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MECHANISM OF ENZYME ACTION

V. DEMONSTRATION OF AN INITIAL STEP OF ENZYME–SUBSTRATE COMPLEX USING D-AMINO-ACID OXIDASE AND D-LACTATE OR D-MANDELATE

KUNIO YAGI, TAKAYUKI OZAWA AND MAKOTO NAOI
Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya (Japan)

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SUMMARY

Upon forming a complex with D-lactate or D-mandelate, the absorption spectrum of D-amino-acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) became similar to that of the enzyme–benzoate complex. Under anaerobic conditions, the complexed enzyme was fully reduced. The enzymic oxidation of D-lactate was confirmed by the stoichiometric relation between the pyruvate formed and the O_2 consumed. The rate of oxidation was very small; it was estimated to be one thousandth of the rate for D-alanine. It was discovered that L-lactate was a competitive inhibitor, D-mandelate was oxidized by this enzyme and L-mandelate was a competitive inhibitor.

The characteristic spectral change observed immediately after the addition of D-lactate or D-mandelate indicated an initial complexing between the enzyme and the substrate; this demonstration had not yet been successful.

Complex formation of the enzyme with D-lactate or with its enantiomer was demonstrated by measuring circular dichroic spectra.

INTRODUCTION

In studying the reaction mechanism of the enzyme using D-amino-acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3], a purple complex composed of equimolar amounts of the enzyme and the substrate moieties was crystallized[1], and its properties were investigated[2]. The complex, which has a diffuse absorption band in the vicinity of 550 mμ, was involved in the equilibrium of the anaerobic reaction[3] and was identical to the rapidly appearing purple species found by a stopped-flow technique[4]. The nature of this complex was assigned to be a sort of charge-transfer complex, *viz.* an inner complex according to the definition of MULLIKEN[5]. Accordingly, the initial complex of the oxidized enzyme and the substrate was supposed to occur in the reaction sequence before the purple complex. However, its demonstration is

Abbreviation: CD, circular dichroism.

difficult even by a stopped-flow technique because it is supposed to turn into the purple complex very rapidly, as far as D-amino acids are used as substrate. This difficulty may be eliminated by applying the "slow reaction method"[6] which is, in the present case, to evolve some suitable substrate that is oxidized slowly enough to demonstrate the initial step. After extensive screening, D-lactate and some other hydroxy acids were found to be suitable because their oxidation rates by this enzyme are very small.

Using these D-hydroxy acids, the spectrophotometric and circular dichroic (CD) demonstration of the primary complex between the enzyme and the substrate is described. Some characteristics of the complex are discussed in comparison with those of the enzyme–benzoate complex, an enzyme–substrate complex model[6]. A preliminary report for part of this paper has appeared[7].

MATERIALS AND METHODS

D-Amino-acid oxidase was prepared by the method of YAGI *et al.*[8]. The concentration of the enzyme was determined spectrophotometrically using the molar absorbance coefficient of $1.13 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$ at 455 mμ of the enzyme-bound FAD. The hydroxy acids were obtained from Sigma Chemical Co., and no contamination of L-lactate in the D-lactate sample was confirmed by use of L-lactate dehydrogenase. Crystalline catalase (39 000 units/mg) and L-lactate dehydrogenase (360 units/mg) were furnished from Boehringer Co.

The changes in the absorption spectra of the enzyme upon addition of lactate or mandelate were recorded with a Beckman DK-2A spectrophotometer. CD spectra were measured with a JASCO ORD/UV recorder with a CD attachment.

O_2 consumption due to the oxidation of the substrate was measured polarographically with a Beckman oxygen sensor. The measurements were carried out at 20° and at pH 7.5 as reported previously[8].

Lactate and pyruvate in the reaction mixture were identified by using a high-voltage paper electrophoresis according to MICHL[9]; it was performed at 0° for 120 min with a potential gradient of 40 V/cm by use of an electrolyte, pyridine–acetic acid–acetone–water (1.5:5.5:15:78, by vol.). After the electrophoresis, spots were located by spraying an aniline–sugar reagent* followed by subsequent heating at 140°. Quantitative determination of pyruvate was performed according to KACHMAR AND BOYER[10].

