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Octamer-dimer Transitions of Mitochondrial Creatine Kinase in Heart Disease

Sibylle Soboll[1], Dieter Brdiczka[2], Dietmar Jahnke[1], Anja Schmidt[1], Uwe Schlattner[3], Silke Wendt[3], Markus Wyss[4] and Theo Wallimann[3]

[1]*Institut für Physiologische Chemie I, Heinrich-Heine-Universität, D-40225 Düsseldorf, Germany,* [2]*Fakultät für Biologie, Universität Konstanz, D-78434, Konstanz, Germany,* [3]*Institut für Zellbiologie, ETH Hönggerberg, HPM F42, CH-8093 Zürich, Switzerland, and* [4]*F. Hoffmann-La Roche Ltd., VM4 Bldg. 241/865, CH-4070 Basel, Switzerland*

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S. Soboll, D. Brdiczka, D. Jahnke, A. Schmidt, U. Schlattner, S. Wendt, M. Wyss and T. Wallimann. Octamer-dimer Transitions of Mitochondrial Creatine Kinase in Heart Disease. *Journal of Molecular and Cellular Cardiology* (1999) **31**, 857–866. Mitochondrial creatine kinase (Mi-CK) occurs in dimeric and octameric forms, both *in vitro* and *in vivo*. The Mi-CK octamer, however, is the predominant form *in vivo* and is important for various functions of the protein. In the present study we show for the first time a significant decrease of the octamer/dimer ratio *in vivo*, related to ischemia-induced damage, and a similar decrease of octamer stability *in vitro*, induced by peroxynitrite (PN) radicals. We used animal models to induce ischemia in two different ways: acute ischemia in intact heart (Langendorff perfusion) and chronic ischemia *in vivo* (LAD-infarction). In both models, impairment of heart function and mitochondrial energy metabolism was associated with a significant decrease of Mi-CK octamer/dimer ratios and of Mi-CK activities. These findings, together with recent data showing that the formation of PN is induced in ischemia and that Mi-CK is a prime target of peroxynitrite (PN)-induced damage, suggest that oxygen radicals generated during ischemia and reoxygenation could be an important factor for the decreased octamer stability. To test this hypothesis, we studied the effect of PN on pure Mi-CK *in vitro*, both on dissociation of octamers and reassociation of dimers. At 1 mM PN 66% of Mi-CK octamers dissociated into dimers, whereas octamerization of PN-modified dimers was already completely inhibited at 100 μM PN. Our data indicate that PN-induced damage could be responsible for the octamer-dimer transition of Mi-CK in ischemia. A loss of Mi-CK octamers would impair the channeling of high energy phosphate out of mitochondria and hence heart function in general.

Key Words: Cardiomyopathy; Energy metabolism; Mitochondrial creatine kinase.

Introduction

Mitochondrial creatine kinase (Mi-CK; E.C. 2.7.3.2) is a central enzyme of energy metabolism and especially important for oxidative muscle like heart (for reviews see Wyss *et al.*, 1992; Schlattner *et al.*, 1998). Impaired creatine kinase (CK) function is of significance for the development of different pathological states and may be also relevant for myocardial diseases (Hamman *et al.*, 1995; Gross *et al.*, 1996; Liao *et al.*, 1996).

Mi-CK is localized in the mitochondrial intermembrane space along the cristae membranes as well as in mitochondrial contact sites (Brdiczka *et al.*, 1990). It has preferential access to ATP, which is generated by oxidative phosphorylation and exported via the adenylate translocator (mitochondrial adenine nucleotide translocase (ANT)) (Brdiczka and Wallimann, 1994). The enzyme then catalyses the reversible transphosphorylation between ATP and creatine to ADP and phosphocreatine. Liberated ADP is preferentially re-taken up into the mitochondrial

Please address all correspondence to: S. Soboll, Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany.

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matrix via ANT to stimulate oxidative phosphorylation (Saks *et al.*, 1985). Phosphocreatine leaves the mitochondria via the mitochondrial porin to build up a high energy phosphate buffer in the cytosol, which is then used by cytosolic CK isoforms to restore local ATP levels (CK shuttle) (Wallimann, 1994; for reviews see Wallimann *et al.*, 1992; Schlattner *et al.*, 1998).

