Contribution of Cytochromes and Proteins to the Effect of Ascorbic Acid on Artificial and Microsomal Hydroxylation Systems containing Oxygen and Hydrogen Peroxide

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(Received 18 April 1977)

Hydroxylation systems containing cytochromes, proteins and ascorbic acid were studied at physiological pH (7.4) under O₂ or N₂ with added H₂O₂. Proteins inhibited aromatic hydroxylation of *p*-nitrophenol or oxidative demethylation of ethylmorphine in ascorbic acid-containing systems incubated under O₂, but strongly activated the systems containing H₂O₂. Cytochrome *c* and partially purified cytochrome P-450 from rat liver microsomal preparations activated the system in either O₂ or H₂O₂. The systems needed ascorbic acid (or other enol structures) for activation. Cytochrome iron participated probably in the activation of O₂, whereas cytochrome protein participated in the free-radical activation of H₂O₂ (or of O₂).

An understanding of oxidation by artificial oxidation systems is important because of similarities to biological systems responsible for drug hydroxylation. A free-radical e.p.r. signal appears in the mixture of peroxidase and H₂O₂ or in a liver microsomal preparation and organic peroxide (Duke, 1968; Hrycay & O'Brien, 1972; Kadlubar *et al.*, 1973; Rahimtula & O'Brien, 1975; Dionisi *et al.*, 1975). A dimer of morphine (Hosoya & Brody, 1957) and a dimer of *p*-nitrophenol (J. Chrastil & J. T. Wilson, unpublished work) were apparent metabolites formed from morphine and *p*-nitrophenol by liver homogenates *in vitro*. These reactions can be explained by a free-radical mechanism. H₂O₂ (added *in vitro* to liver microsomal preparations) could replace the requirement for O₂, but it could not replace NADPH (Gillette *et al.*, 1957; Buhler & Mason, 1961). Ascorbic acid is an important, if not essential, factor in biological microsomal hydroxylation systems (Fishman, 1961; Zannoni *et al.*, 1972). But ascorbic acid is a free-radical activator, and it is probable that the hydroxylation in the liver microsomal system is accomplished by an intermediate free-radical mechanism.

Oxidation systems containing iron and H₂O₂ or O₂ are well known in chemistry (Fenton, 1894, 1895, 1896). Compounds such as catechol, alloxan, ninhydrin, ascorbic acid, dihydroxymaleic acid,

dioxosuccinic acid, crotonic acid and cinnamic acid have enediol or similar structures and are strong activators of these oxidation systems (Ruff, 1899; Udenfriend *et al.*, 1954; Swern, 1971). Intermediate free radicals are formed and participate in hydroxylation activity exhibited by these systems. Unfortunately, these systems were not studied at physiological pH (7.4) in the presence of cytochromes and proteins.

The present study examines the participation of protein, cytochrome structures and ascorbic acid in a free-radical-generating system for drug oxidation in artificial systems and under conditions *in vitro* utilizing certain components of the microsomal system at physiological pH.

Materials and Methods

Reagents

L-Ascorbic acid, FeSO₄, Fe₂(SO₄)₃, H₂O₂, MgSO₄ and sodium phosphates were obtained from Baker Chemical Co., Phillipsburg, NJ, U.S.A. Cysteine, reduced glutathione, glucose 6-phosphate, hydroxylamine, NADPH, haemoglobin, peroxidase (horseradish), albumin (bovine), catalase (bovine liver) and cytochrome *c* (horse heart, 10% in reduced form) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A.

Rat liver microsomal preparations

Rat liver homogenates were prepared from decapitated animals (Fisher, male, 70 days old) by

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adding 2 vol. of 1.15% (w/v) KCl solution buffered with 0.1M-Tris/HCl, pH7.4, to 1 part of liver (v/w). The liver was then homogenized in a glass homogenizer with Teflon pestle. The homogenate was centrifuged at 9000g for 20min. The 9000g-supernatant fraction was centrifuged at 100000g for 1h and the supernatant was carefully separated from the microsomal pellet. The latter was washed with 1.15% KCl/0.1M-Tris/HCl and centrifuged again at 100000g for 1h. The pellet was homogenized in 0.2M-phosphate buffer, pH7.4 (5.44g of KH_2PO_4 /litre + 27.88g of K_2HPO_4 /litre, pH adjusted by KOH and H_3PO_4), and the volume was adjusted so that 1 ml of microsomal suspension was equivalent to 0.33g of liver.

