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Change in the charge on flavin-adenine dinucleotide by forming a model of the enzyme–substrate complex of D-amino acid oxidase

In previous papers[1,2], it was reported that a model of the enzyme substrate complex of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3), *viz.* the apo-enzyme FAD-benzoate complex, could be obtained in crystalline and form that the absorption spectrum of the crystalline product has a characteristic shoulder at 490 mμ. However, MASSEY *et al.*[3] reported that the holo-enzyme of the oxidase already has such a shoulder at 490 mμ. We have now re-examined in detail whether the shoulder at 490 mμ is characteristic for the ES model.

The apo-enzyme was prepared from hog kidney by the method reported previously[4] and dissolved in pyrophosphate buffer (0.0167 M, pH 8.3). The holo-enzyme was reconstructed from the apo-enzyme and FAD.

Since the shoulder at 490 mμ was observed only in the ES model, not in the holo-enzyme, the difference spectrum of the ES model relative to that of the holo-enzyme was recorded. This was found to be positive at the wavelengths longer than 458 mμ, with a peak at 497.5 mμ. In the presence of sufficient benzoate ($1 \cdot 10^{-4}$ M) and a limiting amount of FAD ($3.4 \cdot 10^{-5}$ M), the difference of the absorbancy at 497.5 mμ ($\Delta A_{497.5}$) was found to depend on the concentration of the apo-enzyme as shown in Fig. 1. Plots of $\Delta A_{497.5}$ against concentration of the apo-enzyme gave a sigmoid

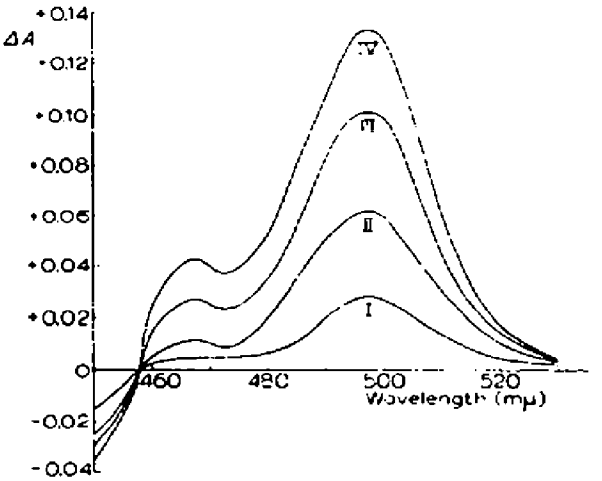

Fig. 1. Difference spectra of the ES model against the holo-enzyme in pyrophosphate buffer (0.0167 M, pH 8.3), 25°. The curves were obtained with solutions of the apo-enzyme, FAD ($3.4 \cdot 10^{-5}$ M) and benzoate ($1 \cdot 10^{-4}$ M) as test samples, solutions of the apo-enzyme and FAD ($3.4 \cdot 10^{-5}$ M) as references. Concentration of the apo-enzyme: I, $0.8 \cdot 10^{-5}$ M; II, $1.6 \cdot 10^{-5}$ M; III, $2.6 \cdot 10^{-5}$ M; IV, $5.2 \cdot 10^{-5}$ M.

curve. The same positive peak was also observed in the difference spectrum of the ES model relative to the holo-enzyme when a sufficient amount of benzoate, a limiting concentration of the apo-enzyme and varying concentration of FAD or a sufficient amount of the apo-enzyme, a limiting concentration of FAD, and varying concentration of benzoate were used. In both cases, $\Delta A_{497.5}$ depended on the concentration

of the varying component. Plots of $\Delta A_{497.5}$ against FAD concentration or against benzoate concentration also gave sigmoid curves.

The maximum height of the peak at 497.5 mμ, $(\Delta A_{497.5})_{max.}$, increased in direct proportion to the concentration of restricted component, *i.e.* to the concentration of the ES model formed in the mixture, as shown in Fig. 2.

As reported previously[4], the isoalloxazine moiety of FAD can combine with the apo-enzyme or benzoate with a shift of its spectrum to longer wavelength. In these cases, however, the shoulder at 490 mμ was not observed, indicating that the shoulder

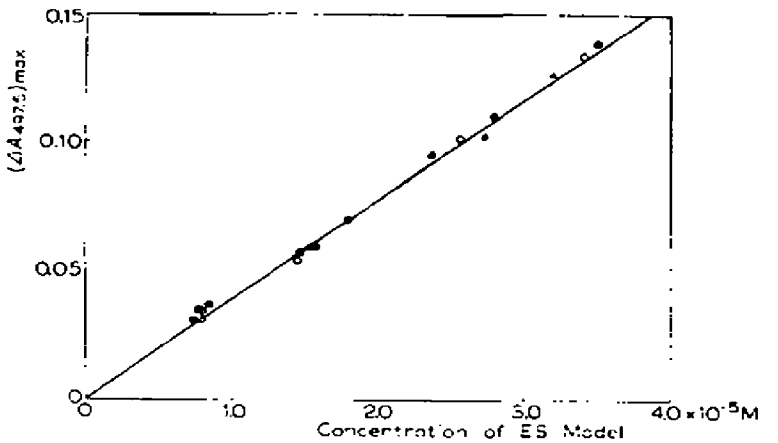

Fig. 2. $(\Delta A_{497.5})_{max.}$ plotted against concentration of the ES model. The ES model was constituted from a restricted concentration of the component indicated (● ●, the apo-enzyme; ○ ○, FAD; △ △, benzoate) and sufficient amounts of other two components ($1 \cdot 10^{-6}$ M).

is specific for the ES model. Moreover, the present experiment revealed that $\Delta A_{497.5}$ depends on the degree of the ES model formation. Considering that the appearance of $\Delta A_{497.5}$ is due to perturbations of the chromophore in the isoalloxazine moiety of FAD, it is concluded that upon ES model formation a change in the charge of some ionizable groups near the chromophore takes place. Although a definite conclusion on the the mode of the binding cannot be safely drawn, the results suggest the occurrence of hydrogen bonding or charge transfer between the isoalloxazine moiety of the holo-enzyme and the bound benzoate.

It is suggested that the shoulder at 490 mμ of the holo-enzyme reported by MASSEY *et al.*[3] is due to contamination of the holo-enzyme benzoate complex, since they used benzoate in the preparation of their enzyme .

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