Free Rad. Res., Vol. 21, No. 6, pp. 387–397
Reprints available directly from the publisher
Photocopying permitted by license only

FREE RADICAL INACTIVATION OF RABBIT MUSCLE CREATINE KINASE: CATALYSIS BY PHYSIOLOGICAL AND HYDROLYZED ICRF-187 (ICRF-198) IRON CHELATES

CARIN THOMAS*, ANITRA C. CARR and CHRISTINE C. WINTERBOURN

Free Radical Research Unit, Department of Pathology, Christchurch School of Medicine, PO Box 4345, Christchurch, New Zealand

(Received April 29th, 1994; in revised form June 9th, 1994)

Creatine kinase is a sulfhydryl containing enzyme that is particularly susceptible to oxidative inactivation. This enzyme is potentially vulnerable to inactivation under conditions when it would be used as a diagnostic marker of tissue damage such as during cardiac ischemia/reperfusion or other oxidative tissue injury. Oxidative stress in tissues can induce the release of iron from its storage proteins, making it an available catalyst for free radical reactions. Although creatine kinase inactivation in a heart reperfusion model has been documented, the mechanism has not been fully described, particularly with regard to the role of iron. We have investigated the inactivation of rabbit muscle creatine kinase by hydrogen peroxide and by xanthine oxidase generated superoxide or Adriamycin radicals in the presence of iron catalysts. As shown previously, creatine kinase was inactivated by hydrogen peroxide. Ferrous iron enhanced the inactivation. In addition, micromolar levels of iron and iron chelates that were reduced and recycled by superoxide or Adriamycin radicals were effective catalysts of creatine kinase inactivation. Of the physiological iron chelates studied, Fe(ATP) was an especially effective catalyst of inactivation by what appeared to be a site-localized reaction. Fe(ICRF-198), a non-physiological chelate of interest because of its putative role in alleviating Adriamycin-induced cardiotoxicity, also catalyzed the inactivation. Scavenger studies implicated hydroxyl radical as the oxidant involved in iron-dependent creatine kinase inactivation. Loss of protein thiols accompanied loss of creatine kinase activity. Reduced glutathione (GSH) provided marked protection from oxidative inactivation, suggesting that enzyme inactivation under physiological conditions would occur only after GSH depletion.

KEY WORDS: Creatine kinase, iron-dependent oxidative damage, superoxide anion radical, adriamycin radical, ICRF-198, xanthine oxidase.

INTRODUCTION

Creatine kinase (CK) (ATP: creatine phosphotransferase, EC 2.7.3.2) is a sulfhydryl containing enzyme that is susceptible to oxidative inactivation.[1-4] Inactivation of thiol enzymes is considered to play an important role in oxidative cell injury and has been observed in muscle cells exposed to Adriamycin or other oxidative stresses as well as in a heart reperfusion model.[1,4,5,6] Although the susceptibility of thiol

To whom correspondence should be addressed: Christine C. Winterbourn Free Radical Research Unit, Department of Pathology, Christchurch School of Medicine, PO Box 4345 Christchurch, New Zealand. Tel: (64 3) 364-0564 Fax: (64 3) 364 1083.

*Present address: Department of Biochemistry and Biophysics, Oregon State University, Coruallis, Oregon 97331-7305, USA.

enzymes to oxidative inactivation is well documented, the mechanism of the process and particularly the role of iron are not clearly established.[7] Iron catalyzed reactions are thought to be important in oxidative injury, by promoting lipid peroxidation and the generation of hydroxyl radical (·OH) or reactive ferryl species that can cause DNA damage, enzyme inactivation, and cellular dysfunction.[8] The efficiency of the iron catalyst depends on its chelated form.[8,9] Various chelators have been studied including physiological compounds such as citrate and adenine nucleotides, and non-physiological compounds such as desferrioxamine and ICRF-187 (ADR-529, *d*-1,2-bis(3,5-dioxopiperazine-1-yl)propane).[10,11] Desferrioxamine is commonly used to implicate iron in oxidative cell injury because it renders iron inert and will thwart iron-dependent cell damage.[12] ICRF-187 is a compound of particular interest because of its therapeutic value in the treatment of cardiotoxicity associated with Adriamycin therapy.[13] The mechanism by which ICRF-187 alleviates Adriamycin-induced cardiotoxicity is unknown. However, it is known to enter cells where its diacid diamide hydrolysis product, ICRF-198 (ADR-925, *dl-N,N'*-carboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane), is generated.[14] ICRF-198 is a strong chelator with a high affinity constant for iron.[15] ICRF-198 has been shown to inhibit Adriamycin-dependent lipid peroxidation,[11,16] but to promote ·OH production with an efficiency comparable to that of Fe(ethylenediaminetetraacetic acid), Fe(EDTA).[17]

