Biochemistry

 Volume 15, Number 23 November 16, 1976

# Inhibition of $(Na^+,K^+)$Adenosine Triphosphatase and Its Partial Reactions by Quercetin†

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ABSTRACT: The bioflavonoid, quercetin, inhibited the $(Na^+,K^+)$adenosine triphosphatase purified from the electric organ of electric eel (*Electrophorus electricus*) or from lamb kidney. An analysis of its mode of action revealed that the formation of phosphoenzyme from $P_i$ but not from ATP was inhibited. Quercetin increased the amount of ADP-sensitive phosphoenzyme ($E_1$–P), indicating an inhibition of the conversion of $E_1$–P to the ADP-insensitive form ($E_2$–P). The rate of dephosphorylation of the phosphoenzyme formed from ATP was slowed by quercetin. These results suggest that quercetin inhibits the formation of $E_2$–P from either $P_i$ or $E_1$–P as well as the hydrolysis of the phosphoenzyme. Its mode of action is therefore different from that of ouabain and other inhibitors of the $(Na^+,K^+)$adenosine triphosphatase.

The bioflavonoid quercetin, which is widely distributed in plants, inhibits the $(Na^+,K^+)$ATPase[1] of the plasma membrane of calf heart (Carpenedo et al., 1969), $F_1$, the ATPase of bovine heart mitochondria (Carpenedo et al., 1969; Lang and Racker, 1974), $CF_1$, the chloroplast ATPase (Deters et al., 1975), the ATPase of *Escherichia coli* (Futai et al., 1974), and the $(Ca^{2+})$ATPase of sarcoplasmic reticulum.[2]

Suolinna et al. (1974, 1975) reported that low concentrations of quercetin suppressed the high glycolytic activity of Ehrlich ascites tumor cells by inhibiting the $(Na^+,K^+)$ATPase of the plasma membrane which is responsible for the regeneration of the ADP and $P_i$ required for glycolytic activity. However, $^{86}Rb$ uptake was not inhibited under these conditions. These results suggested that quercetin increased the efficiency of operation of the $Na^+,K^+$ pump and prompted us to investigate the mode of action of this inhibitor on a purified preparation of $(Na^+,K^+)$ATPase. In this paper we report on the effect of quercetin on partial reactions catalyzed by purified plasma membrane $(Na^+,K^+)$ATPase.

## Experimental Procedure

*Materials.* Radioactive orthophosphate ($^{32}P_i$), obtained from ICN Pharmaceuticals, Inc., was incubated at 100 °C in 2 N HCl for 45 min and stored at −20 °C. Prior to use it was diluted with phosphoric acid and neutralized with Tris base. [$\gamma$-$^{32}$P]ATP was prepared as described (Nelson et al., 1972a). (1,2-Cyclohexylenedinitrilo)tetraacetic acid was obtained from J. T. Baker Chemical Co. Quercetin was obtained from Sigma and recrystallized from ethanol-water. The $(Na^+,K^+)$ATPase from lamb kidney was purified as described elsewhere (Fagan and Racker, 1976). Electric eels were purchased from Paramount Research Supply, Ardsley, N.Y.

*Purification of $(Na^+,K^+)$ATPase from the electric organ of Electrophorus electricus.* Step 1. Microsomes were isolated essentially as described (Albers et al., 1963), suspended in 200 ml of water instead of Tris buffer and stored at −70 °C. About 2.2 g of protein was obtained from 300 g of the electric organ.

Step 2. After thawing, the suspension was diluted to 5 mg of protein per ml in 30 mM imidazole hydrochloride (pH 7.5), 250 mM sucrose, and 1 M NaCl and was stirred at 0 °C for 20 to 30 min. After centrifugation at 170 000*g* for 30 min in a Spinco 60 Ti rotor, the microsomal pellet was suspended in 30 mM imidazole hydrochloride (pH 7.5) and 250 mM sucrose and kept at −70 °C.

Step 3. The protein concentration of the washed microsomes was adjusted with the sucrose-imidazole buffer to 5 mg per ml. After addition of 10% deoxycholate to a final concentration of 0.025% and solid NaCl to yield 1 M NaCl, the mixture was stirred for 30 min at 0 °C, and centrifuged at 7000*g* for 20 min in a Spinco 60 Ti rotor. The supernatant was carefully removed and saved. The precipitate was suspended in the sucrose-NaCl-imidazole buffer in about half the volume of the original supernatant. The suspension was again centrifuged at 7000*g* for 20 min. The supernatant was removed and combined with the first supernatant. The combined supernatants were centrifuged at 170 000*g* for 60 min in a Spinco 60 Ti rotor. The supernatant was discarded and the pellet was suspended in 100

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[1] Abbreviations: $(Na^+,K^+)$ATPase, $(Na^+,K^+)$adenosine triphosphatase; $F_1$, coupling factor 1 (ATPase); $CF_1$, chloroplast coupling factor 1; $(Ca^{2+})$ATPase, $(Ca^{2+})$adenosine triphosphatase; CDTA, (1,2-cyclohexylenedinitrilo)tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $P_i$, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

[2] Unpublished observations.

