MECHANISM OF CONVERSION OF THE PURPLE INTERMEDIATE INTO THE FULLY REDUCED FORM OF D-AMINO-ACID OXIDASE

Kunio Yagi and Morimitsu Nishikimi

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya, Japan

Received December 23, 1968

In a previous study (Yagi and Ozawa, 1964) on the mechanism of action of D-amino-acid oxidase [D-amino acid : oxygen oxidoreductase (deaminating), EC 1.4.3.3] crystallization of the purple complex was achieved under anaerobic conditions and the crystals were found to contain equimolar amounts of the coenzyme FAD and the substrate moiety which was readily convertible into the products on aeration. This purple complex was found to exist in the enzymic equilibrium under anaerobic conditions (Yagi, Okamura et al., 1968). Using stopped-flow technique, it has been identified as the long-wavelength absorbing intermediate appearing in the rapid reaction (Yagi et al., to be published). This intermediate was considered to be a sort of charge-transfer complex between the FAD moiety of the enzyme and the substrate (Yagi, Okamura et al., 1967), viz. an 'inner' complex according to the definition of Mulliken (1952). This assignment was mainly based upon the appearance of a characteristic broad absorption band in the vicinity of 550 mμ and upon the conversion of the essentially diamagnetic complex into a paramagnetic enzyme species through unpairing of electrons involved (Yagi, Okamura et al., 1967). In this paper, possible electronic interaction occurring in the purple complex is proposed on the basis of the results obtained by the investigation of kinetic behavior of the complex under alkaline conditions. Interpreting the data in terms of the proposed interaction, mechanism for the conversion of

the purple intermediate into the fully reduced form is discussed.

EXPERIMENTAL

Holoenzyme of D-amino-acid oxidase was prepared as reported previously (Yagi, Naoi et al., 1967). For the kinetic study of the purple intermediate, a stopped-flow apparatus built according to Gibson and Milnes (1964) was used. The light path of the observation chamber was 20 mm. Each 0.3 ml of 1.1×10^{-4} M (with respect to FAD) enzyme solution and of 5.0×10^{-2} M D-alanine solution were mixed at 24°C. Both solutions (in M/60 pyrophosphate buffer) were previously made anaerobic by bubbling with argon gas washed with alkaline pyrogallol solution. Monochromator and detector system of Shimadzu spectrophotometer was used and output from photomultiplier was recorded as transmittance by use of a Beckman recorder (response time, 1.0 sec; chart speed, 2.54 cm/min). In order to examine pH dependence of the reaction, pH of the reaction system was changed by varying pH of the substrate solution alone before mixing, while pH of the enzyme solution was maintained at 8.3. Rate-pH profile of the reaction could be examined by this procedure, because pH change caused by the mixing was definitely faster than the reaction to be observed. Since pH of the reaction mixture could not be measured directly in the stopped-flow apparatus, it was determined indirectly by measuring the mixture of equal volumes of the enzyme solution and the substrate solution of various pH's by use of a Hitachi-Horiba type F-5 pH meter.

RESULTS AND DISCUSSION

The transmittance change at 550 mμ, caused by the reaction of D-amino-acid oxidase with D-alanine under anaerobic conditions at pH 8.50, is shown in Fig. 1 (left). It can be seen that the transmittance decreases instantaneously and subsequently increases slowly. This transmittance change shows the rapid formation of the purple intermediate and its gradual conversion into the fully reduced enzyme as described by Massey and Gibson (1964). At

