SHORT COMMUNICATIONS

BBA 63338

Detection of complex of D-amino-acid oxidase and D-alanine by rapid scan spectrophotometry

The existence of complexes of D-amino-acid oxidase (D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3) and benzoate, pyruvate or phenylpyruvate *etc.* was proved spectrophotometrically by the titration of the enzyme with these materials[1-4]. The characteristic change of the absorption spectrum of the enzyme during the titration is seen in a sharpening of peaks at the 420, 450 and 490 mμ absorption bands as well as, in some cases, a red shift of these peaks. It was KUBO AND SHIGA[1] who first observed a complex formation of D-amino-acid oxidase and D-alanine. They measured spectral changes with time at various wavelengths and recorded the spectrum of the complex at a fixed time. MASSEY AND GIBSON[5] used the same method to obtain kinetic constants of the reaction, but they did not determine the spectrum of the initial complex. In order to determine the precise spectrum of the short lived initial complex, a new approach has been desired. Though the time scan method is advantageous for the kinetic analysis and the determination of the spectra of longer lived intermediates, this method seems unreliable for our present purpose. Later YAGI, OZAWA AND NAOI[6] reported a complex of the enzyme and D-lactate. Since the reduction of enzyme with D-lactate was much slower than that with D-alanine, the measurement of spectral change was easy. However, when we take into consideration its extremely low reactivity, D-lactate did not seem to be a proper substrate of the enzyme.

The purpose of this communication is to report the complex formation of D-amino-acid oxidase and D-alanine, as observed by a rapid scan spectrophotometer, equipped with an analogue data recorder used for external memory.

The benzoate-free D-amino-acid oxidase was prepared as described previously[7-9]. The D-alanine used was the product of General Biochemicals. A Hitachi RPS-2 rapid scan spectrophotometer was used for measuring absorption spectra. The scan time of wavelength from 420 to 520 mμ is 150 msec, and its frequency is 3 cycles/sec. Signals of the absorption against time, $A = A(t)$, and that of wavelength, $\lambda = \lambda(t)$, were recorded simultaneously in each channel of the TEAC 351-F data recorder. After the reaction, some of the stored spectra were selected by a sweep controller and displayed on Hitachi V-018 memoriscope, reducing the tape speed to one fourth of the recording speed.

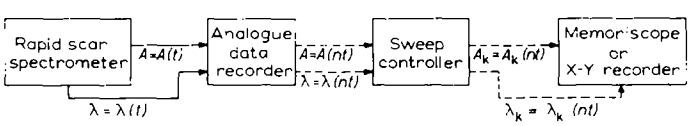

Fig. 1. Block diagram of spectrophotometer used. Solid line indicates recording of signals of absorption with time, $A = A(t)$, and wavelength, $\lambda = \lambda(t)$. The dotted line shows the reproduction of $A = A(nt)$ and $\lambda = \lambda(nt)$, where n indicates the rate of reduction speed. Desired absorption spectra were selected by the sweep controller and displayed on the memoriscope.

Fig. 1 shows the block diagram of our equipment. The analogue recorder, serving as an external memory, allowed us to follow and store all informations of rapidly changing spectra, and to reproduce them repeatedly by reducing the speed of display. This method has a draw-back that it is an off-line system in data processing, but nevertheless it made it much easier to detect the absorption spectrum of a short life complex. Also it has become unnecessary to reduce the rate of enzymatic reaction using abnormal experimental conditions.

The result is shown in Fig. 2. After aerobic addition of D-alanine to D-amino-acid oxidase, an immediate spectral change was observed. The spectrum is quite similar to that of other complexes of D-amino-acid oxidase. While oxygen in the solution was being consumed, the spectrum remained unchanged. Later, however, after the anaerobiosis (30 sec) a bleaching of flavin started to make, first, a purple form, and then a reduced form of the enzyme[10].

