Thiophosphoryl Guanine Nucleotide Analogues Inhibit the (Na^+,K^+)-ATPase†

Diana L. McGill‡

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138

Received February 5, 1991; Revised Manuscript Received April 12, 1991

ABSTRACT: The effects of several guanine nucleotide analogues on (Na^+,K^+)-ATPase activity of membranes isolated from several tissues were analyzed to determine if a G-protein might be involved in the hormonal regulation of the (Na^+,K^+)-ATPase. Submillimolar concentrations of GTPγS, but not GMPPNP, inhibit rat skeletal muscle and axolemma, but not kidney, (Na^+,K^+)-ATPase activity. Furthermore, GDPβS does not reverse GTPγS inhibition, but rather itself slightly inhibits (Na^+,K^+)-ATPase activity. Dithiothreitol can block and reverse GTPγS inhibition of skeletal muscle (Na^+,K^+)-ATPase; the results obtained with axolemma membranes are complicated by the inhibition of (Na^+,K^+)-ATPase activity in these membranes by DTT. Results showing that high membrane concentrations can mute the inhibitory action of GTPγS suggest that a minor contaminant in GTPγS preparations is responsible for inhibiting (Na^+,K^+)-ATPase activity. Neither vanadate, a heavy metal, GDP, phosphate, nor thiophosphate, however, is responsible for this inhibition, and the inhibitory activity elutes with GTPγS from Sephadex G-10 columns. It is concluded that GTPγS or a structural derivative of GTPγS inhibits the (Na^+,K^+)-ATPase, in a tissue-specific manner, not by interaction with a G-protein as a GTP analogue, but through a direct chemical interaction with the (Na^+,K^+)-ATPase or some regulatory protein. The terminal SH group of the nucleotide analogue is probably required for this interaction.

The (Na^+,K^+)-ATPase[1] is an integral membrane protein that is responsible for maintaining potassium and sodium gradients across the plasma membranes of eukaryotic cells (Cantley, 1981; Jorgensen & Andersen, 1988). The enzyme exists as an αβ dimer. The α chain is responsible for catalytic activity; the β chain is not known to function in catalytic turnover, but may be necessary for the proper assembly and insertion of the dimer into plasma membranes (Hiatt et al., 1984; Takeyasu, 1988; Horowitz et al., 1990a). In rats, there are two known isoforms of the β chain, β1 and β2 (Mercer et al., 1986; Young et al., 1987; Gloor et al., 1990; Martin-Vasallo et al., 1989), and three isoforms of the α chain, α1, α2, and α3 (Shull et al., 1986). The expression of these isoforms is tissue specific (Sweadner, 1979; Lytton et al., 1985; Hsu & Guidotti, 1989; Urayama et al., 1989; Young & Lingrel, 1987). The three α chains have different sensitivities to ouabain, NEM, and pyrithiamin (Sweadner, 1979; Urayama & Sweadner, 1988; Hsu & Guidotti, 1989, Matsuda et al., 1984). In addition, they appear to be differentially regulated by various hormones (Horowitz et al., 1990b; Schmitt & McDonough, 1988; Orlowski & Lingrel, 1990; Lytton, 1985; Brodsky, 1990).

The mechanism of regulation of the (Na^+,K^+)-ATPase by some hormones has been elucidated. Several agents stimulate (Na^+,K^+)-ATPase activity by increasing sodium ion flux into cells (Fehlmann & Freychet, 1981; Rosic et al., 1985; Smith & Rozengurt, 1978; Mendoza et al., 1980). Other hormones, such as thyroid hormone (Lo & Edelman, 1976; Schmitt & McDonough, 1988) and aldosterone (Geering et al., 1982; Verrey et al., 1987), increase the Na^+ and K^+ pumping capacity of cells by increasing the number of (Na^+,K^+)-ATPase molecules in the plasma membranes. Insulin stimulates the (Na^+,K^+)-ATPase by an as yet unidentified mechanism, not involving increases in intracellular sodium concentrations or in pump number (Clausen & Hansen, 1977; Clausen & Kohn, 1977; Resh, 1982; Resh et al., 1980; Lytton, 1985; McGill and Guidotti, unpublished results).

Recent evidence suggests the involvement of G-proteins in at least some insulin-regulated pathways. Pertussis toxin treatment of adipocytes inhibits insulin binding and increases the $K_{0.5}$ of insulin stimulation of glucose uptake by approximately 10-fold (Ciaraldi & Maisel, 1989). Because of these and similar results and because it has recently been suggested that dopamine regulation of kidney (Na^+,K^+)-ATPase may be mediated by a G-protein (Aperia et al., 1987; Bertorello & Aperia, 1989), the possible involvement of G-proteins in the regulation of the (Na^+,K^+)-ATPase from a variety of tissues was investigated.

As an initial step in this study, various guanine nucleotide analogues were tested for their ability to regulate the (Na^+,K^+)-ATPase activity from rat skeletal muscle, axolemma, and kidney. This approach in assaying for G-protein-mediated regulation has been used in a variety of systems [for review, see Gilman (1987)]. In this report, it is shown that GTPγS inhibits (Na^+,K^+)-ATPase activity from axolemma and skeletal muscle, but not from kidney; however, GMPPNP has little effect. Moreover, rather than blocking GTPγS inhibition, GDPβS itself inhibits ATPase activity. These results are not expected if the effects of GTPγS are mediated via a G-protein. Features of GPTγS inhibition of (Na^+,K^+)-ATPase activity are presented in this report.

Materials and Methods

Materials. Male CD rats (125–175 g) were obtained from Charles River Breeding Laboratories. Guanylyl imidodiphosphate (GMPPNP), GDPβS, GTPγS, GDP, and GTP were obtained from Boehringer Mannheim Biochemicals as the lithium salts. Thiophosphoryl chloride was obtained from

† This research was supported by NIH Grant HL08893 to G. Guidotti. D.L.M. was supported during part of the course of this work by a fellowship from the National Science Foundation.

‡ Present address: Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati Medical Center, 231 Bethesda Ave. (ML 524), Cincinnati, OH 45267-0524.

[1] Abbreviation: (Na^+,K^+)-ATPase or (Na^+,K^+) pump, sodium and potassium ion activated ATP phosphohydrolase.

0006-2960/91/0430-6727$02.50/0

Aldrich for the synthesis of thiophosphate, according to the method of Yasuda and Lambert (1954). Ouabain, Na_2ATP, dithiothreitol, NADH, lactate dehydrogenase, pyruvate kinase, and phosphoenolpyruvate were from Sigma. All other reagents were of analytical grade or better.

Membrane Preparations. Rat kidney microsomal membranes were prepared according to Jorgensen (1974). Axolemma plasma membranes were prepared according to Devries et al. (1978). Plasma membranes were prepared from rat hindlimb muscle according to the method of Barchi et al. (1979). Stripped synaptosomal membranes were kindley provided by Jeff Brodsky (Brodsky, 1990). Protein concentrations were determined according to Peterson (1977) using bovine serum albumin as a standard.

