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The Enzyme-like Reaction Catalyzed by Flavin-Reduced Keratin Systems

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The reduced keratin (RK), which was obtained by the reductive cleavage of human hair, was found to act as an apoenzyme and to furnish the reaction field for flavin adenine dinucleotide (FAD): the absorbance of the succinic acid–FAD–RK system in solution increased in the region of 200—250 nm with the lapse of time. From this system fumaric acid was isolated. FAD was found to form a complex with RK and the FAD–RK complex exhibited a catalytic activity for the dehydrogenation reaction of succinic acid to fumaric acid. FAD was reduced in this reaction and the reduced FAD was oxidized by oxygen. It was found that the process of the dehydrogenation reaction obeyed the Michaelis-Menten kinetics; the equilibrium constants of the complex formation between FAD and RK were determined by measuring the initial velocities of the dehydrogenation reaction. The binding site of FAD and RK in the complex was discussed.

It has been known that a coenzyme locates in the active center of an enzyme and exhibits a catalytic activity which can not be realized without an apoenzyme.[1,2] As a plausible speculation, we would assume that an apoenzyme might furnish a coenzyme with a reaction field, in which the coenzyme could exhibit the catalytic activity. If this assumption is true, we can expect that a catalytic reaction may occur in a solution containing the coenzyme combined with a compound which could produce the reaction field, like apoenzyme.

Keratin is known to be one of the albuminoids and contains 10—15% cystine residues, which can be reduced to form thiol groups.[3] The physico-chemical properties of RK obtained by the reductive cleavage of human hair have already been reported.[4] It was expected in the viewpoint of practical use that RK might be utilized as a catalyst. Alcohol dehydrogenase and succinate dehydrogenase are known to be thiol enzymes.[3] The former contains nicotinamide adenine dinucleotide (NAD) and the latter FAD as the coenzyme, respectively. Supposing a thiol group can stimulate NAD and FAD to exhibit the catalytic activity, RK may furnish the reaction field for NAD and FAD.

Aiming at the preparation of an artificial enzyme and the practical use of keratin in our study, preliminary experiments were made for an ethanol–NAD–RK system and a succinic acid–FAD–RK system. No reaction was found to occur in the former case. On the other hand it was found that the dehydrogenation of succinic acid to fumaric acid occurred in the latter case as follows:

$$\mathrm{FAD + RK \rightleftharpoons FAD{\cdot}RK} \qquad (1)$$

$$\mathrm{FAD{\cdot}RK + Succinic\ acid \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FAD{\cdot}RK{\cdot}Succinic\ acid\ complex \xrightarrow{k_2} FADH_2RK + Fumaric\ acid} \qquad (2)$$

$$\mathrm{FADH_2RK + 1/2O_2 \longrightarrow FAD{\cdot}RK + H_2O} \qquad (3)$$

The dehydrogenation of succinic acid catalyzed by a FAD and FAD-protein system has not been known so far. Takemoto *et al.* have reported the redox reaction of isoalloxazine residues which are introduced in the vinyl polymer, but they did not discuss the reaction field in the polymer.[5]

This paper will present the results of spectrophotometric studies for the verification of Eqs. 1—3 and the isolation of the reaction product.

Calculation

The association constant, K, in Eq. 1 is expressed as

$$K=\frac{[\mathrm{FAD{\cdot}RK}]}{[\mathrm{FAD}][\mathrm{RK}]} \qquad (4)$$

where [FAD], [RK], and [FAD·RK] are the concentrations of free FAD, free RK, and the FAD–RK complex. The value of K can be determined by Eq. 10, which is obtained by the following derivation. If the reaction in Eq. 2 obeys the Michaelis-Menten kinetics and makes the rate-determining step, then the initial velocity, v_0, of the succinate dehydrogenation could be expressed as:

$$v_0=k_2[\mathrm{FAD{\cdot}RK{\cdot}S}] \qquad (5)$$

where [FAD·RK·S] is the concentration of the three component complex of FAD, RK, succinic acid.