In contrast to cytosolic CK, which is exclusively dimeric, Mi-CK forms both octameric and dimeric molecules that are interconvertible (Wyss *et al.*, 1992). Although the specific activity of octamers and dimers is very similar (Kaldis and Wallimann, 1995), a growing body of evidence suggests that, *in vivo*, the octamer is the true functional unit. Freshly isolated Mi-CK, reflecting most closely the *in vivo* situation, is largely octameric (Wyss *et al.*, 1992). The high local concentration of Mi-CK in the mitochondrial intermembrane space and the high affinity binding of Mi-CK to mitochondrial membranes (Rojo *et al.*, 1991; Stachowiak *et al.*, 1996) and other interaction partners like mitochondrial porin (Brdiczka *et al.*, 1994) favor the association of Mi-CK dimers into octamers and stabilize the octamer. Only octameric Mi-CK is able to interact simultaneously with two opposing membranes (Rojo *et al.*, 1991) and forms complexes with ANT and porin in the so-called mitochondrial contact sites between outer and inner mitochondrial membranes (Beutner *et al.*, 1996, 1998). These contact sites may play an important role for efficient energy export into the cytosol by direct channeling of substrates and products in between the interaction partners (reviewed in Ovádi, 1995; Schlattner *et al.*, 1998; Stachowiak *et al.*, 1998b).

Destabilization of the Mi-CK octamer leads to an impairment of Mi-CK function, as demonstrated recently. Mi-CK mutant proteins with reduced octamerization rate were expressed in neonatal rat cardiomyocytes having very low endogenous Mi-CK activity. The transformants showed low octamer/dimer ratios and reduced membrane binding of Mi-CK, together with very poor rates of creatine-stimulated respiration as compared to cells transformed with wild-type protein (Khuchua *et al.*, 1998). The octameric state of Mi-CK is also essential for its protective effect on the opening of the mitochondrial permeability transition pore. This effect of Mi-CK is lost when octamers are dissociated by the components of the transition state analogue complex (TSAC) (O'Gorman *et al.*, 1997). Taken together, Mi-CK octamer-dimer transitions could play a crucial role in the regulation of mitochondrial energy metabolism and cellular apoptosis. However, it is not known if such a transition can occur *in vivo* and what the potential cellular triggers may be.

A number of studies has shown that cytosolic MM-CK and Mi-CK are main targets of reactive oxygen species (ROS) (Mekhfi *et al.*, 1996; Wolosker *et al.*, 1996), especially H_2O_2 (Yuan *et al.*, 1992; Nathan and Xie, 1994) and peroxynitrite (PN) (Konorev *et al.*, 1998; Stachowiak *et al.*, 1998a). Recent data also indicated an effect of PN on the octamer/dimer ratio of Mi-CK (Stachowiak *et al.*, 1998a). PN, the reaction product of nitric oxide (NO) and $O_2^{\bullet -}$, is an oxidant that irreversibly modifies lipids and proteins at aromatic amino acid side chains and sulfhydryl groups. Therefore ROS, and especially PN, may not only affect CK activity, but also lead to a destabilization of the rather weak dimer/dimer interfaces keeping together the Mi-CK octamer.

In the present study we have directly addressed the question of whether the impairment of heart function after ischemia is related to a destabilization of Mi-CK octamers. It is known that oxygen deficiency and reoxygenation of the heart following infarction lead to enhanced oxidative stress and increased levels of O_2^-, NO and PN in mitochondria (Kaul *et al.*, 1993; Kehrer, 1993; Liu *et al.*, 1997). Under these conditions, Mi-CK octamers might dissociate as an effect of radical damage. In perfused heart treated with PN, iodoacetamide or S-nitrosoactetylcysteine (an NO-donor), a decrease in CK activity was correlated with a reduction in heart contractile reserve and function (Hamman *et al.*, 1995; Gross *et al.*, 1996; Schulz *et al.*, 1997). Thus, ROS-induced modifications of CK should be considered as an important factor for impaired cardiac performance.

To analyse ischemic hearts, we used two animal models to induce different types of ischemia. We applied acute ischemic conditions to isolated perfused rat heart and chronic ischemia by using infarcted rat hearts *in vivo*. In parallel, the influence of PN radicals on the octamer/dimer ratio of Mi-CK was analysed *in vitro* with pure, recombinant Mi-CK. Our studies show for the first time that the Mi-CK octamer is destabilized *in vivo* during episodes of ischemia and reoxygenation. We propose that this is due to the effect of NO and PN on the Mi-CK molecule, as evidenced *in vitro.*

Materials and Methods

Materials

All enzymes and coenzymes were obtained either from Boehringer (Mannheim, Germany) or from Sigma Chemie (München, Germany). Chemicals

were delivered by Merck (Darmstadt, Germany) and were of the highest purity available. Cellulose polyacetate strips were from Schleicher & Schüll (Dassel, Germany) and Nembutal from Ceva (Düsseldorf, Germany). Rabbit polyclonal antibodies against chicken MM-CK and Mi-CK were prepared in the Institute of Cell Biology, ETH Zürich, Switzerland. Anti-rabbit IgG peroxidase conjugate was from Sigma Chemie (München, Germany). Chicken Mi_b-CK was heterologously expressed and purified according to Furter *et al.* (1992). Peroxynitrite was a gift from Prof. W. Koppenol, Laboratorium für Anorganische Chemie, ETH Zürich, Switzerland.

