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Oxidation of reduced cytochrome *c* by hydrogen peroxide

Implications for superoxide assays

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Hydrogen peroxide, formed directly or as a product of superoxide dismutation, can oxidize ferrocytochrome *c* at rates comparable to those at which ferricytochrome *c* is reduced by superoxide. This reoxidation can significantly affect estimates of rates and amounts of superoxide production using absorbance changes for cytochrome *c* at 550 nm as the assay. The oxidation can be inhibited by catalase.

Ferrocytochrome *c* oxidation; Hydrogen peroxide; Superoxide assay

1. INTRODUCTION

Reduction of cytochrome *c* (FeIII) by superoxide, O_2^-, is a rapid, single-electron process which can be monitored with great sensitivity and specificity by measuring the absorbance change at 550 nm [1,2]. Because of its simplicity, this is often the assay of choice for estimating rates of superoxide production in cell systems and is widely used for studies of the respiratory burst in macrophages and polymorphonuclear leukocytes [3–7]. The assay was first described in studies with milk xanthine oxidase [8], and was important for characterization of superoxide dismutase [9]. It is known [10], but not always acknowledged, that the conversion efficiency is less than unity: at best a quarter of the superoxide produced will reduce cytochrome *c* because of rapid, spontaneous dismutation at normal pH [11]. Consequently, both rates and extents of reduction are lower limits for rates and extents of superoxide production.

Here, we present data which show that cytochrome *c* (FeII) can, and will, be reoxidized by H_2O_2. Since H_2O_2 is the major product of O_2^- dismutation, this can significantly affect the rates and conversion efficiencies estimated from the 550 nm absorption changes. Studies with the well-characterized xanthine-xanthine oxidase system show that addition of catalase prevents the reoxidation and provides a less ambiguous assay.

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2. EXPERIMENTAL

Xanthine (lot no.4216JL) from Aldrich, as well as sodium benzoate, ascorbic acid and hydrogen peroxide solution from Fisher, were used as received. Catalase (EC 1.11.1.6) from bovine liver (lot no.19C-8170) was dissolved in PBS (Gibco) at pH 7.2 as a concentrated solution. Xanthine oxidase (EC 1.1.3.22) from buttermilk (grade I; lot no.74F-3844) was dialyzed for 15–24 h at 4°C against several changes of PBS to activate the enzyme. Typically better than a third of the nominal activity was recovered. Cytochrome *c* (FeIII) from horse heart (type VI; lot no.65F-7277) was kept as a concentrated solution in PBS. The enzymes and cytochrome *c* were all obtained from Sigma.

A typical assay was performed in a total volume of 0.75 ml in a cuvette and contained 10–100 mU xanthine oxidase, 25–100 μM xanthine, and 50 μM

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cytochrome *c*. Catalase (50 μg/ml) and sodium benzoate (50 mM) were added in excess.

All absorbance data were collected using an HP 8451A diode array spectrophotometer with data collection at regular time intervals. The amounts (in nmol) of xanthine reduced, uric acid produced and cytochrome *c* reduced were calculated from the absorbance changes using the appropriate extinction coefficients: for example, for uric acid:

$$A_{290}(t) = (n_u(t)E_u + n_x(t)E_x)/V$$
$$= (n_u(t)(E_u - E_x) + n_x(0)E_x)/V$$

so that

$$n_u(t) = \{(A_{290}(t) - [x]_0E_x)V/(E_u - E_x)\}$$

where E is the extinction coefficient, n the number of moles, V the total volume and the subscripts designate uric acid (u) and xanthine (x). Also, $[x]_0$ represents the initial concentration of xanthine in the solution. For the present calculations we used the following values for the molar extinction coefficients (at pH 7.2): cytochrome *c* (FeIII), 8.9×10^3 $M^{-1} \cdot cm^{-1}$ at 550 nm; cytochrome *c* (FeII), 29.9×10^3 $M^{-1} \cdot cm^{-1}$ at 550 nm; uric acid, 10.8×10^3 $M^{-1} \cdot cm^{-1}$ at 290 nm; xanthine, 1.5×10^3 $M^{-1} \cdot cm^{-1}$ at 290 nm.

