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Enzyme-like Reaction Catalyzed by Flavin-Reduced Keratin Systems. II. Binding Site in FAD-Reduced Keratin Complex[1)]

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Synopsis. In order to confirm the binding site in the FAD-reduced keratin (RK) complex, polarographic and fluorometric studies were carried out and the FAD–RK complex was isolated. An adenine moiety of FAD was found to form a 1 : 1 complex with RK.

It has been reported in a previous paper that complex formation between FAD and reduced keratin (RK) occurs and that the complex catalyzes the dehydrogenation of succinic acid to fumaric acid.[1)] The binding site of FAD and RK in the complex was discussed and an arginine residue of RK and an adenine moiety and a pyrophosphate linkage of FAD were suggested as probable paths of ionic bond formation. This paper presents the results obtained by polarographic and fluorometric studies and by the isolation of the FAD–RK complex which throw light on the binding site in the FAD–RK complex.

Experimental

The RK used was prepared by the reductive cleavage of human hair with 2-mercaptoethanol and urea in a sodium hydroxide solution in accordance with the usual method.[2)] Molecular weight; 2800 (by viscometry), S-content; 2.7% as thiol groups; 0.36 mgeq/g (PCMB method[3)]) (1.0 thiol group/molecule of RK). The other chemicals used were the same as described in a previous paper.[1)]

Measurements of absorption and fluorescence spectra were made with a Shimadzu spectrophotometer UV-200 and a JASCO fluorescence spectrophotometer FP-4, respectively. The polarograms were determined with a dropping mercury electrode.

Results and Discussion

Polarography. From the dissimilar behavior between FAD and FMN with RK, it was expected that FAD and RK in solution would give different polarograms from FMN and RK in solution.

The sample solutions contained 2.5 mM of FAD or FMN and the requisite amount of RK in a 0.1 M phosphate buffer (pH 5.6 and 7.0). The results are shown in Figs. 1 and 2. The half-wave potentials of FAD without RK in solution at pH 5.6 and 7.0 were observed at −0.38 and −0.44 V *vs.* SCE at 25 °C. The same values were obtained when FMN containing solutions were measured. The wave height of the half-wave potential decreased when RK was introduced in the FAD or FMN solution. Another half-wave potential was found at −0.69 V *vs.* SCE on the polarograms of the FAD–RK system at pH 5.6 The wave height at the second half-wave potential increased with increasing RK concentration. The total of the two wave heights

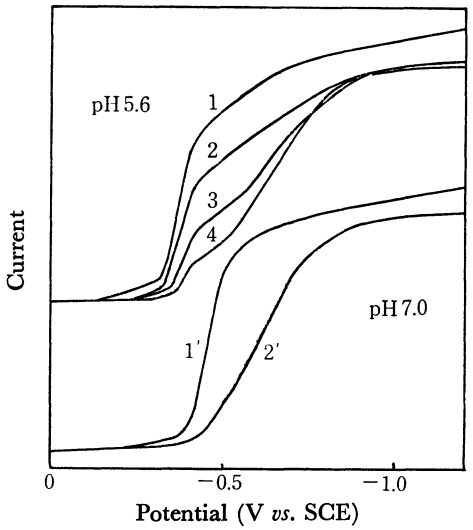

Fig. 1. FAD–RK polarography.
FAD 2.5 mmol/l
RK 1,1′: 0, 2: 0.07, 3: 0.14, 4,2′: 0.71 mmol/l

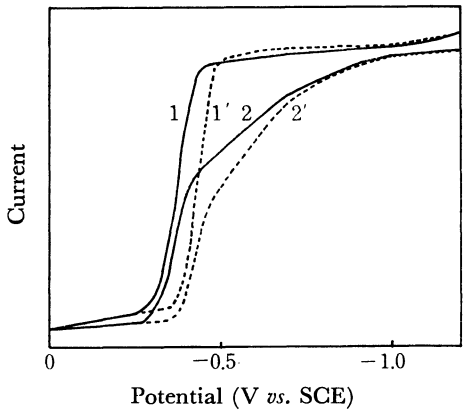

Fig. 2. FMN–RK polarography.
— pH 5.6, ---- pH 7.0
FMN 2.5 mmol/l
RK 1,1′: 0, 2,2′: 0.71 mmol/l

at the first and second half-wave potentials was found to remain constant.

