Biochimica et Biophysica Acta, 661 (1981) 120–123
Elsevier/North-Holland Biomedical Press

BBA 69363

EVIDENCE FOR BINDING OF NAD DIMERS TO NAD-DEPENDENT DEHYDROGENASES

ALESSANDRO FINAZZI-AGRÒ [a], LUCIANA AVIGLIANO [a], VINCENZO CARELLI [b,*], FELICE LIBERATORE [b] and ANTONIO CASINI [b]

[a] *Istituto di Chimica Biologica and* [b] *Cattedra di Chimica Farmaceutica Applicata, University of Rome, Rome (Italy)*

(Received February 24th, 1981)

Key words: NAD dimer; NAD dependence; Dehydrogenase; Coenzyme binding

The binding of dimers of nicotinamide adenine dinucleotide, $(NAD)_2$, to lactate, malate and alcohol dehydrogenase has been studied by the fluorescence quenching technique. While the alcohol dehydrogenase shows a low binding ability, malate and lactate dehydrogenases have been found to bind $(NAD)_2$ in a specific way with high affinity. Malate dehydrogenase binds $(NAD)_2$ more than NADH. All three dehydrogenases are inhibited by $(NAD)_2$, which behaves as a competitive inhibitor with respect to both NAD^+ and NADH. These results show that $(NAD)_2$ is bound to the nucleotide-specific binding site of the dehydrogenases. $(NAD)_2$ was found to stoichiometrically react with ferricyanide at variance with NADH. The specific interactions with the NAD-dependent dehydrogenases and the ability to enter in monoelectronic redox cycles suggest possible physiological roles for $(NAD)_2$.

Introduction

Recently the isolation and characterization of a mixture of three diastereoisomeric dimers of 4,4′-tetrahydrobipyridine type, $(NAD)_2$, arising from the one-electron electrochemical reduction of NAD^+ has been reported [1]. It was also shown that the supernatant of a standard mitochondrial preparation from rat liver is able to catalyze the oxidation of this dimeric product by molecular oxygen. In order to evaluate the role of this compound in the biological redox process we have investigated the possible binding of $(NAD)_2$ to some NAD-dependent dehydrogenases. In fact it is well known that NAD^+ and NADH specifically bind to dehydrogenases and that the catalytic mechanism of these enzymes requires, in addition, binding of the specific substrates in a ternary complex [2]. It is therefore conceivable that $(NAD)_2$ might also require similar binding before undergoing biological redox processes. In the present paper we report the results of this investigation.

* To whom correspondence should be addressed.

Materials and Methods

The enzymes lactate dehydrogenase, malate dehydrogenase and alcohol dehydrogenase were obtained as purified samples in $(NH_4)_2SO_4$ suspension from Boehringer-Mannheim, F.R.G. Polyacrylamide gel electrophoresis according to Davis [3] was routinely used to check the purity of these samples. Other chemicals were purchased in the purest available form and used without further purification. $(NAD)_2$ prepared according to Carelli et al. [1], had an ϵ_{260} of 31 600 $cm^2 \cdot M^{-1}$ and ϵ_{340} of 6 400 $cm^2 \cdot M^{-1}$ in H_2O. It was stored in the dry form at −20°C and dissolved just before use.

Enzymic activity of the three dehydrogenases was tested as described [4]. The reaction between $(NAD)_2$ or NADH and ferricyanide was studied by recording the absorption changes at 340 or at 420 nm with a Beckman ACTA III spectrophotometer.

Thin-layer chromatograms were performed on silica plates and developed with *n*-propanol/ammonia/water (20 : 20 : 3 v/v).

Coenzyme binding was determined by the quench-

0005-2744/81/0000–0000/$02.50

ing of protein intrinsic fluorescence upon addition of NADH or $(NAD)_2$ to the dehydrogenases. Fluorescence spectra were taken at 20°C with a FICA 55 corrected spectrofluorimeter. The binding was also studied by dialysis experiments which were performed at 4°C in 0.25 M phosphate buffer, pH 7.1, using protein concentrations in the range 10–50 μM. The spectrophotometric determinations of $(NAD)_2$ inside (protein compartment) and outside the dialysis bag were done after relatively short time intervals due to the instability of $(NAD)_2$ in these conditions.

