THE ABILITY OF REDUCED NICOTINAMIDE MONONUCLEOTIDE TO FUNCTION AS A HYDROGEN DONOR IN THE GLUTAMIC DEHYDROGENASE REACTION

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While pyridine nucleotide-linked dehydrogenases have been shown to catalyze the transfer of hydrogen from a number of analogs of DPNH, all of these analogs have been dinucleotides. We show here that beef liver glutamic dehydrogenase can catalyze the transfer of a hydrogen atom from reduced nicotinamide mononucleotide (NMNH) in a reaction quite analagous to the one normally catalyzed by that enzyme.

METHODS AND MATERIALS

NMNH, sodium salt, labeled Type III: approx. 90% pure, NMN, labeled 95% - 100%, Crotalus adamanteus venom phosphodiesterase, and L-glutamic dehydrogenase, Type III, were products of Sigma Chemical Company. L-glutamic dehydrogenase was dialyzed against phosphate buffer and filtered through 0.45 μ Millipore filters before use.

Kinetic measurements were made on a Gilford model 2000 recording spectrophotometer; spectra were determined on a Cary 14 spectrophotometer. 10 mm path length cells were used in both cases.

The extinction coefficient of NMNH at 336 mμ was determined by a differential method. The change in A_{336} of DPNH upon complete hydrolysis with venom phosphodiesterase was added to the total A_{336} of DPNH. From this value, an extinction coefficient of 6.8×10^3 $M^{-1}cm^{-1}$ for NMNH is calculated, based on an extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ for DPNH at 340 mμ reported by Horecker and Kornberg (1948).

α-Ketoglutarate was determined by a modification of the method of Friedemann and Haugeń (1943). The reaction was stopped by immediate deproteinization with 10% v/v CCl_3COOH. The 2,4-dinitrophenylhydrazine solution was incubated with an aliquot of the reaction mixture for 25 minutes,

and the absorbancy at 520 mμ of the alkaline Na_2CO_3 solution compared with that of known amounts of the unincubated mixture.

Reaction mixtures which had incubated sufficiently to give a substantial drop in absorbancy at 336 mμ were chromatographed in a Gordon Misco Multiple tube apparatus. 2 ml of absolute ethanol were added to 3 ml of the kinetic mixture. The solution was centrifuged and from 50 to 125 μl of the supernatant spotted side by side with L-glutamate on Whatman No. 4 filter paper, 4 cm wide and 0.2 mm thick. A mixture of 336 ml anhydrous ethanol and 164 ml H_2O (solvent A, Brenner, et al., 1965) was allowed to flow down the paper until the solvent front had moved approximately 20 cm (a little over 2 hours), and the paper was dried and treated with ninhydrin.

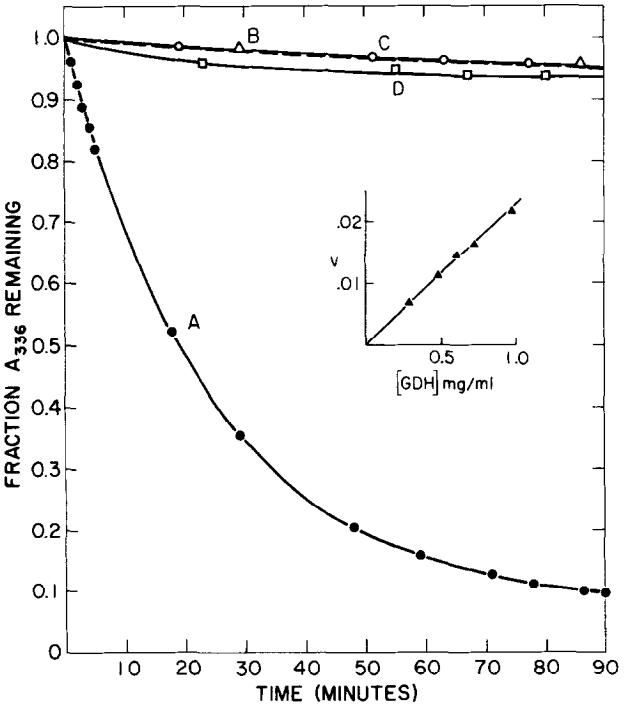

Fig. 1. The enzymatic oxidation of NMNH. Each reaction mixture contained components at the following concentrations except as otherwise indicated: 77 μM NMNH (initial absorbancy .518), 0.1 M NH_4Cl, 3.4 mM α-ketoglutarate, 0.8 mg/ml glutamic dehydrogenase, 0.1 M potassium phosphate buffer, pH 7.6, temperature 25° C; ordinate calculated as fraction of initial absorbance of NMNH remaining.

