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Differential Effects of Peroxynitrite on Human Mitochondrial Creatine Kinase Isoenzymes

INACTIVATION, OCTAMER DESTABILIZATION, AND IDENTIFICATION OF INVOLVED RESIDUES*

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Creatine kinase isoenzymes are very susceptible to free radical damage and are inactivated by superoxide radicals and peroxynitrite. In this study, we have analyzed the effects of peroxynitrite on enzymatic activity and octamer stability of the two human mitochondrial isoenzymes (ubiquitous mitochondrial creatine kinase (uMtCK) and sarcomeric mitochondrial creatine kinase (sMtCK)), as well as of chicken sMtCK, and identified the involved residues. Inactivation by peroxynitrite was concentration-dependent and similar for both types of MtCK isoenzymes. Because peroxynitrite did not lower the residual activity of a sMtCK mutant missing the active site cysteine (C278G), oxidation of this residue is sufficient to explain MtCK inactivation. Mass spectrometric analysis confirmed oxidation of Cys-278 and further revealed oxidation of the C-terminal Cys-358, possibly involved in MtCK/membrane interaction. Peroxynitrite also led to concentration-dependent dissociation of MtCK octamers into dimers. In this study, ubiquitous uMtCK was much more stable than sarcomeric sMtCK. Mass spectrometric analysis revealed chemical modifications in peptide Gly-263–Arg-271 located at the dimer/dimer interface, including oxidation of Met-267 and nitration of Trp-268 and/or Trp-264, the latter being a very critical residue for octamer stability. These data demonstrate that peroxynitrite affects the octameric state of MtCK and confirms human sMtCK as the generally more susceptible isoenzyme. The results provide a molecular explanation of how oxidative damage can lead to inactivation and decreased octamer/dimer ratio of MtCK, as seen in neurodegenerative diseases and heart pathology, respectively.

Creatine kinases (CK)[1] are key enzymes in energy metabolism by catalyzing the reversible transphosphorylation of creatine by ATP to yield phosphocreatine (PCr) and ADP. The CK system is present in cells with high and fluctuating energy demands, such as skeletal and cardiac muscle, brain, and other neuronal tissues, where a mitochondrial and a cytosolic isoform are coexpressed (for reviews, see Refs. 1–4). In striated muscle, cytosolic muscle-type CK is expressed together with sarcomeric mitochondrial CK (sMtCK), whereas in brain and many other tissues, the cytosolic brain-type CK is found together with ubiquitous mitochondrial CK (uMtCK). In contrast to the exclusively dimeric cytosolic CK, the mitochondrial isoenzymes (MtCK) can form cube-like octameric molecules (5, 6). MtCK is bound to the outer leaflet of the inner mitochondrial membrane and located in the intermembrane space, where it cross-links both mitochondrial membranes, forming contact sites (7, 8), as well as along the cristae membranes (9). In contact sites, MtCK forms functional complexes with the adenylate translocator of the inner and porin of the outer mitochondrial membrane, as evidenced, for example, by creatine stimulation of mitochondrial respiration (10) or plasmon resonance spectroscopy (11). In mainly oxidative tissue, this microcompartment facilitates the vectorial transport of high-energy phosphate from the sites of energy production in the mitochondrial matrix to cytosolic sites of energy consumption. PCr is generated by MtCK using mitochondrial ATP (for review see Ref. 12) and transported into the cytosol, where ATP pools are replenished by the reverse reaction of cytosolic CK, which is partially located in the vicinity of cellular ATPases. The octameric structure of MtCK was shown to be crucial for this transport function of the "PCr-shuttle," because reduced octamer stability of N-terminally mutated MtCK (13) transfected into rat neonatal cardiomyocytes resulted in decreased creatine-stimulated mitochondrial respiration (14).

