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Discrimination between two kinetic mechanisms for the monophenolase activity of tyrosinase

In a recent paper (Naish-Byfield and Riley, 1992), an oxymetric study of the oxidation of monohydric phenol substrates by tyrosinase was carried out. The results of this investigation were explained by means of a kinetic mechanism which does not consider another one proposed in the literature (Cabanes et al., 1987). Both mechanisms are similar, and are shown in Figure 1.

Both mechanisms taken into account the occurrence of E_{met}, E_{deoxy} and E_{oxy} forms of tyrosinase, as well as the lack of activity of E_{met} on monophenols. This means that a portion of tyrosinase is scavenged from the catalytic turnover as a dead-end complex in the steady-state of the monophenolase activity of tyrosinase. The only difference between these mechanisms is that in the first one the E_{oxy} form in the presence of monophenol generates *o*-quinone and the E_{deoxy} form in a single step (Naish-Byfield and Riley, 1992), whereas in the second one the generation of E_{deoxy} from E_{oxy} and monophenol requires two steps (Cabanes et al., 1987; Rodríguez-López et al., 1992).

In the mechanism of Figure 1(a) there are three steps of *o*-quinone production, whereas there are two in the scheme of Figure 1(b). However, the stoichiometry between substrates (monophenol and oxygen) and product (aminechrome) cannot be used to discriminate between both mechanisms, because it does not vary.

The following characteristics of the kinetic behaviour of the enzyme must be satisfied by the proposed mechanism.

(i) There is a lag period in the expression of the monophenolase activity.

(ii) The lag period increases when the substrate concentration increases.

(iii) The lag period decreases when the enzyme concentration increases.

Moreover, in order to start the catalysis on monophenols, the presence of a minimum quantity of enzyme in the oxy form is necessary in both mechanisms, since the met form is inactive on these substrates.

The two mechanisms could be distinguished by examining the influence of two types of monophenol substrates, those leading to stable and those leading to unstable quinones, on the lag phase of the reaction.

Monophenols which generate quinones that evolve to other more stable compounds, either by the nucleophilic addition of water to generate a hydroxylated compound (i.e. *p*-cresol, phenol etc.), or those than suffer a 1,4-Michael addition of the amino group of its side chain (i.e. tyrosine, tyramine etc.), have in common the ability to regenerate their corresponding *o*-diphenol by means of the chemical reactions coupled to the enzymically generated quinones (see bottom of Figures 1a and 1b). For this reason, when using this type of substrate it is not possible to discriminate between the two proposed mechanisms, because both can explain the properties mentioned above.

When the monophenol used gives rise to stable quinones, the sequence of chemical reactions does not take place, and therefore the mechanism described in Figure 1(a) does not predict point (i), that is, there is no lag period, since the enzyme would act in the cycle shown in Scheme 1, and this cycle predicts a low activity, but with no lag period. The experimental results using mushroom

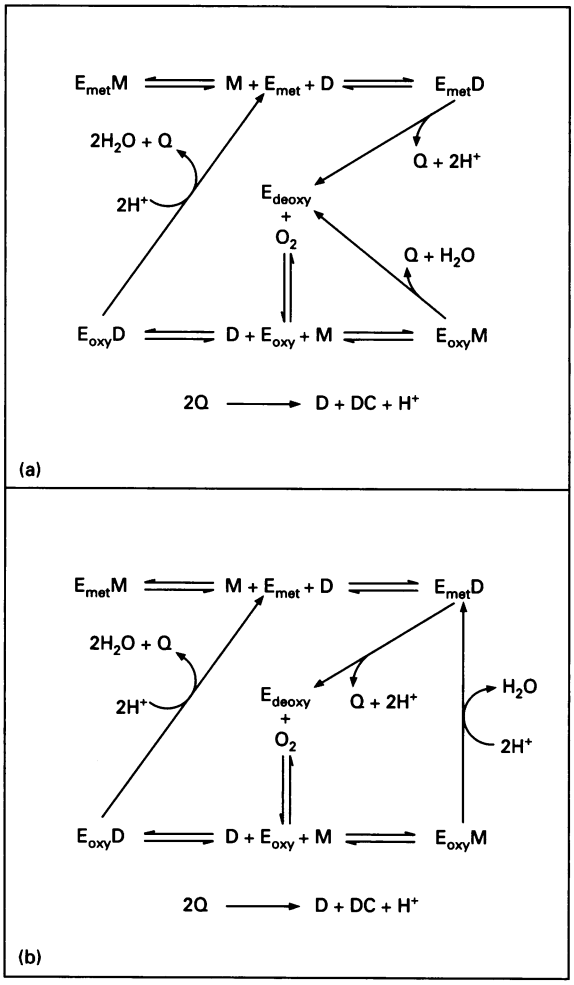
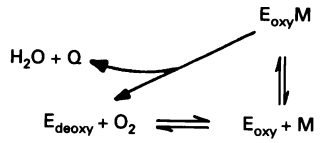


Figure 1 Kinetic mechanisms suggested for tyrosinase

(**a**) Naish-Byfield and Riley (1992). The symbols E_{met}, E_{deoxy}, E_{oxy}, M, D, Q and DC denote the met form of the enzyme, the deoxy form of the enzyme, the oxy form of the enzyme, the monophenol, the diphenol, the *o*-quinone and the aminechrome, respectively. (**b**) Cabanes et al. (1987).