When the condition of the reactions of Eqs. 2 and 3 reach the steady state, the following equation is given:

$$\frac{\mathrm{d}[\mathrm{FAD{\cdot}RK{\cdot}S}]}{\mathrm{d}t}=k_1[\mathrm{FAD{\cdot}RK}][\mathrm{S}]-(k_{-1}+k_2)[\mathrm{FAD{\cdot}RK{\cdot}S}]=0 \qquad (6)$$

The Michaelis constant, K_m, is expressed as:

$$K_\mathrm{m}=\frac{k_{-1}+k_2}{k_1} \qquad (7)$$

Therefore from Eqs. 6 and 7 we obtain,

$$K_\mathrm{m}=\frac{[\mathrm{FAD{\cdot}RK}][\mathrm{S}]}{[\mathrm{FAD{\cdot}RK{\cdot}S}]} \qquad (8)$$

where [S] is the concentration of succinic acid. Let F, R, and S be the concentration of total FAD, total RK, and total succinic acid. And let R and S be greater then F, and S be constant; then

$$\left.\begin{aligned}&\mathrm{F}=[\mathrm{FAD}]+[\mathrm{FAD{\cdot}RK}]+[\mathrm{FAD{\cdot}RK{\cdot}S}]\\&\qquad\ll \mathrm{R}\fallingdotseq[\mathrm{RK}]\\&\mathrm{F}\ll \mathrm{S}\fallingdotseq[\mathrm{S}]\end{aligned}\right\} \qquad (9)$$

From Eqs. 4, 5, 8, and 9, the following equation is obtained:

$$\frac{1}{v_0}=\left(\frac{K_\mathrm{m}}{k_2\mathrm{FS}K}\right)\left(\frac{1}{\mathrm{R}}\right)+\frac{1}{k_2\mathrm{F}}\left(1+\frac{K_\mathrm{m}}{\mathrm{S}}\right) \qquad (10)$$

From Eq. 10, a plot of $1/v_0$ *vs.* $1/\mathrm{R}$ yields a straight line, from which the value of K can be determined.

Experimental

Materials. The RK was prepared in the same way as was described in the previous paper.[4] The molecular weight of RK was 4500 (by viscometry). Thiol group: 0.21 mg-eq/g and S content: 2.7%. All the other chemicals were of reagent grade or the best commercially available.

The Measurements of Initial Velocities. Stock solutions of 2.5×10^{-5} M FAD, 2.5×10^{-4} M RK, and various concentrations of succinic acid in 1/30 M phosphate buffer were prepared. The requisite amounts of FAD and RK stock solutions were poured into 10-ml volumetric flasks and enough 1/30 M phosphate buffer were added to reach the 10-ml mark. The sample solution was prepared by pipetting 4 ml of FAD-RK solution out of the volumetric flask and adding it into 1 ml of succinic acid solution. The reference solution contains the same concentration of FAD and RK as the sample solution, except that succinic acid is omitted. The initial velocities were determined from the slopes of absorbance-time curves which were obtained by measuring the absorbance change at 225 nm ($\varepsilon=5.7\times10^3$) and 240 nm ($\varepsilon=2.1\times10^3$) for fumaric acid with the lapse of time. Measurements of absorption spectra were made with a Shimadzu spectrophotometer UV-200 at controlled temperatures.

Results and Discussion

Absorbance-Time Relation for the Flavin–RK–Succinic Acid Systems. Various flavins and/or RK were dissolved into 1/30 M phosphate buffer (pH 7.5) and then succinic acid was added. With these solutions differential absorption spectra were measured. The absorbance of each solution was found to increase in the region of 200—250 nm and the absorption maximum at 206 nm appeared in the case of the FAD-solution (inserted figure in Fig. 1). The results for the absorbance (at 225 nm) *vs.* time relation are shown in Fig. 1. The initial velocity was calculated from the slope of each line at zero time in Fig. 1. No reaction occurred without flavin. The initial velocities in the solution containing riboflavin (RF), flavin mononucleotide (FMN), and FAD were obtained to be 4×10^{-8}, 2×10^{-8}, and 5×10^{-7} M/min, respectively. It could be thought that the adenine moiety of FAD might combine with RK to form a complex. A little activity (2×10^{-8} M/min) was observed in the case of FAD-succinic acid solution without RK. From this fact, it could be speculated that RK might furnish FAD with the reaction field, as was expected. Paper chromatogram (1-butanol : formic acid : water=10 : 2 : 15)[6] of the succinic acid–FAD–RK system showed one spot ($R_f=0.73$), assigned to be succinic acid, before reaction. Another spot ($R_f=0.84$), assigned to be fumaric acid, appeared with the lapse of time. Maleic acid ($R_f=0.46$) was not found. From the results of absorption spectra and paper chromatography it was suggested that succinic acid was dehydrogenated to form fumaric acid in this reaction.

