THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 27, Issue of July 3, pp. 16694–16699, 1998
Printed in U.S.A.

Mitochondrial Creatine Kinase Is a Prime Target of Peroxynitrite-induced Modification and Inactivation*

(Received for publication, September 22, 1997, and in revised form, April 8, 1998)

Olaf Stachowiak‡, Max Dolder‡, Theo Wallimann‡§, and Christoph Richter¶

From the ‡Institute of Cell Biology, Swiss Federal Institute of Technology, ETH Zürich, CH-8093 Zürich, Switzerland and ¶Laboratory of Biochemistry I, ETH Zürich, CH-8092 Zürich, Switzerland

The reaction of peroxynitrite (PN) with sarcomeric mitochondrial creatine kinase (Mi_b-CK; EC 2.7.3.2) was observed at different stages of complexity (i) with purified Mi-CK, (ii) with enzyme bound on isolated mitoplasts, and (iii) within intact respiring mitochondria. Creatine-stimulated respiration was abolished by PN concentrations likely to be physiological and far before the respiratory chain itself was affected, thus demonstrating that Mi-CK is a prime target for inactivation by PN in intact mitochondria. The inactivation by PN of Mi-CK was reversed by 22% with 2-mercaptoethanol. More remarkable protective effects were noticed with the full set of CK substrates, *e.g.* 30 and 50% protection with MgATP plus creatine and MgADP plus phosphocreatine, respectively, but not with each substrate alone. These data indicate an involvement of the active-site Cys-278 residue of Mi-CK in this process. Furthermore, changes in endogenous tryptophan fluorescence intensity and spectral changes after reaction of Mi-CK with PN suggest additional modifications of Trp and Tyr residues. PN-inactivated Mi-CK can no longer be efficiently converted into dimers by incubation with reagents inducing a transition state analog complex at the active site. Thus, obviously, upon reaction of octameric Mi-CK with PN, the octamer-dimer equilibrium of Mi-CK is also affected. The consequences for cellular energy homeostasis and calcium handling are discussed.

Peroxynitrite ($ONOO^-$, PN[1]), the product of the reaction between nitrogen monoxide (NO) and the superoxide anion (O_2^-), reacts with lipids, aromatic amino acids, or metalloproteins, *e.g.* complex I, II, and III of the respiratory chain and aconitase (1,2) and is also a powerful oxidant of thiols. If the latter are vicinal, a disulfide can be formed (3,4). Another possible reaction is the nitration of tryptophan (5) and tyrosine residues favored at neutral pH and involving Fe^{3+} ions (6), the latter of which can form potent nitrating complexes in the presence of EDTA. A causal link between protein and enzyme nitration by PN and several neurodegenerative diseases as well as aging has been postulated. For example, some patients with amyotrophic lateral sclerosis (ALS) carry mutated Cu/Zn superoxide dismutase, leading to the stabilization of PN and thereby resulting in enhanced nitration of important target enzymes (7). Additionally, reactive oxygen species have been implicated in a variety of myocardial diseases, especially in ischemia/reperfusion injury, which is accompanied by free radical generation (8, 9). Nevertheless, PN also serves an important physiological function in macrophages, where it is present in high concentrations and helps to eliminate targets prone to degradation (10).

Earlier it was shown that the cytosolic muscle isoform of CK (MM-CK) is inactivated by H_2O_2 (11) and also that a decrease in heart mitochondrial Mi-CK activity was due to the latter reactive oxygen species (12). In a skinned cardiac muscle fiber assay, myofibrillar MM-CK was recently shown to be the main target of reactive oxygen species in the myofibrillar compartment (13), indicating that a decreased cytosolic CK activity induced by reactive oxygen species after ischemia and reperfusion could be of clinical/pathological significance. This is supported by the fact that inhibition of the CK reaction by iodoacetamide in isolated perfused rat heart induced serious alterations of cardiac function (14). The target of iodoacetamide- and reactive oxygen species-induced reactions on CK is likely to be the same highly reactive active-site cysteine (15, 16).

Perfusion of isolated rat heart with the NO donor *S*-nitrosoacetylcysteine led to a decrease in heart contractile reserve and inhibition of cardiac CK by 65%, which, however, was reversible *in vitro* after the addition of the reducing agent dithiothreitol, indicating that the "essential" cysteine 278 residue of CK was the possible target for nitrosothiol modification (17). These data indicate that CK is a target for NO or its congeners and accords with the fact that skeletal muscle MM-CK bound to the sarcoplasmic reticulum and also the CK-mediated Ca^{2+} uptake by the Ca^{2+}-ATPase are inhibited by the NO donor *S*-nitrosoglutathione, suggesting that nitrogen monoxides, including NO^+, NO·, and NO^-, inactivate CK, most likely by promoting nitrosylation of the critical sulfhydryl group(s) of the enzyme (18). The inactivation of creatine kinase in skeletal muscle was shown to be enhanced by high amounts of Fe^{3+} (19). In skeletal and heart muscle as well as in brain, mitochondria are the major source of potentially toxic oxygen-derived free radicals (20), especially after ischemia and reflow episodes. The newly discovered NO synthase isoform, which is indeed localized in mitochondria as suggested (21, 53, 54), would produce NO within the mitochondria, a notorious source of O_2^-. Mitochondria are therefore a major source of PN in the cell. In the present study, we investigated whether the mitochondrial CK isoform would be a vulnerable target of this free radical at different levels of complexity, (i) as purified enzyme, (ii) when

* This work was supported by an ETH graduate student grant (to O. S.), by Swiss National Science Foundation Grant 31-33907.92 (to T. W.), and by private sponsoring (Careal Holding, Zürich) (to M. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 0041-1-6333392; Fax: 0041-1-6331069; E-mail: walliman@cell.biol.ethz.ch.

[1] The abbreviations used are: PN, peroxynitrite; ALS, amyotrophic lateral sclerosis; CK, creatine kinase; Cr, creatine; PCr, phosphocreatine; IC_{50}, half-maximal inhibition constant; 2-ME, 2-mercaptoethanol; Mi_b-CK, sarcomeric mitochondrial creatine kinase; MM-CK, muscle isoform of creatine kinase; ROS, reactive oxygen species; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TSAC, transition state analog complex.

bound to isolated mitoplasts, or (iii) within respiring mitochondria.