In most experiments the sample was continuously illuminated with a broad band of excitation, and the absorbance was detected with a diode array detector. In some experiments the illumination source was blocked except during the actual absorbance reading.

3. RESULTS

Fig.1 illustrates the time course of production of uric acid and the simultaneously measured reduction of cytochrome *c* in the absence and presence of catalase. Although the rate of oxidation of xanthine to uric acid is unaffected by the catalase, the rate of reduction of cytochrome *c* is slightly enhanced, and the re-oxidation observed after about 3 min is totally prevented by the enzyme. This effect of catalase is observed for a range of xanthine concentrations, and is proportionally greater at higher initial concentrations of xanthine presumably because the hydrogen peroxide concentration created by spontaneous dismutation reaches a larger value more rapidly.

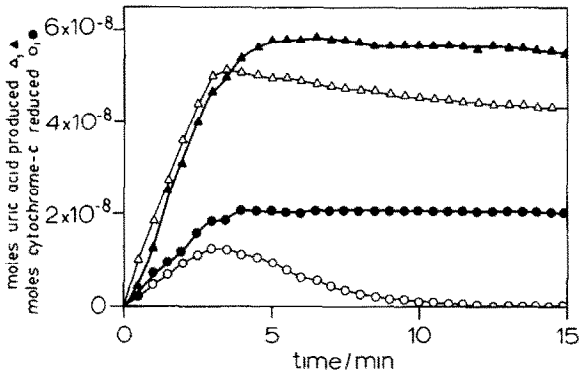

Fig.1. Uric acid produced (△, ▲) and cytochrome *c* reduced (○,●), both expressed in mol, as a function of time after initiation of xanthine oxidation by xanthine oxidase without (△,○) and with (▲,●) catalase present. In all cases the samples contained 50 μM xanthine, 50 μM cytochrome *c* and 50 mU/ml xanthine oxidase. Catalase was added at 50 μg/ml.

It has been shown that catalase can react directly with superoxide [12], but it is its reaction with hydrogen peroxide which prevents the re-oxidation in the present case. Hydrogen peroxide added directly to a solution of ferrocytochrome *c*, made by reduction with ascorbic acid, causes a rapid, concentration-dependent re-oxidation, as monitored by the absorbance decrease at 550 nm. The rates and corresponding pseudo-first-order rate constant for several hydrogen peroxide concentrations are listed in table 1.

Addition of catalase prevents re-oxidation by

Table 1

Rates of oxidation (*r*) of ascorbic acid-reduced cytochrome *c* by hydrogen peroxide[a]

$[H_2O_2]$ (M)	r ($M \cdot s^{-1}$)	k'[b] (s^{-1})
25×10^{-6}	3.16×10^{-9}	1.26×10^{-4}
50×10^{-6}	6.00×10^{-9}	1.20×10^{-4}
100×10^{-6}	11.7×10^{-9}	1.17×10^{-4}
200×10^{-6}	28.4×10^{-9}	1.42×10^{-4}
	mean:	1.26×10^{-4}

[a] Hydrogen peroxide is added to 0.75 ml PBS at pH 7.2 with 50×10^{-6} M cytochrome *c* reduced completely by 25×10^{-6} M ascorbic acid

[b] Pseudo-first-order rate constant calculated as $r = k'[H_2O_2]$. The rate constant $k = k'$[cytochrome *c*] = 6.3×10^{-9} $M^{-1} \cdot s$

removing the hydrogen peroxide produced in the spontaneous dismutation of superoxide, but does not affect the rate at which superoxide is produced. The effect of catalase on the rate of cytochrome *c* reduction arises from the inhibition of re-oxidation at early times. Fig.2 shows that while the cytochrome *c* reduction is sensitive to the rate of superoxide production, controlled here by the amount of xanthine oxidase, the rates are underestimated by at least a factor of five.

