INVESTIGATION OF π-BONDING IN ENZYMIC REACTIONS

I: ENZYME-SUBSTRATE COMPLEX BETWEEN CATECHOL AND TYROSINASE*

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The formation of an ES-complex as the initial step in the interaction between an enzyme and a substrate is generally accepted and has been demonstrated indirectly by spectral means.(1) The current notion underlying the linkage between a metalloenzyme and its substrate is one of a coordination bond between the metal ion and the functional group of the substrate. Recently, however, it has been found that the π-system in double or triple bonds or aromatic rings overlaps with the 3d orbital of transition metal elements to form π-complexes, even in water media.(2) Therefore the possibility of π-bond formation between a metalloenzyme and an aromatic, olefinic or acetylenic substrate must be considered.

The present investigation was initiated to establish a method for observing the formation of an air-sensitive ES-complex using ultraviolet spectroscopy. The system of tyrosinase(3), cupric chloride and catechol was chosen for study. Although it is known that copper exists as cuprous ion in tyrosinase(4), tyrosinase can also be activated by cupric ion.(5) The normal rapid reaction of an oxidase, such as tyrosinase, can be easily arrested by using an oxygen-free system thereby eliminating the use of an inhibitor, which would complicate the ultraviolet spectrum.

Ultraviolet spectroscopic measurements(6) were made from 220 mμ - 900 mμ. The following wavelengths proved to be especially important: 220 mμ - 320 mμ, 370 mμ - 400 mμ and 450 mμ - 860 mμ. Absorption at these wavelengths

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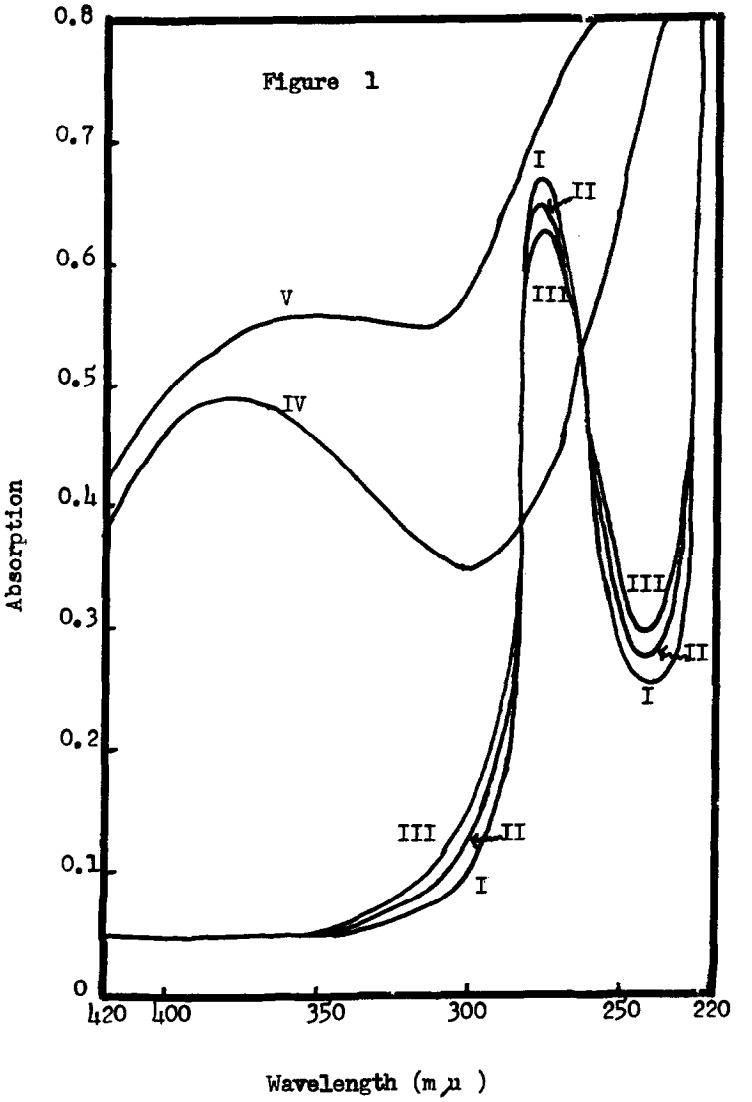
is attributed to catechol, the normal reaction products of catechol, mainly o-quinone which is formed in the early stages of the reaction, and cupric ion, respectively.

A solution of tyrosinase and cupric chloride in a quartz cell designed with a long neck, and a solution of catechol in a small Schlenk tube were flushed with pure nitrogen to expel the oxygen dissolved in the solutions. After bubbling for 30 minutes, the solution of catechol was added to the quartz cell, which was sealed quickly under nitrogen. All UV measurements were performed at room temperature. A buffer, which might complicate the ultraviolet spectrum, was not used in these experiments since the pH remained at 6.7 throughout the course of the reaction. The results obtained in the first two wavelength regions described above are shown in Fig. 1.

