Enzyme-like Reaction Catalyzed by NAD$^+$–Reduced Keratin Systems

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Reduced keratin, which was obtained by reductive cleavage of human hair, was found to form a 1 : 1 complex with nicotinamide adenine dinucleotide (NAD$^+$). The complex exhibited catalytic activity for the oxidation of glyceraldehyde to form glyceric acid. It was found from the results of pH titration, polarograms and NMR measurements that the NAD$^+$-reduced keratin complex binds to glyceraldehyde to form an intermediate, which is susceptible to oxidation by oxygen. It was concluded from kinetic studies that this catalytic reaction showed a homotropic effect.

In a previous paper, it was reported that reduced keratin (RK) was found to form a 1 : 1 complex with flavin adenine dinucleotide (FAD) and that the complex exhibits catalytic activity for dehydrogenation of succinic acid to form fumaric acid.[1] It was suggested that the FAD–RK complex is formed by the interaction between arginine residues of RK and an adenine moiety and the pyrophosphate linkage of FAD. From this conclusion, it is expected that NAD$^+$, which has a nicotinamide moiety in place of an isoalloxazine moiety of FAD, might form a complex with the RK and act as an enzyme-like catalyst.

It is well known that there are many dehydrogenases in which NAD$^+$ and mercapto group are located in the active center of an enzyme, such as alcohol dehydrogenase, aldehyde dehydrogenase, *etc.*

Since it is expected that the NAD$^+$–RK system possesses catalytic activity like that of an enzyme, various substrates such as ethanol, monosaccharide (glucose, mannose, xylose), methyl mandelate, glyceraldehyde 3-phosphate and glyceraldehyde were examined. The formation of NADH (the reduced form of NAD$^+$) was observed spectrophotometrically when glyceraldehyde was added as a substrate into a solution containing NAD$^+$ and RK. The results of kinetic studies of the catalytic reaction between glyceraldehyde and the NAD$^+$–RK complex will be presented in this paper.

Experimental

Materials. The RK used was prepared by the procedure described in a previous paper.[2] The molecular weight of the RK was 4500 (measured by viscometry). Mercapto group: 0.2 mgeq/g of protein. S content: 2.7%. The glyceraldehyde, NAD$^+$, dichlorophenolindophenol (DCIP), and other chemicals used were of reagent grade or the best commercially available.

Equipment and Measurements. The equilibrium constant and the maximum number of bonds in the NAD$^+$–RK system were determined by the dialysis-equilibrium method[3] as follows: Into a 50-ml Ehrenmyer flask containing 20 ml of 0.2—5.0$\times 10^{-3}$M NAD$^+$ in a 0.1 M phosphate buffer (pH 8.0) (or 20 ml of a buffer solutoin as a reference solution without NAD$^+$), a cellulose tube containing 10 ml of 1.0$\times 10^{-4}$ M RK in a 0.1 M phosphate buffer (pH 8.0) was inserted. The cellulose tube used was made by the Visking Company. The flasks were allowed to stand at 35 °C for 24 h. The NAD$^+$ concentration in the flasks were determined spectrophotometrically after 24 h.

The UV absorption spectra were determined with a Shimadzu spectrophotometer UV-200. The anode polarograms were obtained using a Yanaco platinum rotating-electrode polarograph equipped with a Hokuto Denko LS-1D linear scanning unit, and PS-500B potentiostat and a Toa Electronics SXR-1A X-Y recorder. The NMR spectra were measured with a JEOL JNM-FX60.

Glyceric acid was identified using paper chromatography with a mixture of 1-propanol and concentrated aqueous ammonia (6 : 4) as the developer, and using NMR.

Results

The Formation of the NAD$^+$–RK Complex. The binding number, n, and the concentration of free NAD$^+$, [NAD$^+$], were determined using the method of Klotz *et al.* Plots of $1/n$ *vs.* $1/[\mathrm{NAD}^+]$ are shown in Fig. 1. A straight line was obtained. The values of the equilibrium constant, K, and the maximum number of bonds were calculated from the intercept and the slope of this line to be 1.2×10^3 M^{-1} and 1.3 (35 °C), respectively.

The Reaction Product. RK, NAD$^+$, and glyceraldehyde were dissolved into 100 ml of a 0.02 M phoshpate buffer (pH 8.0) to produce 1.0×10^{-4}, 1.0×10^{-3}, and 1.0×10^{-3} M solutions and the mixtures were allowed to stand at 35 °C. The absorption spectra of the mixtures were measured in the 230—400 nm region 0, 24, 48, and 68 h after perparation. The reference solution contained the same concentrations of RK and glyceraldehyde as the sample solution, except that no NAD$^+$ was present. The results are shown in Fig. 2. The absorption maximum at 340 nm, which was assigned to NADH, was found to increase with the lapse of time.

