THE STEREOCHEMISTRY OF CALF SPLEEN NAD-GLYCOHYDROLASE-CATALYZED NAD METHANOLYSIS

Marc PASCAL and Francis SCHUBER*

Laboratoire de Physiologie Végétale, Institut de Botanique de l'Université Louis Pasteur, 28, rue Goethe, 67000 Strasbourg, France

Received 14 April 1976

1. Introduction

NAD glycohydrolases catalyze the hydrolysis of NAD at the nicotinamide–ribose linkage. The mammalian NAD (P) glycohydrolases (EC 3.2.2.6) function also as transglycosidases and have been extensively used to prepare NAD analogs [1]. Few studies have concerned the mechanism of the reactions catalyzed by this class of enzymes.

The coenzyme properties of some analogs synthetized by pyridine base exchange indicate that the β-configuration at the anomeric carbon remains unchanged in the transglycosidation [2,3]. This result, and the possibility to observe transglycosidation with imidazole derivatives, led Alivisatos to suggest that the reactions catalyzed by the NAD-glycohydrolases consist of a double displacement, the nucleophilic catalyst being an imidazole residue of the enzyme [4]. Our study on the kinetic parameters as a function of pH failed to confirm this point [5].

Steady-state kinetic studies have demonstrated the multistep nature of the reaction catalyzed by a solubilized calf spleen NAD-glycohydrolase. In the case of NAD hydrolysis the reaction was consistent with an Ordered Uni Bi mechanism, the formation of an enzyme ADP ribosyl intermediary complex (E-ADPR) being rate determining [6]. In analogy to the mechanism of action of some glycosidases (e.g. lysozyme [7]) alternative reaction pathways to the previously proposed double displacement can be suggested for the NAD-glycohydrolase. The reactivity of the intermediate E-ADPR versus nucleophiles, including methanol, was in favour of an oxocarbonium ion. In the present study we have determined the stereochemistry of the methanolysis of NAD with the aim to gain new information on the nature of the chemical steps catalyzed by the NAD-glycohydrolase. The reaction was found to proceed with over 99% retention of configuration.

*To whom correspondence should be addressed

2. Materials and methods

2.1. *Chemicals*

β-NAD, snake venom *(Crotalus atrox)*, phosphatase acid (potato) type IV, were purchased from Sigma Chemical Co; all other chemicals were Merck products. DEAE cellulose (DE-52) was obtained from Whatman.

2.2. *Enzyme*

Calf spleen NAD-glycohydrolase was solubilized and purified according to the procedure previously published [5]. It had values of specific activity about 7 units per mg of protein under standard assay conditions (1.0 mM NAD, pH 7.4, 0.1 M NaCl and 37°C).

2.3. *N.m.r. spectra*

The n.m.r. spectra (in D_2O) were obtained at 250 MHz using trimethylsilyl-3-propanesulfonic acid, sodium salt as internal reference. The chemical shifts τ are expressed in p.p.m. and the coupling constants J in Hz.

2.4. *Synthesis of α and β methyl-D-ribofuranosides*

The methyl-ribosides were prepared according to literature [8]. The α and β-isomers were separated

by silica gel column chromatography using ethylacetate–n-propanol–water (5:3:1 by vol.) as eluant. The predominant β-isomer was eluted first. Both isomers were identified by their n.m.r. spectrum and characterized as follows:

Methyl-β-D-ribofuranoside : mp 77.5–78°C (recrystallized from ethylacetate); $[\alpha]_D^{20}$ – 54.2° (c = 3.5 in water). Literature [8] mp. 79–80°C; $[\alpha]_D^{20}$ – 50° (c = 2.0 in water). R_F = 0.64; n.m.r., τ 6.56 (singlet, β-OCH_3) 5.78–6.39 (multiplet, H_2, H_3, H_4 and H_5) 5.06 (broad singlet, anomeric proton).

Methyl-α-D-ribofuranoside : oil; $[\alpha]_D^{20}$ + 106.5° (c = 3.4 in water); R_F = 0.47; n.m.r., τ 6.53 (singlet, α-OCH_3) 5.83–6.36 (multiplet, H_2, H_3, H_4 and H_5) 4.97 (doublet $J_{1,2}$ = 4.35, anomeric proton).

