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INHIBITION OF GILL ($Na^+ + K^+$)-ATPase IN RAINBOW TROUT (*SALMO GAIRDNERI*) BY A PURINE DISULFIDE ANALOG OF ADENOSINE TRIPHOSPHATE

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Summary

The effect of the adenosine triphosphate analog, 6,6′-dithiobis(inosinyl imidodiphosphate), $(sIMP\text{-}PNP)_2$, was tested on the ouabain-sensitive ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and the ouabain-insensitive Mg^{2+}-ATPase in microsomes prepared from gill tissue of sea-water-adapted rainbow trout, *Salmo gairdneri*. The ($Na^+ + K^+$)-ATPase was completely inhibited by low concentrations of $(sIMP\text{-}PNP)_2$ (6 μM) but the Mg^{2+}-ATPase was unaffected by the inhibitor at concentrations as high as 28 μM, supporting the suggestion that the two activities represent separate enzymes. The specificity of inactivation could be demonstrated both at a physiological temperature (13°C) and at 37°C. The rates of inactivation were similar at both temperatures. Inactivation of the ($Na^+ + K^+$)-ATPase by $(sIMP\text{-}PNP)_2$ was reversed by dithiothreitol, suggesting that the inhibitor forms a mixed disulfide with sulfhydryl groups on the enzyme. The inability of substrate (either ATP or its analog, adenyl-5′-yl imidodiphosphate) to protect against inactivation suggests that $(sIMP\text{-}PNP)_2$ is reacting with sulfhydryl groups which are not associated with the active site.

Introduction

The ouabain-sensitive ($Na^+ + K^+$)-ATPase is believed to play an essential role in active ion transport across biological membranes [1—3]. There is evidence that sulfhydryl groups are associated with the active center of the enzyme from rabbit kidney [4] and the active site of the enzyme from beef brain [5].

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Abbreviations: ($Na^+ + K^+$)-ATPase, ($Na^+ + K^+$)-activated adenosine triphosphatase (ATP phosphohydrolase EC 3.6.1.3); Mg^{2+}-ATPase, Mg^{2+}-dependent adenosine triphosphatase; sIMP-PNP, 6-thioinosinyl imidodiphosphate; $(sIMP\text{—}PNP)_2$, 6,6′-dithiobis(inosinyl imidodiphosphate); AMP-PNP, adenyl-5′-yl imidodiphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Patzelt-Wenczler and Schoner [6] reported that the disulfide form of thioinosine triphosphate, $(sITP)_2$, inactivated the $(Na^+ + K^+)$-ATPase from beef brain microsomes by reacting with a sulfhydryl group in the substrate binding site. Evidence was presented showing that inactivation proceeds via the formation of a reversible central complex between the enzyme and inhibitor before a slower irreversible inactivation step occurs [6].

Recent studies of another purine disulfide analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate), $(sIMP\text{-}PNP)_2$, have shown its utility in labeling specific cysteines in skeletal [7—9] and cardiac [10,11] myosin. Blocking these cysteines inhibits both ATP cleavage and actin binding [12]. Although $(sIMP\text{-}PNP)_2$ does not react at the active site [9], it does react at a specific binding site involving the so-called alkali light chains of myosin.

The possibility that $(sIMP\text{-}PNP)_2$ might be an active site-specific reagent for the membrane-bound $(Na^+ + K^+)$-ATPase and/or Mg^{2+}-ATPase led to the investigation of its reaction with these enzymes in microsomes prepared from gill tissue of sea water-adapted rainbow trout (*Salmo gairdneri*). Previous studies [13] have shown these two gill enzymes to be similar to those studied in other tissues. This paper reports that $(sIMP\text{-}PNP)_2$ is a potent inhibitor of the trout gill $(Na^+ + K^+)$-ATPase but is without effect on the Mg^{2+}-ATPase. However, the evidence presented here suggests that $(sIMP\text{-}PNP)_2$ is reacting with sulfhydryl groups which are not associated with the active site.