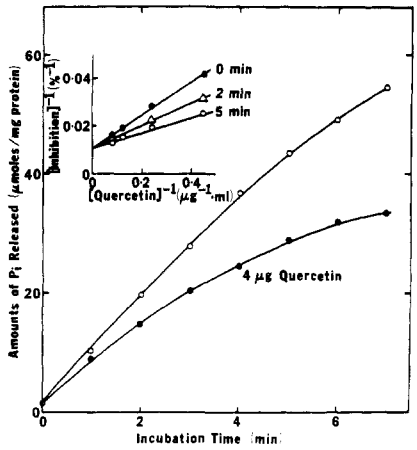


FIGURE 1: Time course of ATP hydrolysis by the $(Na^+,K^+)$ATPase in the presence and the absence of quercetin. The reaction mixture (8 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 60 mM NaCl, 4 mM [$\gamma$-$^{32}$P]ATP ($1.4 \times 10^4$ cpm/$\mu$mol), and 240 $\mu$g of the eel enzyme. After incubation at 37 °C with and without 32 $\mu$g of quercetin for the indicated time periods, 1.0-ml aliquots were added to 50 $\mu$l of 5 N trichloroacetic acid. After centrifugation, the released $^{32}P_i$ was measured as described under Experimental Procedure. (○—○) Without quercetin; (●—●) with quercetin. Inset: Effect of incubation of $(Na^+,K^+)$ATPase with quercetin. The mixture (1.0 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 60 mM NaCl, 30 $\mu$g of the eel enzyme, and quercetin, in amounts indicated. After incubation at 37 °C for 0, 2, or 5 min, 50 $\mu$l of 80 mM [$\gamma$-$^{32}$P]ATP ($9.2 \times 10^3$ cpm/$\mu$mol) was added to the mixture. After further incubation at 37 °C for 3 min, 50 $\mu$l of 5 N trichloroacetic acid was added and released $^{32}P_i$ was measured.

ml of the sucrose–imidazole buffer and the suspension was centrifuged at 170 000$g$ for 30 min. This washing procedure was repeated at least three times to remove all NaCl. The final pellet consisted of a turbid upper and a clear lower layer. The turbid layer was pipetted off and suspended in sucrose–imidazole buffer to 10 mg of protein per ml and stored at −70 °C. The clear layer, which contained no ATPase activity and no protein, was discarded. The recovery of ATPase varied with different preparations between 40 and 95% of the activity present in the microsomes. The specific activity of the purified ATPase varied between 7.0 and 14 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$, when the activity was measured by $^{32}P_i$ release from [$\gamma$-$^{32}$P]ATP. When assayed with a ATP regenerating system in a spectrophotometric assay (Barnett, 1970), the specific activity was about twice as high. The purity of the preparations was estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern (Weber and Osborn, 1969) to be 70–90%, assuming that the enzyme was composed of two subunits with molecular weights of 93 000 and 47 000 (Dixon and Hokin, 1974).

The $(Na^+,K^+)$ATPase was very stable when stored at −70 °C; there was no loss of activity after several months of storage.

*Assay of ATPase.* $(Na^+,K^+)$ATPase activity was measured by the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP (Nelson et al., 1972a). The reaction mixture (1.0 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 60 mM NaCl, 4 mM [$\gamma$-$^{32}$P]ATP ($9.2 \times 10^3$ cpm/$\mu$mol), and 30 $\mu$g of the enzyme protein. After 3 min incubation at 37 °C, 50 $\mu$l of 5 N trichloroacetic acid was added and the denatured protein was removed by centrifugation.

*Assay of Phosphoenzyme Formation with ATP.* The reaction mixture (1.0 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 60 mM NaCl, 0.8 mM [$\gamma$-$^{32}$P]ATP ($1.8 \times 10^7$ cpm/$\mu$mol), and 50 $\mu$g of the enzyme protein. After incubation at 37 °C for 5 s, the reaction was stopped by addition of 0.5 ml of cold 5 N trichloroacetic acid containing 20 mM $P_i$. After centrifugation at 9200$g$ for 10 min in a Sorvall type SS-34 rotor, the precipitate was suspended in 2 ml of cold 0.5 N trichloroacetic acid containing 20 mM $P_i$, and collected by filtration through a type E fiberglass filter (Gelman Instrument Co., Ann Arbor, Mich.). After washing with 10 ml of cold 0.5 N trichloroacetic acid containing 20 mM $P_i$, the filter was dried under an infrared lamp and the radioactivity was measured with a Nuclear Chicago gas flow counter.