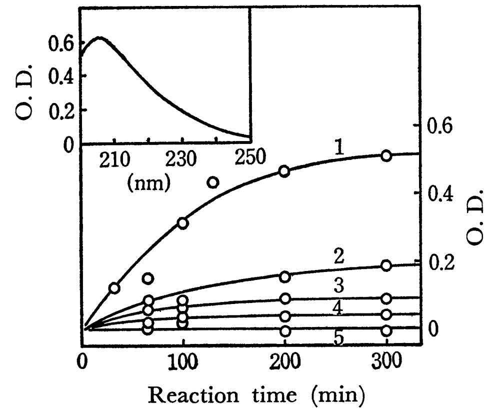

Fig. 1. The absorbance change during reaction.
1/30 M Phosphate buffer (pH 7.5), 35 °C. FAD, FMN, RF: 1.0×10^{-5} M, RK: 1.3×10^{-4} M, Succinic acid (S): 1.0×10^{-4} M. 1: S-FAD-RK, 2: S-RF-RK, 3: S-FMN-RK, 4: S-FAD, 5. S-RK.

The absorbance (at 450 nm) of the succinic acid–FAD–RK system, through which nitrogen gas was passed, was found to decrease with the lapse of time and increase again by the aeration. It can be thought that FAD may be reduced to form reduced FAD ($FADH_2$) during the reaction and $FADH_2$ may be reoxidized by oxygen. These results suggested that Eq. 2 might be the rate-determining step in the reaction of Eqs. 2 and 3.

Isolation of Fumaric Acid. Fumaric acid was isolated from the solution of the succinic acid–FAD–RK system as follows: Into distilled water, 5 mmol (590 mg) succinic acid, 1 mmol FAD, and 1.3 mmol RK were dissolved, and sodium hydroxide solution was added to adjust to pH 7.0. Finally, the total volume of the mixture was set to 1000 ml by adding distilled water. The solution was allowed to stand at 25 °C with occasional aeration. The reaction was broken off after 4 days because of the coagulation of RK. The conversion of succinic acid to fumaric acid was estimated to be nearly 20% by the determination of absorption spectra. The solution of the succinic acid–FAD–RK system was concentrated by evaporation and adjusted to pH below 1 by adding hydrochloric acid. Precipitation occurred. Precipitates were separated by centrifugation and then suspended in methanol. The methanol solution separated from insoluble matter was evaporated. The extraction with ethyl ether was carried out and ether was removed from the extract. A white crystal from the residual matter was obtained by recrystallization from water. Yield: 23 mg. The yield can be raised if the separation processes are repeated. The elemental analysis: Found: C, 41.0; H, 3.8%. Calcd for fumaric acid ($C_4H_4O_4$), C, 41.4; H, 3.5%. NMR: 6.84 ppm, singlet in CD_3OD (succinic acid: 2.57, maleic acid; 6.40 ppm).

It was found that a higher yield of fumaric acid could not be obtained because of the activity change of the FAD–RK system during the reaction or the preservation in solution. A stock solution of 1/30 M phosphate buffer (pH 7.5) containing 1.1×10^{-5} M FAD and 1.4×10^{-4} M RK was prepared and divided into two parts. One was bubbled by air, and nitrogen gas was passed through the other. Both were preserved at 25 °C. After the requisite time each was mixed with 1/30 M phosphate buffer (pH 7.5) containing succinic acid and the mixture was aerated. After

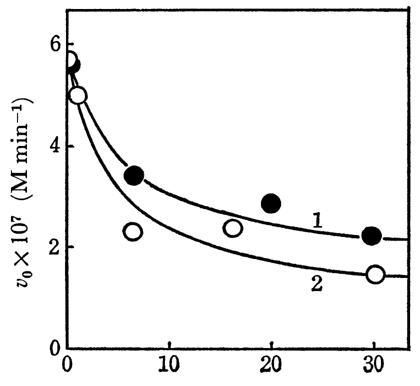

Fig. 2. Activity changes of FAD-RK systems during preservation. 1: Nitrogen gas passed. 2: Aerated.

that the initial velocity of the reaction was calculated by measuring the absorbance change at 225 nm. The mixture contained 1.0×10^{-5} M FAD, 1.3×10^{-4} M RK, and 1.0×10^{-4} M succinic acid. The activities were found to decrease during the preservation, as showin in Fig. 2. The activity change in the solution containing oxygen was larger. Oxidation of RK by oxygen might be the cause of the activity change.

pH Profile. The initial velocities at different pH were determined at 35 °C to obtain the optimum pH for the dehydrogenation of succinic acid. The sample solutions were composed of 1.0×10^{-5} M FAD, 1.0×10^{-4} M RK, and 2.0×10^{-4} M succinic acid in 1/30 M phosphate buffer (pH 6.6—8.4). The optimum pH was found to be in the region of pH 7.5—7.7 (Fig. 3).