Partial purification of cytochrome P-450 from rat liver microsomal preparations

Cytochrome P-450 was partially purified from phenobarbital-pretreated rats by the method of Imai & Sato (1974). The red band eluted from the DEAE-cellulose column was used as the 'P-450 fraction'. The volume of this fraction was adjusted to 200ml by adding 0.1M-potassium phosphate buffer, pH7.4. The protein content was determined by the method of Lowry *et al.* (1951) with albumin as a standard. Cytochromes P-450 and P-420 were determined by the methods of Omura *et al.* (1965) and Sato & Zannoni (1976). The P-450 fraction contained (in 1ml) 0.70mg of protein, 4.2nmol of cytochrome P-450 and 1.2nmol of cytochrome P-420; thus there was 6.0nmol of cytochrome P-450/mg of protein.

Incubation mixtures

All incubation mixtures contained the substrate *p*-nitrophenol (1mM) or ethylmorphine (1mM) in 0.5M-potassium phosphate buffer, pH7.4. Reactants (H_2O_2 , ascorbic acid, cytochromes, proteins etc.) were present in the final concentrations shown in each Figure legend. The biological system utilized liver microsomal preparations corresponding to 0.067g of liver/ml of incubation mixture. In the experiments, 10ml of the incubation mixture was incubated under O_2 in a shaking water-bath incubator at 37°C.

Measurement of the metabolism of p-nitrophenol and ethylmorphine

4-Nitrocatechol, which is the main product of the microsomal hydroxylation of *p*-nitrophenol *in vitro*, was measured by the method described by Chrastil & Wilson (1975a). After the corresponding incubation time, 2ml of an incubation mixture was mixed with 2ml of 5% (w/v) trichloroacetic acid and 20ml of

peroxide-free diethyl ether. The mixture was shaken for 5min, centrifuged (10min, 1000g) and 15ml of ether layer was transferred into tubes containing 3ml of 1M-NaOH. After 5min of shaking followed by centrifugation (5min, 1000g), the A_{510} of the lower layer was read with a Gilford 300-N spectrophotometer. The unincubated mixture, which contained *p*-nitrophenol (1mM) and 4-nitrocatechol (0.1mM), was similarly extracted and used as a standard. Formaldehyde, which is a product of the oxidative demethylation of ethylmorphine, was determined by two methods as described by Nash (1953) and by Chrastil & Wilson (1975b). The results of the first method are influenced by the presence of ascorbic acid and those of the second method by H_2O_2 . Results obtained by both methods, together with incubated blanks, were used for calculation of formaldehyde. The two reactions *p*-nitrophenol $\rightarrow$ 4-nitrocatechol, ethylmorphine $\rightarrow$ formaldehyde were used as typical examples for aromatic-hydroxylation and oxidative-demethylation reactions respectively.

Determination of the initial rate v_0

The initial rate v_0 was determined by the fifth-order regression analysis of the corresponding time curves. v_0 was the slope at zero time, which was represented by the second regression constant A_1 (after the first derivation of the higher-order equation, $A_0 = 0$, and $A_1 = \text{slope at zero time}$).

Statistical differences

All values used in the Figures are the means of two separate experiments. The largest standard deviations from the mean were $\pm 5\%$.

Results

Reactions carried out under O_2 in the absence of H_2O_2

In the absence of ascorbic acid. Biological components, such as NADPH, cysteine, cytochrome *c* or cytochrome P-450 (partially purified fraction), in the artificial system at physiological pH did not significantly activate the hydroxylation of *p*-nitrophenol under O_2 (Fig. 1).

