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# INHIBITION OF THE CATALYTIC SUBUNIT OF PHOSPHORYLASE PHOSPHATASE BY OXALYL THIOESTERS AND ITS POSSIBLE RELEVANCE TO THE MECHANISM OF INSULIN ACTION

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SUMMARY: Oxalyl thioesters, especially S-oxalylglutathione, are shown to be effective inhibitors of the catalytic subunit of phosphorylase phosphatase. The amount of inhibition was found to be time dependent and partially reversed by thiols, thus suggesting that at least part of the inhibition is due to oxalylation of an enzymic thiol group. The possibility that the inhibition of the phosphatase by oxalyl thioesters may be important in vivo and that oxalyl thioesters may be functioning as negative intracellular messengers for insulin is discussed. 

In a recent publication (1), one of the present authors has proposed that oxalyl thioesters ($RSCOCOO^-$) may be important regulators of intracellular enzyme activities in animals. Oxalyl thioesters are formed as the direct products of the likely physiological reaction catalyzed by L-α-hydroxy acid oxidase (1-5) and could reasonably be formed by further metabolism of the products obtained from the suspected physiological reactions catalyzed by D-amino acid oxidase (1,6,7) and D-aspartate oxidase (1,8). Considerable circumstantial evidence (1,9,10) suggests that the products (or their further metabolites) of these enzymic reactions may be involved in controlling metabolism in animals, and a particularly good correlation implies that some may participate as intracellular mediators for insulin and other hormones. If oxalyl thioesters function in this regard then the correlations indicate (1) that they should have the opposite effect on intracellular enzymes that insulin does.

Interest in oxalyl thioesters as possibly important intracellular mediators was stimulated by our recent finding (1,11) that the oxalyl group of oxalyl thioesters is non-enzymically transferred to other thiols very rapidly under physiological conditions. For example, with 0.1 to 1 mM reactants, the oxalyl exchange between thiols occurs in seconds to minutes at 25°C and pH 7.4. These results immediately suggest a mechanism by which oxalyl thioesters could be controlling the activities of enzymes; the transfer of oxalyl groups

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to enzymic thiols should also occur (eq. 1), and the modified enzyme might be

$$RS\text{-}\overset{O}{\overset{\|}{C}}\text{-}COO^- + ESH \rightleftharpoons RSH + ES\text{-}\overset{O}{\overset{\|}{C}}\text{-}COO^- \quad (1)$$

expected to have altered catalytic activity as is the case when various enzymes having sensitive thiol groups are reacted with other reagents (as examples, with organomercurials, disulfides, iodoacetamide, etc.). Until now, however, the effects of oxalyl thioesters on enzymes had not been investigated. Thus, to determine whether a mechanism of metabolic control based on oxalyl thioesters as mediators is at all reasonable, we have begun a program to investigate their effects on specific enzymes.

In the present communication, we report results illustrating the effects of oxalyl thioesters on the catalytic subunit of phosphorylase phosphatase, isolated by the ethanol precipitation method from bovine heart (12). This enzyme was chosen for initial investigation because of considerable evidence that insulin causes the activation of protein phosphatases (13,14). Thus, from our correlations (1) it seemed possible that oxalyl thioesters might inhibit the phosphatase. As reported here, that is indeed observed.

## EXPERIMENTAL PROCEDURE

Materials. Unless specified otherwise, commerical materials were used as received. Pantetheine and N-succinylcysteamine were prepared by reducing the disulfides as described by Gorecki and Patchornick (15). The N,N'-disuccinylcystamine precursor of the latter compound was synthesized in this laboratory by Robert Ryall by reacting cystamine with succinic anhydride. Rabbit muscle phosphorylase b and phosphorylase kinase were purchased from Sigma and [$\gamma$-$^{32}$P]-ATP (24 Ci/mmol) from ICN Pharmaceuticals. The procedure of Antoniw et al. (16) was used to prepare $^{32}$P-labeled phosphorylase a. The catalytic subunit of phosphorylase phosphatase was purified from bovine heart by the method of Killilea et al. (12) through the Sephadex G-75 step; the specific activity of the purified enzyme, measured as described by Killilea et al. (12), was 1200 units/mg. Protein was determined by the method of Bradford (17) using bovine serum albumin (BSA) as standard.

