A Study of the Problems of Inactivation of Creatine Kinase in Serum

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Addition of sulfhydryl groups to the reaction mixture, to stabilize serum creatine kinase (creatine phosphokinase, CPK), results in apparent increases in the activity of this enzyme in many sera. In addition, in sera from patients just after myocardial infarction, assays for sulfhydryl-activated CPK have a different clinical pattern than do those for CPK assayed without sulfhydryl activators: activities are greater and remain abnormally high longer in assays in which the enzyme is sulfhydryl activated. If the assay is done without sulfhydryl activators present, technical difficulties appear, because CPK in serum is rapidly inactivated at room temperature. CPK is apparently inactivated in at least two ways. One, which is irreversible, is inhibited by albumin; the other, which is reversible by sulfhydryl groups, seems to result from the presence of substances in the pooled serum used that are both protein bound and free.

Additional Keyphrases progressive muscular dystrophy • sulfhydryl groups as activators • Cleland's reagent • inactivator in serum • myocardial infarction • AutoAnalyzer

For several years CPK,¹ which catalyzes the reversible reaction creatine + ATP ⇌ creatine phosphate + ADT, was determined in our laboratory by the manual method of Tanzer and Gilvarg (1), as modified by Vincent and Rappaport (2), as an aid in the investigation of progressive muscular dystrophy and other muscle diseases (3–7). With the general acceptance of increased serum CPK activity as a more sensitive indicator of acute myocardial infarction (8, 9) than SGOT and LDH, there has been a very substantial increase in the clinical demand for CPK assays. Because the Tanzer and Gilvarg procedure does not readily lend itself to automation and because of our long experience with “AutoAnalyzers,” we decided to use the automated procedure of Siegel and Cohen (9). Early in our preliminary studies we realized that the results of the two procedures not only correlated inadequately, but that in the method of Siegel and Cohen the CPK activity apparently remained elevated above the normal range for seven to eight days after infarct, in contrast to the Tanzer and Gilvarg method, with which CPK activity values declined to essentially normal within three to four days.

In addition to the rather wide differences in assay conditions and the fact that they measure the CPK-catalyzed reaction in opposite directions, a major difference in the Siegel and Cohen assay is that the reaction system includes sulfhydryl groups, to activate the enzyme, an addition not made in the Tanzer and Gilvarg method.

Although unexpected results of CPK assays have been sporadically reported, the wide variety of methods used and the presence or absence of sulfhydryl groups in the various assay systems make interpretation of these results difficult. This paper reports a study of the effect of sulfhydryl groups on the enzyme assay as well as the clinical interpretation of the results obtained.

Materials and Methods

Creatine phosphate, disodium salt; ADP, sodium salt; 2-mercaptoethanol; and CPK from rabbit muscle, substantially salt free, were obtained from the Sigma Chemical Co., St. Louis, Mo.

¹ Nonstandard abbreviations used: CPK, creatine phosphokinase (ATP: creatine phosphotransferase, EC 2.7.3.2); SGOT, serum glutamate oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1); LDH, lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27); NADH, dithiothreitol (Cleland's reagent); AA, AutoAnalyzer; T-G, Tanzer-Gilvarg.

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The serum pool used in experiments to study the effect of thiol groups on partially purified rabbit muscle CPK was prepared by mixing sera from patients with CPK activity less than 50 AA units/liter as measured by the AutoAnalyzer technique described below, with 5 mmol of DTT added as activator. The pooled serum had 30 AA units/liter CPK as measured by the activated system.

The automated procedure of Siegel and Cohen (9), modified as described in the standard CPK method for the AutoAnalyzer (Method N-76, preliminary, Technicon Corp., Tarrytown, N.Y. 10591, July, 1970), was used with the following changes: The cam used gave a sampling rate of 60 per hour with a sample-to-wash ratio of 1:1. For routine clinical determinations we used, per liter, 10 mmol of sodium creatine phosphate hexahydrate (260 mg/72 ml) and 3.0 mmol of ADP sodium salt (94 mg/72 ml). The basis for these modifications is described in the results. Incubation was timed from the instant that the sample entered the substrate stream to the instant that the enzymatic reaction was stopped by adding N-ethylmaleimide (12 min in our system). Part of the incubation was unavoidably carried out at room temperature, but most of it was at 37°C, in the incubator coils. Creatine solutions (50 to 10,000 μmol/liter) were used as primary standards, and commercially obtained serum controls were run at the start and after every 10 samples for quality control. One AA unit is defined as 1 μmol of creatine formed per minute under the above conditions.

