BBA 65805

THE REDUCTION OF FERRICYTOCHROME *c* DURING THE ENZYMIC OXIDATION OF 3,4-DIHYDROXYPHENYLALANINE

DENIS KERTESZ

Laboratoire de Biochimie Médicale, Faculté de Médecine et de Pharmacie, Marseille (France)

(Received May 1st, 1968)

SUMMARY

Ferricytochrome *c* was found to be reduced during the oxidation of DOPA by polyphenoloxidase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1). For each DOPA oxidised, 4 ferricytochrome *c* were reduced and ferricytochrome *c* appeared to compete with the second molecule of oxygen consumed during the oxidation of DOPA. The formation of reducing equivalents was preceded by the formation of oxidising equivalents (*o*-quinones) as occurs during the enzymic oxidation of the catechol. HCN added, or anaerobiosis established before the oxidation of DOPA, inhibited the reduction of ferricytochrome *c*; HCN added, or anaerobiosis established later, increased the extent of reduction. Tyrosine, tyramine, epinephrine and dihydroxytyramine behaved similar to DOPA.

INTRODUCTION

It was shown earlier[1,2] that ferrocytochrome *c* is rapidly oxidised in the presence of catalytic amounts of catechol and polyphenoloxidase* (*o*-diphenol:O_2 oxidoreductase EC 1.10.3.1). Further studies, however, indicated that the behaviour of cytochrome *c* in the presence of DOPA and polyphenoloxidase is quite different. Although the redox potentials of the catechol (E_0 = 792 mV) and of DOPA (E_0 = 800 mV) are nearly identical[3], it was found that in the presence of the latter and of the enzyme a significant amount of ferricytochrome *c* became reduced.

It will be reported in this paper that during the enzymic oxidation of certain *o*-dihydroxyphenols, the formation of oxidising equivalents[4–7] is followed by the formation of reducing equivalents of a potential low enough to reduce ferricytochrome *c*.

MATERIALS AND METHODS

Polyphenoloxidase was prepared from mushrooms according to a previously published procedure[8] slightly modified. The modification consisted essentially in the substitution of the gel filtration on Sephadex by chromatography on DEAE-Sephadex.

* The trivial name of this enzyme proposed by the Enzyme Commission is catechol oxidase.

The preparation appeared homogeneous in the ultracentrifuge. The specific activity, at the moment of preparation was 7800 enzymic units (for definitions see ref. 8). The enzyme was stored as a dilute solution 0.2 mg/ml, in Na_2HPO_4 5 mM, for more than a year in the frozen state (−20°) and subsequently for several months in the liquid state (+3°). The loss of activity during this time was small, 10–25%.

The ferricytochrome *c* was Sigma type III; it contained 7% of ferrocytochrome *c*. Ferrocytochrome *c*, 98% reduced, was prepared according to YONETANI[9] under argon. DOPA and hydroxytyramine were obtained from Calbiochem; catalase and epinephrine from Sigma; tyrosine and tyramine from Hoffmann-La Roche; myoglobin from Seravac.

All the water used was deionised and doubly redistilled afterwards on an automatic quartz still.

Unless otherwise stated, the quantity of a reactant is expressed as a final concentration in the reaction mixture. Usually 5- to 50-μl portions of appropriately concentrated solutions of the reactants were added to a phosphate–EDTA buffer (see legends of figures) to obtain a final volume of 3 ml.

The oxidation and reduction of cytochrome *c* was followed at 550 mμ with a Cary-14 recording spectrophotometer, the temperature being between 20 and 23°. After correction for the contribution of the colour of oxidised DOPA to the absorption (see RESULTS), the extinction coefficients of VAN GELDER AND SLATER[10] were used.