RESULTS

Enzymic oxidation of D-*lactate*

It was found that D-lactate obviously consumed O_2 upon mixing with D-amino-acid oxidase, and rate of O_2 uptake was directly proportional to the concentration of the enzyme. The reaction product was identified as pyruvate by the aniline–sugar reagent after high-voltage paper electrophoresis. The O_2 uptake and the amount of pyruvate formed are summarized in Table I. An equimolar relation was found between the amounts of pyruvate formed and of O_2 consumed in the reaction system in the absence of catalase. In the presence of catalase, however, the molar amount of O_2 consumed decreased to half that of pyruvate formed. This fact suggests that H_2O_2

* 10 mg of glucose and 5 mg of arabinose were dissolved in 3 ml of ethanol and then mixed with 0.5 ml of aniline and 3 ml of *n*-butanol.

TABLE I

STOICHIOMETRY OF THE ENZYMIC OXIDATION OF D-LACTATE

The reaction systems contained 1 mmole D-lactate, 1 μmole FAD, 170 μmoles $Na_4P_2O_7$ (pH 7.5) and 1 μmole enzyme, with respect to FAD, in a total volume of 10 ml. The reaction was carried out at 20°.

Reaction system	*O_2 uptake* (μmole/min)*	*Pyruvate formed** (μmole/min)*
Catalase (−)	0.40	0.42
Catalase (+)***	0.20	0.42

* Measured with the oxygen electrode (Beckman oxygen sensor).
** Measured by 2,4-dinitrophenylhydrazone method[10].
*** 50 units of catalase were added to the reaction system.

is another product of the oxidation of D-lactate by this enzyme, as in the case of the oxidation of D-amino acids.

The figure in Table I represents the value of molecular activity of the enzyme (in terms of enzyme-bound FAD) with D-lactate under given conditions. It is about one thousandth of the value with D-alanine. It was confirmed that only the D-isomer of lactate is susceptible to the enzymic oxidation. Using its L-isomer neither O_2 consumption nor pyruvate formation could be detected.

The optimum pH for the oxidation of D-lactate was found to be about 7.5 by measuring the rate of pyruvate formation. The Michaelis constant (K_m) at pH 7.5

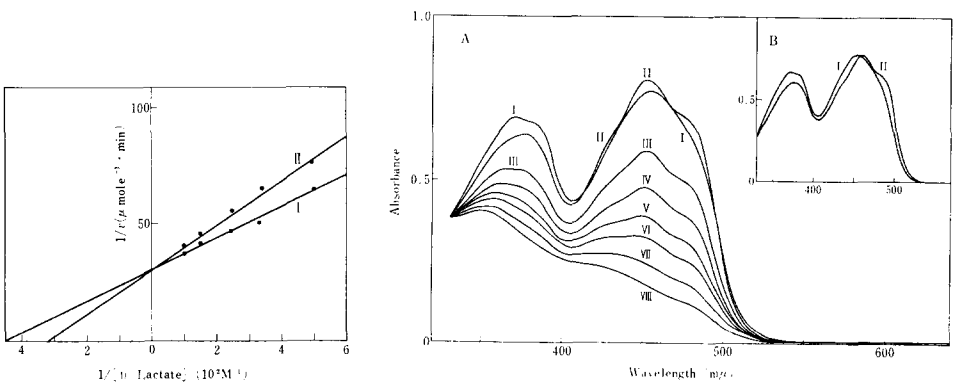


Fig. 1. Lineweaver–Burk plots of the oxidation of D-lactate in the absence and presence of L-lactate. The reaction was carried out at 37° and the product, pyruvate, was assayed by 2,4-dinitrophenylhydrazone method[10]. Curve I, the reaction mixture contained D-lactate, the enzyme (14.3 mμ moles with respect to FAD) and FAD (20 mμ moles) in 0.017 M $Na_4P_2O_7$ buffer (pH 7.5) (total vol. 5 ml); Curve II, L-lactate was added to the reaction mixture, at a final concn. of 2 mM.

