 s^{-1}). We have shown that this is not due to the presence of ADP in the ATP. Nevertheless, the thiophosphorylated intermediate in the native state (not in the acid denatured state) is much more stable than the phosphorylated intermediate.

The observed reduction in K_m for the substrate (35 μM for [γ-S]ATP, 430 μM for ATP) in the overall enzymatic reaction may be due to a general reduction in rate constants of partial reactions. The K_m can be formulated as:

$$K_m = \frac{K_d}{1 + k_{sat}(1/k_1 + 1/k_2 + 1/k_3)}$$

where K_d is the dissociation constant for the E_2K-substrate complex, k_{sat} the rate constant for the $E_2K \rightarrow E_1K$ transition at saturating substrate level, k_1 the rate constant for (thio)phosphorylation, k_2 that for the $E_1 \sim P(S) \rightarrow E_2$-P(S) transition and k_3 that for the K^+-stimulated de(thio)phosphorylation [30]. For ATP the pertinent rate constants are 54, 180, 75 and 230 s^{-1}, respectively [19,30]. For [γ-S]ATP $k_1 = 0.4\ s^{-1}$, k_2 (considered to be equal to the apparent rate constant of K^+-stimulated dephosphorylation) = 0.5 s^{-1} and $k_3 = 3\ s^{-1}$ (see next section of Discussion). Considering that the specificity of E_2K for nucleoside triphosphate binding is not subject to large variations [18], we may divide the equation for [γ-S]ATP by that for ATP thereby canceling K_d. The sum of the reciprocal rate constants for [γ-S]ATP hydrolysis is more than 200-times larger than for ATP hydrolysis. So, even if k_{sat} for [γ-S]ATP would be only 1/10 of that for ATP, the K_m value for [γ-S]ATP would be about 12-times as low as that for ATP, as we indeed find to be the case.

Estimation of the ADP-sensitive fraction of the thiophosphoenzyme

The massive reduction in rate constants, when [γ-S]ATP instead of ATP is used as substrate, can also explain why the steady state balance between the two conformational states $E_1 \sim PS$ and E_2-PS

is poised towards $E_1 \sim PS$. We have estimated the percentage $E_1 \sim PS$ from the total thiophosphorylated intermediate following a chase with excess non-radioactive ADP by taking the tangent to the plot of $\log(E_1 \sim P^{35}S + E_2\text{-}P^{35}S)$ vs. time through $t = 0$. The extent to which the decrease in $E\text{-}P^{35}S$ follows the tangent is taken as the $E_1 \sim PS$ content.

The tangent through $t = 0$ fits the curve up to 10–25% residual thiophosphorylated intermediate in Fig. 7A, indicating that other rate constants have little influence in this part of the curve and that $E_1 \sim PS$ constitutes 75–90% (average 83%) of the total thiophosphorylated intermediate. Hence, the apparent rate constant for ADP-induced dethiophosphorylation (0.35 s^{-1}, Fig. 7A), after correction for spontaneous dephosphorylation of $E_2\text{-}P^{35}S$, should be multiplied by the factor 1/0.83 to give the real k_4 value of 0.41 s^{-1} (see Appendix).

This means that the rate constants for the forward (average 0.4 s^{-1}) and backward reaction (0.41 s^{-1}) in thiophosphorylation are equal. The rate constant of the K^+-stimulated dethiophosphorylation should be higher. We arrive at an average apparent rate constant of 0.54 s^{-1}. However, since $[E_2\text{-}P^{35}S]_0$ is approx. 17% of the total EPS, the intrinsic k_3 value may become at least 3 s^{-1} (see Appendix). In spontaneous dethiophosphorylation the rate constants for the hydrolysis of $E_1 \sim P^{35}S$ and $E_2\text{-}P^{35}S$, in view of their monoexponential decline, might be the same. So here the apparent rate constant equals the true constant.

Conclusions

Substitution of one of the terminal phosphorus oxygens in ATP by a sulphur atom leads to a substantial reduction of the rate constants of the partial reactions in the $(Na^+ + K^+)$-ATPase mechanism and to a predominantly $E_1 \sim PS$ type phosphoenzyme. This makes [γ-S]ATP a useful substrate to follow a sequence of partial enzyme reactions on a time scale of second, as first suggested by Eckstein [31]. For $(Na^+ + K^+)$-ATPase it is of particular use for the study of reactions involving the ADP-sensitive phosphointermediate such as the ADP-ATP exchange mechanism. The only disadvantage, acid lability of the thiophosphorylated intermediate, can be overcome by rapid handling after acid denaturation.

