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How relevant is the reoxidation of ferrocytochrome *c* by hydrogen peroxide when determining superoxide anion production?

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In a recent publication [(1987) FEBS Lett. 210, 195–198] the authors claim the use of cytochrome *c* to detect superoxide anion underestimates the real rate of superoxide anion formation on the basis that: (i) the rate of uric acid formation by xanthine oxidase is about 4-fold faster than the rate of cytochrome *c* reduction and (ii) hydrogen peroxide formed upon dismutation of the superoxide anion generated by xanthine oxidase is capable of reoxidizing ferrocytochrome *c*. That paper may have been misleading for readers not very familiar with the field of oxygen radicals, since both assumptions are, in fact, incorrect. In this report we demonstrate that the build up in concentration of H_2O_2 during most reactions in which superoxide anion is being produced is not enough to affect the rate of cytochrome *c* reduction. Our results suggest that the authors may have been misled by an artifact due to exposure of the samples containing H_2O_2 to UV light, which generates hydroxyl radicals by photolysis.

Cytochrome *c* oxidation; Hydrogen peroxide; Superoxide generation; Kinetics

1. INTRODUCTION

Vandewalle and Petersen [1] published a paper suggesting that the use of ferricytochrome *c* for detecting superoxide anion underestimates the actual rate of superoxide generation. Their conclusions are based on the comparison between the rates of cytochrome *c* reduction and uric acid formation in a system containing xanthine and xanthine oxidase. These authors erroneously assumed that the oxidation of 1 mol of xanthine by xanthine oxidase generates 2 mol superoxide anion, which in turn disproportionate spontaneously to form 1 mol of H_2O_2. In 1970, Fridovich [2] showed that only 20% of the oxygen consumption by xanthine oxidase at pH 7.0 was utilized to form superoxide anion directly, while the other 80% was reduced to H_2O_2 in an apparently concerted two-electron step. The proportion of superoxide anion formed during the autoxidation of xanthine oxidase increases significantly with pH [2], reaching a maximum at low concentrations of xanthine and high oxygen concentrations [2,3]. Fridovich [2] also showed that cytochrome *c* (16 μM) scavenged all the superoxide anion being formed by a system containing xanthine (0.4 mM) and xanthine oxidase (5.3 nM). The kinetic basis for this is that the rate constant for the reaction between cytochrome *c* and superoxide anion is sufficiently high (10^5 $M^{-1} \cdot s^{-1}$, [4]) to allow micromolar concentrations of cytochrome *c* to compete efficiently with the spontaneous dismutation of superoxide radicals [2].

The conclusion by Vandewalle and Petersen [1] that only 25% of the superoxide formation may be detected with cytochrome *c* is erroneous, and may be misleading for other investigators trying to determine rates of oxygen radical generation by the cytochrome *c* reduction method. This method has been used by many investigators for determining rates of superoxide formation, and is the basis for quantifying superoxide dismutase activity [5].

Vandewalle and Petersen also mention that ferrocytochrome *c* may be reoxidized by H_2O_2, which results in an even greater underestimation of superoxide anion. The significance of this 'perox-

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idase' activity associated with cytochrome *c* has been examined in the present paper by evaluating some kinetic parameters of the reaction between cytochrome *c* and H_2O_2. The advantages of using cytochrome *c* for monitoring superoxide generation are also discussed.

2. MATERIALS AND METHODS

Cytochrome *c* (types III and VI) and xanthine oxidase (type I) were purchased from Sigma (St Louis, MO). Some experiments were carried out using xanthine oxidase purified in our laboratory by the method of Waud et al. [6]. All reactions were carried out at 30°C in 50 mM phosphate buffer containing 1 mM EDTA (pH 7.4) and were monitored using a Perkin Elmer Lambda 3A spectrophotometer. Cytochrome *c* was reduced by addition of 0.5 mg/ml sodium dithionite, followed by a passage through a Sephadex G-25 column in order to remove the excess dithionite.

Exposure of samples to UV radiation was carried out using standard quartz cuvette (3 ml, 10 mm lightpath) which were placed at three inches from a model R-52 UV lamp from Ultraviolet Products, Inc. (San Gabriel, CA). These are low pressure Hg lamps, in which 90% of the emission occurs at 254 nm. The changes in absorbance at 550 nm were determined periodically using a Perkin Elmer Lambda 3 spectrophotometer. After every determination (10–15 s) the samples were placed back under the UV lamp. The changes in absorbance with time were plotted and the rates of cytochrome *c* oxidation were calculated from the slopes.

