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TRANSIENT KINETIC STUDIES OF DOPA OXIDATION BY POLYPHENOLOXIDASE

DENIS KERTESZ, MAURIZIO BRUNORI, ROMANO ZITO AND ERALDO ANTONINI

Laboratory of Medical Biochemistry, Faculty of Medecine, Marseille (France) Regina Elena Institute for Cancer Research, and Centre for Molecular Biology of C N R , Institute of Biochemistry, University of Rome (Italy)

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SUMMARY

The oxidation of DOPA (*o*-dihydroxyphenylalanine) to dopachrome as catalyzed by polyphenoloxidase (*o*-diphenol O_2 oxidoreductase, EC 1 10 3 1 , also called tyrosinase) has been followed in transient-state experiments

The progress of the reaction depends on the wavelength of observation and in the spectral region where dopachrome is the major absorbing species (*e g* 480 nm) the appearance of the product occurs with a marked lag-period On the other hand, at 390 nm the time course of the spectral change corresponds to a pseudo-first order process with no evidence of a lag The dependency of the apparent rate constants on enzyme concentration has been also investigated While the pseudo-first-order rate constant reckoned at 390 nm is linearly dependent upon the enzyme concentration, the rate of appearance of dopachrome (*i e* the reaction rate at 480 nm) tends to approach a limiting value of about 0 8 sec^{-1} as the enzyme concentration is increased

In substantial agreement with a mechanism postulated by other authors[4,8,9], the results reported here can be described by a simple scheme involving three reaction steps (characterized by k_1, k_2 and k_3) and four species (*i e* S = substrate, P_1 and P_2 = intermediate products and P_3 = dopachrome)

$$S \xrightarrow[\text{enzymatic}]{k_1} P_1 \xrightarrow[\text{non-enzymatic}]{k_2} P_2 \xrightarrow[\text{enzymatic}]{k_3} P_3$$

The reaction with O_2 is shown not to be rate limiting under the conditions used The non-enzymatic transformation of $P_1 \rightarrow P_2$ becomes rate limiting at high enzyme concentrations (k_2 approx 0 8 sec^{-1}) Minimum estimates of the rate constants for the enzyme–substrate reaction have been also obtained

Kinetic studies of the reactions catalysed by polyphenoloxidase (*o*-diphenol O_2 oxidoreductase, EC 1 10 3 1, also called tyrosinase) have up till now been performed only under steady state conditions[1,2] Aiming to gain more detailed information on the individual steps involved in the catalytic mechanism of polyphenoloxidase, we

have initiated transient kinetic studies of the polyphenoloxidase system in our laboratories The most useful approach in these cases is to resolve the overall catalytic process by direct observation of events associated with binding and release of substrate and products occurring on the enzyme This approach has been successfully applied to other oxidase systems However, polyphenoloxidase offers special difficulties for this type of study since no obvious change in physical properties of the protein, such as changes in absorption spectra, takes place on mixing the enzyme with O_2 or with the oxidizable substrates[3] Therefore information on the pre-steady phase of the catalysis could only be obtained by following the time course of spectral changes associates with product formation at very high enzyme concentrations, with the hope of identifying significant rate limiting steps under these conditions

The present experiments have been performed using DOPA (*o*-dihydroxyphenylalanine) as a substrate for polyphenyloxidase, the main justification for this choice being that the oxidation product of DOPA (dopachrome) is a well-characterized and relatively stable compound which displays favorable spectral properties, with absorption maxima at approximately 300 and 480 nm (ref 4)

The preparation of the enzyme was described earlier[5,6], freshly prepared, the enzyme had a specific activity of 9000 enzyme units/mg, during its long storage (more

