Macro Creatine Kinase: Determination and Differentiation of Two Types by Their Activation Energies

Wolfgang Steinl, Jürgen Bohner, Roswitha Steinhart, and Manfred Eggstein

Determination of the MB isoenzyme of creatine kinase in patients with acute myocardial infarction may be disturbed by the presence of macro creatine kinase. The relative molecular mass of this form of creatine kinase in human serum is at least threefold that of the ordinary enzyme, and it is more thermostable. Here we describe our method for determination of macro creatine kinases and an easy-to-perform test for differentiating two forms of macro creatine kinase, based on their distinct activation energies. The activation energies of serum enzymes are mostly in the range of 40-65 kJ/mol of substrate. Unlike normal cytoplasmatic creatine kinases and IgG-linked CK-BB (macro creatine kinase type 1) a second form of macro creatine kinase (macro creatine kinase type 2) shows activation energies >80 kJ/mol of substrate. The exact composition of macro creatine kinase type 2 is still unknown, but there is good reason to believe that it is of mitochondrial origin.

Additional Keyphrases: immunoinhibition • heat inactivation • mitochondrial creatine kinase • myocardial infarction

Determination of the MB isoenzyme of creatine kinase (CK; EC 2.7.3.2) is valuable in the diagnosis of myocardial infarction. Unfortunately, the methods for differentiating it (electrophoresis, ion-exchange chromatography, immunoinhibition) used in the routine or emergency laboratory may give misleading results in some rare cases when the sample also contains macro creatine kinase. Macro CK shows true- i.e., creatine phosphate dependent-enzyme activity and thus macro CK is defined as a form with substantially increased molecular mass as compared with normal-size CK (1). During isoenzyme electrophoresis macro CK may migrate cathodally or anodally to CK-MM; sometimes it comigrates with CK-MM or even CK-MB (2). During ion-exchange chromatography macro CK may simulate false positives for CK-MB (3), and with the immunoinhibition test macro creatine kinases that cannot be inhibited by anti-M antibodies may result in an unusually high ratio of "CK-MB" vs total CK activity. As compared with the isoenzymes CK-MB and CK-BB, macro CK is clearly more stable towards thermal inactivation and shifts in pH (4). Therefore, in sera with an unusually high amount of apparent CK-MB as determined by the immunoinhibition test, demonstration of heat-stable creatine kinase activity gives good reason to believe that macro CK is present.

Further studies on the nature of macro CK showed the existence of at least two different forms, which can easily be detected by their activation energies: type 1 macro CK is IgG-linked CK-BB with a binding site between CK-BB and IgG located on the Fab region of the immunoglobulin G molecule (5).2

Macro CK type 2 cannot be inhibited by anti-M antibodies or bound to IgG-specific Protein A-Sepharose, migrates cathodally to CK-MM or comigrates with CK-MM during electrophoresis (2), and is released into the bloodstream only during severe illness.

Here we describe an easy-to-perform procedure for differentiating macro CK type 1 from macro CK type 2, based on their distinct activation energies, we present a decision sequence for cases with high total-CK activities and a ratio of CK-MB to total-CK that exceeds 0.25, and we give some more information that shows the similarity between macro CK type 2 detected in human serum and the macromolecular form of CKmit isolated from human tissues.

Materials and Methods

Enzyme assay: Total creatine kinase activity was measured with the "CK-NAC" reactivated kit (no. 128357; Boehringer, Mannheim, F.R.G.) (6). For the assays we used an Eppendorf photometer (M 1101) with recorder or an ACP 5040 analyzer (Eppendorf Gerätebau, Hamburg, F.R.G.) Assay conditions were: 10 μL of sample, 250 μL of reagent, 10 μL of substrate, and temperature 25 °C.

Immunoinhibition test: Residual CK activity after inhibition of the CK-M subunits was determined with "NAC reactivated" reagents (nos. 14109, 14110, 14112; E. Merck, Darmstadt, F.R.G.) (7).

Manual procedure: Run 1 (sample blank): 20 μL of sample and 500 μL of reagent are mixed. During the 10-min incubation the reaction is monitored, to detect residual adenylate kinase (EC 2.7.4.3) activity. Run 2 (test): the actual reaction is started by adding 20 μL of creatine phosphate solution. The creatine kinase activity is monitored continuously at 334 nm and 25 °C for at least 5 min.

