CCA 1247

MITOCHONDRIAL CK (EC 2.7.3.2) IN THE HUMAN HEART

R.A. WEVERS, M.W.F.J. MUL-STEINBUSCH and J.B.J. SOONS *

Department of Clinical Chemistry, Faculty of Pharmacy, Utrecht State University, St. Antonius Hospital, Jan van Scorelstraat 2, 3583 CP, Utrecht (The Netherlands)

Summary

CK_m, a mitochondrial form of CK, can be isolated from human cardiac muscle. It occurs in two forms, separable on the basis of the difference in net charge. Both molecules have a dimeric structure and consist of two identical subunits. Upon incubation in a human serum, the CK isozyme with the faster cathodal mobility is rapidly converted into the slower form, which has an isoelectric point of 6.94. This makes it impossible to separate this slow CK_M form from CK MM (isoelectric point: 6.86) by means of any method that separates on a basis of differences in net charge. Furthermore the two molecules have an almost identical molecular weight. The only difference between the two molecules we have been able to detect is the different behaviour against anti-M antibodies.

Introduction

It was Jacobs [1] who discovered in 1964 the existence of a mitochondrial variant of the CK isozymes. Later on Sobel [2] suggested a dimeric structure for mitochondrial CK (=CK_m), merely on the basis of the molecular weight. Scholte [3] discovered the intermembrane position of CK_m and one year later, in 1974, Saks [4] made suggestions about its functional role. In the same publication he mentioned the existence of two mitochondrial CK forms. Recently, Hall [5] found that these two forms could be interconverted and showed this to be consistent with a reversible oxidation of sulphhydryl groups.

Very little research has been done on human mitochondrial CK. This paper describes the isolation of small amounts of CK_m from human heart muscle. Hybridization experiments were carried out to prove the dimeric structure of CK_m. Furthermore it became clear why no one has been able to detect CK_m in

---

* To whom correspondence should be addressed.

human serum until now. The implications of a possible occurrence of CK_m in human serum for the quantitative MB-determination are discussed.

Materials and methods

Extracts
All extracts were made from human tissues obtained within 24 h post mortem.

CK BB
An extract containing CK BB was obtained by mincing and homogenizing the medulla of the cerebrum for 3 min in 1 : 5 (w/v) 0.01 mol/l Tris/HCl (pH 7.4) with a glass-teflon Potter-Elvehjem homogenizer. The extract was freed from other CK forms possibly present, using anion exchange chromatography with DEAE-cellulose as discussed by Nealon [6].

Isolation of mitochondria
Mitochondria from human heart muscle were isolated by differential centrifugation. Tissue was homogenized in 4 volumes of 0.01 mol/l Tris/HCl (pH 7.4) with a glass-teflon Potter-Elvehjem homogenizer. In this highly hypotonic medium, the CK_m that is attached to the inner mitochondrial membrane, remains associated with the membrane pellet. Intermembrane markers such as adenylate kinase appear in the supernatant [7]. A brief and gentle homogenization procedure turned out to be essential. The homogenate was centrifuged for 10 min at $1700 \times g$. The mitochondria were removed from the resulting supernatant at $12\,000 \times g$ for 10 min. The mitochondrial pellet was washed in the Tris/HCl medium until the CK activity in the supernatant was negligible. Marker enzymes for the cytosol (LDH) and for mitochondria (GLDH) were used to check the differential centrifugation procedure.

CK_m extraction from mitochondria
CK_m was extracted from the mitochondrial pellet with a 0.05 mol/l Na_2HPo_4/NaH_2Po_4 buffer (pH 7.4) containing 0.2% bovine serum albumin and 0.005 mol/l glutathione [4,7]. The mitochondria were incubated for 30 min at 4°C. Ultracentrifugation in a Beckman Spinco J.L. 26 at $100\,000 \times g$ for 30 min yielded a clear supernatant containing CK_m.

Electrophoresis
Agarose electrophoresis, as described earlier [8], was used. The nitro blue tetrazolium staining technique has also been described before [8]. By omitting creatine phosphate from the incubation mixture it was proved that all the bands referred to in this paper are really CK bands.

Hybridization experiment
The hybridization experiment was performed according to Levy and Lum [9]. CK isozymes possibly contaminating the mitochondrial CK extract or the CK BB extract were removed by anion exchange chromatography [6] before the hybridization experiment. Subunit dissociation was achieved with 8 mol/l

urea for 3 h at 24°C. During this period the activity of the hybridization mixture decreased to 10% of the original activity. Subsequently the mixture was dialysed against 2000 ml 10 mmol/l Tris/HCl buffer (pH 8.0) containing 0.1 mol/l 2-mercaptoethanol (Calbiochem no. 4449). The recovery of the original activity was 65%.

