Adenylate Kinase Inhibition by Adenosine 5'-Monophosphate and Fluoride in the Determination of Creatine Kinase Activity

Franco Melattini, Giuliano Giannini, and Paolo Tarl

The current methods for the determination of creatine kinase (EC 2.7.3.2) activity are derived from Oliver's method, in which AMP is used to decrease interference by adenylyl kinase (EC 2.7.4.3). Recently, Szasz et al. and Rosano et al. described methods in which diadenosine pentaphosphate and fluoride, respectively, are used to reduce this interference. However, diadenosine pentaphosphate does not sufficiently inhibit such activity of hepatic origin, while fluoride alone can only inhibit it at concentrations at which the fluoride tends to precipitate as MgF₂. Finally, Szasz et al., the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physi-ology, and the German Society for Clinical Chemistry have proposed methods in which both AMP and diadenosine pentaphosphate are used to inhibit adenylate kinase. We have found that by using low concentrations of AMP and fluoride together, we can greatly diminish this interference without significant loss of creatine kinase activity and with no precipitation of MgF₂.

The usefulness of determining CK₁ activity as an aid to early diagnosis of myocardial infarction and progressive muscular dystrophy has already been well established (1, 2).

Present studies are chiefly aimed at resolving technical problems and at defining optimal assay conditions (3-6). However, the basic method for the determination of CK activity is still that described by Oliver (7), which includes the use of the reaction catalyzed by CK, in the reverse direction (the more favorable thermodynamically):

\[
\text{ATP + creatine} \rightleftharpoons \text{creatinine phosphate + ADP}
\]

This is coupled with side reactions leading to the formation of substances that can be measured spectrophotometrical-

\[
\text{ATP} + \text{d-glucose} \rightleftharpoons \text{ADP} + \text{d-glucose-6-phosphate}
\]

\[
\text{D-Glucose-6-phosphate} + \text{NADP}^+ \rightleftharpoons \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+
\]

However, the above-mentioned system is affected by AK activity (which is always present in normal sera, particularly in hemolyzed samples), which catalyzes the formation of ATP according to the reaction:

\[
\text{2ADP} \xrightarrow{\text{AK}} \text{AMP} + \text{ATP}
\]

Therefore, the presence of AK causes an apparent artifi-
cially high value for CK activity.

Oliver (7) and others (3, 8) noticed that AK could be in-
hibited by AMP in concentrations five- to 10-fold that of ADP. However, at these concentrations, AMP also slightly inhibits CK activity (9), and this effect cannot be ignored. Later, some modifications were proposed, in which AMP was used in lower concentrations in order not to inhibit CK appreciably while causing sufficient, even if incomplete, inhibition of AK. Thus, the concentration of AMP was decreased from 20 mmol/liter to 10 mmol/liter (3) and then to 5 mmol/liter (6). However, with an AMP concentration of 5 mmol/liter CK is inhibited little, but the AK is not sufficiently inhibited (6, 9).

Recently, Rosano et al. (9) proposed the use of fluoride, while Szasz et al. (10), the Scandinavian Committee on Enzymes (11), and the German Society for Clinical Chemistry (12) suggest the use of both AMP and diadenosine pentaphosphate.

We studied the possibility of using AMP and fluoride to-
gether, and found that is possible to inhibit AK effecti-
vely with very low concentrations of them. Under the proposed conditions, the inhibition of CK is negligible and the precipitation of fluoride is avoided.

Materials and Methods

Creatine phosphate, ADP, and AMP were from Prochifar, Milan, Italy; HK (from yeast) and G-6-PD (from Leuconostoc mesenteroides) were from P&L Biochemicals Inc., Milwaukee.
Wisc. 53205; N-acetylcycteine, D-glucose, potassium fluoride, imidazole, and acetic acid were all from E. Merck, Darmstadt, Germany.

Normal and abnormal sera (myocardial infarction) and hemolysate from human erythrocytes were our sources of CK and AK.

AK of hepatic, cardiac, and muscular origin was obtained from liver, heart, and muscle homogenates according to Szasz et al. (10); AK from human platelets was prepared according to Szasz et al. (13). AK activity was determined with the reagent for CK, except that creatine phosphate and AMP were omitted (10).

Table 1 shows the concentration of reagents in the reaction mixture. Serum, 50 μl, was added to 3 ml of the reagent, which had been pre-warmed to 37 °C, and the reaction rate was immediately recorded at 340 nm, to check the lag-phase for each serum. A Beckman-25 spectrophotometer with thermostatic cuvet holder was used. The reaction temperature was 37 °C for the determination of both AK and CK activities.

The data were examined by Student's t-test, linear regression, and other elementary statistical methods (14).

Results

We assessed the inhibition of AK and CK activities by AMP and fluoride.

Without fluoride, AK was inhibited by 95% by AMP greater than 20 mmol/liter (Figure 1). Without AMP, 95% inhibition was reached with 25 mmol of fluoride per liter (Figure 2). However AMP 20 mmol/liter also inhibits CK activity by more than 10%, and fluoride 25 mmol/liter inhibits it by about 3.5% (Figure 3).