No matter which chelator is involved in iron catalyzed reactions, a reductant is required for continuous redox cycling of the iron. Superoxide and Adriamycin radicals can reduce iron, but their abilities to promote iron-catalyzed oxidative injury depend on the chelator.[9,10,18,19] Previous studies have shown that CK can be inactivated by hydrogen peroxide[3] and by superoxide,[1] although it appears that whereas the bovine and rat heart enzymes are susceptible to superoxide, rabbit muscle CK is not. In studies involving xanthine oxidase, the mechanism has not been fully resolved and a role for iron not identified.[4,20] Ferric iron can inactivate CK, but this appears to be a direct reaction with iron rather than one involving reactive oxygen species.[21]

In this study we examined the inactivation of rabbit muscle CK by hydrogen peroxide and catalytic levels of iron with or without EDTA, ICRF-198, ATP or citrate. Xanthine oxidase was used to generate superoxide and Adriamycin radicals which serve as iron reductants. Our results show that inactivation of CK by hydrogen peroxide is enhanced in the presence of ferrous iron. Moreover, iron and iron chelates which are reduced and recycled by superoxide or Adriamycin radicals are effective catalysts of enzyme inactivation. Scavenger studies implicate ·OH as the oxidant which mediates iron-dependent CK inactivation.

MATERIALS AND METHODS

Biochemicals were of the highest quality available from Sigma Chemical Co. (St Louis, MO, USA), Boehringer Mannheim (Penzberg, Germany), or BDH Chemicals Ltd (Poole, UK). Adriamycin was purchased from Farmitalia Carlo Erba Ltd, desferrioxamine from Ciba-Geigy and chelex resin from Biorad Laboratories (Richmond, CA, USA). Rabbit muscle CK, lactate dehydrogenase, and pyruvate kinase were obtained from Boehringer Mannheim. Superoxide dismutase (SOD) from bovine erythrocytes (5,100 U/mg protein), catalase from bovine liver (25,000 U/mg) and xanthine oxidase from buttermilk (1.1 U/mg) were from Sigma. ICRF-198 (ADR-925, *dl-N,N'*-dicarboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane) was provided as a gift from Adria Laboratories (Dublin, OH, USA).

Xanthine oxidase system. A xanthine oxidase system was used to generate superoxide, and the same system in the presence of Adriamycin was used to generate Adriamycin radicals.[22] Inactivation reactions were carried out in chelex-treated 10 mM sodium phosphate buffer, pH 7.4, in plastic test tubes. Iron contamination of xanthine oxidase was minimized by mixing the enzyme with EDTA and separating on a G-25 sephadex column.[9] Protease contamination of the xanthine oxidase preparations was routinely tested and found to be negligible. The simplest reaction mixture contained, at final concentrations, hypoxanthine (100 to 150 μM), CK (100 μg/mL; 1.2 μM) and xanthine oxidase. Xanthine oxidase activity was measured as superoxide dismutase inhibitable cytochrome *c* reduction at 550 nm and an amount yielding 3 to 4 μM superoxide per minute was added to start the reaction. The 1% extinction coefficient, $E_{280} = 8.76$, was used to standardize CK concentration.[23] Further additions to the xanthine oxidase system were, at final concentrations, $FeSO_4$(2 μM), Adriamycin (30 μM), either EDTA (40 μM), ICRF-198 (40 μM), ATP (200 μM) or citrate (200 μM), and at the concentrations stated, formate, benzoate, mannitol, or GSH. Iron chelates were prepared by premixing 2 μM $FeSO_4$ in equimolar HCl with the chelator. Reaction mixtures were incubated under air at 37°C for 30 min, or the time indicated. The mixture was then diluted tenfold in ice cold 5 mM glycine buffer, pH 9.0, and CK activity was determined immediately.

CK assay. The activity of rabbit muscle CK was determined at 37°C in a coupled enzyme assay using pyruvate kinase and lactate dehydrogenase as described by Tanzer and Gilvarg[24] with minor modifications. The assay mixture contained, at final concentrations, ATP (2.1 mM), NADH (0.3 mM), phosphoenolpyruvate (0.5 mM), $MgSO_4$(7.0 mM), creatine (38.7 mM), K_2CO_3(45.3 mM), lactate dehydrogenase (3.75 U/mL), and pyruvate kinase (1.75 U/mL) in a 292 mM glycine buffer at pH 8.9. CK (0.2 μg/mL) was added to start the reactions. As the reactions proceed, creatine is converted to phosphocreatine with concomitant NADH oxidation. NADH oxidation was monitored at 340 nm and linear rates over 2–6 min were used for analysis. Specific activity for control creatine kinase ranged between 59.0 and 68.5 U/mg where one unit converts 1 μmole of creatine to phosphocreatine per min at 37°C and pH 8.9.

Reversal of CK inactivation with GSH. The xanthine oxidase system was used as described above except that the reaction was stopped by the addition of allopurinol (100 μM). The reaction mixtures were divided into two equal portions and GSH (250 μM–1 mM) was added to one of these. The samples were then incubated for a further 15 minutes at 37°C before carrying out the CK activity assay as described.