Results and Discussion

The binding of $(NAD)_2$ to alcohol, lactate and malate dehydrogenase can be followed by fluorimetry as previously described for NADH [5]. In fact binding of NADH to dehydrogenases causes a relevant quenching of the protein intrinsic fluorescence. This quenching has been attributed to the 340 nm absorption band of the nucleotide, which overlaps the protein intrinsic fluorescence peak and makes it a good acceptor for intramolecular energy transfer if bound to a protein. $(NAD)_2$ too shows an absorption at 340 nm but at variance with NADH, is not fluorescent [6], even at 77 K nor in the presence of dehydrogenases. Fig. 1 shows the quenching of intrinsic fluorescence observed when malate, lactate or alcohol dehydrogenase is titrated with NADH or $(NAD)_2$. Malate and lactate dehydrogenase appear to bind $(NAD)_2$ with fairly high affinity (K_{diss} = 1.5 μM and 5 μM, respectively) which in the case of malate dehydrogenase is even higher than that for NADH. NAD^+, which binds to dehydrogenase without a large effect on fluorescence, reduced the quenching effect of $(NAD)_2$, indicating a competition of the two nucleotides for the same site. It was impossible to determine a binding constant of $(NAD)_2$ to alcohol dehydrogenase by fluorescence quenching since this method does not allow us to reach high enough concentrations of protein or nucleotide due to the increasing weight of inner filter effects [7].

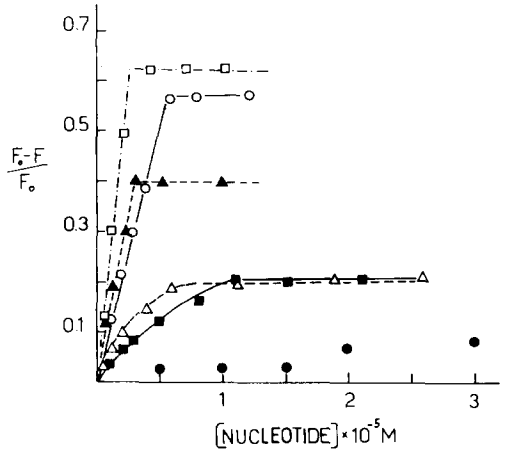

Fig. 1. Fluorimetric titration of dehydrogenases with NADH or $(NAD)_2$. Malate dehydrogenase (0.5 μM, ▲, △), lactate dehydrogenase (0.8 μM, ■, □) and alcohol dehydrogenase (2 μM, ●, ○) were titrated with NADH (open symbols) or $(NAD)_2$ (full symbols) at 20°C in 0.1 M phosphate buffer at pH 7.1. The fluorescence determinations were made immediately after the addition of the nucleotide to the enzyme solutions. The excitation wavelength was 280 nm. The emission wavelengths were 310 nm (malate dehydrogenase), 345 nm (lactate dehydrogenase) and 338 nm (alcohol dehydrogenase). The inner filter effects were corrected for by parallel titrations of *N*-acetyltyrosine or *N*-acetyltryptophan ethyl esters.

In agreement with previous reports [6,8,9], we found that $(NAD)_2$ is unable to act as a coenzyme with the dehydrogenases tested. It rather inhibits these enzymes as shown in Fig. 2, where the enzymic activity of malate and lactate dehydrogenase is plotted as a function of the concentration ratio $(NAD)_2$/NADH. A Dixon plot of alcohol dehydrogenase inhibition by $(NAD)_2$ at different NAD^+ concentrations and saturating concentration of EtOH is shown in Fig. 3.

A K_i' of approx. 40 μM was obtained in this set of

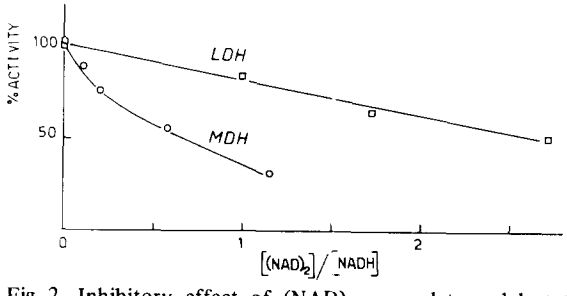

Fig. 2. Inhibitory effect of $(NAD)_2$ on malate and lactate dehydrogenase. Malate dehydrogenase (MDH) activity was measured in 0.2 M Tris-HCl buffer, pH 8/50 μM NADH/600 μM oxalacetate/50 pm enzyme. Lactate dehydrogenase (LDH) activity was measured in 0.2 M Tris-HCl buffer, pH 8/25 μM NADH/600 μM pyruvate/1 nm enzyme. The $(NAD)_2$ concentration was varied as indicated. The activity was measured at 25°C.