EXPERIMENTAL PROCEDURES

Materials

PN was synthesized according to Koppenol *et al.* (23) and provided by Drs. J. S. Beckman and R. Kissner and stored at −80 °C. Its concentration was determined photometrically at 302 nm in 0.1 M sodium hydroxide (ϵ_{mM} = 1.67) daily before use. Buffers and salts were purchased from Sigma, and nucleotide phosphates were from Boehringer Mannheim. Chicken hearts were freshly obtained from a local poultry slaughterhouse and transported on ice.

Methods

Overexpression and Isolation of Mi-CK—Chicken sarcomeric mitochondrial creatine kinase (Mi_b-CK) was produced by overexpression of the pRF23 vector in the *Escherichia coli* strain BL21(DE3)pLysS. The protein (specific activity = 120 units/mg) was purified by a combination of Blue-Sepharose affinity chromatography and cation exchange chromatography on a Mono S HR5/5 fast protein liquid chromatography column (Amersham Pharmacia Biotech) as described by Furter and colleagues (24). Concentrated Mi_b-CK (5 mg/ml) was stored in Mono S elution buffer at −80 °C. CK activity was measured by the pH-stat assay (25) determining the rate of ATP formation (reverse reaction). For substrate protection assays, the Mi-CK forward and backward reaction was measured using a coupled optical test as described by Wallimann *et al.* (26). Protein concentration was determined using the Bio-Rad assay (27) using bovine serum albumin as standard.

Isolation of Mitochondria—Mitochondria from chicken heart were isolated by differential centrifugation after mechanical disruption of the tissue in isotonic buffer (MSH: 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, pH 7.4) as described by Kottke *et al.* (28). Mitoplasts were generated by swelling of isolated mitochondria in water for 5 min at 4 °C. Mitoplasts were sedimented, washed, and resuspended in the appropriate buffer/water.

Respirometry—Respiration measurements were performed with a Cyclobios oxygraph (A. Paar, Innsbruck, Austria) at 25 °C in 2 ml of MSH buffer containing 5 mM $MgCl_2$, 1 mM EGTA, 10 mM $NaP_{i,}$, and 5 μM rotenone. Mitochondria with a total protein content of 150 μg were used for each experiment. Peroxynitrite at a final concentration of 350 μM was administered to the mitochondria before succinate (final concentration 5 mM) was added to initiate state 4 respiration. Stimulation of oxidative phosphorylation was achieved by administration of 25 μM or 1 mM ATP (maximum state 3 respiration). Oxygen consumption was measured in the presence or absence of 17 mM creatine.

Fluorescence Measurements—Fluorescence emission spectra were obtained using a SPEX Fluorolog-2 instrument. Fluorescence intensity was measured with a single photon counter equipped with a Peltier cooling unit. The excitation wavelength was set to 287 nm (Mi-CK-Trp excitation maximum), and detection wavelength was varied from 300 to 500 nm, with 1-nm increments. The protein concentration was 0.15 μM. The excitation slit width was set to 0.7 nm, and the emission slit width was set to 1.7 nm. All measurements were carried out at 25 °C in a stirred 1-cm quartz cuvette put into a thermostated sample holder. The buffer used was 10 mM TES, 50 mM NaCl.

Peroxynitrite Administration—PN was diluted in 0.1 M NaOH and added under extensive stirring to the Mi-CK (or mitochondrial) solution. As a control, the same amounts of NaOH without PN were added. Mi-CK inactivation experiments were carried out in phosphate-buffered saline (150 mM NaCl, 10 mM NaP_i, pH 7.2), mitochondria were suspended in MSH buffer, mitoplasts in H_2O (ultrapure). Whenever possible, the presence of 2-mercaptoethanol (2-ME) in the buffer was avoided (it competes with protein SH groups for PN), except for respiration measurements, where a reducing environment was necessary to keep mitochondria active.

Octamer Dissociation—To achieve octamer dissociation, Mi-CK stock solution (>95% octamers) was incubated in TSAC mixture (transition state analog complex; final concentrations: 4 mM ADP; 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3) and the respective buffer at a protein concentration of 0.2 mg/ml for at least 2 h at 4 °C.

In detail, a solution of octameric Mi-CK (0.2 mg/ml) was made 500 μM in PN. After reaction for 5 min, TSAC reagents were added to one aliquot, and the mixture was left at 4 °C for 2 h before gel permeation chromatography. Untreated Mi-CK, Mi-CK treated with PN or TSAC reagents only, served as controls (see Fig. 8).

Gel Permeation Chromatography—The oligomeric state of Mi-CK was determined on a Superose 12 fast protein liquid chromatography column (Amersham) at 4 °C in running buffer (50 mM NaP_i, 150 mM NaCl, 0.2 mM EDTA, 2 mM 2-ME). The flow rate was 0.8 ml/min. Peak areas in the elution profile were quantified by graphical integration.

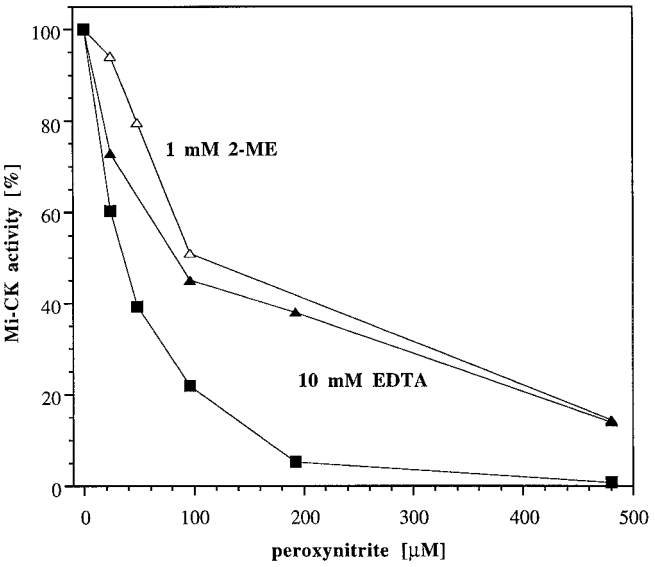

FIG. 1. **Bolus inactivation of purified Mi_b-CK with peroxynitrite.** Representative plots without protecting agent (*squares*) in the presence of 2-ME (*open triangles*) and in the presence of EDTA (*black triangles*) are shown. Mi-CK concentration was 0.4 mg/ml, and the specific activity was 120 units/mg. Peroxynitrite was taken from a 100 mM stock solution in 0.1 M NaOH. Inactivation was performed at 25 °C by adding up to 500 μM peroxynitrite (final concentration) to the Mi-CK solution (in phosphate-buffered saline). After 5 min, all inactivation mixtures were analyzed in the pH-stat for Mi-CK activity (23).