TABLE I: Effect of the Enzyme Concentration on Inhibition of ATPase Activity by Quercetin.[a]

| Protein Concn in Assay ($\mu$g) | ATPase Act. − Quercetin | ATPase Act. + Quercetin |
|---|---|---|
| | [$\mu$mol min$^{-1}$ (mg of protein)$^{-1}$] | |
| 10 | 7.6 | 1.8 |
| 30 | 8.6 | 3.7 |
| 50 | 8.7 | 4.8 |
| 100 | 8.4 | 5.8 |

[a] The reaction mixture (1.0 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 20 mM KCl, 60 mM NaCl, and the enzyme in amounts indicated. After incubation at 37 °C for 1 min with and without quercetin (8 $\mu$g), 50 $\mu$l of 80 mM [$\gamma$-$^{32}$P]ATP ($2.0 \times 10^4$ cpm/$\mu$mol) was added. $^{32}P_i$ released was measured as described under Experimental Procedure.

*Assay of Phosphoenzyme Formaton with $P_i$.* The reaction mixture (2.0 ml) contained 50 mM Tris-Mes (pH 6.5), 5 mM $MgCl_2$, 20 mM KCl, 1 mM [$^{32}$P]Tris-$P_i$ (pH 6.5) ($4.6 \times 10^7$ cpm/$\mu$mol), and 0.1 mg of the enzyme protein. After incubation at 0 °C for 2 min, 1.0 ml of cold 5 N trichloroacetic acid containing 20 mM $P_i$ was added and the radioactivity was measured as described above. The identity of the phosphorylated peptide formed from $P_i$ with that formed from ATP was demonstrated by Post et al. (1975).

*Assay of Phosphoenzyme Formation with ATP and Rate of Dephosphorylation.* After incubation of the reaction mixture (2.0 ml) containing 50 mM Tris-Mes (pH 6.5), 5 mM $MgCl_2$, 120 mM NaCl, 80 $\mu$M [$\gamma$-$^{32}$P]ATP ($1.6 \times 10^8$ cpm/$\mu$mol), and 0.15 mg of the enzyme protein at 0 °C for 10 s, either 0.2 ml of 0.1 M ATP or 0.2 ml of 0.2 M CDTA was added to stop further incorporation of radioactivity. At various time intervals 1.0 ml of cold 5 N trichloracetic acid containing 20 mM $P_i$ was added and the radioactivity measured as described above.

*Assay of $E_1$-P Formation.* $^{32}$P-labeled phosphoenzyme formed with ATP was measured as described above. To measure ADP-sensitive phosphoenzyme ($E_1$-P) further incorporation of radioactivity was stopped by addition of 0.2 ml of 0.2 M Tris-CDTA (pH 6.5) with and without 20 mM ADP (Post et al., 1969) and residual $^{32}$P-labeled phosphoenzyme was measured at various time intervals. From the difference in residual phosphoenzyme in the presence and absence of ADP, the amount of $E_1$-P was calculated.

*Measurement of Protein Concentrations.* Protein was determined after solubilization of the enzyme with 0.8% deoxycholate according to Lowry et al. (1951) with crystalline bovine serum albumin as standard.

## Results

*Inhibition of $(Na^+,K^+)$ATPase by Quercetin.* Quercetin inhibited the $(Na^+,K^+)$ATPase purified from the electric

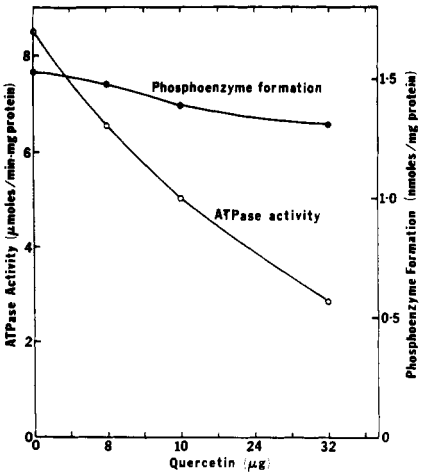


FIGURE 2: Effect of quercetin on phosphorylation of $(Na^+,K^+)$ATPase by ATP. The reaction mixture contained, in 0.9 ml, 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 60 mM NaCl, 50 $\mu$g of the eel enzyme, and quercetin in amounts indicated. After incubation at 37 °C for 2 min, 0.1 ml of 8 mM [$\gamma$-$^{32}$P]ATP ($1.8 \times 10^7$ cpm/$\mu$mol) was added. After further incubation at 37 °C for 5 s, 0.5 ml of cold 5 N trichloroacetic acid containing 20 mM phosphoric acid was added. Radioactivity bound to the acid-denatured protein was measured as described under Experimental Procedure. The ATPase activity was also measured under identical conditions except that 20 mM KCl and 4 mM [$\gamma$-$^{32}$P]ATP ($2.0 \times 10^4$ cpm/$\mu$mol) was added instead of 0.8 mM [$\gamma$-$^{32}$P]ATP and the ATPase activity was measured at 37 °C for 2 min without preincubation of the enzyme with quercetin.