Mitochondrial sMtCK and uMtCK isoenzymes are highly homologous, sharing about 85% of identical amino acids, a further 5% of conservative replacements (15) and the same overall fold in their molecular structure (6, 16). The active site in particular is highly similar in all CKs and even in the other related guanidino kinases (17, 18). Despite this homology, human MtCK isoenzymes differ in many properties, including substrate affinity, substrate binding synergism, membrane interaction, and octamer stability (19, 20).

MtCK is known to be very susceptible to oxygen radical damage (21). Nitric oxide inhibits creatine kinase activity in solution as well as in adult rat ventricular myocytes. This inhibition can be reversed by the addition of dithiothreitol (22). In contrast, the inhibition of CK by superoxide anions (O_2^-) (23, 24) and peroxynitrite ($ONOO^-$) is irreversible (25, 26). Inactivation of cytosolic and mitochondrial CK would interrupt the "PCr-shuttle" and would have severe effects on the energetics of work performance and Ca^{2+} homeostasis in muscle similar to those seen in double-knockout mice lacking both the

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[1] The abbreviations used are: CK, creatine kinase; PCr, phosphocreatine; MtCK, mitochondrial creatine kinase; sMtCK, sarcomeric mitochondrial creatine kinase; uMtCK, ubiquitous mitochondrial creatine kinase; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight.

cytosolic and mitochondrial CK (27). $ONOO^-$ is the product of the nearly diffusion-controlled reaction between NO and O_2^-, both of these compounds being produced by mitochondria (28, 29). Because MtCK is located near the production site of $ONOO^-$, a powerful oxidant, MtCK represents a prime target for $ONOO^-$-induced damage (30). $ONOO^-$ is known to play a role in cells under conditions of oxidative stress, as is the case in heart disease and after ischemia/reperfusion (31, 32), as well as in certain neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease (for review, see Ref. 33). All these pathologies are known to show a compromised CK system (34–38). Brain-type CK was identified as a specific target of protein oxidation in Alzheimer's disease (39). Several reactions of $ONOO^-$ with amino acid side chains are described in the literature, including nitration of tyrosine (40, 41) and tryptophan (40, 42), as well as oxidation of methionine (43, 44) and cysteine (45). Formation of nitrotyrosine serves as a marker for $ONOO^-$-induced damage in tissues (for review, see Ref. 46).

In the present study, we addressed the question of whether $ONOO^-$ administered *in vitro* has differential effects on enzymatic activity and octamer stability of the two human MtCK isoenzymes. Divergent properties of human sMtCK and uMtCK have already been reported (19, 20, 47) and may be relevant to the different pathologies in heart and neuronal tissue, where an impaired CK system is involved. For a molecular description of $ONOO^-$-induced MtCK damage, mass spectrometry was applied to identify those amino acid residues that are modified by $ONOO^-$.

EXPERIMENTAL PROCEDURES

Chemicals—All enzymes and coenzymes were obtained from Roche Molecular Biochemicals (Rotkreuz, Switzerland), α-cyano-4-hydroxycinnamic acid was from Aldrich (Buchs, Switzerland), and 2,5-dihydroxybenzoic acid was from Fluka (Buchs, Switzerland). Further chemicals were purchased from standard suppliers and were of the highest purity commercially available. Human MtCK isoforms (19) and chicken sMtCK were heterologously expressed according to a protocol described previously (48). $ONOO^-$ was a generous gift from Prof. W. Koppenol (Laboratorium für Anorganische Chemie, ETH Zürich, Switzerland).

$ONOO^-$ Administration—The concentration of $ONOO^-$ stock solution, synthesized from the reaction of gaseous NO with solid potassium superoxide (49), was determined photometrically at 302 nm in 10 mM NaOH (ϵ_{m_m} = 1.67) before use. $ONOO^-$, in a volume of 20 ml of 10 mM NaOH, was added to 200 μl of protein solution containing 0.5 mg/ml MtCK in 100 mM Na_3PO_4, 150 mM NaCl, pH 7.2, to reach final $ONOO^-$ concentrations between 1 and 1000 μM. To the control, 20 μl of NaOH but no $ONOO^-$ was added, which led to a pH-shift in the protein solution by <0.05 pH-units.