Results

Preliminary standardization of automated procedures. Optimum concentrations of creatine phosphate and ADP in the assay system were determined by varying each of these individually while maintaining all other reagents constant. Human serum with CPK activity of about 300 AA units/liter was used as the enzyme source in all experiments described in this section. The Km value for creatine phosphate, as calculated from a Lineweaver–Burk plot, was 2.0 mmol/liter, that for ADP was 0.2 mmol/liter.

On the basis of these values and other published results (10, 11) one would assume that an optimal concentration for creatine phosphate would be 20 mmol/liter and for ADP 3 mmol/liter. When sera from 20 patients, which ranged in CPK activity from 20 to 700 AA units/liter, were assayed with creatine phosphate at 10 mmol/liter instead of 20 mmol/liter, the value for the activity decreased by less than 7%; because creatine phosphate is expensive, we continued to use 10 mmol/liter in the substrate mixture for routine assays of patient sera. In all other studies described below we used 20 mmol of creatine phosphate per liter as substrate.

CPK activity decreases rapidly in serum during storage, even at low temperatures. Since the demonstration by Okinaka et al. (12) and Kar and Pearson (13) that the added sulfhydryl groups not only prevent this rapid inactivation but actually stimulate CPK activity, even in fresh serum, most methods direct that such compounds be added either to the serum or to the working substrate. To circumvent the reported instability of cysteine (10), Siegel and Cohen substituted 2-mercaptoethanol as the sulfhydryl source.

We determined CPK activity in sera with abnormally high enzyme activities at concentrations of 2-mercaptoethanol ranging from 0.1 mmol/liter to 8 mmol/liter. The enzyme activity continued to increase with increasing concentrations and maximum activation was apparently not obtained at the highest concentration tested, indicating that 6.5 mmol of 2-mercaptoethanol per liter, as used in the Siegel and Cohen procedure, is suboptimal. Increasing the concentration to 9 mmol/liter interfered with the color development. This, combined with the unpleasant odor of mercaptoethanol, prompted us to try other compounds. Of the compounds tested, DTT appeared to be most suitable. At first we rather empirically used a concentration of 1 mmol/liter (14); we later established that maximum activation was obtained with a DTT concentration of not less than 4 mmol/liter. A concentration of 5 mmol/liter was therefore used in all subsequent work to guarantee that the system was saturated with thiol groups.

Effect of thiol groups on serum CPK activity. Sera from 150 hospital patients, with CPK activity from 0 to 1.5 T-G units/liter of serum, which is considered the normal range in our laboratory, were assayed by the automated procedure, with 5 mmol of DTT per liter of substrate. The 95% limits, as computed by the nonparametric percentile estimate method (15), were 20 to 190 AA units/liter. This range is substantially higher than that reported in the standard AutoAnalyzer method N-76, which is 0–110 AA units/liter. We found, however, that most of the sera in which the CPK activity was greater than 120 AA units/liter were from patients who had had blood drawn repeatedly for several days before the CPK deter-
minations. The 95% limits for CPK, measured in the absence of activation in the same 150 sera, were 5 to 60 A.A. units/liter.