Of compounds known to initiate production of free radicals, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and hydroxylamine were less active than ascorbic acid (in the same concentration) (results not shown).

In the presence of ascorbic acid. Ascorbic acid was an efficient reactant with regard to production of 4-nitrocatechol from *p*-nitrophenol under O_2 (Fig. 1). The addition of protein to the mixture containing ascorbic acid inhibited the hydroxylation,

Fig. 1. Hydroxylation of *p*-nitrophenol by artificial systems incubated under O₂

The incubation mixtures with *p*-nitrophenol were prepared and 4-nitrocatechol was measured as described in the Materials and Methods section. Reactants were present at the following concentrations: *p*-nitrophenol, 1 mM; ascorbic acid, NADPH, cysteine, 10 mM; cytochrome *c*, cytochrome P-450, protein (albumin), 10 μM. Globulin gave results similar to those with albumin. ●, Ascorbic acid + cytochrome *c* + O₂; ■, ascorbic acid + cytochrome P-450 + O₂; ▲, ascorbic acid + O₂; ○, ascorbic acid + protein + O₂; □, (NADPH, cysteine, cytochrome *c* or cytochrome P-450) + O₂.

Fig. 2. Initial rate of the hydroxylation of *p*-nitrophenol when incubated under O₂ with added ascorbic acid (10 mM) and either albumin or cytochrome *c*

*v*₀ is initial rate calculated from time-course plots with each concentration of protein or cytochrome *c*.

whereas the addition of cytochrome *c* or the partially purified fraction of cytochrome P-450 strongly increased the activity of the mixture with ascorbic acid. The inhibition by protein and the activation by cytochrome *c* were concentration-dependent (Fig. 2).

Fig. 3. Hydroxylation of *p*-nitrophenol by artificial systems containing ascorbic acid and H₂O₂

The reaction conditions were the same as in Fig. 1. H₂O₂ concentration was 10 mM. ●, Ascorbic acid + cytochrome *c* + H₂O₂; ■, ascorbic acid + cytochrome P-450 + H₂O₂; ▲, ascorbic acid + H₂O₂; ○, ascorbic acid + protein + H₂O₂; □, (NADPH, cysteine, cytochrome *c* or cytochrome P-450) + H₂O₂.

Fig. 4. Initial rate of the hydroxylation of *p*-nitrophenol with added ascorbic acid and H₂O₂ and with either albumin or cytochrome *c*

Reaction conditions were as in Figs. 1 and 3.

Reactions carried out under N₂ in the presence of H₂O₂

In the absence of ascorbic acid. NADPH, cysteine, cytochrome *c* and liver cytochrome

Fig. 5. Effect of rat liver microsomal preparations on the artificial system containing ascorbic acid, H_2O_2 or NADPH

Ascorbic acid content in liver microsomal preparation was $65 \mu\text{g/g}$ of liver. Incubation mixtures consisted of microsomal preparation (0.067 g of liver/ml of incubation mixture), glucose 6-phosphate ($4 \mu\text{mol/ml}$), MgCl_2 ($10 \mu\text{mol/ml}$) and potassium phosphate buffer, pH 7.4 (0.5 M). The concentration of H_2O_2 , ascorbic acid or NADPH in the incubation mixture was 10 mM . The microsomal preparation depleted of ascorbic acid contained less than $6 \mu\text{g}$ of ascorbate/g of liver. Ascorbic acid was depleted by bubbling O_2 for 30 min at pH 9 or by polarography (ascorbic acid is oxidized at positive half-wave potential $+0.05$ to 0.25 V , far enough from other sensitive components). ●, NADPH+ascorbic acid+microsomal preparation; ■, NADPH+microsomal preparation; ▲, ascorbic acid+microsomal preparation; ▼, ascorbic acid; ○, H_2O_2 +microsomal preparation; □, NADPH+microsomal preparation (ascorbic acid-depleted); Δ, H_2O_2 +(NADPH or microsomal preparation).