RESULTS

Spectrophotometric course of the enzymic oxidation of DOPA and the contribution of the oxidation products to the absorbance at 550 mμ

The spectrophotometric course of the oxidation of DOPA was studied by MASON[11]. Under the conditions of the experiments reported here (enzyme in excess, DOPA in concentrations below saturation) the formation of dopachrome, measured at its maximum absorption at 480 mμ, is very rapid; taking into account the extinction coefficient given by MASON[11] ($\varepsilon = 3600$), about 85% of DOPA was oxidised to dopachrome in less than 5 min. The general course of events agreed with the findings of MASON (although we have never seen the development of a real maximum at 540 mμ); the absorption at 480 mμ subsequently decreased and the absorption between 500 and 600 mμ increased, both very slowly. After several hours a slight turbidity developed (melanine), which could be removed by high-speed centrifugation.

The contribution of oxidised DOPA to the absorption at 550 mμ can be calculated with sufficient accuracy by measuring periodically the increase of absorption at 541.5 and 556.7 mμ, where ferri- and ferrocytochrome *c* are isosbestic, and then taking the arithmetical mean of these increases (which are significantly different only during the first 10 min). Curve 5 of Fig. 1 was calculated in this manner; it shows a good agreement with curves registered directly.

Reduction of ferricytochrome c during the oxidation of DOPA and the stability of the reducing equivalents formed

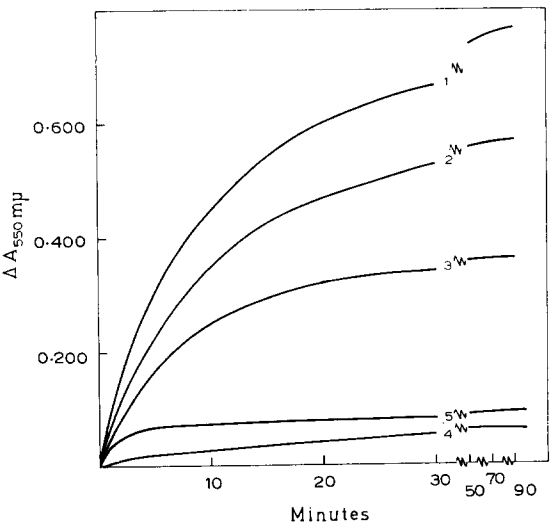
The increase of absorbance at 550 mμ of ferricytochrome *c* in the presence of DOPA and the enzyme and of DOPA alone is reported in Fig. 1.

Clearly a large fraction of ferricytochrome *c* was reduced in the presence of the

enzyme and DOPA, respectively 42, 48.5 and 57%, while in the presence of DOPA without the enzyme, the amount reduced was very small. The ferrocytochrome *c* obtained was very stable and did not appear to autoxidise; it was rapidly oxidised by ferricyanide or by catalytic amounts of catechol + polyphenoloxidase[1].

Inspection of Fig. 1 shows that the oxidation of DOPA in dopachrome was more rapid than the reduction of ferricytochrome *c* and that almost the whole of the reduction occurred after this oxidation. In consequence, we attempted to add ferricytochrome *c* to DOPA already oxidised. Ferricytochrome *c* was therefore added to solutions where DOPA had been in contact with the enzyme for 5, 10, 20 and 40 min.

It was found that in the first three samples the extent of reduction was practically



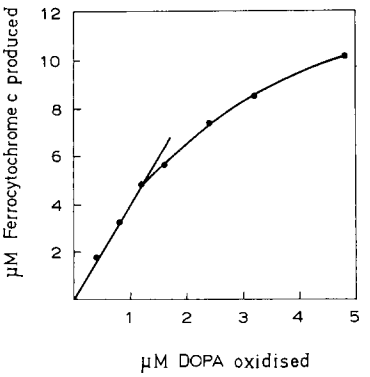


Fig. 1. The reduction of ferricytochrome *c* during the oxidation of DOPA. Phosphate buffer 66 mM (pH 6.8), EDTA 2 mM, DOPA 48 μM. Curves 1, 2 and 3 contain yet 0.66 μg/ml polyphenoloxidase (omitted in 4). Curve 1, contains 67.5 μM; Curve 2, 45 μM; Curves 3 and 4, 22.5 μM ferricytochrome *c*. Curve 5 is the contribution of oxidised DOPA to the absorption at 550 mμ, calculated from the increase of the absorbances at two isosbestic points of cytochrome *c* (see RESULTS).

