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Characterization of isoforms of human mitochondrial creatine kinase by isoelectric focusing

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Abstract

High enzyme activity of mitochondrial creatine kinase (creatine-*N*-phosphotransferase, mCK, EC 2.7.3.2) was detected in serum from a patient with advanced carcinoma of the rectum and its isoforms were characterized by means of isoelectric focusing (IEF). Three forms of mCK, membrane-bound (p*I* 6.9–7.0), octameric (p*I* 7.0–7.9) and dimeric (p*I* 6.7, 6.8, 6.9 and 7.0), were detected in the fresh serum. These three forms of mCK were converted to five dimeric isoforms, and these were characterized as one reduced form (p*I* 7.0) and four oxidized (p*I* 6.6, 6.7, 6.8 and 6.9) forms upon treatment with urea, hydrogen peroxide or 2-mercaptoethanol (2-ME). The C-terminal of the mCKs was concluded to be a lysine residue because the mCKs treated with carboxypeptidase B migrated to positions closer to the anode than did those not treated with carboxypeptidase B. Therefore, four bands were concluded to represent one reduced-delysined isoform (p*I* 6.4) and three oxidized-delysined isoforms (p*I* 6.1, 6.2 and 6.3). The broad octameric mCK band disappeared and a narrow band focused at p*I* 6.8–6.9 appeared upon probable delysination of the mCKs. Thus, the number of lysine residues at the C-terminal of the octamer was concluded to be variable due to variable catalysis by carboxypeptidase N in the plasma. mCKs seemed to be inactivated during conversion from a membrane-bound form to dimeric oxidized-delysined forms via the octameric, dimeric reduced and oxidized forms.

Keywords: Creatine kinase; Isoenzymes

1. Introduction

The fourth isoenzyme of creatine kinase (creatine *N*-phosphotransferase, CK, EC 2.7.3.2), mitochondrial CK (mCK), was reported in 1964 [1]. Although mCK catalyzes the same reactions as the cytosolic isoenzymes, it behaves differently in some respects: the isoelectric point of mCK is strongly basic [2], its antigenicity differs from those of the native cytosolic CKs [3], and it exists in two oligomeric forms, as a dimer and as an octamer [4–6]. The octameric nature of native mCK and the formation of mCK dimers were confirmed by gel permeation chromatography, which revealed a molecular mass of 371±3 kDa for the octameric form and 79.7±0.8 kDa for the dimeric form [7]. K_m (creatine) value is higher for the octameric than for the dimeric form of mCK [8]. Thus, at low creatine concentrations, the dimer is kinetically favored for the forward direction of the reaction (phosphorylcreatine synthesis) compared with the octamer [8]; the octamer is favored at high mCK concentrations and low pH [9,10]. Equilibrium

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substrate combinations (MgADP+MgATP+creatine+phosphorylcreatine) or formation of a transition-state-analogue complex (composed of mCK, MgADP, creatine and nitrate) result in complete and relatively fast dissociation of octamers into dimers [11]. mCK is detected in sera of patients with acute myocardial infarction and malignant tumors. It is considered as being released into the circulation from severely necrotic cells, and its presence in the plasma is considered to indicate a poor prognosis [12]. In the present study, the isoforms of mCK in the serum of a patient with carcinoma of the rectum were characterized by means of isoelectric focusing (IEF). Here we present our results followed by a discussion of their clinical and biochemical implications.

2. Experimental

2.1. Materials

Materials employed here included the following: Monotest CK NAC, Monotest CK-MB and marker proteins for IEF and molecular masses (Boehringer-Mannheim, Mannheim, Germany), DEAE Sephacel, Sephadex G-200 superfine, agarose and ampholytes for IEF (Pharmacia-LKB Biotechnology, Bromma, Sweden), anti-CK-BB antiserum (Research Plus Inc., Bayonne, NJ, USA), anti-human IgG, anti-IgA and anti-IgM antisera (Dako A/S, Glostrup, Denmark), carboxypeptidase B (Sigma Chemical, St. Louis, MO, USA) and Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). Carboxypeptidase B was from porcine pancreas and was chromatographically purified, with an activity of 195 I.U./g protein. One unit was defined by the manufacturer as the amount which hydrolyzes one μmol of hippuryl-L-arginine per min at pH 7.65 at 25°C.

