THE USE OF ACETYLATED FERRICYTOCHROME C FOR THE DETECTION OF SUPEROXIDE RADICALS PRODUCED IN BIOLOGICAL MEMBRANES

Angelo AZZI, Cesare MONTECUCCO and Christoph RICHTER

C.N.R. Unit for the Study of Physiology of Mitochondria, and Institute of General Pathology, University of Padova, Italy.

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SUMMARY

Acetylation of 60% of lysine residues of horse heart ferricytochrome c results in more than 95% decrease of its ability to be reduced by mitochondrial and microsomal reductases and to become oxidized (after chemical reduction) by mitochondrial oxidase. The ability of acetylated ferricytochrome c to be reduced by O_2^- radicals is maintained, making this derivative useful for the detection of O_2^- radicals in biological systems containing cytochrome c reductases or oxidases. Mitochondrial membranes can reduce acetylated ferricytochrome c at a rate of 0.5 nmoles.min^{-1}.mg^{-1}. Such a reaction is 82% inhibited by 2.8 x 10^{-8}M superoxide dismutase.

INTRODUCTION

The techniques currently employed for the detection of superoxide radicals are the co-oxidation of epinephrine to adrenochrome (1), the reduction of nitrobluetetrazolium (2) or ferricytochrome c (3), the reduction of tetranitromethane (4) and polarography (5).

The choice of the technique to be employed in a complex biological system, such as membranes, is particularly complicated due to the presence of a number of interfering reactions. The use of epinephrine has the disadvantage, for example, that not only co-oxidation, but also auto-oxidation can lead to the formation of adrenochrome (6). The presence of a nitrobluetetrazolium reductase in mitochondria, microsomes and nuclei does not permit the use of this dye as an indicator of superoxide radicals (7,8). Complications are also found with cytochrome c, due to a widespread presence in membranes (mitochondria, microsomes and nuclei) of cytochrome c reductases and oxidases.

It is known that acetylation of some lysyl residues of

ferricytochrome c destroyes its ability to undergo enzymatic reduction and oxidation (9), while the hemoprotein is still able to be reduced by chemical agents such as dithionite and has almost negligible autooxidation (9). If acetylated cytochrome c derivative were still able to be reduced by superoxide radicals, it would be of large use in biological systems for detection of O_2^- species, since it has a high extinction coefficient and a relatively high rate constant (10^6 M^{-1} sec^{-1}) with O_2^- radicals (10).

MATERIALS AND METHODS

Ferricytochrome c was acetylated according to the procedure of Minakami et al. (9). At 0°C, 50 mg of cytochrome c were dissolved in 5 ml of a half-saturated solution of sodium acetate. Under stirring ten times excess acetic anhydride with respect to the lysine groups was added, and the reaction was carried out for 30 minutes. The solution was dialyzed in a 5/8 inch Thomas dialyzing tubing at 0°C for 12 hours against 1l of distilled water with four changes of water. Acetylated cytochrome c was stored at - 20°C.

The extent of acetylation was determined by the ninhydrin method (11). The percent modification is given by

$$\% \text{ acetylation} = 100 \quad (1 - \frac{S \text{ acetylated}}{S \text{ native}})$$

where S is the slope of the plot of absorbance at 570 nm versus the concentration of native or acetylated cytochrome c.

The concentration of cytochrome c was determined in the reduced form at 550 nm using the extinction coefficient $\varepsilon_{mM} = 27.7$. Ferrocytochrome c was obtained by reduction with a few grains of dithionite followed by a gel filtration on Sephadex G-25 M. The cytochrome was eluted with degassed destilled water and collected under N_2.

Succinate-oxidase was measured polarographically, with a Clark-type electrode. Cytochrome c depleted mitochondria (1.5 mg/ml) were incubated in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl pH 7.4 and 1 µM rotenone. The concentration of native or acetylated cytochrome c was 5 µM. The reaction was initiated by the addition of 5 mM succinate, and the activity expressed as nmoles O_2 consumed.min^{-1}.mg^{-1}.

Succinate-cytochrome c reductase activity was measured in a Hitachi-Perkin Elmer spectrophotometer in 250 mM Sucrose, 10 mM Tris-HCl pH 7.4, 1 mM KCN, and 10.7 µM cytochrome c. The reaction was started by adding 3 mM succinate. Protein concentration was 180 µg/ml.

