A new approach for using cofactor dependent enzymes: example of alcohol dehydrogenase

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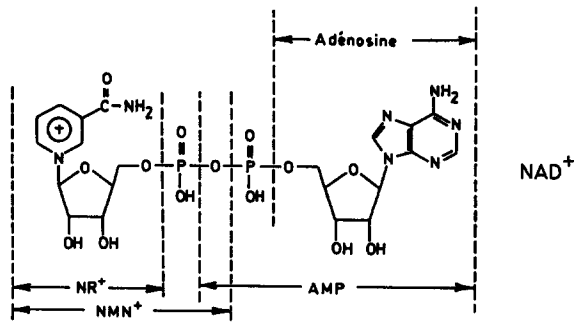
The use of enzymes requiring a cofactor as substrate in organic synthesis is still a problem since the cofactors are expensive. This study deals with a new approach consisting of using fragments of NAD^+. Three fragments of NAD(H) are examined. The activities of NMN^+ and NMNH are greatly improved by the addition of adenosine in ethanol oxidation and in cyclohexanone reduction, respectively. Nicotinamide monocucleoside is not active in the ethanol oxidation but the addition of AMP promotes this reaction.

NAD(H) NMN(H) Nicotinamide mononucleoside Alcohol dehydrogenase Enzymatic oxidation Enzymatic reduction

1. INTRODUCTION

In recent years, considerable efforts have been made to develop cofactor enzymatic reactions as an alternative to conventional chemical synthesis. In particular, deshydrogenases have been proposed to perform oxidoreduction reactions [1–3]. The use of cofactor-requiring enzymes in organic synthesis remains limited, however, due to the cofactor cost. A solution to this problem may be the use of easily available cofactor fragments provided that they are biologically active. For instance, nicotinamide mononucleotide (NMN^+) is able to perform oxidation reactions catalyzed by certain dehydrogenases [4–5], but its activity is low. Before considering analogs simpler than NMN^+, we decided (i) to explore the possibilities for improving its activity and (ii) to look for nicotinamide mononucleoside (NR^+) as an electron sink enzymatically active.

We relate our experiments along these lines with horse liver alcohol dehydrogenase (LADH). A possibility for improving NMN^+(H) activity in dehydrogenation (reduction) reaction was to complement the medium with the missing part of the cofactor, adenosine (AD) or adenosine-5′-phosphate (AMP), as these fragments have been demonstrated to be competitive inhibitors of NAD^+(H)-dependent enzymatic reactions [6].



2. MATERIALS AND METHODS

Horse liver alcohol dehydrogenase (EC 1.1.1.1) and nucleotide pyrophosphatase (EC 3.6.1.9) were from Sigma. NAD^+, NADH, $AMP_{5'}$, $ADP_{5'}$ and adenosine were from Boehringer France, NMN^+ was prepared by enzymatic cleavage of NAD^+ using a nucleotide pyrophosphatase [7] and purified by ion exchange chromatography on Dowex 1 × 8 (formate form) and further exclusion chromatography on Sephadex G10. NMNH was obtained by

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reduction of NMN^+ with sodium hyposulfite. NR^+ (bromide form) was prepared according to [8].

The enzymatic catalyzed reactions were carried out in a UV cuvette containing 1 ml of the reaction mixture. The reaction using NMN(H) was followed by the appearance or disappearance of the absorbance at $\lambda = 336$ nm, using $\epsilon = 6800\ M^{-1} \cdot cm^{-1}$. The reactions using NR^+ were followed by the appearance of the absorbance at $\lambda_{max} = 336$ nm. $\epsilon = 6800\ M^{-1} \cdot cm^{-1}$ was determined by reduction of NR^+ with sodium hyposulfite. The reaction mixtures contained 0.1 M of pyrophosphate buffer (pH 8.6), 2.5×10^{-6} M of LADH and: (i) for the oxidation of ethanol by NMN^+: 5×10^{-3} M NMN and 0.17 M ethanol; (ii) for the reduction of cyclohexanone by NMNH: 2×10^{-4} M NMNH and 0.1 M cyclohexanone; (iii) for the oxidation of ethanol by NR^+: 2.7×10^{-3} M NR^+ and 0.85 M ethanol.

