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# SHOWDOMYCIN, A NUCLEOTIDE-SITE-DIRECTED INHIBITOR OF $(Na^+ + K^+)$-ATPase

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## SUMMARY

Showdomycin [2-(β-D-ribofuranosyl)maleimide] is a nucleoside antibiotic containing a maleimide ring and which is structurally related to uridine. Showdomycin inhibited rat brain $(Na^+ + K^+)$-ATPase irreversibly by an apparently bimolecular reaction with a rate constant of about 11.0 $l \cdot mol^{-1} \cdot min^{-1}$. Micromolar concentrations of ATP protected against this inhibition but uridine triphosphate or uridine were much less effective. In the presence of $K^+$, 100 μM ATP was unable to protect against inhibition by showdomycin. These observations show that showdomycin inhibits $(Na^+ + K^+)$-ATPase by reacting with a specific chemical group or groups at the nucleotide-binding site on this enzyme. Inhibition by showdomycin appears to be more selective for this site than that due to tetrathionate or N-ethylmaleimide. Since tetrathionate is a specific reactant for sulfhydryl groups it appears likely that the reactive groups are sulfhydryl groups. The data thus show that showdomycin is a relatively selective nucleotide-site-directed inhibitor of $(Na^+ + K^+)$-ATPase and inhibition is likely due to the reaction of showdomycin with sulfhydryl group(s) at the nucleotide-binding site on this enzyme.

## INTRODUCTION

Showdomycin [2-(β-D-ribofuranosyl)maleimide] is a nucleoside antibiotic [1] structurally related to uridine (Fig. 1) and containing an aglycone moiety structurally related to maleimide (Fig. 1) [2]. Maleimides are active alkylating agents for SH groups on peptides or protein, although maleimides will also react at much lower rates with some amines and amino acids [3]. Since showdomycin is a structural analog of uridine, is an irreversible inhibitor of a number of enzyme systems [1], and the enzyme $(Na^+ + K^+)$-ATPase contains a cysteine residue at or near its active site for phosphorylation [4], we investigated showdomycin as a possible nucleotide-site-directed inhibitor (affinity label) for $(Na^+ + K^+)$-ATPase. This possibility is all the more attractive because showdomycin may be phosphorylated to showdomycin-5'-phosphate [5], which may be expected to show even greater site-directed specificity than showdomycin.

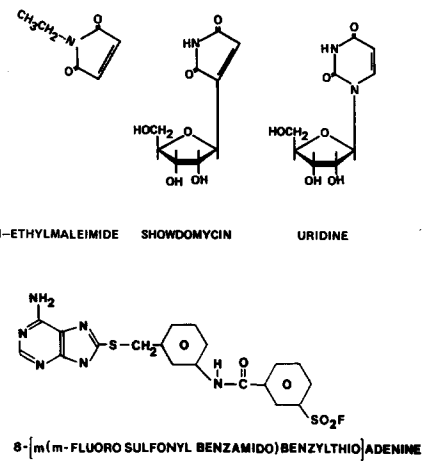



Fig. 1. Structural formulae of *N*-ethylmaleimide, showdomycin, uridine and the AMP analogue 8-[*m*(*m*-fluorosulfonylbenzamido)benzylthio]adenine.

The results obtained show that showdomycin is an irreversible inhibitor of $(Na^+ + K^+)$-ATPase inhibiting this enzyme in much the same way and with about the same rate constant as *N*-ethylmaleimide. Inhibition by showdomycin was hindered by low concentrations of ATP, but not by UTP. ATP in the presence of $K^+$ was much less effective in preventing inhibition by showdomycin. These observations show that ATP protects against showdomycin inhibition by binding at the high-affinity nucleotide-binding site(s) on this enzyme and suggest that this protection was due to direct shielding of the showdomycin-binding site(s) by ATP. Experiments with sodium tetrathionate support the concept that the group with which showdomycin interacts in the nucleotide-binding site is a sulfhydryl group. The data suggest that showdomycin is a much more specific nucleotide-site-directed inhibitor of $(Na^+ + K^+)$-ATPase than *N*-ethylmaleimide.