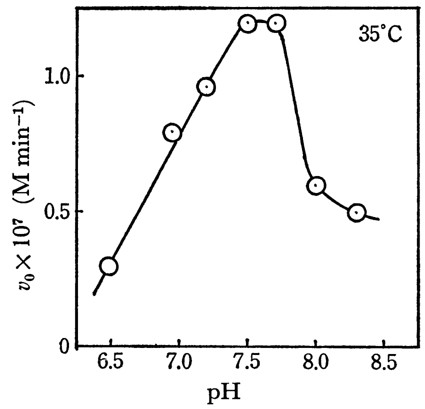

Fig. 3. pH profile.

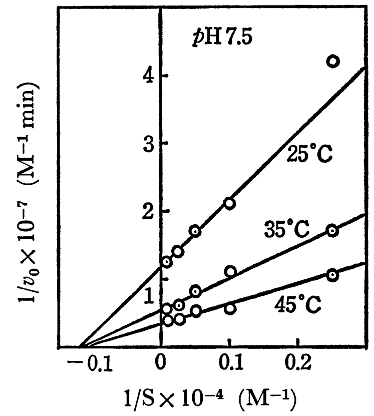

Fig. 4. Lineweaver-Burk plots.

Michaelis-Menten Kinetics. The final concentrations of FAD, RK, and succinic acid in 1/30 M phosphate buffer (pH 7.5) were 1.0×10^{-5}, 1.0×10^{-4}, and 0—10×10^{-4} M, respectively. The initial velocities were measured at different temperatures. The Lineweaver-Burk plots fell on straight lines (Fig. 4), which means that the reaction obeys Michaelis-Menten kinetics. From the straight lines the values of K_m and V_{max} were calculated. The results are summarized in Table 1. The reaction velocity at 60 °C decreased lower than that at 45 °C.

TABLE 1 MAXIMUM VELOCITIES, MICHAELIS CONSTANTS, AND EQUILIBRIUM CONSTANTS

	25 °C	35 °C	45 °C
V_{max} (M min^{-1})	8.7×10^{-8}	2.0×10^{-7}	3.3×10^{-7}
K_m (M)	8.7×10^{-5}	9.4×10^{-5}	1.0×10^{-4}
K (M^{-1})	3.8×10^3	3.7×10^3	3.5×10^3

The K_m values of the succinic acid–FAD–RK complex are about half as big as that of the succinate dehydrogenase.[7)]

Equilibrium Constant of FAD–RK Complex. The final concentrations of FAD, succinic acid, and RK in 1/30 M phosphate buffer (pH 7.5) were 1.0×10^{-5}, 2.0×10^{-4}, and 0—2.0×10^{-4} M, respectively. Plots of $1/v_0$ *vs.* 1/R gave straight lines, as shown in Fig. 5, from which the assumptions for the derivation of Eq. 10 seemed to be verified. The values of K were calculated (Table 1) by using Eq. 10 with the K_m values and slopes and intercepts obtained from the straight lines. In the solution containing 1.0×10^{-5} M FAD, 1.0×10^{-4} M RK, and 1.0×10^{-3} M succinic acid, 80% (25 °C), 79% (35 °C), and 77% (45 °C) of FAD forms the succinic acid–FAD–RK complex, whose concentration can be calculated from K_m and K values. In turn, the values of k_2 can be obtained to be 1.1×10^{-2} (25 °C), 2.6×10^{-2} (35 °C), and 4.4×10^{-2} (45 °C) min^{-1} from Eq. 5 with the values of V_{max}. These values are 10^5 time smaller than that of succinate dehydrogenase.[7)] The activation energy was determined to be 13 kcal/mol from an Arrhenius plot, using the values of k_2 (Fig. 6). The values of enthalpy change and entropy change of the formation

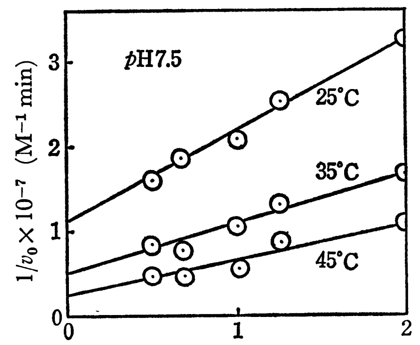

Fig. 5. Plots of $1/v_0$ *vs.* 1/RK.