Oxalyl Thioester Synthesis and Characterization. S-Oxalyl-N-acetylcysteamine was synthesized as outlined by Quayle (8) and S-oxalyl-N-octanoylcysteamine by the method of Koch and Jaenicke (19). S-Oxalyl-CoA, S-oxalylglutathione, S-oxalylpantetheine and S-oxalyl-N-succinylcysteamine were prepared by a procedure similar to that used for S-oxalyl-CoA by Quayle (20) but with some modifications. To 285 µmoles RSH in 5 mL 50 mM PIPES buffer, pH 7.5 were added 570 µmoles S-oxalyl-p-thiocresol in 5 mL ether. After adjusting the pH to 7.5 the mixture was vigorously stirred at room temperature for 15 min. Following removal of the ether layer and lowering the pH to 3.5 with 1 N HCl, the aqueous solution was first extracted several times with ether and subsequently filtered through 0.25 cm of Darco G-60 activated charcoal on a fine scintered glass funnel. When tested with Ellman's reagent (21) the resulting solution had less than 1% free thiol relative to the amount of oxalyl thioester present. Furthermore, each oxalyl thioester showed only one spot on tlc (visualized by uv) when chromatographed on Eastman cellulose plates; none of the corresponding thiol (visualized using Ellman's reagent) or S-oxalyl-p-thiocresol was detectable. In the chromatographic system n-butanol-acetic acid-water (40:30:30) the observed $R_f$ values are: oxalylglutathione, 0.32; oxalyl-CoA, 0.50; S-oxalyl-N-succinylcysteamine, 0.46; and S-oxalylpantetheine, 0.50. When stored at -20°C in approximately 20 mM concentrations at pH 3.5 the oxalyl

thioesters are stable for at least a month. When needed they were diluted to the appropriate concentration with 50 mM PIPES buffer, pH 7.4, under which conditions they are stable for hours to days at room temperature. However, above pH 9 at room temperature they hydrolyze at measurable rates. The concentrations of the oxalyl thioester solutions were initially determined by reacting with excess hydroxylamine and titrating the released thiol with Ellman's reagent (21) assuming an extinction coefficient at 412 nm of 14.15 $mM^{-1}$ (22). It was found that all of the oxalyl thioesters, except oxalyl-CoA, have an absorption at 260 nm with an extinction coefficient of 2900 $M^{-1}$ $cm^{-1}$ so subsequently their concentrations could be determined from this absorption. In the case of oxalyl-CoA the strong adenine absorption at 260 nm precludes accurate determination of its concentration by this latter method.

Enzyme Assays. Enzyme activity was determined by measuring $^{32}Pi$ released from $^{32}P$-labeled phosphorylase a. Each assay solution (0.1 mL) contained 50 mM PIPES buffer, pH 7.4, 0.5 mM EDTA, 5 mM theophylline, 0.2 mg/mL $^{32}P$-labeled phosphorylase a and ca 7 ng phosphorylase phosphatase. All assays were initiated by adding phosphatase and were run at 30°C for 5 min. (less than 10% conversion of substrate). After the reactions were quenched with 0.1 mL 25% TCA, 0.1 mL of a BSA solution (7 mg/mL) was added as a coprecipitant and the resulting mixture allowed to sit for 20 min. on ice before pelleting the protein by centrifugation. An aliquot of the supernatant was added to 10 mL of Ready Solve EP (Beckman) scintillation cocktail and counted using a Beckman LS7500 liquid scintillation counter.

In a typical inhibition study, the phosphatase (0.17 µg in a total volume of 0.12 mL) was incubated at 30°C with the indicated concentration of oxalyl thioester (and glutathione when present) in 50 mM PIPES buffer, pH 7.4, containing 0.5 mM EDTA and 5 µg BSA (to stabilize the phosphatase). At the indicated times 5 µL aliquots were removed and assayed as above. Control incubations and assays were performed with everything being the same except that no oxalyl thioester was present. All rates are expressed as relative to these controls which at each incubation time was taken as 100% activity. However, the phosphatase loses less than 10% of its activity when incubated for 30 min. under the above conditions in the absence of oxalyl thioester. The number of counts obtained from blank assays containing everything but phosphatase were subtracted in all cases; this is less than a 3% correction of control assays.

## RESULTS

Summarized in Table 1 are some data indicating that various oxalyl thioesters inhibit the catalytic subunit of phosphorylase phosphatase. The amount of inhibition is time dependent suggesting that it is not due to simple binding of the inhibitor to the enzyme but rather to some reaction occurring between the enzyme and inhibitor. The enzyme shows considerable specificity with S-oxalylglutathione (GS-Ox) being several fold better as an inhibitor than any of the other compounds tested. However, even a poor inhibitor (for example, S-oxalyl-N-acetylcysteamine) will lead to extensive inhibition if its concentration and incubation time with the enzyme are increased sufficiently.