Mechanized procedure: The instrument (ACP 5040) was programmed as follows: incubation interval, 10 min; sampling rate, one per minute; wavelength, 334 nm; temperature, 25 °C. Channel 1 (run 1, blank): 10 μL of sample and 250 μL of reagent. Channel 2 (run 2, test): 10 μL of sample, 250 μL of reagent, and 10 μL of substrate.

For both procedures, the residual CK activity after inhibition of the CK-M subunits was calculated by multiplying each ΔA/min by 2 × 4369 and subtracting the result of run 1 (blank) from the result of run 2. If not stated otherwise, the results for "CK-B" activity were multiplied by 2 × 4369 to calculate the CK-MB activity; this is why samples containing high amounts of macro CK, CK-BB, or CKmit will give paradoxical results up to 2.0 if the ratio of "CK-MB" to total CK is calculated.

Exclusion chromatography: A 26 × 0.9 cm column packed with Sephacryl S-300 (Pharmacia, Upsala, Sweden) was used with a mobile phase containing, per liter, 50 mmol of Tris (pH 7.0), 50 mmol of NaCl, and 200 mg of NaN3. The sample volume was 200 μL, the flow rate about 3 mL/h, and fraction

---

2 Up to now we have been unable to detect significant amounts of immunoglobulin-linked CK-MB, CK-MM, or CKmit in human sera.
volume 440 µL. Post-column CK activities were determined for total CK and by using the immuno inhibition test, and blanks were corrected for in all fractions. To increase the sensitivity of the CK assay, the temperature we used was 37 °C and sample volume was increased fivefold.

Sera studied: In the sera of 56 patients admitted to our hospital with an unusually high “CK-MB”/total-CK ratio as determined by the immunoinhibition test, macro CK could be detected by exclusion chromatography (Figure 1). The sera were stored frozen at -20 °C, without any additives.

Twenty-two normal sera showing no macro CK were taken from the routine laboratory and directly analyzed.

Isoenzyme purification: Human skeletal muscle, heart, and uterus, obtained at autopsy or surgery, were used as tissue sources of CK-MM, CK-MB, CK-BB, and C小微企业. Stock pools of individual CK isoenzymes were prepared as previously described (5). CK小微企业 from human heart and skeletal muscle was purified according to Wevers et al. (8), except that we omitted all SH-reagents from the buffers. This purification step in our hands usually resulted in a CK小微企业 preparation containing a macromolecular form of CK小微企业 (Figure 1) with a relative molecular mass (Mr) of about 250 000 and, to a lesser degree, normal-size CK小微企业 with a Mr of about 80 000. The possibility of contamination by other CK isoenzymes was excluded by electrophoresis, isoelectric focusing, and immuno inhibition.

Anion-exchange chromatography: Before ion-exchange chromatography, we desalted samples on a mini-column (a Pasteur pipette) packed with Sephadex G-25 (Pharmacia). Ion-exchange chromatography was performed on a 4 X 0.9 cm column of DEAE-Sepharose Cl-6B (Pharmacia) equilibrated with “weak” buffer. The sample volume was 200 µL. For elution of the CK isoenzymes a linear gradient was generated by continuously mixing 30 mL of “weak” buffer (1 mmol/L Tris, pH 8.5, 50 mg/L NaNO3) and 30 mL of “strong” buffer (50 mmol/L Tris, 500 mmol/L NaCl, pH 7.0, 200 mg/L NaNO3). The fraction volume was 440 µL, the flow rate about 3 mL/h.

CK activity in the eluate was determined as described above.

Stability of enzyme activity: We determined the decrease in enzyme activity of macro CK and CK小微企业 (diluted with normal, inactivated serum) caused by thermal inactivation and shifts in pH during storage at 37 °C as described (4). We assayed two preparations of CK小微企业, derived from human heart muscle, showing different proportions of the macromolecular form (75 and 55% of total CK小微企业 activity), as determined by exclusion chromatography. The proportion of the macromolecular CK小微企业 was decreased from 75 to 55% by incubation with 100 mmol of 2-mercaptoethanol per liter, according to Hall et al. (9). Directly before diluting the 2-mercaptoethanol-treated CK小微企业 with inactivated serum we removed most of the 2-mercaptoethanol by use of a concentrator B15 (Amicon Corp., Lexington, MA 02173).