(Na^+,K^+)-ATPase Activity Measurements. All experiments were carried out at 37 °C in buffer A (20 mM imidazole hydrochloride, pH 7.4) with the listed ionic conditions. Most activity determinations were made by measuring the release of P_i colorimetrically according to Ames (1966). Buffer A plus 5 mM $MgCl_2$, 20 mM KCl, and 100 mM NaCl, referred to as buffer B, was used, unless otherwise noted. Membranes were incubated in 225-μL aliquots for 20 min at 37 °C with all necessary reagents except ATP. A 25-μL aliquot of 20 mM Na_2ATP was added to start the reaction. Five minutes later, 300 μL of 10% SDS was added to stop the reaction and a 750-μL aliquot of ascorbic acid/molybdate solution was added for color development (Ames, 1966). A standard curve of inorganic phosphate was always used in conjunction with ATPase measurements for the determination of amounts of P_i released by ATPase activity. All activities presented have been corrected for any non-ouabain-inhibitable ATPase activity.

In experiments measuring ATP concentration dependence of ATPase activity, the coupled enzyme method (Josephson & Cantley, 1977), using buffer B and omitting dithiothreitol, was used. ATPase activity was monitored at 340 nm on a Beckman DU50 spectrophotometer using the Kinetics Soft-Pac.

Occasionally, ATPase activity was determined by measuring the relese of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$, essentially according to Goldin (1977), again using buffer B, using only isobutyl alcohol for extractions.

Partial Reactions. (A) p-Nitrophenylphosphatase Activity Measurements. Because high concentrations of NaCl inhibit (Na^+,K^+) pump pNPPase activity [Skou (1974) and data not shown] but are necessary for GTPγS inhibition of the (Na^+,K^+)-ATPase, the following protocol was designed to determine the sensitivity of pNPPase activity to GTPγS. Axolemma membranes (10 μg) were incubated in the presence or absence of 250 μM GTPγS in 125 μL of buffer A with 5 mM $MgCl_2$, 0 mM KCl, and 40 mM NaCl, with or without 2 mM ouabain, for 20 min at 37 °C. To start the reaction, the mix was diluted 2-fold into a solution with the following final composition: 20 mM imidazole hydrochloride/5 mM $MgCl_2$/25 mM KCl/20 mM NaCl/5 mM *p*-nitrophenyl phosphate. The reaction was stopped 7 min later by the addition of 600 μL of 10% SDS/1 N NaOH at a 5:1 ratio and the absorbance read at 410 nm. A standard curve of *p*-nitrophenol was treated equally and used to determine the amount of *p*-nitrophenol released by the (Na^+,K^+)-ATPase.

(B) Na^+-ATPase Activity. Na^+-ATPase activity of the pump was measured according to Gorga (1985) with modifications. Skeletal muscle membranes (10 μg) were incubated in a volume of 240 μL containing buffer A/1 mM $MgCl_2$/100 mM NaCl with or without 250 μM GTPγS for 20 min. A 10-μL aliquot of $[\gamma\text{-}^{32}P]ATP$ (final 1 mM, 2 μCi) was added and the ATPase reaction continued for 15 min. The reaction was terminated by the addition of 750 μL of 0.5% ammonium molybdate in 1 N H_2SO_4. The $[^{32}P]P_i$ was extracted with 1 mL of isobutyl alcohol, 0.7 mL of which was counted. The amount of $[^{32}P]P_i$ released in the presence of 2 mM ouabain was subtracted from all values.

Measurement of [EP]. The measurement of [EP] was essentially according to Gorga (1985). Skeletal muscle membranes (10 μg) were incubated for 30 min at 37 °C in buffer with the following composition: buffer A/5 mM $MgCl_2$/100 mM NaCl/±250 μM GTPγS/±25 mM KCl. The reactions were then cooled on ice for 2 min, at which point $[\gamma\text{-}^{32}P]ATP$ was added (100 μM, 10 μCi). The reaction was stopped 30 s later and the sample assayed for [EP] as described (Gorga, 1985).

Fractionation of GTPγS. (A) Sephadex G-10. Sephadex G-10 was swollen in H_2O and packed into a 5-mL, 1 cm × 20 cm column, which was equilibrated with buffer A. A 100-μL aliquot of 15 mM GTPγS was applied to the column, and 5-drop (220-μL) fractions were collected. To assay for GTPγS, 25-μL aliquots were diluted into 1 mL of H_2O and the absorbance was read at 253 nm. To assay for P_i, 50-μL aliquots were diluted with 250 μL of H_2O and the amount of P_i was determined colorimetrically (Ames, 1966), measuring absorbance at 820 nm. To determine (Na^+,K^+)-ATPase inhibiting capacity of each fraction, 25 μL of the fractions was added to a total final volume of 250 μL containing either skeletal muscle or axolemma membranes. After a 20-min preincubation at 37 °C, (Na^+,K^+)-ATPase activity was determined colorimetrically as described above.

(B) HPLC. Nucleotide analogues were fractionated on HPLC essentially according to Pogolotti and Santi (1982) with minor modifications. The high concentration buffer contained 500 mM instead of 250 mM $NH_4H_2PO_4$, and a 1.5 mL/min flow rate was used instead of 3 mL/min. The buffer was graduated from 100% low concentration to 100% high concentration in 5 min and the elution continued for an additional 45 min with high concentration buffer. Under these conditions, guanine nucleotides elute at the following times: GMP, 8 min; GDP, 13 min; GTP, 18 min; and GTPγS, a broad peak eluting from 31 to 34 min.

Results and Discussion

The effects of guanine nucleotide analogues were tested on the (Na^+,K^+)-ATPase in kidney microsomes, skeletal muscle, and axolemma, in order to determine whether or not there are tissue-specific and isoform-specific effects. These membranes contain predominantly the α1, α2 and α1, and α2 and α3 isoforms, respectively (Sweadner, 1979; Lytton et al., 1985; Young & Lingrel, 1987; Hsu & Guidotti, 1989; Urayama & Sweadner, 1988). The precedents for this approach are the greater ouabain affinity and the greater sensitivity to NEM and pyrithiamin of the α2 and α3 isoforms as compared to the α1 isoform of the (Na^+,K^+)-ATPase (Sweadner, 1979; Matsuda et al., 1984).

Shown in Figure 1 are measurements of (Na^+,K^+)-ATPase activities in these three membranes with increasing concentrations of GTPγS or GMPPNP. It is evident that GTPγS has a pronounced inhibitory action on (Na^+,K^+)-ATPase activity with an approximate half-maximal inhibition concentration of 100–300 μM; GMPPNP, on the other hand, has relatively little effect on (Na^+,K^+)-ATPase activity even at a concentration of 1 mM. In addition, niether GDP nor GTP as these concentrations inhibits (Na^+,K^+)-ATPase activity (data not shown). If assayed at concentrations of above 2 mM,

