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ELEMENTAL SULFUR: A NOVEL INHIBITOR OF ADENYLATE KINASE

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Elementary sulfur (S-8) is a novel, highly specific and potent inhibitor of adenylate kinase (AK). The inhibition of AK by S-8 is completely reversed by dithiothreitol and is accompanied by several fold increases in enzymatic activity. Sulfhydryl group interaction with S-8 does not appear to be the mechanism of AK inhibitor and the results of comparative inhibition studies with N-ethyl maleimide (NEM) indicates that NEM also does not interact with the sulfhydryl group of AK.

Serendipitous inhibitions of adenylate kinase (EC 2.7.4.3) by elemental sulfur (S-8) derives from investigations of the properties of an inhibitor of an adenylate kinase (AK) from tuna found in mammalian sera which yields S-8 when made alkaline and heated (1). Inhibitions of AK by S-8 are very specific and very potent. Creatine phosphokinase (CPK) and several other enzymes readily inhibited by sulfhydryl reagents are not inhibited by S-8, and CPK activity can be quantified completely with AK when S-8 is present. A comparison of AK and CPK inhibitions using N-ethyl maleimide (NEM) suggests that the sulfhydryl group of AK is also not involved in this inhibition. Inhibition of CPK by NEM is not reversed by dithiothreitol (DTT) while the inhibitions of AK by NEM and S-8 are completely reversed with marked a stimulation of activity. A mechanism of AK inhibition by S-8 that involves its lipophilic domains is discussed.

MATERIALS AND METHODS

Commercial products were obtained from the Sigma Chemical Co., unless noted otherwise, and are Myokinase, Grade III, referred to in the text as rabbit muscle AK or RMAK; Creatine Phosphokinase, Type II; and Diagnotic Kit No. 45-UV was used for CPK assays. Other products and methods are given elsewhere (2).

Sulfur stock solutions contained 100 μmoles/L in absolute ethanol. Dilutions of the stock solution were with water and controls contained an ethanol

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volume equivalent. No inhibition by ethanol was evident up to 35 percent by volume.

RESULTS

The rate inhibition of rabbit muscle AK (RMAK) by S-8 shown in Figure 1 is slow requiring 2 hrs of 96 percent inhibition.

The details of the inhibition kinetics are currently under study. The addition of DTT or similar sulfhydryl compounds to the inhibited RMAK results in a marked stimulation of activity as shown, in this instance in excess of 5-fold. Also shown is the 2-fold stimulation by DTT of uninhibited RMAK. The inhibition is very potent since inhibition of RMAK by S-8 can be detected within nanomolar ranges. Based upon the chemistry of sulfur (3,4), an interaction with sulfhydryl group of RMAK was expected. Therefore, enzymes known to be sensitive to inhibitions by sulfhydryl reagents were tested for inhibitions by S-8. Hexokinase, pyruvate kinase, phosphoglycerate kinase, CPK, and lactate dehydrogenase were not inhibited by S-8 at concentrations several times that required to inhibit RMAK completely. The AK isozymes from liver were also not inhibited by S-8. Since the differential inhibition of AK in the presence of CPK might be of some immediate clinical interest, a preliminary study was made. Table I shows that CPK, either the brain or the muscle isozyme, is not

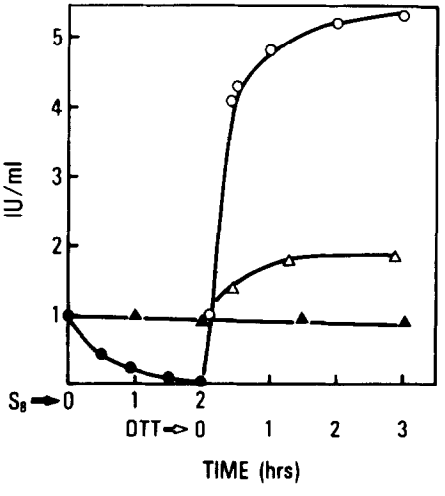

Figure 1. Inhibition of RMAK by S-8 and reversal of inhibition by DTT. The inhibited mixtures at 25°C contained 0.9 IU of RMAK; 100 μmoles/L K-PO_4, pH 7.0; and 9 μmoles/L S-8 initially (black arrow). Activity was measured at times indicated by (●). At 2 hr, mixtures were made 10 mmoles/L DTT (open arrow) and RMAK activity was measured at times indicated by (o). Triangles (▲) indicate RMAK controls without S-8 or DTT and the open triangles (△) indicate RMAK controls with DTT.

Table 1

Quantitative determination of CPK in the presence of AK and S-8

Condition[b]	Enzyme Activity (IU/ml)[a] CPK	AK[c]	CPK + AK
Control	2.49	0.72	3.44
10 μmoles/L S-8	2.32	0	2.40

[a] See Materials and Methods. All enzyme measurements are by the CPK assay system.
[b] Reaction mixtures were at 25°C; contained 100 mmoles/L $K\text{-}PO_4$, pH 7.0; and were incubated 0.5 hr and then enzyme activities were determined.
[c] AK activity is 2.2 times greater when measured by an AK assay system (6).

inhibited by S-8 and that a quantitative determination of CPK activity in the presence of AK obtains.