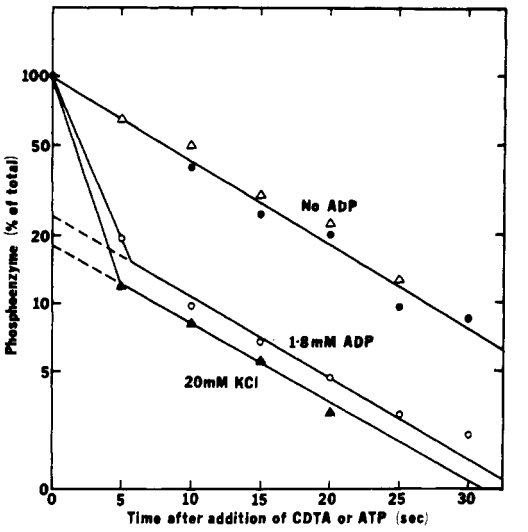


FIGURE 3: Dephosphorylation of the phosphoenzyme formed by [$\gamma$-$^{32}$P]ATP. The reaction mixture (2.0 ml) contained 50 mM Tris-Mes (pH 6.5), 5 mM $MgCl_2$, 120 mM NaCl, 40 $\mu$M [$\gamma$-$^{32}$P]ATP ($1.6 \times 10^8$ cpm/$\mu$mol), and the eel enzyme (150 $\mu$g). After 10 s of incubation at 0 °C, 0.2 ml of 0.2 M Tris-CDTA (pH 6.5) with and without 20 mM ADP was added to measure the dephosphorylation according to the procedures described under Experimental Procedure. The dephosphorylation was also measured after adding 0.2 ml of 0.1 M ATP with and without 0.22 M KCl which was added in place of Tris-CDTA (pH 6.5). Phosphoenzyme formed under these conditions was about 1.8 to 2.0 nmol/mg of enzyme protein. The rate constant for the dephosphorylation was calculated from the slope. (●—●) Added Tris-CDTA; (○—○) added Tris-CDTA with ADP; (△—△) added ATP; (▲—▲) added ATP with KCl.

organ of electric eel. As shown in Figure 1, inhibition of ATP hydrolysis increased with time. It can be seen from the inset that incubation of the enzyme prior to assay markedly increased the inhibition. The apparent $K_i$ values with and without the preincubation (5 min) were calculated to be 2.4 and 5.9 $\mu$g per ml, respectively, from the double-reciprocal plots of data obtained. To assay the effect of quercetin on phosphoenzyme formation much larger amounts of protein had to be used than for the ATPase assay. The effect of quercetin at increasing protein concentration was therefore tested. As shown in Table I, with 100 $\mu$g of the enzyme 8 $\mu$g of the quercetin inhibited 30%, whereas this amount inhibited 77% of ATPase activity with 10 $\mu$g of enzyme. Since the partial reaction had to be tested under different ionic conditions, the effects of $K^+$, $Na^+$, and ATP concentrations on the inhibition by quercetin were also examined. Variations in the concentrations of these ions had no pronounced effect on the inhibition of ATP hydrolysis by quercein with either the enzyme from electric eel or from lamb kidney.

*Effect of Quercetin on Phosphorylation by ATP.* The $(Na^+,K^+)$ATPase was phosphorylated by ATP in the presence of $Na^+$ and $Mg^{2+}$ (Albers et al., 1963). As shown in Figure 2, phosphorylation was much less sensitive to quercetin than the ATPase activity. Whereas 32 $\mu$g of quercetin inhibited 71% of the ATPase activity, phosphoenzyme formation was inhibited less than 15%. Similar results were obtained with the kidney enzyme. Addition of KCl reduces the yield of phosphoenzyme with ATP (Albers et al., 1963). Again, quercetin had little effect on phosphoenzyme formation in the presence of 10 mM KCl.

*Effect of Quercetin on the Formation of Phosphoenzyme with ATP.* According to the hypothesis proposed by Albers et al. (1968) and Post et al. (1969), the first phosphorylated intermediate of the $(Na^+,K^+)$ATPase is $E_1$-P which can be dephosphorylated by ADP. $E_1$-P is converted to $E_2$-P, which cannot be dephosphorylated by ADP. In the course of studying the effect of quercetin on the accumulation of $E_1$-P and $E_2$-P in the presence of ATP, it was noted that the concentration of $Na^+$ during assay had a pronounced effect on the relative concentrations of $E_1$-P and $E_2$-P. In Figure 3, the procedure of measuring $E_1$-P formation is shown. The amount of phosphoenzyme sensitive to ADP was determined from the data after correction for the rate of spontaneous hydrolysis by extrapolation as indicated in the figure. It also can be seen from this figure that this rate is the same irrespective of whether formation of radioactive enzyme is stopped by addition of unlabeled ATP or by a chelator of $Mg^{2+}$. As can be seen from Figure 4, NaCl had a pronounced effect on the yield of $E_1$-P. At 300 mM NaCl, virtually all of the phosphoenzyme was in the $E_1$-P form, whereas at 10 mM most of it was in the $E_2$-P form. As summarized in Table II, about 18% of total phosphoenzyme formed at 8 mM NaCl was $E_1$-P. Addition of 32 $\mu$g of quercetin to the reaction mixture increased the amount of $E_1$-P to 54%. In the presence of 100 mM NaCl, almost all of the phosphoenzyme was $E_1$-P and quercetin had little or no effect.