Fig. 2. The stoichiometry of the reduction of ferricytochrome *c* during the oxidation of DOPA. Ferricytochrome *c* 23.5 μM, phosphate buffer 66 mM (pH 6.8) EDTA 2 mM. The concentration of DOPA, oxidised as in Fig. 1, is reported on the abscissa.

identical to that reported in Curve 3 of Fig. 1, where DOPA was added, under identical conditions, after the ferricytochrome *c*; even in the fourth sample, the extent of reduction was only slightly diminished, by about 10%.

The stability of the reducing equivalents produced allows great experimental freedom. Polyphenoloxidase can be added to a solution of DOPA, the solution distributed into two cuvettes and ferricytochrome *c* added to the sample, while the other is used as a reference. Proceeding in this manner it can be verified that the isosbestic points of cytochrome *c* remain invariant and correction is no longer necessary.

Stoichiometry of the reduction

A constant amount of ferricytochrome *c* was added to decreasing amounts of oxidised DOPA and the extent of the reaction was measured for 2 h and remeasured after 4 h (Fig. 2). The stoichiometry is clearly four ferricytochrome reduced for each DOPA oxidised. The efficiency of the reduction decreases with increasing DOPA concentration. It appears therefore, that there is a competitition for the reducing equivalents between ferricytochrome *c* and oxygen. When the former is in great excess, about 20 to 1, the reducing equivalents reduce exclusively the ferricytochrome *c*; if this ratio is smaller, the reducing equivalents reduce also oxygen.

Effect of HCN

As is well known, polyphenoloxidase is inhibited by HCN (ref. 12). If HCN was added at the beginning of the reaction, practically no ferricytochrome was reduced, because the oxidation of DOPA was inhibited and no reducing equivalents were produced. If HCN was added 5 min after the beginning of the reaction, after the end of the first phase of the oxidation of DOPA, the yield in reduced cytochrome *c* was increased significantly (Fig. 3).

Reduction of ferricytochrome c under argon and in oxygen

If anaerobiosis was set up before the beginning of the reaction, the reduction of ferricytochrome *c* remained moderate. If anaerobiosis was established after the end

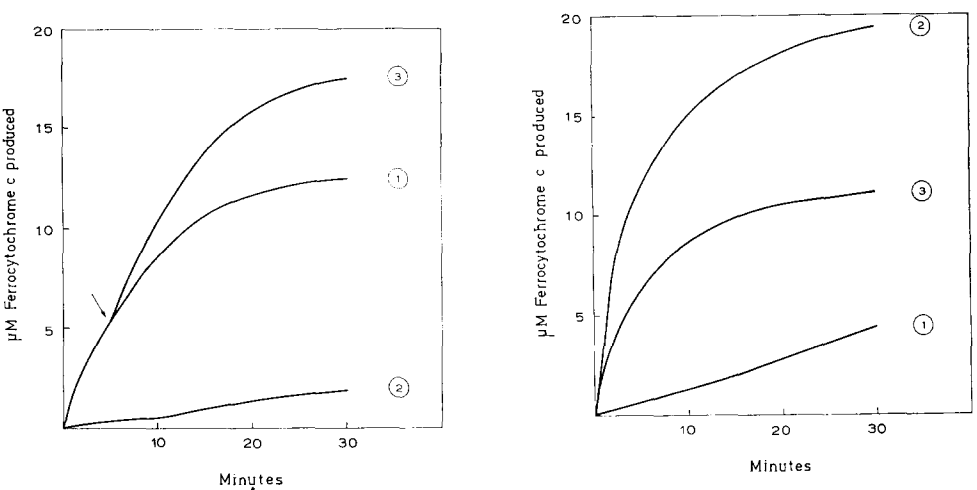


Fig. 3. The effect of HCN on the reduction of ferricytochrome *c* Ferricytochrome *c* 23.5 μM, phosphate buffer 66 mM (pH 6.8), EDTA 2 mM, DOPA 40 μM, polyphenoloxidase 0.66 μg/ml. Curve 1, no HCN; Curve 2, HCN 1 mM added at time 0; Curve 3, HCN 1 mM added at 5 min.

