Xanthine oxidase-catalysed oxidation of paracetamol

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Paracetamol was polymerized in a reaction mixture containing xanthine oxidase, xanthine and paracetamol. This polymerization reaction was not inhibited by allopurinol or KCN, indicating that neither the molybdenum sites nor the iron–sulphur centres of the enzyme were involved in this catalytic activity. Removal of the flavin centres from the enzyme, however, completely abolished paracetamol oxidation. Spectroscopic measurements suggested that in the simultaneous presence of both paracetamol and H_2O_2 a peroxyflavin intermediate was formed, which is presumably responsible for the paracetamol polymerization reaction.

INTRODUCTION

In recent studies it was shown that paracetamol protected human erythrocytes against t-butyl hydroperoxide-induced oxidative stress, presumably by a reaction with t-butyl hydroperoxide-derived radicals [1]. A similar reaction of paracetamol with highly reactive radicals has been implicated by Reiter & Wendel in the protection of liver cell microsomal fraction against NADPH-induced lipid peroxidation [2], suggesting a more general activity of paracetamol as a radical scavenger. In this context it seemed appropriate to study the effect of paracetamol on the $\text{OH}^\bullet$ -generating system xanthine oxidase/xanthine/ Fe^{3+} -EDTA. During these studies it appeared that paracetamol dimers and some higher polymers were formed in the presence of xanthine oxidase and xanthine. The dimers exhibited the typical fluorescence characteristics of biphenyl derivatives [3].

A similar polymerization of paracetamol is catalysed by low concentrations of horseradish peroxidase in the presence of H_2O_2 . At higher concentrations of the enzyme also larger polymers are formed [4]. It has been shown that this polymerization is caused by the one-electron oxidation of paracetamol to NAPSQI, with subsequent dimerization of two molecules of NAPSQI [5,6]. Also, the two-electron oxidation of paracetamol to NAPQI, as catalysed, e.g., by cytochrome P-450 [7], may ultimately lead to paracetamol dimerization, as a result of comproportionation of NAPQI with paracetamol, yielding two molecules of NAPSQI that will subsequently dimerize [5].

It seemed likely that the observed xanthine oxidase-catalysed polymerization of paracetamol would proceed essentially via one of these two routes. As described in the present paper, however, the reaction mechanism with xanthine oxidase appeared to be different.

MATERIALS AND METHODS

Xanthine oxidase, horseradish peroxidase, SOD, catalase and NADH were obtained from Boehringer

(Mannheim, Germany). ^3H -labelled paracetamol (> 99.5% radiochemically pure) was purchased from NEN Research Products (Boston, MA, U.S.A.).

Inactivation of xanthine oxidase by allopurinol was performed as described by Massey *et al.* [8]. Modification of the iron–sulphur centres was done by incubation with KCN, in accordance with Massey & Edmondson [9]. For removal of the flavin centres from the enzyme the method described by Topham *et al.* [10] was followed.

Experiments with xanthine oxidase were performed at 20 °C in 0.1 M-sodium pyrophosphate buffer, pH 8.5, containing 0.3 mM-EDTA. In experiments with horseradish peroxidase 50 mM-sodium phosphate buffer, pH 7.4, was used. Anaerobic conditions were obtained by leading Ar (less than 0.3 p.p.m. O_2) through the solution. If not otherwise stated the peroxidase system contained, in final concentrations, 1.8 units of horseradish peroxidase/ml, 1.5 mM- H_2O_2 and 1.0 mM-paracetamol, whereas the xanthine oxidase system contained 0.05 unit of xanthine oxidase/ml and 0.15 mM-xanthine. When present, other reagents were added to the following final concentrations: Fe^{3+} -EDTA, 0.01 mM; paracetamol, 1 mM; deoxyribose, 1 mM; cytochrome c, 0.05 mM; catalase, 250 units/ml; SOD, 1000 units/ml.

Xanthine oxidase-catalysed oxidation of xanthine was monitored by measurement of uric acid production [11]. NADH oxidation was measured spectrophotometrically, at 340 nm. Release of $\text{O}_2^{\bullet -}$ radical anions was measured as SOD-inhibitable cytochrome c reduction [12]. H_2O_2 concentrations were determined by adding catalase to the reaction mixture and measuring O_2 generation with a YSI oxygen monitor. $\text{OH}^\bullet$ -induced degradation of 2-deoxyribose was measured by the thiobarbituric acid method [13]. Paracetamol dimerization was measured fluorimetrically (λ_{exc} , 325 nm, λ_{em} , 425 nm). Further analysis of the paracetamol polymerization products was performed by h.p.l.c. by the procedure of Potter *et al.* [4]. Covalent binding of paracetamol to bovine serum albumin was measured as described by Nelson *et al.* [14]. Reduction of Fe^{3+} to Fe^{2+} was monitored by the bathophenanthroline method [15].