2.2. Specimens

Serum samples were obtained from a 58-year-old man with advanced carcinoma of the rectum (moderately differentiated adenocarcinoma) and multiple metastatic liver and pulmonary carcinomas. The patient had been treated with 5FU by continuous intravenous infusion but not with adriamycin. Total CK and non-CK-M-subunit activities in the patient's serum, obtained on different days, were 231–489 I.U./l and 205–455 I.U./l, respectively, and percent activity of non-CK-M-subunit activity against total CK activity was 80–99%. CK in the patient's serum was detected as a broad band on the cathodal side of the CK-MM position on a Cellogel membrane.

2.3. Assay methods

Serum CK and non-CK-M-subunit activities were determined with Monotest CK NAC and Monotest CK-MB, respectively, at 37°C on a Hitachi 7450 analyzer. Reference intervals of CK and non-CK-M-subunit activities were those for men, 40–240 I.U./l and 2.0–11.0 I.U./l, respectively.

IEF was carried out on supporting matrices using an IEF system (Joko, Tokyo, Japan). The agarose plate used (124×258×0.5 mm) consisted of 1% agarose and 2% ampholytes (pH range 5–9). Five μl of serum sample was applied to the agarose plate, and 0.2 mol/l citric acid and 0.5 mol/l ethanolamine were used as anode and cathode solutions, respectively. Focusing was carried out at 4°C, at 300 V for 15 min, 600 V for 30 min, 900 V for 30 min and finally 1200 V for 45 min (power: 0.8–4.8 W). The focused mCK isoforms were stained with a mixture of one pack of Monotest CK NAC, 75 mg/l of nitroblue tetrazolium an 7.5 mg/l of phenazine methosulfate at 37°C for 30 min.

2.4. Isolation of mCK and preparation of anti-mCK antibody

Human cardiac muscle was homogenized in 10 mmol/l Tris–HCl buffer, pH 7.4, and centrifuged at 1700 *g* for 10 min at 4°C, and the supernatant was further centrifuged at 12 000 *g* for 10 min [13]. The pellet was washed with Tris–HCl buffer until CK activity in the supernatant was negligible, and a mitochondrial pellet was finally obtained by centrifugation at 12 000 *g* for 10 min at 4°C. The mitochondria were suspended in 50 mmol/l phosphate buffer, pH 7.4, incubated at 4°C for 60 min and centrifuged at 105 000 *g* for 30 min at 4°C. The supernatant containing mCK was chromatographed on a DEAE Sephacel column of 0.9×15 cm that had been equilibrated with 50 mmol/l Tris–HCl buffer,

pH 7.4, containing 30 mmol/l NaCl, and the effluent was applied to a Sephadex G-200 superfine column [13]. mCK fractions eluted from the column were concentrated and approximately 5 mg of mCK was emulsified with an equal volume of complete Freund's adjuvant.

One ml of the emulsion was injected intramuscularly into the back of a rabbit and booster injections were given with adjuvant on days 7, 14 and 28. Seven days after the last booster injection, the rabbit was bled from the ear vein, and the serum containing anti-human mCK antibody was collected. Non-specific antibodies in the anti-serum were absorbed by incubation with normal human serum at 37°C for 2 h followed by incubation at 4°C for 24 h and centrifugation. CK activity and complements in the antiserum were inactivated by heating at 56°C for 30 min. The anti-mCK antiserum was confirmed to react with only mCK and not CK-MM or CK-BB by the Ouchterlony test.