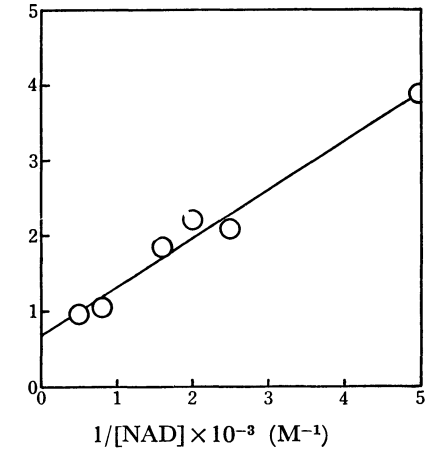


Fig. 1. The $1/n$ - $1/[\mathrm{NAD}]$ plots for the dialysis equilibrium.

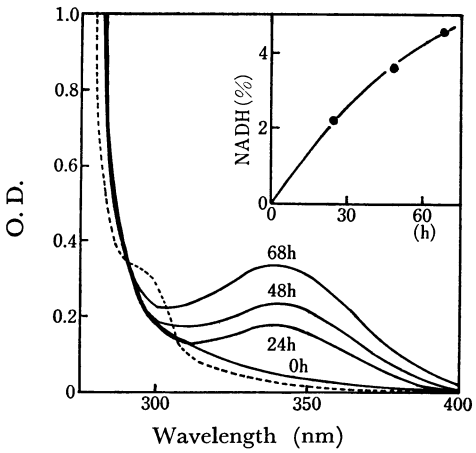


Fig. 2. Absorption spectra.
——: pH 8.0, ----: pH 7.0.

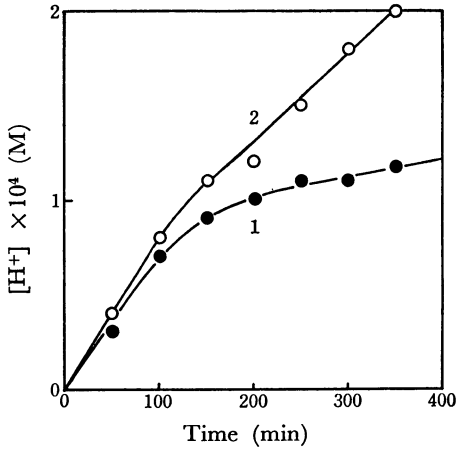


Fig. 3. The alkaline titration curves.
—○—: Oxygen bubbling,
—●—: nitrogen bubbling.

In the case of a pH 7.0 mixture, an absorption shoulder appeared at 290 nm and no absorption maximum was observed at 340 nm. On the paper chromatogram of the mixture 68 h after preparation, only one spot was observed at R_f=0.25, which is agreement with an authentic sample of glyceric acid (R_f=0.26).

Into 100 ml of distilled water, 1.0×10^{-4} M of RK, 1.0×10^{-4} M of NAD$^+$ and 1.0×10^{-3} M of glyceraldehyde were dissolved and adjusted to pH 8.0 by adding a sodium hydroxide solution. The mixture was bubbled with oxygen at 35 °C for 40 h, and the pH of the mixture was maintained at 8.0 by adding a sodium hydroxide solution. It was estimated from alkaline titration that 70% of the glyceraldehyde was converted to produce acid for a period of 40 h. The reaction mixture was dialyzed against distilled water, concentrated by evaporation, and then treated with an ion exchange resin (SE-sephadex-C50 H type) to remove any sodium ions. The solution was decolored by active charcoal and then evaporated resulting in a sticky syrup. The NMR spectrum of the syrup in deuterium oxide was found to be in agreement with that of glyceric acid (a multiplet at 3.68 (CH_2) and another at 4.32 (CH) ppm using the signal from tetramethylsilane as the external reference).

It is concluded from these results that glyceraldehyde was oxidized to form glyceric acid and NAD$^+$ was reduced to form NADH in the solution containing glyceraldehyde, NAD$^+$, and RK. The catalytic reaction could not proceed in either case because one component of the glyceraldehyde, NAD$^+$, and RK system had been omitted. Glyceraldehyde 3-phosphate, which is a compound similar to glyceraldehyde, was not oxidized by the NAD$^+$–RK system.