RESULTS AND DISCUSSION

The sensitivity of Mi-CK toward PN was tested at increasing levels of complexity. Purified Mi-CK (Fig. 1) was inactivated to more than 90% at concentrations of 200 μM PN, and the inactivation plot was monophasic with an IC_{50} of 35 μM. Inactivation was reduced by 10 mM EDTA (Fig. 1), likely because it competes for PN with the enzyme (EDTA can undergo oxidative decarboxylation). Preincubation of the enzyme with 1 mM 2-ME also significantly reduced Mi-CK inactivation (Fig. 1), indicating the involvement of sulfhydryl groups in the inactivation reaction, a phenomenon that also has been shown for the superoxide-induced inactivation of Mi-CK (12). A full protection of Mi-CK by 2-ME could not be achieved, showing a certain specificity of PN for target residues of the enzyme. Indeed, there was only a partial reactivation (regain of maximally 22% enzyme activity) of Mi-CK upon the addition of 2-ME to the PN-inactivated enzyme (Fig. 2), which also proved the irreversibility of some modifications. A most likely candidate out of the 8 sulfhydryl groups per Mi-CK monomer is Cys-278, which is conserved in all known CK sequences. This residue was shown to be highly reactive and may serve as a "hinge" between both substrate binding sites conferring synergistic substrate binding but is not directly involved in catalysis (16). However, a striking loss in enzymatic activity was observed when replacing the cysteine residue (Cys-278 is deprotonated in the active enzyme) with uncharged residues like glycine. The missing negative charge in the mutant enzyme can be partially replaced by Cl^- ions, which leads to a severalfold increase in activity as well as the replacement of the uncharged amino acid in the Cys-278 mutant by the corresponding negatively charged amino acid (16).

When PN was added to mitoplasts, the inactivation of Mi-CK was biphasic, with IC_{50} values of about 30 and 60 μM (Fig. 3). In the absence of the outer mitochondrial membrane, there is probably a partial release of tightly bound CK from the high

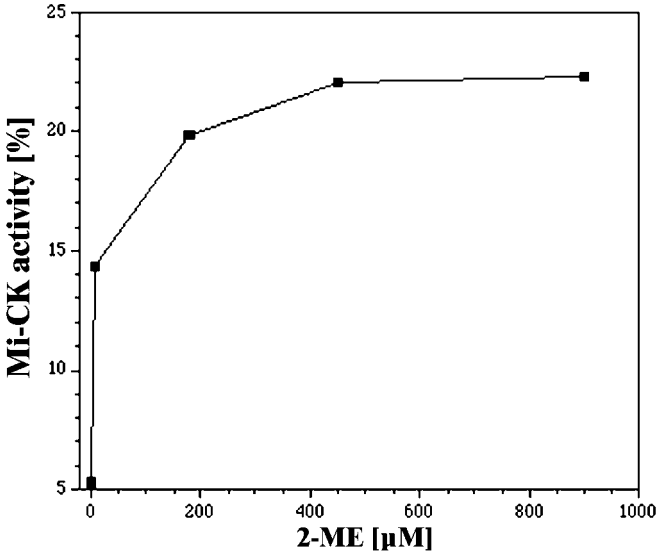

FIG. 2. **Reactivation of peroxynitrite-inactivated Mi-CK (0.4 mg/ml) with 2-ME after bolus addition of 500 μM peroxynitrite.** The maximum activity that could be restored on application of 2-ME was 22% of original CK activity.

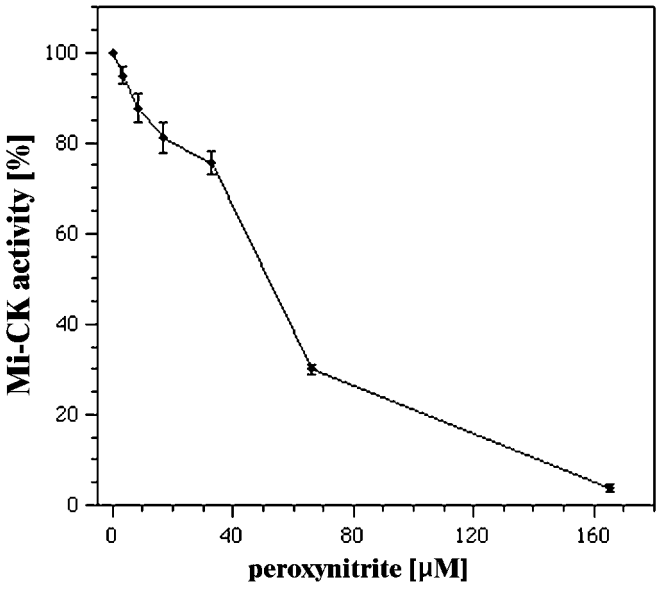

FIG. 3. **Inactivation of mitoplasts with peroxynitrite.** Mitoplasts solutions containing a total amount of 0.5 mg/ml mitochondrial protein were used for each inactivation experiment. *Error bars* represent S.D.