The R_F values are given for a silica gel thin-layer chromatography (TLC) using ethylacetate–n-propanol–water (3:2:2, by vol.) as solvent. In the same system D-ribose had R_F = 0.41. The sugars were detected with a 1-naphtol spray.

2.5. *Transformation of methyl-ADP riboside into methyl-riboside*

Preparation of methyl-ADP riboside: A 0.05 M sodium phosphate buffer pH 7.4 containing 16 units of NAD glycohydrolase was incubated at 25°C in the presence of 4.6 mM NAD and 4.3 M methanol. The reaction (final volume 350 ml) which was stopped after 18 h was essentially complete. The reaction mixture was concentrated by evaporation and transferred on a 1.6 × 20 cm DEAE cellulose column which was previously equilibrated with water. The column was washed successively with water and a 10 mM NH_4HCO_3 solution. The ADP ribose and methyl-ADP riboside mixture was then eluted using a 0.2 M NH_4HCO_3 solution. The pooled fractions were evaporated and the salts eliminated by lyophilization. Methyl-ADP riboside accounted for 70% of the mixture; n.m.r. spectra, τ 6.62 (singlet, $-OCH_3$).

Preparation of methyl-riboside 5-phosphate: To 970 mmol of the nucleotides mixture dissolved in water (20 ml) were added a 1 M $NaHCO_3$ solution (7.5 ml) and a 0.3 M magnesium acetate solution (2.5 ml); the mixture was incubated for 6 h at 37°C in the presence of snake venom (50 mg).

Preparation of methyl-riboside: After treatment with the snake venom the mixture was acidified to pH 5.3 with HCl then a 0.5 M sodium acetate solution (6 ml) was added. The reaction mixture was finally incubated at 37°C overnight in the presence of acid phosphatase (12 mg). A TLC revealed the complete disappearance of the methyl-riboside 5-phosphate spot and the presence of a compound which migrated like methyl β-ribofuranoside. The proteins and the salts were successively eliminated by filtration on an UM-2 Amicon membrane and a passage on a Dowex 50, H^+ column. After evaporation the methyl ribosides were separated from ribose by a silica gel column chromatography (see above); after isolation the fraction was analyzed by n.m.r.

As a control we prepared α and β methyl-riboside 5-phosphate [10]; both isomers were found to be substrates for the acid phosphatase.

3. Results and discussion

Hydrolysis of NAD catalyzed by calf spleen NAD-glycohydrolase and the partition of the intermediary complex E-ADPR using methanol as acceptor are found in table 1. Methanolysis versus hydrolysis increases with increasing concentrations of methanol; it becomes largely predominant when [MeOH] = 5 M. From the data of table 1 the ratio of the secondary rate constants: methanolysis/hydrolysis can be calculated. Using $[H_2O]$ = 55 M one finds that, compared to water, methanol reacts faster with the intermediate E-ADPR by two orders of magnitude. This ratio (average value = 95) is essentially independent of the methanol concentration, in the range used for this study. It follows that the enzyme active site has no special affinity for methanol and is

Table 1
Partition by methanol during calf spleen NAD glycohydrolase catalyzed NAD hydrolysis

Methanol (M)	Methanolysis / Hydrolysis + Methanolysis
0.25	0.29
0.50	0.54
1.00	0.62
2.00	0.74
5.00	0.97

The reactions were performed at 37°C and pH 7.4. The contribution of methanolysis versus hydrolysis was determined as described in Materials and methods.

not saturated by the acceptor. It is therefore expected that methanol, like water, will behave as a solvent in its reaction with E-ADPR. As a corollary, the rates of reaction of methanol and water with E-ADPR can be compared.