The normal reaction product in air of the three components revealed a peak at 390 mμ (IV). In these experiments, however, no peak was observed in this region (I, II, III) even if eight times the concentration of tyrosinase was used to accelerate the reaction as long as oxygen was excluded. In addition, this oxidation reaction was so fast in air that a very small amount of oxygen, even if it remained in the sealed cell, should be consumed at an early stage of the reaction.

The catechol peak which appeared at 276 mμ gradually decreased with time, while the intensity of absorption on both sides of this peak increased (I, II, III). After the measurement under nitrogen was completed, aeration of the sample revealed the same absorption curve (V) as was determined in air, indicating that the enzyme is still active. On the other hand, in the two-component systems of tyrosinase and cupric chloride or catechol and cupric chloride, no change was observed with time. These results rigidly establish that the change in the ultraviolet spectrum of the three-components system is not due to the formation of a secondary two component complex formation between the substrate or the enzyme and cupric chloride.

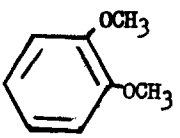


Ultraviolet absorption of three-component System: Tyrosinase, Cupric Chloride and Catechol.

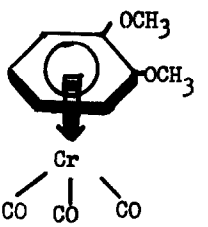
Concentration (in 3.25 ml. of solution):
Catechol, 1.5×10^{-4} mmole; $CuCl_2$ 0.5×10^{-3} mmole;
tyrosinase, 0.25 mg.
Curve: I, Start under nitrogen; II, After 1 hour (N_2);
III, After 2 hours (N_2); IV, Aeration; V, 1-5 hours after aeration.

On the basis of these results, it appears that an ES-complex is formed by tyrosinase, copper and catechol and as a consequence, the point of maximum absorption shifts from that of catechol to another wavelength.

Since free catechol exists in excess in the reaction mixture, the change in the ultraviolet absorption curves from I to III via II, as shown in Figure I, is most likely due to an increase in the total amount of the ES-complex. Our hypothesis that the binding in this case between E and S may involve a new chemical bond, a π-bond, has led us to investigate the ultraviolet absorption spectra of an aromatic compound, veratrole (A) and one of its π-complexes, veratrole chromium tricarbonyl (B) as supporting evidence for the formation of an ES-π-complex. Although this system employs chromium rather than copper as in the case of tyrosinase a change in the ultraviolet absorption curve due to an increase in the amount of aromatic π-complex present might be expected to be similar to the change observed in the tyrosinase system.



(A)

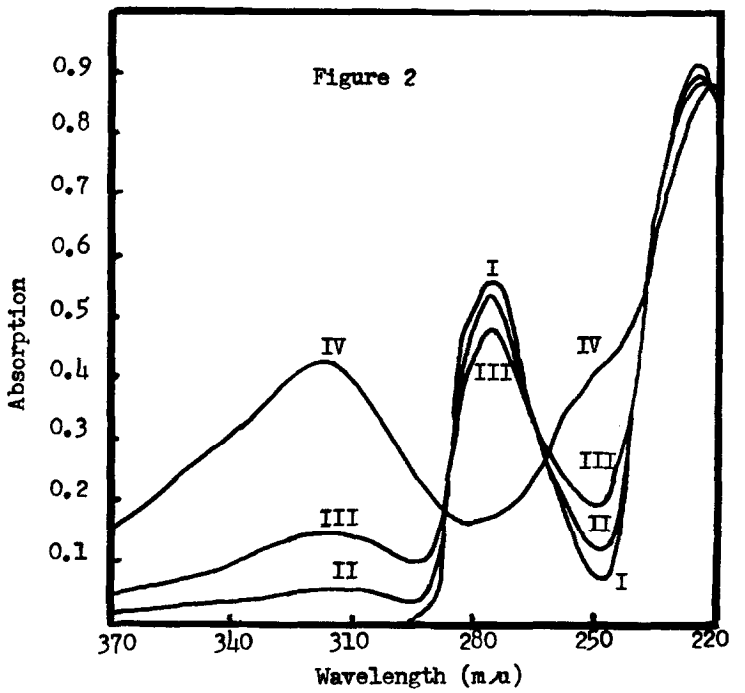


(B)

As shown in Figure 2, the maximum absorption of veratrole chromium tricarbonyl is at 317 mμ, while that of veratrole is at 276 mμ. The absorption of mixtures of the two with increasing amounts of the π-complex reaction to veratrole reveals a decrease of intensity at 276 mμ due to veratrole and an increase of intensity on both sides of this peak (II, III). This change in ultraviolet absorption is quite similar to that in the solution of tyrosinase, cupric chloride and catechol previously described.