On using AMP and fluoride together, we found that AK activity was 95% inhibited with only 2 and 6 mmol/liter, respectively (Figure 4). Figure 5 reports the combinations of AMP and fluoride to inhibit AK by specific percentages. At the concentrations adopted, CK was inhibited by not more than 3% (Figure 6).

The proposed method was verified by determining the CK activity in normal sera with and without increasing amounts of added AK from different sources. These samples were analyzed by the described method, and also with use of 5 mmol of AMP per liter (6) and 5 mmol of AMP per liter plus 10 μmol of diadenosine pentaphosphate per liter (10–13). No signifi-
significant differences ($P > 0.05$) were found between results for normal samples and those with AK added on using the proposed reagent and the reagent with AMP plus diadenosine pentaphosphate. In contrast, there was a highly significant difference ($P < 0.01$) when using the method with AMP (5 mmol/liter) alone; the values we obtained for the samples with AK were about 50% higher than those of the corresponding samples without added AK.

We also tested the extent of inhibition of erythrocyte, platelet, hepatic, cardiac, and muscular AK activity by AMP (2 mmol/liter) plus fluoride (6 mmol/liter). Table 2 shows the results. The extent of inhibition ranged from 72 to 98%, but with human material it was never less than 95%.

Results of precision studies are shown in Table 3.

The comparison between the proposed reagent (y) and that suggested by the Scandinavian Committee (11), performed on 28 samples with activities ranging from 13 to 650 U/liter, gave the following data:

correlation coefficient ($r$) = 0.990
linear regression: $y = 0.999x - 10.1$
mean $x = 252.586$ U/liter
mean $y = 242.156$ U/liter

The proposed method, with use of the composition indicated in Table 1, is stable for as long as 55 h at $8^\circ$C and for as long as 24 h at $25^\circ$C.

(We use a sample volume fraction of 0.016; that used in refs. 11 and 12 is 0.04.)

**Table 2. Effect of AMP (2 mmol/liter) and Fluoride (6 mmol/liter) on Adenylate Kinase Activity from Different Sources**

<table>
<thead>
<tr>
<th>Source</th>
<th>Szasz method (10)</th>
<th>plus AMP, 2 mmol/liter and fluoride 6 mmol/liter *</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td></td>
<td>U/liter</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>864</td>
<td>205</td>
<td>76</td>
</tr>
<tr>
<td>Muscle</td>
<td>3064</td>
<td>843</td>
<td>72</td>
</tr>
<tr>
<td>Heart</td>
<td>1755</td>
<td>483</td>
<td>72</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>265</td>
<td>73</td>
<td>72</td>
</tr>
<tr>
<td>Muscle</td>
<td>1981</td>
<td>389</td>
<td>80</td>
</tr>
<tr>
<td>Heart</td>
<td>989</td>
<td>189</td>
<td>81</td>
</tr>
<tr>
<td>Monkey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1687</td>
<td>355</td>
<td>79</td>
</tr>
<tr>
<td>Muscle</td>
<td>36 335</td>
<td>6154</td>
<td>83</td>
</tr>
<tr>
<td>Heart</td>
<td>4433</td>
<td>613</td>
<td>86</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1133</td>
<td>58</td>
<td>95</td>
</tr>
<tr>
<td>Muscle</td>
<td>7802</td>
<td>245</td>
<td>97</td>
</tr>
<tr>
<td>Heart</td>
<td>1275</td>
<td>49</td>
<td>96</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>978</td>
<td>40</td>
<td>96</td>
</tr>
<tr>
<td>Platelets</td>
<td>285</td>
<td>7</td>
<td>98</td>
</tr>
</tbody>
</table>

* The concentrations of AMP and fluoride shown were added to the reagent used for AK determination according to the method for CK (10), omitting creatine phosphate and AMP.
Table 3. Between-Days Precision of the Proposed Method \( (n = 20) \)

<table>
<thead>
<tr>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.0</td>
<td>3.45</td>
<td>8.6</td>
</tr>
<tr>
<td>39.15</td>
<td>14.03</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Discussion

The simultaneous action of low concentrations of AMP and fluoride improves the performance of the system for determining CK activity. AK from different tissue-sources is highly inhibited (Table 2), while avoiding CK inhibition. In fact, the proposed reagent inhibits the CK activity to the same extent as fluoride, 25 mmol/liter (Figures 3 and 6), but without any precipitation of MgF\(_2\), even at low temperatures. Moreover, we observed that AMP and fluoride, when used together, show a different behavior towards AK, compared with that found when these substances are used separately (Figures 1, 2, 4).

In order to check the stability as regards MgF\(_2\) precipitation, the reagent was subjected to temperature changes: transfer from the refrigerator at 5 °C to the bath thermostated at 37 °C and vice versa, freezing and thawing, and continuous storage for six months at 5, 20, and 37 °C. Obviously, in these conditions, the enzymatic activity of HK and G-6-PD is destroyed, except in the case of the reagent stored at 5 °C; however, in no case was any precipitation of MgF\(_2\) found.

It should also be noted that no lag periods exceeding 1 min were found with the reagent and method proposed. This may be attributed to our use of very low concentrations of the AMP and fluoride inhibitors.

References