The stereochemistry of NAD methanolysis catalyzed by the enzyme was studied quantitatively. As there exists no reference for determining the stereochemistry of methyl-ADP ribofuranoside, the methanolysis product, this nucleotide was transformed by successive treatment with a phosphodiesterase (snake venom) and an acid phosphatase into the known methyl-ribofuranoside. Comparison of the n.m.r. spectra of authentic samples of α- and β-methyl-D-ribofuranosides and the methyl-ribofuranoside obtained from the enzymatic reaction revealed that the latter had a β-configuration. The high sensitivity of the physical method used allowed to conclude that its stereochemical purity was superior to 99%. In conclusion, in the calf spleen NAD glycohydrolase catalyzed cleavage of the nicotinamide–ribose bond the configuration of the anomeric carbon is retained to an extent of at least 99%.

At a molecular level the cleavage of the nicotinamide–ribose bond of NAD can proceed according to several pathways.

(a) Double displacement which results in a retention of configuration at the C-1′ of the ribose. In the intermediary complex ADPR is covalently linked to a nucleophilic side chain of the enzyme.

(b) Unimolecular decomposition of NAD(A-1 mechanism) resulting in the formation of an oxocarbonium ion which can be stabilized by the formation of an ion-pair with a residue of the enzyme, e.g. carboxylate. (This ion-pair could be in spontaneous equilibrium with a covalent intermediate e.g. acylal).

(c) General base catalysis involving the anchimeric assistance of the neighbouring 2-hydroxy group and formation of an 1,2-epoxyde.

The approach we took in the present study is distinct from that of the stereochemistry of the transglycosidation reaction. In the pyridine base exchange the acceptor pyridine would be expected to bind to the active site, identically with the original nicotinamide leaving group, and therefore would lead to a transglycosidation product of retained configuration. In the case of hydrolysis and methanolysis, the nucleophiles are small molecules and we have seen that they have no apparent affinity for the active site; water and methanol would therefore be expected to behave as true solvents and to quench the intermediate E-ADPR. The stereochemistry of this latter reaction would be only governed by the nature of the intermediate. It follows that isolation of a small but significant amount of the α isomer in the methanolysis, in addition to the β isomer, would have constituted a direct proof for the existence of the oxocarbonium ion (pathway b). The observed complete retention of configuration does not allow one to distinguish between the pathways a, b, and c for the NAD glycohydrolase catalyzed NAD hydrolysis. However our result excludes the occurrence of a single displacement by water (A-2 mechanism) with inversion of configuration. The anomeric retention of configuration implies also in case (b) that an assymetric environment is provided by the enzyme i.e. the approach of the nucleophile being sterically controlled (α-face hidden). In the context of this work the higher nucleophilicity of methanol, compared to water, towards the intermediary complex in β-galactosidase catalyzed reaction, was given as evidence in favour of a reaction through an oxocarbonium ion [11].

References

[1] Colowick, S. P., Van Eys, J. and Park, J. H. (1966) in: Comprehensive Biochemistry (Florkin, M. and Stotz, E. H. eds.) Vol 14, pp. 1–98 Elsevier Publishing Co. Amsterdam.
[2] Kaplan, N. O., Ciotti, M. M. and Stolzenbach, F. E. (1956) J. Biol. Chem. 221, 833–844.
[3] Anderson, B. M. and Kaplan, N. O. (1959) J. Biol. Chem. 234, 1226–1232.
[4] Alivisatos, S. G. A. (1959) Nature, 183, 1034–1037.
[5] Schuber, F. and Travo, P. (1976) Eur. J. Biochem. in the press.
[6] Schuber, F., Travo, P. and Pascal, M. (1975) 10th International Congress FEBS (Paris) Abstract 608.
[7] Imoto, T., Johnson, L. N., Worth, A. C. T., Phillips, D. C. and Rupley, J. A. (1972) in: The Enzymes (Boyer, P. D. ed.) 3rd Edn., Vol 7 pp. 665–868, Academic Press, New York.
[8] Barker, R. and Fletcher, Jr., H. G. (1961) J. Org. Chem. 26, 4605–4609.
[9] Secemski, I. I., Lehrer, S. S. and Lienhard, G. E. (1972) J. Biol. Chem. 247, 4740–4748.
[10] Switzer, R. L. and Simcox, P. D. (1974) J. Biol. Chem. 249, 5304–5307.
[11] Sinnott, M. L. and Viratelle, O. M. (1973) Biochem. J. 133, 81–87.