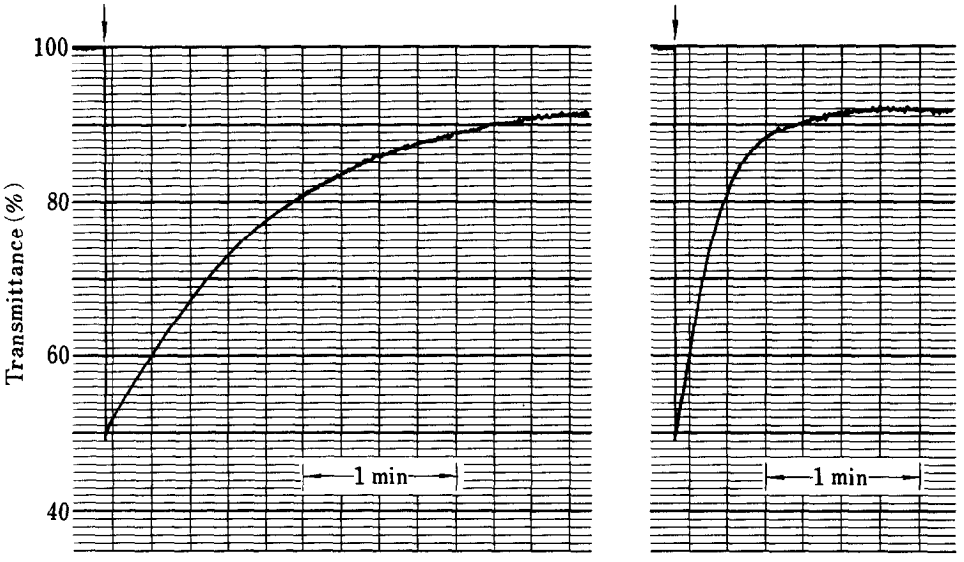

Fig. 1. Reaction of D-amino-acid oxidase with D-alanine under anaerobic conditions. By use of a stopped-flow apparatus, each 0.3 ml of 1.1×10^{-4} M (with respect to FAD) D-amino-acid oxidase solution and of 5.0×10^{-2} M D-alanine solution were mixed under anaerobic conditions at 24°C. Arrow shows the mixing. The final concentrations of the reactants became half of the above values. Transmittance change at 550 mμ was followed by a pen recorder (response time, 1.0 sec). After the mixing, pH's of the reaction system were 8.50 (left) and 9.75 (right).

---

higher pH of the reaction system, the amount of the purple intermediate formed in the initial rapid phase did not change, but the rate of its conversion into the fully reduced form increased. As an example a reaction trace at pH 9.75 is shown in Fig. 1 (right). These results provided the foundation to study the interrelation between rate of the conversion of the purple intermediate and pH of the medium. The kinetic runs were made at various pH's. Transmittance at 550 mμ was transformed into absorbance, and log A_{550} was plotted versus time. As a result, it was found that the plots gave straight lines, indicating that the reactions are of the first order. From slopes of these lines, first order rate constants, k_{obs}, were evaluated and plotted versus pH as shown in Fig. 2. Using D-proline as substrate, a similar rate-pH profile was also observed for the conversion of the purple intermediate into the fully reduced enzyme, though the rate was larger than that found for D-alanine.

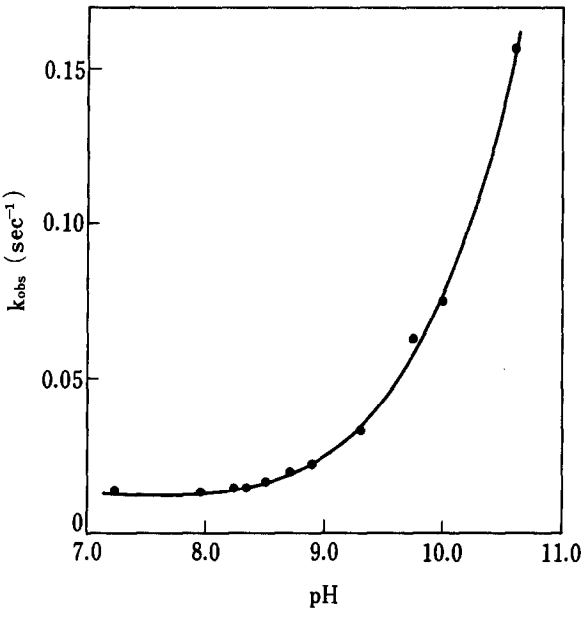

Fig. 2. Rate-pH profile for the conversion of the purple intermediate into the fully reduced enzyme under anaerobic conditions. First order rate constants of the reaction at various pH's were evaluated from the plots of log A_{550} *versus* time. Details of the procedure are described in the text.

The rate-pH profile shown in Fig. 2 suggests that the conversion of the purple intermediate into the fully reduced enzyme involves a proton-transfer which occurs in the intermediate. Since the proton-transfer process is relevant to the full reduction of the FAD moiety of the enzyme coupled with the oxidation of the substrate, it is possible to consider that the abstraction of a proton is provoked in the substrate moiety prior to the oxidation of the substrate. This idea should be discussed on the basis of the electronic interaction occurring between the substrate and the enzyme in the purple intermediate. The substrate D-amino acid, which has a lone pair on nitrogen atom, is an example of what Mulliken (1952) terms 'onium donor', and the isoalloxazine ring, the electron affinity of which is fairly strong (Pullman and Pullman, 1959), serves as an excellent electron acceptor. In fact, participation of the nitrogen lone pair is verified by the result that the energy of the long-wavelength absorption of the purple complex is dependent on the ion-

ization potential of the substrate's amino group (Yagi and Nishikimi, 1969). Considering also the properties of the purple complex mentioned in the introductory part of this paper, it could be postulated that the lone pair of the substrate's nitrogen atom is involved in charge-transfer interaction with a proper position of the isoalloxazine ring of the FAD moiety in the purple complex, as shown by the left formula in Fig. 3.