Abbreviations used: NAPQI, N-acetyl-p-benzoquinoneimine; NAPSQI, N-acetyl-p-benzosemiquinoneimine; SOD, superoxide dismutase.

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RESULTS

Oxidation of paracetamol by horseradish peroxidase

In the presence of horseradish peroxidase and H_2O_2 dimerization of paracetamol took place, as reflected by the development of the characteristic biphenyl fluorescence ($\lambda_{\text{exc.}}$ maximum 325 nm and $\lambda_{\text{em.}}$ maximum 425 nm), in accordance with earlier studies [6]. Cytochrome *c* was reduced when added to the reaction mixture. Both paracetamol dimerization and cytochrome *c* reduction were unaffected by SOD. H.p.l.c. analysis of the reaction products revealed that about 90% of the oxidized paracetamol was recovered as the paracetamol dimer, 2% as the *N*-acetaminophen dimer, 7% as the trimer and 1% as the tetramer. Only the dimer and the trimer exhibited fluorescence.

In a standard reaction mixture containing horseradish peroxidase, H_2O_2 , paracetamol, Fe^{3+} -EDTA and deoxyribose, no thiobarbituric acid-reactive products were formed. When bathophenanthroline was added to the mixture, an increasing absorbance at 530 nm was observed, indicating reduction of Fe^{3+} to Fe^{2+} . Also, this reduction was unaffected by SOD. Finally, in the presence of horseradish peroxidase and H_2O_2 , covalent binding of [^3H]paracetamol to serum albumin was observed. Maximal binding was achieved after 10 min and amounted to 4.6 nmol/mg of protein, in good agreement with the results reported by Nelson *et al.* [14].

Influence of paracetamol on xanthine oxidase-catalysed oxidation of xanthine and generation of $\text{OH}^\bullet$ radicals

In a reaction mixture containing xanthine oxidase, xanthine, Fe^{3+} -EDTA and deoxyribose a rapid generation of $\text{OH}^\bullet$ radicals occurs, as indicated by the formation of thiobarbituric acid-reactive products. This is caused by the formation of both H_2O_2 and $\text{O}_2^{\bullet-}$ during the catalytic activity of xanthine oxidase, with subsequent generation of $\text{OH}^\bullet$ radicals in an iron-catalysed

Haber-Weiss reaction [12,13,16,17]. Addition of paracetamol (1 mM) to the reaction mixture caused an almost complete inhibition of deoxyribose degradation. In Fig. 1(a) xanthine oxidation, cytochrome *c* reduction and H_2O_2 accumulation are shown in a standard xanthine oxidase/xanthine/cytochrome *c* mixture. Cytochrome *c* reduction was completely inhibited by SOD. After addition of paracetamol xanthine oxidation was slightly inhibited (18%), whereas both cytochrome *c* reduction and H_2O_2 accumulation were abolished (Fig. 1b). Addition of catalase (arrow in Fig. 1b) restored the velocities of xanthine oxidation and cytochrome *c* reduction to the initial values. The restored cytochrome *c* reduction was completely suppressed by SOD (results not shown).

Polymerization of paracetamol by xanthine oxidase

When paracetamol was incubated with xanthine oxidase, no reaction took place. After addition of xanthine or hypoxanthine, however, a fluorescence with the characteristics of the paracetamol dimer developed. No fluorescence developed under anaerobic conditions. Thus paracetamol is apparently dimerized in the simultaneous presence of xanthine oxidase and xanthine or hypoxanthine. This dimerization was strictly H_2O_2 -dependent, as it was abolished by catalase. Therefore, in further experiments paracetamol was incubated with xanthine oxidase and H_2O_2 , but without xanthine. Again dimerization was observed, but now under both aerobic and anaerobic conditions. At very low paracetamol concentrations the reaction velocity was proportional to the square of the paracetamol concentration. With h.p.l.c. analysis about 94% of oxidized paracetamol was recovered as the dimer, 5% as the trimer and 1% as the tetramer. No reaction took place when xanthine oxidase

Fig. 1. Uric acid (curves A), H_2O_2 (curves B) and $\text{O}_2^{\bullet-}$ (curves C) generation

(a) Standard xanthine oxidase/xanthine/cytochrome *c* reaction mixture. (b) As (a), with paracetamol added; catalase was added at the point indicated by the arrow.

