The Inhibition of Manganese Superoxide Dismutase by Cacodylate

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Manganese superoxide dismutase is inhibited by cacodylate buffer when assayed using the xanthine–xanthine oxidase system for generating superoxide radicals. No inhibitory effect was observed when the enzyme activity was measured using superoxide generated by pulse radiolysis. Further investigations revealed that the inhibitory effect is due to a cacodylate anion radical produced by the interaction of hydroxyl radicals (generated by the xanthine–xanthine oxidase system) and cacodylate anions. The formation of cacodylate radicals by hydroxyl radical activation of cacodylate was investigated by electron paramagnetic resonance spectroscopy. The biochemical use of cacodylate buffers, where hydroxyl radical production is suspected, may generate the formation of highly reactive cacodylate radicals which are potentially toxic to enzyme systems.

Introduction

Superoxide dismutase (E.C. 1.15.1.1) is an important enzyme in the line of defence against the toxicity of oxygen-centred radicals in living species. Three forms of the enzyme have been characterised. These are the copper/zinc, manganese and iron forms. The enzyme with a copper/zinc prosthetic group is with two exceptions [1, 2] a eukaryotic enzyme, the manganese enzyme is found in both prokaryotes and eukaryotes, whilst the iron-containing enzyme is essentially a prokaryotic enzyme although it has recently been reported in a eukaryotic alga [3] and in a higher plant [4].

Diethyldithiocarbamate [5] is a well-characterised inhibitor of the copper/zinc enzyme besides cyanide which does not inhibit the other forms of the enzyme. The manganese and iron forms of the enzyme are usually distinguished on the basis of their differential inhibition by hydrogen peroxide and azide [6].

During an investigation concerning anion effects on the activity of manganese superoxide dismutase, we observed that the activity of this enzyme is diminished in the presence of cacodylate buffer. The observed inhibitory effect of cacodylate on manganese superoxide dismutase is reported in this work.

Experimental

Manganese superoxide dismutase from *Bacillus stearothermophilus* was a kind gift from Dr. John Walker (MRC Laboratory for Molecular Biology, Cambridge, England) and was further purified on Ultrogel AcA 44 (LKB Instruments Ltd., Surrey, England). Enzyme concentration was determined using the extinction coefficient $E^{1\%}_{280} = 13.2$ [7]. Copper/zinc superoxide dismutase was purified from bovine erythrocytes [8].

Enzyme activity was assayed by either following the reduction of cytochrome c by the xanthine–xanthine oxidase system, or by following the disappearance of superoxide generated by pulse radiolysis at 250 nm in air-equilibrated buffer solutions containing 100 m*M* formate. Hydroxyl radicals were also generated by pulse radiolysis in nitrous oxide equilibrated solutions.

Electron paramagnetic resonance (epr) spectra were recorded in a Varian E104, X band epr spectrometer with a Varian E900-3 data acquisition system. A 200W mercury lamp was used for ultraviolet radiation.

Generation of superoxide radicals, O_2^-, and hydroxyl radicals, $OH^\bullet$, in the xanthine–xanthine oxidase system can be followed by spin trapping using the spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) [9]. By locking on to a peak in the DMPO superoxide spin adduct, 5,5-dimethyl-2-hydroperoxypyrrolidino-1-oxy (DMPO–OOH) epr spectrum and on to a peak in the DMPO hydroxyl spin adduct, 5,5-

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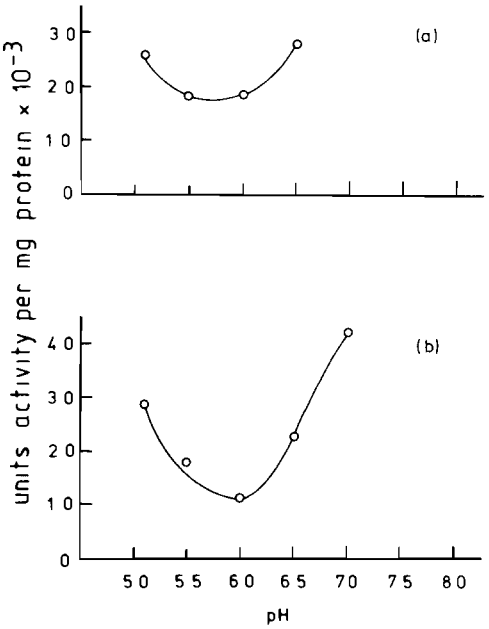

Fig. 1. Activities of (a) copper/zinc superoxide dismutase and (b) manganese superoxide dismutase in the presence of 0.1 *M* cacodylate buffer at various pH values.