P-450 (partially purified fraction) did not activate the hydroxylation of *p*-nitrophenol when H_2O_2 was added (Fig. 3). The free-radical initiators $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and hydroxylamine were less active than ascorbic acid in artificial systems with H_2O_2 at pH 7.4 (results not shown).

In the presence of ascorbic acid. Ascorbic acid was a very efficient activator of the hydroxylation of *p*-nitrophenol in the presence of H_2O_2 . In contrast with the system incubated under O_2 , the addition of protein increased the hydroxylation activity of the ascorbic acid+ H_2O_2 (Fig. 3). Also, the addition of cytochrome *c* or cytochrome *P*-450 increased hydroxylation of *p*-nitrophenol by the ascorbic acid+ H_2O_2 system, but the degree of enhancement was much less than that for incubation under O_2 (Fig. 1). Additionally, high concentrations of protein and cytochrome *c* inhibited the initial rate of the reaction (Fig. 4), with a profile dissimilar to that observed for the system incubated under O_2 (Fig. 2).

Effect of rat liver microsomal preparation

Microsomal preparation, in the presence of ascorbic acid or H_2O_2 , increased the hydroxylation activity under O_2 compared with that with the compound alone (Fig. 5). Ascorbic acid also gave an enhancing effect when added to a mixture containing NADPH and microsomal preparation. Depletion of ascorbic acid in the microsomal preparation produced low activity even with addition of NADPH. This is in contrast with activity observed when NADPH was added to microsomal preparations that were not depleted of ascorbic acid. These results show that the combination of NADPH+ascorbic acid+microsomal preparation is the most effective for drug oxidation and that the microsomal system does not have an absolute requirement for NADPH for *p*-nitrophenol hydroxylation.

Oxidative demethylation of ethylmorphine

Ethylmorphine was used as a substrate for oxidations by the same artificial systems as described for *p*-nitrophenol, and formaldehyde was determined (see the Materials and Methods section) as a final product of the reaction. The characteristic kinetic profiles for ethylmorphine (results not shown) were similar to those for *p*-nitrophenol hydroxylation.

Discussion

It is well known that the catalytic oxidation by O_2 or H_2O_2 is a free-radical mechanism. It has been proved with so many inorganic and organic chemical reactions (Swern, 1971) that we do not believe that the systems with cytochromes or proteins can be exceptions from this rule. For example, not only peroxidase but also cytochrome *P*-450 catalyses the H_2O_2 -dependent hydroxylation of drugs (Nordblom *et al.*, 1976) in the absence of NADPH or O_2 .

From our experiments with artificial systems at physiological pH (7.4) it was apparent that cytochrome *c* and partially purified *P*-450 fraction were only weak activators of the free-radical hydroxylation system in the presence of O_2 or H_2O_2 (Figs. 1 and 3). Addition of ascorbic acid was needed for activation when these cytochromes were present. Cytochrome *c*, or purified cytochrome *P*-450, activated the mixture containing ascorbic acid+ O_2 and also the mixture ascorbic acid+ H_2O_2 (Figs. 1 and 3). On the other hand, albumin (and other proteins) increased the hydroxylation activity of ascorbic acid+ H_2O_2 (Figs. 3 and 4), but inhibited the activity of ascorbic acid+ O_2 (Figs. 1 and 2). Thus it is apparent that protein participated in the free-radical activation of H_2O_2 , but it did not participate in (or impeded) the activation of O_2 .

Table 1. *Some characteristic reactions of H₂O₂, ascorbic acid, NADPH and cytochromes in artificial and biological systems*
 Abbreviations: M, liver microsomal preparation; Cyt, cytochrome (cytochrome *c* or cytochrome *P*-450); Prot, protein (albumin, globulin or cytochrome protein); AA, ascorbic acid; Org-Per, organic peroxide (cumene hydroperoxide); DAA, dehydroascorbic acid.

