Sulfur Specifically Inhibits Adenylate Kinase in Assays for Creatine Kinase

Percy J. Russell, Joe Conner, and Stephen Sisson

Elemental sulfur is a specific and potent inhibitor of the muscle-type isoenzyme of adenylate kinase (EC 2.7.4.3). We find inhibition by sulfur and by diadenosine pentaphosphate to be similarly potent and specific. Some properties of inhibition of adenylate kinase isoenzymes by sulfur are given. The adenylate kinase isoenzymes from skeletal muscle, brain, and heart muscle are inhibited by sulfur; those from liver and kidney are not. Other enzymes not inhibited by sulfur include the isoenzymes of creatine kinase (EC 2.7.3.2). We show that creatine kinase can be measured in serum when adenylate kinase is inhibited by sulfur, and that the sensitivity and specificity of this inhibition are of the same order as the inhibition of serum adenylate kinase activity by AMP plus diadenosine pentaphosphate.

Additional Keyphrases: enzyme activity, isoenzymes, variation, source of diadenosine pentaphosphate as inhibitor

Measurement of total creatine kinase (CK) activity in serum provides a very sensitive index to myocardial damage (1-3), even better when CK-MB isoenzyme activity is measured (4, 5). Measurement of serum CK activity is complicated by adenylate kinase (AK), which is present in all tissues. AK activity in serum originates both from myocardial tissue and from hemolyzed erythrocytes. To circumvent such interference, investigators have used sample-blank determinations or some more-or-less-specific inhibitors of AK (9), among them fluoride (6, 7) and AMP (8), both of which also inhibit CK activity to some extent. Diadenosine pentaphosphate (Ap5A) highly specifically inhibits AK activity from muscle and erythrocytes (9), particularly in the presence of some AMP (10).

We show here that elemental sulfur (S8) is a novel and highly specific inhibitor of the AK isoenzymes found in heart, skeletal muscle, and erythrocytes (11), and that it resembles Ap5A in its pattern of inhibitions of the various AK isoenzymes (9). We present some comparisons between inhibition of rabbit skeletal muscle AK by S8 and by Ap5A. Some other characteristics of the inhibition by S8 are shown, including its inability to inhibit CK. Advantages of using S8 in the presence of CK activity include obviation of AMP, high specificity of the inhibition, and the low concentration of (low-cost) S8 required.

Materials and Methods

AK assay. We measured AK activity essentially according to Adams (12), as detailed elsewhere (11). The 1-mL assay mixture contained, per liter: 20 mmol of potassium phosphate (as pH 7.0 buffer), 0.3 mmol of phosphoenolpyruvate, 0.4 mmol of NADH, 8.0 mmol of ATP, 8.0 mmol of AMP, 20 mmol of MgCl2, and sufficient pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) that the coupling system was not rate limiting. The reaction was started by adding MgCl2 after mixing the assay mixture with a sample possessing AK activity. Reaction rates were determined by measuring the decrease in absorbance of NADH at 340 nm. The molar absorptivity of NADH, 6.22 × 103, was used to convert absorbance to micromoles of product formed. One unit (U) of enzyme activity is equivalent to the formation of 1 μmol of ADP per minute at 25 °C under the specified assay conditions.

CK assay. CK activity was measured at 25 °C with a kit (no. 45-UV; Sigma Chemical Co., St. Louis, MO 63178).

Inhibition of AK activity in the presence of CK activity. Incubations of AK or CK with S8 were either in human serum or in potassium phosphate buffer (0.1 mol/L, pH 7.0) that contained 4 mg of bovine serum albumin per milliliter; incubations of AK with Ap5A were the same. After the incubations, we measured the enzyme activities remaining. We used Ap5A as its ammonium salt (Sigma Chemical Co.).