*Effect of Quercetin on Phosphoenzyme Formation by $P_i$.* In the presence of $Mg^{2+}$, the $(Na^+,K^+)$ATPase was phosphorylated by $P_i$ (Albers et al., 1968; Lindenmayer et al., 1968; Tanigushi and Post, 1975). Dephosphorylation of this phosphoenzyme was not stimulated by ADP (Post et al., 1975). The effect of quercetin on the formation of the phosphoenzyme was tested in the presence and absence of 20 mM KCl since an effect of $K^+$ on $E_2$-P formation has been reported (Post et al., 1975; Kuriki et al., 1976). As shown in Table III, quercetin reduced the level of phosphoenzyme formed both in the presence and in the absence of $K^+$. Thus, $K^+$ had no significant effect on the inhibition by quercetin. Since these studies were carried out at 0.5 mM $P_i$, which was not saturating, it was

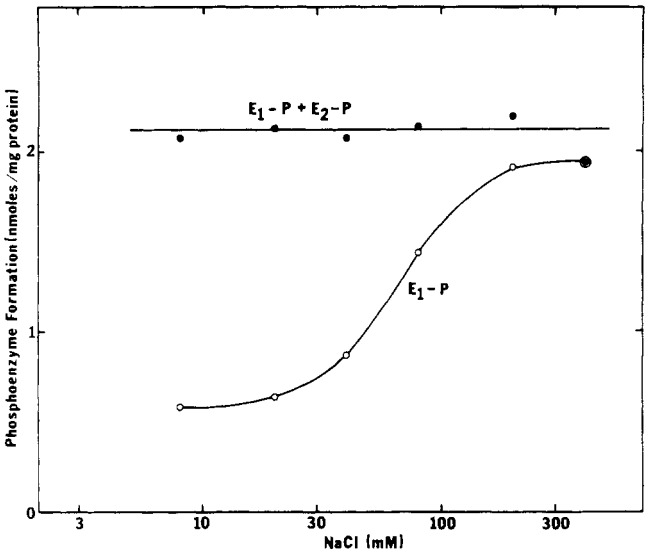


FIGURE 4: Effect of NaCl concentration on formation of $E_1$-P. The reaction mixture (0.5 ml) containing 50 mM Tris-Mes (pH 6.5), 5 mM $MgCl_2$, 80 $\mu$M [$\gamma$-$^{32}$P]ATP ($1.0 \times 10^8$ cpm/$\mu$mol), 600 $\mu$g of enzyme, and NaCl at the indicated concentrations was incubated at 0 °C. After 15 s, dephosphorylation was started by addition of 0.2 ml of solution containing 0.2 M Tris-CDTA (pH 6.5) and 20 mM ADP as described under Experimental Procedure. Logarithmic values of the data thus obtained were plotted against time and the straight lines were extrapolated as shown in Figure 3 thereby determining the fraction of ADP-sensitive phosphoenzyme ($E_1$-P). (●—●) Total phosphoenzyme; (○—○) $E_1$P.

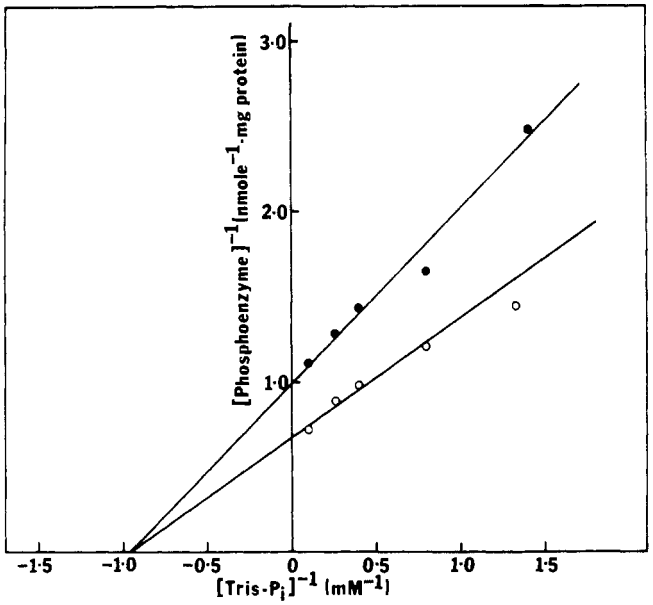


FIGURE 5: Effect of $P_i$ concentration on phosphoenzyme formation in the presence and the absence of quercetin. The eel enzyme (0.1 mg) was incubated in 2.0 ml of 50 mM Tris-Mes (pH 6.5), 10 mM $MgCl_2$, 20 mM KCl with and without 20 $\mu$g of quercetin at 0 °C for 2 min and phosphorylation was started by adding [$^{32}$P]Tris-$P_i$ (pH 6.5) ($4.6 \times 10^7$ cpm/$\mu$mol). After incubation at 0 °C for 1 min, 1.0 ml of cold 5 N trichloroacetic acid containing 20 mM phosphate was added and radioactivity bound to the acid-denatured protein was determined. (○—○) Without quercetin; (●—●) with quercetin.

possible that the quercetin inhibition was caused by an increase in the apparent $K_m$ value for $P_i$. In order to test this possibility, a titration with $P_i$ was performed at a saturating $Mg^{2+}$ concentration (10 mM). As shown in Figure 5, a double-reciprocal plot of the data did not suggest a major change in the apparent $K_m$ for $P_i$ in the presence of quercetin.