Fig. 2. Xanthine oxidase-catalysed oxidation of NADH

Curve A, standard xanthine oxidase/NADH mixture, aerobic conditions. Curve B, as for curve A, with paracetamol and H_2O_2 added, aerobic conditions. Curve C, as for curve B, anaerobic conditions.

was omitted from the reaction mixture. Addition of desferrioxamine did not affect the polymerization reaction, excluding the possibility that contaminating iron would be involved in the reaction.

In a solution containing xanthine oxidase, H_2O_2 , paracetamol and cytochrome *c*, no cytochrome *c* reduction could be detected. In a reaction mixture containing xanthine oxidase, paracetamol, albumin and either H_2O_2 or xanthine, no covalent binding of paracetamol to albumin took place.

NADH is oxidized by xanthine oxidase under aerobic but not under anaerobic conditions (Fig. 2). Addition of xanthine or paracetamol or H_2O_2 did not affect the NADH oxidation rate significantly. If paracetamol and H_2O_2 were added simultaneously, however, the NADH oxidation rate increased strongly. Strikingly, in this mixture NADH oxidation also occurred under anaerobic conditions (Fig. 2).

Modification of xanthine oxidase with either allopurinol or KCN abolished xanthine oxidation, but did not affect paracetamol polymerization in the presence of H_2O_2 . Removal of the flavin centres with hyperosmotic salt solution, on the other hand, blocked both xanthine oxidation and paracetamol polymerization. Xanthine oxidation could be restored by adding artificial electron acceptors such as ferricyanide, as shown before [18]. These electron acceptors did not restore paracetamol polymerization, however (results not shown).

Spectral measurements

On the addition of xanthine to an anaerobic solution of xanthine oxidase, the immediate reduction of the enzyme was reflected spectroscopically by decreased absorbance both around 450 nm and around 550 nm (Fig. 3). Addition of H_2O_2 had no influence on the spectra. Also, addition of paracetamol to xanthine oxidase alone did not affect the spectrum of the enzyme. When H_2O_2 was also present, however, the spectrum changed immediately, with a new maximum at 372 nm (Fig. 3).

Fig. 3. Absorbance spectra of an anaerobic solution of xanthine oxidase

---, Inactive enzyme; ·····, xanthine added; —, paracetamol and H_2O_2 added.

Scheme 1. Scheme of the one-electron oxidation and two-electron oxidation and the proposed xanthine oxidase-catalysed oxidation of paracetamol

DISCUSSION

As described in the Results section, incubation of paracetamol with only xanthine oxidase did not lead to any reaction. When xanthine or hypoxanthine was also present, however, polymerization of paracetamol occurred. The reaction should be ascribed to a peroxidase-like activity of xanthine oxidase, as catalase completely abolished the reaction, whereas, conversely, polymerization was supported by H_2O_2 in the absence of xanthine or hypoxanthine, both aerobically and anaerobically.

Two different enzymically catalysed pathways leading to paracetamol polymerization have been described (Scheme 1). The one-electron oxidation, as catalysed by horseradish peroxidase, leads primarily to the formation of NAPSQI, as shown by e.s.r. studies [14,19]. Subsequently two molecules of NAPSQI will either dimerize, or disproportionate, yielding paracetamol and NAPQI [6]. As shown in the present study, both cytochrome *c* and Fe^{3+} were reduced in the reaction mixture. Apparently this was not caused by the generation of $\text{O}_2^{\cdot-}$, as the reductions were SOD-insensitive. Therefore the reducing agent must be the NAPSQI radical itself. The NAPSQI-induced reduction of Fe^{3+} to Fe^{2+} should, in

the simultaneous presence of H_2O_2 , lead to the production of $\text{OH}^\bullet$, via the iron-catalysed Haber-Weiss reaction. However, in a mixture of peroxidase, Fe^{3+} -EDTA, paracetamol, H_2O_2 and deoxyribose, no ($\text{OH}^\bullet$ -induced) degradation of deoxyribose was observed. Most probably paracetamol acted as a highly efficient $\text{OH}^\bullet$ scavenger under these conditions, in accordance with previous conclusions concerning the scavenging properties of paracetamol [1,2].