Heat-inactivation test: After determining the CK activity by immuno inhibition test, we heated 500 µL of each serum at 45 °C in a water bath for 20 min, cooled the samples to room temperature, and once more determined the residual CK activity by use of the immuno inhibition test. All values were corrected for blanks. As described elsewhere (4), under these conditions (pH 7.4-7.7) CK-MB and CK-BB lose most of their activities (>50%), whereas macro CK is almost unaffected.

Determination of Activation Energies

Principle: The determination of the apparent activation energy according to Arrhenius (10) is based on the dependence of enzyme activity on temperature:

\[ \ln k_{\text{cat}} = -E_a/RT + \ln A = \ln (U/L) \]

where \( k_{\text{cat}} \) is the constant of the rate of the enzyme-catalyzed reaction; \( E_a \) is the activation energy (kJ/mol); R is the gas constant (8.31 X 10^{-3} kJ/mol X degree); T is the absolute temperature (K); and A is the collision factor. Under the test conditions used (constant amount of enzyme, totally saturated with substrate) \( k_{\text{cat}} \) can be replaced by U/L. The collision factor is assumed to be constant between 293 and 313 K.

Assay procedure: To assess the dependence of the enzyme activity on temperature, we determined CK activity at various temperatures, ranging from 19 to 54 °C. Except for temperature, all other assay conditions remained unchanged. CK activities of the normal sera were determined by the above-mentioned method for total CK activity. In all other cases we used the immuno inhibition test, to avoid interferences by normal-size CK-MM. After \( \ln (U/L) \) is plotted vs 1/T, the slope \( b \) of the linear part of the curve (Figure 2) can easily be determined by regression analysis, and the apparent activation energies are calculated by the following equations:

\[ -E_a/RT = b/T \] and \( E_a = -8.31 \times 10^{-3} \times b \) (kJ/mol)

For routine purposes the activation energies of macro CKs can be calculated after determining the enzyme activity of the sample with use of the immuno inhibition test at 25, 30, and 37 °C. If interferences by CK-MB or CK-BB are possible, activation energies should be determined only after heat inactivation of the thermolabile B subunits.

Results

Macro CK can be shown to be present in human sera by exclusion chromatography. With the method described here, it is possible to detect macro CK activities as low as about 10 U/L (25 °C), and to differentiate from CK isoenzymes...
of normal size (Figure 1). However, the two types of macro CK cannot be discriminated from one another in this way, because macro CK usually shows an $M_r$ of about 300 000 (1). Moreover, some sera containing macro CK type 2 show activities for components with $M_r$ exceeding 700 000 (Figure 1, upper part, fractions 15–17) and (or) measurable amounts of free, normal-size CK-BB.

Figure 2 illustrates the dependence of enzyme activity on temperature of the assay. The relationship between ln(U/L) and $1/T$ is linear between 25 and 34 °C for all creatine kinases we investigated. The mean apparent activation energies shown in Table 1 were calculated by using only data pairs from the linear part of the curves. For routine purposes, fewer analyses than this need be done: three determinations—at 25, 30, and 37 °C—suffice to recognize the presence of macro CK type 2. At 37 °C we already see a slight deviation from the straight line of the Arrhenius plot towards lower activities; therefore the activation energies calculated from only these three data pairs are about 10–20% too low. Nevertheless, a good separation into two groups was achieved for the 56 sera containing macro CK (Figure 3).