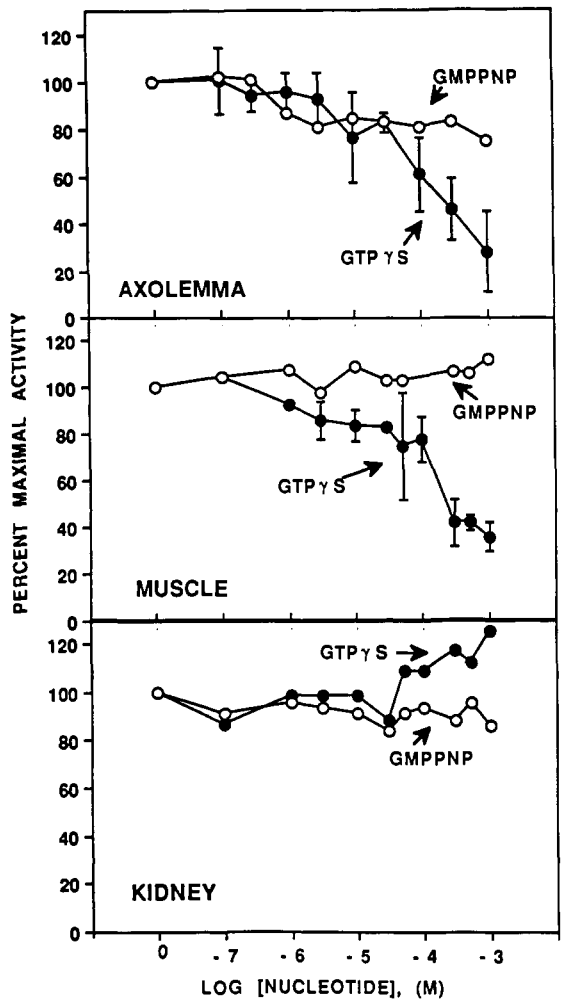

FIGURE 1: Determination of guanine nucleotide sensitivity of (Na^+,K^+)-ATPase activity from three tissue sources. Axolemma, skeletal muscle, and kidney microsomes were incubated with various concentrations of GMPPNP (○) or GTPγS (●) for 20 min. Ouabain-inhibitable (Na^+,K^+)-ATPase activity was determined colorimetrically as described under Materials and Methods. Results shown are either the determinations from single experiments or the average ± SE of $n = 5$ (axolemma, GTPγS) or $n = 2$ (muscle, GTPγS) experiments. Activities with no nucleotide analogues in units of μmol/(mg·min) are 2.06 ± 0.11, 1.42 ● 0.07, and 0.52 for axolemma, skeletal muscle, and kidney, respectively.

GMPPNP, GDP, and GTP do slightly inhibit (Na^+,K^+)-ATPase activity from all tissues studied (data not shown), presumably because at the ATP concentration used in these reactions (2 mM) there is some competition for nucleotide binding to the pump. This effect of GTP on the (Na^+,K^+)-ATPase has been noted (Stekhoven et al., 1983).

It is noteworthy that sensitivity to GTPγS is specific for membranes containing either the α2 or α2 and α3 isoforms of the (Na^+,K^+)-ATPase; the (Na^+,K^+)-ATPase activity of kidney microsomes is unaffected by these concentrations of GTPγS. It was of interest do determine if the (Na^+,K^+)-ATPase in kidney membranes is resistant to GTPγS inhibition because the α1 isozyme itself is resistant or because the membranes do not supply all the necessary components for inhibition. Skeletal muscle and stripped synaptosomal membranes were used to distinguish between these two possibilities because, unlike axolemma membranes, they contain quantities of the α1 isozyme high enough from which to assay activity. The ouabain dependence of (Na^+,K^+)-ATPase activity in skeletal muscle and synaptosomal membranes in the presence or absence of 250 μM GTPγS was determined, and the results

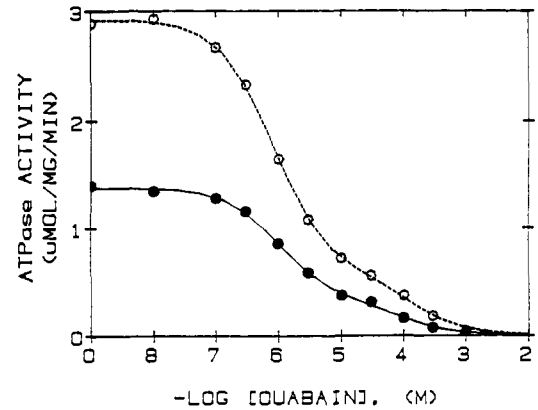

FIGURE 2: Determination of isozyme specificity of GTPγS inhibition. Stripped synaptosomal membranes (2 μg) were incubated with various concentrations of ouabain in the absence (○) or presence (●) of 250 μM GTPγS for 20 min. Activity was determined as described for Figure 1.

Table I: Quantitation of the Effects of GTPγS on Partial Reactions Catalyzed by (Na^+, K^+)-ATPase[a]

reaction	control	250 μM GTPγS	% inhibition
[EP] (pmol/μg)	0.74	0.31	58
Na^+-ATPase [nmol/(mg·min)]	49	24	51
phosphatase [nmol/(mg·min)]	233 ± 10	98 ± 2	58

[a] Membranes were incubated in the presence or absence of 250 μM GTPγS for 20 min at 37 °C. The catalysis of the listed partial reactions by the pump were assayed as described under Materials and Methods.

for synaptosomal membranes are presented in Figure 2. (The results obtained with skeletal muscle membranes are not shown but are similar to those obtained with synaptosomal membranes.) The activity of the α1 isozyme and the α2/α3 isozymes is inhibited by GTPγS.

The sequences of the α1 isozyme in brain and kidney are identical (Shull et al., 1985, 1986), so it is unlikely that the tissue specificity of GTPγS inhibition of the α1 isozyme results from differences in structure of the (Na^+,K^+)-ATPase. Although not proved directly, the lack of effect of GTPγS on kidney microsomal (Na^+,K^+)-ATPase probably does not result from a breakdown of GTPγS by microsomes for two reasons. First, (Na^+,K^+)-ATPase purified from kidney microsomes [specific activity 5 μmol/(mg·min)] is also unaffected by GTPγS (data not shown). Second, less than 5% of GTPγS (as measured by release of phosphate) is hydrolyzed by kidney microsomes (data not shown). It is likely that additional regulatory molecules that are present in axolemma and skeletal muscle membranes but are absent from kidney microsomal membranes are responsible for the inhibitory action of GTPγS.

Nonetheless, it was of interest to study in more detail the effects of GTPγS on the (Na^+,K^+)-ATPase. Therefore, the sensitivity of the partial reactions of the (Na^+,K^+)-ATPase to GTPγS was determined. The data presented in Table I demonstrate that, in addition to inhibiting (Na^+,K^+)-ATPase activity, GTPγS also inhibits Na^+-ATPase activity, *p*-nitrophenylphosphatase activity, and the formation of [EP]. These GTPγS effects are similar to the effects of other compounds, such as vanadate (Cantley, 1981), which inhibit the cycling of the (Na^+,K^+)-ATPase.

There is, in addition, a strict requirement for the presence of sodium ions in the preincubation mixture in order for GTPγS to inhibit the (Na^+,K^+)-ATPase (data not shown). This fact was determined by preincubating axolemma membranes for 20 min with or without GTPγS with increasing

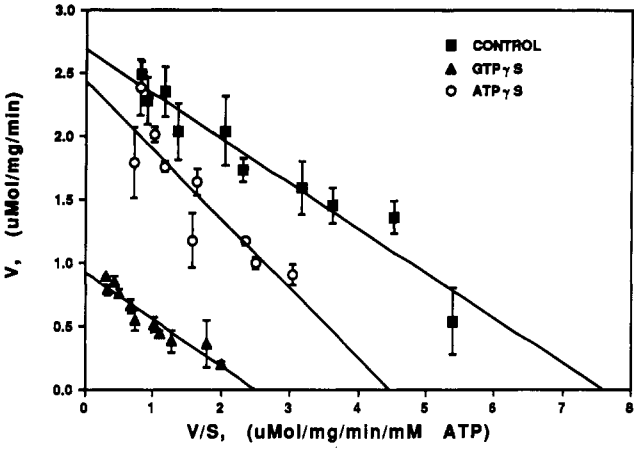

FIGURE 3: Effect of ATPγS and GTPγS on the ATP concentration dependence of (Na^+,K^+)-ATPase activity of skeletal muscle. Skeletal muscle membranes were incubated either under control conditions (■), with 250 μM ATPγS (○), or with 250 μM GTPγS (▲) for 20 min. ATP was added at various concentrations, and ouabain-inhibitable (Na^+,K^+)-ATPase activity was determined according to the coupled enzyme method in order to maintain constant ATP concentrations. Data points were fit by using least-squares analysis. The parameters derived from the fit are as follows: control, $n = 3$, V_{max} = 2.68 μmol/(mg·min), K_m = 0.35 mM ATP; ATPγS, $n = 2$, V_{max} = 2.43 μmol/(mg·min), K_m = 0.54 mM ATP; GTPγS, $n = 3$, V_{max} = 0.92 μmol/(mg·min), K_m = 0.37 mM ATP.