Fig. 2. Change in the absorption spectrum of the enzyme by addition of D-lactate or pyruvate. A. Curve I, the enzyme (69 μM with respect to FAD) in 3.0 ml of 0.017 M $Na_4P_2O_7$ buffer (pH 8.3); Curve II, immediately after the aerobic addition of 100 μmoles of D-lactate. Under anaerobic conditions, the spectrum changed from Curve II to VIII. From Curve III to VI, each measurement was carried out at 30 min interval. B. Curve I, the enzyme (69 μM with respect to FAD) in 3.0 ml of the same buffer; Curve II, the enzyme added with 300 μmoles of pyruvate. Reactions were carried out at 15°.

of the enzyme for D-lactate was determined to be 2.2 mM from the intercept of $1/[S]$ axis of a Lineweaver–Burk plot obtained from the rate of pyruvate formation (Fig. 1, Curve I). L-Lactate was found to inhibit the oxidation in competition with the D-isomer, and the inhibition constant (K_i) was calculated to be 4.9 mM (Fig. 1, Curve II).

D-Mandelate was also found to consume O_2 when mixed with the enzyme. The oxidation rate of D-mandelate was 1.5 times larger than that of D-lactate. L-Mandelate was, like L-lactate, found to be a competitive inhibitor.

The reversibility of the reaction of D-lactate oxidation was confirmed by observing the formation of lactate from the reduced enzyme and pyruvate. The enzyme (38 μM with respect to FAD) was reduced with D-alanine (0.03 M) in a Thunberg-type cell under anaerobic conditions (gas phase, argon), and the reduced enzyme was incubated with pyruvate (0.03 M) at 20° and at pH 7.5 for 1 h. The reaction was terminated by adding 1.0 ml of 20% (v/v) $HClO_4$ to 3.0 ml of the reaction mixture. The supernatant was neutralized and applied to a high-voltage paper electrophoresis. Spots were located with the aniline–sugar reagent as described before. As a result, one of the products was identified to be lactate. The lactate in the neutralized solution was further examined by using L-lactate dehydrogenase; reduction of NAD^+ was checked spectrophotometrically, and it was found that the product was not attacked by the dehydrogenase, indicating that the lactate formed is of the D-form.

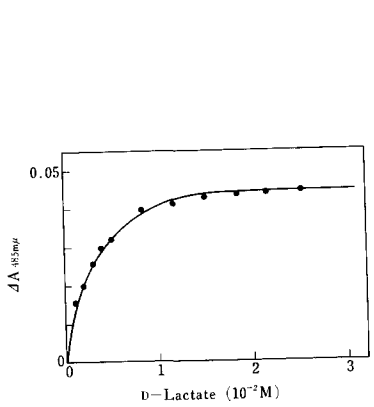
These results show that D-lactate and D-mandelate are newly found substrates of D-amino-acid oxidase although their oxidation rates are extremely smaller than those of D-amino acids.

Change in the absorption spectrum of the enzyme upon addition of lactate or mandelate

The absorption spectrum of the enzyme (Fig. 2A, Curve I) changed into Curve II immediately after adding D-lactate under aerobic conditions. The peak at 370 mμ of the enzyme was shifted to 380 mμ with some hypochromism and with the disappearance of the fine structure. On the other hand, the peak at 455 mμ was shifted to 452 mμ with some hyperchromism, and the emergence of shoulders at 480 mμ and at 430 mμ was observed. It is noted that this change is, in general, similar to the change found in the formation of the enzyme–benzoate complex[11], an enzyme–substrate complex model, and also to that of the enzyme–pyruvate complex[12*] (Fig. 2B). However, when examined in detail, the spectral change of the enzyme upon addition of D-lactate is obviously different from that of the enzyme–pyruvate complex; chiefly in that the absorption peak at longer wavelengths is blue-shifted accompanying hyperchromism in the former case whereas red-shifted without any change in absorbance in the latter case (*cf.* Fig. 2A, Curves I and II with Fig. 2B, Curves I and II).