Incubation of CK_m in serum

The mitochondrial extract was incubated in sera low in CK activity. The activity of these sera was below the detection limit of the electrophoresis procedure under the conditions used. Various incubation temperatures and times were used.

Density gradient focusing

Isoelectric focusing experiments were carried out as described before [10].

Enzyme activity determination

All enzyme determinations were performed at 35°C on a LKB reaction rate analyser. CK was measured using *N*-acetyl cysteine as an activator (Baker reagents: nos. 3171/3/7). For LDH the recommendations of the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology were followed (Baker reagents: nos. 3163/4). GLDH was assayed using the method described by Ellis et al. [11] (Baker reagents: nos. 3121/3118/2412).

Inhibition with anti-M inhibiting antibodies

Experiments with inhibiting anti-M antibodies from Merck Darmstadt (nos. 14326/7) were carried out at 24°C and were further dealt with in accordance with the directions for use given by Merck.

Precipitation with anti-M precipitating antibodies

Samples that were measured in the precipitation test were diluted with 0.05 mol/l NaCl in 0.05 mol/l Tris/HCl buffer (pH 7.5). The CK activity of the dilutions was approximately 70 I.U./l. The precipitation test was carried out according to the method of Würtzberg [12], although some modifications were necessary in order to regain an acceptable percentage of the original activity of an antiserum blank after the overnight incubation at 4°C. Instead of the borate buffer a 0.05 mol/l Tris/HCl buffer (0.05 mol/l NaCl) was used. Furthermore the NAC concentration (Baker no. 0460) in the PEG-NAC solution was reduced to 0.130 g NAC in 100 ml buffer. The antiserum used is anti-creatine kinase MM (von Hammel) (Merck no. 11642).

Results

CK_m isolation

Table I shows the CK activities as well as the activity of the marker enzymes during the various stages of CK_m isolation. For this experiment 23 g of heart tissue were homogenized. The Potter-Elvehjem homogenizer forms a critical step in the procedure. It should be used briefly and gently in order to avoid disruption of the mitochondrial membrane.

TABLE I

	CK (units)	LDH (units)	GLDH (units)
12 000 g supernatant	4720	10 555	117
wash 1	129	359	2
wash 2	12	10	1
wash 3	3	0	0
wash 4	1	0	0
100 000 g supernatant	60	0	4

CK_m heterogeneity

The electrophoretic pattern of a mitochondrial extract indicates the presence of two CK isozymes (Fig. 1). This is consistent with the work of Hall [5] on beef heart mitochondria. We also experienced the influence of reducing substances on CK_m. 2-Mercaptoethanol, for instance, clearly converts the isozyme with the faster cathodal mobility into the slower form. Consequently we call these two forms $CK_m(ox)$ and $CK_m(red)$ respectively. We have never succeeded, however, in showing the reversible character of the conversion. The use of several weak and strong oxidizing substances only resulted in a loss of activity and not in the conversion from $CK_m(red)$ to $CK_m(ox)$.

Inhibiting antibodies

Fig. 2 shows that the $CK_m(red)$ isozyme has almost the same mobility as CK MM_3 [10]. In order to prove the absence of CK MM in the mitochondrial extract, inhibiting anti-M antibodies were used. Table II shows that the two CK_m forms are not inhibited by anti-M.

Isoelectric point

The isoelectric point of $CK_m(red)$ was determined by density gradient focusing. In eight experiments a mean pI of 6.94 ± 0.04 was measured. The activity
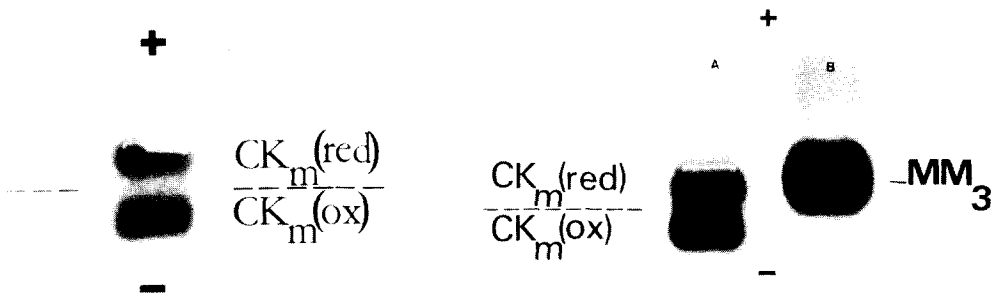

Fig. 1. CK electrophoretogram. Agarose electrophoresis, 50 mmol/l sodium barbital buffer, pH 8.0, 85 V, 45 min. Mitochondrial extract.