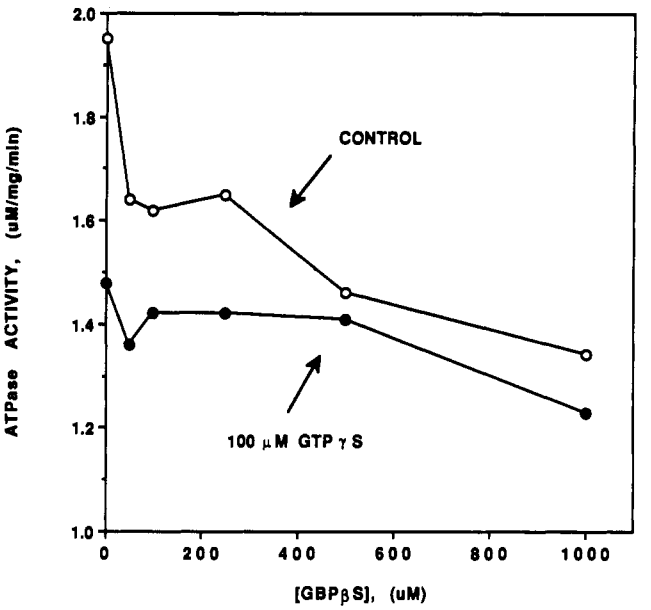

FIGURE 4: Effect of GDPβS on axolemma (Na^+,K^+)-ATPase activity in the presence or absence of GTPγS. Axolemma membranes (6 μg) were incubated without (○) or with (●) 100 μM GTPγS and increasing concentrations of GDPβS for 20 min. Ouabain-inhibitable (Na^+,K^+)-ATPase activity was determined colorimetrically.

concentrations of Na^+. Sodium chloride was then added with the ATP to start the reaction so that all tubes had 100 mM NaCl present for the ATPase measurement. While basal (Na^+,K^+)-ATPase activity remains constant at all preincubation $[Na^+]$ tested, at $[Na^+]$ less than 5 mM, GTPγS is unable to inhibit ATPase activity (data not shown). Maximal inhibition is noted at sodium concentrations of 50 mM or higher. This lack of inhibition at low sodium concentrations is not simply an effect of low osmotic strength of the buffer; the addition of neither sucrose nor choline chloride to equalize osmotic conditions allows GTPγS inhibition of (Na^+,K^+)-ATPase activity (data not shown). Because the $K_{0.5}$ for Na^+ of axolemma (Na^+,K^+)-ATPase activity is much less than that for GTPγS inhibition, it cannot be determined if GTPγS has any effect on the sodium dependence of ATPase activity. The requirement for sodium ions as well as a requirement for Mg^{2+} ions (data not shown) in the preincubation mixture may indicate a need for the (Na^+,K^+)-ATPase to exist in the E_1 conformation in order to be inhibited. Alternatively, the ions may be required to maintain either GTPγS or some other protein in conformational states necessary for interaction with the (Na^+,K^+)-ATPase.

Although it seems unlikely that low concentrations of GTPγS could inhibit the (Na^+,K^+)-ATPase by interfering with binding of ATP, this possibility was addressed directly. The effects of both GTPγS and ATPγS were determined on the affinity of skeletal muscle (Na^+,K^+)-ATPase of ATP. It has already been established that AMPPNP inhibits (Na^+,K^+)-ATPase activity competitively with ATP (Stekhoven et al., 1983), so the prediction was that ATPγS would also competitively inhibit the (Na^+,K^+)-ATPase. Figure 3 shows an Eadie–Hofstee plot of the effects of these nucleotide analogues on skeletal muscle (Na^+,K^+)-ATPase activity in the presence of various concentrations of ATP. Magnesium was added at the same concentration as ATP in order to eliminate the fluctuation of Mg^{2+} as a possible cause of changes in ATPase activity. As predicted, ATPγS inhibits the (Na^+,K^+)-ATPase competitively, increasing the K_m for ATP from 0.35 to 0.54 mM, with little effect on V_{max}. In contrast, GTPγS inhibits (Na^+,K^+)-ATPase activity noncompetitively, decreasing V_{max} from 2.68 to 0.92 μM/(mg·min) without affecting the K_m for ATP.

Even though the activity of the (Na^+,K^+)-ATPase is affected as described above, there is no evidence that GTPγS interacts directly with the pump. On the contrary, the fact that α1 is inhibited in some membranes but not others suggests that an accessory protein or lipid is involved in this regulation. An obvious possibility is that a G-protein is involved in this phenomenon. While there is evidence that G-proteins can be preferentially activated by GTPγS as compared to GMPPNP (Rasenick et al., 1989), there are no examples of a G-protein that is completely insensitive to GMPPNP. Because the activity of the (Na^+,K^+)-ATPase is not inhibited by GMPPNP (Figure 1), the involvement of a G-protein in the described GTPγS effect becomes suspect. Nonetheless, the effects of GDPβS were analyzed on this system; GDPβS can block the effects of GTPγS on G-proteins (Gilman, 1987). Axolemma membranes were incubated in the presence or absence of 100 μM GTPγS, with increasing concentrations of GDPβS for 20 min, at which point ATPase activity was measured. The results in Figure 4 show that GDPβS itself inhibits axolemma (Na^+,K^+)-ATPase activity at submillimolar concentrations: the effects of GTPγS and GDPβS together, however, do not appear to be completely additive. These results do not rule out the involvement of a G-protein; GDPβS, like GTPγS and GMPPNP, has been shown to activate adenylate cyclase in some systems (Rasenick et al., 1989). It is more likely, however, because of the lack of effect of GMPPNP, that GTPγS and GDPβS inhibit (Na^+,K^+)-ATPase activity by similar mechanisms not involving G-protein, but perhaps through a direct interaction with the pump or another molecule.