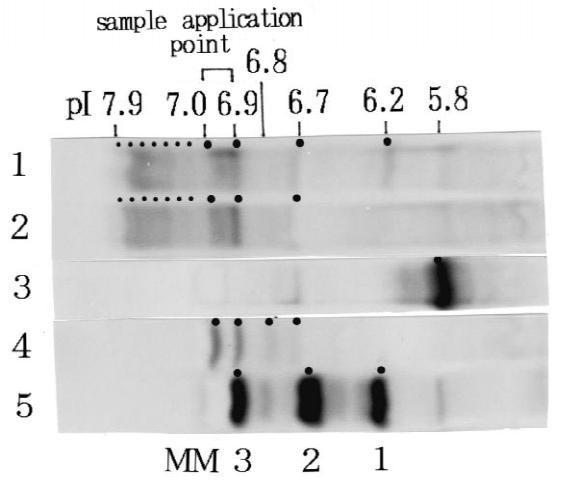

Fig. 1. CK isoform patterns in serum of the tumor patient obtained by isoelectric focusing. (1) An original pattern of fresh serum CKs. (2) After reaction of patient's serum with anti-CK-M subunit antibody. The CK at p*I* 6.2 was inactivated. (3) After reaction with anti-mCK antibody. The atypical CKs were reacted with anti-mCK antibody. (4) After treatment with 2 mol/l urea at 27°C for 15 min. Four CK bands newly appeared instead of the broad bands. (5) Marker isoforms of CK-MM.

3. Results

Fig. 1 shows IEF patterns of CKs in serum of the patient. Four sharp bands focused at p*I* 6.2, 6.7, 6.9 and 7.0 and broad bands at p*I* 7.0–7.9 and p*I* 6.9–7.0 (the sample application point) were detected in fresh serum (lane 1). Since the CK bands were not observed when creatine phosphate was eliminated from the reaction mixture, the bands were concluded to represent true CKs. The CKs did not react with anti-CK-BB, anti-human IgG, anti-IgA or anti-IgM antibodies. After reaction of the CKs with anti-CK-M subunit antibody, the band focused at p*I* 6.2 disappeared; however, all other bands remained at their original positions (lane 2). Thus, the bands focused at p*I* 6.2 was concluded to represent CK-MM1 by comparison with p*I*s of markers (lane 5), and the other bands were concluded to represent neither CK-MM, CK-MB, CK-BB nor macro CK type 1. All of the following experiments were performed with the patient's sera after complete inactivation of CK-MM activity by reaction with anti-CK-M subunit antibodies.

Lane 3 of Fig. 1 shows reaction of the CKs with anti-mCK antibody. All CK bands showed reaction with anti-mCK antibody and migrated to the anodal side, and a strong CK band appeared at p*I* 5.8. Activation energy of the CKs and residual CK activity after heating of the serum at 45°C for 20 min were 145 kJ/mol and 89.9%, respectively. After treatment of the CKs with 2 mol/l urea, four CK bands newly appeared at p*I* 6.7, 6.8, 6.9 and 7.0, whereas the broad bands (p*I* 7.0–7.9 and p*I* 6.9–7.0) disappeared (lane 4). After treatment with 0.5 mol/l 2-mercaptoethanol (2-ME), the IEF pattern of the CKs did not change, whereas the enzyme activities increased.

Fig. 2 shows results of column chromatography of the CKs in the patient's serum using a Sephadex G-200 superfine column. CK activity was detected in 4S, 7–19S and 19S fractions, and relative molecular masses of the CKs were estimated to be 80 000, 350 000 and more than 1 000 000, respectively. The bands focused at p*I* 6.7, 6.8, 6.9 and 7.0 were detected in the 4S fraction (lane 1 of Fig. 3), the broad band at p*I* 7.0–7.9 in the 7–19S fraction (lane 2) and the band at the sample application point in the 19S fraction (lane 3).