Fig. 2. CK electrophoretogram. Agarose electrophoresis, 50 mmol/l sodium barbital buffer, pH 8.0, 85 V, 45 min. A, mitochondrial extract; B, CK MM_3.

TABLE II

	Delta E/min before inhibition	Delta E/min after inhibition
CK MM$_3$	71 mEx	1 mEx
CK$_m$ (red)	45	45
CK$_m$ (red + ox)	52	53

in the CK$_m$(red) peak could not be inhibited with anti-M antibodies. In a series of five experiments the pI of CK MM$_3$ turned out to be 6.86 ± 0.03. As a consequence of this small difference in pI, it is impossible to separate CK$_m$(red) and CK MM$_3$ electrophoretically. The overall recovery of CK$_m$ in the isoelectric focusing experiments ranged from 72—82%. It was not possible to detect a separate peak of activity for CK$_m$(ox) in the column eluates. Apparently the CK$_m$(ox) was reduced during the isoelectric focusing. Fig. 3 shows the elution pattern of the density gradient.

Incubation of CK$_m$ in serum

When CK$_m$(red + ox) was incubated at 37°C in a serum low in CK activity, the CK$_m$(ox) form was converted into the reduced form within three hours (Fig. 4). Because of this it would be practically impossible to detect CK$_m$ after a myocardial infarction by an electrophoretic technique. Upon incubation of
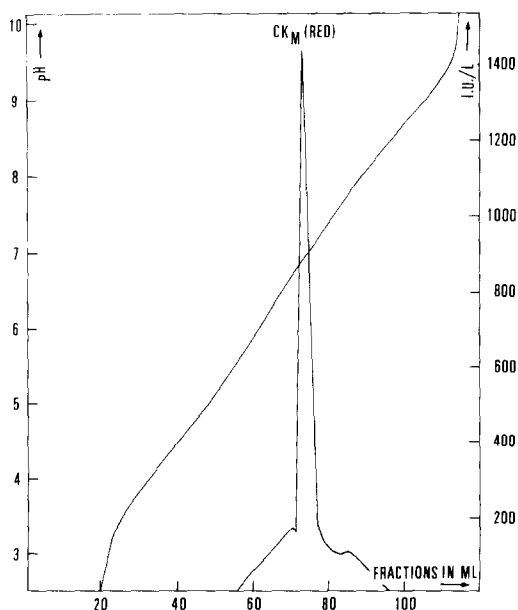

Fig. 3. Isoelectric focusing of a mitochondrial extract in a sucrose density gradient. Ampholines 6 ml, pH range 3.5—10; 65 h, 2°C. Current, voltage and power limited at 5 mA, 700 V and 1 W, respectively. 120 fraction of 1 ml.
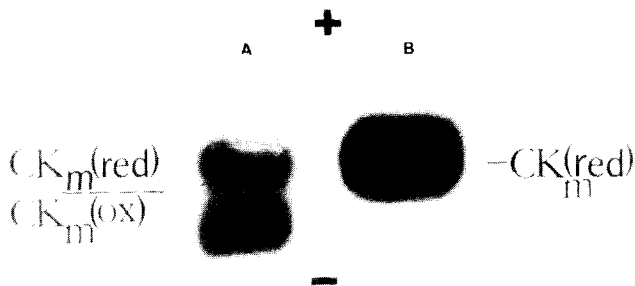
108

Fig. 4. CK electrophoretogram. Agarose electrophoresis, 50 mmol/l sodium barbital buffer, pH 8.0, 85 V, 45 min. A, mitochondrial extract; B, mitochondrial extract after an incubation of 3 h at 37°C in a serum low in CK activity.

$CK_m(ox)$ in the Na_2HPo_4/NaH_2Po_4 extraction buffer, $CK_m(ox)$ was completely converted into $CK_m(red)$ within 25 days at 4°C.

