Volume 262, number 2, 275–278 FEBS 08268 March 1990

Immunological determination of the oligomeric form of mitochondrial creatine kinase in situ

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Received 22 December 1989; revised version received 9 February 1990

Whereas factors governing the interconversion of the two oligomeric forms of mitochondrial creatine kinase are relatively well known, few informations are yet available on the actual form in situ. Antibodies against purified pig and rabbit heart mitochondrial creatine kinase were obtained. The former exhibits a marked specificity for the dimer while the second reacts with both dimer and octamer. They allowed to demonstrate that no dimer can be detected in mitochondria and that CKm occurs naturally exclusively as an octamer. We present arguments that the larger part, if not the totality, of the octamer is membrane-bound rather than soluble in the intermembrane space. However, these findings do not refute the previously proposed models for the regulation of CKm activity in the mitochondrion but urge to envisage a more complex one.

ELISA analysis; Immunoinhibition; Mitochondrial creatine kinase; Octameric structure; (Rabbit heart mitochondria)

1. INTRODUCTION

Mitochondrial creatine kinase (CKm) is thought to play a prominent role in the exportation of the mitochondrially synthesized ATP towards the cytosolic utilization sites in high energy-consuming mammalian cells [1]. Whereas the cytosolic isoenzymes exclusively occur as dimers, namely MM, MB or BB resulting from the association of two types of subunits, the structure of CKm is heterogeneous: a unique feature of CKm is its ability to form an octamer (CKm_2) in addition to a dimer (CKm_1). Several authors reported a reversible octamer-dimer conversion when the enzyme is incubated in dilute solution at alkaline pH or in the presence of substrates forming either an active combination (Mg-ATP-creatine) or a dead-end complex (Mg-ADP-NO_3-creatine) [2–5].

Mitochondrial phosphate extracts at neutral pH contain mainly the octamer [2,3,5] and its conversion to dimer occurs after solubilization only. Furthermore, it has been demonstrated that CKm_2 was the only form able to reassociate to mitochondria depleted from their endogenous CKm thus indirectly showing that it might be the actual membrane-bound one [3]. Direct proof that the membrane-bound form is the octamer was given by radiation inactivation [6] and cross-linking [7].

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Abbreviations: CKm, mitochondrial creatine kinase; CKm_1, dimeric structure; CKm_2, octameric structure

However, the fact that CKm is solubilized by physiological concentrations of nucleotides [8] and that octamer-dimer conversion is reversible and under control of substrates led some authors to postulate that the equilibrium (bound octamer/soluble octamer/soluble dimer) is an element for the regulation of CKm activity according to the physiological state of the mitochondrion [2,5,9].

In the present work, we raised antibodies against purified pig heart CKm and characterized a form-specificity that was used to show that no CKm_1 is detectable in mitochondria. A second antiserum obtained against purified rabbit heart CKm recognized both forms but provided direct proof that octamer is the only membrane-bound form and allowed us to estimate the amount of CKm_2 naturally present in rabbit heart mitochondria.

2. MATERIALS AND METHODS

Rabbit heart CKm was purified as previously described [10]. Pig heart CKm was prepared by a similar procedure in which the second chromatographic step was replaced by an anion exchange on DEAE-Sephacel (Pharmacia) equilibrated with 20 mM Tris-acetate, 0.1 mM EDTA, 1 mM DTT, pH 7.4. Elution was performed by a 0–1 M ammonium bicarbonate linear gradient in the same buffer. Purified enzymes were stored at −80°C after lyophilisation. They were resuspended in 10 mM Hepes, 1 mM DTT, pH 7.4 a few hours before use.

Antisera were raised by intradermic injections of purified pig heart and rabbit heart CKm into a rabbit and a guinea pig, respectively. The immunization protocol involved 4 injections of 130 μg enzyme at days 0, 14, 30 and 50. Bleedings were performed at day 58 and the sera were cleared up by centrifugation.