3. RESULTS AND DISCUSSION

Addition of H_2O_2 to a solution containing 20 μM type VI ferrocytochrome *c* oxidized this hemoprotein in a concentration-dependent manner (fig.1). The rate of cytochrome *c* oxidation by H_2O_2 was approx. 2-fold faster when type III instead of type VI cytochrome *c* was used (fig.1). The difference in the rates of ferrocytochrome *c* oxidation may be the consequence of minor differences in the tertiary structure of the two cytochromes, which make the type III form more reactive towards H_2O_2. It is worth mentioning that type VI cytochrome *c* is prepared using a technique which avoids the use of perchloric acid while the type III variety is precipitated with perchloric acid. The rates shown in fig.1 indicate that, at a constant concentration of 20 μM ferrocytochrome *c*, addition of H_2O_2 will cause cytochrome *c* oxidation at a rate of approx. 0.01%/min per μM H_2O_2. This reaction will therefore be insignificant at concentrations of H_2O_2 below 0.1 mM, as would be the case in virtually all applications where rates of superoxide production would be measured.

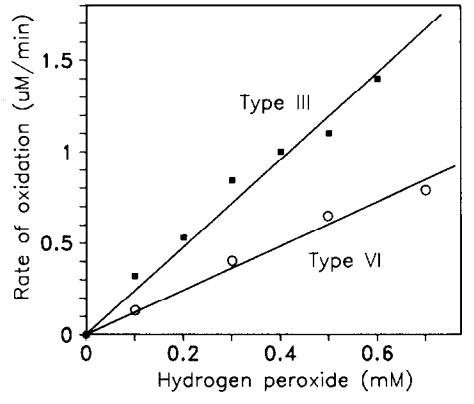

Fig.1. Oxidation of type III or type VI ferrocytochrome *c* (20 μM) by added H_2O_2. The reaction was carried out in a buffer containing 50 mM phosphate and 0.1 mM DTPAC (pH 7.8).

The proposal by Vandewalle and Petersen [1] that this peroxidatic reaction is catalyzed by a contaminant cytochrome *c* peroxidase [1] is not a possibility. Cytochrome *c* peroxidase is found in yeast mitochondria [7] but not in mammalian, avian or plant mitochondria [8], thus being impossible to appear as a contaminant of horse heart cytochrome *c*. Other contaminant peroxidases also seemed unlikely since passage of the reduced cytochrome *c* solution through a G-100 Sephadex column did not eliminate the oxidation of cytochrome *c* by H_2O_2 (not shown). Finally, microperoxidase activity (a 1.6–1.9 kDa derivative from cytochrome *c* [9]) may also be ruled out since it should have been separated from cytochrome *c* after passage through Sephadex G-25 or G-100. Although heme and several hemoproteins may act as peroxidases, it is also possible that this reaction is just a Fenton-like reaction in which ferrocytochrome *c* catalyzes the homolytic scission of H_2O_2, which in turn may react with cytochrome *c*.

How significant is the oxidation of ferrocytochrome *c* by H_2O_2 when determining superoxide anion production? In many cases H_2O_2 is produced only by virtue of the dismutation of superoxide anion [5,8]. When that is the case, if ferricytochrome *c* is present, there will be no H_2O_2 formation since superoxide anion is oxidized back to molecular oxygen upon reacting with ferricytochrome *c*. Thus, in those cases the superoxide dismutase-inhibitable rate of ferrocytochrome *c* accumulation will reflect the scavenging of all the generated superoxide anion.

Even if H_2O_2 were being produced simultaneously with superoxide, its rate of production would have to exceed that of superoxide by more than 100-fold to measurably affect the initial rate of net cytochrome reduction. For example, the rate of superoxide formation by xanthine oxidase in the standard assay for superoxide dismutase activity is approx. 1 μM/min [5]. Considering that at pH 7.0, 80% of the oxygen being consumed generates H_2O_2 directly [2], the rate of the peroxide formation should be 4 μM/min. Thus, according to the rate constants derived from fig.1, it may be estimated that the rate of ferrocytochrome *c* oxidation by accumulated H_2O_2 would compete equally with the rate of cytochrome *c* reduction by superoxide anion only if the concentration of H_2O_2 were around 1 mM. That would be approx. 4 h after the reaction started. This point, of course, would never be reached, as the xanthine would be exhausted when the H_2O_2 reached 0.05 mM.