## MATERIALS AND METHODS

Rat brain $(Na^+ + K^+)$-ATPase prepared by the method of Akera and Brody [6] with minor modifications [7] was used throughout. The protein concentration of these enzymes was estimated by the method of Lowry et al. [8], and phosphorylation, [$^3$H]ouabain binding, and the $(Na^+ + K^+)$-ATPase activity of these membranes were measured as previously described by Tobin et al. [9].

The experiments reported in this communication in general deal with the rate of inhibition of $(Na^+ + K^+)$-ATPase by irreversible inhibitors. In a typical experiment about 1 mg of the enzyme was incubated in 50 mM Tris buffer, pH 7.4, with 1 mM EDTA. EDTA was added to block phosphorylation of the enzyme by ATP when this was present in the experimental system. The required drug concentrations were then added and the mixture incubated at 37 °C with constant shaking. At the indicated times after addition of the drug, 0.1-ml aliquots of the incubation mixture were removed, added to the standard $(Na^+ + K^+)$-ATPase reaction mixture and assayed for 5 min at 37 °C. Thus, the drug-enzyme mixture was diluted 10-fold and

therefore the concentration of showdomycin in the assay system never exceeded 0.1 mM. Under these conditions inhibition of $(Na^+ + K^+)$-ATPase activity by this drug was negligible (Fig. 2). Unless otherwise noted the experimental points presented are the means of three or more experiments on different enzymes with the vertical bars indicating the S.E. values. To allow comparison of experiments performed on different enzymes zero-time incubations in each experiment were arbitrarily designated 100 % and other values expressed as a percentage of this.

Showdomycin and formycin B were obtained from Calbiochem Ltd, La Jolla, Calif. *N*-Ethylmaleimide and the nucleoside substrates were from Sigma Ltd, St. Louis, Mo. Sodium tetrathionate was obtained from K and K Labs, Plainview, N.Y. 8-[*m*(*m*-Fluorosulfonylbenzamido)benzylthio]adenine, an AMP analogue (Fig. 1) was a gift of Dr D. J. Graves of Iowa State University [10].

## RESULTS

Preincubation of $(Na^+ + K^+)$-ATPase with showdomycin in the absence of ATP potentiated inhibition of the enzyme by showdomycin as shown in Fig. 2. When showdomycin was added to the $(Na^+ + K^+)$-ATPase assay mixture in the presence of ATP only about 20 % inhibition of enzyme activity was observed. However, if the enzyme, showdomycin and ions were pre-incubated together for 10 min in the absence of ATP, and then ATP was added to the mixture to start the enzyme assay up to 80 % inhibition of enzyme activity was observed. The experiment suggests that showdomycin produces a relatively stable inhibition of $(Na^+ + K^+)$-ATPase and that development of this inhibition is hindered by the presence of ATP.

Because a stable enzyme-inhibitor complex is of primary importance in affinity labeling, we tested the ability of successive "washings" to produce reactivation of the showdomycin-inhibited enzyme. Table I shows that after three washings no

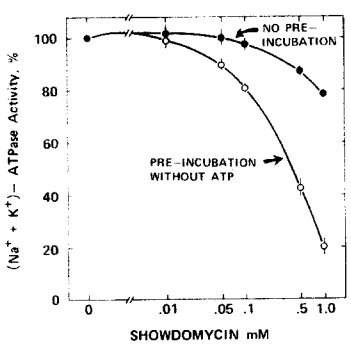



Fig. 2. Showdomycin inhibition of $(Na^+ + K^+)$-ATPase: effect of preincubation with showdomycin. About 40 $\mu$g of rat brain enzyme were preincubated with 100 mM $Na^+$, 5 mM $Mg^{2+}$, 15 mM $K^+$ and 50 mM Tris buffer, pH 7.4, at 37° C. The solid circles (● — ●) indicate $(Na^+ + K^+)$-ATPase activity observed when showdomycin was added with ATP to start the enzymatic assay. The open circles (○ — ○) show inhibition observed when the enzyme was preincubated with the indicated concentrations of showdomycin for 10 min prior to addition of ATP. Activities are plotted as a percentage of that observed in the absence of showdomycin, which averaged 207±11 $\mu$mol $P_i$/mg protein per h. ATPase activities observed in the presence of $10^{-4}$ M ouabain were deducted as ouabain-insensitive ATPase. All points are the means of four experimental determinations ±S.E.