TABLE II: Effect of Quercetin on Formation of $E_1$-P and Total Phosphoenzyme by ATP.[a]

| Additions | Total Phosphoenzyme | | $E_1$-P | |
|---|---|---|---|---|
| | − Quercetin | + Quercetin | − Quercetin | + Quercetin |
| NaCl (8 mM) | 2.0 | 1.6 | 0.3 | 0.9 |
| NaCl (100 mM) | 2.1 | 1.6 | 1.6 | 1.5 |

The eel enzyme (0.1 mg) was incubated in 1.8 ml of 55.5 mM Tris-Mes (pH 6.5), 5.55 mM $MgCl_2$, and 8.9 or 111 mM NaCl with and without 32 $\mu$g of quercetin at 0 °C for 2 min. After addition of 0.2 ml of 0.8 mM [$\gamma$-$^{32}$P]ATP ($9.0 \times 10^7$ cpm/$\mu$mol), the mixture was further incubated at 0 °C for 10 s. Dephosphorylation of the $^{32}$P-labeled phosphoenzyme was measured by adding 0.2 ml of 0.2 M Tris-CDTA (pH 6.5) with and without 20 mM ADP. After incubation at 0 °C, the remaining phosphoenzyme was measured at the indicated time intervals. The amount of $E_1$-P formed was calculated from semilogarithmic plots.

TABLE III: Effect of Quercetin on the Formation of Phosphoenzyme by $P_i$.

| Quercetin ($\mu$g) | $(Na^+,K^+)$ATPase Act. ($\mu$mol min$^{-1}$ (mg of protein)$^{-1}$) | Phosphoenzyme Formed with $P_i$ | |
|---|---|---|---|
| | | Without $K^+$ | With $K^+$ |
| | | (nmol/mg of protein) | |
| 0 | 8.3 | 0.30 | 0.44 |
| 8 | 6.3 | 0.22 | 0.29 |
| 16 | 4.5 | 0.13 | 0.20 |
| 32 | 2.4 | 0.09 | 0.14 |

[a] Phosphorylation was carried out as described in the legend of Figure 5, except that the reaction mixture contained 5 mM $MgCl_2$ and 0.5 mM [$^{32}$P]Tris-$P_i$ ($4.1 \times 10^7$ cpm/$\mu$mol). After incubation of the enzyme with and without the indicated amounts of quercetin at 37 °C for 1 min, the reaction mixture which had been chilled at 0 °C and [$^{32}$P]Tris-$P_i$ was added. After 2 min, phosphoenzyme formation was measured as described under Experimental Procedure. The ATPase was measured under the same conditions with 0.1 mg of enzyme as described in the legend of Table I.

*Effect of Quercetin on Dephosphorylation of the Phosphoenzyme Formed by ATP.* After the formation of phosphoenzyme with [$\gamma$-$^{32}$P]ATP, addition of a large excess of ATP makes it possible to measure the dephosphorylation of the phosphoenzyme (see Figure 3). As shown in Figure 6, the phosphoenzyme decayed with a constant of 2.0 min$^{-1}$ in the absence of $K^+$. In the presence of quercetin the constant was about 0.6 min$^{-1}$ after an initial burst of dephosphorylation. The latter was probably caused by the relatively slow interaction between quercetin and the enzyme. In the presence of $K^+$, dephosphorylation was accelerated but the effect of quercetin was not substantially altered.

In Table IV several experiments performed with the lamb kidney enzyme are summarized. It can be seen (experiment 1) that, at concentrations of quercetin that inhibited ATP hydrolysis by 40%, there was no effect on phosphoenzyme formation from ATP. As in the case of the eel enzyme, phosphoenzyme formation from $P_i$ was strongly inhibited when the reaction was performed either at 0 °C (experiment 2) or at 37 °C (experiment 3). These experiments also demonstrate the marked temperature dependency for the accumulation of