Both GTPγS and GDPβS contain terminal SH groups, while GMPPNP does not; therefore, the possibility that this inhibitory effect was due to some oxidation/reduction phenomenon needed to be addressed. Matsuda et al. (1984) demonstrated that the brain α+ (α2/α3) isozyme of the (Na^+,K^+)-ATPase is much more sensitive to pyrithiamin than is the kidney α1 isoform. It has also been established that

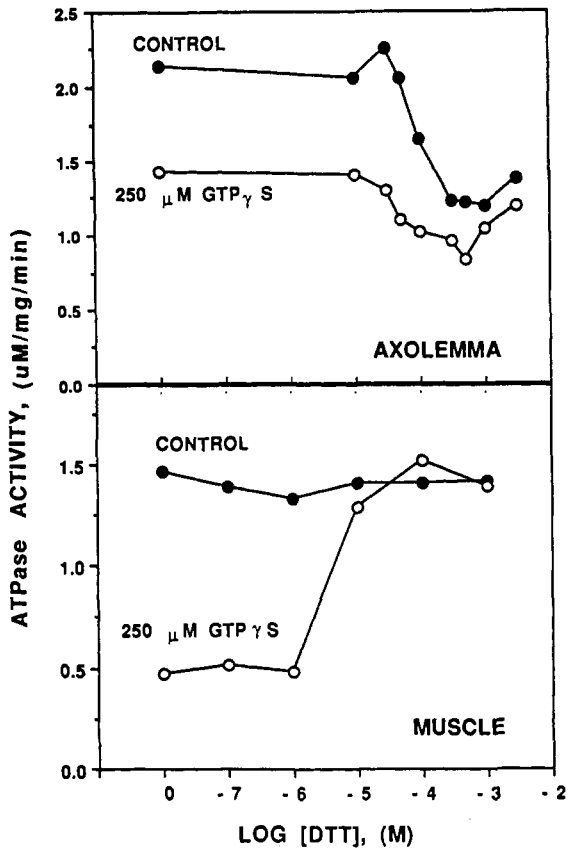

FIGURE 5: Effects of increasing concentrations of dithiothreitol on both (Na^+,K^+)-ATPase activity and GTPγS inhibition of (Na^+,K^+)-ATPase activity. Axolemma or skeletal muscle membranes were incubated in the absence (●) or presence (○) of 250 μM GTPγS with increasing concentrations of dithiothreitol. Ouabain-inhibitable (Na^+,K^+)-ATPase activity was determined colorimetrically.

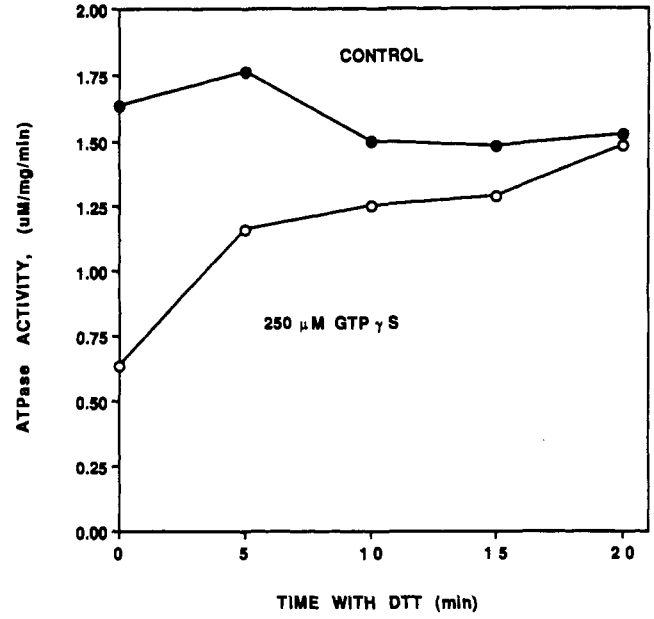

FIGURE 6: Reversal of GTPγS inhibition of skeletal muscle (Na^+,K^+)-ATPase activity by dithiothreitol. Skeletal muscle membranes were incubated without (●) or with (○) 250 μM GTPγS for 40 min before the addition of ATP. Dithiothreitol (1 mM) was either not added (time = 0) or added 20, 15, 10, or 5 min prior to the addition of ATP. Ouabain-inhibitable (Na^+,K^+)-ATPase activity was determined colorimetrically.

brain (Na^+,K^+)-ATPase is more readily inhibited by NEM than is the kidney enzyme (Sweadner, 1979). To compare the effects of GTPγS to another SH-containing compound, membranes from different tissues were preincubated in the presence of either 250 μM GTPγS or 1 mM DTT. The (Na^+,K^+)-ATPase of kidney microsomes and skeletal muscle membranes is unaffected by DTT. Surprisingly, the (Na^+,K^+)-ATPase in axolemma membranes is inhibited by DTT (45 ± 8% inhibition, $n = 7$) nearly as much as by GTPγS (55 ± 5% inhibition, $n = 15$). This inhibition by DTT was unexpected; the buffers used in many assays of (Na^+,K^+)-ATPase activity contain DTT (Barnett, 1970; Josephson & Cantley, 1977). Moreover, while only axolemma (Na^+,K^+)-ATPase is significantly inhibited by DTT, both skeletal muscle and axolemma (Na^+,K^+)-ATPase are inhibited by GTPγS. It is, therefore, unlikely that DTT and GTPγS inhibit (Na^+,K^+)-ATPase activity by the same mechanism, but it is possible that GTPγS inhibits due to a different SH-dependent interaction.

To determine whether DTT can block the inhibition of (Na^+,K^+)-ATPase activity by GTPγS, axolemma and skeletal muscle membranes were incubated with or without 250 μM GTPγS with increasing concentrations of DTT for 20 min prior to the addition of ATP. Figure 5 shows that skeletal muscle (Na^+,K^+)-ATPase is not inhibited by up to 1 mM DTT; however, concentrations of DTT as low as 10 μM block the inhibition of (Na^+,K^+)-ATPase activity by GTPγS.

The DTT inhibition of axolemma (Na^+,K^+)-ATPase activity is concentration dependent, with some inhibition at 0.1 mM DTT and maximal inhibition at 0.3 mM DTT. Although there is GTPγS inhibition of axolemma ATPase activity at low concentrations of DTT (10 μM), the sum of GTPγS and DTT inhibition at 1 mM DTT is not additive. These results could be interpreted to suggest that DTT and GTPγS inhibit axolemma (Na^+,K^+)-ATPase by the same mechanism and the reason that the effects are not additive at higher concentrations of DTT is that maximal inhibition has been reached. Alternatively, these data could result from DTT having two effects on axolemma (Na^+,K^+)-ATPase: inhibition of (Na^+,K^+)-ATPase activity and blockage of inhibition by GTPγS in a manner similar to the effect demonstrated in muscle membranes. Dithiothreitol may inhibit only the α3 isozyme of the (Na^+,K^+) pump but block GTPγS inhibition. Addition studies on the nature of the DTT effect on axolemma (Na^+,K^+)-ATPase are currently underway in this laboratory.

Because DTT blocks the effect of GTPγS on skeletal muscle (Na^+,K^+)-ATPase, one possibility is that GTPγS inhibits the (Na^+,K^+)-ATPase by forming a disulfide link with either the pump or some other regulatory protein. [This hypothesis is consistent with the finding that inhibition by GTPγS is slow to occur, with a $T_{1/2}$ for inhibition of skeletal muscle and axolemma (Na^+,K^+)-ATPase of 10 min; data not shown.] If this is true, DTT should not only block GTPγS inhibition, it should also reverse it, possibly in a time-dependent manner. To test the reversibility of GTPγS inhibition by DTT, several tubes containing muscle membranes were incubated with or without 250 μM GTPγS for a total of 40 min before the addition of ATP. Dithiothreitol was either not added (time = 0 in Figure 6) or added, at a final concentration of 1 mM, 5, 10, 15, or 20 min prior to the addition of ATP. Figure 6 demonstrates that DTT can reverse GTPγS inhibition and that this reversal is time dependent, with a half-time of approximately 10 min. These results are consistent with the idea that GTPγS forms a disulfide with a protein as a inhibitory mechanism of the (Na^+,K^+)-ATPase. However, the possibility that DTT merely changes the conformation of some protein to a state that is not longer sensitive to inhibition by GTPγS cannot be ruled out.