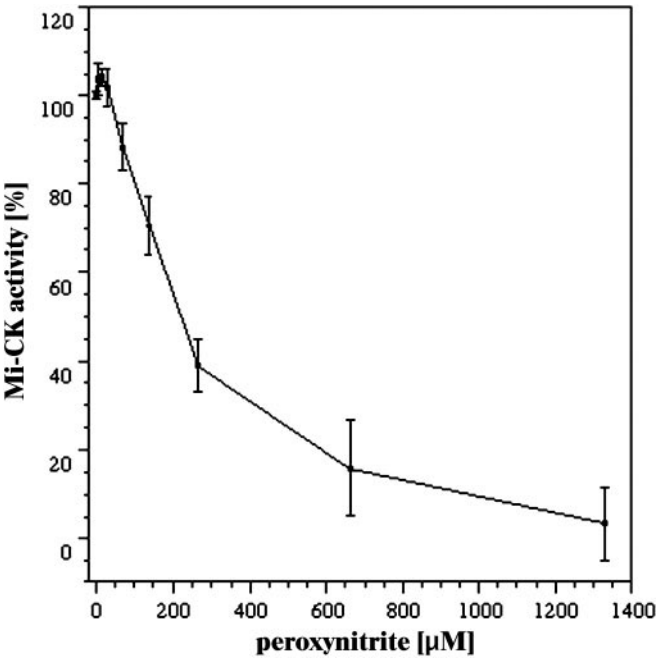

FIG. 4. **Inactivation of respiring mitochondria with peroxynitrite.** Mitochondrial solutions containing 0.4 mg/ml total protein were inactivated by bolus addition of peroxynitrite. Each inactivation was carried out separately; *error bars* represent S.D.

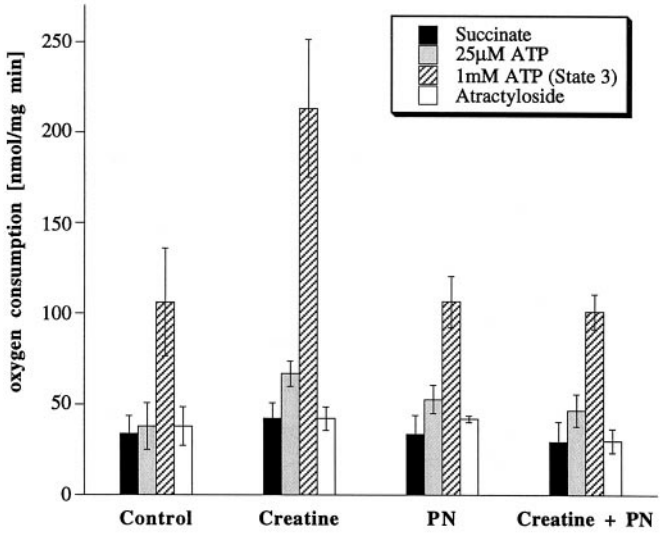

FIG. 5. **Respiration analysis of peroxynitrite-treated mitochondria and untreated control mitochondria.** Mitochondria were treated with 350 μM peroxynitrite before the addition of succinate and then measured in the Oxygraph. After peroxynitrite addition, creatine stimulation of respiration was completely abolished, whereas the respiratory chain was rendered still fully functional.

affinity, inner membrane binding site to the low affinity, peripheral binding site (29), where the enzyme is more accessible to PN and therefore easier to inactivate. Rebinding studies of octameric Mi-CK to mitoplasts revealed a very tightly and a loosely bound fraction of CK (30). Similarly, binding studies of Mi-CK to model membranes (29) showed that in membranes containing 16% cardiolipin, 66% of Mi-CK stays tightly bound under nonequilibrium conditions. This is close to the amount of Mi-CK (75%) that is more resistant to PN in mitoplasts.

In the presence of the outer mitochondrial membrane, *i.e.* in intact mitochondria, Mi-CK is less sensitive to PN inactivation. There, initiation of inactivation requires more than 40 μM PN and is monophasic, and IC_{50} is 200 μM (Fig. 4). This finding indicates that the outer membrane and its constituents effectively intercept some of the added PN, and that in mitochondria all Mi-CK may be present in just one binding state.

To further demonstrate the exquisite and specific sensitivity of Mi-CK to PN in mitochondria, we measured oxidative phosphorylation under conditions where provision of ADP by Mi-CK is the rate-limiting step in state 3 respiration (Fig. 5). The presence of creatine strongly stimulates state 3 respiration. Preincubation of mitochondria with 350 μM PN completely prevents this stimulation but does not affect the respiration in the absence of creatine. The latter is proven by the oxygen consumption data measured for ATP-stimulated respiration in the presence of succinate before (Fig. 5, *Control*) and after PN treatment (Fig. 5, *PN*), which are almost identical. In addition, the PN-treated mitochondria still showed an atractyloside-induced decline of O_2 consumption, indicating that mitochondrial respiration was still coupled to ATP generation. Thus, oxidative phosphorylation is not compromised by PN under conditions where Mi-CK is inac-

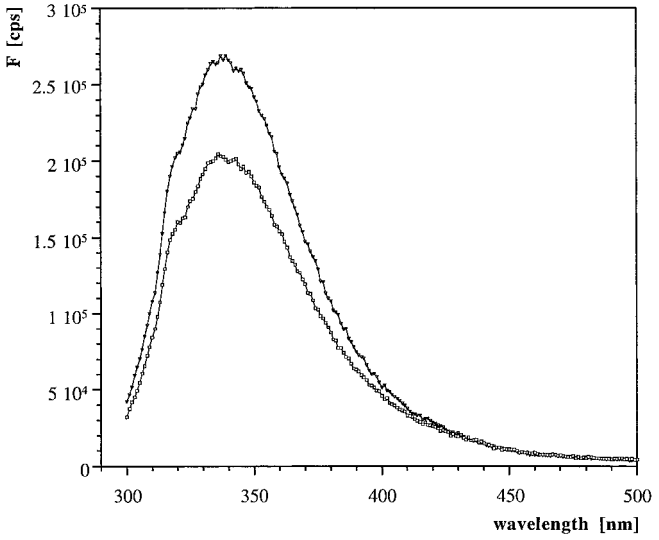

FIG. 6. **Corrected tryptophan emission spectra of native and peroxynitrite-treated Mi_b-CK.** The *upper curve* shows the spectrum of 0.15 μM octameric before the addition of 500 μM peroxynitrite, the *lower curve* displays the spectrum of the modified protein after PN treatment. PN-induced loss of fluorescence (*F*) quantum yield was determined to 27%. the temperature was set at 30 °C, and the excitation wavelength was set at 287 nm to specifically excite tryptophan residues.