Since enzymes sensitive to sulfhydryl reagents were not inhibited by S-8 and AK was, a comparative study of several inhibitors was undertaken. Of the inhibitors, NEM evoked interest because DTT reverses its inhibition of RMAK and has no effect on CPK inhibition. Figure 2 shows the inhibition patterns obtained when RMAK or CPK is titrated against fixed concentrations of NEM. The pattern shown for the CPK titration is typical for an irreversible inhibition or pseudo-irreversible inhibition (5). Such a pattern is consistent with the formation of a thioether by CPK and NEM interaction. The pattern shown for the AK titration is typical of a reversible inhibitor and suggests a dissociation of the enzyme-inhibitor complex. Apparently, NEM inhibits CPK by a mechanism that differs from the inhibition of AK. Although not shown, the patterns obtained for the titration of AK in fixed S-8 concentration is identical with that given for the AK versus NEM in Figure 2.

DISCUSSION

The inhibition of AK isozymes by S-8 appears novel with respect to sensitivity, specificity, and mechanism. The sensitivity of RMAK to inhibition by S-8 is within the nanomolar range and the specificity of the inhibition by S-8 is high since several kinases and other enzymes were not inhibited. A mechanism of inhibition that involves sulfhydryl groups seems likely based on our

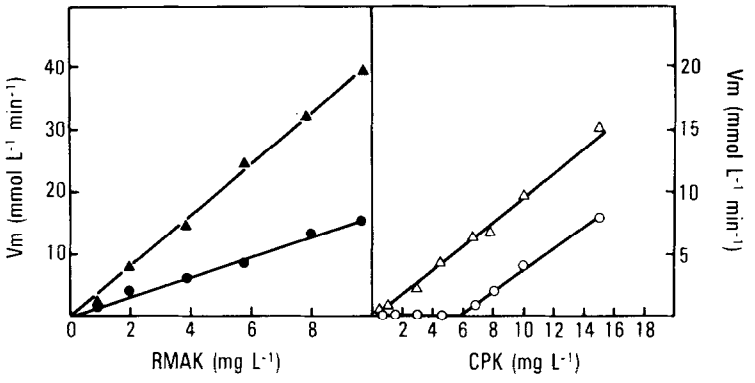

Figure 2. Inhibitions of RMAK and CPK by NEM. Mixtures for inhibitions of RMAK shown contained 125 mmoles/L $K\text{-}PO_4$, pH 6.9 and 0.20 mmoles/L NEM, indicated by (●). Controls without NEM are indicated by (▲). RMAK activities were determined after 1 hr at 25°C. Conditions for inhibitions of CPK were similar except that NEM was 0.05 mmoles/L, indicated by (o) and the controls (△). Protein determinations were by the method of Bradford (13).

observation that DTT obliterates the absorption at 263 nm (6,7) and therefore interacts with sulfhydyl groups, and based on the chemistry of S-8 (3,4). However, such a mechanism is negated by the inability of S-8 to inhibit enzymes sensitive to inhibition by sulfhydryl reagents and by preliminary data (not shown) indicating that S-8 does not block the detection of the sulfhydryl groups of AK or bovine serum albumin by Ellman's reagent (8). An outgrowth of study of S-8 as an inhibitor of RMAK is that NEM appears to inhibit RMAK in a novel manner also. The inhibition of enzymes by NEM is generally considered to involve sulfhydryl groups with th formation of a thioether (9), but the pattern of inhibition of RMAK by NEM shown in Figure 2 is similar in form to the inhibition of S-8 (not shown) and is indicative of reversible inhibition. By contrast, the inhibition of CPK by NEM, also shown in Figure 2 is consistent with the formation of a thioether. We propose that NEM and S-8 inhibit RMAK by interacting with or adsorbing to a hydrophobic domain of AK (10) and that DTT reverses inhibition by the removal of NEM to form a thioether and by disruption of the ring structure of S-8 (4), respectively.

The reversals of S-8 inhibition of RMAK by DTT, and also by glutathione or mercaptoethanol, result in recoveries of activities several times the initial (see Figure 1). One possible explanation is the co-existence of active and inactive conformers. The existence of at least two distinct crystalline conformations of AK isozyme has been reported (11) and we propose the

existence of active and inactive conformers of RMAK in solution that interconvert slowly. We propose further that the inhibited RMAK-S-8 complex is ordered into a single conformation, that the rate of removal of S-8 from the inhibited complex by DTT is faster than the rate of conformational change, and that the most enzymatically active conformer then presents in relatively high concentration upon reactivation by DTT. The kinetics of the inhibition, of the activation, and of the recovery from stimulation are currently under investigation.

Of pragmatic and clinical interest is the sensitivity of RMAK to inhibition by S-8 and the inability of S-8 to inhibit CPK under similar conditions. The inhibition of RMAK by low concentrations of S-8 permits a quantitative estimate of CPK (see Table I). In our studies, S-8 alone was equal to or more effective than the substrate analog AP5A (12) as a differential inhibitor of AK in the presence of CPK.

Finally, it is intriguing to speculate about an intracellular AK control mechanism involving S-8 and glutathione, but our search for S-8 in mammalian tissues and fluids was essentially negative. There remains the possibility of intracellular control of AK activity by lipophilic molecules at the site on the enzyme that S-8 occupies during inhibition.

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