Isolation and Langendorff perfusion of rat hearts

Rats weighing 220–250 g and fed *ad libitum* were anesthetized by intraperitoneal injection of 75 mg pentobarbital Na (Nembutal) per kg body weight. Subsequently the thorax and the abdomen were opened and the vena cava inferior and the vena portae were ligated. The vena portae was cannulated, the vena cava inferior opened and the heart perfused through the liver with Krebs-Henseleit bicarbonate buffer (KH) (containing 1.8 mM $CaCl_2$, 10 mM glucose and oxygenated with 95% O_2-5% CO_2 v/v, 37°C; Krebs and Henseleit, 1932), with a flow rate of 7 ml/min. After 2 min of perfusion the heart was excised, put into ice-cold KH and perfused retrogradely via an aortic cannula with KH for 20 min in the Langendorff mode (Langendorff, 1895) with a flow rate of 15 ml/min. One group of hearts was made ischemic by reducing the flow rate to 2 ml/min for 30 min, while controls were continuously perfused at 15 ml/min. Mitochondria were isolated immediately after the treatment.

Ligation of the left anterior descending coronary artery (LAD-infarction)

Male Wistar rats (200–250 g) were anesthetized as above, intubated through the larynx and artificially ventilated. The thorax was opened on the left side and the heart exposed. The left anterior descending coronary artery was ligated (Fishbein *et al.*, 1978) approximately 2 mm below the coronary ostium and the wound partially closed. One hour later the ligature was removed and the wound fully closed. Thereafter the animals were allowed to recover. Sham-operated rats were treated in the same way, except that the ligature was not pulled tight. Following coronary artery ligation, the underperfused myocytes necrotize and are replaced by scar tissue. The LAD-rats were prepared and functional parameters were measured by Dr Paul Rounding (Institut für Herz-Kreislaufforschung, Bayer AG, Wuppertal, Germany). For isolation of mitochondria, the animals were re-anesthetized after 3 weeks and the hearts removed and put into ice-cold KH. Mitochondria were isolated directly from the area surrounding the infarction center (LAD, *c.* 20% of the total ventricular tissue), from the remaining parts of the infarcted ventricles (rest) and from ventricles of sham-operated rats (control).

Isolation and incubation of heart mitochondria

Hearts or heart samples were put into ice-cold medium immediately after removal. Mitochondria were isolated from ventricular tissue essentially according to Jacobus and Saks (1982) in a medium containing 0.25 M sucrose, 10 mM Tris-HCl, 5 mM KH_2PO_4, 20 mM KCl, 0.2 mM ethylene-dinitrilotetra-acetic acid (EDTA), pH 7.2, and 1 mg of trypsin per 3 g of tissue wet weight. Respiratory control ratios were determined in an oxygraph chamber (volume 1.5 ml) with a Clark-type oxygen electrode in the absence of $MgCl_2$. As respiratory substrates, 2.5 mM glutamate and 2 mM malate were used. State 3 respiratory rates were in the range of 250–300 nmol/min × mg of mitochondrial protein at 37°C.

The specific activity of Mi-CK was measured as the rate of PCR synthesis according to Forster *et al.* (1974) with slight modifications. Values were corrected for interference of ATPases present in the mitochondrial preparation by measuring the activity in a parallel sample in the absence of creatine.

Cellulose polyacetate electrophoresis of Mi-CK

Mi-CK octamers and dimers were separated by native cellulose polyacetate electrophoresis on 25.5 × 1.45 cm cellogel strips in phenobarbital buffer (pH 8.6) for 90 min at 150 V and room temperature (RT). Freshly isolated mitochondria were diluted in phenobarbital buffer to 2.5 U/ml, corresponding to approximately 5 mg/ml protein. To avoid any artifact which may be introduced by extraction procedures, 5 μl samples of this suspension were directly applied to the cellogen strip. The Mi-CK bands were visualized by a coupled enzymatic assay in an overlay gel as described by Wallimann *et al.* (1985). Gels were scanned for the determination of the relative amounts of Mi-CK

octamers and dimers. For validation of this quantification technique it was assured that the dependency of spot intensity on mitochondrial protein concentration was the same for octamers and dimers and that the protein concentration transferred onto the strips was always in the linear dose-response range.