On ion-exchange chromatography as described, macro CK type 2 of human serum is eluted at an NaCl concentration of about 100 mmol/L (Figure 4). It co-elutes with the high-molecular-mass form (Figure 1, lower part) of mitochondrial

![Diagram](image)

**Table 1. Activation Energies of Creatine Kinases**

<table>
<thead>
<tr>
<th>Patient Rud.M. (Macro creatine kinase type 1, IgG-linked CK-BB)</th>
<th>Activation energy (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK-BB</td>
<td>53 ($n = 4, r = 0.9963$)</td>
</tr>
<tr>
<td>Patient Fot. P. (Macro creatine kinase type 2)</td>
<td></td>
</tr>
<tr>
<td>CK_{mit} (heart)</td>
<td>142 ($n = 6, r = 0.9971$)</td>
</tr>
<tr>
<td>CK_{mit} (skeletal muscle)</td>
<td>142 ($n = 4, r = 0.9998$)</td>
</tr>
<tr>
<td>CK_{mit} (skeletal muscle)</td>
<td>130 ($n = 3, r = 0.9746$)</td>
</tr>
</tbody>
</table>

* Number of data pairs ($n$) of the linear part of the curve used for to calculate the slope and regression coefficient ($r$) are shown in parentheses.
CK from human heart is markedly influenced by the proportion of CK_mak, that is in the macromolecular form. We find a decrease of stability if the proportion of the macro form is decreased from 75 to 55%: at the more alkaline pH values, the CK activity is zero after storage for only 3 h at 37 °C.

Discussion

In patients suspected of having an acute myocardial infarction, laboratory diagnosis may be complicated by atypical CK isoenzyme patterns during electrophoresis and ion-exchange chromatography or by an unusually high apparent CK-MB/total-CK ratio in the immunoinhibition test. The usual reason for these problems is that adenylate kinase, CK-BB, and(or) macro creatine kinase are present in such sera. Adenylate kinase is easily detected if the usual tests for the determination of the CK activity are performed without adding the substrate creatine phosphate. The presence of macro CK can be reliably demonstrated by exclusion chromatography. Previous results of our ongoing study on the prevalence of macro CK indicate that—regardless of their diseases—about 0.5% of all patients admitted to our hospital show both above-normal values for total CK and the presence of macro CK in their sera. The patients with macro CK type 1 are generally elderly women with various diseases; those with macro CK type 2 are regularly severely ill patients, often, but not invariably, patients with malignant neoplasms.

If the presence of macro CK is shown by exclusion chromatography, the two types of macro CK can then be further differentiated by determining activation energies. All of our patients' sera that contained macro CK and showed an activation energy <70 kJ/mol proved to be immunoglobulin-linked CK-BB, whereas all sera with activation energies >80 kJ/mol were classified as macro CK type 2, a macro CK not formed by IgG-linked CK isoenzymes (2). Some of these macro CK type 2 sera also contained free CK-BB of normal size. Determination of activation energies will be biased by the presence of normal-size CK-BB, but this is avoided by inactivating the thermolabile CK-B subunits as described at elevated temperature before the activation energy is measured. Then only thermostable CK-M subunits and macro CK remain active, and the residual catalytic activity is determined by use of the immunoinhibition test in which all CK-M subunits are inhibited and therefore possible interferences by these CK-M subunits are circumvented. In the case of sera
with high concentrations of macro CK, determination of the activation energy presents no problems, but in those cases where the macro CK activity is only 10 U/L (immunoinhibition test, 25 °C) the imprecision intrinsic to the measurement of the enzyme activity may cause erroneous conclusions. In such sera, the enzyme activity should be measured in duplicate, and the sample volume may be increased. The occurrence of both macro CK or "atypical" CK and CK-BB in the same sample has been reported (11-15). In contrast to Urdal et al. (15, patient no. 5), we have never seen both macro CK-BB and free CK-BB in the same sample during electrophoresis.

If the immunoinhibition test is used to determine CK-MB and exclusion chromatography is not available, the heat-inactivation test and the determination of the activation energies should be combined and used in a two-step decision tree. This combination may help to differentiate CK-MB activities, as determined by immunoinhibition, that amount to >25% of an above-normal total CK activity (Figure 6). The results will be unequivocal in all cases showing considerable amounts of macro CK type 1 or type 2 and additionally only CK-MM or adenylate kinase in their sera. In those rare cases with both macro CK type 2 and high amounts of CK-BB, the heat-inactivation test only can give one part of the whole answer: depending on the decrease in CK activity (50%, more or less) one will interpret the results as either macro CK or heat-labile isoenzyme, and therefore will only recognize the main component of both unusual CK activities in this serum. Full information about the CK activities in this sample can only be acquired by using more efficient—and therefore time consuming—techniques such as chromatography and electrophoresis with high resolution and sensitivity. Nevertheless, a second sample from patients suspected of myocardial infarction should be collected during the first 20 h after onset of acute symptoms, in order to observe whether enzyme activity is increasing, peaking, or decreasing (16).