In the presence of O_2, the above-mentioned spectral change caused by the addition of D-lactate remained unchanged until O_2 in the medium was consumed. At 20°, the spectrum did not change for at least 10 min when the solution was open to air. However, under anaerobic conditions (gas phase, argon), it turned gradually into the fully reduced form (Fig. 2A, Curve VIII) through the states shown by Curves III–VII. The fact that the spectrum at the initial step of this conversion is obviously different from that of the enzyme–pyruvate complex suggests that the spectral change

* Details of the enzyme–pyruvate complex will appear in the forthcoming paper of this series.



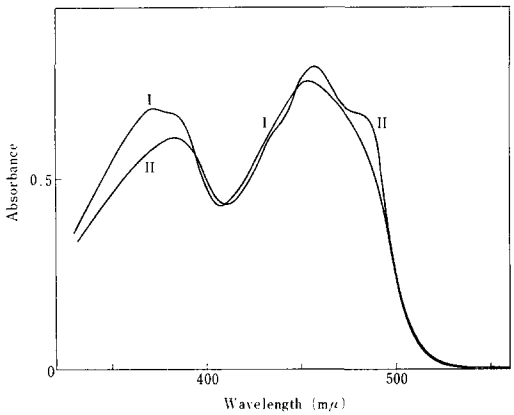


Fig. 3. The differences in the absorbance of the enzyme–D-lactate to the enzyme at 485 mμ plotted against the concentration of D-lactate. The enzyme was 58 μM with respect to FAD.

Fig. 4. Change in the absorption spectrum of the enzyme by addition of L-lactate. Curve I, the enzyme (67 μM with respect to FAD) in 3.0 ml of 0.017 M $Na_4P_2O_7$ buffer (pH 8.3); Curve II, the enzyme added with 100 μmoles of L-lactate.

from Curve I to Curve II caused by to the formation of an oxidized enzyme–substrate complex (DISCUSSION).

The absorption spectrum obtained immediately after the aerobic addition of D-lactate was apparently isosbestic with that of the uncomplexed enzyme at 414 and at 496 mμ (Fig. 2A, Curves I and II), and the increase in the absorbance at 485 mμ upon addition of D-lactate depended on the concentration of D-lactate, as shown in Fig. 3. The dissociation constant graphically obtained from these data was 2.4 mM.

L-Lactate provoked a remarkable change in the absorption spectrum of the enzyme (Fig. 4, Curve II). The spectrum observed was similar to that of the initial step of the enzyme–D-lactate mixture (*cf.* Fig. 2A, Curve II) as well as to that of the enzyme–benzoate complex. It was noticed, however, that the direction of the shift of the peak at 455 mμ in the case of L-lactate was opposite to that in the case of D-lactate. Moreover, in contrast to the case of D-lactate, the spectrum remained unchanged under anaerobic conditions. It was, therefore, considered that L-lactate can form a complex with the enzyme but is not oxidized by the enzyme. It was confirmed further by the fact that neither the O_2 uptake nor the formation of a reaction product, pyruvate was observable when L-lactate was incubated with the enzyme for more than 120 min.

D-Mandelate and its enantiomer were found to form similar complexes with the enzyme as in the case of D- and L-lactate, respectively. Immediately after the aerobic addition of D-mandelate, the absorption spectrum of the enzyme changed into one similar to the spectrum of the enzyme complexed with D-lactate (*cf.* Fig. 5, Curve II with Fig. 2A, Curve II). The peak at the longer wavelength was red-shifted (from 455 to 460 mμ) with hyperchromism, and the three-banded structure appeared. This red shift was not found for the enzyme–D-lactate complex. With L-mandelate, the peak at 455 mμ of the enzyme shifted to a direction opposite to that in the case of D-mandelate (Fig. 5, Curve III). It is noticed that Curves I, II and III in Fig. 5A are isosbestic at