Immunodetection of CK in subcellular fractions of the rat heart

The purity of subcellular fractions was checked by immunodetection of CK isoforms. Samples having about equal CK activities, e.g. isolated mitochondria (10 μg protein per lane), cytosolic fractions (16 μg protein per lane), and purified MM-CK (1 μg protein per lane), were subjected to SDS-PAGE (Lämmli, 1970) in 4% stacking and 10% running gels. After semi-dry blotting of the proteins onto polyvinylidine difluoride (PVDF) membranes, CK isoforms were detected with polyclonal rabbit anti-chicken MM-CK or anti-chicken Mi-CK antibody (100-fold dilution each) and peroxidase-coupled anti-rabbit IgG (1000-fold dilution) with 4-chloro-1-naphthol as substrate.

Investigation of isolated Mi_b-CK

To achieve octamer dissociation of pure Mi_b-CK, a stock solution was diluted to a final concentration of 0.3 mg/ml in phosphate buffered saline (PBS) (150 mM NaCl, 100 mM Na_3PO_4, pH 7.2) and transition state analogue mixture (TSAC) (final conc.: 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3). This mixture was incubated for 3 h at RT, which leads to almost complete dimerization of Mi-CK octamers (Gross and Wallimann, 1993). The Mi-CK dimer solution was treated with PN by adding slowly, while vortexing, different amounts of PN (final conc.: 10–1000 μM in a volume of 30 μl 0.01 M NaOH) to 300 μl aliquots of Mi-CK dimer (Stachowiak *et al.*, 1998a). As a control, one aliquot was treated with 30 μl 0.01 M NaOH containing no PN. After reaction for 5 min, reoctamerization was induced by addition of 25 mM EDTA to chelate Mg^{2+} from MgADP, which is necessary for the formation of TSAC. The mixture was incubated overnight at RT (Gross and Wallimann, 1993). Aliquots of octameric Mi-CK (0.3 mg/ml) were treated with PN in the same way and the samples were incubated overnight at RT to reach equilibrium. The oligomeric state of Mi-CK was determined by HPLC (BioCAD, Perseptive Biosystems) on a Superose 12 column (Pharmacia) at RT, using phosphate buffer (50 mM Na_3PO_4, 150 mM NaCl, 2 mM 2-mercaptoethanol (BME), 0.2 mM EDTA) and a flow rate of 0.8 ml/min. The oligomeric species were quantified by numerical integration of the peak areas in the elution profile (software from Perseptive Biosystems).

Statistical analysis

All values are expressed as mean $\pm$ SEM. Differences between the control and the experimental groups were compared using Student's *t*-test. Differences were considered significant at the $P<0.05$ level.

Results

Detection of Mi-CK octamers and dimers in isolated mitochondria

Following native cellulose acetate electrophoresis of mitochondrial samples, a specific enzymatic assay coupled to a color reaction revealed two Mi-CK bands (Fig. 1A, lane 2). Suspension of mitochondria in medium containing 1.2 M urea, which is known to dissociate octamers into dimers (Wyss *et al.*, 1992) resulted in the disappearance of the more cathodically migrating (octamer) band and in a concomitant increase in the more anodically migrating (dimer) band (Fig. 1A, lane 1). In addition, urea and detergents led to a disappearance of CK-activity at the starting point, which is due to CK remaining attached to mitochondrial remnants. However, this CK fraction had no influence on the measurement of octamer/dimer ratios, since after the addition of Triton X-100 to samples the spot at the starting point disappeared but the octamer/dimer ratio remained unchanged (results not shown).

Earlier studies have shown that the spontaneous conversion of octamers into dimers is very slow unless urea or substrates of the CK reaction are added (Wyss *et al.*, 1992). Since the isolation medium for mitochondria contained no CK substrates or other chemicals known to influence octamer/dimer transitions, a change in the octamer/dimer ratio during the isolation procedure is unlikely. In keeping with this conclusion, samples stored for several hours to days at 4°C revealed no significant change in the octamer/dimer ratio from the original (results not shown).