Conversion of rabbit CKm_2 to CKm_1 was achieved by an overnight incubation of the enzyme in Tris-barbital buffer, pH 8.8 [3]. Dimer

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00145793/90/$3.50

and octamer were separated by cellulose acetate electrophoresis and CK activity detected as described in [3]. CKm was immunodetected on a nitrocellulose blot obtained by a Southern-like procedure after Cellogel electrophoresis. ELISA 96-well plates (Nunc, Denmark) were coated with pure CKm or mitochondrial extracts by an overnight adsorption at 4°C. Mitochondria were diluted at 2 mg protein/ml in TBS (10 mM Tris, 0.9% NaCl, pH 7.4) which NaCl concentration (154 mM) leads to a 100% solubilization of CKm [8]. After a 10000 × *g* centrifugation for 2 min, supernatants were directly transferred to the wells. For blotting as well as for ELISA immunodetections, saturation of nonspecific binding sites was carried out by PBS-3% BSA for 1–2 h. Working dilutions for rabbit antiserum and guinea pig antiserum were respectively 1/100 and 1/500 in PBS-0.05% Tween. Immune complexes were revealed by peroxidase-conjugated anti-rabbit IgG (Biosys, France) and anti-guinea pig IgG (Sigma) using either ABTS as substrate for ELISA or 4-chloro-naphthol for immunoblots.

Immunoinhibition experiments were performed at 30°C on 0.1 mg/ml purified CKm or 6 mg/ml mitoplasts prepared by the digitonin method [8] in a nonsolubilizing isotonic mixture (10 mM Hepes, 0.25 M sucrose, 1 mM DTT, pH 7.4) containing the anti-rabbit CKm antiserum (working dilution 1/3). Aliquots were taken up periodically and assayed spectrophotometrically at 340 nm by following phosphocreatine synthesis with PK and LDH as auxiliary enzymes.

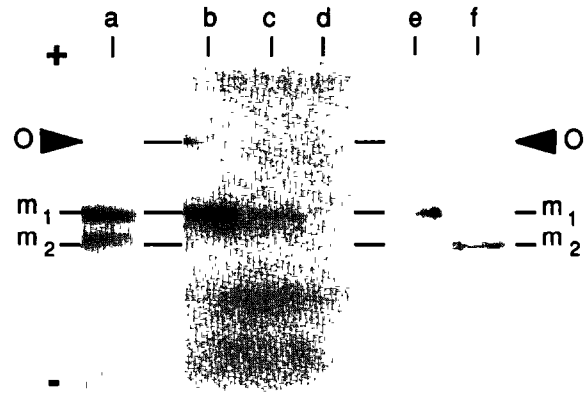

Fig.1. Immunodetection on nitrocellulose blots after a 50 min cellulose acetate electrophoresis of 80 ng of purified pig CKm_1 (b), rabbit pH-induced CKm_1 (c,e) and CKm_2 (d,f). (a) Detection of enzymatic activity of a 4 mIU rabbit dimer-octamer mixture. (b,c,d) Anti-pig CKm antiserum. (e,f) Anti-rabbit CKm antiserum.

3. RESULTS

3.1. *Characterization of anti-pig and anti-rabbit heart CKm reactivities on both forms*

Pig heart CKm is solubilized mainly as the dimeric form [3] and purified enzyme is usually obtained as homogeneous CKm_1 preparations. The antiserum obtained by using pig CKm as antigen exhibits a marked specificity for the dimer, either the spontaneously formed pig one or the pH-induced rabbit one (fig.1b,c). On the contrary, rabbit octamer that migrates more cathodically does not react with this antiserum (fig.1d). This apparent dimer specificity is demonstrated with native enzymes only. If the nitrocellulose blot is allowed to dry both forms are recognized as a result of denaturation and emergence of epitopes hidden in the native structures (not shown). This specificity for the dimer also exists for enzymes immobilized on the polystyrene surface of ELISA wells (fig.2A). It should be noted that pig CKm_1 is quantitatively as immunoreactive as rabbit CKm_1 (not shown).

On the other hand, the antiserum we prepared against a homogeneous CKm_2 rabbit antigen revealed absence of such a form-specificity. Immunorevelation of nitrocellulose-blotted native enzymes (fig.1e,f) and ELISA analysis (fig.2B) show that both CKm_1 and CKm_2 are recognized. Octameric CKm nevertheless gives a higher response than dimer (fig.2B). It is not possible to determine if this absence of form-specificity is due to dissociation of CKm_2 or CKm_1 after injection in the host animal or if antibodies are generated against similar epitopes on the surfaces of both isoenzymes.

None of these antisera exhibit any cross-reactivity with cytosolic isoenzymes MM and BB (not shown) thus confirming previous data [11–13].