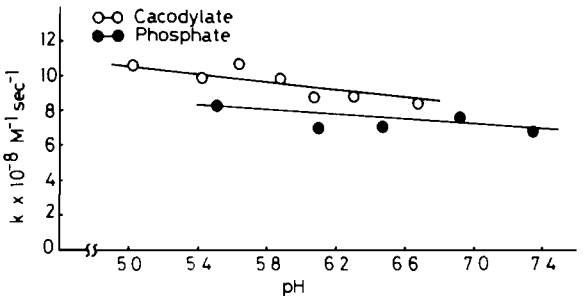

Fig. 2. Variation in the rate constant for disproportionation of superoxide (k) by manganese superoxide dismutase in the presence of 0.1 *M* cacodylate and 0.1 *M* phosphate at various pH values.

dimethyl-2-hydroxyl-pyrrolidino-1-oxy (DMPO–OH) epr spectrum generation of O_2^- and $OH^\bullet$ can respectively be followed over the course of the xanthine–xanthine oxidase reaction. The effect of cacodylate buffer on these spin adduct reaction profiles was used to demonstrate any interactions between cacodylate and O_2^-, $OH^\bullet$ or the xanthine–xanthine oxidase radical generating system.

Results and Discussion

The activities of copper/zinc and manganese superoxide dismutase measured by the xanthine–xanthine oxidase assay are given in Fig. 1. The effect of 0.1 *M* cacodylate buffer on the copper/zinc enzyme is shown in Fig. 1a, whilst the effect on the manganese enzyme is shown in Fig. 1b. Appreciable inhibition is observed with the manganese but not with the copper/zinc enzyme. This inhibition appeared to be due to cacodylate and not to a pH effect since no inhibition of the manganese enzymes was observed in 0.1 *M* tris-acetate over the same pH range.

When the manganese superoxide dismutase was assayed by pulse radiolysis no significant change in rate constants was observed (Fig. 2) over the same pH range in the presence of either 0.1 *M* cacodylate or 0.1 *M* phosphate. This result confirms that the activity of the *Bacillus stearothermophilus* manganese superoxide dismutase is independent of pH over the range investigated in the presence of cacodylate. Thus the effect observed in the xanthine–xanthine oxidase assay is not due directly to cacodylate although it is mediated by cacodylate. It is interesting to point out that the maximum inhibitory effect is observed around pH 6.0 close to the pK of cacodylate which is 6.2. It thus appears that the cacodylate anion is interacting with products of the xanthine–xanthine oxidase reaction other than O_2^- to form an active species which acts as an inhibitor of the activity of manganese dismutase and to a lesser extent of the copper/zinc enzyme. Besides O_2^-, the xanthine–xanthine oxidase reaction is also known to produce $OH^\bullet$ radicals and hydrogen peroxide [10]. We found the same level of inhibition in the presence of 100 Units of catalase, indicating that the inhibitory species is not a peroxo form of cacodylate. Almost no inhibitory effect was, however, observed in the presence of 5 m*M* ethanol, a well known scavenger of $OH^\bullet$ radicals, although ethanol itself was found to cause inhibition of the enzyme. Manganese superoxide dismutase has previously been described to be inhibited by organic solvents [11]. Mannitol, another $OH^\bullet$ radical scavenger, was also found to protect against cacocylate inhibition although it was not as effective as ethanol. It therefore appears that $OH^\bullet$ radicals are interacting with cacodylate anions to form a cacodylate anion radical which then acts as the inhibitory species of manganese superoxide dismutase activity.

When cacodylate at pH 6.1 is reacted with $OH^\bullet$ radicals generated by pulse radiolysis, the formation of a species with an absorption around 300 nm is evident after 4 μs (Fig. 3). The absorption decays very rapidly and completely disappears at 300 μs indicating that the species is very short-lived. In the presence of 2.9×10^{-3} *M* manganese superoxide dismutase no absorption maximum is observed indicating that the radical formed interacts to form an adduct with the enzyme (Fig. 3). The adduct formed also decays very rapidly. The absorption