There remained some possibility that this spectrum did not show the complex of an enzyme substrate, but that of an enzyme–product. As pyruvate was formed during oxygen consumption, this spectral change may be considered due to enzyme–

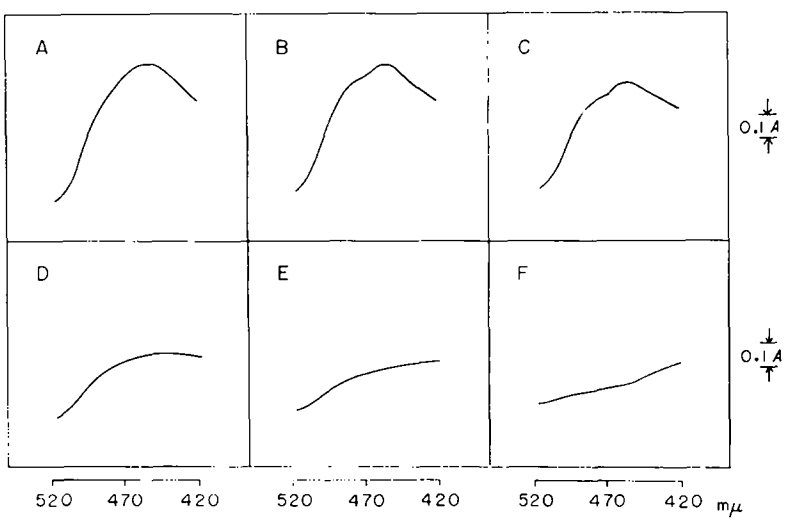

Fig. 2. Change of the absorption spectrum of D-amino-acid oxidase ($6 \cdot 10^{-5}$ M) by aerobic addition of D-alanine ($2.5 \cdot 10^{-3}$ M) at 15° (pH 7.0). A. Before addition of substrate. B. 1.2 sec after addition. C. 37 sec. D. 42 sec. E. 45 sec. F. 49 sec. By the measurement of oxygen consumption in the same experimental condition, anaerobiosis was confirmed to be attained at 30 sec after starting of the reaction.

pyruvate complex. However, the dissociation constant of enzyme and pyruvate was estimated as about 10^{-2} M, whereas oxygen content in the solution was $2 \cdot 10^{-4}$ M and flavin of the enzyme $6 \cdot 10^{-5}$ M. Therefore, the possible amount of produced pyruvate was calculated as $2.6 \cdot 10^{-4}$ M (ref. 1). When $5 \cdot 10^{-4}$ M pyruvate was added aerobically to the enzyme, no spectral change could be observed. The complex between the oxidized enzyme and imino acid was also considered[5]. According to the scheme of MASSEY AND GIBSON[5], in anaerobic condition there is no possibility of the existence

of oxidized enzyme–imino acid complex. As shown in Fig. 2 (C and D), the shoulder peak of absorption at 490 mμ could be still observed, after the oxygen in solution was exhausted as indicated by the measurement of oxygen consumption. Therefore, the possibility of the existence of the above complex seems to be negative. Thus the complex should be considered to be that of the enzyme and substrate.

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Betaine aldehyde dehydrogenase: assay and partial purification

Betaine aldehyde dehydrogenase (betaine-aldehyde:NAD oxidoreductase, EC 1.2.1.8) is the enzyme responsible for the oxidation of betaine aldehyde to betaine. The activity of this enzyme has been previously measured either manometrically[1] or spectrophotometrically[2]. The present communication describes a procedure for the partial purification and the necessary conditions for the fluorometric assay of the enzyme.

Assay. The buffer substrate was prepared just prior to each analysis and contained the following constituents at the indicated final concentrations: 100 mM Tris buffer (pH 8.1); 5 mM Cleland's reagent (Calbiochem.); 1 mM NAD^+; and 4 mM betaine aldehyde.

The betaine aldehyde was prepared from 2,2-diethoxy-ethyltrimethylammonium iodide (Aldrich, D8370) as described by ROTHSCHILD AND GUZMAN-BARRON[3]. The stock solution was approx. 400 mM and was stored at −20°.

1 μl of water or enzyme (equivalent to 0.4 μg protein) was added to a pointed tube of 2.5 mm internal diameter containing 10 μl of ice-cold incubation mixture. The