Hybridization

A CK BB and a CK_m extract were freed from CK MB and other possibly present contaminating CK forms by anion exchange chromatography. The activity of a mixture of 2 ml CK BB (3500 I.U./l) and 2 ml $CK_m(red + ox)$ (3000 I.U./l) decreased to 10% of the original activity after 3 h at a concentration of 8 mol/l urea. After the subunit recombination, an artificial hybrid CK form was clearly detectable (Fig. 5). The hybrid moved slightly less toward the anode than CK MB. Apart from this hybrid form, CK BB and $CK_m(red)$ were also detectable. Probably because of the presence of 2-mercaptoethanol the $CK_m(ox)$ form could not be detected any more. This hybridization experiment
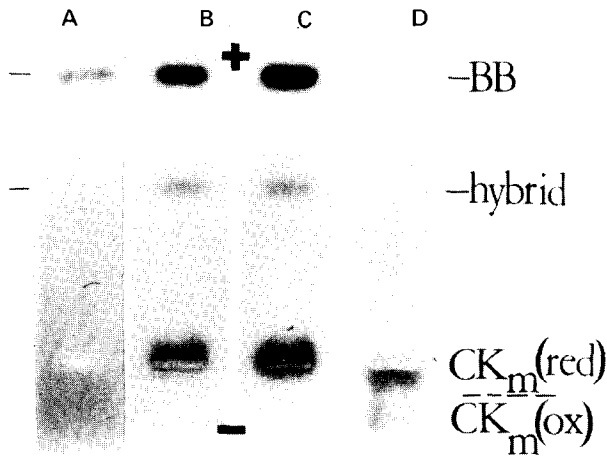

Fig. 5. CK electrophoretogram. Agarose electrophoresis, 50 mmol/l sodium barbital buffer pH 8.0, 85 V, 45 min. A, CK BB used for hybridization; B = C, Result after subunit recombination; D, CK_m)red + ox) used for hybridization.
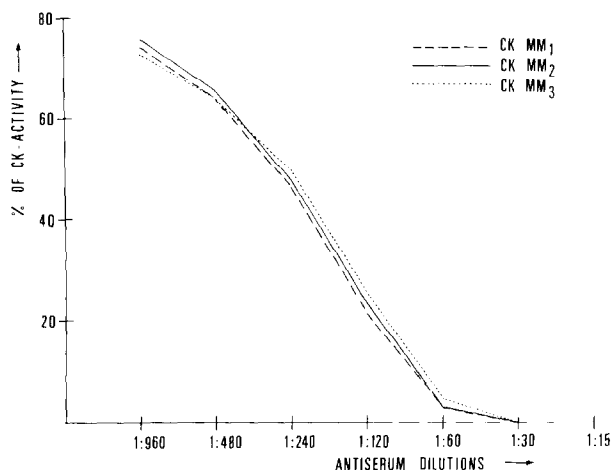
Fig. 6. Immuno-precipitation of creatine kinase MM isozymes. The residual activity after the incubation with antiserum is plotted as a percentage of the activity of an antiserum blank after overnight incubation.

proves the dimeric structure already proposed by Sobel [2] on a basis of the molecular weight. It also proves the presence of two similar subunits in the CK_m molecule.

Precipitating anti-M antibodies

Three MM isozymes were isolated from a human serum with increased CK activity, using density gradient focusing as described in a previous article [10]. The mean activity of the antiserum blank after the overnight incubation was 98% of the activity prior to the incubation. The three isozymes show an identical behaviour with dilutions of precipitating anti-M antibodies (Fig. 6). This experiment proves that no antigenic determinant of CK MM is involved in the modification process of the enzyme observed in serum.

Discussion

In working with human heart muscle extracts, we saw a CK isozyme with cathodic mobility. This finding was consistent with the work of Sanders [13]. The isozyme turned out to have a mitochondrial origin. The isolation of mitochondria even proved it possible to isolate two forms of CK_m. These behaved just like the two CK_m isozymes, isolated from beef heart mitochondria by Hall [5]. We therefore assume a redox conversion between the two isozymes.

Hybridization experiments proved the dimeric structure of CK_m, as already suggested by Sobel [2] on the basis of the molecular weight. The hybridization experiments also prove that the molecule consists of two similar subunits. The molecular weight of CK_m, determined by using Sephadex G-150 gel filtration, is equal to the molecular weight of the other CK isozymes [2]. This makes it impossible to separate CK_m from the other CK forms with a technique that separates on the basis of the molecular weight.

The reduced form of human CK_m has an isoelectric point of 6.94. This is slightly higher than the isoelectric point of CK MM, which is 6.86 [10]. There-

fore, it is also virtually impossible to separate $CK_m(red)$ and MM_3 with any technique that separates on the basis of differences in net charge.

Two mitochondrial forms of CK were found in human heart muscle. In skeletal muscle, however, we have only been able to detect the $CK_m(red)$ form. This could be the result of differences in internal milieu between mitochondria from heart and skeletal muscle. On the other hand this finding leaves open the possibility of different functional roles for the two isozymes.