Fig. 4 shows alteration of IEF patterns of the 4S CKs by oxidation and reduction. After treatment of

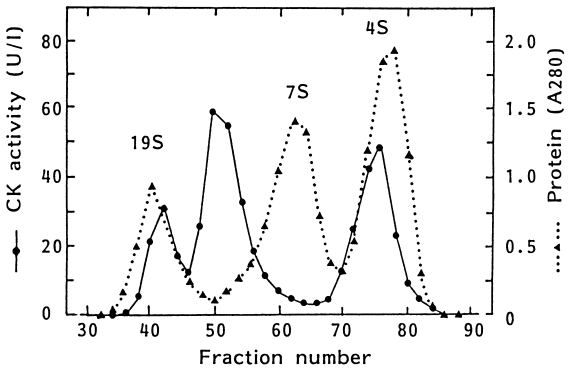

Fig. 2. Elution patters of mCK and serum proteins from a Sephadex G-200 superfine column. mCK activities were detected in 4S, 7–19S and 19S fractions.

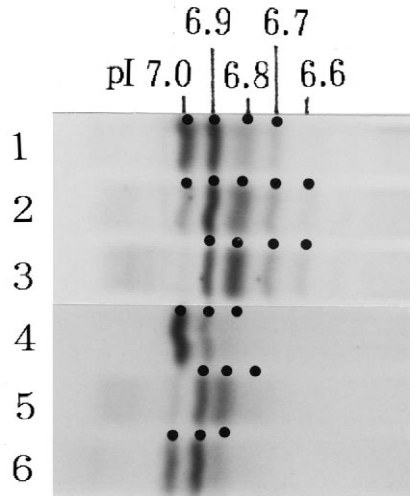

Fig. 4. Conversion of mCK isoforms by oxidation and reduction with hydrogen peroxide (1–3) and 2-ME (4–6), respectively. (1) Patient's serum before treatment. (2) After treatment with 0.1% hydrogen peroxide at 27°C for 15 min. (3) After treatment with 0.2% hydrogen peroxide at 27°C for 15 min. mCK at p*I* 7.0 disappeared and enzyme activities at p*I* 6.6, 6.7, 6.8 and 6.9 increased. (4) The patient's serum before treatment. (5) After treatment with 0.2% hydrogen peroxide. (6) After treatment with 200 mmol/l 2-ME at 27°C for 15 min following treatment with 0.2% hydrogen peroxide.

the CKs with 0.1% and 0.2% hydrogen peroxide, the band at p*I* 7.0 gradually disappeared and enzyme activities of the CKs focused at p*I* 6.8 and 6.7 increased, and an additional band appeared at p*I* 6.6 (lanes 1–3). The original CK IEF pattern including the band focused at p*I* 7.0 was again observed upon treatment of the hydrogen-peroxide-treated CKs with 200 mmol/l 2-ME (lane 4–6).

Fig. 5 shows alteration of the CK IEF pattern upon delysination. After treatment of the 7–19S fraction (lane 1) with 900 I.U./l carboxypeptidase B, a strong band newly appeared at p*I* 6.8–6.9 (lane 2), and the broad band disappeared. The 4S CKs (lane 3) migrated to p*I* 6.1, 6.2, 6.3 and 6.4 upon treatment with 900 I.U./l carboxypeptidase B (lane 4).

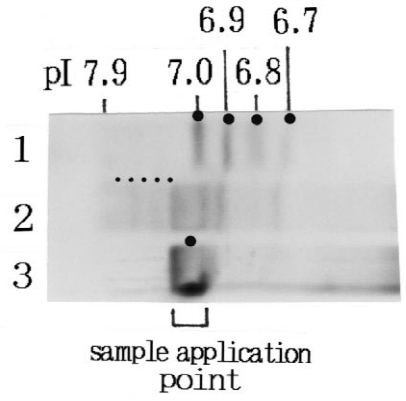

Fig. 3. Isoelectric focusing patterns of mCK in the effluents from Sephadex G-200 superfine column. (1) 4S fraction. Four mCKs were detected at p*I* 6.7, 6.8, 6.9 and 7.0. (2) 7–19S fraction. The broad band at p*I* 7.0–7.9 was detected. (3) 19S fraction. The mCK at the sample application point was detected.