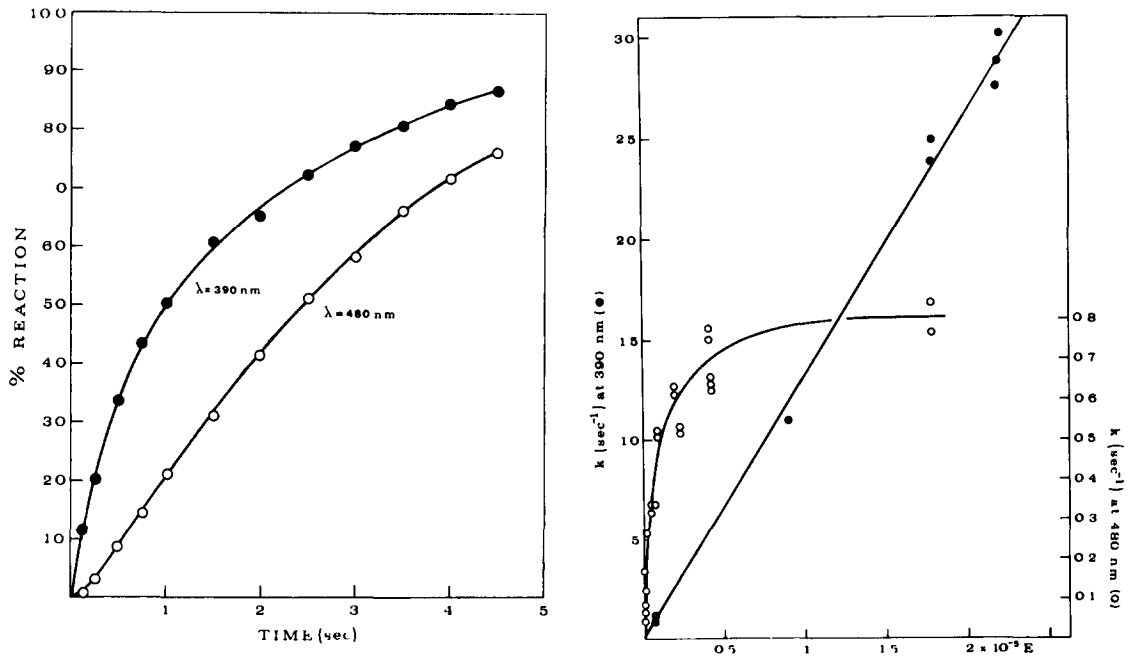


Fig 1 Time course of the reaction observed on mixing polyphenoloxidase (concn , 0 78 10^{-6} Cu equiv/l) with DOPA (concn , 5 10^{-5} moles/l) in the presence of air (O_2 concn , 2 7 10^{-4} moles/l) The two curves refer to two identical experiments carried out at two different wavelengths (*i e* λ = 390 and 480 nm) Other conditions pH 6 8, 0 1 M potassium phosphate buffer at 20°

Fig 2 Dependence on polyphenoloxidase concentration (in Cu equiv/l) of the apparent first order rate constant for the oxidation of DOPA in the presence of air The different symbols refer to the rate constant for the reaction as reckoned at 390 nm (●) or 480 nm (○) Other experimental conditions as in Fig 1

than a year) at −20°, it lost about 30% of its original activity As is well known[1,2,5], the enzyme is a tetramer, (mol wt 128 000) containing 4 atoms of copper In this paper the concentration of the enzyme is given in copper equivalents Kinetic experiments were performed with a Gibson–Durrum rapid mixing apparatus equipped with a 2-cm observation tube

Fig 1 shows the time course of the reaction calculated from the absorbance changes observed on mixing the enzyme with DOPA in the presence of oxygen At 480 nm, where dopachrome has an absorption maximum, the time course is characterised by a lag, the successive progress of the reaction corresponding closely to a pseudo-first-order process This apparent first-order rate constant tended to approach a limiting value corresponding to about 0 8 sec^{-1} as the enzyme concentration increased (Fig 2) At low concentrations, approaching those usually employed is steady state experiments, the rate of dopachrome formation increases linearly with enzyme concentration[7]

These initial results indicated that the reaction was complex and that a limiting step dominated the overall rate of appearance of dopachrome In order to clarify the situation, the time course of the optical density changes was followed as a function of wavelength at different enzyme concentrations The results are summarized in Fig 3 It is evident that more than one spectral species appears in the reaction and specifically that a species with a maximum differential absorption at approximately 390 nm precedes the formation of the final product

The time course of the formation of this species corresponds to a pseudo-first-

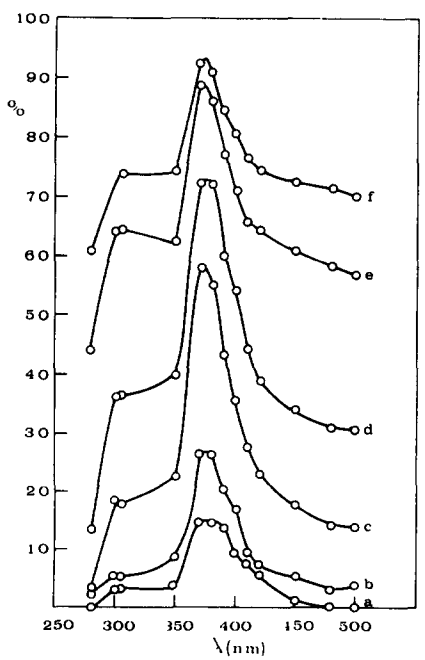
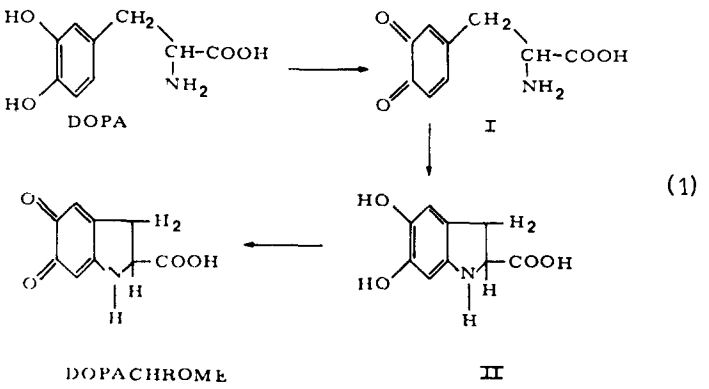