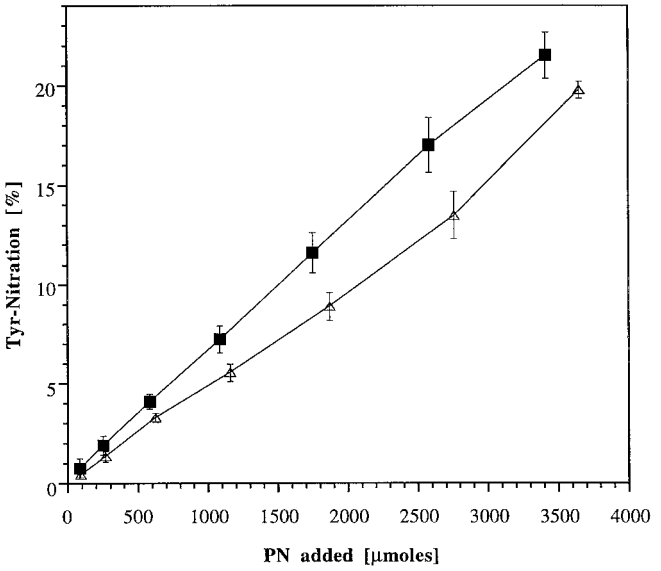

FIG. 7. **Tyrosine nitration of Mi_b-CK after the addition of increasing amounts of peroxynitrite.** 320 μg of octameric Mi_b-CK were diluted 1:10 in phosphate-buffered saline and exposed to increasing amounts of peroxynitrite; all additions were performed in time intervals of 2 min. Tyrosine nitration was determined in the absence (*triangles*) and presence of 20 μM $FeCl_3$, 0.4 mM EDTA (*squares*) by measuring the OD_{428} readings for each titration step and calculating the percentages of tyrosines nitrated. An extinction coefficient of 4.1 l/mmol cm for 3-nitrotyrosine was assumed for all calculations (35). All values were corrected for dilution.

tivated to about 70% as shown by the corresponding *in vitro* inactivation studies in intact mitochondria (see also Fig. 4). These findings bear far-reaching consequences for the energy homeostasis of a cell, *e.g.* Mi-CK, which is primarily responsible for the synthesis of PCr, is inactivated as a prime target of PN and cannot longer serve this function. Therefore, the cell becomes depleted of PCr, which is used to maintain high phosphorylation potentials at sites of energy demand and embodies an important energy and pH buffer. Regarding the Ca^{2+} handling capabilities of the cell, a permanent loss of PCr, which is used by sarcoplasmic reticulum-bound CK to fuel the sarcoplasmic reticulum Ca^{2+}-ATPase (31), may be fatal, as PN also stimulates the release of Ca^{2+} from mitochondria (3). Both effects may lead to a strikingly higher Ca^{2+} concentration in the cytosol and may cause chronic Ca^{2+} overload, which can lead to reduced cellular and mitochondrial function and eventually cause apoptosis and necrosis (32,33).

The reaction of isolated Mi-CK with PN was further investigated with biophysical techniques. We analyzed tryptophan fluorescence, because tryptophan residues are essential for Mi-CK activity (Trp-223; Ref. 34), and oligomeric state (Trp-264; Ref. 34). PN treatment lowered the intensity of tryptophan fluorescence emission by 27% (Fig. 6) without changing the position of the emission peak, indicating that it was not solvent exposure of internal tryptophan residues caused by unfolding of Mi-CK that was responsible for the reduced quantum yield. These findings support the idea that a destructive interaction between PN and tryptophan takes place, one example being an irreversible oxidative ring-opening of tryptophan residues yielding *N*-formylkynurenine. Because mutants affecting Trp-223 were shown to be completely inactive enzymatically (34) but here some enzymatic activity could still be recovered after incubation of PN-treated Mi-CK with 2-ME, presumably by deblocking some PN-modified active-site Cys-278 residues (Fig. 2), the modification of the active-site Trp-223 was not quantitative at the concentrations of PN used. Thus, the inactivation of CK activity observed under the conditions of PN treatment described herein is due to a combination of modifications of Cys-278 as well as Trp-223 active-site residues. We also found that the decrease in tryptophan emission intensity cannot be ascribed to fluorescence quenching by nitrite, the major product formed when PN decomposes, by constructing the appropriate Stern-Vollmer plots (not shown). Nitrite only contributed a few percent to the quenching, assuming the highest possible concentration arising from the decay of PN. Another residue likely to be modified is tyrosine, which is converted to 3-nitrotyrosine after the addition of PN (35–37). As Mi-CK contains 11 tyrosine residues per monomer, we examined tyrosine nitration and calculated the percentage of modified residues (Fig. 7). The data obtained suggest that under physiological conditions, tyrosine nitration is not the primary trigger of Mi-CK inactivation because a cumulative amount of 2 mmol of PN was necessary to modify on average one tyrosine residue per Mi-CK monomer (10% tyrosine nitration). Astonishingly, the preincubation of the enzyme in 50 mM $NaHCO_3^-$ (a source for the CO_2 adduct of PN) did not facilitate the nitration reaction at all, in contrast to tyrosine in solution (36). Nitration yield was significantly enhanced in the presence of Fe^{3+}-EDTA (Fig. 7), which is in good accordance with the results of Ramezanian *et al.* (6), who demonstrated the high nitration efficiency of chelated Fe^{3+}.