Heart muscle contains much CK_m. Saks [4], for instance, has shown that rat heart contains more CK_m than CK MB. Therefore it is strange that no CK_m can be detected in serum after a myocardial infarction, while the presence of CK MB can be clearly shown. Still we may expect to find mitochondrial proteins in the circulation after irreversible muscle damage. Boyde [14], for example, has described the occurrence of the mitochondrial isozyme of aspartate transaminase in serum after myocardial infarction. The observation that $CK_m(ox)$ is rapidly converted into $CK_m(red)$ in serum at $37°C$ is interesting in this connection. As mentioned above, this $CK_m(red)$ has the same electrophoretic behaviour as CK MM_3, so that the presence of CK_m in serum would remain unobserved in electrophoresis. The similar behaviour of $CK_m(red)$ and CK MM in several separation techniques might be the reason that no one has been able to show the presence of CK_m in serum after a myocardial infarction. Another possible explanation is the binding of CK_m to the inner mitochondrial membrane. Jacobus [7] already showed that CK_m remains associated with the mitochondrial membrane, under circumstances where other mitochondrial proteins, like adenylate kinase, appear in the supernatant. Adenylate kinase is an intermembrane marker, which is not bound to one of the mitochondrial membranes.

The possible presence of CK_m in serum could have implications for the determination of CK MB. When column chromatography on mini-column anion exchangers [6,15] is used, CK_m will appear in the same fraction as CK MM. As a consequence, the quantity of CK MB, determined in this way, will not be influenced by the presence of CK_m. This is not the case when the inhibition method with inhibiting anti-M antibodies is used. Inhibition experiments in this paper indicate that CK_m is not inhibited by anti-M antibodies. The result will be a CK MB value which is too high. Witteveen [16] indeed described tremendous discrepancies in MB value between column chromatography and the inhibition method. These discrepancies are found for heart extracts and not for human sera. A possible destruction of mitochondria in the Ultra Turrax homogenizer can cause this finding. The suggestion [16] that the three CK MM isozymes, described elsewhere [8], are responsible for this phenomenon is not correct. MM_1, MM_2 and MM_3 show a similar behaviour with anti-M antibodies.

As long as there is no definite proof whether either of the two CK_m forms is present in the circulation after myocardial infarction, the quantitative CK MB determination with anti-M antibodies remains questionable.

References

1 Jacobs, H., Heldt, H.W. and Klingenberg, M. (1964) Biochem. Biophys. Res. Commun. 16, 516—521
2 Sobel, B.E., Shell, W.E. and Klein, M.S. (1972) J. Mol. Cell. Cardiol. 4, 367—380

3   Scholte, H.R., Weijers, P.J. and Wit-Peters, E.M. (1973) Biochim. Biophys. Acta 291, 764—773

4   Saks, V.A., Chernousova, G.B., Voronkov, Iu.I., Smirnov, V.N. and Chazov, E.I. (1974) Circ. Res. Suppl. III 34/35, 138—148

5   Hall, N., Addis, P. and DeLuca, M. (1977) Biochem. Biophys. Res. Commun. 76, 950—956

6   Nealon, D.A. and Henderson, A.R. (1975) Clin. Chem. 21, 392—397

7   Jacobus, W.E. and Lehninger, A.L. (1973) J. Biol. Chem. 248, 4803—4810

8   Wevers, R.A., Olthuis, H.P., Van Niel, J.C.C., Van Wilgenburg, M.G.M. and Soons, J.B.J. (1977) Clin. Chim. Acta 75, 377—385

9   Levy, A.L. and Lum, G. (1975) Clin. Chem. 21, 1691

10 Wevers, R.A., Wolters, R.J. and Soons, J.B.J. (1977) Clin. Chim. Acta 78, 271—276

11 Ellis, G. and Goldberg, D.M. (1972) Clin. Chem. 18, 523—527

12 Würtzberg, U., Hennrich, N., Orth, H.D. and Lang, H. (1977) J. Clin. Chem. Clin. Biochem. 15, 131—137

13 Sanders, J.L., Joung, J.I. and Rochman, H. (1976) Biochim. Biophys. Acta 295, 549—554

14 Boyde, T.R.C. (1968) Enzym. Biol. Clin. 9, 385—392

15 Mercer, D.W. (1974) Clin. Chem. 20, 36—40

16 Witteveen, S.A.C.J., Hollaar, L. and Van der Laarse, A. (1978) Clin. Chim. Acta 83, 297—301