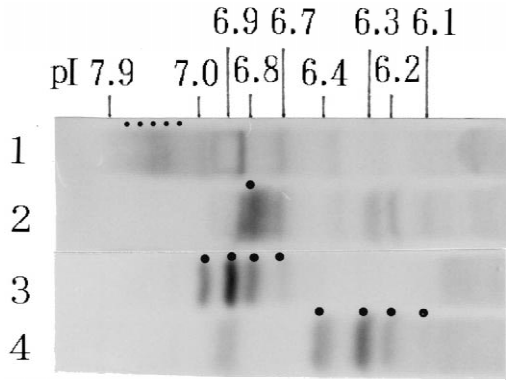

Fig. 5. Conversion of mCK isoforms by delysination with 900 I.U./ml carboxypeptidase B at 37°C for 15 min. (1) An octameric form before treatment. (2) After treatment, octameric delysined form. (3) Dimeric forms before treatment. (4) After treatment, dimeric delysined form.

4. Discussion

The atypical CKs detected in the patient's serum reacted with anti-mCK antibodies but not with anti-CK-BB, anti-CK-MM, anti-human IgG, anti-IgA or anti-IgM antibodies. Thus, the antigenicity of the CKs corresponded to that of mCK. The p*I*, of 6.7–7.9, of the CKs was nearly the same as the p*I*, of 6.86–8.01, of mCK isolated from human cardiac muscle by SiragEldin et al. [14]. The activation energy and heat stability of the atypical CKs agreed with the criteria proposed by Stein et al. [15] for mCK determination. Thus, the atypical CKs in the patient's serum were judged to be mCKs.

mCKs are found exclusively in the mitochondrial compartment, attached to the inner membrane [6,7,16]. Imaging of mCKs by scanning electron microscopy revealed a banana-shaped structure for the dimer, and a cubical structure for the octamer [4]. Due to its cubical structure, the octamer binds more strongly to the inner membrane of mitochondria than the dimer [4]. The three-dimensional structure of mCK octamers has been clarified and the positively charged amino acids at the four-fold faces of the octamer possibly interact with negatively charged mitochondrial membranes [6,7]. The dimer and octamer are released from mitochondrial membranes into circulation in the absence of detergent, in vivo.

From the present results of IEF performed on fractions collected from column chromatography, the bands focused at p*I* 6.7, 6.8, 6.9 and 7.0 were concluded as representing the dimeric form and the broad band at p*I* 7.0–7.9, the octameric form, based on their molecular masses which were estimated to be 80 000 and 350 000, respectively (Fig. 6). mCK found at the sample application point seemed to be the form which binds to the mitochondrial membrane, because its molecular mass was greater than 1 000 000. The finding of a mCK with such a high-molecular-mass in the patient's serum suggests that the mCKs were probably released into the circulation due to necrosis of the tumor cells.

In RNA blot hybridization analysis of human tissue, mCK mRNA was detected in jejunum but not in liver or lung tissue [17]. Therefore, the mCK in the patient's serum are thought to have originated from rectal carcinoma cells and not from metastatic liver or pulmonary carcinoma cells. Cardiolipin is a membrane receptor for mCK [6,18], and the binding of mCKs to cardiolipin-containing liposomes was reported to be inhibited by adriamycin [19]. Adriamycin had not been administered to our patient; however, the relationship between the presence of mCKs in the serum and chemotherapy in cancer patients would be an interesting subject for future study.