Determination of Octamer Content—The oligomeric state of MtCK was determined by gel permeation chromatography on a Superose 12 column (Pharmacia) at room temperature in running buffer (50 mM Na_3PO_4, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.2 mM EDTA, pH 7.0) at a flow rate of 0.8 ml/min. Peak areas in the elution profile were quantified by graphical integration using Biocad Sprint HPLC software (Applied Biosystems, Foster City, CA).

CK Activity Determination—The specific CK activities were assayed photometrically in the reverse reaction, using the glucose-6-phosphate-dehydrogenase/hexokinase-coupled enzyme assay as described previously (19) at room temperature (22 °C).

Tryptic Digest of MtCK—$ONOO^-$-treated MtCK, as well as native MtCK, were dialyzed against 50 mM NH_4HCO_3 and 20 mM methylamine, pH 8.0, before adding urea to a final concentration of 1 M. The proteins were digested for 16 h at 37 °C by adding trypsin in an enzyme-to-protein ratio of 1:100. The reaction was stopped by freezing the samples.

Mass Spectrometric Analysis—Mass spectra were performed on a Voyager-DE Elite MALDI-TOF (Applied Biosystems) in positive ion reflector mode, using either α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid in a 2:1 mixture of acetonitril, 0.1% trifluoroacetetic acid in H_2O. For sample preparation, 0.5 μl of the digested protein solution was mixed without further preparation with 0.5 μl of the matrix solution directly on target and the solution was air-dried. Post-source decay spectra were recorded on an Axima-CFR MALDI-TOF (Kratos/Shimadzu, Manchester, UK) with a curved field reflection.

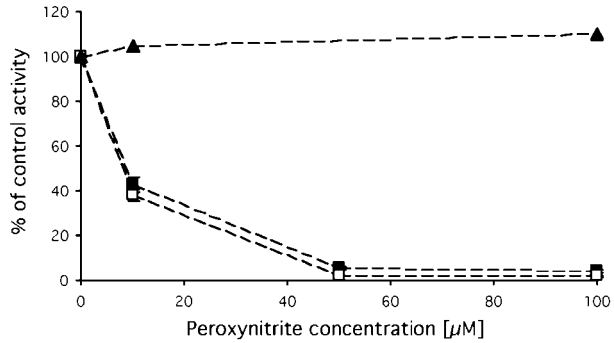

FIG. 1. **Dose response of peroxynitrite-induced inactivation of human MtCK isoenzymes.** Enzymatic activity of human uMtCK (*filled squares*), human sMtCK (*open squares*), and chicken sMtCK C278G mutant (*filled triangles*) is expressed in percentage of control activity to allow direct comparison of inactivation patterns. Note that in absolute terms, mutant C278G has a residual CK activity of ~3 to 5% compared with wild-type enzyme. IC_{50} values were determined by graphical estimation. Data are given as mean value of four independent experiments; *error bars* are within the boundaries of symbols. Note that both human MtCK isoenzymes show very similar inactivation kinetics by peroxynitrite, whereas the C278G active site cysteine mutant is resistant toward this reagent.

RESULTS

Inactivation of MtCK by Peroxynitrite—The sensitivity of human sMtCK and uMtCK toward $ONOO^-$ was first compared with respect to enzymatic activity. Activity of both human MtCK isoenzymes, as determined immediately after $ONOO^-$ administration, decreased in a dose-dependent fashion at already very low $ONOO^-$ concentrations (Fig. 1). The IC_{50} was determined by graphical estimation to be about 8 μM. This inactivation was not reversed by subsequent addition of 20 mM dithiothreitol, which reduces disulfides and reverses *S*-nitrosylation (22).