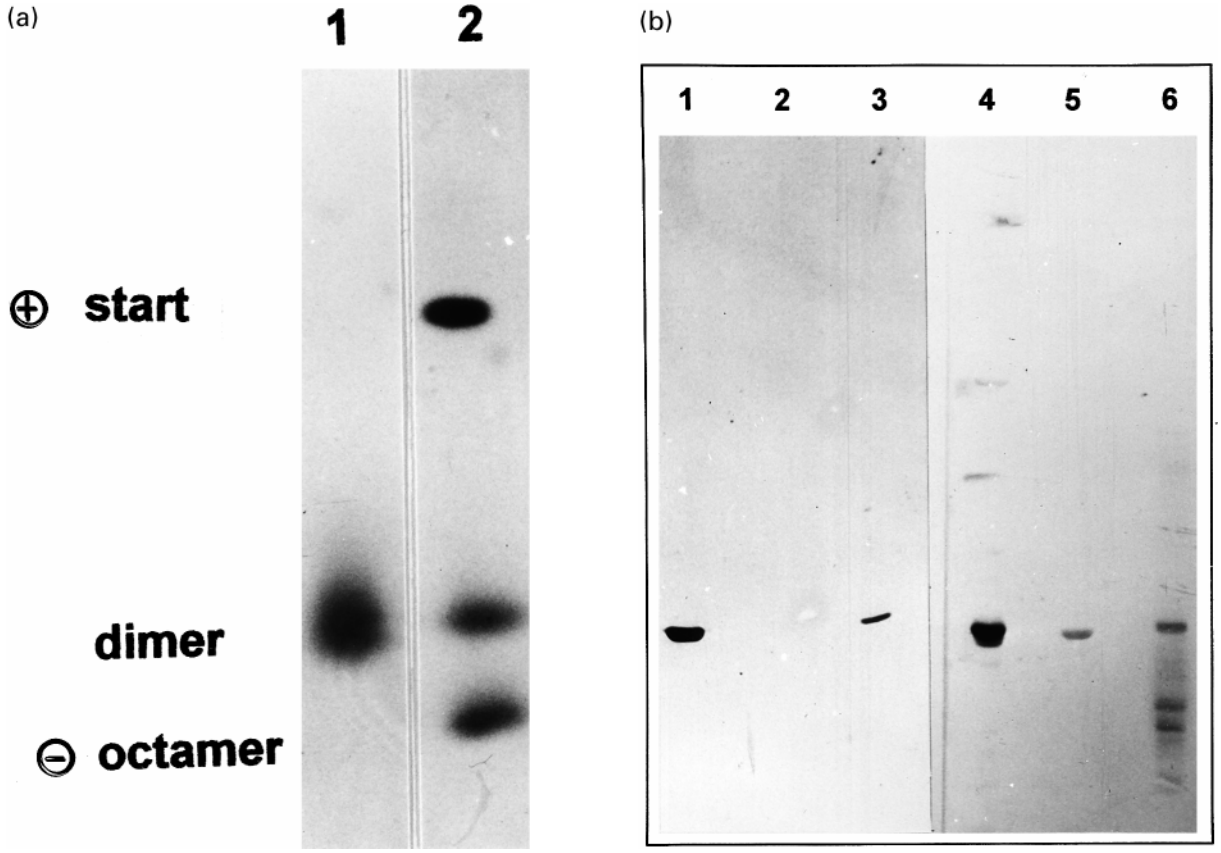

Figure 1 Cellulose polyacetate electrophoresis of Mi-CK oligomers from rat heart mitochondria. Mi-CK octamers and dimers were visualized by a CK activity-dependent colour reaction (see Materials and Methods). (a) Influence of urea on the oligomeric state of Mi-CK in rat heart mitochondria: lane 1, sample treated with 1.2 M urea for 1 h resulting in full conversion of Mi-CK octamers into dimers; lane 2, untreated control sample. (b) Western blot of an SDS-polyacrylamide gel loaded with purified MM-CK (lane 1), isolated rat cardiac mitochondria (lane 2), and heart muscle cytosol (lane 3), were immunostained with anti-chicken MM-CK antibody (1,2,3). Same loaded with isolated rat heart mitochondria (lane 4), purified cytosolic MM-CK (lane 5) and cardiac muscle cytosol (lane 6) after immunostaining with anti-chicken Mi-CK antibody. Note the absence of MM-CK in the preparations of rat heart mitochondria (lane 2), but the cross-reactivity of our rabbit anti-chicken Mi-CK antibody with purified MM-CK (lane 5).

Since cytosolic MM-CK may comigrate with dimeric Mi-CK in cellulose acetate electrophoresis under certain conditions, we examined the purity of our mitochondrial samples by SDS-PAGE and Western blot using polyclonal rabbit antibodies against chicken MM-CK or Mi-CK. In mitochondrial fractions, anti-Mi-CK antibody detected only one band, which corresponds to the size of Mi-CK monomers (Fig. 1B, lane 4), whereas no MM-CK contamination was detectable with anti-MM-CK antibody (Fig. 1B, lane 2). The latter antibody reacted perfectly, however, with pure MM-CK (Fig. 1B, lane 1) and our cytosolic fraction (Fig. 1B, lane 3). In this setup the anti-Mi-CK antibody showed some cross-reaction with cytosolic MM-CK (Fig. 1B, lanes 5,6). These experiments demonstrate that cellulose acetate electrophoresis is well suited to discriminate between Mi-CK dimers and octamers in mitochondrial fractions. Furthermore, the relative amounts of both oligomers can be obtained by scanning the electrophoresis strips, since the dependency of spot intensity (corresponding to enzyme activity) on protein concentration was the same for octamers and dimers (results not shown). This is in accordance with a previous report showing that both Mi-CK oligomers display very similar specific activities (Kaldis and Wallimann, 1995).