Reactions supported by ascorbic acid or NADPH:

Ascorbic acid (non-enzymic)

1. + O₂ = H₂O₂
2. + H₂O₂ = HO• (and/or HOO•)
3. + Catalase = inhibition
4. + Org-Per = Org-OH + HO•
5. + CytFe³⁺ = CytFe²⁺

NADPH (enzymic)

1. + O₂ = H₂O₂
2. + DAA = AA
3. + Sugars = AA
4. + Cystine = Cysteine
5. + CytFe³⁺ = CytFe²⁺

Oxidation of substrate without liver microsomal preparations:

Under O₂

- O₂ + Cyt = Low
 O₂ + NADPH = Low
 O₂ + AA = High
 O₂ + AA + Cyt = High
 O₂ + Prot = Low

Under N₂

- N₂ + H₂O₂ + Cyt = Low
 N₂ + H₂O₂ + NADPH = Low
 N₂ + H₂O₂ + AA = Very high
 N₂ + H₂O₂ + AA + Cyt = Very high
 N₂ + H₂O₂ + AA + Prot = Very high

Oxidation of substrate in the presence of liver microsomal preparations:

Under O₂

- O₂ + M = No
 O₂ + M + NADPH = High
 O₂ + M + AA = High
 O₂ + M(AA depleted) + NADPH = Low
 O₂ + M + AA + NADPH = Very high

Under N₂

- N₂ + M + H₂O₂ = Low
 N₂ + M + Org-Per = High
 N₂ + M(AA depleted) + Org-Per = Low
 N₂ + M + Org-Per + NADPH = High
 N₂ + M + Org-Per + AA = Very high

Characteristic findings for the artificial and biological hydroxylation systems can be summarized thus (see also Table 1).

1. Artificial systems in phosphate buffer at physiological pH (7.4) containing solubilized cytochromes required H₂O₂ (or O₂ with conversion into H₂O₂ or activated O₂ radical) and ascorbic acid for optimum hydroxylation activity.

2. Ascorbic acid very efficiently activated hydroxylation in the mixtures with H₂O₂, but was much less active with O₂ alone. The activity with O₂ probably resulted from the reactions ascorbic acid + O₂ → H₂O₂ + ascorbic acid free radical; H₂O₂ + ascorbic acid → HO• + HO•; HO• + H₂O₂ → HOO•; HO• + RH → R• + H₂O; R• + HO• → R-OH etc.

3. Cytochromes but not pure proteins (albumin, globulin etc.) efficiently activated the system ascorbic acid + O₂, probably by facilitating the formation of H₂O₂.

4. Cytochromes, but also pure iron-free proteins (albumin, globulin etc.), supported very efficiently the formation of free radicals by the reaction H₂O₂ + ascorbic acid → HO• + HO•; R• + HO• → R-OH; (where R is a substrate such as *p*-nitrophenol, ethylmorphine etc.).

From points 1-4 it can be stated that cytochrome iron (which, however, demonstrates no hydroxylation activity for *p*-nitrophenol or ethylmorphine

under O₂ or with H₂O₂) efficiently participates in the generation of activated O₂ and H₂O₂:

On the other hand, protein (alone or cytochrome molecular protein) in the presence of ascorbic acid efficiently activates the reaction:

Free radicals must be the source of hydroxylation activity, since R• + HO• → R-OH.

It is thus apparent that cytochrome iron and cytochrome protein participate in the hydroxylation system probably in two different steps of the reaction. Cytochrome iron participates in the activation of O₂ and cytochrome protein in the free-radical activation of H₂O₂. Ascorbic acid is required for optimum activity, presumably through free-radical activation.

Although the situation in the liver microsomal system is, as expected, more complex and subject to modulation, it seems probable that hydroxylation by a liver microsomal system, one that contains the same obligatory factors as the artificial systems, is accomplished by a fundamentally similar mechanism.

This work was supported by National Institutes of Health grants GM-15431 and GM-21949. J.T.W. is the recipient of a Research Career Development Award from the National Institutes of Health (K4-HD42539).

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