Sulfur. The sulfur we used was designated "sulfur, sublimed (powder)" (Mallinckrodt, St. Louis, MO 63134). It is indistinguishable as an inhibitor from a more purified preparation ("sulfur, 99.99%, Gold Label"; Aldrich Chemical Co., Milwaukee, WI 53201). Stock solutions in absolute ethanol containing 1.5 mmol of S8 per liter are stable for several weeks at 4 °C. AK activities are inhibited by ethanol in concentrations >30 percent by volume (6.5 mol/L) after incubation for several hours at 25 °C. All experiments contained proper controls for ethanol concentration. The dilutions of S8 stock solutions were so great that turbidity was not a problem in the experiments.

Rabbit skeletal muscle AK. The commercial preparation from Sigma Chemical Co., designated "myokinase, grade III from rabbit muscle," is the source of the AK used unless otherwise specified. When subjected to isoelectric focusing, the rabbit muscle AK usually showed a single isoenzyme with an isoelectric point (pl) value near 9.4. Occasionally, preparations contained a minor AK isoenzyme with a pl value near 7.0 that constituted at most 3% of the activity. We used the commercial rabbit muscle AK preparation without further purification.

CK. We used commercial CK preparations (Sigma Chemical Co.) in these studies: "creatin phosphokinases Type I, Type III, and Type IV" from rabbit muscle, bovine heart, and rabbit brain, respectively. Unless otherwise specified, we used CK type III in these experiments. The CK isoenzyme preparations are not inhibited by S8 (13).

Results

Inhibition patterns of S8. The inhibition patterns of some human AK isoenzymes by S8 were as follows:

<table>
<thead>
<tr>
<th>Inhibition (%) in extract of</th>
<th>Skeletal muscle</th>
<th>Heart muscle</th>
<th>Erythrocytes</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.3 ± 2.4</td>
<td>95.0 ± 1.0</td>
<td>97.6 ± 2.1</td>
<td>1.6 ± 2.3</td>
<td>4.4 ± 2.1</td>
<td></td>
</tr>
</tbody>
</table>

Tissue extracts were prepared as previously reported (14) and adjusted by dilution to 1000 U of AK activity per liter. The incubations were for 2 h at 25 °C. Final concentrations of S8 were 20 μmol/L.

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1 Nonstandard abbreviations: AK, adenylate kinase (AMP:ATP phosphotransferase, EC 2.7.4.3); CK, creatine kinase (ATP:creatin N-phosphotransferase, EC 2.7.3.2); CK-MB, an isoenzyme of CK; and Ap5A, diadenosine pentaphosphate (Ap1, Ap2,Ap3-diadenosine-5') pentaphosphate.

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Similar inhibition patterns of AK isoenzymes obtain for tissues from cat, rabbit, cow, dog, and rat. In general, inhibitions of AK by S₈ follow patterns similar to the sulfhydryl reagent inhibitors (11, 15) and Ap₅A (9).

Skeletal muscle-type AK isoenzymes are slowly but nearly completely inhibited at nanomolar concentrations of S₈ (13), a process greatly accelerated at the micromolar concentrations of S₈ used in our studies, as Figure 1 shows.

Protective effects of the substrates. Most of the standard procedures for determining CK activity that involve use of Ap₅A as an inhibitor of AK activity also include AMP to augment the inhibition (8, 10, 16). Under certain conditions, the nucleotide substrates protect AK from inhibition by S₈, as shown in Figure 2. Under the conditions given, 6.3 mmol of AMP per liter completely prevented the inhibition of rabbit muscle AK at concentrations of S₈ that inhibit more than 90% of unprotected AK activity. The nucleotide substrates prevent or moderate the inhibition of AK by S₈ under the conditions given in Figure 2, but they cannot reverse it. For example, heart- or muscle-type AK isoenzymes inhibited by S₈ remain inhibited to the same extent after incubation for longer than 18 h with concentrations of AMP or ATP that completely protect from inhibition.

Comparisons of rabbit muscle AK inhibition by Ap₅A and S₈. Figure 3 shows that S₈ at 1.3 μmol/L or Ap₅A at 2.1 μmol/L inhibit 50% of rabbit muscle AK activity under the conditions given.