Materials and Methods

Animals. Fresh water rainbow trout, weighing from 150 to 300 g, were obtained from a commercial hatchery and kept unfed in a holding tank at 13°C. Fish were adapted to sea water, also at 13°C, as previously described [13].

Preparation and assay of enzyme. The enzyme solution consisted of NaI-treated microsomes prepared as previously described [13] except that dithiothreitol was omitted both during homogenization of gill tissue and from the final enzyme solution. The enzyme assay reaction mixture contained 30 mM HEPES (made to pH 7.1 with Tris), 5.0 mM $MgCl_2$, 3.2 mM Tris/ATP, 100 mM NaCl, 20 mM KCl, and enzyme (0.1—0.2 mg/ml). Both sodium and potassium were omitted in the determination of Mg^{2+}-ATPase activity. After a 10 min equilibration, the assay was started by addition of Tris/ATP. A 30 min preincubation of the reaction mixture (minus ATP) at 37°C was used for the 13°C assays. This treatment was found to lower Mg^{2+}-ATPase activity by 85% while decreasing the total activity ($(Mg^{2+} + Na^+ + K^+)$-ATPase) by less than 20%, thus enhancing the ouabain-sensitive $(Na^+ + K^+)$-ATPase [13]. The final volume was 1.0 ml. The reaction was run at 13°C or 37°C for 10—30 min and then stopped by addition of 1.0 ml 15% trichloroacetic acid. Precipitated protein was removed by centrifugation at 27000 × *g* for 5 min and a 1.0—1.5 ml fraction of the supernatant taken for determination of inorganic phosphate by the method of Fiske and Subbarow [14]. Protein was determined by a slightly modified method of Lowry et al. [15] using bovine plasma albumin as a standard.

Chemicals. $(sIMP\text{-}PNP)_2$ was prepared by sodium triiodide oxidation of the 6-thioinosinyl imidodiphosphate [16]. Concentrations of $(sIMP\text{-}PNP)_2$ were determined spectrophotometrically as described by Greene and Yount [10].

AMP-PNP was prepared and characterized as previously described [17] and was greater than 96% pure. Tris/ATP was prepared from the disodium salt of ATP (Calbiochem) by a previously described method [13]. HEPES and dithiothreitol were from Calbiochem.

Results

In most of the experiments reported here an assay temperature of 37°C was used which is much higher than the 13°C adaptation temperature of the trout. However, the temperature optimum of the $(Na^+ + K^+)$-ATPase in sea water-adapted trout is about 45°C; that of the Mg^{2+}-ATPase is about 15—24°C (see reference 25). Since no attempt was made to relate the results reported in this paper directly to physiological function in the fish, this assay temperature (37°C) was chosen to maximize the $(Na^+ + K^+)$-ATPase activity but still permit the detection of significant levels of Mg^{2+}-ATPase (see Table II, ref. 13). At 37°C, as at lower temperatures, $5 \cdot 10^{-4}$ M ouabain inhibited the $(Na^+ + K^+)$-ATPase but had no effect on the Mg^{2+}-ATPase (Table I).

Effect of (sIMP-PNP)$_2$

The effect of increasing concentrations of $(sIMP\text{-}PNP)_2$ on Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities at 37°C is shown in Fig. 1. The Mg^{2+}-ATPase is unaffected by $(sIMP\text{-}PNP)_2$ within the concentration range studied. However the $(Na^+ + K^+)$-ATPase activity (i.e. $(Mg^{2+} + Na^+ + K^+)$-ATPase minus the Mg^{2+}-ATPase) is quite sensitive to this inhibitor. Essentially complete inhibition of the enzyme occurs in 10 min at a $(sIMP\text{-}PNP)_2$ concentration of about 6 μM. Inhibition could be reversed by adding excess dithiothreitol to $(sIMP\text{-}PNP)_2$-treated enzyme (not shown).

