PREPARATION OF 3-CHLOROACETYLPYRIDINE ADENINE DINUCLEOTIDE: AN ALKYLATING ANALOGUE OF NAD^+

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1. Introduction

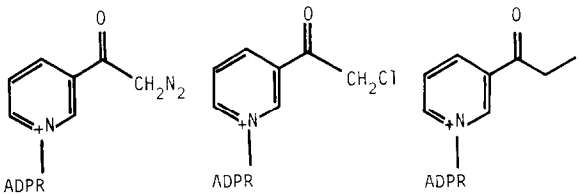
Analogues of coenzymes with reactive groups have been extensively used to detect the presence of functional amino acid residues in the catalytic site of dehydrogenases. Thus, alkylating analogues of NAD^+ have been synthesized: the group at C-3 of nicotinamide of NAD^+ has been replaced by a diazonium group [1], a diazoacetate [2] or a bromoacetyl group, with an aliphatic chain instead of the ribose [3–6] and the adenine has been replaced by a bromoacetylimidazole [6, 7]. As basic residues are invoked in the mechanism of the dehydrogenase for the removal of the proton located at the alcohol during the hydrogen transfer [8, 9], it was tempting to attack this basic group from the coenzyme side, by the chloroacetyl group, replacing at C-3 the amide group of the pyridinium ring. Our goal was to synthesize the 3-chloroacetylpyridine adenine dinucleotide analogue of NAD^+. This is quite similar to 3-acetylpyridine adenine dinucleotide, which is active as hydrogen acceptor with many dehydrogenases. The similarity of the structure may lead to the activity of this analogue as hydrogen acceptor, as well as to alkylation. This activity is therefore a good indication for the alkylation of a residue in the active site.

The preparation of this analogue, and of the 3-propionylpyridine adenine dinucleotide are described here and the preliminary results on the alkylation of some dehydrogenases are briefly reported.

2. Materials and methods

NAD^+ glycohydrolase (3.2.2.6) from pig brain, alcohol dehydrogenases from horse liver (LADH) and yeast (YADH) and the NAD^+, NAD^+ glycohydrolase (3.2.2.5) from *Neurospora Crassa* are products from Sigma. Glutamate dehydrogenase from beef liver is product from Boehringer.

The octopine dehydrogenase has been kindly provided by Dr. A. Olomucki and 17 β-hydroxysteroid dehydrogenase from human placenta by Prof. B. Descomps.



1 2 3

Fig. 1. ADPR = Adenosine diphospho ribose. 1: 3-diazoacetylpyridine adenine dinucleotide; 2: 3-chloroacetylpyridine adenine dinucleotide; 3: 3-propionylpyridine adenine dinucleotide.

2.1. *Preparation of 3-diazoacetylpyridine adenine dinucleotide 1*[☆☆]

This analogue is prepared from NAD^+ by enzymatic exchange as follows: NAD^+ (1 g), 3-diazoacetylpyridine (1 g) [10] and pig brain NAD^+ glycohydrolase (0.3 g) in bidistilled water (75 ml) is left at pH 2.6 and 37°C. The progress of the exchange is followed

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☆☆ By the same manner, we have prepared the 4-diazoacetylpyridine adenine dinucleotide.

by UV spectroscopy. The absorption at 365 nm corresponding to the cyanide adduct of 3-diazoacetylpyridine adenine dinucleotide in a 1.0 N KCN solution is measured. After about 8 hr, this value is at its maximum. The DPNase and diazoacetylpyridine are removed from the medium respectively by centrifugation and continuous extraction with ether during 24 hr. The medium is then chromatographed on Dowex 1×2 (200–400 Mesh, 2×60 cm, formiate form) linear gradient of formic acid 0–0.4 N. The fractions for which the ratio of the absorptions at 260 nm and at 307 nm is about 3, are combined and concentrated to ca. 100 ml under reduced pressure. The pH is brought to 7.1 with 0.1 M Tris–HCl buffer. The residual NAD^+ is hydrolysed by the NAD^+ glycohydrolase from *Neurospora Crassa* [11]: 0.6–1 unit of this enzyme in 0.1 M Tris–HCl buffer pH 7.1 (50 ml) is added to the above solution. After the total disappearance of NAD^+ (followed by enzymatic reduction with ethanol and YADH), the solution is chromatographed as described above. Fractions containing the analogue are combined, evaporated under reduced pressure to a small volume and finally liophilised. The analogue obtained is stored at −20°C under nitrogen, the overall yield with respect to NAD^+ is 13%.