Thiol oxidation. Xanthine oxidase dependent inactivation of CK was carried out as described above and the reaction was stopped with the addition of allopurinol (100 μM). A minimal amount of sample was removed for the CK activity assay. Dithiobisnitrobenzoic acid (DTNB) (60 μM) was added to the remainder of the reaction mixture and A_{412} determined. Thiol loss was calculated as percent of controls to which xanthine oxidase was not added. Reagent concentrations in the xanthine oxidase system were increased in order to detect thiols and were, at final concentrations, hypoxanthine (300 μM), CK (400 μg/mL; 4.8 μM) and xanthine oxidase (8 μM superoxide/min). The extinction coefficient, $1.41 \times 10^4\ M^{-1}\ cm^{-1}$ at A_{412}, was used for quantitative estimates of thiol content.

RESULTS

Inactivation of rabbit muscle CK by hydrogen peroxide and superoxide or Adriamycin radical generating systems was examined. As demonstrated previously,[3] hydrogen peroxide alone caused inactivation in a dose dependent manner (Figure 1). Higher H_2O_2 concentrations, 200 and 400 μM H_2O_2, decreased CK activity to 44.5 and 17.5 percent of control, respectively. Fe^{2+} added with H_2O_2 (Fenton's reagent) enhanced the inactivation of CK over that observed by H_2O_2 alone (Figure 1). Addition of desferrioxamine (200 μM) to the incubation medium or dialysis of the CK enzyme preparation against EDTA and then desferrioxamine did not alter its inactivation by H_2O_2, indicating little or no contribution of contaminating iron.

The xanthine oxidase system in the absence of iron inactivated CK to approx. 60 percent of control activity (Figure 2). Addition of desferrioxamine (100 μM) to the xanthine oxidase system had no effect on this CK activity, nor did iron when chelated to desferrioxamine. When $FeSO_4$ was added in the absence of desferrioxamine, however, there was additional inactivation of CK that was dependent on the iron concentration (Figure 2). SOD did not protect in the absence of iron, but in the presence of iron it did protect, abrogating all iron-dependent inactivation (Table 1). These results indicate that superoxide radicals were required for iron-dependent inactivation of CK. Catalase protected CK from inactivation by the xanthine oxidase system both in the presence and absence of iron, indicating a requirement for hydrogen peroxide. Addition of catalase and SOD together gave nearly complete protection in both systems. Protection by catalase indicated that iron alone had no effect on CK.

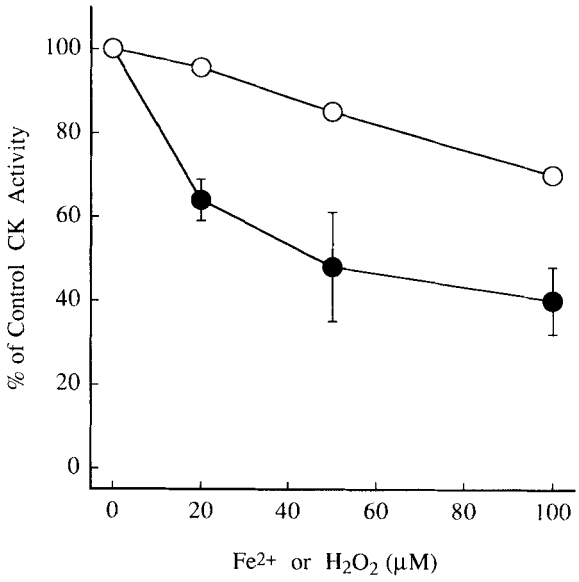

FIGURE 1 Inactivation of CK by (○) H_2O_2 and (●) Fenton's reagent. Values are means and ranges of two or three sets of duplicate determinations. The reaction mixture contained CK (100 μg/mL), H_2O_2, and $FeSO_4$ or no further addition in a 10 mM sodium phosphate buffer, pH 7.4. The Fenton's reagent reaction mixture contained equimolar $FeSO_4$ and H_2O_2 except at 20 μM $FeSO_4$ which contained 50 μM H_2O_2. Reaction mixtures were incubated for 30 min at 37°C.

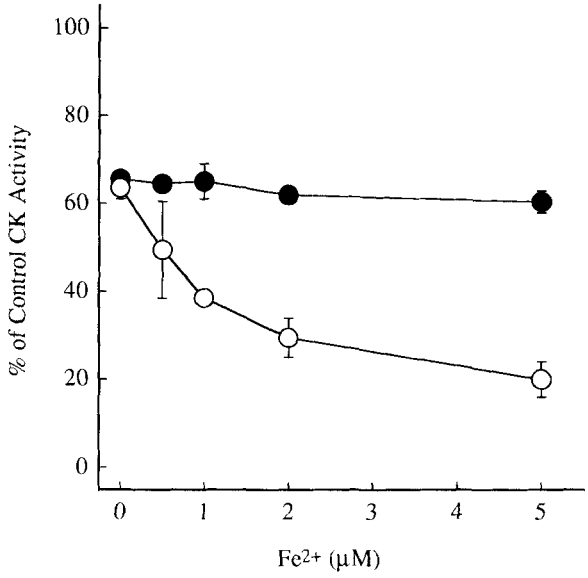

FIGURE 2 Inactivation of CK by the xanthine oxidase system and the effect of iron. Values are means and ranges of two sets of duplicates. Reaction mixtures contained xanthine oxidase, hypoxanthine, CK and (○) $FeSO_4$, or (●) $FeSO_4$ and desferrioxamine (100 μM).