A value similar to the one calculated above for the rate constant may be obtained from the results of Vandewalle and Petersen [1], who reported an oxidation rate of 0.7 μM/min for the reaction between 50 μM ferrocytochrome *c* and 0.1 mM H_2O_2 (or 0.014%/min per μM H_2O_2). These authors, however, found that a H_2O_2-dependent reaction began to cause significant reoxidation of ferrocytochrome *c* only 3 min after the oxidation of xanthine had started. This suggests that there was an additional H_2O_2-dependent reaction interfering with their assay of cytochrome *c* reduction. The spectrophotometer used by these authors illuminates the sample with a relatively intense beam of polychromatic light (200–700 nm) and detects changes in absorbance, after dispersion, by a photodiode array. Exposure of H_2O_2 to 254 nm radiation causes photolysis of the oxygen-oxygen bond, resulting in the production of two molecules of hydroxyl radical, one of the most potent oxidizing species known [10]. Indeed, the authors considered that hydroxyl radical may be generated in their system, and they proposed a photodecomposition of uric acid as the source of hydroxyl radical. Uric acid, in fact a good scavenger of hydroxyl radical [11], seems to react with cytochrome *c* when exposed to UV irradiation in the absence of H_2O_2 (table 1). Addition of 10 mM benzoate did not completely inhibit the reaction between cytochrome *c* and uric acid, suggesting a more complicated mechanism than that proposed by these authors. In the presence of H_2O_2 the rate of reaction between uric acid and cytochrome *c* increased significantly, indicating a major role for H_2O_2 photolysis in the reoxidation of cytochrome *c*.

Table 1

Effect of UV irradiation on the rate of ferrocytochrome *c* oxidation in the presence of different reagents

Treatment	Cytochrome *c* oxidation (μM/min)
Cyt *c* + uric acid	0
Cyt *c* + uric acid + UV	1.7
Cyt *c* + uric acid + UV + benzoate	1.1
Cyt *c* + uric acid + H_2O_2 + UV	4.0
Cyt *c* + H_2O_2	0.7
Cyt *c* + H_2O_2 + UV	2.6
Cyt *c* + H_2O_2 + UV + benzoate	0.2
Cyt *c* + UV (no H_2O_2)	0.7
Cyt *c* + UV + benzoate	0.2

The reactions were carried out at room temperature. The concentrations of different reagents were: cytochrome *c* (Cyt *c*), 20 μM; uric acid, 0.1 mM; H_2O_2, 100 μM and benzoate 5 mM. Irradiation with a polychromatic ultraviolet (UV) light was carried out as described in section 2

To demonstrate the production of hydroxyl radicals by the photolysis of H_2O_2, a solution containing 100 μM H_2O_2 and 20 μM ferrocytochrome *c* was exposed to UV radiation with or without added hydroxyl radical scavengers (table 1). The results indicate that when exposed to UV radiation hydroxyl radicals formed from H_2O_2 photolysis may react with reduced cytochrome *c* in a reaction that may be inhibited by addition of benzoate. Even in the absence of H_2O_2, when exposed to a polychromatic source of UV light, reduced cytochrome *c* may be reoxidized, probably through water photolysis (table 1).

These experiments suggest that similar reactions may occur when using a diode array spectrophotometer in which the samples are exposed to continuous UV radiation from a deuterium lamp. Although this problem may be minimized by exposing the samples intermittently during short periods of time, Vandewalle and Petersen [1] performed most of their experiments under continuous illumination of their samples.

The aim of this paper is to stress that

cytochrome *c* is an excellent reagent for quantification of superoxide generation or superoxide dismutase activity in the presence of xanthine and xanthine oxidase [5], a reaction in which the inhibition of an initial rate of superoxide generation is monitored. Other choices such as acetylated or succinylated cytochrome *c* derivatives tend to reoxidize much faster than the native protein [12] and should be used only when cytochrome oxidase or cytochrome *c* reductases are present. Addition of catalase [1] may be required only if the concentration of H_2O_2 in the medium builds up to a millimolar range, reoxidizing the reduced form cytochrome *c*. For example, a recent article [13] shows that when monitoring superoxide generation by macrophages stimulated with phorbol myristate acetate, there is no difference in the rate of ferricytochrome *c* reduction if catalase is present during the first 10 min of the reaction. After that, the rate of cytochrome *c* reduction is faster in the presence of catalase, than when catalase is not added. In most cases, however, when superoxide anion is the precursor of H_2O_2, or when monitoring initial rates of superoxide generation upon addition of a given substrate, addition of catalase will be completely useless.

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