No increase of the absorption at 340 nm was observed after addition of ethanol and yeast alcohol dehydrogenase. By electrophoresis according to [6], no other product was detected. The UV spectrum of the 3-diazoacetylpyridine adenine dinucleotide is: λ_{max} 258.5 nm (ϵ = 18200) and λ_{max} 307 nm (ϵ = 9100) (Tris–HCl buffer 0.1 M pH 7.1). KCN N in water: ϵ_{258} = 16 000, ϵ_{365} = 13 200. NMR C_{13}: see table 1.

2.2. *Preparation of 3-chloroacetylpyridine adenine dinucleotide 2* ☆

3-Diazoacetylpyridine adenine dinucleotide (15 mg) is dissolved in aqueous solution (0.7 ml), 2 M in lithium chloride, 0.2 M in chlorhydric acid. The disappearance of the absorption band at 307 nm is complete after 4 hr. The excess of lithium and chloride ions are removed by chromatography on Biogel P_2 (2×60 cm, elution with water). The analogue is eluted after the void volume. This solution of 3-chloroacetylpyridine adenine dinucleotide is used as such. It may be stored in frozen solution for four to five days. On electrophoresis [6], one single spot is detected. The UV spectrum is λ_{max} 260 nm (ϵ = 18 000). NMR C_{13}: see table 1.

The stability of the 3-chloroacetylpyridine adenine dinucleotide in aqueous solution has been determined at pH 7.0 and pH 8.8 at 20°C. The pH was kept constant by addition of 0.02 N sodium hydroxide solution. The half life is 3.5 mn at pH 8.8. The final volume of base added corresponds to one equivalent of the coenzyme analogue. At pH 7.0, the half life is about 4 hr. The reduced form of this analogue has the following spectral characterisation: λ_{max} 258.5 nm (ϵ = 13 300) and λ_{max} 374.5 (ϵ = 10 000). NMR C_{13}: see table 1.

2.3. *Preparation of 3-propionylpyridine adenine dinucleotide 1* ☆☆

This analogue is prepared by enzymatic transglucosidation in a similar manner to that described above. The yield with respect to NAD^+ is about 15 to 20%.

UV: ϵ_{259} = 16300 (Tris–HCl buffer 0.1 M pH 7.1)

reduced form: ϵ_{259} = 14000 (same buffer); $\epsilon_{362.5}$ = 9350

KCN M in water: ϵ_{259} = 13470; $\epsilon_{343.5}$ = 7800

NMR C_{13}: see table 1.

3. Discussion

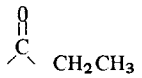
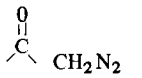
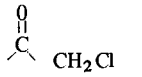
Before the instability of the chloroacetyl group in neutral and basic pH was known, the preparation of 3-chloroacetylpyridine adenine dinucleotide by enzymatic exchange catalysed by the NAD^+ glycohydrolase was attempted.

$NAD^+ + PyX \rightarrow PyX\text{–}AD^+ +$ nicotinamide

☆ We have prepared also the 4-chloroacetylpyridine adenine dinucleotide.