When reduced iron (2 μM) was incubated with CK in the absence of xanthine oxidase, no inhibition of CK activity was observed.

Since the reactivity of iron in free radical reactions depends on its chelated form, we examined the ability of iron chelates to inactivate CK in the xanthine oxidase system. None of the chelators alone affected inactivation, but when complexed to iron, all increased the extent of CK inactivation. Fe(citrate) and Fe(ICRF-198) catalyzed the inactivation of CK with comparable efficiency to non-chelated iron, whereas inactivation by Fe(ATP) and Fe(EDTA) was much enhanced (Table 2). Fe(ADP) produced results similar to Fe(ATP) (data not shown). Time dependence curves (Figure 3) show the much more rapid inactivation of CK with Fe(ATP) than with Fe(citrate). However, when 500 μM Mg^{2+} was added together with Fe(ATP),

TABLE 1
Effects of catalase and SOD on xanthine oxidase and iron-dependent inactivation of CK

Additions	CK activity (% control)	
	XO system	XO system + 2 μM Fe^{2+}
None	64.5 ± 5.5	32.0 ± 1.0
SOD (10 μg/mL)	70.0 ± 3.0	66.5 ± 0.5
CAT (10 μg/mL)	84.5 ± 1.5	78.5 ± 4.5
SOD + CAT	98.5 ± 1.5	94.0 ± 1.0

Values are means and ranges of two sets of duplicates. Results are presented as % control activity to which xanthine oxidase was not added. The inactivation reaction was carried out at 37°C for 30 min as described in the Materials and Methods. CAT, catalase; XO, xanthine oxidase.

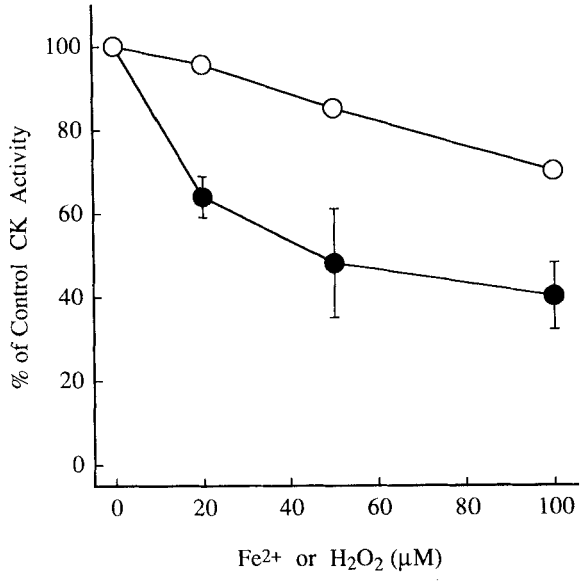

FIGURE 3 Time course of CK inactivation by the xanthine oxidase system plus iron and iron chelates. Values are means and ranges of two sets of duplicates. Xanthine oxidase without iron, (●); with Fe, (○); with Fe(ATP), (△); with Fe(citrate), (▲). Concentrations were 2 μM $FeSO_4$, 200 μM ATP and 200 μM citrate.

inactivation of CK was not as pronounced (28.5 ± 1.5% of control, $n = 2$, cf. Table 2).

Adriamycin radicals have been shown to be better reductants than superoxide for some iron chelates.[18] Therefore, we investigated whether CK inactivation was enhanced if Adriamycin radicals were generated. Adriamycin was added to the xanthine oxidase system. This produces Adriamycin radicals which can subsequently react with oxygen to give superoxide.[22] Adriamycin alone did not affect the activity of CK, but addition to the xanthine oxidase system in the absence of iron increased CK inhibition by approximately 15% (Table 2). This increase corresponded to a 15% rise in the SOD inhibitable cytochrome *c* reduction rate measured in the presence of Adriamycin (data not shown) and probably reflects the slightly faster rate of radical production. Adriamycin in the presence of the iron chelates did not generally induce any greater CK inactivation than superoxide (Table 2), except with Fe(ICRF-198), when Adriamycin slightly enhanced inactivation. These results indicate that generation of Adriamycin radicals causes no greater inhibition of CK than when superoxide is the only radical produced.

Whether ·OH generated from the Haber-Weiss reaction or the Adriamycin-radical driven Fenton reaction was responsible for iron chelate-dependent CK inactivation was investigated by adding scavengers. Neither formate, benzoate, nor mannitol had any effect on the inactivation seen with xanthine oxidase in the absence of iron, indicating no role for ·OH under these conditions (data not shown). Formate and mannitol protected CK from inactivation by all iron chelates, with increased protection observed at higher scavenger concentrations (Table 3). Formate was generally more efficient than mannitol, as expected for competition for ·OH. Benzoate,

TABLE 2
Effect of iron chelates on iron-dependent inactivation of CK by the xanthine oxidase system in the presence and absence of Adriamycin