TABLE I

RESISTANCE OF SHOWDOMYCIN INHIBITION OF $(Na^+ + K^+)$-ATPase TO WASHING

Rat brain ATPase in 50 mM Tris buffer, pH 7.4, was incubated with or without 2 mM showdomycin for 30 min at 37 °C and at the end of this period its $(Na^+ + K^+)$-ATPase activity was assayed. The enzyme preparations were then centrifuged three times at $35000 \times g$ for 20 min, each time resuspending in 4.0 ml of 50 mM Tris buffer, pH 7.4. Before the centrifugation and after each resuspension, aliquots were taken and assayed for $(Na^+ + K^+)$-ATPase activity. Enzyme activity is expressed as a percentage of that observed in the uninhibited enzyme at the end of the 30-min preincubation period, which averaged 269 $\mu$mol $P_i$/mg protein per h. All points are the means $\pm$S.E. values of three experimental determinations with different enzymes.

| | $(Na^+ + K^+)$-ATPase activity (%) | | | |
|---|---|---|---|---|
| Wash No. | 0 | 1 | 2 | 3 |
| −Showdomycin | 100.0 | 94.0±3.0 | 87.6±3.9 | 86.5±4.9 |
| +Showdomycin | 9.7±1.6 | 10.6±1.9 | 9.2±2.0 | 9.4±1.8 |

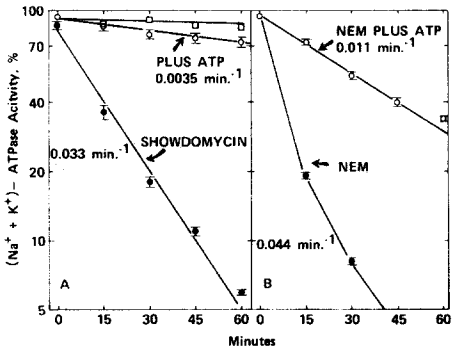
significant recovery of enzyme activity was observed, suggesting that the showdomycin-enzyme complex is irreversible under these conditions. Table II shows that preincubation of showdomycin with mercaptoethanol or addition of mercaptoethanol to the reaction medium essentially prevented inhibition of the enzyme by showdomycin. In other experiments addition of mercaptoethanol to the enzyme in the presence of showdomycin reduced the rate of inhibition of the enzyme by showdomycin but did not reverse inhibition. These experiments suggest that showdomycin inhibits this enzyme by virtue of its chemical reactivity [1, 2] with SH groups. In other experiments formycin B, a better analogue of adenosine than showdomycin [11] but not reactive with SH groups failed to produce significant inhibition of $(Na^+ + K^+)$-ATPase. 8-[*m*-(*m*-Fluorosulfonylbenzylamido)benzylthio]adenine, which reacts with the

TABLE II

PREVENTION OF SHOWDOMYCIN INHIBITION OF THE $(Na^+ + K^+)$-ATPase BY MERCAPTOETHANOL

About 1 mg of rat brain enzyme was incubated with 1 mM EDTA in 50 mM Tris buffer, pH 7.4 at 37 °C. At zero time 1 mM showdomycin, 1 mM mercaptoethanol or 1 mM each of a solution of showdomycin and mercaptoethanol which had been preincubated together for 30 min at 23 °C were added to the enzyme. At the indicated times aliquots of the enzymes were taken out and assayed for $(Na^+ + K^+)$-ATPase activity. An appropriate amount of mercaptoethanol was added to those aliquots which contained showdomycin alone. All values are a percentage of the $(Na^+ + K^+)$-ATPase activity at zero time, which averaged 172±16 $\mu$mol $P_i$/mg protein per h. Data points are the means of three separate experiments ±S.E.