Target of Peroxynitrite in the MtCK Active Site—Cysteine 278 is a highly conserved residue in the active site of CK, which is known to be very reactive and essential for substrate binding and synergism in all guanidino kinases (18, 50). To test whether MtCK inactivation was caused by oxidation of this residue, a glycine replacement mutant (C278G) of chicken sMtCK (50) was treated with $ONOO^-$ in the same manner. Chicken sMtCK is almost identical to human sMtCK (97% sequence identity) and shows exactly the same dose-response curve for $ONOO^-$-inactivation. The residual enzymatic activity of the C278G mutant, which is about 3–5% compared with wild type depending on the assay conditions, was completely unaffected by $ONOO^-$ (Fig. 1).

Octamer Dissociation of MtCK by Peroxynitrite—In parallel experiments, sMtCK and uMtCK solutions with an initial octamer content of more than 80% were treated with increasing concentrations of $ONOO^-$ and kept overnight at room temperature to reach a stable octamer-dimer equilibrium. Subsequent gel permeation chromatography was performed to determine the oligomeric state of the isoenzymes as a function of peroxynitrite concentration (Fig. 2). Although $ONOO^-$ administration destabilized both MtCK isoenzymes and favored their dissociation into dimers, uMtCK was significantly more stable than sMtCK. Whereas for the latter, a concentration of only about 240 μM $ONOO^-$ was sufficient to dissociate 50% of the initial octamer concentration into dimers (C_{50} value), this concentration was about 790 μM for the brain-type uMtCK (Fig. 2).

MALDI-TOF Analysis of Peroxynitrite-modified MtCK Residues—To further identify the MtCK residues modified by $ONOO^-$, human uMtCK, human sMtCK, and chicken sMtCK

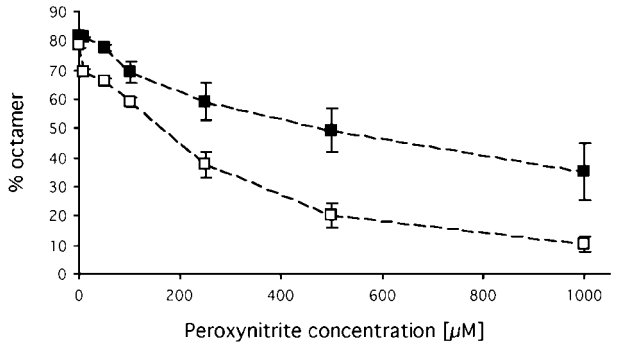

FIG. 2. **Dose response of peroxynitrite-induced octamer dissociation of human MtCK isoenzymes.** Octamer content of human uMtCK (*filled squares*) and sMtCK (*open squares*) as measured by integration of elution peaks from high performance liquid chromatography gel filtration chromatography. C_{50} values were determined by graphical estimation. Data are given as mean ± S.E. of three independent experiments. Note that in terms of octamer destabilization by $ONOO^-$, human sMtCK is much more susceptible than human uMtCK.

were treated with increasing concentrations of $ONOO^-$ as before and, after exhaustive digestion by trypsin, subjected to mass spectrometric analysis by MALDI-TOF. Chemically modified peptides of $ONOO^-$-treated MtCK were identified by their higher molecular mass compared with peptides derived from untreated control protein. Fig. 3 summarizes all peptides that could be unambiguously identified by mass spectrometry in the native, as well as the $ONOO^-$-treated human isoenzymes (highlighted in *gray* in Fig. 3*A*), aligned with the amino acid sequences. Some theoretically predicted fragments that could harbor putative $ONOO^-$-sensitive residues (Fig. 3*A*, *bold capital letters* in *white* areas) were not covered by the mass spectrometric analysis. However, we did not pursue their identification, because they are neither located in the active site or at the dimer/dimer interface nor have any other known functions for the enzyme been attributed to them. From the MtCK peptides recovered by MALDI-TOF, only three were modified by $ONOO^-$ treatment (Fig. 3*A*, highlighted in *black* with residues marked by *arrowheads*): (i) peptide Gly-263–Arg-271 containing two tryptophans, Trp-264 and Trp-268, (ii) peptide Leu-272–Arg-287 containing the active site Cys-278, and (iii) peptide Ser-340–Arg-360, including the C-terminal Cys-358. The cysteine residues in the latter two fragments were modified by single and double oxidation. Thus, combined with results described above for the C278G mutant, modification of Cys-278 is sufficient to explain the loss of enzymatic MtCK activity upon peroxynitrite treatment.