When serum CPK was determined daily on patients after myocardial infarction, we found that CPK activities remained above the normal range for seven to 10 days after the infarct. This is in contrast to the serum CPK values obtained with the Tanzer and Gilvarg procedure, in which increased activities were observed within 6 to 12 h of the infarct, reached a maximum approximately 29 h after the infarct, and returned to normal within 72 h, at which time SGOT and LDH activities were still usually elevated. Because the Tanzer and Gilvarg procedure differs from the Siegel and Cohen procedure in several ways—including the direction in which the reaction is measured, the pH and temperature of the reaction, as well as the absence or presence of a sulfhydryl reagent—we tried to ascertain the cause of the discrepancy by assaying by the Siegel and Cohen procedure, with and without added DTT. Figure 1 shows the results obtained on one such patient. Increase in enzyme activity was obtained both in the presence and absence of DTT, though by the second day the increases were over threefold greater in the presence of DTT, and enzyme activity was essentially within the normal range by the third day in the absence of DTT, while in its presence it remained elevated for at least ten days. Similar results were obtained with several other patients, although in most cases elevations in the presence of DTT did not persist beyond seven to eight days.

Effect of thiol groups on partially purified CPK from rabbit muscle. The unavoidable time lag between collection of blood from a patient and determination of serum CPK activity, as well as the unknown effects of this on the enzyme molecule, precluded use of serum for the detailed study of inactivation of CPK and its reactivation by sulfhydryl groups. We therefore decided to use partly purified CPK from rabbit muscle, despite the known differences in kinetic behavior and structure between rabbit and human CPK (16).

Rabbit muscle CPK was very rapidly inactivated at room temperature when suspended in physiological saline. Addition of DTT to the reaction mixture had very little effect (Table 1). Similar results were obtained when imidazole buffer (0.2 mmol/liter, pH 6.8) was substituted for saline.

Table 2 shows the stabilizing effect of human serum albumin. In the presence of 80 g of albumin per liter, activity is unchanged for as long as 6 h at room temperature, and there is only a moderate loss at albumin concentrations of 20 or 40 g/liter, which is reversed in the presence of DTT.

Data presented in Table 3 indicate the presence of a substance in serum that inactivates CPK. There was considerable inactivation of rabbit muscle CPK suspended in a serum pool that had an albumin concentration of 35 g/liter; 75 g/liter protected the activity only a little more. DTT partially reactivated the enzyme in both cases. It is unlikely that there is interference owing to the presence of specific drugs that may have been administered to patients, since serum from a large number of patients was pooled, but this possibility cannot be totally discounted.

Attempts at characterization of the factor in serum responsible for inactivation were only partly successful. Results are summarized in Table 4. Heating serum pool at 80°C for approximately 5 min, which should remove most enzymatic activity, did not affect its capacity to inactivate rabbit muscle CPK even in the presence of added albumin. If pooled serum is dialyzed against saline for 24 h or ultrafiltered through a membrane filter that eliminates macromolecules, it continues to inactivate rabbit muscle CPK. Apparently the inactivating substance is both protein bound and freely diffusible.

Discussion

Results obtained with rabbit muscle CPK seem to indicate that the enzyme undergoes two types of inactivation, one being irreversible and prevented by the presence of albumin and therefore usually not important in clinical assays unless the serum sample is diluted. Our results indicate that saline or imidazole buffer should not be used as a diluent. When enzyme activities are high, salt-free albumin solution should be used as a
Table 1. Inactivation of Rabbit Muscle CPK Suspended in Saline

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Residual activity, %</th>
<th>-DTT</th>
<th>+DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>25</td>
<td></td>
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<tr>
<td>6</td>
<td>0</td>
<td>10</td>
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* Results in this and subsequent tables are means of at least three determinations.

Table 2. Effect of Human Albumin on Inactivation of Rabbit Muscle CPK

<table>
<thead>
<tr>
<th>Albumin concn., g/l</th>
<th>-DTT 3 h</th>
<th>+DTT 3 h</th>
<th>-DTT 6 h</th>
<th>+DTT 6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>86</td>
<td>102</td>
<td>69</td>
<td>96</td>
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<tr>
<td>40</td>
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<td>90</td>
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<tr>
<td>80</td>
<td>101</td>
<td>97</td>
<td>101</td>
<td>99</td>
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Table 3. Inactivation of Rabbit Muscle CPK in Various Diluents

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Residual activity, %, after 3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>-DTT</td>
</tr>
<tr>
<td>Albumin, 40 g/l</td>
<td>85</td>
</tr>
<tr>
<td>Serum pool</td>
<td>27</td>
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<tr>
<td>Serum pool + albumin, 40 g/l</td>
<td>44</td>
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</tbody>
</table>