Since GS-Ox is such a good inhibitor, the characteristics of its inhibition were studied in further detail. Shown in Fig. 1A are more extensive data on the effects of inhibitor concentration and time of incubation. These data illustrate, firstly, that even low concentrations of GS-Ox can lead to extensive inhibition; it just takes longer. They also show

TABLE 1
The Effects of Oxalyl Thioester Structure, Concentration and Incubation Time on the Activity of the Catalytic Subunit of Phosphorylase Phosphatase

| Inhibitor structure; the S-oxalyl derivative of: | Inhibitor Concentration[a] mM | % Catalytic activity after incubation with inhibitor[b] for 14 min | 34 min |
|---|---|---|---|
| none | 0 | (100) | (100) |
| glutathione | 0.025 | 81 | 61 |
| | 0.050 | 76 | 47 |
| | 0.10 | 52 | 33 |
| | 0.20 | 32 | 20 |
| | 1.0 | 25 | 14 |
| coenzyme A | 0.10 | 85 | 68 |
| pantetheine | 0.10 | 100 | 100 |
| | 0.20 | 91 | 85 |
| N-acetylcysteamine | 0.10 | 97 | 87 |
| | 0.20 | 92 | 79 |
| | 0.50 | 80 | 63 |
| | 1.0 | 71 | 46 |
| | 2.0 | 58 | 34 |
| N-octanoylcysteamine | 0.10 | 83 | 57 |
| | 0.20 | 65 | 38 |
| N-succinylcysteamine | 0.10 | 82 | 80 |
| | 0.20 | 74 | 66 |

[a]Concentration in the incubation solution.
[b]At 30°C and pH 7.4; see Experimental for details

that high concentrations of the GS-Ox do not inhibit the phosphatase completely even after incubation for extended periods of time. Such results imply that, either the modified enzyme still retains some catalytic activity, or that the phosphatase preparation is heterogeneous with two or more enzymes having differing sensitivities.

Because the concentration of glutathione (GSH) in cells is of the order of 1 mM, experiments were performed to determine how such concentrations would affect the inhibition of the phosphatase by GS-Ox. In control experiments it was shown that 1 to 5 mM GSH has no effect on the phosphatase in the absence of GS-Ox. Illustrated in Fig. 1B are some data obtained when both are present. With 1 and 5 mM GSH present during the incubation with the GS-Ox (filled squares and triangles) inhibition is still obtained but the amount of inhibition ultimately observed is less as the GSH concentration is increased. Interestingly, if the GSH is added after the incubation with GS-Ox has proceeded for 30 min. in the absence of GSH, the activity increases (open squares and triangles) to approximately the same ultimate value (the data in Fig. 1b seem to indicate some overshoot; whether this is real or experimental error requires further investigation). Such results initially suggested that the equilibrium of eq. 1 was being established but experiments where cysteamine (open circles, Fig. 1b) or L-cysteine (not illustrated, but the results are essentially the same as with cysteamine) is added instead of GSH indicate that the explanation is not quite so simple. When excess