Iron Chelate Added	CK activity (% control)	
	XO system	XO system + Adriamycin
None	71.5 ± 1.5	56.5 ± 0.5
$FeSO_4$	33.0 ± 9.0	39.0 ± 6.0
Fe(ATP)	6.0 ± 2.0	7.0 ± 5.0
Fe(citrate)	30.5 ± 5.5	28.5 ± 9.5
Fe(EDTA)	4.5 ± 1.5	10.5 ± 0.5
Fe(ICRF-198)	37.7 ± 1.5	30.0 ± 2.0

Values are means and ranges of two sets of duplicates. Results are presented as % control activity to which xanthine oxidase was not added. The inactivation reaction mixture, prepared by premixing chelators with iron before adding the remaining reagents, was incubated at 37°C for 30 min under air as described in the Materials and Methods. Cytochrome *c* reduction rates in the presence of Adriamycin were 15% higher than in its absence. Concentrations were 2 μM $FeSO_4$, 200 μM ATP, 40 μM EDTA, 200 μM citrate, 40 μM ICRF-198, 30 μM Adriamycin.

TABLE 3
Effect of the hydroxyl radical scavengers, formate, benzoate, and mannitol, on iron-dependent inactivation of CK

			% CK Activity Remaining After Treatment			
		No Iron	Fe(ATP)	Fe(EDTA)	Fe(citrate)	Fe(ICRF-198)
Xanthine Oxidase System						
HX/XO		65.0 ± 5.3	3.6 ± 0.5	5.0 ± 1.5	31.4 ± 6.8	42.0 ± 10.8
+ Formate	1 mM		22.6 ± 4.9	47.6 ± 8.3	49.6 ± 3.3	59.3 ± 1.6
	20 mM		53.5 ± 1.8	62.2 ± 3.4	59.8 ± 1.8	58.3 ± 0.8
+ Benzoate	1 mM		4.5 ± 1.4	11.2 ± 3.8	36.3 ± 1.3	49.3 ± 6.9
+ Mannitol	1 mM		6.1 ± 0.7	36.0 ± 7.2	45.7 ± 3.2	55.6 ± 0.5
	20 mM		51.5 ± 4.3	54.4 ± 1.6	60.5 ± 2.9	56.8 ± 0.6
Xanthine Oxidase System + Adriamycin (30 μM)						
HX/XO/Adr		61.6 ± 5.2	13.2 ± 3.1	10.2 ± 5.5	34.0 ± 9.7	29.6 ± 10.7
+ Formate	1 mM		28.8 ± 2.5	35.2 ± 3.2	44.3 ± 0.2	45.1 ± 3.7
	20 mM		50.8 ± 6.1	43.0 ± 6.3	50.2 ± 5.2	44.2 ± 8.9
+ Mannitol	1 mM		15.4 ± 3.7	23.3 ± 5.7	34.9 ± 4.4	34.7 ± 4.8
	20 mM		45.8 ± 4.4	43.6 ± 4.2	44.9 ± 5.3	44.7 ± 9.3

Values are means and ranges of two or four sets of duplicates. Results are presented as % of CK activity remaining after treatment in the xanthine oxidase system. The inactivation reaction was carried out at 37°C for 30 min as described in the Materials and Methods. Iron and chelate concentrations were 2 μM $FeSO_4$, 200 μM ATP, 40 μM EDTA, 200 μM citrate, 40 μM ICRF-198. HX, hypoxanthine; XO, xanthine oxidase; Adr, Adriamycin.

however, exhibited less protection than expected based on rate constants. This may be due to inactivation of CK by the oxidizing benzoate radical itself. Protection of CK activity by the scavengers was also observed in the non-chelated iron catalyzed reaction (data not shown). In the presence of Adriamycin, CK activity was protected by formate and mannitol, with formate generally being more effective (Table 3). Absolute levels of protection tended to be slightly less with Adriamycin, but overall

the results with and without Adriamycin were similar and consistent with ·OH being responsible for the iron-dependent inactivation.

CK is a dimeric enzyme that contains one ionized thiol group per subunit.[25] Although the reactive cysteine thiol is nonessential for catalysis, it appears to facilitate dual substrate binding and is required for maximum enzyme activity.[26] Since thiols are sensitive to attack by oxidants, we evaluated the effect of iron-dependent generation of oxidants on the thiol content of CK. Analysis using DTNB gave a thiol content of CK incubated under control conditions that corresponded precisely to amounts calculated on the basis of its concentration (9.6 μM DTNB detected thiols in 4.8 μM CK). As shown in Table 4, loss of CK activity was accompanied by a loss of thiol content.

Addition of 250 μM GSH to the xanthine oxidase reaction system markedly protected CK from inactivation in the presence and absence of iron and the iron chelates. In most cases, only 6 to 12% of CK activity was lost when GSH was present. The exception was a loss of 22% activity when inactivation was catalyzed by Fe(ATP). These observations indicated that glutathione either protected CK from inactivation or that it reversed the inactivation. When GSH was added after the xanthine oxidase reaction was stopped after 30 min with allopurinol, and incubation continued for a further 15 min, only a small increase in activity was recorded. With all of the iron chelates, 250 μM GSH produced no reversal of inactivation (2.6 ± 3.9%, $n = 6$) and 1 mM GSH produced minimal reversal (9.6 ± 4.5%, $n = 6$). Hence, GSH was much more effective if present during the inactivation process.