Ferrocytochrome c oxidase was tested by adding 210 µg/ml of a suspension of cytochrome c depleted rat liver mitochondria to both sample and reference cuvettes containing 250 mM sucrose, 10 mM Tris-HCl pH 7.4. 8.8 µM ferrocytochrome c were also present in the sample cuvette. The decrease in absorbance at 550 nm

was plotted on semilogarithmic paper as a function of time and the first order velocity constant calculated from the slope of the plot.

NADPH-cytochrome c reductase was assayed in a medium like that for succinate-cytochrome c reductase (without KCN) and the reaction was started by adding 1 mM NADPH. The protein concentration of microsomes was 235 µg/ml.

Rat liver mitochondria (13), beef heart mitochondrial fragments (14) and microsomes (15) were prepared by standard procedures. Cytochrome c (type VI, from horse heart) was from Sigma Chemical Co. acetic anhydride from Merck, Darmstadt, xanthine oxidase from Boehringer. All other chemicals were commercially available reagent pure products.

RESULTS AND DISCUSSION

Reduction of native and acetylated ferricytochrome c by superoxide radicals.

Superoxide radicals, generated by the xantine-xantine oxidase system (16) were able to reduce native cytochrome c (Fig. 1), the rate of reduction being progressively higher at increasing cytochrome c concentration until a maximum was reached at about 50 µM cytochrome c. When the same experiment was carried out with

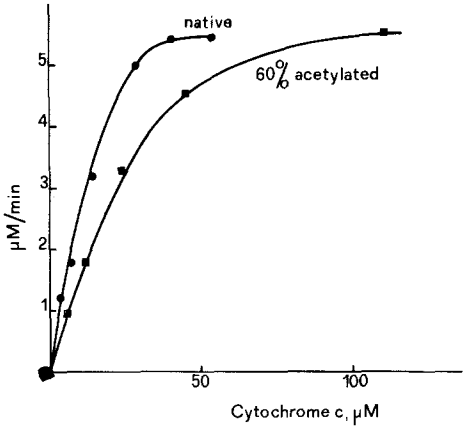

Fig. 1 - Reduction of native and acetylated cytochrome c by O_2^- radicals.

Experimental conditions: cytochrome c (native or acetylated) was dissolved in 50 mM phosphate buffer containing 10 mM KCl and 200 µM xantine, saturated with oxygen, pH 7.4. Reduction of cytochrome c (at 550 nm, in a Hitachi-Perkin Elmer spectrophotometer Mod. 124) was started by the addition of 17 µg/ml xantine oxidase.

acetylated cytochrome c about twice as much cytochrome was necessary to attain the maximum rate of reduction, identical to that of native cytochrome c. This finding indicates that the ability of acetylated cytochrome c to become reduced by O_2^- radicals is only slightly decreased, when the concentration of cytochrome c was rate limiting. However if excess acetylated cytochrome c is present the rate of its reduction became of the zero order, being presumably limited by the rate of radicals production.

Thus, acetylated cytochrome c appears to be competent in detecting O_2^- radicals formation. The reduction of both native and acetylated cytochrome c was highly sensitive to superoxide dismutase, 92% inhibition of the reduction beeing obtained under the above conditions at 2.5×10^{-8}M superoxide dismutase.

Enzymatic reduction and oxidation of acetylated cytochrome c.

The ability of acetylated cytochrome c to transfer electrons in the respiratory chain of mitochondria was tested by assaying its efficiency in reconstituting succinate oxidase activity of cytochrome c extracted mitochondria (Table I). The rate of oxygen consumption of cytochrome c extracted mitochondria, supplemented with succinate in the presence of excess acetylated cytochrome c was less than 3.3 % of the rate in the presence of native cytochrome c.

The succinate-cytochrome c reductase activity, measured by the zero order rate constant of the reduction of excess ferricytochrome c (at 550 nm) by cytochrome c extracted mitochondria, in the presence of succinate, was more than 30 folds higher for native than for acetylated cytochrome c.

The oxidase activity of cytochrome c depleted mitochondria using acetylated ferrocytochrome c as the substrate (a first order reaction with respect to cytochrome c), had a rate constant of 4% with respect to the native hemoprotein.