Mitochondria from Langendorff-perfused rat hearts

Control hearts perfused in the Langendorff mode displayed a rate pressure product of $18\,100 \pm 1100$ mmHg/min. Ischemic hearts stopped beating. Compared to control hearts, they showed a significant decrease in the octamer/dimer ratio and the specific activity of Mi-CK as well as in the respiratory control ratio of mitochondria

Table 1 Influence of ischemia on the activity and octamer proportion of Mi-CK in isolated perfused rat hearts.

	Control (10)	Ischemia (7)
Respiratory control ratio	7.0 ± 0.4	3.2 ± 0.5*
Mi-CK activity	717 ± 64	412 ± 44*
Octamer proportion (%)	90 ± 4	79 ± 3*

Mi-CK activity in mU/mg mitochondrial protein. The respiratory control ratio was determined in the absence of $MgCl_2$.
* Significantly different from control, $P<0.05$.

(Table 1). Reperfusion of ischemic hearts for 30 min had no further significant influence on the octamer/dimer ratio of Mi-CK (results not shown).

Mitochondria from infarcted and non-infarcted areas of LAD rats

Three weeks after surgery, rats subjected to coronary ligature displayed functional impairment (measured *in situ*) when compared to sham-operated controls (left ventricular dp/dt_{max}: ligature 5900 ± 750; sham 9860 ± 500 mmHg/s; left ventricular end diastolic pressure: ligature 14 ± 2; sham 3 ± 1 mmHg).

Like in ischemic Langendorff rat hearts, a significant decrease was observed in the octamer/dimer ratio of Mi-CK, in the specific Mi-CK activity and in the respiratory control ratio of mitochondria isolated from the zone surrounding the infarction center as compared to the remaining ventricular tissue (rest) in LAD rats (Fig. 2, Table 2). However, the changes were more pronounced than in mitochondria from Langendorff-perfused ischemic hearts. There were no differences in the measured parameters between the remaining ventricular tissue from LAD rats and ventricular tissue from sham-operated control rats.

Effect of peroxynitrite on the octamer/dimer ratio of pure Mi-CK

The octamer content of native Mi-CK is dependent on protein concentration, pH and temperature. Under our experimental conditions the absolute octamer content was 65%; this value was taken as reference value and set to 100% (Fig. 3). Overnight incubation of octameric Mi-CK with PN led to a spontaneous dissociation into dimers not seen in controls incubated without PN. While the octamer

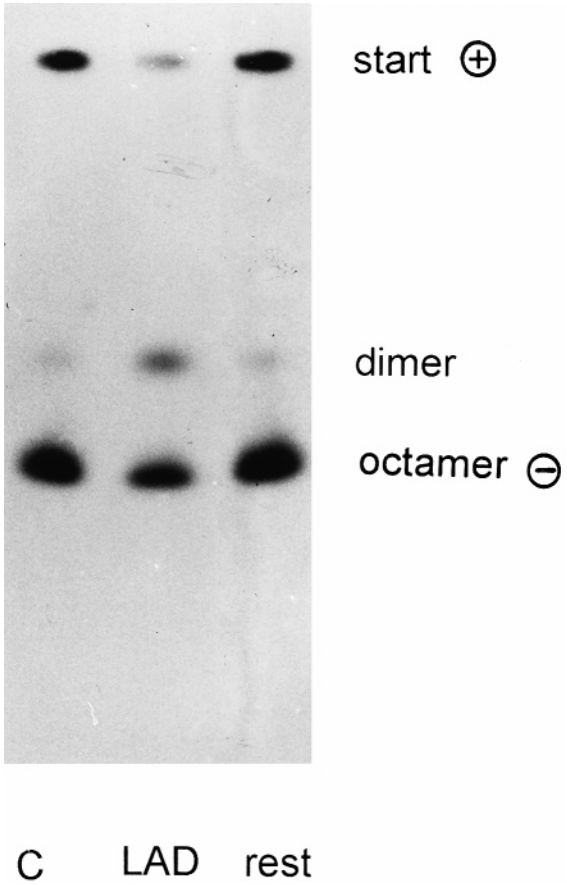

Figure 2 Effect of ligation of the left anterior descending coronary artery on the proportions of Mi-CK octamers and dimers in rat heart mitochondria. LAD, mitochondria isolated from the infarcted area; rest, from the non-infarcted area; c, from sham-operated rats.