The modified peptide Gly-263–Arg-271 is located at the dimer/dimer interfaces of the octameric MtCK isoenzymes, as revealed by the x-ray structures of sMtCK (6) and uMtCK (16). All mass shifts occurring with this peptide (Fig. 4) can be explained by reactions of $ONOO^-$ with methionine and tryptophan. The difference of 16 Da would point to oxidation of methionine Met-267, whereas further mass shifts are caused by nitration of Trp-264 and/or Trp-268. The mass shift of 45 Da corresponds to nitration of a tryptophan residue, whereas the mass shift of 29 Da can be explained by the loss of one oxygen from the nitro group. This loss of oxygen can occur as a laser-induced photochemical decomposition, as has been shown for the similar aromatic side chain nitro-tyrosine (51). Finally, the mass shift of 61 Da can be explained by the nitration of one tryptophan plus the additional oxidation of methionine (Fig. 4). Because peptide Gly-263–Arg-271 contains only Trp-264 and Trp-268, but no tyrosine, this modification-pattern unambiguously identifies tryptophan nitration. All these latter modifications are at or near the dimer/dimer interface and show the same concentration dependence as octamer dissociation (compare Figs. 2 and 4). This strongly indicated that these modifications are involved in octamer dissociation. In fact, Trp-264 in particular has been identified by site-directed mutagenesis to be crucial for octamer stability (52). To identify which of the two tryptophans in the peptide Gly-263–Arg-271 was modified, we applied postsource decay MALDI-TOF with human sMtCK. When mass selection was set to pass the unmodified parent ion at a measured *m/z* of 1255 (calculated 1254.4), all b- and y-fragments could be detected, leaving no doubt about the identity of this peptide. However, postsource decay analysis of the nitration-modified parent ion did not reveal a single modified tryptophan. By contrast, we detected a b-ion (b_4) that is a unique fragment to the peptide modified at Trp-264 but also a y-ion (y_6) that is unique to the peptide modified at Trp-268, suggesting that both Trp-264 and Trp-268 are partially modified.

DISCUSSION

In this study, we show that both human mitochondrial CK isoenzymes are very sensitive to $ONOO^-$-induced damage but differ in part in the dose-dependence of the deleterious modifications. The concentration for half-maximal inactivation was 8 μM $ONOO^-$ for both sMtCK and uMtCK, whereas concentration for half-maximal octamer dissociation was only 240 μM $ONOO^-$ for sMtCK but was 790 μM for uMtCK. We could also identify the MtCK residues involved in $ONOO^-$ damage, namely active site Cys-278, as well as Met-267, Trp-264, and/or Trp-268 at the dimer/dimer interface.

Half maximal inactivation of human MtCK isoenzymes occurred at very low $ONOO^-$ concentrations (*i.e.* 8 μM), which corresponds to a $ONOO^-$:MtCK monomer-ratio of 0.7:1. This is in line with earlier results on chicken sMtCK, showing inactivation with an IC_{50} of 35 μM (30); the slightly decreased IC_{50} value in our study could be attributable to species-specific differences or to the stronger buffering capacity used. Cytosolic muscle CK was reported to be inactivated with an IC_{50} of 2.5 μM $ONOO^-$ (26). However, their protein concentrations were much lower (0.4 μg/ml, compared with the 0.5 mg/ml used here), resulting in a significantly higher $ONOO^-$:protein ratio of about 50:1 at the IC_{50} value. It is well known that $ONOO^-$ added in a single bolus can decompose very quickly in water without reacting with the highly diluted protein. Our work has taken into account the high MtCK concentration in the mitochondrial intermembrane space and the limited stability of MtCK octamers below a concentration of 0.5 mg/ml (20).