The results obtained with veratrole and its π-complex, veratrole chromium tricarbonyl, support the hypothesis that the change of the curve in the tyrosinase system with time (curves I, II, III in Fig. 1) may be due to an increase in the amount of an ES-π-complex.

The change in ultraviolet spectrum of the three-component tyrosinase system in the region between 450 mμ and 850 mμ is shown in Figure 3.



Ultraviolet Absorption of Veratrole and Veratrole Chromium Tricarbonyl (VCT)
Concentration: Veratrole, 1.42×10^{-4} M/L; VCT, 0.51×10^{-4} M/L
Solvent: Methanol
Curve: I, Veratrole; II, Mixture of Veratrole (90) and VCT (10); III, Mixture of Veratrole (70) and VCT (30); IV, VCT

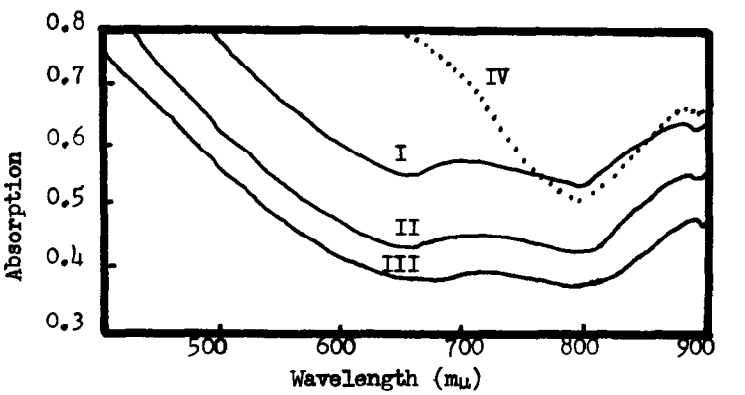


Figure 3

Ultraviolet absorption of three component System

Concentration (in 3 ml. of sample); Catechol, 0.1 mmole; $CuCl_2$ 0.03 mmole; tyrosinase 2 mg.
Curve: I, after 25 minutes under nitrogen; II, after 1 hr. (N_2); III, after 2 hrs. (N_2); IV aeration (diluted 1:50)

In the experiments in this region, a much higher concentration of the three components was used than that employed in the region between 220 mμ and 520 mμ shown in Fig. 1. At the higher wavelengths catechol has no absorption and tyrosinase shows less absorption especially around 700 mμ where the absorption of cupric ion is observed.

The intensity of absorption of the three-component tyrosinase system decreased with time under nitrogen (I, II, III) in the region 450 - 860 mμ. However, no change with time was observed under nitrogen in the two-component systems of tyrosinase and cupric chloride or catechol and cupric chloride. Aeration of the above three-component system which had been observed under nitrogen immediately led to increased absorption which was beyond the absorption scale used. Measurement of absorption was therefore made in a 1:50 dilution of the original solution (IV). If the change of ultraviolet spectrum (I, II, III) was due to oxygen, then the intensity of absorption should be increased as is shown from the result of aeration. Therefore, the decrease of absorption under nitrogen may have been due mainly to the decrease of cupric ion concentration in formation of the ES-complex. Comparable experiments have been conducted on the two component systems of tyrosinase and catechol under a nitrogen atmosphere. Results similar to those from the three component system shown in Figures 1 and 3 were obtained although changes in the amount of absorption were very slight. These changes were presumably due to the small amount of copper inherent in the enzyme. The slowness of the changes in absorption spectra in the experiments shown in Figure 1 and 3 may be due to a) time necessary for stable π-bond formation or b) increase in amount of E-S π-complex. π-Bond formation between E and S may play an essential role in the fast catalytic oxidation of catechol by tyrosinase. It has been demonstrated previously by a number of workers that catalysis of the reactions unsaturated compounds by transition elements is generally activated by the initial hybridization of π-electron and the 3d orbital of metal atom. If this hybridization reaches to the ground state and forms a stable π-complex,

catalysis is considerably slowed down[2c]. The details of the π-complex mechanism of tyrosinase oxidation will be discussed elsewhere.

In conclusion, the normal reaction of an oxidase such as tyrosinase can be arrested in an oxygen-free atmosphere and the bonding behavior between the enzyme and substrate can be observed by ultraviolet spectroscopy. The change of the absorption with time resembles that obtained with an established π-bonding system. This suggests that the ES-complex formed between tyrosinase, cupric chloride and catechol in an atmosphere of nitrogen, likewise involves π-bonding.

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(6) 220-520 mμ: Bausch and Lomb, 505 Spectrophotometer.
450-900 mμ: Beckman DK-2 Spectrophotometer.