Table 2 Influence of LAD infarction on the activity and octamer proportion of Mi-CK in rat ventricular tissue.

	Control (20)	Infarcted area (10)	Rest area (10)
Respiratory control ratio	6.7 ± 0.2	5.2 ± 0.6†	6.4 ± 0.3*
Mi-CK activity	639 ± 53	359 ± 48†	577 ± 71*
Octamer proportion (%)	83 ± 2	61 ± 5†	82 ± 2*

$n = 4–8$.
* Not significant.
† Significantly different from control, $P<0.05$. For further details see Table 1.

content was still 95% at 50 μM PN, it decreased to 80% at 100 μM PN and to only 34% at 1000 μM PN (Fig. 3).

Similar to the initial octamer content (see above), the reoctamerization rate of TSAC-dissociated, dimeric Mi-CK depends on different factors (Gross

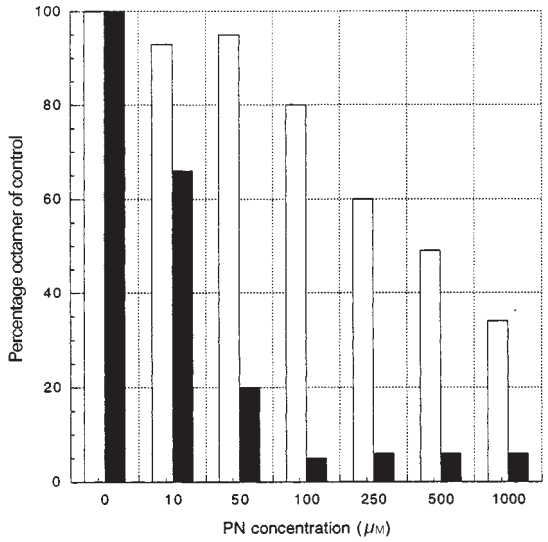

Figure 3 Influence of peroxinitrite on octamer-dimer transition of isolated Mi-CK. Octameric or dimeric Mi-CK was treated with PN and incubated at RT overnight to reach equilibrium or reoctamerized by addition of EDTA and incubation overnight, respectively. The octamer content of the samples was determined by gel permeation chromatography. (□) PN-treated octamer; (■) PN-treated dimer after reoctamerization.

and Wallimann, 1993). Under our experimental conditions the control incubated without PN octamerized to a final octamer content of 40%; this value was set to 100% and the dimer samples treated with PN were compared to the latter (Fig. 3). A PN concentration as low as 10 μM was sufficient to reduce the final octamer content to 66%. At concentrations above 100 μM PN, almost no reoctamerization of Mi-CK dimers was observed (Fig. 3).

Discussion

A growing body of evidence supports the hypothesis that the octamer is the true functional unit of Mi-CK *in vivo* and that dissociation of the octamer leads to impairment of Mi-CK function, especially creatine stimulation of oxidative phosphorylation, channeling of PCR out of the mitochondria, and protection of the mitochondrial permeability pore opening (O'Gorman *et al.*, 1997; Khuchua *et al.*, 1998). In this study, we demonstrate that Mi-CK octamers are destabilized during heart disease *in vivo* as well as by radical treatment *in vitro*. This is the first report showing an influence of such factors on octamer/dimer ratios.

Ischemia significantly decreases the octamer proportion of Mi-CK in both animal models: the isolated, Langendorff-perfused ischemic rat heart and the infarcted rat heart *in vivo*. The decrease is more pronounced in the heart *in vivo* 3 weeks after infarction as compared to the perfused heart after 30 min of ischemia. Since controls show a very high octamer content and transition from the octameric state of Mi-CK to the dimeric one is a very slow process (Gross and Wallimann, 1993), the measured octamer/dimer ratios should reflect the *in vivo* situation and should not be influenced by sample preparation. Octameric Mi-CK in intact mitochondria is remarkably stable, remaining at about 90% even after 1 h of anoxic incubation or incubation with inhibitors of mitochondrial ATP-synthesis (results not shown). Therefore, it appears reasonable to assume that it is not the lack of energization of mitochondria that is causing dimerization of Mi-CK.

