myeloperoxidase (MPO) levels, into the ileum (62, 75, and 76% at 0.1, 0.3, and 1 mg/kg, respectively) and lung (42, 56, and 61% at 0.1, 0.3, and 1 mg/kg, respectively) (Fig. 3, A and B).

These results with M40403 are consistent with observations from transgenic mice overexpressing human SOD (Cu and Zn) (*19*), indicating that neutrophil infiltration in the lung and intestine in the SAO model is prevented. Likewise, administration of PEG SOD also exhibited a protective effect in this model (*20*). Furthermore, M40403 (n = 8), but not M40404 (n = 4), both given at 1 mg/kg, prevented the fall in blood pressure seen after reperfusion (*8*) and increased the survival time (90 ± 5% survival at 4 hours for rats treated with M40403 versus 0% survival at 4 hours in untreated rats and those treated with M40404).

In summary, our results demonstrate that M40403 is a stable SOD mimic with therapeutic activity in models of inflammation and ischemia. In addition to the direct effects of $O_2^{\bullet-}$ in these models, there are likely to be indirect effects mediated by the formation of PN. It is possible that some of the beneficial anti-inflammatory and cytoprotective effects of M40403 are due to the prevention of PN formation by the removal of $O_2^{\bullet-}$ before it reacts with NO (*21*). The mechanism or mechanisms by which $O_2^{\bullet-}$ modulates events such as neutrophil influx at inflamed sites or cytokine production and release have yet to be defined.

Understanding the signal transduction mechanisms used by free radicals to modify the course of disease will undoubtedly elucidate important molecular targets for future pharmacological intervention. SOD mimics such as M40403 can serve as tools to dissect these mechanisms. In addition, these molecules may have potential for the treatment of diseases ranging from acute and chronic inflammation to cardiovascular disease and cancer.

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Anaerobic Microbes: Oxygen Detoxification Without Superoxide Dismutase

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Superoxide reductase from the hyperthermophilic anaerobe *Pyrococcus furiosus* uses electrons from reduced nicotinamide adenine dinucleotide phosphate, by way of rubredoxin and an oxidoreductase, to reduce superoxide to hydrogen peroxide, which is then reduced to water by peroxidases. Unlike superoxide dismutase, the enzyme that protects aerobes from the toxic effects of oxygen, SOR does not catalyze the production of oxygen from superoxide and therefore confers a selective advantage on anaerobes. Superoxide reductase and associated proteins are catalytically active 80°C below the optimum growth temperature (100°C) of *P. furiosus*, conditions under which the organism is likely to be exposed to oxygen.

Aerobic organisms have an efficient metabolism as compared to that of most anaerobes because of the high reduction potential of molecular oxygen, which serves as the terminal electron acceptor for respiration. This advantage comes with a price, because both the chemical and the metabolic reduction of oxygen result in the production of highly toxic and reactive oxygen species (*1*, *2*). The univalent reduction product of oxygen, superoxide (O_2^-), reacts with hydrogen peroxide in the presence of transition metals to produce the reactive hydroxyl radical $OH^{\cdot}$, which is most likely responsible for the toxic effects of molecular oxygen (*3*). Aerobic organisms have developed mechanisms to protect themselves from oxygen toxicity. These involve the enzymes superoxide dismutase (SOD) (Eq. 1) (*4*), catalase (Eq. 2) (*5*), and nonspecific peroxidases (Eq. 3) (*5*, *6*).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \quad (3)$$

For normal growth, aerobic organisms require molecular oxygen at near-atmospheric concentrations (21% v/v), whereas anaerobic organisms vary in their responses to oxygen, ranging from the extremely sensitive methanogens (*7*) to the more aerotolerant, sulfate-reducing *Desulfovibrio* (*8*), some species of which may be microaerophilic (*9*, *10*). Although most anaerobes inhabit ecosystems that are periodically exposed to air, they are unlikely to contain SOD or catalase, because both enzymes generate molecular oxygen (Eqs. 1 and 2) and thereby potentially prop-

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agate the production of reactive oxygen species. Although there are some exceptions (*11*), SOD and catalase genes are not generally present in anaerobes, as illustrated by their absence from the complete genome sequences now available for the anaerobic organisms *Methanococcus jannaschii* (*12*), *Archaeoglobus fulgidus* (*13*), *Pyrococcus horikoshii* (*14*), *P. abysii* (*15*), and *Thermotoga maritima* (*16*), as well as the incomplete genome of *Clostridium acetobutylicum* (*17*). So what defense mechanism against reactive oxygen species do these organisms possess? Our data suggest that anaerobes contain an enzyme involved in oxygen metabolism: superoxide reductase (SOR).