Curve A - complete reaction mixture
Curve B - α-ketoglutarate omitted
Curve C - glutamic dehydrogenase omitted
Curve D - NH_4Cl omitted

The dependence of the reaction rate on glutamic dehydrogenase concentration. v represents initial velocity in ΔA/min/mg/ml of glutamic dehydrogenase. Other conditions as described for Curve A.

RESULTS AND DISCUSSION

The enzymatic catalysis of the decrease in absorbance of NMNH is demonstrated in Figure 1.

While NMNH in the absence of enzyme undergoes a slow spontaneous loss of absorbancy at 336 mμ (as shown in Figure 1C), the rate is increased about 25 fold in the presence of glutamic dehydrogenase, NH_4^+, and α-keto-glutarate (Figure 1A).

In the absence of any one of these components, the rate of the reaction is that of NMNH alone (Figures 1C, 1D, 1E). The inset to Figure 1 shows that the initial velocity of the reaction is proportional to the enzyme concentration.

The reaction cannot be due to traces of DPNH. The absorption spectrum of the initial NMNH solution is shown in Figure 2A; that of the solution after the reaction is shown in Figure 2B. After the enzymatic reaction, ninety-two percent of the absorbance at 336 mμ has disappeared and the characteristic 264 mμ peak of NMN has appeared. The 260 mμ absorbance of the original solution is too small to permit the existence of more than a tiny fraction of the amount of DPNH required to account for the reaction.

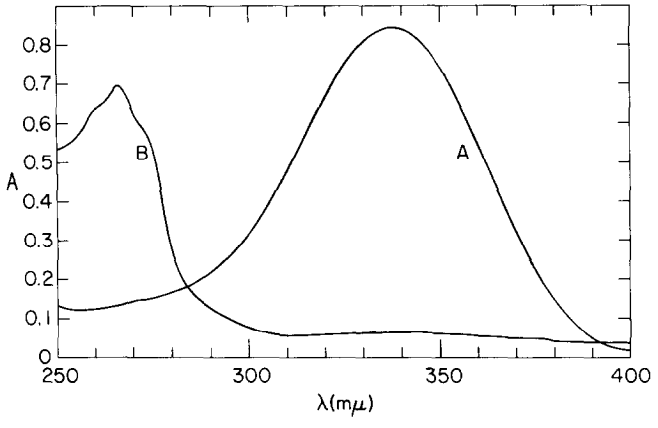

Fig. 2. Absorption spectra of reactants and products of the enzymatic oxidation of NMNH. (A) 150 μM NMNH in buffer vs. buffer. (B) 150 μM NMNH, 0.1 M NH_4Cl, 10 mM α-ketoglutarate and 0.84 mg/ml glutamic dehydrogenase vs. same mixture with NMNH omitted, incubated for 2 hours at 25° C. Other conditions as in Figure 1.

Proof of the stoichiometry of the enzyme catalyzed reaction is provided in Table I, which shows that the disappearance of a given amount of NMNH (as measured by absorbance at 336 mμ) is accompanied by the loss of a

Table I. The stoichiometry of the enzymatic oxidation of NMNH.

Time	αKG μ moles			NMNH μ moles			$\frac{\Delta \alpha KG}{\Delta NMNH}$ net
	Initial	Final	Δ	Δ Total	Δ Blank	Δ Net	
105 min.	130	72	58	81	18	63	0.92
105 min.	260	153	107	131	18	113	0.95
123 min.	260	141	119	140	14	126	0.95
123 min.	390	227	163	177	14	163	1.00