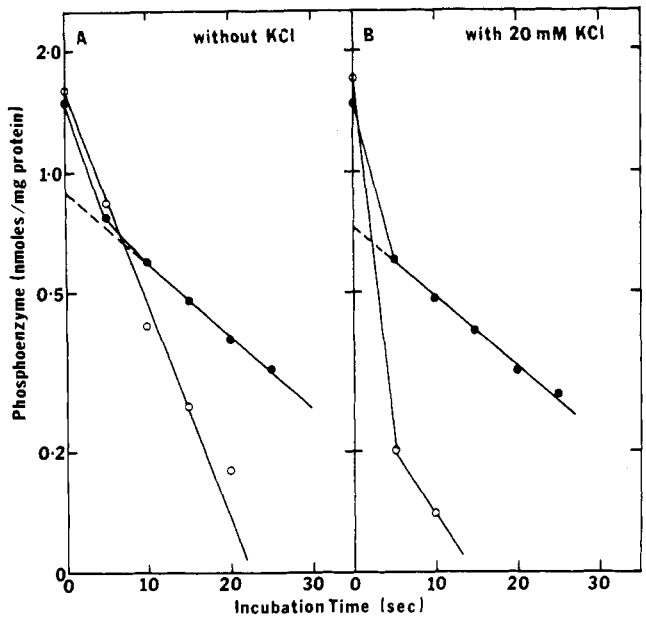


FIGURE 6: (A) Effect of quercetin on dephosphorylation of the phosphoenzyme formed by ATP. The eel enzyme (0.1 mg) was incubated in 1.8 ml of 50 mM Tris-Mes (pH 6.5), 5 mM $MgCl_2$, and 60 mM NaCl with 32 $\mu$g of quercetin at 0 °C. After 2 min, first 0.2 ml of 0.8 mM [$\gamma$-$^{32}$P]ATP ($1.6 \times 10^8$ cpm/$\mu$mol) and, 10 s later, 50 $\mu$l of 0.1 M ATP were added to stop further incorporation of radioactivity into the protein. The rate of dephosphorylation was measured at the indicated time periods by addition of 1.0 ml of cold 5 N trichloroacetic acid containing 20 mM phosphoric acid at the indicated time intervals. The radioactivity bound to the denatured protein was measured as described under Experimental Procedure. (B) The experimental procedure was the same as described under A, except that 50 $\mu$l of 0.8 M KCl containing 0.1 M ATP was added to the reaction mixture to measure dephosphorylation. (○—○) Without quercetin; (●—●) with quercetin (32 $\mu$g).

TABLE IV: Effect of Quercetin on ATP Hydrolysis and Phosphoenzyme Formation by Lamb Kidney ATPase.[a]

| Expt No. | Added Quercetin ($\mu$g) | ($Na^+$,$K^+$)ATPase Act. (nmol $min^{-1}$ mg of protein)$^{-1}$) | Phosphoenzyme Formed (pmol/mg of protein) |
|---|---|---|---|
| 1 | 0 | 33.0 | 220 |
| | 10 | 26.2 | 211 |
| | 20 | 23.8 | 222 |
| | 40 | 19.5 | 223 |
| 2 | 0 | | 3.2 |
| | 5 | | 2.6 |
| | 20 | | 1.7 |
| 3 | 0 | | 32.1 |
| | 4 | | 21.9 |
| | 12 | | 17.9 |
| | 20 | | 9.6 |

[a] (Experiment 1) The reaction mixture (2.0 ml) contained 0.1 M Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 0.7 mg of kidney enzyme, 4 mM [$\gamma$-$^{32}$P]ATP ($6.5 \times 10^7$ cpm/$\mu$mol), and quercetin as indicated. After incubation for 5 min at 0 °C, the reaction was stopped by adding 0.3 ml of cold 5 N trichloroacetic acid. Assays were performed as described under Experimental Procedure. (Experiment 2) The reaction mixture (1.0 ml) contained 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.05 mM [$^{32}$P]Tris-$P_i$ ($5.7 \times 10^8$ cpm/$\mu$mol), 1 mg of kidney enzyme, and quercetin as indicated. After incubation at 0 °C for 4 min, assays for phosphoenzyme were performed as described under Experimental Procedure. (Experiment 3) The reaction mixture (1.0 ml) contained 0.1 M Tris-HCl, 5 mM $MgCl_2$, 0.05 mM [$^{32}$P]Tris-$P_i$ ($6.6 \times 10^7$ cpm/$\mu$mol), 0.7 mg of the enzyme, and quercetin as indicated. After incubation at 37 °C for 5 min, assays were performed as described under Experimental Procedure.

phosphoenzyme from $P_i$. With ATP as substrate, virtually identical amounts of phosphoenzyme were formed at 0 and at 37 °C.

## Discussion

The data presented in this paper indicate that quercetin is a novel inhibitor of ($Na^+$,$K^+$)ATPase. It prevents the accumulation of $E_2$-P from either $E_1$-P or $P_i$ in the presence of enzyme and $Mg^{2+}$. In contrast, ouabain markedly increased the formation of $E_2$-P from $P_i$ (Albers et al., 1968; Lindenmayer et al., 1968) presumably by stabilization of $E_2$-P.