With the standard SOD assay (*18*), we measured high SOD activity [18 U/mg at 25°C (pH 7.8)] in cell-free extracts of the strictly anaerobic hyperthermophile *P. furiosus* (*19*). After purification of the putative SOD by multistep column chromatography, analysis by denaturing gel electrophoresis revealed a single protein of molecular weight ~14,000, which contained iron (0.5 atoms/mol) as indicated by direct chemical analysis (*20*). Using NH_2-terminal amino acid sequence information, the gene encoding this protein was cloned from the *P. furiosus* genome (*20*). The gene encodes a protein of 124 amino acid residues (14,323 daltons) and is located 14 base pairs downstream of the gene encoding rubredoxin, a small (5895 daltons), mononuclear, iron-containing redox protein previously purified from *P. furiosus* (*21*). The complete amino acid sequence of the putative SOD showed 40% identity to the mononuclear iron-containing COOH-terminal region (93 residues) of desulfoferrodoxin, a redox protein previously purified from the bacterium *Desulfovibrio desulfuricans* (*22*), and 50% sequence identity to a redox protein termed neelaredoxin, previously purified from the mesophile *D. gigas* (*23*). It was recently reported that both neelaredoxin and desulfoferrodoxin possess SOD activity (1200 and 70 U/mg at 25°C, respectively) (*24*).

Both the native and recombinant (*20*) forms of the putative SOD from *P. furiosus* exhibited high activity (~4000 U/mg) in the standard SOD assay at 25°C, which is comparable to that measured with bovine SOD under the same conditions (3400 U/mg). However, there is a fundamental difference in the properties of the two enzymes (Fig. 1). Although in both cases the rate of cytochrome c reduction decreased with increasing amounts of enzyme, excess "SOD" from *P. furiosus* (Fig. 1B), in contrast to excess bovine SOD (Fig. 1A), caused a reoxidation of reduced cytochrome c. This required the presence of superoxide: The putative SOD from *P. furiosus* did not oxidize cytochrome c when the cytochrome was chemically reduced by means of sodium dithionite. However, the cytochrome was oxidized if xanthine oxidase and xanthine were subsequently added to generate superoxide. Thus, the *P. furiosus* protein appears to function as a cytochrome c–superoxide oxidoreductase, or SOR, according to Eq. 4 [where cyt $c_{red(ox)}$ is the reduced (oxidized) form of cytochrome c]. This differs from the SOD reaction (Eq. 1) in that molecular oxygen is not produced.

$$O_2^- + 2H^+ + \text{cyt } c_{red} \rightarrow H_2O_2 + \text{cyt } c_{ox} \quad (4)$$

The high apparent SOD activity of *P. furiosus* SOR in the xanthine oxidase–cytochrome c assay was not reflected in three other SOD assays (*25*), where the activities were reduced by at least 95% (Table 1). Moreover, the apparent SOD activity of SOR decreased by about 98% when the acetylated form of cytochrome c was used (*26*), whereas that of bovine SOD was largely unaffected (Table 1). Acetylation has no effect on the superoxide-dependent reduction of cytochrome c, but it does interfere with protein-protein interactions (such as SOR–cytochrome c), which are needed for SOR but not for SOD activity (*26*).