Besides the inhibitory effect of PN on CK activity, this radical also drastically affected the octamer/dimer equilibrium, as shown by gel permeation chromatography (Fig. 8): Although native Mi-CK was to 93% octameric, after PN treatment, the octamer content was decreased to 83.7%, which could be caused by modification of the tryptophan residue critical for the dimer/dimer interface (Trp 264; Ref. 34). The TSAC quantitatively dissociates native Mi-CK into dimers (38) by the induction of a conformational change shown to take place as a consequence of Mg-nucleotide binding (39). When adding TSAC to PN-pretreated Mi-CK, the action of PN on Mi-CK was most striking. Although the control (native Mi-CK + TSAC) dissociated into dimers to an extent of 96.6%, the PN-exposed Mi-CK almost completely persisted in the octameric state (84.4% octamers), *i.e.* at almost the same percentage that was determined for

peak Nr.	composition	octamer [%]
1.	native Mi-CK	92.4
2.	Mi-CK+PN	83.8
3.	Mi-CK+PN+TSAC	84.4
4.	native Mi-CK+TSAC	3.4

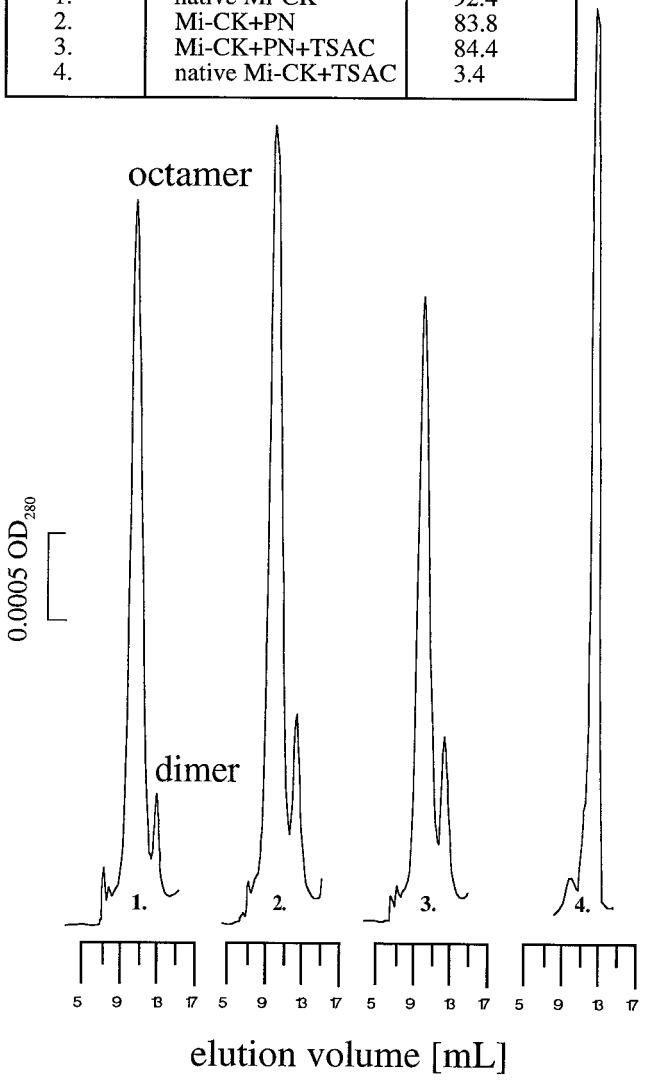

FIG. 8. **Gel filtration profiles of native and PN-pretreated Mi-CK in the presence and absence of TSAC.** Octameric Mi-CK was treated with 500 μM PM shortly before the addition of the TSAC mixture (transition state analog complex-forming reagents: 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3), which usually induces a fast dimerization of octameric Mi-CK (see peak Nr. 4 of control sample). 50 μg each of the protein samples (1–4) were applied to a Superose 12 column after a 2-h incubation (vol = 23 ml), calibrated according to described procedures (38), from which the Mi-CK octamer and dimer eluted at 11 and 13 ml, respectively. Transiently generated tetramers running intermediary between the two peaks shown in the figure are extremely short-lived and can hardly be detected by this method (34, 38). Note: PN treatment of octameric Mi-CK alone after 1–2 h before loading the sample onto the gel permeation columns induced a small but significant spontaneous decay into Mi-CK dimers (from 92.4 to 83.8% octamer content, compare peaks 1 and 2). However, TSAC-induced dimerization via binding of the CK substrates to the active site and thus inducing a conformational change (39), normally seen in control Mi-CK (peak 4), is completely blocked by PN treatment before the addition of the TSAC substrate mixture (peak 3).

PN-treated Mi-CK without TSAC. Analysis of the PN-modified protein on an SDS gel showed that it was not due to covalent cross-linking of Mi-CK, because its monomer band was by far most prominent (data not shown), although a small amount of "artificial" covalently linked Mi-CK dimers was detected. Obviously, active-site residues like Trp-223 and Cys-278, which have been shown to take part in the catalytic process, have been modified by PN. As a result, TSAC and the native sub-

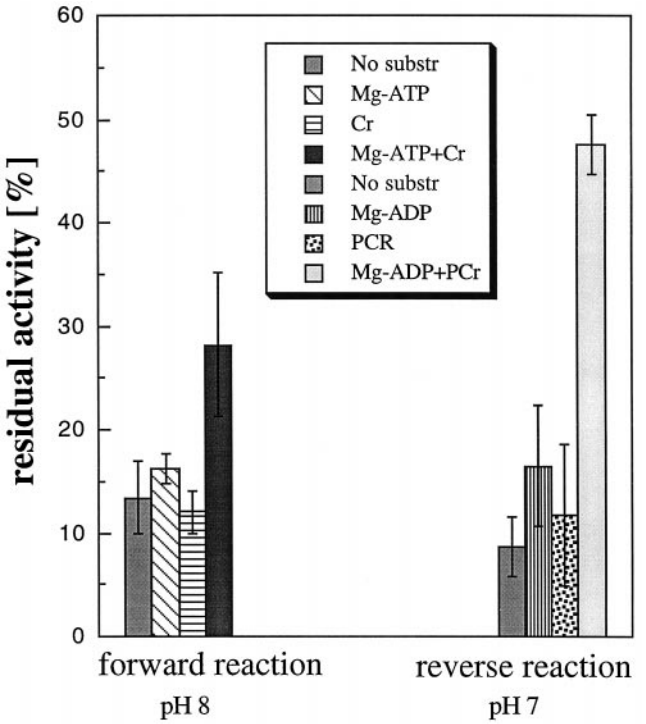

FIG. 9. **PN-induced inactivation of Mi-CK in the presence of different substrates.** Mi-CK was preincubated with 25 mM Mg-ADP, Mg-ATP, Cr, PCr, Mg-ADP + PCr (reverse reaction, pH 7), and Mg-ATP + Cr (forward reaction, pH 8) in MSH buffer. Subsequently, 350 μM PN in 0.1 M NaOH was added to achieve inactivation of Mi-CK. Mi-CK activity was measured according to Wallimann and co-workers (24). Values are expressed as percentage activity of the unmodified enzyme (control). *Error bars* represent S.D.