It is known that sulfur- or nitrogen-containing compounds are readily absorbed on the surface of dropping mercury.[4)] Therefore, RK can very possibly be absorbed by mercury. The absorbed RK may interact with free FAD on the mercury surface and the reduction of FAD may be hindered so that the wave height at the first half-wave potential is decreased. Less influence on the wave height was observed for a solution containing FMN, for which complex formation with RK

was weaker than that for FAD. The second half-wave potential was assigned to the reduction of the FAD bound to RK. It is probably not possible that the reduction of free FAD is facilitated by the desorption of RK from the mercury surface at more negative potentials, because the second half-wave potential appeared at the same point, although the RK concentration had increased.

In the presence of an excess of RK, only the 1 : 1 complex is formed with RK. Then the equilibrium constant can be determined from the wave heights of the first and second half-wave potentials. The equilibrium constant was calculated to be about 2800 M^{-1} at pH 5.6 from Table 1.

Table 1. Equilibrium constant of the FAD–RK complex

FAD (mM)	RK (mM)	Wave height $(E_{1/2})_1$	Wave height $(E_{1/2})_2$	Equilibrium constant (M^{-1})
0.5	1.8	0.6	2.4	2.9×10^3
1.0	1.8	1.3	4.8	2.6×10^3
1.5	1.8	1.9	6.9	3.0×10^3

pH 5.6, 25 °C

Fluorescence Spectra. It was confirmed from the fluorescence spectra that the behavior of the interaction of FAD with RK was different from that of FMN with RK. The fluorescence intensity of FAD with RK (pH 7.0) at 522 nm (excited by 468 nm light) was found to be 7% higher than that of FAD without RK (Fig. 3). The fluorescence intensity of FMN was about 4 times stronger than that of FAD. It is known that the adenine moiety of FAD quenches the fluorescence caused by the intramolecular interaction.[5] It can be explained that quenching by RK may result in a decrease in the fluorescence intensity in the FMN–RK solution. In the FAD–RK case it is speculated that the interaction between the adenine moiety and isoalloxazine moiety may weaken when the adenine moiety bonds to RK, *i.e.*, the reduced influence of the adenine moiety on the isoalloxazine moiety decreases the quenching.

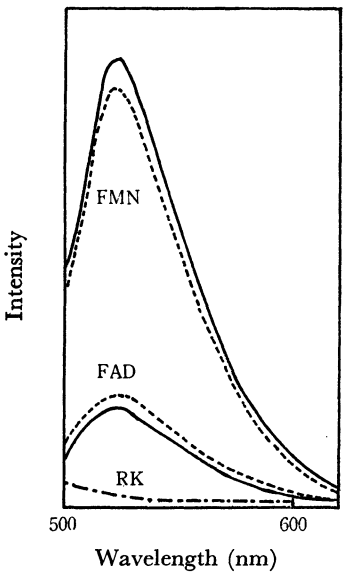

Fig. 3. Emission spectra of flavin–RK systems.
—— Flavin only, ------ Flavin–RK
Flavin 6 μmol/l, RK 3 μmol/l
λ_{EX}=468 nm, pH 7.0

Isolation of the FAD–RK Complex. Attempting further confirmation of complex formation in solution, the FAD–RK complex was isolated. Dissolving 0.071 mmol of RK and 0.11—0.42 mmol of FAD or FMN into distilled water and adding 1 M hydrochloric acid to adjust to pH 4, precipitation occurred. The precipitates were separated by centrifugation, washed by distilled water and dried. The determination of RK and flavin in the complex was carried out by analysis of the sulfur content and measurement of the absorption at 450 nm. It is seen from the results in Table 2 that a 1 : 1 complex was obtained from the FAD–RK solution. The FMN content in the precipitates obtained from the FMN–RK solution was found to be variable. These results confirm that the adenine moiety of FAD is a bonding site for RK.

Table 2. Analysis of flavin–RK complexes

	Flavin (mmol)	Flavin RK	Yield (%)	RK (mmol/g)	Flavin (mmol/g)
FAD	0.11	1.5	49	0.26	0.22
	0.42	5.9	50	0.30	0.26
FMN	0.11	1.5	37	0.33	0.09
	0.42	5.9	39	0.32	0.19

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