| | $(Na^+ + K^+)$-ATPase activity (%) | | | |
|---|---|---|---|---|
| Minutes post showdomycin | 15 | 30 | 45 | 60 |
| Showdomycin | 32.7±0.9 | 16.1±0.8 | 9.8±0.7 | 6.5±0.6 |
| Showdomycin+mercaptoethanol | 92.1±2.4 | 84.2±0.9 | 77.6±2.4 | 76.8±1.1 |
| Showdomycin preincubation with mercaptoethanol | 86.8±3.4 | 79.0±1.9 | 76.8±1.9 | 68.9±0.6 |



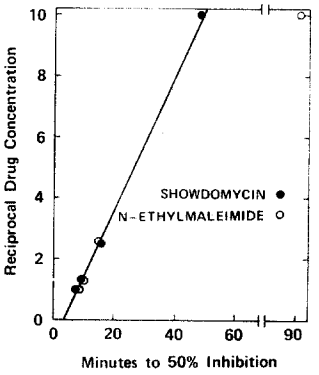




Fig. 3. Protection by ATP against $(Na^+ + K^+)$-ATPase inhibition by maleimide derivatives. About 1 mg of rat brain enzyme was incubated with 1 mM *N*-ethylmaleimide or 1 mM showdomycin in the presence of 1 mM EDTA, with and without 5 mM ATP at 37 °C. At the indicated times an aliquot of the enzyme was removed and assayed for $(Na^+ + K^+)$-ATPase activity. The solid circles (●—●) show the time course of enzyme inhibition in the absence of added ATP, the open circles (○—○) enzyme inhibition in the presence of 5 mM ATP. The open squares (□—□) in Panel A show enzyme activity in the presence of ATP and absence of showdomycin. Enzyme activities are plotted as a percentage of that observed in the presence of ATP and inhibitor at zero minutes incubation, which averaged $234 \pm 5$ μmol $P_i$/mg protein per h. All points are the means of three experimental determinations, with ATPase activity in the presence of $10^{-4}$ M ouabain deducted as background.

Fig. 4. Concentration dependence of rate of inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide and showdomycin. The experimental protocol was as in Fig. 3 except that the concentrations of *N*-ethylmaleimide or showdomycin were varied between 0.1 and 1.0 mM. The times for 50 % inhibition at these concentrations were estimated graphically and plotted against reciprocal drug concentrations. From this relationship the rate constant for $(Na^+ + K^+)$-ATPase inhibition by showdomycin was estimated as $11.01 \cdot mole^{-1} \cdot min^{-1}$ [13]. All experimental points are the means of four separate determinations.

AMP-binding site of phosphorylase *b* [10] also failed to inhibit this enzyme in other experiments (data not shown).

Inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide is characteristically a slow process which is partially prevented by the presence of ATP in the system [12]. Fig. 3 shows a comparison of the rates of inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide and showdomycin and the ability of ATP to protect against inhibition by these agents. Rat brain $(Na^+ + K^+)$-ATPase was relatively slowly inhibited by 1 mM showdomycin, 90 % inhibition taking 45 min to develop. Inhibition by *N*-ethylmaleimide [12] also developed at about the same rate. The time course of inhibition by showdomycin was exponential and 5 mM ATP reduced the rate of inhibition about 10-fold. In contrast, ATP was markedly less effective in protecting against inhibition by *N*-ethylmaleimide. Because *N*-ethylmaleimide produced about three times more inhibition of this enzyme in the presence of ATP than showdomycin, the data suggest that showdomycin is at least three times more specific for the ATP protected sites than *N*-ethylmaleimide.