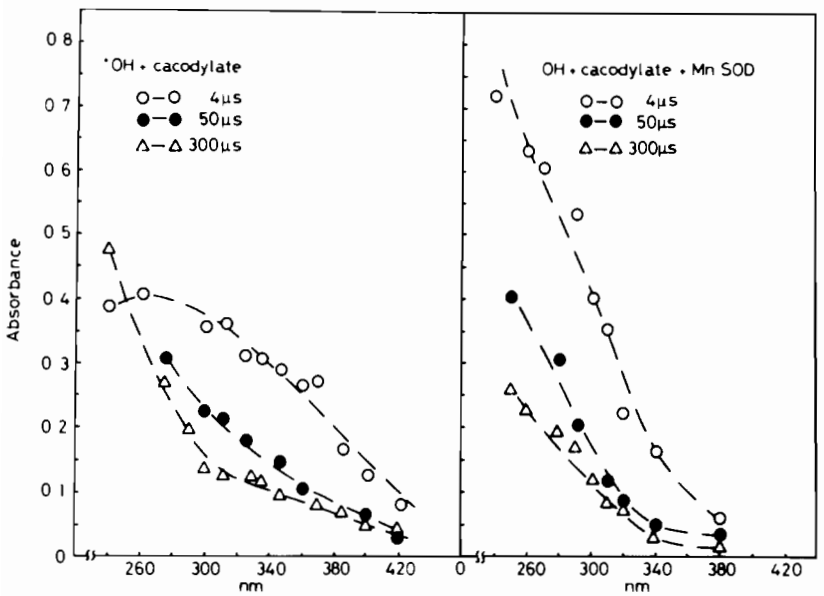

Fig. 3. Absorption spectra of solutions pulsed with hydroxyl radicals in the presence of 0.1 *M* cacodylate and in the presence of 0.1 *M* cacodylate and 50 μg of manganese superoxide dismutase.

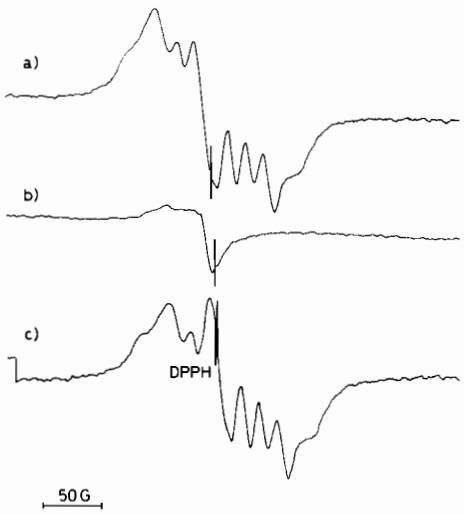

Fig. 4. Photolysis of hydrogen peroxide in the presence of cacodylate. The reaction mixtures were for spectrum (a) 1 m*M* H_2O_2 and 50 m*M* cacodylate in D_2O and for spectrum (b) 1 m*M* H_2O_2 in D_2O Spectrum (c) is the difference spectrum (a) – (b). The reaction mixtures were irradiated by ultraviolet light at 77 K.

decreases to the value observed in the absence of the enzyme after 300 μs. The species derived from the cacodylate anion by the interaction with $OH^{\bullet}$ radicals appears to inhibit the enzyme activity by attacking the protein moiety rather than the manganese chromophore. When $OH^{\bullet}$ radicals were reacted with manganese superoxide dismutase in the presence of either cacodylate or phosphate at pH 6.1, the same bleaching effect was observed for the manganese chromophore at 480 nm. The rate constant for the bleaching reaction was found to be $3.5 \times 10^5\ M^{-1}\ s^{-1}$ both with $OH^{\bullet}$ radicals and cacodylate anion radicals.

Spin trapping with DMPO of the radical produced by the xanthine–xanthine oxidase system in the presence and absence of 1 *M* cacodylate yielded identical amounts of DMPO–OOH. The $O_2^{\bar{}}$ production is not significantly affected by cacodylate indicating that cacodylate is not affecting the xanthine–xanthine oxidase radical producing activity. However, a 33% decrease in the concentration of DMPO–OH produced is observed over a 30 minute period in the presence of cacodylate. Cacodylate is therefore competing with DMPO for $OH^{\bullet}$ radicals as no new spin adducts are observed.

Ultraviolet irradiation of aqueous hydrogen peroxide solutions at 77 K produces hydroxyl ($OH^{\bullet}$) and peroxyl ($OOH^{\bullet}$) radicals in an ice matrix [12]. Using deuterated peroxide in D_2O, a higher ratio of $OD^{\bullet}$ to $OOD^{\bullet}$ is produced, as deuterium abstraction from D_2O_2 is less favoured [13] than $H^{\bullet}$ from the H_2O_2. An epr study of the photolysis of D_2O_2 in D_2O in the presence and absence of cacodylate at 77 K was used to determine the presence of any cacodylate-derived epr active species (Fig. 4). Deuterated cacodylate was also investigated under similar conditions to elucidate the structure of any cacodylate radical formed under these conditions. The epr data obtained from this investigation (Fig. 4c) suggest the formation of a cacodylate radical from the interaction between $OH^{\bullet}$ radicals and cacodylate. The epr spectrum of an α-carbon arsenic radical has been reported [14]. The spectral features

obtained for the cacodylate radical are indicative of an α-methylene arsenic radical.

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