Fig 3 Extent of reaction in the oxidation of DOPA by polyphenoloxidase reported as a function of wavelength The different curves (a–f) refer to observations at different times after mixing *i e* (a) 0 125 sec, (b) 0 250 sec, (c) 0 750 sec, (d) 1 5 sec, (e) 3 0 sec, and (f) 4 0 sec Experimental conditions as in Fig 1

order process with no evidence of a lag (Fig 1), the first-order rate constant is, for the progress curve at 390 nm, linearly dependent on enzyme concentration up to very high values (Fig 2)

These kinetic results indicated that the initial product of the polyphenoloxidase catalysed oxidation of DOPA is a compound with an absorption maximum at approximately 390 nm and that the subsequent formation of dopachrome involves a rate limiting step of about 0 8 sec^{-1} under the experimental conditions This interpretation of the kinetic data is fully consistent with a postulated mechanism of DOPA oxidation proposed by RAPER[8,9] on chemical grounds and substantiated later by MASON[4] According to these authors, oxidation of DOPA would involve the following intermediate species



(1)

As is generally characteristic of *o*-quinones[10], Compound I would be the species absorbing at 390 nm, Compound II, on the other hand, would be nonabsorbing at wavelengths higher than 300 nm Direct evidence for the formation of the nonabsorbing species II was obtained in experiments performed at a low O_2 concentration, where the increase in absorption observed at 390 nm was followed by a marked decrease

The time course of the reaction at 390 nm shows no detectable lag even at the highest enzyme concentrations, this may allow a minimum estimate for the rate of formation of the enzyme–substrate complex, which should be higher than 4 10^6 M^{-1} sec^{-1} The time course of the appearance of Compound I in experiments in which a deoxygenated mixture of polyphenoloxidase and DOPA was mixed with an air-equilibrated buffer indicated that the enzyme reacts with oxygen with a very high rate constant

Therefore, the reactions with S and O_2 are never rate limiting under the conditions of the experiments and the main features of the system can be described by the approximate scheme

$$\begin{aligned} &S \xrightarrow{k_1} P_1 \text{ (enzyme catalyzed) } k_1/E = 1\ 3\ 10^6\ sec^{-1}\ Cu\ equiv^{-1} \\ &P_1 \xrightarrow{k_2} P_2 \text{ (non enzymatic) } k_2 = 0\ 8\ sec^{-1} \\ &P_2 \xrightarrow{k_3} P_3 \text{ (enzyme catalyzed) } k_3/E \gg k_1/E \end{aligned} \qquad (2)$$

It may be noted that oxidation of P_2 to P_3 may be non enzymatic, provided

it is fast enough in comparison with the rate of formation of P_1 Indeed, preliminary analog computations in the framework of Scheme 2 gave results fully consistent with the experimental data

A much more detailed study of the system, including study of the effect of several variables on the time course of the appearance of the various intermediates, is in progress However, the results reported here give clear and direct evidence for intermediates formed in the polyphenoloxidase-catalysed oxidation of DOPA, and allow estimation of the rates of the individual steps involved in the overall process

REFERENCES

1 D KERTESZ AND R ZITO, in O HAYAISHI, *Oxygenases*, Academic Press, New York, 1962, p 307
2 H W DUCKWORTH AND J E COLEMAN, *J Biol Chem*, 245 (1970) 1613
3 D KERTESZ, in J PEISACH, P AISEN AND W E BLUMBERG, *The Biochemistry of Copper*, Academic Press, New York, 1966, p 359
4 H S MASON, *J Biol Chem*, 172 (1948) 83
5 D KERTESZ AND R ZITO, *Biochim Biophys Acta*, 96 (1965) 447
6 D KERTESZ, *Biochim Biophys Acta*, 167 (1968) 250
7 M FLING, N H HOROWITZ AND S F HEINEMANN, *J Biol Chem*, 238 (1963) 2045
8 H S RAPER, in F F NORD AND R WEIDENHAGEN, *Ergebnisse der Enzymforschung*, Vol 1, Akademische Verlag, Leipzig, 1932, p 270
9 H S RAPER, *J Chem Soc*, (1938) 125
10 H S MASON, *J Biol Chem*, 181 (1949) 803