DISCUSSION

Creatine kinase is considered to be one of the most vulnerable enzymes to oxidative stress, such as occurs during ischemia/reperfusion or when cells are exposed to redox cycling agents like the anticancer drug Adriamycin.[1,2,5] We have shown that the rabbit muscle enzyme is oxidatively inactivated either by the direct action of H_2O_2 or by an iron catalyzed Fenton-type reaction that also requires H_2O_2. With xanthine oxidase in the presence of iron, a system which generates both superoxide and ·OH, superoxide was required to recycle the iron in the Fenton reaction. Both reactions

TABLE 4
Loss of enzyme activity and protein thiols in iron-dependent inactivation of CK by the xanthine oxidase system

Iron chelate added	CK activity % of control	Thiol Content % of control
None	44.5 ± 6.5	42.0 ± 11.0
$FeSO_4$	36.5 ± 1.5	52.5 ± 5.5
Fe(ATP)	24.0 ± 4.0	39.5 ± 14.5
Fe(citrate)	40.5 ± 5.5	45.5 ± 11.5
Fe(EDTA)	27.5 ± 7.5	37.5 ± 16.5
Fe(ICRF-198)	34.5 ± 7.5	28.0 ± 11.0

Values are means and ranges of two sets of duplicates. Results are presented as % of control to which no xanthine oxidase was added. Inactivation was carried out by incubating CK with the xanthine oxidase system with or without iron chelate at 37°C for 30 min. Thiols were measured using DTNB as described in the Materials and Methods. Iron and chelate concentrations were 2 μM $FeSO_4$, 200 μM ATP, 40 μM EDTA, 200 μM citrate, 40 μM ICRF-198.

were additive, with iron concentrations in excess of 1–2 μM being sufficient for the iron-dependent mechanism to predominate. To the extent that it can be ascertained using scavengers,[27] our results are consistent with ·OH being responsible for iron-dependent inactivation.

The efficiency of iron as a catalyst of CK inactivation depended on its chelated form. Of the physiological and nonphysiological iron chelates that we studied, only Fe(desferrioxamine) was inert. The fastest inactivation was seen with Fe(ATP). The data in Figure 3 suggest that only submicromolar concentrations of Fe(ATP) would be required to cause rapid inactivation well in excess of any direct effect of H_2O_2. Since adenine nucleotides are likely candidates for binding any low molecular weight iron that may be available in the cell, this could provide a mechanism for oxidative injury. Although Fe(ATP) catalyzed inactivation was diminished in the presence of Mg^{2+}, inactivation still exceeded that of H_2O_2. In addition to its efficiency, Fe(ATP) catalyzed inactivation of CK was more resistant to inhibition by ·OH scavengers and GSH than with the other chelates. These effects could be explained if the iron complex binds to the Mg(ATP) substrate binding site, allowing site-localized oxidative attack on the enzyme.

Although the physiological chelates used in this study have been shown to be much better Fenton catalysts when the reaction is driven by Adriamycin radicals than by superoxide,[10,18,19] we found that adding Adriamycin to the xanthine oxidase system gave no obvious enhancement of CK inactivation. However, our results do show that generation of superoxide and H_2O_2, either directly or through redox cycling of Adriamycin, causes the inactivation of CK, and they could be taken as support for the proposal that oxidation of CK contributes to the cardiotoxicity of Adriamycin. ICRF-187 has been shown in clinical trials to provide protection against Adriamycin cardiotoxicity.[13,18] Its mechanism of action has not been elucidated fully, but a strong possibility is that it undergoes intracellular hydrolysis to ICRF-198 which then binds any low molecular weight iron that may be already present or released from ferritin by the Adriamycin radical.[29,30] Fe(ICRF-198) was found to be a good catalyst of CK inactivation, however, suggesting that if the proposed mechanism of action of ICRF-187 is correct, inactivation of CK may not be a crucial cardiotoxic event. The result with ICRF-198 is consistent with previous findings that its iron complex is a good catalyst of ·OH production.[17] This contrasts with the protection given by ICRF-198 against microsomal lipid peroxidation and inactivation of Ca-ATPase mediated by redox cycling of Adriamycin.[11] Iron-dependent inactivation of Ca-ATPase also differed in not being inhibited by ·OH scavengers, suggesting that its inactivation was not simply due to free ·OH.[31]

As has been observed with H_2O_2[3], we found that loss of protein thiols accompanied loss of CK activity after iron-dependent oxidant treatment. Relatively low concentrations of GSH present during oxidant exposure protected against inactivation. This could be due partially to ·OH scavenging, but the greater efficiency of GSH than other scavengers and its more extensive protection suggested that it could also reverse thiol oxidation. Miller *et al.*[20] have observed the formation of mixed disulfides between GSH and CK in the presence of a xanthine oxidase system, which disappeared at higher GSH concentrations. Reversal by dithiothreitol of CK inactivation caused by high ferric iron concentrations has also been observed.[21] We measured only limited regeneration of active enzyme when GSH was added subsequent to the oxidant system. The probable explanation is that initial reversible oxidation of the enzyme is followed by conversion to an irreversible product. This has been observed with glyceraldehyde-3-phosphate dehydrogenase,[32] and is also consistent

with the finding of Korge & Campbell[21] that CK activity was restored by dithiothreitol in their system after a brief 30 sec inactivation period. It is probable, therefore, that GSH protects both by scavenging and by reducing the initial oxidized CK product.