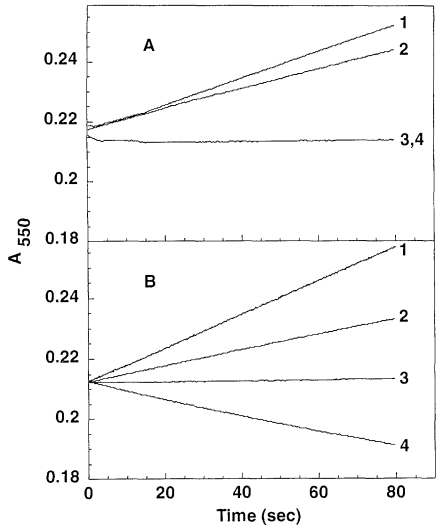

Fig. 1. *Pyrococcus furiosus* superoxide reductase is not a superoxide dismutase. Reactions were performed as described (*18*) in 1-ml cuvettes under aerobic conditions. Superoxide produced by xanthine (0.2 mM) and xanthine oxidase (3.4 μg) directly reduced horse heart cytochrome c (20 μM), as shown by the increase in absorbance at 550 nm (A_{550}) (**A** and **B**, trace 1). Addition of bovine SOD (3.4 μg, 1 U) inhibited the rate of reduction [(A), trace 2]. Excess SOD (40 U) prevented reduction completely [(A), trace 3], and additional SOD (60 U) had no further effect [(A), trace 4]. *P. furiosus* SOR (2.5 μg or 17 nM) also resulted in inhibition of reduction [(B), trace 2], and more SOR (6.2 μg) completely prevented reduction [(B), trace 3]. Addition of excess SOR (15 μg) caused oxidation of the reduced cytochrome c that was present before SOR addition [(B), trace 4]. Time zero is when SOR or SOD was added to the cuvettes (approximately 90 s after addition of xanthine oxidase). Under these conditions, A_{550} = 0.178 for fully oxidized cytochrome c.

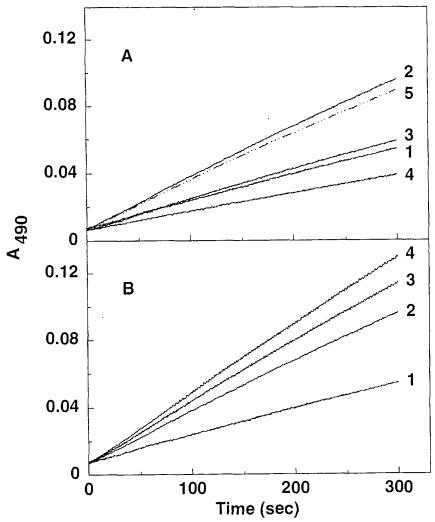

Fig. 2. *Pyrococcus furiosus* SOR is a rubredoxin–superoxide oxidoreductase. Reactions were done as in Fig. 1, except that reduced rubredoxin replaced cytochrome c. Superoxide directly oxidized *P. furiosus* rubredoxin, as shown by the increase in A_{490}. Rubredoxin (28 μM) reduced by the addition of sodium dithionite (42 μM) slowly auto-oxidized upon exposure to air (**A** and **B**, trace 1). Addition of superoxide rapidly increased the rate of oxidation [(A) and (B), trace 2]. Catalase (10 U) had little effect [(A), trace 5], whereas in a separate experiment, bovine SOD (1 U) abolished the effect of superoxide [(A), trace 3], and excess SOD (10 U) slowed down even the spontaneous oxidation of rubredoxin [(A), trace 4]. In contrast, addition of *P. furiosus* SOR (1.2 μg) increased the rate of superoxide-dependent rubredoxin oxidation [(B), trace 3], and the rate increased with additional SOR [1.2 μg; (B), trace 4].

Table 1. Comparison of bovine SOD and *P. furiosus* SOR in different assay systems. Assays were performed as in (*25*, *26*). Specific activity is expressed in units per milligram of protein, where one unit of activity inhibits the superoxide-dependent oxidation or reduction of the indicated electron carrier by 50% at 25°C.

Superoxide source	Superoxide detection method	Specific activity	
		Bovine SOD	*P. furiosus* SOR
Xanthine oxidase	Cytochrome c reduction	3400	4000
Pyrogallol	Pyrogallol oxidation	2300	80
Xanthine oxidase	Epinephrine oxidation	2200	100
Xanthine oxidase	Nitroblue tetrazolium reduction	1800	200
Xanthine oxidase	Acetylated cytochrome c reduction	3400	100

Thus, we suggest that SOR catalyzes a redox reaction; that it has little, if any, SOD activity; and that superoxide reduction is the physiological role of this protein, not only in *P. furiosus* but also in other anaerobic organisms. Homologs of *P. furiosus* SOR are found in almost all of the complete genome sequences that are currently available from anaerobes (*12–16, 27, 28*) and in the incomplete genomes of *Desulfovibrio gigas* (*23*) and *Clostridium acetobutylicum* (*17*). They contain 116 to 138 residues and show 20 to 70% sequence identity to *P. furiosus* SOR, although NH_2-terminal sequence information is available only from the *P. furiosus* and *D. gigas* proteins (*29*). In contrast to the anaerobes, analogs of *P. furiosus* SOR are not present in any of the 16 available genomes of "true" aerobes such as *Synechocystis* sp. (*30*) or facultative anaerobes such as *Escherichia coli* (*31*). In agreement with the role for SOR proposed here, superoxide reduction was postulated as the mechanism by which an SOD-deficient *E. coli* strain was complemented by desulfoferrodoxin, because SOD activity could not be detected in cell extracts (*32*).