In earlier studies, it was speculated that modifications of the highly reactive active site cysteine (Cys-278 in MtCK, Cys-283 in cytosolic CK) cause enzymatic inactivation (26, 30). Here, we show unambiguously the modification of this residue and its role in inactivation, using MALDI-MS analysis of $ONOO^-$-modified wild-type enzyme and activity measurements of a C278G replacement mutant. As shown previously, site-directed mutagenesis of Cys-278 leads to significant but not complete inactivation of the enzyme and, most importantly, to a complete loss of substrate synergism (50). This residue is located in the active site of MtCK (6), and its homologue in the transition state structure of the closely related arginine kinase is in direct contact with the reactive guanidino group of the arginine substrate (18). Mutagenesis studies and the CK x-ray structures identified another catalytically important residue in the active site of MtCK that can potentially be modified by peroxynitrite, the active site Trp-223 (6, 52). However, peptide Gly-211–Arg-231 containing this tryptophan showed no mass shift after $ONOO^-$ exposure, demonstrating that Trp-223 is not modified by $ONOO^-$, similar to inactivation by radiation (21). Because the active site is highly conserved throughout all CK isoen-

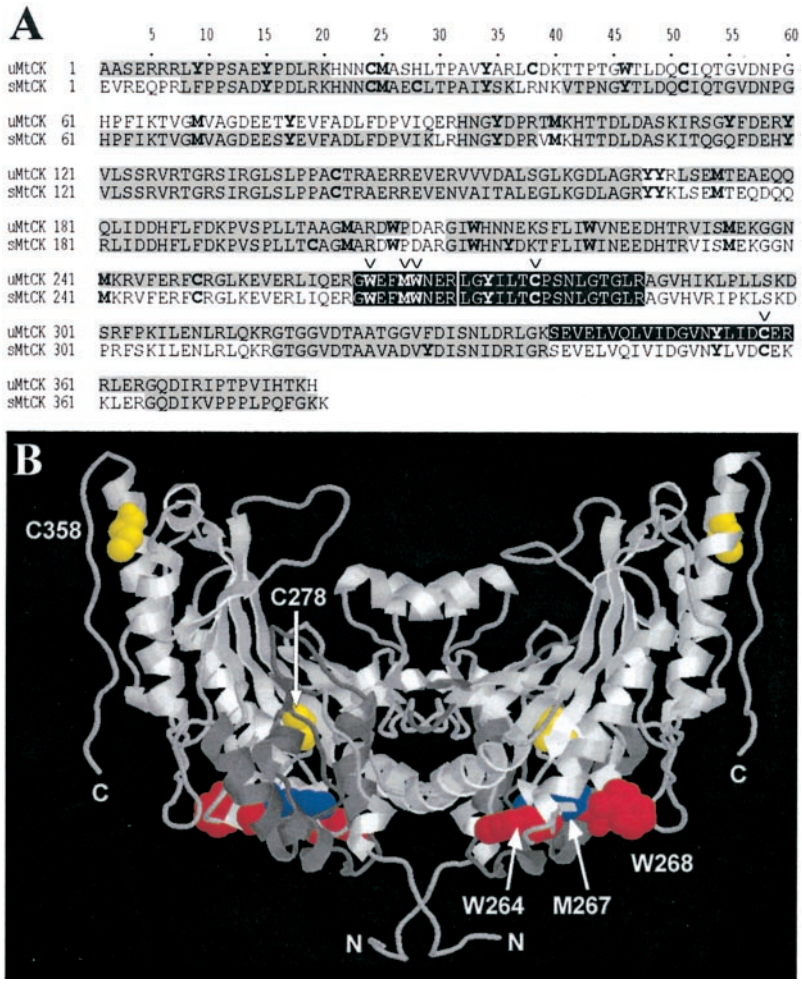

FIG. 3. **Peptides identified by MALDI-TOF in untreated and peroxynitrite-treated human sMtCK and uMtCK.** *A*, amino acid sequence alignment of human uMtCK and human sMtCK (15). Peptides recovered from both native unmodified and $ONOO^-$-treated protein are highlighted in *gray*; those peptides found to be modified by $ONOO^-$ are highlighted in *black*. Residues potentially susceptible to $ONOO^-$-induced damage are *bold*, and the modified residues identified by MALDI are marked by *arrowheads*. *B,* molecular structure of a dimer of human uMtCK (16), showing the spatial localization of $ONOO^-$-modified residues (prepared with Rasmol 2.6 (61)). The dimer/dimer-interface