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Appendix

Kinetics of de(thio)phosphorylation following a chase with excess non-radioactive substrate

The train of events following (thio)phosphorylation can be schematically represented by:

$$E_1 + ATP \rightleftharpoons E_1 \sim P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2\text{-}P \xrightarrow{k_3} E_2 + P_i$$
$$\searrow ADP$$

Chasing with non-radioactive ATP (with or without K^+) or with non-radioactive ADP interrupts the supply of radioactive (thio)phosphate and the scheme depicting the decrease of $E\text{-}^{32}P$ or $E\text{-}P^{35}S$ can be symbolized by:

$$D \xleftarrow{k_4} A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_3} C$$

A is the (thio)phosphorylated intermediate in the E_1 conformation, B the intermediate in the E_2 conformation, D is E_1 and C is E_2; k_3 refers to spontaneous or K^+-stimulated de(thio)phosphorylation, k_4 to spontaneous or ADP-stimulated de(thio)phosphorylation. The decrease in A and B (concentrations of A and B) follows the equations:

$$-\frac{dA}{dt} = (k_1 + k_4)A - k_2 B$$

$$-\frac{dB}{dt} = (k_2 + k_3)B - k_1 A$$

Solving the equations via a second-order differential equation of the form

$$A'' + k_n A' + \{k_1 k_3 + (k_2 + k_3)k_4\}A = 0$$

after reduction to

$$\lambda^2 + k_n\lambda + k_1k_3 + (k_2 + k_3)k_4 = 0$$

by substitution of $e^{\lambda t}$ for A, leads to the roots

$$\lambda_{1,2} = -0.5\,k_n \pm \left[0.5\,k_n^2 - 4\{k_1k_3 + (k_2 + k_3)k_4\}\right]^{1/2}$$

where

$$k_n = k_1 + k_2 + k_3 + k_4$$

The integrated forms for A and B are:

$$A = C_1 \cdot e^{\lambda_1 t} + C_2 \cdot e^{\lambda_2 t}$$

$$B = \frac{A' + (k_1 + k_4)A}{k_2} = \left(\frac{\lambda_1 + k_1 + k_4}{k_2}\right) C_1 \cdot e^{\lambda_1 t} + \left(\frac{\lambda_2 + k_1 + k_4}{k_2}\right) C_2 \cdot e^{\lambda_2 t}$$

The constants C_1 and C_2 are solved by setting $t = 0$. The integrated form for $A + B$ at any time following the chase becomes

$$A_t + B_t = \frac{(k_4 + \lambda_2)A_0 + (k_3 + \lambda_2)B_0}{\lambda_2 - \lambda_1} \cdot e^{\lambda_1 t} + \frac{(k_4 + \lambda_1)A_0 + (k_3 + \lambda_1)B_0}{\lambda_1 - \lambda_2} \cdot e^{\lambda_2 t}$$

in which A_0 and B_0 represent A and B at zero time. Setting $A_t + B_t = y$ and writing q and r, respectively, for the fractions in the right hand part of the equation, one obtains

$$y = qe^{\lambda_1 t} + re^{\lambda_2 t}$$

Converting this to a logarithmic form yields

$$\ln y = \ln\left(qe^{\lambda_1 t} + re^{\lambda_2 t}\right)$$

Differentiation to t according to

$$\frac{d \ln y}{dt} = \frac{d \ln y}{dy} \cdot \frac{dy}{dt}$$

gives the slope of the tangent to the plot of $\ln(A_t + B_t)$ vs. time. For $t = 0$, this plot provides the apparent rate constant of the decrease in $A + B$. At any time:

$$\frac{d \ln y}{dt} = \frac{\lambda_1 qe^{\lambda_1 t} + \lambda_2 re^{\lambda_2 t}}{qe^{\lambda_1 t} + re^{\lambda_2 t}}$$

This shows that the slope changes with time, more specifically it should be downward concave (the second derivative is positive), as is usually observed in our experiments.

This only holds when $k_3 \neq k_4$. When $k_3 = k_4$, the factor r becomes zero ($\lambda_1 = -k_3 = -k_4$) and the factor q then equals $A_0 + B_0$ ($\lambda_2 = -(k_1 + k_2 + k_4)$), giving rise to a monoexponential decline in $A + B$. This phenomenon is observed in spontaneous dethiophosphorylation, but not in spontaneous dephosphorylation (Figs. 7A and B).
The slope at zero-time under any condition is:

$$\frac{d \ln y}{dt_0} = \frac{\lambda_1 q + \lambda_2 r}{q + r} = -\frac{k_4A_0 + k_3B_0}{A_0 + B_0}$$

With this equation we calculate the rate constant k_4 of ADP-stimulated dethiophosphorylation, using for $d \ln y/dt_0$ the value of $-0.35\ s^{-1}$, for $A_0/(A_0 + B_0)$ the value of 0.83 ($B_0/(A_0 + B_0) = 0.17$) and for $k_3 = 0.04\ s^{-1}$ (see Results section). This yields the value of $k_4 = 0.41\ s^{-1}$. For K^+-stimulated dethiophosphorylation $d \ln y/dt_0 = -0.54\ s^{-1}$, the fractions of A_0 and B_0 are the same as above, and $k_4 = 0.04\ s^{-1}$, yielding a value for $k_3 = 3\ s^{-1}$.

The model used to describe EP decomposition following a non-radioactive nucleotide chase is essentially that described by Klodos et al. [24]. It does not contain an $E_1 \sim$ P-ADP complex as indicated and kinetically analyzed by Pickart and Jencks [32] for $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum. Involvement of such a complex is unlikely, since the expected rapid drop in EP [26] after a chase with non-radioactive ATP, corresponding to the abolishment of the $E_1 \sim {}^{32}$P-ADP complex, is not observed upon thiophosphorylation (Fig. 7A).

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