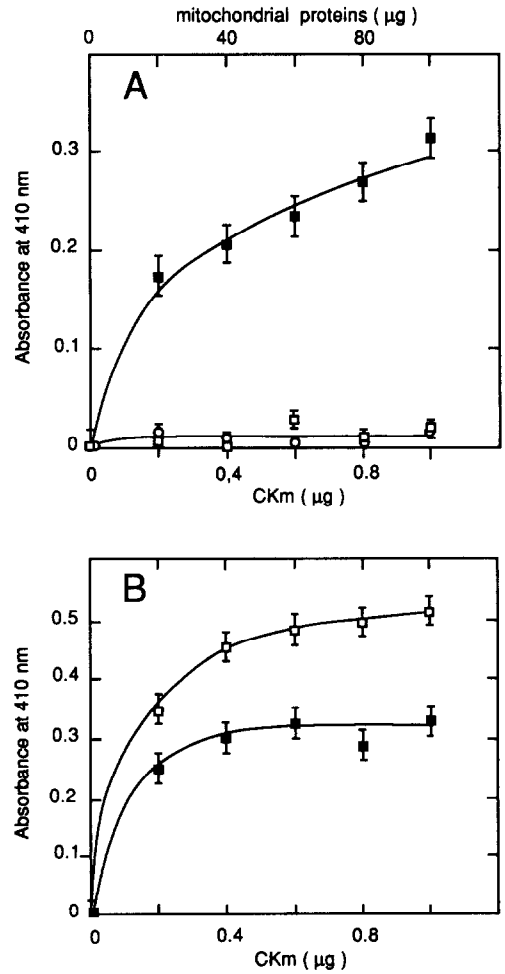

Fig.2. ELISA analysis by anti-pig CKm (A) and anti-rabbit CKm (B) antisera as a function of the incubated amount of protein per well. Purified rabbit CKm_2 (□) and CKm_1 (■). (○) TBS-extracts of rabbit heart mitochondria. Mean values for 4 measurements.

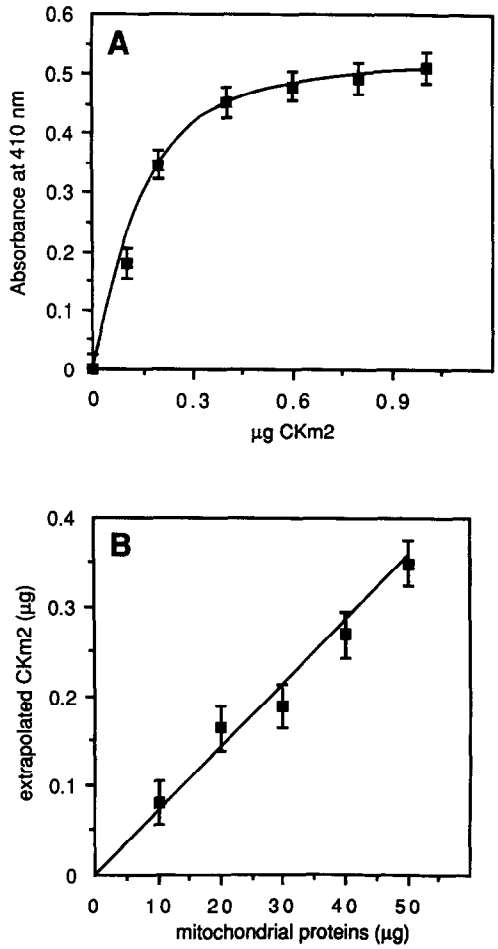

Fig.3. ELISA quantitation of the endogenous CKm in rabbit heart mitochondria. In the same assay a standard absorbance curve for purified CKm_2 was plotted (A) and absorbance of TBS-extracts from 0–60 μg mitochondrial proteins were measured (mean values from 4 measurements). Deduced CKm_2 amounts were plotted in (B), the slope of the regression line gives 7 ± 1 μg/mg mitochondrial proteins.

3.2. *Identification and quantitation of the naturally occurring CKm form in mitochondria*

A large number of intermembrane space proteins, among which CKm abounds, are released by high ionic strength treatment of mitochondria [8]. TBS-extracted proteins were analysed by a direct ELISA in comparison with purified CKm_1 and CKm_2. As the anti-pig CKm antibodies do not react with TBS-extracts (fig.2A) it can be concluded that no soluble or membrane-bound dimer naturally occurs in mitochondria.