that would connect to neighboring dimers in the octameric structure is situated at the lower, convex side of the banana-shaped dimer. Trp-264 (*red*), Met-267 (*blue*) and to minor degree Trp-268 (*red*) are located at the dimer/dimer interface, Cys-278 (*yellow*) identifies the active site, and Cys-358 (*yellow*) is near the octamer face that binds to mitochondrial membranes. Sequences covered by MALDI-TOF analysis are in *white*. Note also the far-reaching N termini of uMtCK that are mainly responsible for higher octamer stability of uMtCK by providing additional polar interactions between the dimers (16, 20).

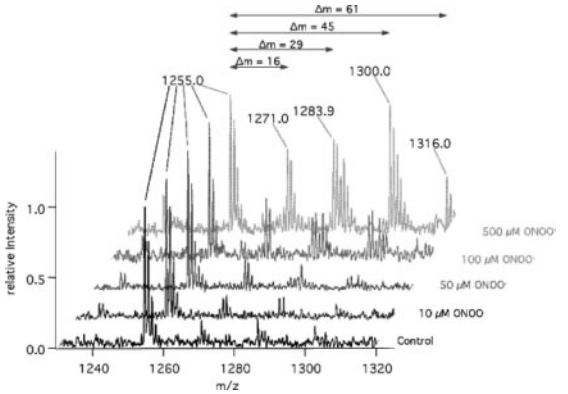

FIG. 4. **MALDI mass spectra of peptide Gly-263–Arg-271 treated with increasing peroxynitrite concentrations.** The molecular masses of species arising from this fragment at *m/z* 1255 due to modification by peroxynitrite are indicated at the *top*, and the difference from the unmodified fragment is given. The unmodified peak from each spectrum was standardized to a relative intensity of 1.0 to show the increases of additional peaks relative to the unmodified species. $ONOO^-$ concentrations are given to the *right*. Note that molecular mass shifts of 45 and 29 Da identify nitro-tryptophan, and the shift of 16 Da indicates methionine oxidation (see text and Ref. 51).

zymes and even the whole group of guanidino kinases (17, 18), it can be tacitly assumed that the active site cysteine is involved as the prime target of $ONOO^-$ in all these kinases. In addition, this homology also explains why there is no difference between the two human MtCK isoenzymes with regard to inactivation. Oxidation at the C-terminal cysteine Cys-358 may lead to reduced membrane binding of MtCK, as we have observed after anthracycline-induced oxidation of human MtCK *in vitro* (47). Anthracyclines are efficient cancer chemotherapeutics, but they also induce oxidative damage of proteins such as MtCK that may cause their well known cardiotoxic side-effects.