Creatine kinase is a dimeric enzyme with two different subunits designated m and b. Adult mammalian skeletal muscle contains only mmCK, whereas heart muscle contains mmCK and mbCK.[25] Since the amino acid sequence which contains the essential cysteine is similar in the m and b subunits of rabbit muscle CK,[25] and since the isolated subunits are catalytically active,[33] the mbCK isozyme may be as susceptible to sulfhydryl oxidation and inactivation as the mmCK isozyme.

In conclusion, we have shown that rabbit muscle CK is inactivated both directly by H_2O_2 and by an iron-catalyzed reaction requiring superoxide or another reductant to recycle the iron. We did not see direct inactivation by superoxide, as has been reported for CK from rat heart.[1] The physiological chelates, Fe(citrate) and Fe(ATP), catalyze the reaction. Intracellular GSH should be protective, so physiological inactivation might not be expected until GSH is depleted. CK inactivation could be one mechanism whereby Adriamycin is cardiotoxic. However, this may not be consistent with the protection that is afforded by ICRF-187, since Fe(ICRF-198) catalyzes CK inactivation and would be expected to give only modest protection from inactivation catalyzed by physiological chelates.

Acknowledgements

This work was supported by the Health Research Council of New Zealand and the New Zealand Cancer Society.

References

1. J.M. McCord and W.J. Russell (1988) Inactivation of creatine phosphokinase by superoxide during reperfusion injury. In *Oxygen Radicals in Biology and Medicine* (eds. M.G. Simic, K.A. Taylor, J.F. Ward and C. von Sonntag), Plenum Press, New York, pp. 869–873.
2. A. Banerjee, M.A. Grosso, J.M. Brown, K.B. Rogers and G.J.R. Whitman (1991) Oxygen metabolite effects on creatine kinase and cardiac energetics after reperfusion. *American Journal of Physiology*, **261**, H590–H597.
3. Y.J. Suzuki, J.D. Edmondson and G.D. Ford (1992) Inactivation of rabbit muscle creatine kinase by hydrogen peroxide. *Free Radical Research Communications*, **16**, 131–136.
4. E.M. Park and J.A. Thomas (1988) *S*-thiolation of creatine kinase and glycogen phosphorylase *b* initiated by partially reduced oxygen species. *Biochimica et Biophysica Acta*, **964**, 151–160.
5. Y. Sato, L. Eddy and P. Hochstein (1991) Comparative cardiotoxicity of doxorubicin and a morpholino anthracycline derivative (KRN8602). *Biochemical Pharmacology*, **42**, 2283–2287.
6. I. Fabregat, J. Satrustegui and A. Machado (1984) Interaction with protein SH groups could be involved in adriamycin cardiotoxicity. *Biochemical Medicine and Metabolic Biology*, **32**, 289–295.
7. N.M. Scherer and D.W. Deamer (1986) Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca^{2+}-ATPase. *Archives of Biochemistry and Biophysics*, **246**, 589–601.
8. S.D. Aust, L.A. Morehouse and C.E. Thomas (1985) Role of metals in oxygen radical reactions. *Free Radicals in Biology and Medicine*, **1**, 3–25.
9. C.C. Winterbourn and H.C. Sutton (1986) Iron and xanthine oxidase catalyze formation of an oxidant species distinguishable from OH: Comparison with the Haber-Weiss reaction. *Archives of Biochemistry and Biophysics*, **244**, 27–34.
10. G.F. Vile, C.C. Winterbourn and H.C. Sutton (1987) Radical-driven Fenton reactions: Studies with paraquat, adriamycin, and anthraquinone 6-sulfonate and citrate, ATP, ADP, and pyrophosphate iron chelates. *Archives of Biochemistry and Biophysics*, **259**, 616–626.
11. G.F. Vile and C.C. Winterbourn (1990) *dl-N,N'*-dicarboxamidomethyl-*N,N'*-dicarboxymethyl-1,2-diaminopropane (ICRF-198) and *d*-1,2-bis(3,5-dioxopiperazine-1-yl)propane (ICRF-187) inhibition