It is important to demonstrate that $(sIMP\text{-}PNP)_2$ inhibits only the ouabain-sensitive ATPase activity in order to confirm that the $(Na^+ + K^+)$-ATPase activity is the only one being affected. This is shown in Table I. The Mg^{2+}-ATPase

TABLE I

EFFECT OF OUABAIN ON TROUT GILL ATPase TREATED WITH $(sIMP\text{-}PNP)_2$

25 μM $(sIMP\text{-}PNP)_2$ was equilibrated with the designated reaction mixture minus ATP for 10 min at 37°C. Ouabain was then added where indicated to give a final concentration of 0.5 mM followed by an additional 10 min equilibration. Assays were run as described in Materials and Methods for 30 min at 37°C using 0.2 mg/ml enzyme.

Expt. No.	$(sIMP\text{-}PNP)_2$	Specific activity (μmol P_i/mg protein per h)			
		—ouabain		+ouabain	
		Mg *	Mg,Na,K **	Mg *	Mg,Na,K **
1	—	4.0	7.0	3.8	4.9
	+	4.0	4.7	3.8	4.5
2	—	3.9	6.8	4.0	5.2
	+	3.9	4.9	4.6	4.8

* Mg^{2+}-ATPase specific activity.

** $(Mg^{2+} + Na^+ + K^+)$-ATPase (total ATPase) specific activity.

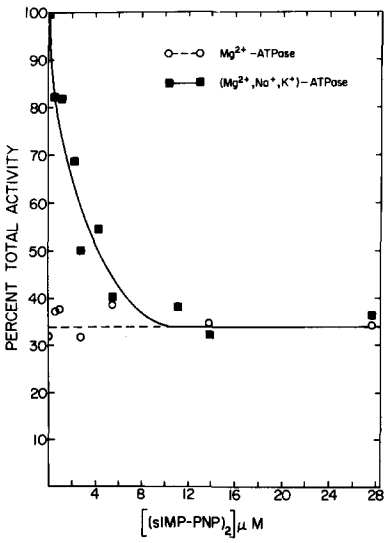

Fig. 1. Effect of $(sIMP\text{-}PNP)_2$ concentration on Mg^{2+}-ATPase and total $(Mg^{2+} + Na^+ + K^+)$-ATPase activity at 37°C. The $(sIMP\text{-}PNP)_2$ was added at the beginning of the 10 min equilibration (see Materials and Methods) and was present during the assay. The values are expressed as percent of control ($(sIMP\text{-}PNP)_2$-free) activity. A representative specific activity for $(Mg^{2+} + Na^+ + K^+)$-ATPase is 35 μmol P_i/mg protein per h. Total ATPase represents activity with 5 mM Mg^{2+}, 100 mM Na^+, and 20 mM K^+. Mg^{2+}-ATPase represents activity with 5 mM Mg^{2+} in the reaction mixture. The results were the same for both 10- and 30-min assays.

activity is unaffected by either ouabain or $(sIMP\text{-}PNP)_2$ whereas the $(Mg^{2+} + Na^+ + K^+)$-ATPase activity remaining after $(sIMP\text{-}PNP)_2$ inactivation is not further reduced by ouabain. Thus the activity observed after inactivation by relatively high concentrations of $(sIMP\text{-}PNP)_2$ is due to the Mg^{2+}-ATPase only. The $(Mg^{2+} + Na^+ + K^+)$-ATPase specific activities in these experiments were lower than normal but the effects of the inhibitors are clear.

Because kinetic parameters are known to vary when some enzymes from ectothermic organisms are assayed at non-physiological temperatures [18], inactivation experiments were also conducted at 13°C, the adaptation temperature of the trout. The results are shown in Fig. 2. As at 37°C, Mg^{2+}-ATPase activity was unaffected by $(sIMP\text{-}PNP)_2$ whereas $(Na^+ + K^+)$-ATPase activity was abolished. Inhibition was complete after a 10 min incubation with a $(sIMP\text{-}PNP)_2$ concentration of about 8 μM, further indicating the marked sensitivity of the $(Na^+ + K^+)$-ATPase to this inhibitor. These results show that the specificity of $(sIMP\text{-}PNP)_2$ inactivation is not altered by a lowering of the reaction temperature. In addition, within experimental error, the rates of inactivation are similar at the two temperatures.