The times for 50 % inhibition of $(Na^+ + K^+)$-ATPase by different concentrations of these agents plotted against drug concentration are shown in Fig. 4. The linear plot for showdomycin suggests that the rate-limiting step in the inhibition reaction is a bimolecular reaction [13], while the non-linear plot with *N*-ethylmalei-

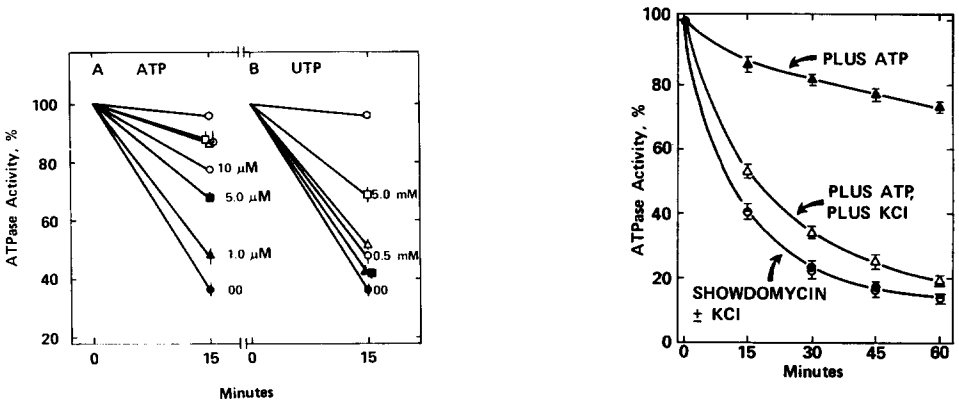



Fig. 5. Effect of nucleotide type and concentration on showdomycin-dependent inhibition of $(Na^+ + K^+)$-ATPase. The experimental conditions are as in Fig. 3 except that the incubation period was 15 min and the concentrations of the nucleotides were varied as indicated. The left-hand panel shows inhibition occurring in the presence of 1 mM showdomycin and 500 $\mu$M (⊙), 100 $\mu$M (□), 50 $\mu$M (△), 10 $\mu$M (○), 5 $\mu$M (■), 1 $\mu$M (▲), and no added ATP (●). The right-hand panel shows inhibition occurring in the presence of 5 mM UTP (□), 1 mM (△), 0.5 mM (○), 0.1 mM (▲), 50 $\mu$M (■) and no added UTP (●). The hexagons (◇) show inhibition in the absence of added nucleotides and showdomycin. Inhibition is calculated as a percentage of that observed in the absence of preincubation, which averaged 196±2 $\mu$mol $P_i$/mg protein per h. All experimental points are the means of four separate determinations ±S.E.

Fig. 6. Prevention by $K^+$ of ATP protection against showdomycin inhibition. The experimental conditions are as in Fig. 3 except that 30 mM KCl was added in the presence or absence of ATP. The solid circles (● — ●) show inhibition of the rat brain enzyme in the presence of 1 mM showdomycin, the open circles (○ — ○) inhibition in the presence of showdomycin plus 3 mM $K^+$. The solid triangles (▲ — ▲) show inhibition in the presence of 0.1 mM ATP while the open triangles (△ — △) show inhibition in the presence of $K^+$ and ATP. $(Na^+ + K^+)$-ATPase activity is plotted as a percentage of that at zero time incubation, which averaged 190±5 $\mu$mol $P_i$/mg protein per h. All points are the means of three separate experiments ±S.E.

mide suggests a more complex reaction. The rate constant for showdomycin inhibition of $(Na^+ + K^+)$-ATPase was about 11.0 $l \cdot mol^{-1} \cdot min^{-1}$.

Recently, Hegyvary and Post [14] have shown that the ATP-binding site(s) on $(Na^+ + K^+)$-ATPase have an apparent affinity for ATP in the order of 0.25 $\mu$M. Therefore, if showdomycin is a nucleotide-site-directed inhibitor of $(Na^+ + K^+)$-ATPase, very low concentrations of ATP should be sufficient to protect the enzyme against inhibition by showdomycin [14]. Fig. 5 shows that half-maximal protection against showdomycin is obtained with approx. 5 $\mu$M ATP. This effect is also nucleotide specific [14] since UTP is about 1000-fold less effective than ATP. In another experiment 5 mM uridine had no protective effect against 1 mM showdomycin. Since the ATP-binding site on $(Na^+ + K^+)$-ATPase has about 1000-fold less affinity for UTP than ATP [14] these characteristics of the protection by ATP correspond well with an action of ATP at the high-affinity nucleotide-binding site(s) of $(Na^+ + K^+)$-ATPase.