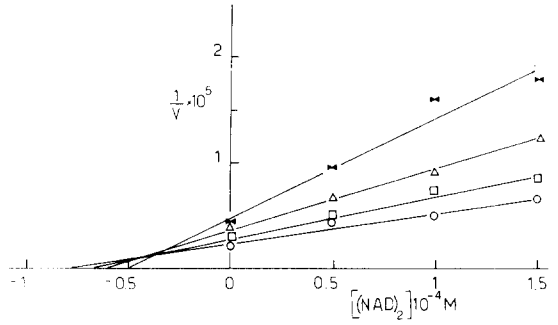

Fig. 3. Dixon plot for inhibition of alcohol dehydrogenase by $(NAD)_2$. Reaction mixture contained 0.2 μM liver alcohol dehydrogenase/75 mM semicarbazide/20 mM glycine/10 mM ethanol/75 mM pyrophosphate buffer, pH 8, and the following amounts of NAD^+: 50 μM (⋈); 100 μM (△); 150 μM (□); 200 μM (○). The incubation was made at 25°C. V is expressed as mol NADH formed/min^{-1}.

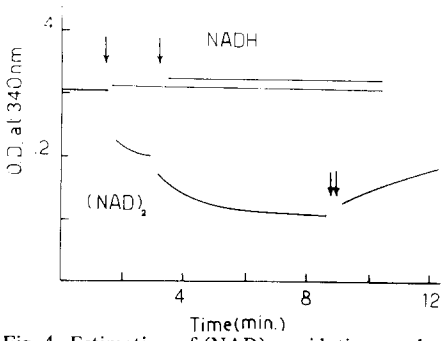

Fig. 4. Estimation of $(NAD)_2$ oxidation product as enzymatically active NAD^+. Reaction mixture contained 45 μM $(NAD)_2$ or 46 μM NADH/75 mM semicarbazide/20 mM glycine in 75 mM pyrophosphate buffer, pH 8. When indicated by a single arrow 30 μM (final concentration) $K_3Fe(CN)_6$ was added. Double arrow indicates the addition to the reaction mixture of 20 mM ethanol and 0.9 μM liver alcohol dehyrogenase.

experiments, which is in keeping with the impossibility of determining the association constant for $(NAD)_2$ binding to alcohol dehydrogenase by fluorimetry. The kinetic data altogether suggest that $(NAD)_2$ is a competitive inhibitor with respect to NAD^+ and NADH. Dialysis experiments, which would allow independent determination of the affinity constants, could not be conducted at the equilibrium, since $(NAD)_2$ slowly decomposes at neutral pH values. However, at short time intervals, when the system is still far from the equilibrium, the $(NAD)_2$ concentration was significantly higher in the protein compartment. All the dehydrogenases tested appear to bind $(NAD)_2$, but with very different binding constants. Malate dehydrogenase shows the greatest binding ability for $(NAD)_2$, alcohol dehydrogenase the lowest, and lactate dehydrogenase an intermediate value, as shown by fluorimetric and kinetic measurements. $(NAD)_2$ appears to bind to the dehydrogenases at the same site of NAD^+ and NADH. In fact fluorimetry and steady-state kinetics show competition between $(NAD)_2$ and the oxidized or reduced pyridine nucleotides. The variable affinity of $(NAD)_2$ for these dehydrogenases could reflect structural differences in nucleotide binding sites, already observed by other techniques, e.g., X-ray crystallography [5]. These differences may influence the reactivity of alcohol and malate dehydrogenase complexes with NADH toward redox agents like O_2^- [10]. The $(NAD)_2$-dehydrogenase complexes do not appear to undergo redox process with their specific substrates. Nevertheless it could well be that the binding of $(NAD)_2$ to dehydrogenases affects its reactivity in redoxy processes. It should be recalled that the dehydrogenases used in the present study were found to inhibit the dimerization of NAD· to $(NAD)_2$ [11]. The lack of reactivity as a dehydrogenase coenzyme does not rule out a possible physiological formation and role of $(NAD)_2$, which can be oxidized by biological systems such as liver homogenates [1] or plant enzymes [12]. $(NAD)_2$ can be also reoxidized to NAD^+ both electrochemically [13] and photochemically [14]. Furthermore, in the present investigation we have found that in contrast with NADH, $(NAD)_2$ reacts stoichiometrically in aqueous solutions at neutral pH with ferricyanide, yielding ferrocyanide and NAD^+ (see Fig. 4). The latter compound was identified both by thin-layer chromatography and by its ability to substitute authentic NAD^+ in the alcohol dehydrogenase reaction. As a whole, these findings indicate a different reactivity of $(NAD)_2$ with respect to either NAD^+ or NADH. Therefore, while $(NAD)_2$ has not been so far identified in vivo, its ability to bind dehydrogenases and to easily undergo single-electron transfer processes could be of physiological meaning.

Acknowledgement

The work was supported by a research grant from CNR, Rome, Italy.

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