The electron carrier rubredoxin, whose gene is adjacent to SOR in *P. furiosus*, was tested as a physiological donor in the SOR assay. In contrast to cytochrome c [midpoint potential (E_m) = 260 mV at 25°C (*33*)], which is reduced by superoxide, rubredoxin [E_m = 0 mV at 25°C (*21*)] is oxidized, in accord with the ability of superoxide to act as either an oxidant or a reductant in biological systems (*2*). Oxidation of colorless reduced rubredoxin was measured by the increase in absorbance at 490 nm. Reduced rubredoxin auto-oxidizes, so exposure of dithionite-reduced rubredoxin to air results in a slow increase in the visible absorbance (trace 1; Fig. 2, A and B). Subsequent addition of superoxide (from xanthine plus xanthine oxidase) causes a rapid increase in the rate of rubredoxin oxidation (trace 2; Fig. 2, A and B) that is inhibited by bovine SOD (trace 3, Fig. 2A), showing that it is a superoxide-dependent oxidation. Addition of catalase did not significantly affect the rate of rubredoxin oxidation (trace 5, Fig. 2A), demonstrating that peroxide plays a minimal role in these reactions. *Pyrococcus furiosus* SOR had the opposite effect from that of SOD and increased the rate of rubredoxin oxidation (trace 3, Fig. 2B). As before, the effect of SOR required superoxide, which is consistent with a redox rather than a dismutation reaction.

These data are consistent with a physiological role for SOR as a superoxide–rubredoxin oxidoreductase according to Eq. 5 [where $Rd_{red(ox)}$ is the reduced (oxidized) form of rubredoxin].

$$O_2^- + 2H^+ + Rd_{red} \rightarrow H_2O_2 + Rd_{ox} \quad (5)$$

Genes encoding rubredoxin are present in all of the anaerobe genomes but one (*P. horikoshii*) (*14*) and the protein has been purified from over a dozen different anaerobic species (*34*). These organisms represent a wide range of different metabolisms and the function of rubredoxin was unknown (*21*, *35*), although a role in oxygen toxicity had been suggested (*36*). In *P. furiosus*, an enzyme termed reduced nicotinamide adenine dinucleotide phosphate (NADPH)–rubredoxin oxidoreductase (NROR) reduces rubredoxin [Michaelis constant (K_m) = 10 μM] using NADPH (K_m = 5 μM) or NADH (K_m = 34 μM) as the electron donor (*37*). Thus, the reducing power for superoxide reduction by SOR is probably provided by NAD(P)H (Fig. 3).

Although the SOR reaction (Eq. 5) does not produce oxygen, it does produce hydrogen peroxide and this must be removed. We could not measure catalase activity in cell-free extracts of *P. furiosus* (*38*), but peroxide conversion is unlikely to occur through catalase activity because this activity, like the SOD reaction, produces oxygen (Eq. 2). Although most of the anaerobic genomes do not contain genes for classical catalases (*5*), most contain genes encoding other enzymes involved in peroxide detoxification (*39*), and thus these organisms have other mechanisms to remove peroxide (Fig. 3). Evidence for additional oxygen-related reactions in anaerobes comes from the finding that most of these genomes also contain genes encoding alkyl hydroperoxide reductase (*40*), which in aerobes is involved in the protection of lipid components against damage from reactive oxygen species, and rubrerythrin (*41*), which in anaerobes can have SOD, ferroxidase, or peroxidase activities (*42*). In both *P. furiosus* (*20*) and *T. maritima* (*16*), the rubrerythrin-encoding gene is adjacent to the genes encoding rubredoxin and SOR.

Pyrococcus furiosus SOR functions efficiently in vitro at 25°C, which is 75°C below the organism's optimal growth temperature (*19*), yet enzymes from hyperthermophiles typically exhibit no or barely detectable activity at such temperatures (*43*). Other than SOR, the only known exception is NROR from *P. furiosus,* which reduces rubredoxin with high efficiency at 25°C [catalytic constant (k_{cat})/K_m = 22,000 mM^{-1} s^{-1}] (*37*). Hence, a combination of SOR, rubredoxin, and NROR would rapidly reduce superoxide, using NAD(P)H as the electron donor (Fig. 3) at temperatures far below the growth temperature of *P. furiosus*. Organisms such as this inhabit marine hydrothermal fluids, and they can survive (cold) exposure to oxygen when hot, anaerobic vent waters mix with the surrounding cold, oxygen-saturated seawater (*44*, *45*). Thus, one would expect that a protective mechanism in anaerobic hyperthermophiles would be constitutively expressed and active at low temperatures, like the SOR system of *P. furiosus*. Regardless of their growth temperature, however, the data presented here indicate that anaerobic microorganisms possess a mechanism for detoxifying reactive oxygen species that is independent of the SOD- and catalase-based system of the aerobic world.