The oxidation reactions were performed at 35°C and the reduction reaction at 25°C.

3. RESULTS AND DISCUSSION

The activities of NMN(H) were studied in the following reactions:

$$CH_3CH_2OH + NMN^+ \xrightarrow{LADH} CH_3CHO + NMNH$$

Fig.1 shows the course of ethanol oxidation by NMN^+ and allows initial rate determination.

$V_{i(NMN^+)} = 1.74\ \mu mol/l$ per min
$V_{i(NMN^+ + AD)} = 9.9\ \mu mol/l$ per min
$V_{i(NMN^+ + AMP)} = 0.98\ \mu mol/l$ per min

These values show an important increase in the enzymatic activity of NMN^+ when AD is added to the medium and an inhibition promoted by AMP. Compared to the NAD^+, the catalytic system using NMN^+ + AD has an activity of 30%.

Fig.2 indicates the course of cyclohexanone reduction by NMNH. The initial rates are:

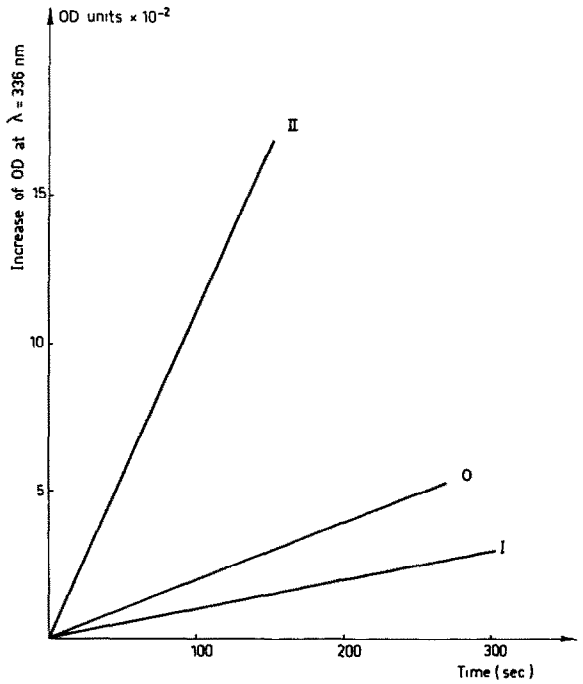

Fig.1. Oxidation of ethanol by NMN^+ catalyzed by LADH. O: NMN^+ alone; I: NMN^+ with 2×10^{-4} M AMP; II: NMN^+ with 10^{-2} M adenosine.

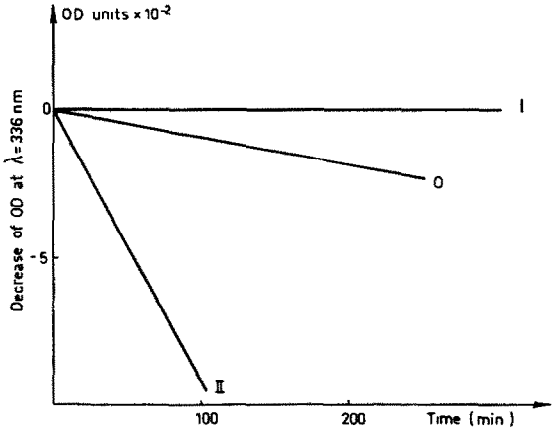

Fig.2. Reduction of cyclohexanone by NMNH catalyzed by LADH. O: NMNH alone; I: NMNH with 5×10^{-4} M AMP; II: NMNH with 10^{-2} M adenosine.