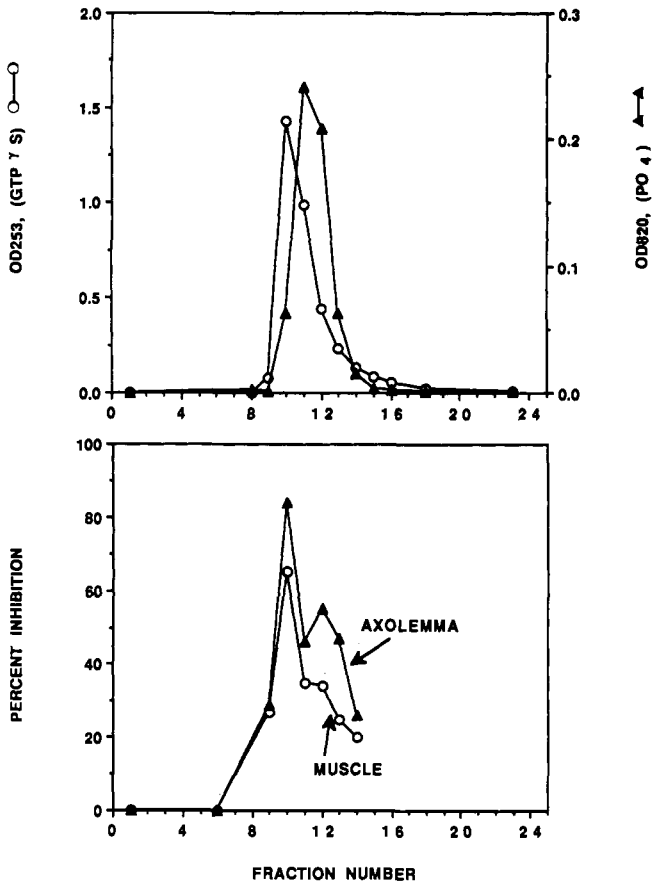

FIGURE 7: Fractionation of GTPγS by Sephadex G-10. An aliquot of GTPγS was separated by using Sephadex G-10 as described under Materials and Methods. The top panel shows the elution profile of GTPγS (O) and PO_4 (▲). The bottom panel illustrates the elution profile of (Na^+,K^+)-ATPase inhibitory action using axolemma (▲) and skeletal muscle membranes (O) as described under Materials and Methods.

A surprising result is that just 10 μM DTT is able to reverse the inhibition of skeletal muscle (Na^+,K^+)-ATPase of 250 μM GTPγS (Figure 5). One possible explanation for this result is that a minor contaminant present in GTPγS preparations is responsible for the inhibitory effect. In order to further ascertain whether the actions of preparations of GTPγS on (Na^+,K^+)-ATPase activity are due to GTPγS itself or some other compound, a Sephadex G-10 column was loaded with GTPγS and the fractions were assayed for inhibitory activity. As illustrated in Figure 7, the inhibiting species elutes with the peak of GTPγS. The shoulder of inhibitory activity noted in fractions eluting after GTPγS could be due to the presence of thiophosphate (discussed below). So, either the inhibitory activity present in preparations of GTPγS is due to GTPγS itself or a contaminating compound elutes with GTPγS.

Some preparations of ATP have been shown to contain trace amounts of vanadate, a potent inhibitor of the (Na^+,K^+)-ATPase (Josephson & Cantley, 1977; Cantley et al., 1977). In addition, vanadate can elute with ATP in a variety of separation systems. For the following reasons, however, vanadate has been ruled out as an inhibitory contaminant in our preparations of GTPγS: (1) norepinephrine does not block the effects of GTPγS (data not shown), (2) DTT does reverse the effects of GTPγS, (3) GTPγS does not inhibit the (Na^+,K^+)-ATPase of kidney microsomes, and (4) ashed GTPγS no longer inhibits the (Na^+,K^+)-ATPase (data not shown). Moreover, because ashed GTPγS does not inhibit (Na^+,K^+)-ATPase activity and because neither EDTA nor EGTA block the inhibitory effects of GTPγS (data not shown), it is unlikely that the inhibition is due to a trace contaminant of heavy metals.

Thiophosphate analogues of GDP and GTP can form disulfides and complicated oxidation products of analogue and thiophosphate itself, the structures of which are dependent on reaction conditions (Goody & Eckstein, 1971). It has been proposed that the disulfide compounds of thiophosphate nucleotide analogues would be very reactive with cysteine residues on proteins (Goody & Eckstein, 1971). Certainly it is possible that such an anomalous GTPγS structure could inhibit (Na^+,K^+)-ATPase activity and closely elute with GTPγS on separation systems. When GTPγS is fractionated by HPLC, the major eluting peak is broad and irregular (data not shown). This peak was first assumed to consist just of GTPγS, but could in fact consist of GTPγS disulfides as well as GTPγS. To test whether such structures of GTPγS are involved in inhibition of the (Na^+,K^+)-ATPase, GTPγS was treated with a variety of sulfhydryl-reactive compounds and tested for the ability to inhibit (Na^+,K^+)-ATPase activity.

When GTPγS was treated with DTT and then separated from DTT by gel filtration, it retained by ability to inhibit skeletal muscle (Na^+,K^+)-ATPase activity (data not shown). Moreover, GTPγS that has been reduced with $NaBH_4$ also retains the ability to inhibit (Na^+,K^+)-ATPase activity (data not shown). These results suggest that a disulfide compound of GTPγS is not responsible for inhibiting the Na^+ pump.

As mentioned previously, the fact that GTPγS and GDPβS but not GMPPNP inhibit (Na^+,K^+)-ATPase activity suggests that the terminal SH group of these compounds might be important in the measured effect. If not important for the formation of a disulfide compound that itself inhibits (Na^+,K^+)-ATPase activity, the free SH group itself may be necessary for the reaction with protein molecules. To test this possibility, GTPγS was treated with NEM and then separated from NEM by Sephadex G-10 gel filtration as described for Figure 8. The bottom panel shows the elution profiles of GTPγS and NEM, as recorded by their absorption profiles at 253 and 302 nM, respectively. The top panel shows the elution profile of inhibitory activity of untreated GTPγS, NEM alone, and NEM treated GTPγS on axolemma (Na^+,K^+)-ATPase activity.

As in Figure 7, the inhibitory activity of the GTPγS preparation elutes with GTPγS. Consistent with work by others (Sweadner, 1979), NEM is shown also to inhibit (Na^+,K^+)-ATPase activity. However, when GTPγS and NEM are incubated together, both lose the ability to inhibit (Na^+,K^+)-ATPase activity. The probable explanation for NEM losing the ability to inhibit activity is that some has reacted with GTPγS and some has been hydrolyzed during the incubation period. The fact that NEM-treated GTPγS no longer inhibits (Na^+,K^+)-ATPase activity directly demonstrates that the free SH group is necessary for inhibitory activity.