Table 4. Effect of Various Treatments to Serum Pool on Inactivation of Rabbit Muscle CPK

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Residual activity, %, after 3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated serum pool</td>
<td>20</td>
</tr>
<tr>
<td>Heated serum pool + albumin (40 g/l)</td>
<td>35</td>
</tr>
<tr>
<td>Dialyzed serum pool</td>
<td>25</td>
</tr>
<tr>
<td>Dialyzed serum pool + albumin (40 g/l)</td>
<td>33</td>
</tr>
<tr>
<td>Ultrafiltrate* of serum pool</td>
<td>38</td>
</tr>
<tr>
<td>Ultrafiltrate of serum pool + albumin (40 g/l)</td>
<td>33</td>
</tr>
</tbody>
</table>

* "Ultrafiltrate" refers to material passing a "Centriflo" ultrafiltration membrane filter cone (cat. No. CF20; Amicon Scientific Systems Division, Lexington, Mass. 02173).

The second form of inactivation, which is reversible by sulfhydryl groups, seems to be mediated through a substance(s) in serum that is both protein bound and free. Perhaps it is calcium, which is present in both forms in serum. Enner and Rosenberg, in 1954 (17), reported that the calcium: enzyme-nitrogen ratio affects the forward CPK reaction. When the calcium to enzyme-nitrogen ratio is greater than 4000 there is a marked inhibition of creatine liberated from creatine phosphate. Several years ago, we noted while using the Tanzer and Gilvarg method, which is the reverse reaction, that EDTA protects plasma CPK activity for a while. This also may be due to reversal of an effect of calcium. However, direct addition of Ca²⁺, at concentrations found in serum, to an albumin solution containing rabbit muscle CPK did not inactivate the enzyme even partly, indicating that the effect is probably very complex.

The increased CPK activity within 4 to 6 h of a myocardial infarction and its return to normal on the 3rd or 4th day is generally considered to be a well-established phenomenon. The experimental evidence on which this is based, however, was obtained with the Tanzer and Gilvarg method in the absence of sulfhydryl groups (8, 18). The extrapolation of these observations to CPK measurement made by other techniques in the presence of activation is not completely justified, as can be seen from the results reported here. Although the initial rapid increase in serum CPK after an infarction is actually accentuated by the use of activators, the return to normal activity takes six to seven days and sometimes even longer. This observation has been made by others (18, 19), but has not been sufficiently emphasized in the literature.

If the in vitro results discussed above can be applied to in vivo situations, a possible reason for the discrepancy between activated and nonactivated serum CPK values after a myocardial infarction is indicated. The enzyme released into the blood stream at the time of an infarct may be inactivated irreversibly as well as, at a relatively much greater rate, by a process that is reversible by sulfhydryl groups. In the presence of sulfhydryl groups, the reversibly inactivated CPK is reactivated and hence a higher value is obtained. Essentially normal activities are restored in serum only when all the circulating enzyme has been irreversibly inactivated.

Performing the assay without added sulfhydryl introduces a major technical problem, because enzyme activity is lost rapidly when serum sets at room temperature. Although this problem can be circumvented if the serum is assayed within 90 min of blood collection, this is not always possible in a clinical laboratory. We have not yet found a compound that prevents inactivation but does not reactivate the enzyme. Therefore, we collect the samples in specially labeled tubes and run the tests as promptly as possible in the absence of activators. If the test cannot be assayed within 90 min, we rapidly freeze the serum and store it frozen until it is assayed. If repeated freezing and thawing is avoided, less than 20% activity is lost by this procedure. Admittedly, we encounter a few false negatives, but we believe...
that we are not confusing the issue with a lot of false positives. Attempting to solve the activation problem by increasing the normal range does not help because there are too many variables, and the effect of activation varies with every serum.

Obviously, we do not have an ideal solution to the problem, and the continued use of activators may be preferred in other laboratories as long as the limitations are borne in mind and interpretation of results obtained by one method are not automatically extrapolated to other methods.

References