Fig. 4. The reduction of ferricytochrome *c* in argon, in air and in oxygen. Curve 1: in the Thunberg cuvette: ferricytochrome *c* 23.5 μM, phosphate buffer 66 mM (pH 6.8), EDTA 2 mM, polyphenoloxidase 0.66 μg/ml; in the cavity of the stopper: DOPA to 40 μM. The tube was evacuated (water pump) and filled with argon 5 times, during a period of 7 min before the addition of the DOPA. Curve 2: in the Thunberg cuvette: DOPA 40 μM, phosphate buffer 66 mM (pH 6.8), EDTA 2 mM, polyphenoloxidase 0.66 mg/ml; in the cavity of the stopper, ferricytochrome *c* 23.5 μM. After 5 min in air, anaerobiosis was established exactly as reported in Curve 1, for 7 min before the addition of ferricytochrome *c*. Curve 3: as in Curve 2, but air was replaced by oxygen instead of argon.

of the first phase of the oxidation of DOPA and before the addition of ferricytochrome *c*, the latter was almost completely reduced. If the air was replaced by oxygen under the same conditions, the amount of ferricytochrome *c* was diminished (Fig. 4).

Influence of pH

The reduction of ferricytochrome *c* was found to be strongly dependent upon pH. Already at pH 5.8 the rate of the reaction became exceedingly slow. The reduction of ferricytochrome *c* was very rapid at pH 7.4. However, because DOPA begins to be autoxidisable above pH 7, with the possible formation of H_2O_2, these studies were limited to pH 6.8 where DOPA, under our conditions, is stable.

Effect of catalase

Catalase, even if added in great excess (the largest amount tried was 415 μg in 3 ml) has no influence whatsoever on the rate or on the extent of the reduction of ferricytochrome *c*.

Effect of EDTA

In the presence of the enzyme, EDTA has no influence whatsoever on the rate or on the extent of reduction. However, because it was found that the small non-enzymic reduction, reported in Curve 4 of Fig. 1 was doubled when EDTA was omitted, this was added systematically to all samples.

Oxidation of ferrocytochrome during the enzymic oxidation of DOPA

In earlier extensive studies[4–7] it was shown that the polyphenoloxidase–*o*-diphenol system oxidises several reducing agents, and in the presence of catechol, it

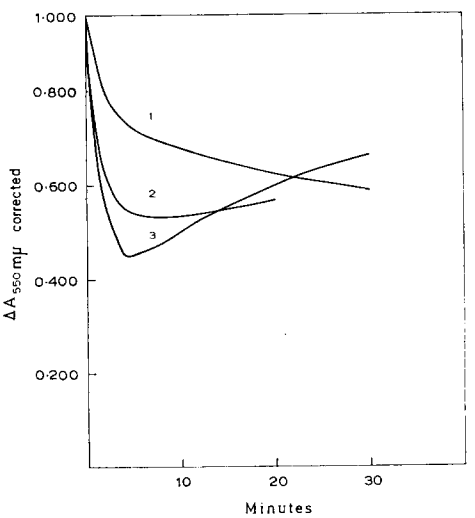


Fig. 5. The oxidation of ferrocytochrome *c* during the enzymic oxidation of DOPA. Ferrocytochrome *c* 47.5 μM, phosphate buffer 66 mM (pH 6.8) EDTA 2 mM, polyphenoloxidase 0.66 μg/μl. DOPA, in Curve 1, 20 μM; in Curve 2, 40 μM; in Curve 3, 60 μM. The highest corrections of the contribution of oxidised DOPA to the absorbance at 550 μM were in Curve 1, 0.020; in Curve 2, 0.040; in Curve 3, 0.070.

also oxidises ferrocytochrome *c* (refs. 1, 2). Because the redox potentials of the catechol–*o*-benzoquinone couple and of the DOPA–dopaquinone couple are nearly identical (see INTRODUCTION) and because the formation of dopaquinone, which is the first product of the oxidation of DOPA[13], must necessarily precede the formation of the reducing equivalents, it seemed interesting to investigate the effect of this system on ferrocytochrome *c* also.

