Heterogeneity of Creatine Kinase Isoenzyme MM in Serum in Myocardial Infarction: Interconversion of the "Normal" and "Abnormal" Sub-Bands by Glutathione

John Williams,1 Katherine M. Williams,2 and Thomas Marshall3

We used isoelectric focusing (IEF) in polyacrylamide gels to investigate the effects of glutathione on the sub-bands of serum creatine kinase (CK; EC 2.7.3.2) isoenzyme MM in acute myocardial infarction. The intensity of the "normal" sub-bands c (pl 7.25), e (pl 6.85), and g (pl 6.50) increased, and that of the "normal" sub-bands 1 (pl 6.91), 2 (pl 6.65), and 3 (pl 6.35) decreased, following serum incubation with reduced glutathione (GSH; final concentration 1.25 mmol/L). Further incubation with oxidized glutathione (GSSG; final concentration 5 mmol/L) reversed this change and restored the original pattern, whereas GSSG at 7.5 mmol/L caused sub-bands c, e, and g to disappear and sub-bands 1, 2, and 3 to be enhanced. Sequential incubation of serum with 2.5 mmol of GSSG and 7.5 mmol of GSH per liter produced the opposite sequence of events; i.e., the "abnormal" sub-bands disappeared then reappeared (and GSH at 10 mmol/L enhanced their reappearance). At higher concentrations, glutathione (GSH or GSSG) impaired the detection of the CK-MM sub-bands after IEF, an effect that was "quenched" by heat-inactivated serum of low CK activity. Likewise, the intensity of tissue CK-MM (corresponding to myocardium extracted into 100 mmol/L Tris HCl buffer, pH 7.4) was greatly enhanced by adding heat-inactivated serum to the tissue extract before IEF. We discuss the significance of these findings for the diagnosis of myocardial infarction.

Additional Keyphrases: CK-MM isoforms • variation, source of isoelectric focusing

Cytoplasmic creatine kinase (CK; ATP-creatine N-phosphotransferase, EC 2.7.3.2), a dimer of two subunits, M and B, exists as three isoenzymes: CK-MM, CK-BB, and CK-MB (1,2). The presence of CK-MM in serum indicates myocardial damage (3,4), as in acute myocardial infarction (AMI), but the CK-MM in serum is also increased in AMI and displays "abnormal" sub-bands on isoelectric focusing (IEF) (5). We have recently investigated the heterogeneity of CK-MM isoforms in serum after AMI and detected three "normal" sub-bands (CK-MM 1–3) and up to 11 "abnormal" sub-bands (CK-MM a–k) (6, 7). The "abnormal" sub-bands were detected only in AMI (7). The response of the patterns to 2-mercaptoethanol indicated conversion of the "normal" to "abnormal" sub-bands, i.e., CK-MM 1 to b; c; CK-MM 2 to d; e; and CK-MM 3 to f, g (7). In the present study we demonstrate reversible interconversion of "normal" and "abnormal" sub-bands in the presence of glutathione.

Materials and Methods

Reagents. Acrylamide and N,N'-methylenediacrylamide were purchased from BDH Chemicals, Poole, Dorset, U.K. Glutathione (GSH and GSSG, Grade II) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. All solutions were freshly prepared with de-ionized water ("Elgastat" purification system).

Apparatus. Flat-bed IEF with pH 5–8 "Pharmalyte" (Pharmacia, Uppsala, Sweden) was performed in a Model 600 electrophoresis system (Shandon Southern Products Ltd., Runcorn, Cheshire, U.K.) with a power supply unit from LKB Instruments (Bromma, Sweden).

Serum samples. Venous blood was processed within 2 h of collection from patients admitted to the Coronary Care Unit, Sligo General Hospital. Total serum CK was measured with a centrifugal analyzer (Cobas Bio; Hoffmann-La Roche & Co. Ltd., Basel, Switzerland), and CK isoenzymes were monitored with a Corning electrophoresis system. Serum aliquots, stabilized with potassium EDTA (5 mmol/L), were stored in liquid nitrogen before IEF of the CK-MM sub-bands (7).

Tissue samples. Myocardial slices were obtained at autopsy 24–48 h after death and stored frozen. The tissue was rinsed in water and 1.3-g aliquots were diced and homogenized in 10 mL of heat-inactivated (60 °C, 30 min) serum with low CK content (from apparently healthy subjects) or Tris HCl buffer (100 mmol/L, pH 7.4). The homogenates were sonicated for 10 min at 4 °C, centrifuged at 2000 × g for 10 min and the supernatants diluted with isotonic saline (NaCl, 150 mmol/L) to give a final CK activity of ≈1000 U/L.