Table 1
^{13}C chemical shifts* of the NAD^+ analogues**

	Adenine ring					Adenine ribose				
	A-2	A-4	A-5	A-6	A-8	A-1′	A-2′	A-3′	A-4′	A-5′
3	117	120	89.7	121.7	114.7	59.0	45.7	41.1	55.1	35.9
1	117.0	120.5	90.1	122.6	114.8	59.1	45.6	41.3	55.2	35.9
2	117.4	120.4	90.1	122.4	115.0	59.0	45.6	41.1	55.1	35.9
	Nicotinamide ring					Nicotinamide ribose				
	N-2	N-3	N-4	N-5	N-6	N-1′	N-2′	N-3′	N-4′	N-5′
3	112.5	107.4	117.8	100.1	114.1	71.3	48.7	41.7	58.3	35.9
1	111.3	107.6	117.5	100.7	114.2	71.4	48.8	41.7	58.3	35.9
2	112.0	110.6	117.4	100	114.1	71.0	48.8	41.7	58.2	35.9

	Other carbons					
3		171.5	CH_2	3.0	CH_3	23.2
1		>150	–CH–	30.9		
2		>180	CH_2Cl	40.6		

* Shifts are in ppm at 25.1 MHz and are referred to a tBuOH standard. All shifts are upfield relative to the standard.
** 3: 3-propionylpyridine adenine dinucleotide; 1: 3-diazoacetylpyridine adenine dinucleotide; 2: 3-chloroacetylpyridine adenine dinucleotide.

However, in the presence of the 3-chloroacetylpyridine☆, the transglucosidation is strongly inhibited. Therefore, for the preparation of the 3-chloroacetylpyridine adenine dinucleotide, an intermediate was used, which is stable enough to be stored, easy to separate from NAD^+ and readily converted to the reactive analogue in high yield. The diazoketone group is a good precursor for chloroketone. The 3-diazoacetylpyridine is exchanged by the transglycosidase. During this preparation it was found that the hydrolysis of NAD^+ to nicotinamide and adenine diphosphoribose is slowed down by low pH and that the exchange reaction of nicotinamide with other pyridines is less sensitive to the pH. Thus, the 3-diazoacetylpyridine adenine dinucleotide can be obtained in a reasonably good yield from NAD^+. Excess of 3-diazoacetylpyridine is extracted with ether in order to avoid appearance of decomposition products during the chromatography.

☆ The 3-chloroacetylpyridine shows a tendency to form oligomers in neutral solution like the 3- and 4-bromoacetylpyridine [12].

The NAD^+ is removed by enzymatic cleavage with the NAD^+ glycohydrolase from *Neurospora Crassa*, which does not seem to act on the 3-diazoacetylpyridine adenine dinucleotide with a rate similar to that for NAD^+.

The 3-diazoacetylpyridine adenine dinucleotide is very slowly reduced by ethanol in presence of alcohol dehydrogenase from horse liver (commercial preparation). No reduction in the presence of yeast alcohol dehydrogenase is detected.

The 3-deazoacetylpyridine adenine dinucleotide is converted to the 3-chloroacetylpyridine adenine dinucleotide by treatment with hydrochloric acid in the presence of lithium chloride.

The structure and purity of these compounds are demonstrated by NMR C_{13} [13, 14]: see table 1. The data are to be compared to those published for NAD^+ [13] and for chloroacetyl and propionyl side chain [14].

The 3-chloroacetylpyridine adenine dinucleotide is active as hydrogen acceptor with the glutamate

dehydrogenase, 17 β-hydroxysteroid dehydrogenase and slightly active with horse liver alcohol dehydrogenase. No such activity was detected with yeast alcohol dehydrogenase or with octopine dehydrogenase.

The following enzymes are inactivated in the presence of 3-chloroacetylpyridine adenine dinucleotide: 17 β-hydroxysteroid dehydrogenase, horse liver and yeast alcohol dehydrogenase.

The lack of reactivity of octopine dehydrogenase with this alkylating analogue is probably due to the fact that this analogue does not enter the active site as suggested by the lack of activity and inhibition with the 3-propionylpyridine adinine dinucleotide. On the other hand, the glutamate dehydrogenase accepts as coenzyme the 3-chloroacetylpyridine adenine dinucleotide and no detectable inhibition occurs. Therefore no basic group is close to the substituent at C-3 in this enzyme. This might imply that no basic group is needed for the oxidation of glutamate.

Further aspects on these analogues and alkylation of dehydrogenases will be reported later.

Acknowledgements

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