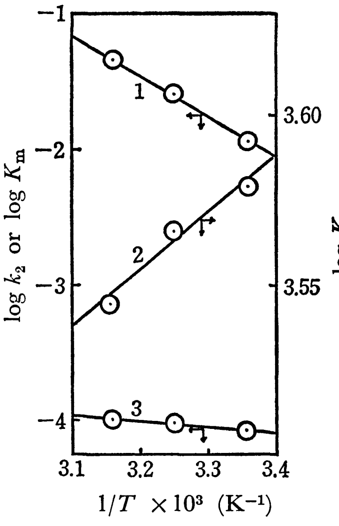

Fig. 6. van't Hoff's plots and Arrhenius plot.
1: $\log k_2$-$1/T$ plot, 2: $\log K$-$1/T$ plot, 3: $\log K_m$-$1/T$ plot.

Table 2. The thermodynamic parameters

	ΔH kcal/mol	ΔS e. u.
FAD–RK complex formation	−0.8	+14
Succinic acid–FAD–RK complex formation	−1.3	+15

of the FAD–RK complex and the succinic acid–FAD–RK complex calculated by the van't Hoff's plots (Fig. 6) are summarized in Table 2.

It is known that the overall entropy changes for the formation of an ES complex in many enzymatic reactions give positive values caused by the elimination process of water molecules. It is plausible that a similar process may occur in the formation of the FAD-RK complex and the succinic acid–FAD–RK complex.

The Amino Acid Residues' Part in Catalytic Activity. Various chemical modification reagents were added to the RK solution in order to modify the specific functional groups in RK, and the amino acid residues which play a part in the catalytic activity were assigned from the changes of the reaction velocity caused by the modification of the amino acid residues.

p-Chloromercuribenzoic acid, *N*-acetylimidazole, and glyoxal were used for the chemical modification reagents of cysteine, tyrosine, and arginine residues, respectively.[8–10] The results are summarized in Fig. 7.

The modification of thiol groups made the FAD–RK system inactive. It is very possible that the thiol group participates in the catalysis. However a question still remains: A bulky group of *p*-mercuribenzoate may hinder sterically the complex formation for the dehydrogenation. The initial velocity in the FAD solution containing RK modified by glyoxal is comparable to that in the FMN–RK system. It seems that the adenine moiety of FAD may not interact with modified RK. The arginine residue in RK may made a binding site for the adenine moiety of FAD. Tyrosine residues may not play any part in the catalytic activity.

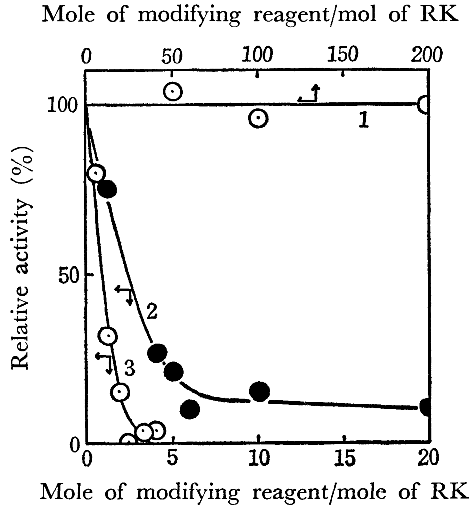

Fig. 7. Effect of the modifying reagents to the catalytic activity of FAD–RK complex.
1: *N*-Acetylimidazole, 2: Glyoxal, 3: *p*-Chloromercuribenzoate.

As a conclusion we suggest that the FAD–RK complex can be formed by the interaction between an arginine residue of RK and an adenine moiety and pyrophosphate linkage of FAD, and that the catalytic activity can be exhibited by the interaction between an isoalloxazine moiety of FAD and a thiol group of RK.

The FAD–RK complex cannot be a model of succinate dehydrogenase, because succinate dehydrogenase is an iron-sulfur flavoprotein and the product of reduced flavin and oxygen is hydrogen peroxide, not water. However, it is significant that the FAD–RK complex exhibits the catalytic activity of an artificial enzyme.

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