$V_{i(NMNH)} = 0.52\ \mu mol/l$ per h
$V_{i(NMNH + AD)} = 7.28\ \mu mol/l$ per h
$V_{i(NMNH + AMP)} = 0$

Once more adenosine appears to be a powerful activator of NMNH whereas AMP completely inhibits the reaction.

In these systems, the high absorbance of NMNH at $\lambda = 336$ nm prevents the use of reaction mixtures containing saturating concentrations of the cofactor, which should lead to higher reaction rates than the observed ones, and no comparison with the activity of NADH was possible.

These results do not allow mechanistic interpretations of the respective roles of AD and AMP during the course of the reactions. We can however attribute the difference observed to the repulsion of the negatively charged phosphate groups of AMP and $NMN^+(H)$ which may give wrong relative positions for the two cofactor fragments in the catalytic site of the enzyme.

NR^+, which is a fragment of NAD^+ smaller than NMN^+, was also assayed in an ethanol oxidation reaction.

$$CH_3CH_2OH + NR^+ \xrightarrow{LADH} CH_3CHO + NRH$$

Fig.3 shows the course of this reaction. It is remarkable that the alcohol oxidation does not occur by NR^+ alone, showing the importance of the phosphoryl group in position 5′ and that the addition of AD, AMP or ADP promotes this reaction. As expressed by the initial rates

$V_{i(NR^+ + AD)} = 0.08\ \mu mol/l$ per min
$V_{i(NR^+ + ADP)} = 0.15\ \mu mol/l$ per min
$V_{i(NR^+ + AMP)} = 1.1\ \mu mol/l$ per min

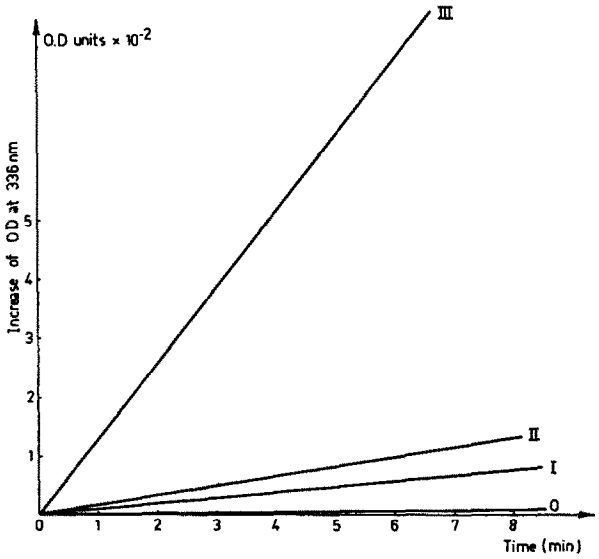

Fig.3. Oxidation of ethanol by NR^+ catalyzed by LADH. O: NR^+ alone; I: NR^+ with 2.2×10^{-2} M adenosine; II: NR^+ with 4×10^{-2} M ADP; III: NR^+ with 4×10^{-2} M AMP.

AMP is the best activator, indicating clearly that one phosphate group linked to the adenosine moiety is essential for the reaction to take place. Compared to the NAD^+, the catalytic system using NR^+ + AMP has an activity of 10%. Why ADP is not as good an activator as AMP is not clear. We can assume, however, that the large pyrophosphate group non-linked to the nicotinamide ribose moiety produces a steric strain in the enzymatic catalytic site.

In conclusion, this work clearly indicates that it is possible to improve significantly the enzymatic activity of $NMN^+(H)$ and also to transform NR^+, which is enzymatically inactive, into an active analog cofactor. In both cases the phenomenon is observed after the addition of molecules which are parts of the non-functional moiety of the $NAD^+(H)$. The presence of one phosphate group linked to either the functional or the non-functional part seems to be essential for the analog to be enzymatically active and the catalytic systems thus obtained have fairly good activities compared to the NAD(H).

These observations open a new means for the design of simple cofactor analogs which will probably be usable in cheap preparative organic synthesis.

ACKNOWLEDGEMENT

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