Differential inhibition of rabbit muscle AK and CK by S₈. Of the several enzymes tested (13), only skeletal muscle-type AK isoenzymes were inhibited by S₈. As Figure 4 shows, S₈ in a wide range of concentrations failed to inhibit CK. The concentration of S₈ required for 50% inhibition of rabbit muscle AK varied in different sera and was greater than for inhibiting AK in phosphate buffer. A 50% inhibition of 1000 U of rabbit muscle AK in 1 L of phosphate buffer required 0.16 μmol of S₈ per liter, while as much as 0.6 μmol of S₈ per liter was required in some human sera. In our experiments, the concentrations of S₈ were great enough to obviate any such inhibition variations of AK.

Quantitative estimation of CK in the presence of AK. Table 1 shows the quantitative estimation of CK activity in the presence of AK activity inhibited by S₈. Because S₈ is hydrophobic, we used ethanol as the solvent; the data indicate that a 200 mL/L final concentration of ethanol has little or no effect on CK or AK activities.

Discussion

CK activity in serum is a widely used index for the identification of a myocardial infarct by serum enzyme analysis (17). Moreover, an accurate, timely estimate of serum CK activity might reflect infarct size (14, 16, 19), so its early determination would be useful in guiding therapeutic strategies (4, 5). Estimates of serum CK activity are rendered falsely high by the presence in serum of AK activity from heart tissue or from the hemolysis of erythro-
cytes (17). Several inhibitors of serum AK activity have been studied (6, 7, 16, 20), of which the most successful, specific inhibitor is the combination of AMP (5 mmol/L) and ApA (10 μmol/L) (10).

We found that S₈ is as specific an inhibitor of AK activity from skeletal muscle, erythrocytes, and heart tissue as the AMP/ApA combination. The similarities of the specificities extend to the inability of S₈ to inhibit AK isoenzymes from liver and kidney, or other kinases (13, 21). Inhibition of AK activity by S₈ does not require AMP to augment the inhibition. In the procedures we developed, the S₈ is incubated with the serum sample before a small aliquot (5 to 40 μL) is assayed for CK activity. At the concentrations of AK activity found in normal serum (22) and during infarctions (21), nanomolar concentrations of S₈ are sufficient for complete inhibitions, but the incubation times required are long (13). In the procedure we report, we used micromolar concentrations of S₈, which reach maximum inhibition within a few minutes. Although the inhibition of AK activity by S₈ is reversed slowly and completely by thiol compounds (13) such as dithioerythritol, with our procedures we observed no reversals of S₈ inhibition of AK activity by the commercial assay mixtures, which contain 20 mmol of dithioerythritol per liter, and no lag periods after the sample was introduced into the assay mixture.

In summary, S₈ at low concentrations effectively, sensitively, and specifically inhibits serum AK activity, giving results comparable with those of AMP plus ApA but at a much lower cost.

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References


Table 1. Inhibition of AK in the Presence of CK* 

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Water control</th>
<th>Ethanol control</th>
<th>S₈*</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK</td>
<td>710 ± 50</td>
<td>600 ± 70</td>
<td>10</td>
<td>98.5</td>
</tr>
<tr>
<td>CK</td>
<td>2230 ± 190</td>
<td>2150 ± 180</td>
<td>2230 ± 80</td>
<td>1.8</td>
</tr>
<tr>
<td>CK-AK</td>
<td>3030 ± 380</td>
<td>2980 ± 360</td>
<td>2170 ± 90</td>
<td>27.8</td>
</tr>
</tbody>
</table>

* All mixtures contained 800 mL of human serum per liter. Incubations were for 1 h at 25 °C, although as little as 20 min suffices under these conditions. Activity remaining was determined by the CK assay system given in Methods and Materials. Each determination was repeated twice, with triplicate analyses each time.

* The S₈ incubation mixtures contained the same concentrations of ethanol as the ethanol control (i.e., 200 mL/L) and 50 μmol of S₈ per liter.

* Calculated based on the average of the water and ethanol controls.

**The AK activity is rabbit muscle-type and was determined by the CK assay system. When measured with an AK assay system (18), its activity is 2.2-fold greater.