The interconversion between the octamer and the dimers is reversible both in vivo and in vitro [9]. The octamer is converted to the dimers in alkaline solution or 1 mol/l urea, and this conversion can be reversed by dialysis against 20 mmol/l phosphate [9]. In the present study, the octamer was converted to the dimers at p*I* 6.6, 6.7, 6.8 and 6.9 upon treatment with 2 mol/l urea. Urea is usually considered to be a hydrogen bond breaker. Thus, the octamer seemed to be formed through hydrogen bonding among dimers. Kaldis et al. suggested that the charged amino acid residues in the N-terminal heptapeptide of mCK play an important role in the formation and stabilization of the octameric molecule, because removal of the N-terminal pentapeptide of mCK by limited proteolysis drastically destabilized the octamer [20].

The dimeric mCKs focused at p*I* 6.6, 6.7, 6.8 and 6.9 were further characterized as being oxidized forms because their enzyme activities increased upon treatment with hydrogen peroxide. The bands were again focused at p*I* 7.0 upon treatment with 2-ME; thus the band at p*I* 7.0 was concluded as representing a completely reduced form. Three isoforms of CK-MB and CK-BB were detected upon treatment with 2-ME or hydrogen peroxide [21]. Quest et al. reported two types of CK-B subunit, the basic and the acidic forms, and that the basic CK-B subunit contained three serine residues more than the acidic CK-B subunit: 21 vs. 18 [22]. The sites of oxidation and reduction of mCK are not clear; however, there may be two oxidation sites in one subunit of mCK, since four dimeric oxidized forms were recognized, and the high-mobility anodal band focused at p*I* 6.6 seemed to represent a completely oxidized dimer.

The IEF patterns of the octamer in the patient's serum showed a broad band at p*I* 7.0–7.9. Upon carboxypeptidase B treatment, this band disappeared and a narrow band appeared at p*I* 6.8–6.9, due probably to delysination of the mCKs. This finding