The two-electron oxidation of paracetamol, as catalysed by cytochrome *P*-450 [6], yields NAPQI (Scheme 1). NAPQI may covalently bind to protein molecules or, in a comproportionation reaction with paracetamol, yield two molecules of NAPSQI, dimerizing before they diffuse [5].

It seemed likely *a priori* that the xanthine oxidase-catalysed dimerization of paracetamol would proceed essentially according to one of these two pathways. Experimental evidence indicates, however, that the reaction mechanism is different. Whereas in the presence of horseradish peroxidase, H_2O_2 and paracetamol an SOD-insensitive reduction of cytochrome *c* occurred, no cytochrome *c* reduction was observed when horseradish peroxidase was replaced by xanthine oxidase. As cytochrome *c* reduction in the former case is caused by NAPSQI, it must be concluded that this radical is not generated in the xanthine oxidase-catalysed reaction. This is convincingly confirmed by e.s.r. studies, demonstrating that NAPSQI was generated in the system horseradish peroxidase/ H_2O_2 /paracetamol, but not with xanthine oxidase/xanthine/paracetamol [14]. Further, both one-electron oxidation and two-electron oxidation of paracetamol lead to covalent binding of the drug to proteins [7,14]. Whereas in the present studies paracetamol binding to albumin in the presence of peroxidase and H_2O_2 was confirmed, no binding was observed with xanthine oxidase and H_2O_2 . These results indicate that neither NAPQI nor NAPSQI is an intermediate in the xanthine oxidase-catalysed polymerization of paracetamol. Therefore it seems probable that in the presence of H_2O_2 xanthine oxidase catalysed the direct dimerization of two molecules of paracetamol (Scheme 1), consistent with the observation that at low concentrations the reaction velocity was proportional to the square of the drug concentration.

Allopurinol inactivates the molybdenum sites of xanthine oxidase [8,20,21], whereas KCN inactivates the iron-sulphur centres [9,10]. In accordance, both allopurinol and KCN completely inhibited xanthine oxidation. These agents did not inhibit paracetamol polymerization in the presence of H_2O_2 , however, indicating that neither the molybdenum nor the iron-sulphur centres were involved in this reaction. Only removal of the flavin centres from the enzyme blocked the enzymic activity with respect to paracetamol, indicating that only these centres are crucial for the polymerization reaction. The loss of activity cannot be attributed to denaturation of the deflavo enzyme, as artificial electron acceptors restored xanthine oxidation but not paracetamol dimerization.

Previous studies have shown that NADH oxidation by xanthine oxidase is also directly mediated by the flavin centres [8]. In this context the observation that NADH oxidation is strongly increased when both H_2O_2 and paracetamol are added is noteworthy. Apparently the flavin centres were activated with respect to NADH

oxidation under these conditions. This was most convincingly demonstrated under anaerobic conditions. Neither paracetamol nor H_2O_2 initiated NADH oxidation by xanthine oxidase under anaerobic conditions. When H_2O_2 and paracetamol were both present, however, NADH oxidation took place (Fig. 2). Apparently O_2 was not required as ultimate electron acceptor under these circumstances.

With xanthine or hypoxanthine as substrate the fully six-electron-reduced enzyme is reoxidized by a four-step sequential removal of electrons, with O_2 as electron acceptor. In the first and second steps two electrons are transferred, yielding two molecules of H_2O_2 and the two-electron-reduced enzyme. In the third and fourth steps one electron is transferred, yielding two $\text{O}_2^{\bullet -}$ radical anions and the fully reoxidized enzyme [22,23]. This is in accordance with the results shown in Fig. 1(a). In a mixture of xanthine oxidase, xanthine and paracetamol, however, neither H_2O_2 accumulation nor $\text{O}_2^{\bullet -}$ formation was observed (Fig. 1b). Nevertheless, H_2O_2 was certainly formed under these conditions, as catalase blocked the xanthine oxidase-induced polymerization of paracetamol. Apparently H_2O_2 was used in this peroxidase-like activity of xanthine oxidase with respect to paracetamol, with the same velocity as it was formed. The lack of $\text{O}_2^{\bullet -}$ accumulation cannot be explained by secondary reactions, as $\text{O}_2^{\bullet -}$ generation was restored to the original rate by addition of catalase (Fig. 1b), blocking paracetamol polymerization. Therefore it seems likely that in the simultaneous presence of xanthine and paracetamol the one-electron-reduced and two-electron-reduced forms of the enzyme (yielding $\text{O}_2^{\bullet -}$ on reoxidation) are not generated as intermediates.