The binding of ATP at its high-affinity site on $(Na^+ + K^+)$-ATPase is also readily displaced by $K^+$ [14]. Fig. 6 shows that while 100 $\mu$M ATP provides near maximal protection against inhibition of the ATPase by showdomycin, this protection is antagonized

by 3 mM $K^+$, while $K^+$ alone had no effect on inhibition of the ATPase by showdomycin. The data show that the $K^+$-induced configurational change in the enzyme has little direct effect on the reactivity of the enzyme with showdomycin but that it very effectively inhibited protection of the enzyme by ATP, presumably due to its ability to displace ATP from this enzyme system [13]. In other experiments with *N*-ethylmaleimide, 3 mM $K^+$ also antagonized the partial protection that ATP afforded against *N*-ethylmaleimide inhibition, while neither $Na^+$ or $K^+$ had any marked effects on the initial rates of inhibition of this enzyme by showdomycin or *N*-ethylmaleimide in the absence of ATP.

Though these experiments make it likely that ATP acts by directly shielding the group reactive with showdomycin the experiments provide no evidence as to the identity of this reactive group [15]. To answer this question we investigated the ability of sodium tetrathionate to inhibit this enzyme. Tetrathionate is a disulfide reagent and is considered unlikely to interact with groups other than sulfhydryl groups [16]. Thus, an ATP-protected inhibition of this enzyme by tetrathionate would support the concept that the ATP-protected reactive group is a sulfhydryl group. Preliminary experiments showed tetrathionate to be a relatively potent inhibitor of $(Na^+ + K^+)$-ATPase, and Fig. 7 shows that 0.4 mM tetrathionate produced about 80 % inhibition of this enzyme within 60 min. The addition of ATP to the reaction medium produced a partial protection against inhibition by tetrathionate much like that observed in the presence of *N*-ethylmaleimide. An essentially similar protection was afforded by 100 $\mu$M ATP and this protection was antagonized by $K^+$ (Table III). The experiment shows that tetrathionate inhibits the $(Na^+ + K^+)$-ATPase by reacting with a sulfhydryl group or groups at or near the ATP-binding site,

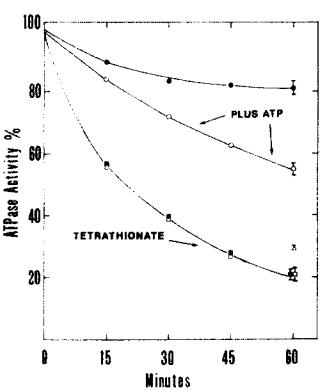



Fig. 7. Tetrathionate inhibition of $(Na^+ + K^+)$-ATPase and protection against inhibition by ATP. The experimental protocol was as in Fig. 3 except that 0.4 mM sodium tetrathionite was substituted for showdomycin. The solid and open squares (■, □) show the rate of inhibition of the enzyme by tetrathionate in the presence of 20 mM $Na^+$ or 20 mM $K^+$, respectively. The open triangle (△) shows the amount of inhibition observed under these conditions in the absence of added cations. The open circles (○ – ○) show inhibition of the enzyme by tetrathionate in the presence of 5 mM ATP while the solid circles (● – ●) show the rate of loss of enzyme activity in the presence of buffer and EDTA. Enzyme activity is expressed as a percentage of that observed after a zero-time preincubation under each of these conditions which averaged 203 pmol $P_i$/mg protein per h. All points at 60 min are the means ±S.E. of four separate experiments.