Reaction Rate. The rate of acid formation was determined by the alkaline titration method. A sample solution was prepared by dissolving glyceraldehyde, NAD$^+$ and RK into distilled water to produce 0.4—6.0×10^{-3}, 2.0×10^{-4}, and 1.0×10^{-4} M solutions and by adding a 0.1 M sodium hydroxide solution to adjust the mixture to pH 8.0. The reaction commenced immediately upon preparation of the sample solution at 35 °C. The alkaline titration was carried out manually to maintain the pH at 8.0. Typical results are shown in Fig. 3. Curves 1 and 2 in Fig. 3 show the results obtained by bubbling nitrogen and oxygen into the sample solution, respectively. The initial rate of acid formation did not appear to be influenced by the presence of oxygen. In the case of nitrogen bubbling, however, the rate of acid formation was found to decrease after the amount of acid produced in the reaction had reached the equivalent amount of RK.

The relation between the initial rate and the concentration of glyceraldehyde is shown by Lineweaver-Burk plots in Fig. 4. The plots are lines which gave two slopes about a turning point at 1.4×10^{-3} M of glyceraldehyde. It is speculated from this fact that same glyceraldehyde was tightly bound to the NAD$^+$–RK complex to be inactivated. The Lineweaver-Burk plot is a straight line in the case that 2.0×10^{-3} M glyceraldehyde 3-phosphate, which was not oxidized, was contained in the NAD$^+$–RK solution before adding glyceraldehyde. The values of the Michaelis constant and the maximum rate of the oxidation of glyceraldehyde were obtained from the intercept and the slope of the line to be 1.1×10^{-3} M and 3.6×10^{-6} M/min, respectively.

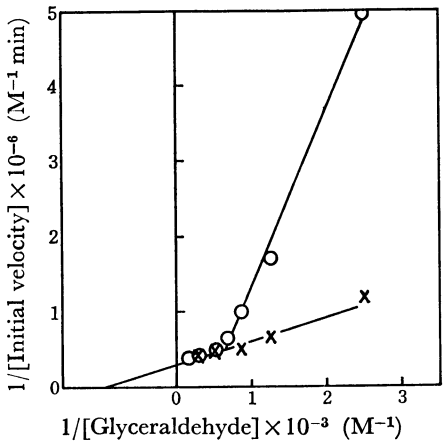


Fig. 4. Lineweaver-Burk plots.
—○—: Glyceraldehyde, —×—: glyceraldehyde with 2.0×10^{-3} M glyceraldehyde-3-phosphate.

Intermediates. This reaction should obey Michaelis-Menten kinetics. The glyceraldehyde–NAD+–RK complex appears to be an intermediate which was susceptible to oxidation by oxygen. In order to verify the existence of the intermediate, a polarographic study and DCIP oxidation were carried out.

The sample solution contained 2.0×10^{-4} M of glyceraldehyde, 2.0×10^{-4} M of NAD+, and 2.0×10^{-4} M of RK in a 0.1 M phosphate buffer (pH 8.0). Polarograms were measured (1) as fast as possible after preparation of the sample solution and (2) after 24 h at 35 °C. The sample solution was bubbled with nitrogen. The polarogam of NADH in the phosphate buffer was obtained and compared with that of the sample solution. The results are shown in Fig. 5. The oxidation wave of NADH was found to appear around +0.66 V *vs.* SCE. In the case of measurement immediately after preparation of the sample solution, no oxidation wave was observed in this region. Polarograms of the sample solutions after 24 h of preservation showed an oxidation wave at +0.19 V *vs.* SCE. It is plausible that this new oxidation wave can be assigned to the intermediates. No oxidation wave for NADH was observed.

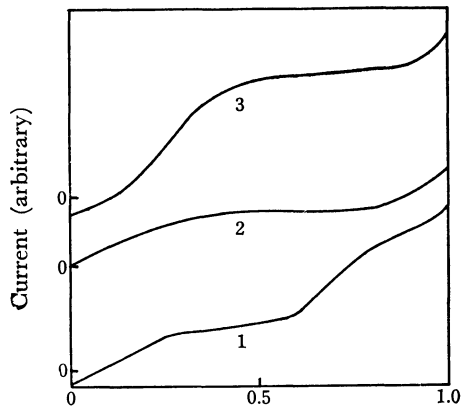


Fig. 5. Anode polarography.
1: NADH solution, 2: the NAD+–RK–glyceraldehyde system immediately after preparation, 3: the same system after 24 h.

DCIP oxidation was carried out by inserting the constituents as given in Table 1 into the Tunberg tube in which oxygen was removed by the passage of nitrogen gas. The conversion ratios of DCIP to reduced DCIP were determined spectrophotometrically at 600 nm after 30 min. DCIP was found to oxidize glyceraldehyde directly (Table 1). The conversion ratio decreased for a solution containing both NAD+ and RK. The DCIP oxidation was thought to be disturbed by the complexation of glyceraldehyde with the NAD+–RK complex.