The nature of the flavin derivative involved in H_2O_2 -supported paracetamol oxidation is still unclear. The spectral studies (Fig. 3), however, are of interest in this context. Addition of xanthine to an anaerobic solution of xanthine oxidase caused spectral changes characteristic for the reduction of the enzyme. Reduction of the iron-sulphur centres is reflected by decreased absorbance around 550 nm, whereas reduction of the FAD centres results in decreased absorbance in the 450 nm region [8,24]. Anaerobic incubation of the enzyme with either H_2O_2 or paracetamol did not induce spectral changes, but incubation with H_2O_2 and paracetamol simultaneously caused a pronounced change of the absorption spectrum, with a new maximum at 372 nm. A similar absorption maximum is observed during the catalytic activity of flavoprotein hydrolases. Experimental evidence indicates that with these enzymes this absorbance maximum should be attributed to an oxygenated intermediate, most probably a flavin peroxide [25-28]. It is conceivable that a similar flavin peroxide intermediate is generated during the xanthine oxidase-mediated peroxidation of paracetamol.

REFERENCES

1. Van der Zee, J., Mulder, G. J. & Van Steveninck, J. (1988) *Chem.-Biol. Interact.* **65**, 15-23
2. Reiter, R. & Wendel, A. (1983) *Biochem. Pharmacol.* **32**, 665-670
3. Bridges, J. W., Creaven, P. J. & Williams, R. T. (1965) *Biochem. J.* **96**, 872-878

4. Potter, D. W., Miller, D. W. & Hinson, J. A. (1985) *J. Biol. Chem.* **260**, 12174–12180
5. Potter, D. W. & Hinson, J. A. (1986) *Mol. Pharmacol.* **30**, 33–41
6. Potter, D. W. & Hinson, J. A. (1987) *J. Biol. Chem.* **262**, 966–973
7. Dahlin, D. C., Miwa, G. T., Lu, A. Y. H. & Nelson, S. D. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1327–1331
8. Massey, V., Komai, H. & Palmer, G. (1970) *J. Biol. Chem.* **245**, 2837–2844
9. Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
10. Topham, R. W., Jackson, M. R., Joslin, S. A. & Walker, M. C. (1986) *Biochem. J.* **235**, 39–44
11. Waud, W. R. & Rajagopalan, K. V. (1976) *Arch. Biochem. Biophys.* **172**, 365–379
12. McCord, J. M. & Day, D. (1978) *FEBS Lett.* **86**, 139–142
13. Gutteridge, J. M. C. (1984) *Biochem. J.* **218**, 983–985
14. Nelson, S. D., Dahlin, D. C., Rauckman, E. J. & Rosen, G. M. (1981) *Mol. Pharmacol.* **20**, 195–199
15. Brumby, P. E. & Massey, V. (1967) *Methods Enzymol.* **10**, 463–474
16. Halliwell, B. (1978) *FEBS Lett.* **92**, 321–326
17. Rowley, D. A. & Halliwell, B. (1982) *FEBS Lett.* **142**, 39–41
18. Komai, H., Massey, V. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1692–1700
19. Mason, R. P. & Fischer, V. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2493–2499
20. Spector, T. (1977) *Biochem. Pharmacol.* **26**, 355–358
21. Spector, T., Hall, W. W. & Krenitsky, T. A. (1986) *Biochem. Pharmacol.* **35**, 3109–3114
22. Porras, A. G., Olson, J. S. & Palmer, G. (1981) *J. Biol. Chem.* **256**, 9096–9103
23. Hille, R. & Massey, V. (1981) *J. Biol. Chem.* **256**, 9090–9095
24. Hille, R. & Massey, V. (1986) *J. Biol. Chem.* **261**, 1241–1247
25. Spector, T. & Massey, V. (1972) *J. Biol. Chem.* **247**, 5632–5636
26. Entsch, B., Ballou, D. P. & Massey, V. (1976) *J. Biol. Chem.* **251**, 2550–2563
27. Ghisla, S., Entsch, B., Massey, V. & Husein, M. (1977) *Eur. J. Biochem.* **76**, 139–148
28. Husein, M. & Massey, V. (1979) *J. Biol. Chem.* **254**, 6657–6666

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