TABLE III

EFFECT OF KCl ON PROTECTION BY ATP AGAINST TETRATHIONATE INHIBITION

Rat brain ATPase was preincubated with 0.4 mM tetrathionate at 37 °C for 30 or 60 min as described in Fig. 7. ATPase activity is expressed as a percentage of that observed in zero-time preincubations under the indicated conditions, which averaged 218±8 μmol $P_i$/mg protein per h. The concentration of ATP was 100 μM and the concentration of KCl was 3 mM where indicated. The data are the means of four experimental determinations ±S.E.

| Preincubation time (min) | Additions | | | |
|---|---|---|---|---|
| | None | ATP | KCl | ATP+KCl |
| 30 min | 37.8±3.4 | 61.5±0.8 | 27.6±0.6 | 31.8±0.7 |
| 60 min | 24.3±1.8 | 49.6±1.0 | 21.7±1.0 | 24.5±0.3 |

presumably the group(s) accessible to showdomycin and *N*-ethylmaleimide inhibition.

## DISCUSSION

In these experiments the nucleoside antibiotic showdomycin produced a time-dependent, irreversible inhibition of rat brain ($Na^+ + K^+$)-ATPase. This inhibition appeared to depend on the chemical reactivity of showdomycin since preincubation with mercaptoethanol or the presence of mercaptoethanol in the incubation medium protected against inhibition. The time course of inhibition by showdomycin was exponential and the rate of inhibition was directly dependent on the concentration of showdomycin. These observations are consistent with the rate-limiting step in inhibition by showdomycin being a bimolecular reaction with one of the reactants present in excess [13]. The observations suggest that showdomycin inhibits this enzyme by interacting with a specific class of chemically reactive groups on this enzyme.

ATP protected against inhibition of this enzyme by showdomycin. This protective action of ATP was observed at very low concentrations of ATP, consistent with the concept that ATP produced this protection by interacting at the high-affinity binding site(s) for ATP on this enzyme [14]. Consistent with this hypothesis, UTP, which binds much less effectively to the high-affinity nucleotide-binding site(s) than ATP [14], was much less effective in protecting against showdomycin inhibition. Uridine itself also did not protect against showdomycin inhibition. Similarly, if ATP was driven off its high-affinity binding site by the addition of $K^+$ to the incubation medium [14] ATP no longer protected this enzyme against inhibition by showdomycin. These observations show that the protective action of ATP against showdomycin inhibition of the ($Na^+ + K^+$)-ATPase depends on ATP interacting with its high-affinity binding sites on this enzyme.

If ATP produces its protective effect by binding at the high-affinity nucleotide-binding site one may next ask whether or not protection is due to a direct steric effect of bound ATP or to an allosteric or configurational effect induced by bound ATP. In this respect the data obtained with $K^+$ are of interest. $K^+$ appears to produce marked configurational changes in ($Na^+ + K^+$)-ATPase, altering its apparent affinities

for $Na^+$, ouabain [17] and ATP [14] and markedly affecting the rate of reaction of $E_2$-P with water [18] and dissociation of [$^3$H]ouabain [19]. These configurational changes, however, did not affect the reactivity of the specific SH groups with showdomycin but only affected the ability of ATP to protect against this inhibition. Similarly, current data suggests that ($Na^+ + K^+$)-ATPase in the presence of EDTA exists in a configuration with a high affinity for ATP, and ATP interacts readily and with apparently minimal configurational effects on native ($Na^+ + K^+$)-ATPase [14]. Similarly $Na^+$ did not affect the rate of inhibition of the enzyme by showdomycin and did not antagonize the protective effect of ATP, consistent with the concept that $Na^+$ produces little configurational change in the $E_1$ or ATP-binding configuration of ($Na^+ + K^+$)-ATPase. Thus, ATP protects against showdomycin inhibition but produces minimal configurational changes in the enzyme, and $Na^+$ does not alter the interaction of the enzyme with ATP. On the other hand, $K^+$ produces marked configurational changes and displaces ATP from its binding site. These observations are consistent with the protective effect of ATP against showdomycin inhibition being a direct steric effect of bound ATP rather than an allosteric or configurational effect.