The rate of $(Na^+ + K^+)$-ATPase inactivation at 37°C, using different concentrations of $(sIMP\text{-}PNP)_2$, is shown in Fig. 3. Inactivation was rapid, dependent on $(sIMP\text{-}PNP)_2$ concentration and, within experimental error, pseudo first-order in enzyme over at least one half-life for all concentrations of inhibitor tested. As can be seen from Fig. 3, some inactivation occurred during the assay even in the presence of a large excess of ATP, since, at zero time, the lines do not intercept at 100% activity. The zero-time values correspond to the end of

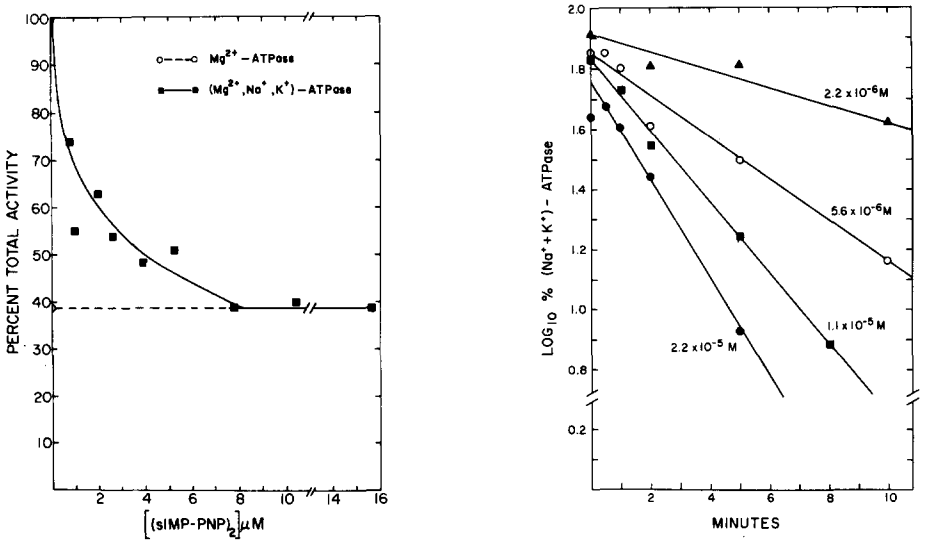

Fig. 2. Effect of $(sIMP\text{-}PNP)_2$ concentration on Mg^{2+}-ATPase and total $(Mg^{2+} + Na^+ + K^+)$-ATPase activity at 13°C. Reaction mixtures were preincubated, without $(sIMP\text{-}PNP)_2$, for 30 min at 37°C. After a 5 min equilibration at 13°C, $(sIMP\text{-}PNP)_2$ was added and 10 min later the reaction was started. The values are expressed as percent of control ($(sIMP\text{-}PNP)_2$-free) activity. Ion additions are the same as in Fig. 1. Assay time was 30 min.

Fig. 3. Semi-log plot of the rate of $(Na^+ + K^+)$-ATPase inactivation at various $(sIMP\text{-}PNP)_2$ concentrations. The inhibitor was added at the given times during the 10 min equilibration. The points on the ordinate were obtained by adding $(sIMP\text{-}PNP)_2$ and ATP (to start the reaction) simultaneously. The reaction was run for 10 min at 37°C.

the inactivation period and the beginning of the assay and were obtained by adding $(sIMP\text{-}PNP)_2$ and ATP simultaneously to the reaction mixture. Also, as the inhibitor concentration increases, the amount of inhibition occurring during the assay increases as seen by the progressively lower intercept points. This suggests that ATP is unable to completely protect the enzyme against inactivation, even when present at concentrations 10^2—10^3 times that of the inhibitor. This observation makes the interpretation of the kinetic data more difficult. In addition, the inhibition which occurs during the assay, at any given inhibitor concentration, does not appear to be uniform since no differences were noted in degree of inhibition for assays of 10 and 30 min (see Fig. 1). Nevertheless, the data in Fig. 3 suggest that inactivation occurs by means of a pseudo first-order process. However, because of the problems just mentioned, a further kinetic analysis of the data, which would reveal more about the mechanism of inactivation [19], was not attempted.