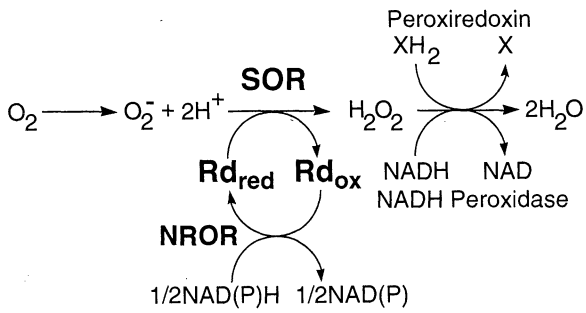

Fig. 3. Model for detoxification of reactive oxygen species in anaerobes such as *P. furiosus*. Abbreviations are as follows: NROR, NAD(P)H–rubredoxin oxidoreductase; Rd_{red}, reduced rubredoxin; Rd_{ox}, oxidized rubredoxin; XH_2, unknown organic electron donor. Enzymes and proteins shown in bold were purified from *P. furiosus*; the others are hypothetical, based on genome sequence analyses.

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The Tyrosine Kinase Negative Regulator c-Cbl as a RING-Type, E2-Dependent Ubiquitin-Protein Ligase

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Ubiquitination of receptor protein-tyrosine kinases (RPTKs) terminates signaling by marking active receptors for degradation. c-Cbl, an adapter protein for RPTKs, positively regulates RPTK ubiquitination in a manner dependent on its variant SRC homology 2 (SH2) and RING finger domains. Ubiquitin-protein ligases (or E3s) are the components of ubiquitination pathways that recognize target substrates and promote their ligation to ubiquitin. The c-Cbl protein acted as an E3 that can recognize tyrosine-phosphorylated substrates, such as the activated platelet-derived growth factor receptor, through its SH2 domain and that recruits and allosterically activates an E2 ubiquitin-conjugating enzyme through its RING domain. These results reveal an SH2-containing protein that functions as a ubiquitin-protein ligase and thus provide a distinct mechanism for substrate targeting in the ubiquitin system.

Specific and precise signaling by RPTKs requires that both the intensity and the duration of the elicited intracellular signals be tightly regulated. To terminate signaling, ubiquitination can mark receptors for degradation (*1*). Free ubiquitin (Ub) is recruited to ubiquitination pathways by the E1 Ub-activating enzyme through the formation of a thioester between a cysteine in E1 and the COOH-terminus of Ub (*2*). Ub is subsequently transferred, also as a thioester, to members of the E2 Ub-conjugating (Ubc) enzyme superfamily. Ub-protein ligases, or E3s, are defined as the pathway components responsible for substrate recognition and for promoting Ub ligation to substrate (*2*). HECT domain–containing E3s (*h*omologous to *E6*-AP *C*OOH-*t*erminus) accept Ub from E2s in the form of a thioester and then catalyze the formation of stable Ub-substrate conjugates (*2*). E3s such as the Skp1/cullin/F box (SCF) and anaphase-promoting complexes (APC) (*2*) do not appear to form thioesters with Ub (*3*). Rather, these complexes act by bringing E2~Ub and substrate together and by allosterically activating E2 (*3*, *4*). The efficiency of poly-Ub chain synthesis may be increased by other factors, such as the E4 proteins (*5*).

c-Cbl, a 120-kD protein that contains a variant SH2 domain, a RING finger, a proline-rich region, and a Ub-associated domain, binds to, and stimulates the ubiquitination of, active platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and colony-stimulating factor–1 receptors (*6–8*). Moreover, the c-Cbl homolog in *Caenorhabditis elegans*, SLI-1, has been genetically defined as a negative regulator of RPTK signaling (*9*). The fact that c-Cbl can function as an adapter protein in tyrosine phosphorylation–dependent signaling raises the possibility that it recruits E3 or E4

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[12] **Complete Genome Sequence of the Methanogenic Archaeon, Methanococcus jannaschii**

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