strates of CK no longer bind properly to the enzyme and thus are no longer able to induce the structural changes required for dimerization (39). These results clearly show that the dimer/octamer equilibrium of Mi-CK, which is presumed to be important for the regulation of Mi-CK-induced contact sites (40) is severely disturbed by PN. More recent data indicate that at higher concentrations of PN, Mi-CK octamers spontaneously tend to dimerize and that re-octamerization of PN-modified Mi-CK dimers is also affected.[2] Such data will certainly be relevant for cellular energetics during ischemia/reperfusion as well as with respect of the fact that Mi-CK octamers, in the presence of creatine or creatine analogues, seem to prevent opening of the mitochondrial permeability transition pore (52), an early event of apoptosis.

To assess possibilities of avoiding Mi-CK inactivation under conditions of excessive NO production and oxidative stress, the protective effect of substrates was examined. For this purpose, Mi-CK was co-incubated with different substrates and combinations thereof and subsequently inactivated by PN (350 μM). The data obtained (Fig. 9) show no remarkable protection effect for substrates and combinations that are not sufficient to keep the enzyme working but bind to the active site (*e.g.* MgATP, MgADP, PCr, Cr), whereas the full set of substrates in both cases, forward and reverse reaction, showed a highly protective effect. These findings can be interpreted with respect to the "hinge" function of Cys-278 postulated by Furter *et al.* (16). As long as only the binding sites for ATP/ADP or PCr/Cr are occupied, no protection can be seen, because Cys-278 is exposed and not participating in the processing of substrate, as no enzymatic reaction takes place. Once the full set of substrates

[2] S. Wendt and T. Wallimann, unpublished observation.

is present, Cys-278 is involved in the substrate binding sites conferring synergistic substrate binding (16) and therefore in contact with substrates. This leads to an enhanced protection of Cys-278 against PN. Both directions of the CK reaction were measured at their optimum pH and, in case of the forward reaction (pH 8), the protective effect is lower, which can be explained by the greater stability of PN at alkaline pH, which gives it more time to find its sulfhydryl target before decaying in solution. We, therefore, conclude that deenergization of mitochondria, resulting in a loss of ATP, which is needed for PCr synthesis in the intermembrane space, is detrimental to CK in the presence of PN, as the enzyme becomes inactivated more easily and therefore further compromises energy homeostasis within the entire cell. Consequently, this mechanism embodies a vicious circle for all energy-demanding processes.

The concentrations of PN used in this study are well within the physiological range. Thus, a bolus of 250 μM PN is equivalent to the constant exposure to a concentration of 1 μM PN for only 7 min (3, 41). The production of PN by activated macrophages inside phagolysosomes can reach about 500 μM (42). The present results show that Mi-CK is readily inactivated by PN at concentrations that do not affect the activity of the respiratory chain. We have previously reported that up to 500 μM added PN activates the specific Ca^{2+} release from rat liver mitochondria, which operates with maintenance of the mitochondrial membrane potential (4). Peroxynitrite, hitherto mainly considered to be a toxic NO congener (43), may thus also be of physiological relevance. This is particularly important in view of the fact that mitochondria are a copious source of O_2^- (44, 45) and, according to most recent data, contain a highly active nitric oxide synthase (22, 53, 54). This makes it very likely that *in vivo* they are primarily challenged by PN formed within the organelles themselves. These data deserve full attention, because they may lead to a clue concerning the formation of the crystalline intramitochondrial inclusions (mainly consisting of Mi-CK octamers) seen in the pathological state of patients with several neuromuscular diseases and mitochondrial myopathies (46–48) and to a better understanding of the factors that trigger apoptosis and/or necrosis like the ATP/ADP ratio (48) and the energetic state of mitochondria (33). Some important conclusions also arise for ischemia/reperfusion damage occurring after organ transplantation and episodes of hypoxia. It has been demonstrated that oxygen-derived radicals attacking the cytosolic CK isoforms are involved in the irreversible cardiac tissue damage found in many patients after ischemia and subsequent reperfusion (13,50) and that mitochondrial proteins are important targets as well (51). Our data clearly show that PN could be a potential culprit for causing ischemia/reperfusion damage, as Mi-CK, being responsible for the maintenance of high phosphorylation potentials in the cell (via PCr delivery), is challenged at quite low bolus concentrations of PN. Our results seem also to be relevant with respect to the recently discovered role of Mi-CK in mitochondrial permeability transition, linking this enzyme and its substrates to early events of apoptosis (52). Thus, a search for compounds that would protect cytosolic as well as mitochondrial CK isoforms from inactivation by oxygen-derived free radicals and that could be supplied with perfusion media may represent a future achievement of possible clinical relevance.

Acknowledgments—Drs. R. Kissner and W. Koppenol are acknowledged for generous gifts of PN. The authors are grateful to Dr. P. Ghafourifar for critically reviewing the manuscript. Dr. U. Schlattner is acknowledged for many scientific discussions.