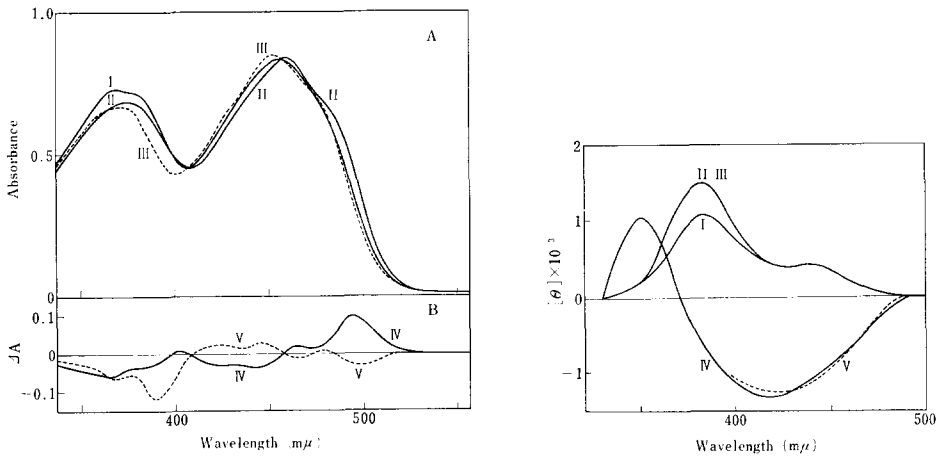


Fig. 5. Change in the absorption spectrum of the enzyme by addition of D- or L-mandelate. A. Curve I, the enzyme (73 μM with respect to FAD) in 3.0 ml of 0.017 M $Na_4P_2O_7$ buffer (pH 8.3); Curve II, the enzyme added with 100 μmoles of D-mandelate; Curve III, the enzyme added with 100 μmoles of L-mandelate; B. Curve IV, difference spectrum of the enzyme–D-mandelate complex *vs.* the enzyme; Curve V, difference spectrum of the enzyme–L-mandelate complex *vs.* the enzyme.

Fig. 6. CD spectra of the enzyme and the complex with D- or L-lactate. Curve I, the enzyme (0.1 mM with respect to FAD) in 3 ml of 0.017 M $Na_4P_2O_7$ buffer (pH 8.3); Curve II, immediately after aerobic addition of 100 μmoles of D-lactate. (The reaction mixture showed the absorption spectrum identical with Curve II in Fig. 2A.); Curve III, the enzyme added with 100 μmoles of L-lactate; Curve IV, after the full reduction of the enzyme with D-lactate. (The reaction mixture showed the absorption spectrum identical with Curve VIII in Fig. 2A.); Curve V, the enzyme was fully reduced with sodium dithionite. Unit of (Θ): degree·cm²/dM.

407 mμ and at 457 mμ. These spectral changes are clearly demonstrated by difference spectra shown in Fig. 5B.

Under anaerobic conditions, the absorption spectrum of the enzyme–D-mandelate complex changed gradually into that of the reduced enzyme; however, the complex with L-mandelate showed no further spectral change even under anaerobic conditions.

CD spectrum of the complex of the enzyme with lactate

CD spectrum of the enzyme was measured immediately after mixing D-lactate aerobically. The spectrum showed an increase in the peak at around 380 mμ (Fig. 6, Curve I to II). Under anaerobic conditions, the spectrum turned gradually into Curve IV which is similar to that of the enzyme reduced with D-amino acids[13] or with dithionite (Curve V). Upon addition of L-lactate, CD spectrum of the enzyme changed into the same one as that of the enzyme–D-lactate complex (Curve III). The spectrum remained unchanged even under anaerobic conditions.

DISCUSSION

Evidences reported in the present paper indicate that the oxidation of D-lactate to form pyruvate is catalyzed by D-amino-acid oxidase, even though its reaction rate

is extremely small as compared with the rate for D-alanine. D-Mandelate was found to be oxidized in the same fashion; thus they should be added to the list of substrates for this enzyme. In the case of these hydroxy acids, the specificity of this enzyme for the optical isomer is noted as being compatible with the case of amino acids.