of Fe^{3+} reduction, lipid peroxidation, and CaATPase inactivation in heart microsomes exposed to adriamycin. *Cancer Research*, **50**, 2307–2310.
12. D.E. Gannon, J. Varani, S.H. Phan, J.H. Ward, J. Kaplan, G.O. Till, R.H. Simon, U.S. Ryan and P.A. Ward (1987) Source of iron in neutrophil mediated killing of endothelial cells. *Laboratory Investigation*, **57**, 37–44.
13. J.L Speyer, M.D. Green, E. Kramer, M. Rey, J. Sanger, C. Ward, N. Dubin, V. Ferrans, P. Stecy, A. Zeleniuch-Jacquotte, J. Wernz, F. Feit, W. Slater, R. Blum and F. Muggia (1988) Protective effect of the bispiperazinedione ICRF-187 against doxorubicin-induced cardiac toxicity in women with advanced breast cancer. *New England Journal of Medicine*, **319**, 745–752.
14. K.M. Dawson (1975) Studies on the stability and cellular distribution of dioxopiperazines in cultured BHK-21S cells. *Biochemical Pharmacology*, **24**, 2249–2253.
15. Z.X. Huang, P.M. May, K.M. Quinlan, D.R. Williams and A.M. Creighton (1982) Metal binding by pharmaceuticals. Part 2. Interactions of Ca(II), Cu(II), Fe(II), Mg(II), Mn(II) and Zn(II) with the intracellular hydrolysis products of the antitumour agent ICRF 159 and its inactive homologue ICRF 192. *Agents and Actions*, **12**, 536–542.
16. L. Gianni, L. Vigano, C. Lanzi, M. Niggeler and V. Malatesta (1988) Role of daunosamine and hydroxyacetyl side chain in reaction with iron and lipid peroxidation by anthracyclines. *Journal of the National Cancer Institute*, **80**, 1104–1111.
17. C. Thomas, G.F. Vile and C.C. Winterbourn (1993) The hydrolysis product of ICRF-187 promotes iron-catalyzed hydroxyl radical production via the Fenton reaction. *Biochemical Pharmacology*, **45**, 1967–1972.
18. G.F. Vile and C.C. Winterbourn (1988) Microsomal reduction of low-molecular-weight Fe^{3+} chelates and ferritin: Enhancement by adriamycin, paraquat, menadione, and anthraquinone 2-sulfonate and inhibition by oxygen. *Archives of Biochemistry and Biophysics*, **267**, 606–613.
19. M.S. Baker and J.M. Gebicki (1984) The effect of pH on the conversion of superoxide to hydroxyl free radicals. *Archives of Biochemistry and Biophysics*, **234**, 258–264.
20. R.M. Miller, H. Sies, E.-M. Park and J.A. Thomas (1990) Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthine oxidase-initiated S-thiolation. *Archives of Biochemistry and Biophysics*, **276**, 355–363.
21. P. Korge and K.B. Campbell (1993) The effect of changes in iron redox state on the activity of enzymes sensitive to modification of SH groups. *Archives of Biochemistry and Biophysics*, **304**, 420–428.
22. D.A. Bates and C.C. Winterbourn (1982) Deoxyribose breakdown by the adriamycin semiquinone and H_2O_2: Evidence for hydroxyl radical participation. *FEBS Letters*, **145**, 137–141.
23. S.A. Kuby, L. Noda and H.A. Lardy (1954) Adenosinetriphosphate-creatine transphosphorylase I. Isolation of the crystalline enzyme from rabbit muscle. *Journal of Biological Chemistry*, **209**, 191–201.
24. M.L. Tanzer and C. Gilvarg (1959) Creatine and creatine kinase measurement. *Journal of Biological Chemistry*, **234**, 3201–3204.
25. D.C. Watts (1973) Creatine kinase (adenosine 5′-triphosphate-creatine phosphotransferase). In *The Enzymes*, 3rd ed. Vol. 8. (ed. P.D. Boyer), Academic Press, London and New York, pp. 383–455.
26. R. Furter, E.M. Furter-Graves and T. Wallimann (1993) Creatine kinase: The reactive cysteine is required for synergism but is nonessential for catalysis. *Biochemistry*, **32**, 7022–7029.
27. S. Goldstein, D. Meyerstein and G. Czapski (1993) The Fenton reagents. *Free Radicals in Biology and Medicine*, **15**, 435–445.
28. R.T. Dorr (1991) Chemoprotectants for cancer chemotherapy. *Seminars in Oncology*, **18**(Suppl 2), 48–58.
29. H.P. Monteiro, G.F. Vile and C.C. Winterbourn (1989) Release of iron from ferritin by semiquinone, anthracycline, bipyridyl, and nitroaromatic radicals. *Free Radicals in Biology and Medicine*, **6**, 587–591.
30. C.E. Thomas and S.D. Aust (1986) Release of iron from ferritin by cardiotoxic anthracycline antibiotics. *Archives of Biochemistry and Biophysics*, **248**, 684–689.
31. G.F. Vile and C.C. Winterbourn (1990) Thiol oxidation and inhibition of Ca-ATPase by adriamycin in rabbit heart microsomes. *Biochemical Pharmacology*, **39**, 769–774.
32. C. Little and P.J. O'Brien (1969) Mechanism of peroxide-inactivation of the sulphydryl enzyme glyceraldehyde-3-phosphate dehydrogenase. *European Journal of Biochemistry*, **10**, 533–538.
33. G.F. Bickerstaff and N.C. Price (1978) Creatine kinase: a review of some recent work on the mechanism and subunit behaviour of the enzyme. *International Journal of Biochemistry*, **9**, 1–8.

Accepted by Professor B. Halliwell