Most of the factors known to dissociate Mi-CK octamers efficiently *in vitro*, like, for example, extreme pH, dilution or stabilization of the transition state of the enzyme, are not very likely to occur during or after ischemia. A recent study, however, points to PN as a potential cellular trigger of octamer destabilization. Physiological concentrations of PN very efficiently target Mi-CK *in vitro* as well as in intact mitochondria, reduce enzymatic activity and induce partial dissociation of Mi-CK octamers (Stachowiak *et al.*, 1998a). PN is the reaction product of NO and superoxide anions ($O_2^{\bullet -}$). Both ROS are generated especially during phases of ischemia and reperfusion in mitochondria due to the activity of mitochondrial nitric oxide synthase (NOS) (Ghafourifar and Richter, 1997) and oxygen reduction in the respiratory chain. NO and other ROS were shown to inactivate cytosolic MM-CK irreversibly (Mekhfi *et al.*, 1996; Konorev *et al.*, 1998) and to decrease cardiac work (Schulz *et al.*, 1997) and contractile reserve (Gross *et al.*, 1996). In our study, mitochondria isolated from the area surrounding the LAD-infarction center were less well coupled and showed decreased Mi-CK activity. This is consistent with damage of mitochondria by high concentrations of ROS.

To clarify the influence of PN on Mi-CK octamer/dimer ratios, we performed a series of experiments *in vitro*. Pure, recombinant Mi-CK was treated with PN levels comparable to the *in vivo* situation (Radi *et al.*, 1991; Beckmann *et al.*, 1994). Our results show that octameric Mi-CK, if allowed to reach thermodynamic equilibrium overnight, is destabilized by PN in a dose-dependent fashion, leading to a significant dissociation of the octamers into dimers at concentrations between 250 and 1000 μM PN. Even more pronouncedly, dimeric Mi-CK pretreated with PN is clearly inhibited in its

octamerization capability. The PN treatment affects Mi-CK dimers much more than octamers, since pretreatment of dimers with 100 μM PN was already sufficient to block reoctamerization.

The results obtained with PN treatments *in vitro* are consistent with the observations made in diseased heart. In the case of the acute model, using excised hearts perfused *in vivo*, the experimental period for production and action of PN is limited to 30 min. Since dimerization is a relatively slow process, this time span leads to an only moderate octamer dissociation. For the same reason, reperfusion for 30 min did not cause significantly more damage (not shown).

By contrast, in the chronic model, where ischemia is applied *in situ* for 1 h and the heart excised after 3 weeks, damage can accumulate for a much longer time span. Radicals may be produced in the infarcted area not only during acute ischemia and upon reoxygenation, but also later due to damage of mitochondria or the fine structure of blood vessels. A delayed, slow progression of irreversible damage is known in cerebral as well as myocardial ischemia and is probably due to increased expression of NOS isoforms (Iadecola *et al.*, 1996; Fukuchi *et al.*, 1998). Although considered to be a constitutive enzyme, upregulation of endothelial NOS (eNOS) (Fukuchi *et al.*, 1998) has been reported after ischemia. Even more relevant for our data, a delayed and persistent upregulation of inducible NOS (iNOS) has been detected in the heart after ischemia (Dudek *et al.*, 1994; Wildhirt *et al.*, 1997; Fukuchi *et al.*, 1998) and during inflammatory response (Ungureanu-Longrois *et al.*, 1995). Under these conditions NO is the triggering factor for PN-mediated oxidative damage finally leading to necrosis and apoptosis (Suzuki *et al.*, 1996; Shimizu-Sasamata *et al.*, 1998). In our chronic model of heart ischemia, as a consequence of such a delayed radical production not only Mi-CK octamer decay would be induced, but also octamerization of newly synthesized Mi-CK dimers could be inhibited.

Mi-CK is nucleus-encoded and imported into mitochondria most likely in the form of unfolded monomers which then dimerize and octamerize in the intermembrane space. During this process, dimeric Mi-CK may be hit by chronic low-dose PN which is efficient at preventing octamerization at 100 μM. Since mitochondrial proteins are rather long-lived with half-lives of up to 20 days (Saikumar and Kurup, 1985), accumulation of damage measured after 3 weeks would lead to a significantly lower octamer/dimer ratio of Mi-CK as compared with the acute ischemic model.

Taken together, these findings suggest a relationship between ischemia, PN production, modification of Mi-CK octamers, octamer dissociation and impaired mitochondrial energy metabolism.

In conclusion, our data show that ischemia significantly decreases the Mi-CK octamer content in heart *in vivo*, a process very likely impairing mitochondrial energy metabolism as recently demonstrated with mutant Mi-CK in cardiomyocytes (Khuchua *et al.*, 1998). Since ischemic conditions also lead to increased acute or chronic production of PN radicals, our *in vitro* data obtained with pure Mi-CK support the idea of radical damage being responsible for the diminished octamer/dimer ratio.

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