REFERENCES

1. Lizasoain, I., Moro, M. A., Knowles, R. G., Darley-Usmar, V., and Moncada, S. (1996) *Biochem. J.* **314,** 877–880
2. Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997) *J. Biol. Chem.* **272,** 20340–20347
3. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *J. Biol. Chem.* **266,** 4244–4250
4. Schweizer, M., and Richter, C. (1996) *Biochemistry* **35,** 4524–4528
5. Padmaja, S., Ramezanian, M. S., Bounds, P. L., and Koppenol, W. H. (1996) *Redox Report* **2,** 173–177
6. Ramezanian, M. S., Padmaja, S., and Koppenol, W. H. (1996) *Chem. Res. Toxicol.* **9,** 232–240
7. Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993) *Nature* **364,** 584
8. Kaul, N., Siveski-Iliskovic, N., Hill, M., Slezak, J., and Singal, P. K. (1993) *J. Pharmacol. Toxicol. Methods* **30,** 55–67
9. Kehrer, J. P. (1993) *CRC Crit. Rev. Toxicol.* **23,** 21–48
10. Suzuki, Y. J., Edmondson, J. D., and Ford, G. D. (1992) *Free Radical Res. Commun.* **16,** 131–136
11. Nathan, C., and Xie, Q. W. (1994) *Cell* **78,** 915–918
12. Yuan, G., Kaneko, M., Masuda, H., Hon, R. B., Kobayashi, A., and Yamazaki, N. (1992) *Biochim. Biophys. Acta* **1140,** 78–84
13. Mekhfi, H., Veksler, V., Mateo, P., Maupoil, V., Rochette, L., and Ventura-Clapier, R., (1996) *Circ. Res.* **78,** 1016–1027
14. Hamman, B. L., Bittl, J. A., Jacobus, W. E., Allen, P. D., Spencer, R. S., Tian, R., and Ingwall, J. S. (1995) *Am. J. Physiol.* **269,** H1030–H1036
15. Korge, P., and Campbell, K. B. (1994) *Am. J. Physiol.* **267,** C357–C366
16. Furter, R., Furter-Graves, E., and Wallimann, T. (1993) *Biochemistry* **32,** 7022–7029
17. Gross, W. L., Bak, M. I., Ingwall, J. S., Arstall, M. A., Smith, T. W., Balligand, J. L., and Kelly, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 5604–5609
18. Wolosker, H, Panizzutti, R., and Englender, S. (1996) *FEBS Lett.* **392,** 274–276
19. Korge, P., and Campbell, K. B. (1993) *Arch. Biochem. Biophys.* **304,** 420–428
20. Ambrosio G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello, M., and Flaherty, J. T. (1993) *J. Biol. Chem.* **268,** 18532–18541
21. Bates, T. E., Loesch, A., Burnstock, G., and Clark, J. B. (1996) *Biochem. Biophys. Res. Commun.* **218,** 40–44
22. Ghafourifar, P., and Richter, C. (1997) *FEBS lett.* **418,** 291–296
23. Koppenol, W., Kissner, R., and Beckman, J. S. (1996) *Methods Enzymol.* **269,** 296–301
24. Furter, R., Kaldis, P., Furter-Graves, E., Schnyder, T., Eppenberger, H. M., and Wallimann, T. (1992) *Biochem J.* **288,** 771–775
25. Wallimann, T., Schlösser, T., and Eppenberger, H. M. (1984) *J. Biol. Chem.* **259,** 5238–5246
26. Wallimann, T., Turner, D. C., and Eppenberger, H. M. (1975) *J. Cell Biol.* **75,** 297–317
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
28. Kottke, M., Adams, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G., and Panfili, E. (1988) *Biochim. Biophys. Acta* **935,** 87–102
29. Stachowiak, O., Dolder M., and Wallimann, T. (1996) *Biochemistry* **35,** 15522–15528
30. Schlame, M., and Augustin, W. (1985) *Biomed. Biochim. Acta* **44,** 1083–1088
31. Rossi, A. M., Eppenberger, H. M., Volpe, P., Cotrufo, R., and Wallimann, T. (1990) *J. Biol. Chem.* **265,** 5258–5266
32. Richter, C. (1993) *FEBS Lett.* **325,** 104–107
33. Richter, C., Schweizer, M., Cossarizza, A., and Franceschi, C. (1996) *FEBS Lett.* **378,** 107–110
34. Gross, M., Furter-Graves E. M., Wallimann, T., Eppenberger, H. M., and Furter, R. (1994) *Protein Sci.* **3,** 1058–1068
35. Bray, M. R., and Clarke, A. J. (1995) *Biochemistry* **34,** 2006–2014
36. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) *Biochemistry* **35,** 7855–7861
37. MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11853–11858
38. Gross, M., and Wallimann, T. (1993) *Biochemistry* **32,** 13933–13940
39. Forstner, M., Kriechbaum, M., Laggner, P., and Wallimann, T. (1996) *J. Mol. Struct.* **383,** 217–222
40. Stachowiak, O., Schlattner, U., Dolder, M., and Wallimann, T. (1998) *Mol. Cell. Biochem.,* in press
41. Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) *Methods Enzymol.* **233,** 229–240
42. Denicola, A., Rubbo, H., Rodriguez, D., and Radi, R. (1993) *Arch. Biochem. Biophys.* **304,** 279–286
43. Darley-Usmar, V., Wiseman, H., and Halliwell, B. (1995) *FEBS lett.* **369,** 131–135
44. Loschen, G., Azzi, A., Richter, C., and Flohe, L. (1974) *FEBS Lett.* **42,** 68–72
45. Turrens, J. F., Beconi, M., Barilla, J., Chavez, U. B., and McCord, J. M. (1991) *Free Radical Res. Commun.* **12,** 681–689
46. Eppenberger, H. M., Perriard, J. C., Rosenberg, U. B., and Strehler, E. (1981) *J. Cell Biol.* **89,** 185–193
47. Stadhouders, A. M., Jap, P., Winkler, H. P., Eppenberger, H. M., and Wallimann, T. (1994) *Proc. Natl. Acad. Sci.* U. S. A. **91,** 5089–5094
48. O'Gorman, E., Beutner, G., Wallimann, T., and Brdiczka, D. (1996) *Biochim. Biophys. Acta* **1276,** 161–171
49. Leist, M., Single, B., Castoldi, A. F., Kiehnle, S., and Nicotera, P. (1997) *J. Exp. Med.* **185,** 1481–1486
50. Banerjee, A., Grosso, M. A., Brown, J. M., Rogers, K. B., and Whitman, G. J. R. (1991) *Am. J. Physiol.* **261,** H590–H597
51. McCord, J. M., and Turrens, J. F. (1994) *Curr. Top. Bioenerg.* **17,** 173–195
52. O'Gorman, E., Beutner, G., Dolder, M., Koretsky, A. P., Brdiczka, D., and Wallimann T. (1997) *FEBS Lett.* **414,** 253–257
53. Giulivi, C., Poderoso, J. J., and Boveri, A. (1998) *J. Biol. Chem.* **273,** 11038–11043
54. Tatoyan, A., and Giulivi, A. (1998) *J. Biol. Chem.* **273,** 11044–11048