It is assumed that the absorption shoulder at 290 nm in the pH 7.0 solution can be assigned to the intermediate, which is more stable in a pH 7.0 solution than in a pH 8.0 solution, because the absorption change at 290 nm is smaller than that at 340 nm. The NMR spectra at pH 7.0 were measured in order to better understand the struture of the intermediate. Proton signals (H4–6) of the nicotinamide moiety of NAD+ in the NAD+–RK solution were found to shift by 0.15—0.25 ppm to higher fields compared to that in NAD+ itself, as is indicated in Table 2. The RK signal may be located on the H4—6 side of the nicotinamide moiety, and that due to glyceraldehyde may be on the H2 side.

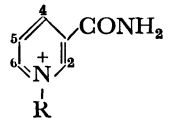
Table 1. DCIP oxidation of various systems

	Formation of $DCIPH_2$
NAD+–RK GCHO (1 : 1 : 10)	24.8 (%)
NAD+–RK (1 : 1)	0.0 (%)
NAD+–GCHO (1 : 10)	43.0 (%)
RK–GCHO (1 : 10)	18.2 (%)
NAD+	0.0 (%)
RK	0.0 (%)
GCHO	45.4 (%)

1.0×10^{-4} M NAD+, 1.0×10^{-4} M RK, 10×10^{-4} M GCHO, 1.0×10^{-4} M DCIP.
pH 8.0, 35 °C, 30 min, GCHO: glyceraldehyde.

Table 2. NMR of NAD+ in an NAD+–RK–GCHO solution

	NAD+	NAD+–RK	NAD+–RK–GCHO
H2	10.55	10.50	10.41
H4	9.67	9.52	9.50
H5	9.50	9.25(s)	9.23(d)
H6	10.05	9.87	9.85



1.0×10^{-3} M NAD+, 1.0×10^{-3} M RK, 1.0×10^{-3} M GCHO in D_2O (pH 7.0), GCHO: glyceraldehyde.

Discussion

It can be speculated from the fact that in Fig. 4 the catalytic reaction of NAD+–RK complex is seen to proceed *via* a similar reaction mechanism, which is called a homotropic effect, as that for tryptophane pyrolase.[4] It is possible for an aldehyde group of glyceraldehyde to form a Schiff base linkage with an amino group of RK. Glyceraldehyde forming the Schiff base linkage may be only slightly oxidized because the active center of the NAD+–RK complex may be located in a position different from that of the Schiff base linkage. Therefore, the actual concentration of glyceraldehyde is reduced. It is surely possible that glyceraldehyde 3-phosphate can bind more easily to RK than to glyceraldehyde, because ionic bond formation of that phosphate group may facilitate the formation of the Schiff base linkage. In the mixture of glyceraldehyde 3-phosphate and glyceraldehyde, the former may preferentially form a Schiff base linkage, so that oxidation may be carried out by a Michaelis-Menten type reaction as follows:

$$\text{NAD}^+\text{RK} + \text{GCHO} \rightleftharpoons \text{NAD}^+\text{RK GCHO} \longrightarrow \text{X} + \text{H}^+, \quad (1)$$

where X and GCHO indicate an intermediate and glyceraldehyde, respectively.

The rate of NADH formation shown in Fig. 2 was

very slow as compared with that of acid formation. Therefore, the conversion of the intermediate in the following reaction is thought to be the rate-determining step:

$$X + H_2O \xrightarrow{A} NADH + RK + GCOOH, \quad (2)$$

where GCOOH denotes glyceric acid.

The intermediate is susceptible to oxidation by oxygen and to an anodic reaction (Figs. 3, 5). Oxygen may participate in the second step in a subsequent reaction. The reaction can be expressed as follows:

$$X + 1/2O_2 \longrightarrow NAD^+RK + GCOO^-. \quad (3)$$

The DCIP oxidation suggests the existence of the ES complex in Fig. 1. Tagaki *et al.* have isolated *N*-benzyl-5-[hydroxy(1-pyridyl)methyl]nicotinamide, which exhibits an absorption maximum at 290 nm, by a reaction between aldehyde and a reduced nicotinamide derivative.[5] They explained that the product obtained may be one of the intermediates in the reaction. The absorption shoulder at 290 nm in Fig. 2 can thus be assigned to a similar intermediate.

Many authors have recently proposed that, at the active center of glyceraldehyde 3-phosphate dehydrogenase, a nicotinamide moiety in NAD^+ combines with an apoenzyme located near Cys-149 and that this center is activated by a mercapto group.[6–9] If the NAD^+–RK complex is assumed to catalyze the oxidation of glyceraldehyde in the same way as glyceraldehyde 3-phosphate dehydrogenase, a mercapto group on RK may play a similar role in the catalytic reaction. However, this assumption is still open to further investigation.

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