In each experiment the initial reaction mixture contained 450 μM NMNH, 0.1 M NH_4Cl, 0.94 mg/ml glutamic dehydrogenase, and the indicated initial concentration of α-ketoglutarate in 0.1 M phosphate buffer, pH 7.6 - 7.9. The control reaction mixture contained the same components with α-ketoglutarate omitted. After incubation at 30° C for the indicated period of time, the absorbance at 336 mμ of aliquots of both the reaction mixture and the control were read. The decrease in NMNH in both cases was determined from the ΔA_{336} using the extinction coefficient of 6.8×10^3 M^{-1} cm^{-1} determined as described under Methods. The total decrease in the reaction mixture was corrected for the spontaneous decomposition of NMNH by subtracting the amount lost in the control. (This correction is actually too large by a few percent, as the average concentration of NMNH in the reaction mixture is from 7% to 18% lower than that in the control solution). The α-ketoglutarate concentration in the initial and final reaction mixtures was then determined as described under Methods, and the loss of α-ketoglutarate was calculated from the difference. No correction from the control reaction was applied to the change in α-ketoglutarate as we have found that even a very large spontaneous loss of absorbancy of NMNH in the presence of α-ketoglutarate does not cause any measurable change in α-ketoglutarate concentration.

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corresponding amount of keto acid (as measured by the 2,4-dinitrophenylhydrazine reaction).

The product of the reaction, L-glutamate, was identified by paper chromatography of the reaction mixture represented by curve A of Figure 1. The average R.F. for seven experiments was 0.39 for the reaction mixture and 0.39 for a sample of L-glutamate at a concentration comparable to that calculated from the NMNH oxidation measured spectroscopically in the same reaction. Both the reaction aliquot and the L-glutamate sample produced lavender spots of comparable intensity. Separate identical chromatograms of each of the initial reagents produced no such spots.

We have also found that the enzymatic reaction is reversible using NMN and L-glutamate. Due to the difficulty of driving the reaction very far in the reverse direction, we are not able to eliminate the possibility of a DPN contaminant by reacting of a major part of the nucleotide, as was done in the forward reaction. However, we are able to demonstrate kinetically that the reverse reaction we observe cannot be due to a contamination of the NMN by DPN. Figure 3A shows the reaction of NMN, L-glutamate and glutamic dehydrogenase. In Figure 3B the identical reaction was allowed to run for six minutes and at that time DPN was added to a final concentration of 25 μM. It can be seen that this small amount of DPN is reduced almost instantly even in the presence of millimolar concentrations of NMN, and that the reaction products completely inhibit the further reduction of NMN (presumably, because of the formation of the relatively tight glutamic dehydrogenase - DPNH - L-glutamate ternary complex). Thus, the reverse reaction demonstrated here cannot be accounted for by a DPN contamination.

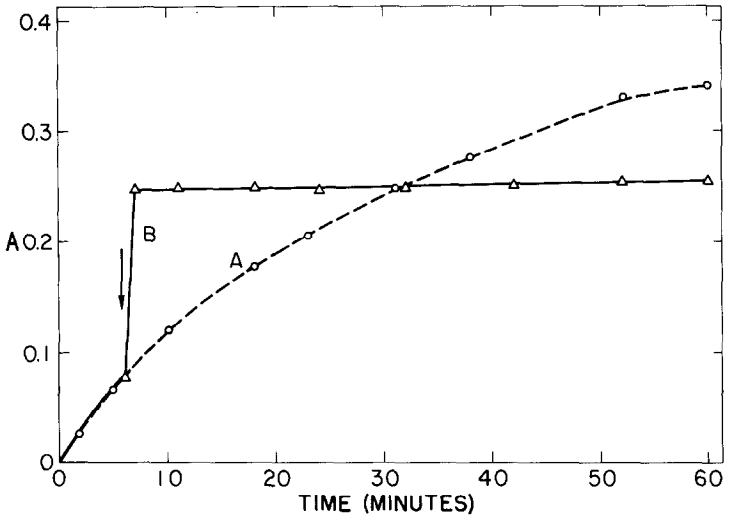

Fig. 3. The effect of a DPN contaminant on the rate of the reduction of NMN catalyzed by glutamic dehydrogenase. The reactions are carried out at pH 9.05, temperature 27° C. The L-glutamate is adjusted to the proper pH with KOH and serves as a buffer for the reaction. (A) 0.25 M potassium L-glutamate, 3 mM NMN, and 0.49 mg/ml glutamic dehydrogenase. (B) The same reaction mixture; 25 μM DPN added at time indicated by arrow.

The demonstration of the participation of a mononucleotide in a pyridine-nucleotide-linked dehydrogenase reaction will now permit studies on the precise role of the adenine moiety in such reactions. A study of the comparative kinetics of NMNH and DPNH in the glutamic dehydrogenase reaction will be published elsewhere.

ACKNOWLEDGMENTS

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