It is tempting to consider the mechanism of the conversion of the purple intermediate into the fully reduced enzyme in the following way. The charge-transfer interaction would withdraw the α-C-H binding electrons of the sub-

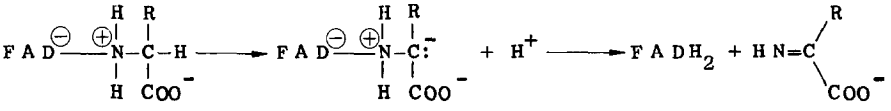

Fig. 3. Possible electronic interaction occurring in the purple complex of D-amino-acid oxidase in relation to the mechanism of acceleration of the conversion of the purple intermediate into the fully reduced enzyme by raising pH. In the purple complex, the lone pair of the substrate's nitrogen atom is considered to be involved in the interaction with FAD moiety of the enzyme. α-Hydrogen atom of the substrate is considered to be bound to the carbon kernel more loosely than hydrogen atom of usual C-H bond (see footnote in the next page). At higher pH, this hydrogen atom may be facilitated to be ionized, and the substrate moiety would be oxidized through the flow of electron(s) to the isoalloxazine ring of the FAD moiety. In this scheme, the primary product of the dehydrogenation of amino acid is expressed as imino acid. However, the possibility of the occurrence of an α-β unsaturated intermediate can not be ruled out.

strate amino acid towards the carbon kernel, and thus facilitate the release of a proton*; this would in turn provoke the flow of electron(s) from the

* In the purple complex, α-C-H bond of the substrate amino acid would be loosened through the charge-transfer interaction as discussed in the text. This seems to promote further the charge-transfer process, resulting in the maximal interaction between the donor and the acceptor (i.e. formation of 'inner' complex). In contrast to this type of complex, the oxidized enzyme forms a green complex with o-aminobenzoate which shows a typical charge-transfer band characteristic of 'outer' charge-transfer complex (Yagi, Ozawa et al., 1968). In this case, the donor has no mobile hydrogen atom bound to the carbon kernel adjacent to the amino group, and therefore seems to fail to interact as strongly as α-amino acid. Consequently, o-aminobenzoate acts as an inhibitor for D-amino-acid oxidase.

substrate moiety to the isoalloxazine ring, resulting in the full reduction of the enzyme coupled with oxidation of the substrate moiety. Considering in this way, it becomes comprehensible why the reduction of the coenzyme moiety of the purple intermediate is accelerated at higher pH which may promote the ionization of the substrate's α-hydrogen**. It seems feasible that the ionization of α-hydrogen is a rate-limiting process in the conversion of the purple intermediate into the fully reduced form of this enzyme.

REFERENCES

Gibson, Q. H., and Milnes, L., Biochem. J., 91, 161 (1964)

Massey, V., and Gibson, Q. H., Federation Proc., 23, 18 (1964)

Mulliken, R. S., J. Phys. Chem., 56, 801 (1952)

Pullman, B., and Pullman, A., Proc. Natl. Acad. Sci. U. S., 45, 136 (1959)

Yagi, K., Naoi, M., Harada, M., Okamura, K., Hidaka, H., Ozawa, T., and Kotaki, A., J. Biochem., 61, 580 (1967)

Yagi, K., and Nishikimi, M., J. Biochem., in press (1969)

Yagi, K., Nishikimi, M., Ohishi, N., and Hiromi, K., to be published.

Yagi, K., Okamura, K., Naoi, M., Sugiura, N., and Kotaki, A., Biochim. Biophys. Acta, 146, 77 (1967)

Yagi, K., Okamura, K., Sugiura, N., and Kotaki, A., Biochim. Biophys. Acta, 159, 1 (1968)

Yagi, K., and Ozawa, T., Biochim. Biophys. Acta, 81, 29 (1964)

Yagi, K., Ozawa, T., Naoi, M., and Kotaki, A., Flavins and Flavoproteins, Ed. by K. Yagi, p. 237, Univ. Tokyo Press (1968)

** In this process, there exists a possibility that some basic side chain of the enzyme protein participates in α-proton abstraction.