If inhibition by showdomycin was due to interactions at a site of sites distinct from the catalytic site one might expect different effects of showdomycin on the partial reactions of ($Na^+ + K^+$)-ATPase whose reaction rate may be modified by distinct subunits of this enzyme. A number of preliminary experiments not presented here suggested that showdomycin inhibition affected [$^{32}$P]ATP binding, E-P formation, [$^3$H]ouabain binding and *p*-nitrophenylphosphatase activity equivalently, consistent with the idea that showdomycin inhibition primarily affects the active center for phosphorylation on this enzyme system.

Inhibition of the ($Na^+ + K^+$)-ATPase by showdomycin shows the same general characteristics as inhibition by *N*-ethylmaleimide (Fig. 4, Refs 12, 20). However, inhibition by *N*-ethylmaleimide did not follow the kinetics of a bimolecular reaction, *N*-ethylmaleimide being less effective than showdomycin at lower concentrations (Fig. 4). The principal difference between *N*-ethylmaleimide and showdomycin observed in these experiments was that ATP protected much less effectively against inhibition by *N*-ethylmaleimide than against inhibition by showdomycin. The data suggest that showdomycin is more specific for the nucleotide-protected site(s) than *N*-ethylmaleimide and that *N*-ethylmaleimide reacts at both the nucleotide-protected site(s) and elsewhere on this enzyme.

As pointed out by Hokin and Dahl [15] many sulfhydryl reactants inhibit the ($Na^+ + K^+$)-ATPase but there is little evidence that the ATP-protected group with which these reagents interact is actually a sulfhydryl group. Further, these authors point out that ATP will also protect against inhibition by reagents which do not react with free sulfhydryl groups [21]. To resolve this question we examined the interaction of tetrathionate with ($Na^+ + K^+$)-ATPase, since tetrathionate is a reagent which is considered specific for sulfhydryl groups [22]. The results show that the pattern of tetrathionate inhibition of this enzyme is similar to that observed with *N*-ethylmaleimide, and partial protection against this inhibition by low concentrations of ATP was observed. Further, this partial protection by ATP was antagonized by $K^+$ like that due to showdomycin and *N*-ethylmaleimide. The results show that the binding of ATP by this enzyme does indeed shield a sulfhydryl group and this sulfhydryl group is probably the group which reacts with showdomycin and *N*-ethyl-

maleimide.

The present data obtained with *N*-ethylmaleimide are in good agreement with those of Skou and Hilberg [20]. These authors observed that 1 mM *N*-ethylmaleimide inhibited ox brain $(Na^+ + K^+)$-ATPase at about the same initial rate as observed by us and reported partial protection against this inhibition by ATP. These authors also observed almost complete antagonism by $K^+$ of the protective effect of ATP against *N*-ethylmaleimide inhibition and similar results have been reported by Banerjee and coworkers [12]. Skou [23] has also recently reported that $Na^+$ blocks the action of $K^+$ on protection by ATP, consistent with the idea that $Na^+$ acts to increase the affinity of the enzyme for ATP in the presence of $K^+$, thus allowing ATP to bind at its protective sites.

Studying the labeling of rabbit kidney $(Na^+ + K^+)$-ATPase by radiolabeled *N*-ethylmaleimide, Hart and Titus [24, 25] showed that all labeling modified by physiological ligands of this enzyme occurred to a peptide of about 98 000 molecular weight, presumably the same peptide as is phosphorylated by ATP in these preparations [26–28]. These workers report at least six binding sites for *N*-ethylmaleimide per active site of $(Na^+ + K^+)$-ATPase, and under the conditions of their experiments only about 10 % of the available reactive sites for *N*-ethylmaleimide were protected by ATP from alkylation. The identification of showdomycin as a nucleotide-site-directed inhibitor of $(Na^+ + K^+)$-ATPase should enable more specific labeling of the nucleotide-protected reactive groups on this enzyme by either showdomycin or its 5′-phosphorylated derivative and many lead to their indentification.

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