Several inhibitors of ($Na^+$,$K^+$)ATPase have been described. Harmaline has been reported to inhibit ($Na^+$,$K^+$)ATPase activity competitively with $Na^+$ (Canessa et al., 1973). Oligomycin blocks the conversion of $E_1$-P to $E_2$-P but does not inhibit the phosphorylation of the enzyme protein by either ATP (Fahn et al., 1968) or $P_i$ (Post et al., 1975). *N*-Ethylmaleimide appears to act similarly (Post et al., 1969; Fahn et al., 1966).

As shown in Figure 1, quercetin did not show its maximum inhibitory effect without prior incubation with the protein, indicating a relatively slow binding to the effective site of the enzyme. The binding of quercetin to the protein is not firm as indicated by the reversal of the inhibition on addition of bovine serum albumin (Suolinna et al., 1975). The interaction of quercetin with serum albumin is a distant disadvantage in our studies of tumor glycolysis and a search for more specific inhibitors of this type is in progress.

On the other hand, at low concentrations, quercetin inhibits ATPase activity of mitochondrial $F_1$ (Lang and Racker, 1974) and chloroplast $CF_1$ (Deters et al., 1975) while in mitochondria it does not interfere either with electron transport or coupled phosphorylation (Suolinna et al., 1974). In this respect the flavonoid reacts like the natural inhibitor of $F_1$ (Pullman and Monroy, 1963) and the $\epsilon$ subunit of $CF_1$ (Nelson et al., 1972b) which are regulatory components of the ATPase complex. It is remarkable that quercetin acts similarly also on the ($Na^+$,$K^+$)ATPase inhibiting the uncoupled ATPase and thereby diminishing glycolysis without interfering with the coupled process of ion translocation. (Suolinna et al., 1975). This phenomenon prompted the current investigation which identified the steps during ATP hydrolysis that are affected by quercetin. Quercetin inhibits the formation as well as the hydrolysis of $E_2$-P catalyzed by the isolated enzyme. The inhibition of hydrolysis may be related to the increased efficiency of pumping (Suolinna et al., 1974). The inhibitor may either induce an altered state in the enzyme that interferes with either the acceptance or release of $P_i$ or may block the site concerned with these reactions.

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# Calorimetric Studies of the Interaction of Magnesium and Phosphate with $(Na^+,K^+)$ATPase: Evidence for a Ligand-Induced Conformational Change in the Enzyme†

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ABSTRACT: The phosphorylation of $(Na^+,K^+)$ATPase from the electric organ of the electric eel is dependent on $Mg^{2+}$. The amount of phosphoenzyme formed was increased by $K^+$ and decreased by $Na^+$. Kinetic analyses indicate that a ternary complex of ATPase, $P_i$, and $Mg^{2+}$ is formed prior to phosphorylation of the protein. Calorimetric studies revealed extraordinarily large enthalpy changes associated with the binding of $Mg^{2+}$ (−49 kcal/mol) and of $P_i$ (−42 kcal/mol), indicating a thermodynamically significant conformational change in the enzyme. The dissociation constant for the binding of $Mg^{2+}$ and $P_i$ derived from calorimetric measurements is in good agreement with the value obtained from the kinetic studies. These results indicate that ion binding induces a conformational change in the enzyme which is a prerequisite for phosphorylation by $P_i$.

Membranous ATPases[1] catalyze energy transformations during ATP-driven ion transport and during ion-flux-driven ATP generation (cf. Racker, 1976). $(Na^+,K^+)$ATPase of the plasma membrane and $(Ca^{2+})$ATPase of the sarcoplasmic reticulum membrane are of particular interest because phosphorylated intermediates are formed under appropriate conditions with either ATP or $P_i$ as substrates (Albers et al., 1963, 1968; Yamamoto and Tonomura, 1967; Lindenmayer et al., 1968; Masuda and de Meis, 1973). The carboxyl group of an aspartate residue of the protein has been identified as the recipient of the phosphate (Degani and Boyer, 1973; Post and Orcutt, 1973). Moreover, the phosphoenzyme formed with $P_i$ was shown to transfer stoichiometrically the phosphoryl group to ADP to form ATP (Taniguchi and Post, 1975; Knowles and Racker, 1975). The net formation of ATP from $P_i$ and ADP is catalyzed by a highly purified $(Ca^{2+})$ATPase under conditions that appear to rule out the formation of an ion gradient across a membrane (Knowles and Racker, 1975). The ATP formed under these conditions does not remain bound to the protein but is released into solution. These observations raised questions relating to the thermodynamics of the reactions involved.

We have proposed (Knowles and Racker, 1975) that the energy required for the condensation of $P_i$ and ADP is derived from the binding of specific ions to the protein. To test this hypothesis, parallel kinetic and calorimetric studies with $(Na^+,K^+)$- and $(Ca^{2+})$ATPases have been carried out. In this paper we report data obtained with the $(Na^+,K^+)$ATPase system. We show that ligand binding to the $(Na^+,K^+)$ATPase monitored by calorimetric measurements can yield meaningful and accurate thermodynamic values for the binding reactions. The magnitude of the enthalpy changes for the binding of the

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Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Tris, tris(hydroxymethyl)aminomethane.