Substrate protection experiments

If $(sIMP\text{-}PNP)_2$ were inactivating the enzyme by interacting with a sulfhydryl group(s) in the ATP binding site, excess substrate should protect the enzyme against inactivation. As mentioned previously, inactivation continues during the assay suggesting that large excesses of ATP are not protecting the enzyme. In an attempt to explore this further, $(sIMP\text{-}PNP)_2$ inactivation was examined in the presence of excess AMP-PNP, an ATP analog differing from the parent com-

TABLE II

EFFECT OF AMP-PNP ON $(sIMP\text{-}PNP)_2$ INACTIVATION OF TROUT GILL $(Na^+ + K^+)$-ATPase

Activity remaining, expressed as percent of control activity, after a 10 min incubation with 4.2 μM $(sIMP\text{-}PNP)_2$ (I) plus three different concentrations of AMP-PNP; 22 μM (II), 72 μM (III), and 167 μM (IV). AMP-PNP and $(sIMP\text{-}PNP)_2$ were added simultaneously. Activity values have been corrected for AMP-PNP inhibition of control activity (see Results).

Expt. No.	I	II	III	IV
1	50	53 (3) *	—	—
2	28	—	45 (17)	—
3	33	34 (1)	56 (23)	34 (1)
4	34	28 (—)	44 (10)	48 (14)
Average percent protection		(1)	(17)	(8)

* The percent protection against $(sIMP\text{-}PNP)_2$ inactivation by AMP-PNP is shown in parentheses. Assays were run as described in Materials and Methods for 10 min at 37°C using 0.1 mg/ml of enzyme.

pound only by a -NH- substitution linking the β and γ phosphates. The -NH- linkage prevents cleavage by a large variety of mammalian enzymes [17]. AMP-PNP is sufficiently similar to ATP that it would be expected to occupy ATP sites on the enzyme but without being hydrolyzed. It was found that AMP-PNP is indeed an inhibitor of the $(Na^+ + K^+)$-ATPase (22 μM AMP-PNP inhibits activity by 5%; 72 μM by 8%; 167 μM by 22%), suggesting competition between AMP-PNP and ATP for the substrate binding site.

The effect of various concentrations of AMP-PNP on $(sIMP\text{-}PNP)_2$ inactivation is shown in Table II. AMP-PNP and $(sIMP\text{-}PNP)_2$ were added simultaneously to the reaction mixtures and 10 min later reactions were started by addition of ATP. The data show that very little substrate protection (less than 20%) occurs in the presence of a 5—35-fold excess of AMP-PNP over $(sIMP\text{-}PNP)_2$. In one experiment (not shown in Table II) microsomes were treated with 3 mM AMP-PNP before addition of 3.8 μM $(sIMP\text{-}PNP)_2$. After 10 min at 37°C the microsomes were isolated by centrifugation and resuspended in the assay solution lacking AMP-PNP and $(sIMP\text{-}PNP)_2$ and assayed for $(Na^+ + K^+)$-ATPase activity. Even at this concentration, AMP-PNP showed little evidence of protecting against $(sIMP\text{-}PNP)_2$ inactivation.