Incubation of serum. Fresh AMI serum (50 μL, without EDTA) was mixed with an equal volume of Tris HCl buffer (100 mmol/L, pH 7.4) containing glutathione (5–100 mmol/L as GSH or 2–50 mmol/L as GSSG) and incubated at room temperature for up to 30 min. In some cases, the incubation mixture was modified after 15 min by adding 100 μL of buffered glutathione (5–40 mmol/L) of the opposite oxidation/reduction state, or 50 μL of heat-inactivated serum of low CK content. In other cases, serum from AMI patients but of low CK content (collected <6 h after infarction) was incubated for 15 min with an equal volume of the heat-inactivated serum. In control incubations, Tris HCl buffer (100 mmol/L, pH 7.4) was substituted for the glutathione and serum additions. The effects of incubation were evaluated by IEF of the CK-MM sub-bands. Values were corrected and sample volumes adjusted to compensate for

1 Biochemistry Department, General Hospital, Sligo, Ireland.
2 School of Pharmaceutical & Chemical Sciences, Edinburgh Building, Sunderland Polytechnic, Sunderland SR1 3SD, U.K.
3 Address correspondence to this author.
4 Nonstandard abbreviations: CK-MM, -MB, -BB, isoenzymes of creatine kinase; AMI, acute myocardial infarction; IEF, isoelectric focusing; pl, isoelectric point; GSH, reduced glutathione; and GSSG, oxidized glutathione.

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Incubations of tissue extracts. Aliquots (100 μL) of myocardium extracted into Tris HCl (as described above) were mixed with an equal volume of Tris HCl buffer or heat-inactivated serum (or a mixture consisting of variable proportions of each) and incubated at room temperature for 15 min before IEF.

Isoelectric focusing. Polyacrylamide gels (130 × 110 × 0.48 mm; T = 5%; C = 3%) containing, per liter, 20 g of Pharmalyte pH 5–8 and 200 g of glycerol were polymerized with N,N,N',N'-tetramethylethylenediamine (0.3 mL/L) and ammonium persulfate (0.4 mg/L). They were focused at 200 V (10 W) for 10 min, then at 1400 V (10 W) for 20 min, and 1400 V (15 W) for 2 h, with 0.5 mol/L sodium hydroxide solution as catholyte and 40 mmol/L DL-glutamic acid solution as anolyte (6, 7). We viewed the fluorescence of CK-MM under ultraviolet light after incubation (37°C, 20 min) with commercial CK reagent ("CK-Nac"; BCL, Lewes, Sussex, U.K.) and drying of the gel (60°C, 20 min) (6, 7).

Results

Figure 1 shows a comparison of the effects of GSH and GSSG on the CK-MM sub-band distribution in serum. The intensity of the "abnormal" sub-bands c (pI 7.25), e (pI 6.85), and g (pI 6.50) increased, and that of the "normal" sub-bands 1 (pI 6.91), 2 (pI 6.65), and 3 (pI 6.35) decreased, when serum was incubated with GSH, 1.25 mmol/L, before IEF (Figure 1, tracks 1 and 2). Conversely, the "abnormal" sub-bands disappeared and the "normal" sub-bands were enhanced after incubation with GSSG, 2.5 mmol/L (Figure 1, tracks 5 and 6). Thus, GSH promoted the "abnormal" distribution pattern and GSSG promoted "normalization." Both effects were reversible to an extent dictated by the glutathione concentration. Sequential incubation with GSSG, 5 mmol/L, reversed the GSH-induced change (Figure 1, track 3), whereas GSSG, 7.5 mmol/L, caused sub-bands c, e, and g to disappear and sub-bands 1, 2, and 3 to be enhanced (Figure 1, track 4). Conversely, sequential incubation with GSH, 7.5 mmol/L, reversed the GSSG-induced change (Figure 1, track 7), and GSH at 10 mmol/L further enhanced sub-bands c, e, and g and diminished sub-bands 1, 2, and 3 (Figure 1, track 8). The final concentrations of GSH and GSSG required to produce these effects varied from serum to serum, but only slightly.

Incubation of serum from AMI patients with higher concentrations of GSSG or GSH impaired detection of the CK-MM sub-bands to an extent dictated by the glutathione concentration (Figure 2). Thus, GSSG at 15 mmol/L reduced the intensity of all of the sub-bands; at 20 mmol/L, the sub-bands were undetectable (Figure 2A, tracks 5 and 7, respectively). Similar effects were observed with GSH at 25 and 37.5 mmol/L (Figure 2B, tracks 7 and 9). In each case adding heat-inactivated serum of low CK content to these incubation mixtures before IEF restored detectable CK-MM activity to an intensity equivalent to that observed in the presence of lower amounts of glutathione (Figure 2A, tracks 1–4; Figure 2B, tracks 3–6). However, added serum did not enhance CK-MM detection at low concentrations of GSSG/GSH (Figure 2A, tracks 2 and 4; Figure 2B, tracks 4 and 6) or reverse the corresponding glutathione-induced changes in sub-band distribution (Figure 2B, tracks 1–4). The "quenching" effect of serum (Figure 2A, tracks 6, 8, and 10; Figure 2B, tracks 8 and 10) was not a dilution effect, because substitution of buffer (100 mmol/L Tris HCl buffer, pH 7.4) for heat-inactivated serum failed to restore CK-MM activity.