On the basis of preceding results we assumed that all endogenous CKm is octameric and thus constructed a standard CKm_2 curve to quantitate CKm in mitochondria (fig.3). The slope of plot 3B, which was drawn after correction with absorbance values obtained on CKm-depleted mitoplasts, permitted to determine a mean value of 7 ± 1 μg CKm_2/mg mitochondrial proteins.

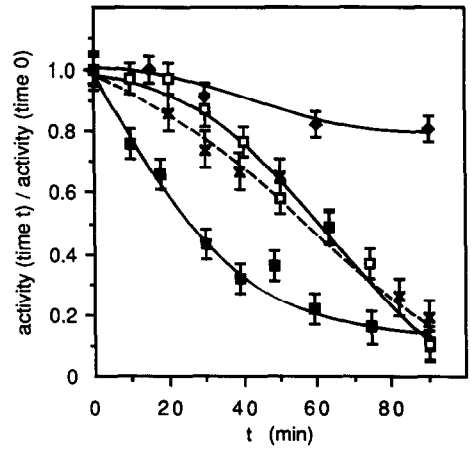

Fig.4. Immunoinhibition of purified and membrane-bound CKm by the anti-rabbit CKm antiserum in an isotonic non-solubilizing medium. Normalized activity values of CKm_1 (■), CKm_2 (□) and mitoplast-bound CKm (×). Initial activities were respectively 4–5, 4–6, and 3–5 IU/ml. (◆) Control CK activity without antibodies.

This technique did not allow to evaluate which proportion of the octamer was originally membrane-bound or soluble in the intermembrane space. However when mitochondria were treated by the nonsolubilizing 10 mM Tris buffer pH 7.4, CKm was not immunodetectable in the supernatant thus showing that no CKm exists in a soluble state in the intermembrane space.

3.3. *Immunoinhibition of mitochondria-bound CKm*

In order to provide additional evidence that membrane-bound CKm is exclusively octameric we tested the sensitivity to immunoinhibition of CKm activity in digitonin-mitoplasts (fig.4). Periodic controls were made (not shown) to check that no CKm solubilization occurred during incubation.

Immunoinhibition profiles (fig.4) clearly show that soluble CKm_2 and membrane-bound CKm were similarly inhibited by antibodies, the kinetics of inhibition being significantly more rapid for the dimer. The initial slight difference between soluble and membrane-bound CKm_2 can be the result of a diminution of the accessibility to essential epitopes in the latter one.

4. DISCUSSION

Previous reports of the use of polyclonal antibodies against CKm provided indications on the presence of CKm in close vicinity to contact sites between inner and outer membranes [5,14] and on a possible association between CKm and translocase [15]. This latter assumption was discussed [5,16]. Arguments were also advanced for the participation of an ionic component [17,18] and cardiolipin [16] in the binding of CKm to mem-

brane. The aim of this work was to determine whether CKm in situ can be detected as octamer alone or as a mixture of dimer and octamer as postulated [2,3,5]. Our immunological data are in good agreement with less sensitive methods reporting that the majority, if not the totality, of CKm is released under octameric form [2,3,5,9]. Whole CKm is undoubtedly octameric in situ but it remained to be elucidated if soluble and membrane-bound octamers may coexist in the intermembrane space. Our experiments with mitochondria of which the outer membranes were ruptured in nonsolubilizing media showed that no, or very minute quantities of CKm were spontaneously released from mitochondria. We thus assume that the great majority of octamer is naturally membrane-bound. We measured about 7 μg CKm_2 per mg mitochondrial proteins, that is to say, about 20 pmol/mg assuming a molecular mass for the octamer of 350 kDa [3,6]. This value confirms our preliminary estimation that CKm may not exceed 1% of mitochondrial proteins [8]. Our data are close to those reported on the basis of activity measurements for chicken heart mitochondria [5,19] but lower than the calculated octamer contents of beef (100 pmol/mg [2]), chicken (230 pmol/mg [20]), and rabbit (214 pmol/mg [20]) heart mitochondria.

We admit, however, that under different and perhaps more physiological conditions a part of CKm_2 may naturally dissociate from the membrane according to the attractive model proposed for the regulation of CKm activity based on the solubilization and conversion of CKm_2 in situ. Perhaps the role of the outer membrane, the lateral mobility of the CKm binding site at the surface of the inner membrane and the localization of nucleotides intermembrane compartments should be taken into account and further investigated.

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