If GTPγS forms a disulfide bond with a protein as the mechanism of inhibition, it would explain why DTT and β-mercaptoethanol (data not shown) reverse and block inhibition of (Na^+,K^+)-ATPase activity. However, attempts at labeling the (Na^+,K^+)-ATPase or other proteins with [^{35}S]GTPγS have been unsuccessful (data not shown). It is not clear, however, that this failure to label a protein reflects a true lack of covalent modification. Labeled [^{35}S]GTPγS contains 10 mM DTT in the packaged form. Although attempts were made to separate the [^{35}S]GTPγS from the DTT, perhaps not all of the DTT was removed and it interfered with the labeling procedure. More experiments are currently underway to resolve this issue.

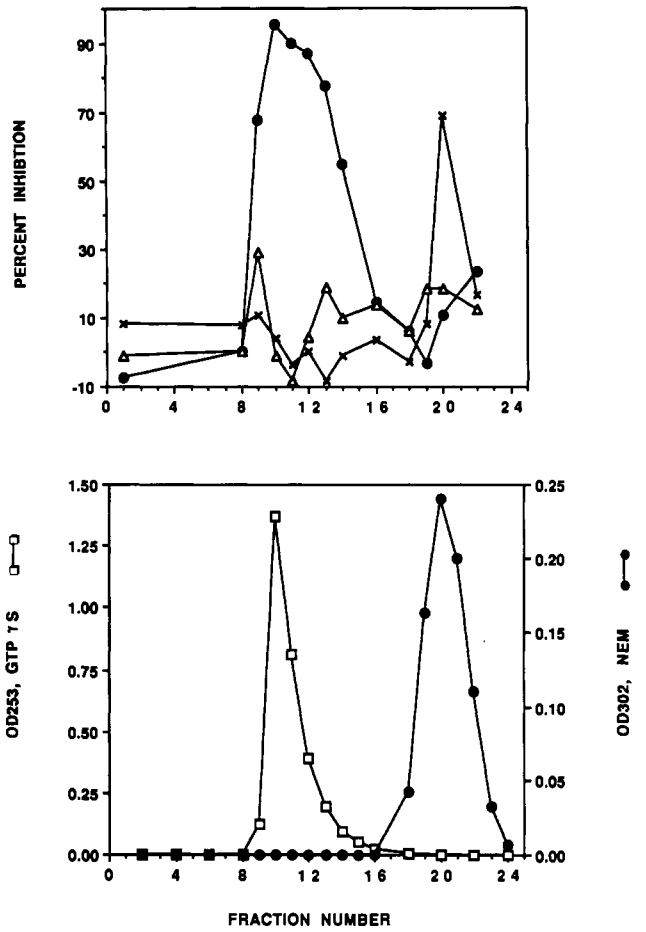

FIGURE 8: Effect of NEM-treated GTPγS on (Na+,K+)-ATPase activity. GTPγS (15 mM) was treated with NEM (70 mM) in a total volume of 100 μL, at 37 °C for 20 min. It was then separated by using Sephadex G-10 as for Figure 7. As controls, GTPγS and NEM were run separately on the column. The bottom panel shows the elution profile of GTPγS (□) and NEM (●). The top panel illustrates the elution profile of (Na+,K+)-ATPase inhibitory action on axolemma membranes for untreated GTPγS (●), untreated NEM (×), and NEM-treated GTPγS (△).

Thiophosphate, a probable contaminant in GTPγS preparations, also contains a free SH group. To determine if thiophosphate can inhibit (Na+,K+)-ATPase activity, thiophosphate was synthesized and, as well as phosphate, was assayed directly for possible inhibitory effects on the (Na+,K+)-ATPase. Phosphate (up to 0.5 mM) does not inhibit while thiophosphate does inhibit (Na+,K+)-ATPase activity slightly (data not shown). This inhibition, however, is only detectable at concentrations of 100 μM thiophosphate and above, but the concentration of thiophosphate contributed by even very old solutions of GTPγS is much less than this at the GTPγS concentrations used in these experiments (data not shown). However, when the fractions from gel filtration are studied for inhibitory capacity, a large amount of GTPγS is loaded on the column. The final concentration of GTPγS in the reaction mixture is usually 400–500 μM, and the concentration of thiophosphate could reach concentration levels necessary for inhibition of (Na+,K+)-ATPase activity. It is concluded that thiophosphate is responsible for the shoulder of inhibitory activity seen in the gel filtration elution profiles (Figures 7 and 8) but that it is not the major inhibiting species present in solutions of GTPγS.

It is interesting that even though ATPγS has a terminal SH group, its effect on the (Na+,K+)-ATPase is very different from the effects of GTPγS (Figure 3). The terminal SH group of ATPγS was not expected to be of importance in the inhibition of (Na+,K+)-ATPase activity because other ATP analogues also competitively inhibit the pump (Stekhoven et al., 1983). If GTPγS interacts directly with the (Na+,K+) pump, one would expect that it binds very differently than ATPγS, perhaps at a different nucleotide site, so that it is positioned to react with an important cysteine residue. If GTPγS reacts with a protein other than the (Na+,K+) pump, it is predicted that this protein does not bind ATPγS. It should be noted in this context that neither ATP, ADP, nor GTP can protect axolemma (Na+,K+)-ATPase from inhibition by GTPγS so the nature of the binding site for this nucleotide analogue is unclear (data not shown). It is obviously of great interest to determine with what molecule GTPγS interacts and in what way this interaction regulates the activity of the (Na+,K+)-ATPase. Further experiments are currently underway to address this issue.

ACKNOWLEDGMENTS

I thank Michael Ho (Harvard University) for his help with the HPLC fractionations. I thank Jeff Brodsky for supplying the stripped synaptosomal membranes. I especially thank Dr. Guido Guidotti, in whose laboratory this work was done, for synthesizing the thiophosphate used in this study and for all the helpful suggestions and conversations throughout the course of this work.

Registry No. ATPase, 9000-83-3; GTPγS, 37589-80-3; GDPβS, 71376-97-1; ATPγS, 35094-46-3; ATP, 56-65-5; Na, 7440-23-5; Mg, 7439-95-4; ouabain, 630-60-4; dithiothreitol, 3483-12-3; thiophosphate, 15181-41-6.