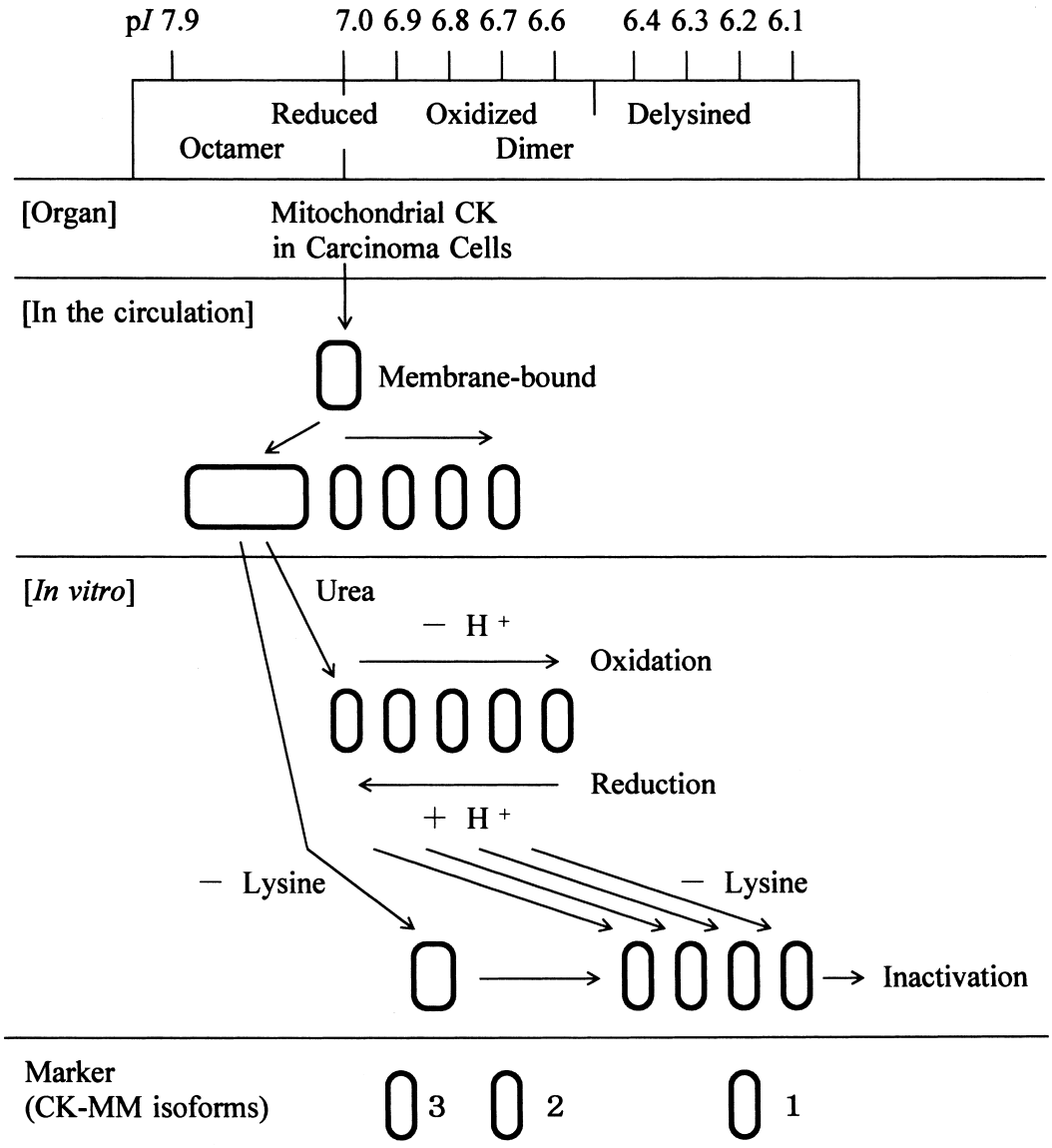

Fig. 6. Summary of the mechanism of appearance and disappearance of mCK isoforms. Membrane-bound mCK, released into the circulation by necrosis of carcinoma cells, is converted into octameric, dimeric reduced and oxidized forms in that order. In vitro, membrane-bound mCK is converted into octameric and dimeric forms upon urea treatment. The dimeric reduced form at p*I* 7.0 is converted into the oxidized forms at p*I* 6.6–6.9 by oxidation, and the conversion is reversible. The octameric and dimeric forms migrate to p*I* 6.8–6.9 and p*I* 6.1–6.4, respectively, upon removal of lysine, and the dimeric completely oxidized-delysined form seems to be inactivated. As the p*I*s of the membrane-bound and the dimeric mCKs overlap those of CK-MM isoforms, the results led to inconsistency of the data obtained by electrophoresis and the immunoinhibition method in routine clinical tests.

suggests that the presence of the broad band reflects the number of lysine residues at the C-terminal of the octamer. The number of lysine residues at the C-terminal of the octamer in the patient's serum is thought to be variable. The activity of carboxypeptidase N in the sera of healthy adults was reported to be 267±45 (mean±SD) I.U./l [23]. Therefore, octameric mCK released into circulation

is probably hydrolyzed resulting in a variable number of lysine residues at the C-terminal. This finding was not observed in the case of the octameric form of mCK purified from human tissue [2] and suggests catabolism of mCK following release into circulation.

Furthermore, a delysined form of the completely oxidized dimer focused at p*I* 6.6 was not detected. This suggests that the delysined dimer was inactivated by complete oxidation. From the aforementioned findings suggesting catabolism of mCK following release into circulation, we suggest that the membrane-bound mCK released into circulation from the carcinoma cells was converted successively to the octamer and dimer, inactivated by removal of lysine and complete oxidation, and then removed from the circulation.

The mCKs in the serum of one patient have been characterized. The p*I*s of 6.2, 6.7 and 6.9 of the dimeric mCKs corresponded to those of CK-MM1, CK-MM2 and CK-MM3, respectively, and the other p*I*s detected were similar to those of the minor bands of CK-MM isoforms [24]. Therefore, we conclude that the dimeric form of mCKs in the serum cannot be distinguished from CK-MM by IEF or conventional electrophoresis. mCKs and CK-MM can be distinguished by reaction with anti-CK-M subunit antibodies following separation by IEF.

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