Scheme 1

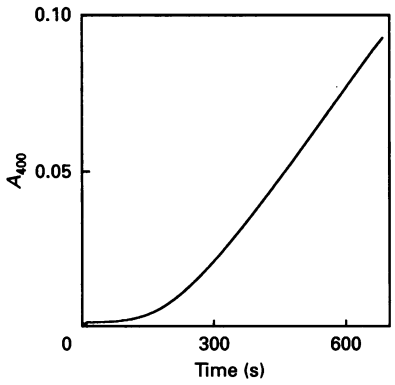


Figure 2 Time course of quinone production by tyrosinase with t-butylphenol at 0.1 mM

In the assay, concentrations of O_2 and tyrosinase of 0.26 mM and 0.3 μM, respectively, were used. The quinone accumulation was monitored spectrophotometrically at 400 nm (ϵ = 1150 $M^{-1} \cdot cm^{-1}$) using a Perkin–Elmer Lambda-2 spectrophotometer interfaced on-line with an Amstrad PC2086 computer. The reaction medium was 0.1 M sodium phosphate buffer, pH 7.0. Temperature was controlled at 25 °C with a Haake D1G circulating bath with a heater/cooler and checked with a Cole–Parmer digital thermometer with a precision of ±0.1 °C.

tyrosinase and t-butylphenol indicate the appearance of a lag period (Figure 2), and therefore the mechanism proposed in Figure 1(a) is not valid and the steps which must occur in the turnover would be those described in Figure 1(b) without the chemical reactions after the enzymic reaction. This gives rise to the lag period and, when the enzyme concentration increases, this process takes less time, resulting in a shorter lag period, and when the monophenol concentration increases, more diphenol is accumulated, the lag period becoming longer.

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Sequences of clostridial ferredoxins: determination of the *Clostridium sticklandii* sequence and correction of the *Clostridium acidurici* sequence

Clostridial low-potential 2[4Fe-4S] ferredoxins (Fds) have been among the first isolated (Mortenson et al., 1962) and characterized (Lovenberg et al., 1963) iron–sulphur proteins. These small (approx. 55 residues) molecules have proved amenable to a variety of structural investigations, which have yielded numerous amino acid sequences (George et al., 1985), a three-dimensional structure (Adman et al., 1976; Backes et al., 1991) and a host of data on the electromagnetic properties and topology of the [4Fe-4S] clusters (Mathews et al., 1974; Moulis et al., 1984).

Recently, these Fds have attracted renewed interest, since they afford useful models, as small and well-defined structures, for the investigation of the influence of the polypeptide chains on the properties of the [4Fe-4S] clusters. Research along these lines may benefit from the availability of molecular variants of 2[4Fe-4S] Fds, which are produced by chemical synthesis of polypeptide chains (Smith et al., 1991) or by using a synthetic gene expressed in *Escherichia coli* (Davasse and Moulis, 1992). Micro-organisms nevertheless remain a valuable source of Fd, the more so since naturally occurring sequences are likely to point out functionally important amino acids. In order to strengthen the basis for such studies, we report here corrections of the sequence of the Fd from *Clostridium acidurici* (Rall et al., 1969), which has long been a benchmark protein in the study of 2[4Fe-4S] Fds (Rabinowitz, 1972), and the newly determined sequence of *Clostridium sticklandii* Fd.

Automated Edman degradation of carboxymethylated *C. acidurici* Fd yielded the complete sequence, with only the three C-terminal residues missing. This sequence displays four changes, as compared with the previously reported one (Rall et al., 1969): D15 has to be replaced by E, D21 by N, Q25 by S, and S28 by D (Figure 1). The first error, in position 15, was initially detected on a 0.18 nm (1.8 Å)-resolution electron-density map derived from X-ray-diffraction data (TranQui et al., 1991) and prompted us to re-sequence the entire protein. Peptides purified from a thermolysin digest of the carboxymethylated ferredoxin allowed the identification of the three C-terminal amino acids (peptide Th-4), and confirmed the four corrections in positions 15, 21 (peptides Th-1 and Th-2), 25 and 28 (peptide Th-3) (Figure 1). The four changes in the sequence do not modify the amino acid analysis of the protein: in both the previously reported sequence (Rall et al., 1969) and the corrected one shown in Figure 1, the four residues come out as 2 Asx, 1 Glx, 1 Ser. This is probably why these sequencing errors have remained undetected in the numerous careful analyses that have been reported in the meantime (Lode et al., 1976).

The evidence for the amino acid sequence of *C. sticklandii* Fd is summarized in Figure 2. The carboxymethylated Fd yielded the sequence down to residue 33, with four undetected residues. The sequences of six overlapping peptides obtained by either chymotrypsin or thermolysin digestion afforded the complete sequence of the protein (Figure 2), which was consistent with amino acid analyses.

The two sequences determined here have been aligned in Figure 3 with those of two other well-characterized clostridial-

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                    10        20        30        40        50  55
                    AYVINEACISCGACEPECPVNAISSGDDRYVIDADTCIDCGACAGVCPVDAPVQA
Cm-Fd               AYVINEACISCGACEPECPVNAISSGDDRYVIDADTCIDCGACAGVCPVDAP---
Th-1                  VINEACISCGACEPECPVNA
Th-2                        ISCGACEPECPVNA
Th-3                                  ISSGDDRY
Th-4                                          VIDADTCIDCGACAGVCPVDAPVQA
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Figure 1 Summary proof of the sequence of *C. acidurici* ferredoxin

C. acidurici (A.T.C.C. 7906, formerly *C. acidi-urici*; Cato et al., 1986) Fd was purified as described by Gaillard et al. (1986). The final sequence is shown in **bold** characters, and the four corrected residues are doubly underlined. The sequences of the carboxymethylated ferredoxin (Cm-Fd) and of four peptides obtained by thermolysin cleavage of Cm-Fd are shown below. Hyphens stand for unidentified residues. The sequence has been deposited in the EMBL Data Library with accession number P00198.