REFERENCES

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–117.
Aperia, A., Bertorello, A., & Seri, I. (1987) *Am. J. Physiol. 252*, F39–F45.
Barchi, R. L., Weigele, J. B., Chalikian, D. M., & Murphy, L. E. (1979) *Biochim. Biophys. Acta 550*, 59–76.
Barnett, R. E. (1970) *Biochemistry 9*, 4644–4648.
Bertorello, A., & Aperia, A. (1989) *Am. J. Physiol. 256*, F57–F62.
Brodsky, J. L. (1990) *Am. J. Physiol. 258*, C812–C817.
Cantley, L. C., Jr. (1981) *Curr. Top. Bioenerg. 11*, 201–237.
Cantley, L. C., Jr., Josephson, L., Warner, R., Yanagisawa, M., Leuchene, C., & Guidotti, G. (1977) *J. Biol. Chem. 252*, 7421–7423.
Ciaraldi, T. P., & Maisel, P. (1989) *Biochem. J. 264*, 389–396.
Clausen, T., & Hansen, O. (1977) *J. Physiol. 270*, 415–430.
Clausen, T., & Kohn, P. G. (1977) *J. Physiol. 265*, 19–42.
Devries, G. H., Matthieu, J. H., Benny, M., Chicheportiche, R., Lazdunski, M., & Dolivo, M. (1978) *Brain Res. 147*, 339–352.
Fehlmann, M., & Freychet, P. (1981) *J. Biol. Chem. 256*, 7449–7453.
Geering, K., Girardet, M., Bron, C., Kraehenbuhl, J.-P., & Rossier, B. C. (1982) *J. Biol. Chem. 257*, 10338–10343.
Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615–649.
Gloor, S., Antonicek, H., Sweadner, K. J., Pagliusi, S., Frank, R., Moos, M., & Schlachner, M. (1990) *J. Cell Biol. 110*, 165–174.
Goldin, S. M. (1977) *J. Biol. Chem. 252*, 5630–5642.
Goody, R. S., & Eckstein, F. (1971) *J. Am. Chem. Soc. 93*, 6252–6257.
Gorga, F. R. (1985) *Biochemistry 24*, 6783–6788.
Hiatt, A., McDonough, A. A., & Edelman, I. S. (1984) *J. Biol. Chem. 259*, 2629–2635.

Horowitz, B., Eakle, K. A., Scheiner-Bobis, G., Randolph, G. R., Chen, C. Y., Hitzeman, R. A., & Farley, R. A. (1990a) *J. Biol. Chem. 265*, 4189-4192.
Horowitz, B., Hensley, C. B., Quintero, M., Azuma, K. K., Putman, D., & McDonough, A. A. (1990b) *J. Biol. Chem. 265*, 14308-14314.
Hsu, Y.-M., & Guidotti, G. (1989) *Biochemistry 28*, 569-573.
Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 53-67.
Jørgensen, P. L., & Andersen, J. P. (1988) *J. Membr. Biol. 103*, 95-120.
Josephson, L., & Cantley, L. C., Jr. (1977) *Biochemistry 16*, 4572-4578.
Lo, C.-S., & Edelman, I. S. (1976) *J. Biol. Chem. 251*, 7834-7840.
Lytton, J. (1985) *J. Biol. Chem. 260*, 10075-10080.
Lytton, J., Lin, J., & Guidotti, G. (1985) *J. Biol. Chem. 260*, 1177-1184.
Martin-Vasallo, P. Dackowski, W., Emanuel, J. R., & Levenson, R. (1989) *J. Biol. Chem. 264*, 4613-4618.
Matsuda, T., Iwata, H., & Copper, J. R. (1984) *J. Biol. Chem. 259*, 3858-3863.
Mendoza, S. A., Wigglesworth, N. M., & Rozengurt, E. (1980) *J. Cell Physiol. 105*, 153-162.
Mercer, R. W., Schneider, J. W., Savitz, A., Emanuel, J., Benz, E. J., Jr., & Levenson, R. (1986) *Mol. Cell. Biol. 6*, 3884-3890.
Orlowski, J., & Lingrel, J. B. (1990) *J. Biol. Chem. 265*, 3462-3470.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.
Pogolotti, A. L., Jr., & Santi, D. V. (1982) *Anal. Biochem. 126*, 335-345.
Rasenick, M. M., Hughes, J. M., & Wang, N. (1989) *Brain Res. 488*, 105-113.
Resh, M. D. (1982) *J. Biol. Chem. 257*, 11946-11952.
Resh, M. D., Nemenoff, R. A., & Guidotti, G. (1980) *J. Biol. Chem. 255*, 10938-10945.
Rosic, N. K., Standaert, M. L., & Pollet, R. J. (1985) *J. Biol. Chem. 260*, 6206-6212
Schmitt, C. A., & McDonough, A. A. (1988) *J. Biol. Chem. 263*, 17643-17649.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691-695.
Shull, G. E., Greeb, J., & Lingrel, J. B. (1986) *Biochemistry 25*, 8125-8132.
Skou, J. C. (1974) *Biochim. Biophys. Acta 339*, 258-273.
Smith, J., & Rozengurt, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5560-5564.
Stekhoven, F. M. A. H. S., Swarts, H. G. P., De Pont, J. J. H. H. M., & Bonting, S. L. (1983) *Biochim. Biophys. Acta 732*, 607-619.
Sweadner, K. J. (1979) *J. Biol. Chem. 254*, 6060-6067.
Takeyasu, K., Tamkun, M. M., Renaud, K. J., & Fambrough, D. M. (1988) *J. Biol. Chem. 263*, 4347-4354.
Urayama, O., & Sweadner, K. J. (1988) *Biochem. Biophys. Res. Commun. 156*, 796-800.
Urayama, O., Shutt, H., & Sweadner, K. J. (1989) *J. Biol. Chem. 264*, 8271-8280.
Verrey, F., Schaerer, E., Zoerkler, P., Paccolat, M. P., Geering, K., Kraehenbuhl, J. P., & Rossier, B. C. (1987) *J. Cell Biol. 104*, 1231-1237.
Yasuda, S. K., & Lambert, J. L. (1954) *J. Am. Chem. Soc. 76*, 5356.
Young, R. M., & Lingrel, J. B. (1987) *Biochem. Biophys. Res. Commun. 145*, 52-58.
Young, R. M., Shull, G. E., & Lingrel, J. B. (1987) *J. Biol. Chem. 262*, 4905-4910.

Nonreductive Interaction of Vanadate with an Enzyme Containing a Thiol Group in the Active Site: Glycerol-3-phosphate Dehydrogenase†

Debbie C. Crans* and Carmen M. Simone
Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523
Received July 24, 1990; Revised Manuscript Received March 20, 1991

ABSTRACT: The inhibitory effects of vanadium(V) were determined on the oxidation of glycerol 3-phosphate (G3P) catalyzed by glycerol-3-phosphate dehydrogenase (G3PDH), an enzyme with a thiol group in the active site. G3PDH from rabbit muscle was inhibited by vanadate, and the active inhibiting species were found to be the vanadate dimer and/or tetramer. The dimer was a sufficiently weak inhibitor at pH 7.4 with respect to G3P; the tetramer could account for all the observed inhibition. The tetramer was a competitive inhibitor with respect to G3P with a K_i of 0.12 mM. Both the dimer and tetramer were noncompetitive inhibitors at pH 7.4 with respect to NAD with K_i's of 0.36 mM and 0.67 mM. G3PDH inhibited by vanadate was reactivated when EDTA complexed the vanadate. The reactivation occurred even after extended periods of incubation of G3PDH and vanadate, suggesting that the inhibition is reversible despite the thiol group in the active site. Analogous reactivation is also observed with glyceraldehyde-3-phosphate dehydrogenase (Gly3PDH). Gly3PDH is an enzyme that previously had been reported to undergo redox chemistry with vanadate. The work described in this paper suggests vanadate will not necessarily undergo redox chemistry with enzymes containing thiol groups exposed on the surface of the protein.

The in vivo and in vitro mechanisms by which the trace element vanadium interacts in biological systems are poorly understood in part due to its complex chemistry (Nechay et al., 1986; Gresser et al., 1987). Aqueous solutions of vanadate in the presence of biological materials will form many vanadium compounds, and several of these are likely to exhibit biological activities. Several mechanisms have recently emerged demonstrating how vanadate can affect various en-

† This work was funded by the NIH.

0006-2960/91/0430-6734$02.50/0