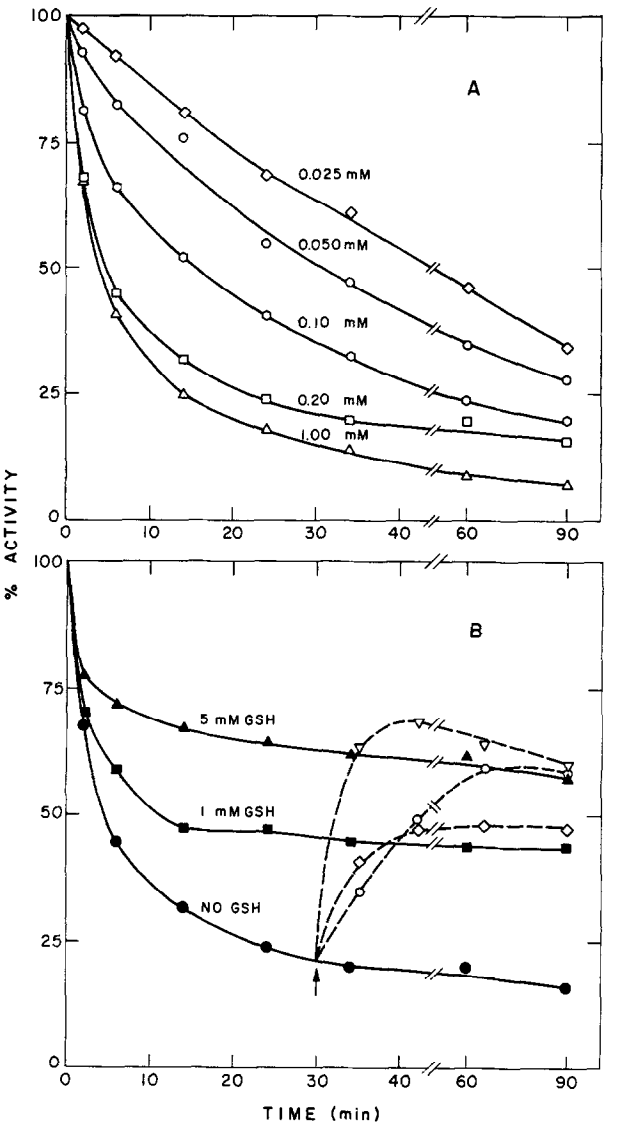


Fig. 1A The effects of S-oxalylglutathione concentration and time of incubation on the activity of the catalytic subunit of phosphorylase phosphatase. All experiments were carried out at 30°C and pH 7.4 using the indicated S-oxalylglutathione concentrations. See Experimental for further details.

Fig. 1B. The effects of glutathione (GSH) concentration on the inhibition of the catalytic subunit of phosphorylase phosphatase by 0.2 mM S-oxalylglutathione at 30°C and pH 7.4. The experiments represented by filled symbols had the indicated GSH concentration present during the entire incubation period. The results represented by open symbols were obtained when aliquots of the incubation mixture containing no GSH were removed at 30 min (arrow) and treated with 1 mM GSH (◇), 5 mM GSH (△) or 2 mM cysteamine (○).

β-mercaptoamine is added all of the oxalyl thioester will be converted in a few minutes to an N-oxalyl derivative (1). Since control experiments show that N-oxalyl-cysteamine has no effect on the phosphatase activity, the observation that cysteamine does not regenerate all of the catalytic activity

of the phosphatase indicates that at least part of the enzyme has been permanently modified in some way by the oxalyl thioester.

## DISCUSSION

The main initial conclusion to be derived from these results is that oxalyl thioesters are effective inhibitors of the catalytic subunit of phosphorylase phosphatase. They thus represent a new type of inhibitor for this enzyme. Although the mechanism of the phosphatase inhibition clearly needs to be investigated further, several of the characteristics suggest that at least part of the inhibition may be due to oxalyl transfer to the enzymic thiol (23,24) as originally hypothesized (1). Evidence for this are the findings that the inhibition is time dependent, that several oxalyl thioesters can cause inhibition (Table 1) and that they react at different rates. However, the observation that the inhibition can be partially but not completely reversed (Fig. 1B) indicates that there may be two different mechanisms operating.

Regardless of the mechanism, however, the fact that oxalyl thioesters do inhibit the phosphatase raises the question whether such inhibition could be important in vivo. The observation that oxalylglutathione is the best inhibitor of those tried lends support to this possibility. The high specificity suggests that the enzyme has evolved a specific binding site for oxalylglutathione, which in turn implies, therefore, that it is a normal effector of the enzyme in vivo. Glutathione is the most abundant thiol in the cell. Consequently, if oxalyl thioesters are formed in cells, as our results with the peroxisomal oxidases suggest (1), then at equilibrium oxalylglutathione would be the most abundant such thioester. Heretofore, oxalyl thioesters have not been directly identified in animals, most probably because no-one has ever looked for them. However, they are well characterized intermediates in bacterial and plant metabolism (20,25,26) and very recently we (27) have obtained preliminary evidence that they are present in animal tissues as well. Consequently, the possibility that the in vivo activity of this protein phosphatase is partially controlled by oxalyl thioesters seems very high. Detectable inhibition is observed even with quite low concentrations of oxalylglutathione although the non-catalyzed rate of inhibition of the catalytic subunit is relatively slow under such conditions. However, it should be kept in mind that in vivo the catalytic subunit is complexed to a regulator (28-30) which may possibly make it even more sensitive to oxalyl thioesters.

Since activation of protein phosphatases appears to be one of the most important intracellular effects of insulin (13,14), the present results add credence to the hypothesis (1) that oxalyl thioesters may be negative intracellular messengers for insulin, i.e., that insulin causes a decrease in their concentration. Mechanisms by which insulin could cause this have been

discussed (1). Because growth factors cause physiological effects very similar to those of insulin (1) presumably oxalyl thioesters may be important negative effectors for them as well. Indeed it seems possible that oxalyl thioesters may form the basis for a whole new system of metabolic control in animals because any enzyme with a reactive thiol group (and there are many) could potentially have its activity modified by such compounds. Their effects on some other enzymes are currently being investigated.

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