Taking the expected enzyme–substrate interaction into account, attention should be focused on the spectral change of the enzyme observed immediately after the addition of these substrates under aerobic conditions. The spectral change of the enzyme due to the mixing with D-lactate is in fact noticeable; the resulting spectrum is, in many respects, similar to the spectrum of the enzyme–benzoate complex[11] in which the enzyme should be in the oxidized form. In addition, the spectrum gradually turned into that of the fully reduced form under anaerobic conditions; thus the spectrum obtained immediately after the mixing of the enzyme with D-lactate reflects the oxidized form of the enzyme.

It is noted from Fig. 2 that the purple intermediate which is observed during the anaerobic reaction of this enzyme with D-alanine is not observable. This is also the case for the oxidation of L-proline by this enzyme[14].

Thus, the reaction sequence may be represented as

$$E_{ox} + S \rightleftharpoons E_{ox} \cdot S \rightleftharpoons E_{red} \cdot P \overset{O_2}{\rightleftharpoons} E_{ox} \cdot P \rightleftharpoons E_{ox} + P \qquad (1)$$

where E_{ox}, E_{red}, S and P denote the oxidized form of the enzyme, the reduced form of the enzyme, the substrate and the product, respectively. In this case E_{red} is assumed to be oxidized in its complexed form*. If E_{red} is oxidized after its liberation from the product,

$$E_{ox} + S \rightleftharpoons E_{ox} \cdot S \rightleftharpoons E_{red} \cdot P \rightleftharpoons E_{red} + P \overset{O_2}{\rightleftharpoons} E_{ox} + P \rightleftharpoons E_{ox} \cdot P \qquad (2)$$

could be the case.

In these formulations, the observed spectrum of the oxidized enzyme immediately after the aerobic addition of D-lactate should be $E_{ox} \cdot S$ or $E_{ox} \cdot P$.

To determine whether $E_{ox} \cdot S$ or $E_{ox} \cdot P$ is compatible with the observed spectrum, the conformity between the spectrum and that of $E_{ox} \cdot P$ was tested. Calculating from the molecular activity for the oxidation of D-lactate (0.4), the amount of the product (pyruvate) formed for 10 min in the reaction medium under given conditions in Fig. 2 is 0.3 mM. Since the dissociation constant of pyruvate from its complex with this oxidase obtained as K_i by kinetic analysis is 0.04 M, the occurrence of 0.3 mM of pyruvate cannot modify the absorption spectrum of the enzyme due to complex formation. Even if the rate of decomposition of $E_{ox} \cdot P$ shown in Eqn. 1 could be assumed to be so small as to accumulate the complex in the reaction sequence, this $E_{ox} \cdot P$ is not compatible with the observed spectrum chiefly because the observed spectrum is delicately but obviously different from that of the enzyme–pyruvate complex as mentioned before.

From these considerations, the observed spectrum should be ascribed to $E_{ox} \cdot S$. The observed results on CD of the enzyme mixed with D-lactate are compatible with

* However, a possibility that O_2 reacts with some intermediate between $E_{ox} \cdot S$ and $E_{red} \cdot P$ which corresponds to the purple intermediate in the case of D-amino acids could not be ruled out.

those of the absorption spectrum and support the interpretation deduced from the latter.

Considering that the present absorption spectrum is essentially similar to the spectrum of the enzyme–benzoate complex[11] or of the enzyme–straight chain fatty acid complex[15], the interpretation given to the enzyme–benzoate complex[16] may be applicable to the enzyme–D-lactate one. Namely, that upon complex formation with the substrate, a hydrophobic environment surrounding the coenzyme chromophore of the enzyme somewhat changed, probably due to the conformational rearrangement in the protein moiety of the enzyme. Such an interpretation may be applied to the complex of the enzyme with L-lactate which provokes a similar spectral change upon mixing with the enzyme. In addition, it is noted that the directions of the shift of the peak at 455 mμ are opposite to each other in D-lactate and its enantiomer as well as in D-mandelate and its enantiomer. The isosbestic points observed in Fig. 5 indicate the equilibrium between the enzyme–D-mandelate and the enzyme–L-mandelate complexes. Considering the competition between D-mandelate and its enantiomer observed in kinetic analysis, it is concluded that they combine with the enzyme in a similar fashion although with some delicate difference. The precise interpretation on this point should be made when the steric structure of this enzyme is further elucidated.

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