The octamer can be considered the physiological form of MtCK. Impaired octamer stability leads to reduced creatine stimulated mitochondrial respiration (14). We show here that $ONOO^-$ also destabilizes the octameric structure of MtCK, albeit at higher concentrations than necessary for inactivation and by affecting human sMtCK much more than human uMtCK. The higher octamer stability of uMtCK could be caused by reduced reactivity toward $ONOO^-$ or an intrinsically higher octamer stability compared with sMtCK. However, the latter mechanism is strongly supported by different observations. A very similar difference in octamer stability between human uMtCK and sMtCK was observed *in vitro* after incubation with transition state analogue complex (20), as well as after treatment with anthracyclines (47). Finally, an exact comparison of the x-ray structures (6, 16) and a direct biophysical approach (20) revealed that the mainly hydrophobic dimer/dimer interfaces of uMtCK are larger than those of sMtCK and contain additional polar interactions that preserve the octameric state of uMtCK. Peroxynitrite not only destabilizes the octameric structure, at much lower concentrations it already prevents formation of octamers from dimers as shown *in vitro* with chicken sMtCK (53). Thus, inactivation and dimerization of MtCK *in vivo* eventually occur simultaneously.

According to our mass spectrometric results, destabilization of octameric MtCK by $ONOO^-$ is caused by the chemical mod-

ification of peptide Gly-263–Arg-271, which is part of the dimer/dimer interface and responsible for hydrophobic stabilization of the MtCK octamer (6, 16). The modifications are clustered and involve oxidation of Met-267 and nitration of Trp-268 and/or Trp-264. Trp-264 is a key residue in the hydrophobic dimer/dimer interaction patch, as revealed by x-ray crystallography (6) and replacement mutants leading to octamer destabilization (52), but modifications rendering the nearby residues such as Trp-268 more polar may also contribute to destabilization. Both tyrosine residues present in the dimer/dimer interface, Tyr-15 and Tyr-34 (6), were not modified by $ONOO^-$ at least in sMtCK, because the peptides containing these residues (Leu-8–Arg-19 and Lys-20–Lys-36, respectively) did not show any mass shift upon $ONOO^-$ treatment.

The CK system plays an important physiological role for cellular energetics in health and disease (for review, see Ref. 54). A compromised CK system, caused, among others, by oxygen radical damage to the enzyme, has been implicated in heart disease (34), as well as in many neurodegenerative diseases such as Huntington's disease (35), amyotrophic lateral sclerosis (36), and Alzheimer's disease (for reviews, see Refs. 33 and 37). In brains of persons with Alzheimer's disease, CK activity decreased, whereas mRNA levels did not change significantly (55, 56) and cytosolic brain-type CK was oxidatively modified (39, 57). Besides the integrity of the active site, the octameric state of MtCK seems also to be crucial for efficient vectorial channeling of high-energy phosphates from mitochondria to the cytosol (4, 14). In addition, octameric MtCK has been shown to stabilize mitochondria from going into permeability transition (58). Thus, a decrease in MtCK octamer content caused by $ONOO^-$ could interrupt the creatine/PCr-shuttle and also lead to early events of apoptosis (59). In animal models of short- and long-term ischemia-reperfusion damage, as well as diseased human heart, a significant decrease of the octamer/dimer ratio was observed (53). Some of the main pathological symptoms of these cardiomyopathies, but also of many neurodegenerative diseases, are caused by reactive oxygen and nitrogen species and involve mitochondria (33, 60). Therefore, it is highly probable that $ONOO^-$-induced modifications at the dimer/dimer interfaces of MtCK octamers, which we have observed *in vitro*, also occur *in vivo*. The fact that uMtCK is significantly more stable than sMtCK could explain why a decreased octamer/dimer ratio seems to be an early event in heart disease, whereas it may occur in brain and neuronal tissue only at a more advanced stage of neurodegeneration.

In summary, this study shows that $ONOO^-$ has severe effects on enzyme activity, as well as on the stability of human MtCK octamers, with sMtCK being more susceptible than uMtCK in the latter case. Among the modified amino acid side chains, we could identify some key residues, such as active site Cys-278 and dimer/dimer interface Trp-264. *In vivo*, molecular damage of MtCK would lead to an interruption of the creatine/PCr-shuttle and therefore to a lower cellular energy state, with all its far-reaching consequences.

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