The results, at a first glance most unusual, are reported in Fig. 5. In the presence of excess ferrocytochrome *c*, the polyphenoloxidase–DOPA system was indeed able to oxidise ferrocytochrome *c*. However, in the presence of greater amounts of DOPA, the rapid oxidation of ferrocytochrome *c* not only ceased abruptly, but the reaction turned back and part of the ferricytochrome *c* produced was slowly reduced again. In the experiment reported in Curve 3 of Fig. 5, from 47 μM ferrocytochrome *c*, 26 μM were oxidised in 4 min, of which 10 μM were reduced back slowly to ferrocytochrome *c*.

Specificity of the reaction

Among other haem-proteins, only metmyoglobin was studied. It was not reduced at all, not even if added under anaerobiosis to oxidised DOPA.

Among the catecholamines and phenols, dihydroxytyramine, epinephrine, tyrosine and tyramine behaved similarly to DOPA; in the presence of the enzyme, they were able to reduce ferricytochrome *c*.

DISCUSSION

According to MASON[11] the oxidation of DOPA (the melanogenesis) proceeds in three phases. In a rapid first phase, through dopaquinone and leucodopachrome, dopachrome is formed and accumulates, with the consumption of 1 molecule of oxygen. During a second and a third phase, which are not necessarily enzymic, a second molecule of oxygen is consumed.

There are several oxido–reduction couples involved in this process. The first, the dopaquinone–DOPA, cannot be responsible for the reduction of cytochrome *c*; it is oxidising, in accordance with its high potential (see Fig. 5 and ref. 1). The dopachrome–leucodopachrome couple also seems to be an unlikely candidate: the effective reducing equivalents appear to be formed after, and not before the dopachrome.

Whatever the mechanism of this reaction is, we have here a direct and effective way of conservating the energy gained during the reduction of oxygen. In the experiment reported in Fig. 4, Curve 2, the original ratio ferricytochrome *c*–ferrocytochrome *c* was 21.9:1.6 and the final ratio 2.7:20.8. We have here an apparent decrease of the redox potential by about 120 mV, that is to say, an apparent increase of the free energy by about 2700 cal.

ACKNOWLEDGEMENTS

I wish to thank Professors R. WURMSER, J. WYMAN, S. LISSITZKY, G. DUCET and my friends at the 'Regina Elena Institute for Cancer Research' in Rome for helpful discussions.

REFERENCES

1 D. KERTESZ AND R. ZITO, *Biochim. Biophys. Acta*, 59 (1962) 752.
2 D. KERTESZ in J. PEISACH, P. AISEN AND W. E. BLUMBERG, *The Biochemistry of Copper*, Academic Press, New York-London, 1966, p. 359.
3 E. G. BALL AND T. T. CHEN, *J. Biol. Chem.*, 102 (1933) 691.
4 D. KERTESZ AND O. AZZOPARDI, *Bull. Soc. Chim. Biol.*, 42 (1960) 1945.
5 D. KERTESZ AND O. AZZOPARDI, *Compt. Rend. Soc. Biol.*, 154 (1960) 270.
6 D. KERTESZ AND O. AZZOPARDI, *Biochim. Biophys. Acta*, 64 (1962) 149.
7 D. KERTESZ AND R. ZITO, *Biochim. Biophys. Acta*, 64 (1962) 153.
8 D. KERTESZ AND R. ZITO, *Biochim. Biophys. Acta*, 96 (1965) 447.
9 T. YONETANI, *Biochem. Prep.*, 11 (1966) 14.
10 B. F. VAN GELDER AND E. C. SLATER, *Biochim. Biophys. Acta*, 58 (1962) 593.
11 H. S. MASON, *J. Biol. Chem.*, 172 (1948) 83.
12 D. KEILIN AND T. MANN, *Proc. Roy. Soc. London, Ser. B*, 127 (1938) 127.
13 H. S. RAPER, *Ergeb. Enzymforsch.*, 1 (1932) 270.