NADPH-ferricytochrome c reductase of rat liver microsomes was 7.7% with acetylated cytochrome c with respect to native cytochrome c. Acetylation of cytochrome c appears to depress drastically the enzymatic reduction and oxidation of cytochrome c both in mitochondria and microsomes, without substantial modifications of its reducibility by superoxide radicals.

Acetylation of cytochrome c is thus a simple and very useful

T A B L E I

ENZYMATIC REDUCTION AND OXIDATION OF NATIVE AND ACETYLATED CYTOCHROME C

Experimental conditions: details concerning the preparations used and the enzymatic tests are reported in the section of Methods.

	cytochrome c			
	native		acetylated	
	rate	%	rate	%
Enzyme system succinate--cyt c reductase+	6.4	100	0.2	3.1
Ferrocytochrome c oxidase (210 µg/ml)++	0.046	100	<0.002	<4
NADPH-cyt c reductase+	10.4	100	0.8	7.7
succinate oxidase+	60	100	<2	<3.3

+ expressed in nmoles cytochrome c reduced.min^{-1}.mg^{-1}

++expressed as first order rate constant, sec^{-1}

modification of the hemoprotein which permits detection of O_2^- radicals even in the presence of mitochondria or microsomes. When these membranes are tested for their ability to produce superoxide radicals, one should, however, be aware that a residual enzymatic reduction or oxidation of cytochrome c is present. In the presence of superoxide dismutase, which prevents cytochrome c reduction by superoxide radicals, such residual activities can be easily calculated.

Measurements of O_2^- radicals production in mitochondrial membranes.

In order to demonstrate the possible applicability of acetylated cytochrome c for the detection of superoxide radicals, mitochondrial membranes were chosen, treated by ultrasonic irradiation,

to release endogenous superoxide dismutase, and washed.

It has been suggested from the oxidation of epinephrine to adrenochrome that O_2^- radicals are produced in mitochondrial membrane fragments (17). In the experiment reported in Fig. 2, mitochondrial membrane fragments were incubated in the presence of antimycin, to prevent oxidation of respiratory carriers by cytochrome c oxidase, and of acetylated ferricytochrome c. Addition of succinate induced a reduction of cytochrome c of 0.5 nmoles.min^{-1}.mg $protein^{-1}$ which is highly sensitive to superoxide dismutase. It appears clearly that O_2^- radicals are formed in the respiratory chain of mitochondria under the above experimental conditions.

CONCLUSIONS

The data reported above indicate that acetylated cytochrome

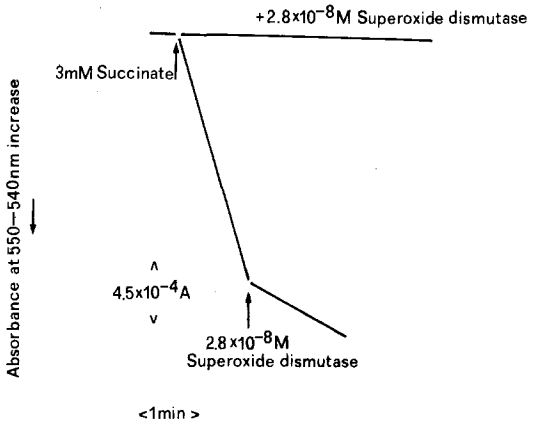

Fig. 2 - Detection of O_2^- radicals in mitochondrial fragments by acetylated cytochrome c.

Experimental conditions: mitochondrial fragments (180 µg/ml) were incubated in 50 mM phosphate buffer, containing 10 mM KCl, 2 µM antimycin, saturated with oxygen, 6.0 µM acetylated cytochrome c, pH 7.4. Radicals production was initiated by addition of 3 mM succinate and monitored by the reduction of acetylated cytochrome c at 550-540 nm, in a dual-wavelength spectrophotometer (constructed in the workshop of the Johnson Research Foundation, Philadelphia).

c can be utilized, similarly to native cytochrome c, for the detection of O_2^- radicals generated from simple enzymatic systems, such as xantine oxidation by xantine oxidase.

The large decrease of enzymatic oxidation and reduction of acetylated cytochrome c makes this derivative useful for the detection of O_2^- radicals in the presence of cytochrome c reductases or oxidases, in particular in biological membranes. In the same systems acetylated cytochrome c can be used advantageously in the presence of an appropriate source of O_2^- radicals, to detect the activity of superoxide dismutase.

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