Enhanced intensity of CK-MM was also detected when tissue CK (isolated by extraction of myocardium into 100 mmol/L Tris HCl buffer, pH 7.4) was incubated with increasing proportions of heat-inactivated serum of low CK content (final volume proportions 0.5–50%) before IEF (Figure 3). The tissue CK-MM was often not detected in the

Fig. 1. Glutathione-induced interconversion of the "normal" and "abnormal" sub-bands of serum CK-MM

AMI serum (total CK activity 2950 U/L) was incubated (before IEF) with either (a) Tris HCl buffer (tracks 1, 2), (b) GSH (final concentration 1.25 mmol/L, tracks 2–4) followed by GSSG (5 and 7.5 mmol/L, tracks 3 and 4, respectively), or (c) GSSG (2.5 mmol/L, tracks 6–8) followed by GSH (7.5 and 10 mmol/L, tracks 7 and 8, respectively). The anodal activity (tracks 3–8) corresponds to sub-bands i–k (6, 7).
absence of serum (Figure 3, track 1), but increasing amounts of serum progressively enhanced the detection without changing the sub-band distribution of the pattern (Figure 3).

To assess whether similar inhibitory effects could be associated with measurement of total serum CK, we added heat-inactivated serum to serum of low CK content collected within 6 h of infarction from AMI patients. The added serum was without effect (Table 1), irrespective of whether the sample was fresh or frozen (with or without EDTA).

**Discussion**

The present study demonstrates reversible interconversion of the "normal" and "abnormal" sub-bands of serum CK-MM in response to glutathione incubation before IEF. This is clinically significant because the increase of total serum CK 3–12 h after infarction is characterized by prominence of the "abnormal" CK-MM sub-bands c, e, and γ (and occasionally a, b, d, f, and h) (7); indeed, Chapelle 8,9 has proposed that cathodal sub-band c represents a tissue CK precursor indicative of myocardial damage after AMI. Our results show that the "abnormal" cathodal sub-band c is enhanced, in vitro, by GSH and diminished by GSSG as a consequence of sub-band interconversion. The mechanism is unknown; however, other isoenzyme patterns are known to be modified during storage by sulphydryl group interaction with glutathione (Enz-SH + GSSG \( \rightleftarrows \) Enz-S-S-G + GSH) (10). This modification causes an anodal shift in isoenzyme distribution, consistent with the effect of GSSG on the "abnormal" sub-bands, but there is no published evidence of CK–glutathione mixed disulfides.

It remains unclear whether our observations, based on in vitro incubation of serum with millimolar amounts of glutathione, are relevant to CK-MM and its isofoms in vivo. The intracellular concentration of GSH is in the millimolar range (11), and GSSG is readily formed from GSH by oxidation and interaction with free radicals (11). Thus, on its release at the site of infarction, CK may be exposed to glutathione at the experimental concentrations we used. However, because of subsequent dilution in blood, the circulating concentrations of glutathione are only micromolar (11,12).

The adverse effect of glutathione on the analysis of CK-MM (Figure 2) may involve scavenging of NADPH (the detection product) by GSSG due to glutathione reductase activity in serum (13). Added serum could "quench" this by forming mixed disulfides with GSSG. These effects are unlikely to be of clinical significance because circulating GSSG is only in the micromolar range (11) and added serum fails to enhance total CK values in serum collected from patients immediately after AMI. However, the impaired detection of CK-MM and the "quenching" effect of added serum may be important in tissue CK-MM studies; i.e., GSSG accumulates in such extracts on storage (10) and the addition of serum greatly enhances detection of the sub-band patterns (Figure 3).

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**Table 1. Effect of Heat-Inactivated Serum on Total CK in Serum Collected from AMI Patients <8 h after Infarction**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Original value</th>
<th>Incubated (37 °C, 15 min) value</th>
<th>+ Serum</th>
<th>+ Buffer (control)</th>
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* Samples 1 and 2 were fresh and did not contain EDTA. Samples 3 and 4 contained EDTA (5 mmol/L) and were stored in liquid nitrogen until assay. All values are corrected for dilution effects. n = 1 each.

**References**