During our studies on CK isoenzyme electrophoresis most of the atypical bands co-migrating with CK-MM and all bands migrating cathodally to CK-MM proved to be macro CK type 2. Earlier reports have dealt with such cathodal bands of CK (8, 11-14, 17-19), but the interpretations of the results are contradictory. Reports of cathodally migrating CK activities mainly referred to the similar behavior of CKmit from human tissues during electrophoresis, and this is why the authors (13, 18, 19) also classified the CK activities in human sera migrating cathodally to CK-MM as CKmit. Whereas Yyu et al. (12) reported their cathodally migrating CK activity to be a macro CK consisting of the isoenzyme CK-MM bound to IgA, Heinz et al. (13) classified their cathodally migrating macro CK as of mitochondrial origin.

Thus we would do well to point out the similar properties of macro CK type 2 found in patients' sera and CKmit isolated from human tissues:

- Both macro CK type 2 and freshly prepared CKmit from heart show greater relative molecular masses than the normal dimers of CK isoenzymes. Only after further purification steps and the use of 2-mercaptoethanol is CKmit converted to a low-molecular-mass CK similar to the usual CK isoenzymes.
- The activation energies of CKmit from human heart and skeletal muscle are in the same high range as the activation energy of macro CK type 2. We have never seen such high values for normal CK isoenzymes or macro CK type 1.
- The stability of macro CK type 2 and of IgG-linked CK-BB with respect to heat inactivation and shifts in pH are comparable. Of the CK activities that cannot be inhibited by anti-M antibodies, only mitochondrial CK from human heart in its high-molecular-mass form showed a comparable stability. This stability is markedly influenced by the proportion of CKmit that is of normal size, an increase in this was associated with a decreased stability over the whole range of pH tested, although the samples still contained 2-mercaptoethanol, which usually protects CK from inactivation. It is as yet unknown what tissues and compartments of the cells release macro CK type 2 into the bloodstream during severe illness. We do not presume that the macro CK type 2 in the sera of our patients had its origin in the heart muscle of which we used the CKmit for comparison. Therefore the moderate differences in stability between macro CK type 2 and CKmit at alkaline pH values may be due to the respective preparation techniques, or the release of enzyme into the blood, or to different characteristics of CK from various organs (or, as in some cases, tumors).
- On ion-exchange chromatography, macro CK type 2 and CKmit in its macromolecular form co-elute at the same ionic strength in the linear gradient, and are discrete from both CK-MM and CK-BB.

---

**Fig. 6. Decision sequence for listing of sera with high total CKI and a ratio CK-MB/CK (Immunoinhibition) exceeding 0.25.**

Heat-inactivation test and determination of activation energies have to be determined by the immunoinhibition test. Only the, in our experience, most probable constellations are mentioned.
• Our investigations (20) on the Michaelis constants of macro CK type 2 and $K_{\text{mik}}$ revealed low values of only 0.5 and 0.7 mmol of creatine phosphate per liter, the lowest values for all CK isoenzymes, whereas the $K_w$ values for CK-BB and macro CK type 1 are 1.1 and 1.4 mmol/L of substrate at 30 °C.

The coincidence of circulating macro CK and a possible myocardial infarction creates troubles with all the methods usually applied for determination of CK-MB. To show how these problems may be overcome without performing time-consuming and expensive analyses, we have presented two tests, which in combination allow one to find the exact interpretation with a high probability. Therefore we suggest the following procedure, which requires no special equipment and can be carried out easily and at any time, 24 h a day:

• take at least two samples during the first 20 h after onset of acute symptoms; assay CK-MB by immunoinhibition if the total-CK activity exceeds the upper limit of normal;
• test the sera with a high proportion (≥25%) of CK-MB as compared to total CK for heat-stable macro CK; and
• determine the activation energy for a further differentiation of macro CK.

References