During continuous measurements of cytochrome *c* reduction and uric acid production, we frequently observe a slow decrease in absorbance at 290 (figs 1,3a) which we interpret as a decomposition of uric acid. The decomposition is not affected by addition of hydrogen peroxide, but intermittent, rather than continuous exposure to 290 nm radiation, decreases the degradation rate by orders of magnitude (fig.3a). Associated with

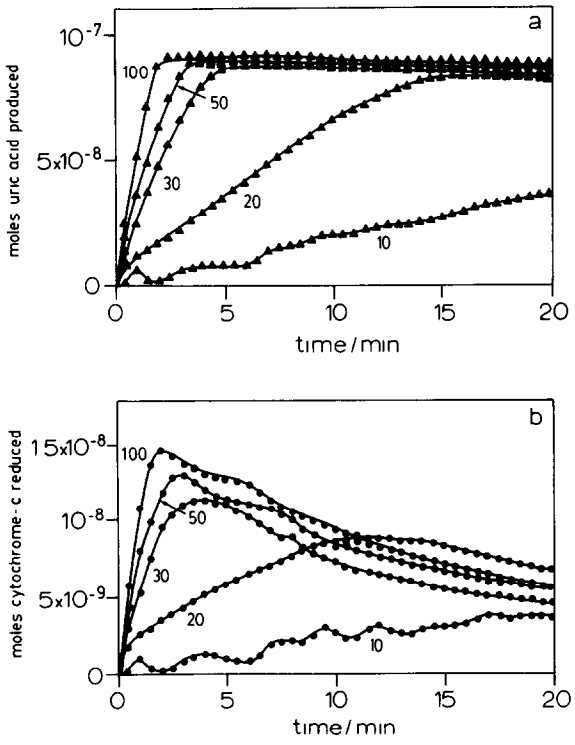

Fig.2. Uric acid produced (a) and cytochrome *c* reduced (b), both expressed as mol, as a function of time after initiation of xanthine oxidation by xanthine oxidase with catalase present. Each curve corresponds to a different xanthine oxidase concentration given as mU/ml. Other concentrations are as indicated in fig.1.

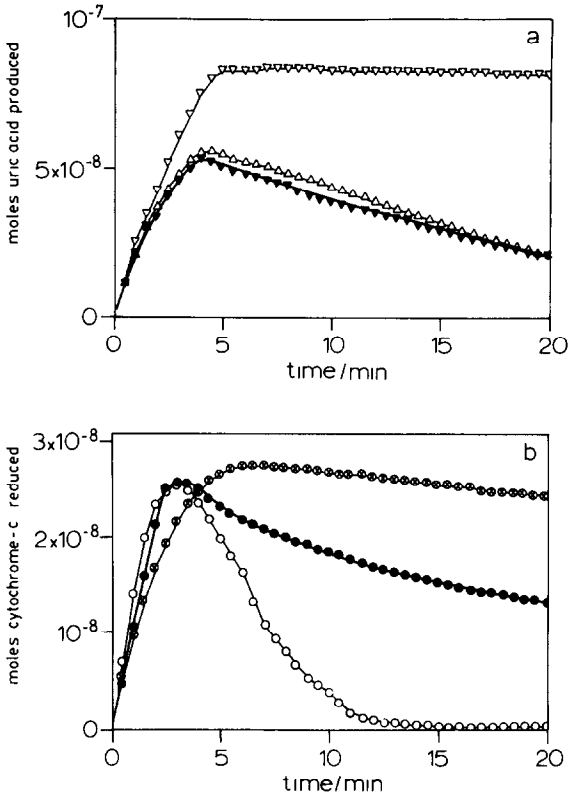

Fig.3. Uric acid produced (a) and cytochrome *c* reduced (b), both expressed as mol, as a function of time after initiation of xanthine oxidation by xanthine oxidase. (a) Intermittent (▽) and continuous (△, ▼) illumination, with (▽, ▼) and without hydrogen peroxide added at 100 μM. Other concentrations are as indicated in fig.1 except xanthine which was at 100 μM. (b) Experiments without catalase (○), with catalase only (●), and with both catalase and sodium benzoate (⊗) with continuous illumination. Sodium benzoate was added at 50 μM and other concentrations were as indicated in fig.1.