Discussion

It has been shown that the purine disulfide analog of ATP, $(sIMP\text{-}PNP)_2$, is a potent and specific inhibitor of trout gill $(Na^+ + K^+)$-ATPase. The lack of an inhibitory effect on the Mg^{2+}-ATPase supports the suggestion, based on purification studies [20], that the two activities represent different enzymes. Inactivation of the $(Na^+ + K^+)$-ATPase by $(sIMP\text{-}PNP)_2$ can be reversed by treatment with dithiothreitol, suggesting that the inhibitor is forming a mixed disulfide with sulfhydryl groups on the enzyme. Further evidence for a disulfide exchange reaction between a similar analog, $(sITP)_2$, and sulfhydryl groups of beef brain $(Na^+ + K^+)$-ATPase was obtained by Patzelt-Wenczler and Schoner [6] using $([\gamma\text{-}^{32}P]sITP)_2$. These workers found that inactivation was accompanied by the incorporation of radioactivity into the membrane protein and that

both inactivation and labeling could be reversed by dithiothreitol. Since $(sITP)_2$ and $(sIMP\text{-}PNP)_2$ differ only in that the latter contains a -NH- substitution between the β and γ phosphates, it is not unreasonable to assume that they would both have similar mechanisms of inactivation.

It was found that substrate protects the beef brain $(Na^+ + K^+)$-ATPase from $(sITP)_2$ inactivation, suggesting that the inhibitor is reacting with sulfhydryl groups in the active site [6]. However, little or no substrate protection against $(sIMP\text{-}PNP)_2$ inactivation was found for the trout gill enzyme. It would not seem likely that two very similar analogs would have two different apparent sites of action on the enzyme. This discrepancy might be due to the different preparations employed or possibly to differences in inactivation conditions. Inactivation of beef brain $(Na^+ + K^+)$-ATPase was conducted in the absence of Mg^{2+}, Na^+, and K^+ whereas these ions were present in the trout gill experiments. Preliminary experiments suggested that inactivation of trout gill $(Na^+ + K^+)$-ATPase by $(sIMP\text{-}PNP)_2$ proceeds more slowly in the absence of Mg^{2+} than in its presence. The deletion of Na^+ and K^+, however, did not slow down the inactivation. Similar effects of Mg^{2+} were found for $(sIMP\text{-}PNP)_2$ inactivation of heavy meromyosin [16].

A preliminary analysis of the pseudo first-order rate constants obtained from Fig. 3, as described by Kitz and Wilson [19], suggests that $(sIMP\text{-}PNP)_2$ inactivation of the trout gill $(Na^+ + K^+)$-ATPase shows saturation kinetics. This would indicate that $(sIMP\text{-}PNP)_2$ rapidly forms a central complex with the enzyme before a slower inactivation step takes place. Such a mechanism has been postulated for $(sITP)_2$ inactivation of beef brain $(Na^+ + K^+)$-ATPase [6]. However, because of the problem of inactivation continuing during the assay, the true mechanism of $(sIMP\text{-}PNP)_2$ inactivation remains unclear. Therefore it could be argued that $(sIMP\text{-}PNP)_2$ was inactivating the enzyme by binding nonspecifically to membrane sulfhydryl groups. This has been suggested for ethacrynic acid inhibition of renal $(Na^+ + K^+)$-ATPase [21] and must be considered since the membrane-bound enzyme used here was not in the purified form (see ref. 20). However, the observations that $(sIMP\text{-}PNP)_2$ inactivation is specific for the $(Na^+ + K^+)$-ATPase and that it occurs at relatively low inhibitor concentrations argue against this interpretation.

Finally it should be noted that other disulfide compounds, which are not analogs of ATP, also inhibit $(Na^+ + K^+)$-ATPase activity. These include dithiobisnitrobenzoic acid [22] and oxidized glutathione [23] but with these reagents both the Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase are inhibited. In this regard the specificity of $(sIMP\text{-}PNP)_2$ is unique. However, the reaction of the monosulfide, sIMP-PNP, with mammalian erythrocyte ghosts shows just the opposite specificity, i.e. sIMP-PNP specifically inactivates the Mg^{2+}-ATPase and is without effect on the $(Na^+ + K^+)$-ATPase [24]. This specificity was lost at high temperatures and pH values above 7.5. These results suggest that the role of sulfhydryl groups in the catalytic mechanisms of these two enzymes needs to be more thoroughly examined.

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