the disappearance of uric acid we often see a corresponding re-oxidation of cytochrome *c* even in the presence of catalase. This re-oxidation can be inhibited by sodium benzoate (fig.3b), a known hydroxyl radical scavenger. It is possibe that a photochemical degradation of uric acid produces hydroxyl radicals, which subsequently react with cytochrome *c* (FeII).

4. DISCUSSION

Reduction of ferricytochrome *c*, observed as an

absorbance change at 550 nm, is a commonly used assay for the rate and extent of production of superoxide in particular in cell systems [3–7]. The assay is rendered specific for superoxide by comparison to a sample containing superoxide dismutase also, frequently directly in a double-beam spectrometer [3]. The assay is good for comparative studies, but because of competition by spontaneous dismutation, the rates and extents of superoxide production are necessarily underestimated [10]. The present data suggest that in many cases the assay can be affected by the presence of hydrogen peroxide, since ferrocytochrome *c* is readily oxidized by this powerful oxidation agent. The problem can be partly alleviated by addition of catalase to the assay system. Interestingly, early studies [9] included catalase but no reasons were presented.

Assays which include superoxide dismutase in a reference cuvette could in principle be distorted in the other direction, since the more rapid production of hydrogen peroxide in the reference would tend to cause re-oxidation of cytochrome *c* reduced by other means. Thus the reference will exhibit a slower reduction than expected. This effect should, however, be minor.

In the present 'pure' system, we presume that the hydrogen peroxide reacts directly with ferrocytochrome *c*. It is nevertheless possible that the commercial enzyme preparations contain minute amounts of cytochrome *c* peroxidase [13]. We have not pursued this point.

In general, the photochemically induced reoxidation of cytochrome *c* should not be a problem in assay systems where only the absorbance changes at 550 nm are monitored with a monochromatic source. The problem arises in this particular system because of the simultaneous irradiation of the uric acid, and because of the sensitivity of uric acid to light. Nevertheless, it could be important in situations where broad-band illumination might cause hydroxyl radical formation.

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REFERENCES

[1] McCord, J.M., Crapo, J.D. and Fridovich, I. (1977) in: Superoxide and Superoxide Dismutases (Michelson, A.M. et al. eds) pp.11–18, Academic Press, New York.
[2] Rosen, G.M., Finkelstein, E. and Rauckman, E.J. (1982) Arch. Biochem. Biophys. 215, 367–378.
[3] Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) J. Clin. Invest. 52, 741–744.
[4] Salin, M.L. and McCord, J.M. (1974) J. Clin. Invest. 54, 1005–1009.
[5] Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1081–1087.
[6] Newburger, P.E., Speier, C., Borregaard, N., Walsh, C.E., Whitin, J.C. and Simons, E.R. (1984) J. Biol. Chem. 259, 3771–3776.
[7] Dri, P., Soranzo, M.R., Cramer, R., Menegazzi, R., Miotti, V. and Patriarca, P. (1985) Inflammation 9, 21–31.
[8] McCord, J.M. and Fridovich, I. (1968) J. Biol. Chem. 243, 5753–5760.
[9] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[10] Fridovich, I. (1970) J. Biol. Chem. 245, 4053–4057.
[11] Bielski, B.H.J. and Arudi, R.L. (1983) Anal. Biochem. 133, 170–178.
[12] Shimizu, N., Kobayashi, K. and Hayashi, K. (1984) J. Biol. Chem. 259, 4414–4418.
[13] Kang, D.S. and Erman, J.E. (1982) J. Biol. Chem. 257, 2775–2779.