THE SYNTHESIS OF N^6-(6-AMINOHEXYL)-NAD$^+$ AND ITS APPLICATION TO AFFINITY CHROMATOGRAPHY

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1. Introduction

Immobilised group-specific supports have been well established as advantageous in the fractionation of groups of enzymes from complex mixtures [1–4]. With many poly-functional cofactors acute synthetic problems have been encountered when attempts were made to immobilise cofactors to the matrix backbone. This has led to the preparation of several functional polymers which are effective in the fractionation of group-specific enzymes but have limited value since the mode of attachment of the cofactor is ambiguous. Nevertheless several preparations of chemically defined polymers have been described [5–7]. Two novel syntheses of a defined N^6-immobilised 5′-AMP have recently been described [8, 9], and successfully used both in the purification of a variety of enzymes [9, 10] and in the examination of some of the parameters relevant to affinity chromatography [11, 12].

To increase the specificity of this support it is desirable to attach the additional portions of the nucleotide associated with the natural cofactor relevant to the enzymes under examination. In this context we now describe what we believe to be the first preparation of a defined NAD$^+$ polymer for affinity chromatography.

2. Materials and methods

2.1. *Materials*

Enzymes and their substrates were purchased from C.F. Boehringer, Mannheim, Germany except for malate dehydrogenase which was obtained from Sigma (London) Chemical Co. Ltd., London S.W. 6., U.K. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia (G.B.) Ltd., London, W.5., U.K. and Dowex-1 and Dowex-50 was from Biorad Labs., California, USA. All other chemicals were either as described previously [9] or of the highest purity available from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

2.2. *General methods*

Ultraviolet spectra were obtained with a Pye-Unicam SP 1800 double beam spectrophotometer. Thin-layer chromatography was carried out on aluminium plates precoated with cellulose (0.1 mm thick) in the following solvent systems: [I] $(NH_4)_2SO_4$ (70 g) plus 100 ml water: 0.1 M NaH_2PO_4, pH 6.0:*iso*-propanol (79:19:2); [II] *iso*-propanol:1% $(NH_4)_2SO_4$ (2:1); [III] Pyridine:H_2O (2:1); [IV] *n*-butanol:pyridine:H_2O (3:2:1.5); [V] Ethanol:1M acetic acid, pH 3.8 with 1M NH_4OH (5:2); [VI] Ethanol:acetic acid:H_2O (174:1:173); [VII] Butanol:acetic acid:H_2O (5:3:2). Two-dimensional thin-layer chromatography was achieved by developing the plates in solvent [I] followed by solvent [II]. Compounds were detected by viewing under ultraviolet light (254 nm), enzymic assay, and/or spraying with group-specific reagents. Electrophoresis was performed on Whatman No. 1 or 3 MM paper in acetic acid: formic acid:H_2O, pH 1.9 (15:5:80) at 60 V/cm for 2 hr. The sample was applied as a narrow band at a position 10 cm from the anode on a 40 cm sheet.

Enzymes were assayey by the methods cited by Barman [13] and protein was measured by the ultraviolet absorption method [14]. Enzyme chromatography on affinity columns (1.0 g moist weight) was performed according to the procedures outlined pre viously [9].

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Table 1
Chromatographic and electrophoretic mobilities of relevant nucleotides.

Solvent	Chromatographic R_f values I	II	III	IV	V	VI	VII	Electrophoretic mobility
Nucleotide								M_L*
N^6-(6-Aminohexyl)-NAD^+	0.48	0.04	0.40	0.03	0.08	0.53	0.07	80
N^6-(6-Aminohexyl)-5'-AMP	0.33	0.23	0.38	0.04	0.30	0.71	0.18	100
Dinicotinamide-dinucleotide	0.70	0.03	0.20	0.01	0.02	0.36	0.03	16
NMN	0.82	0.13	0.30	0.04	0.16	0.57	0.18	16
NAD^+	0.40	0.05	0.48	0.04	0.04	0.42	0.02	16
5'-AMP	0.30	0.20	0.54	0.02	0.16	N.D.	0.19	N.D.

* Mobility relative to N^6-(6-Aminohexyl)-5'-AMP which moved 22 cm towards the cathode. Experimental procedure and solvent composition are given in the text. N.D. = not determined.

2.3. *Preparation of reaction nucleotides*

The synthesis of N^6-(6-Aminohexyl)-5'-AMP was performed according to the method of Craven et al. [9]. Nicotinamide mononucleotide and dinicotinamide-dinucleotide were prepared from nicotinamide adenine dinucleotide after the procedure of Shuster et al. [15] with the following modifications during the purification stages. After Dowex 1-X8 (formate), 200–400 mesh, chromatography the water effluent was lyophilised, redissolved in a minimal volume of water and applied to a Dowex 50-X4 (H^+), 200–400 mesh, column (1.5 × 30 cm). Nicotinamide mononucleotide and dinicotinamide-dinucleotide were eluted with water as an overlapping double peak which was lyophilised and dissolved in water prior to a final purification on Dowex 1-X8 (formate), 200–400 mesh, column (2.5 × 30 cm). Dinicotinamide-dinucleotide was eluted with water from this resin, while nicotinamide mononucleotide elution was effected with 0.02 M formic acid. The purity of the two fractions was established by ultraviolet spectra and by thin-layer chromatography in solvents I to VII; the observed R_f values are given in table 1.

2.4. *Preparation of N^6-(6-Aminohexyl)-NAD^+*

Trifluoroaceticanhydride [15] (0.5 ml) was added to a mixture of N^6-(6-Aminohexyl)-5'-AMP (45 mg) and either nicotinamide mononucleotide (45 mg) or dinicotinamide-dinucleotide (45 mg). The mixture was left overnight (16 hr) in a sealed test tube at room temperature. The anhydride was removed under reduced pressure; the viscous light-brown residue was washed copiously with ether and dried under reduced pressure in a vacuum desiccator over KOH/ P_2O_5. The addition of further trifluoracetic anhydride at that stage followed by another 16 hr reaction period did not enhance the yield of N^6-(6-Aminohexyl)-NAD^+ The straw-coloured residue was dissolved in a minimal volume of water and applied at pH 7.0, adjusted with KOH, to a column (2.5 × 30 cm) of Dowex 1-X8 (formate) resin, 200–400 mesh. The column was washed with water to effect elution of N^6-(6-Aminohexyl)-NAD^+, nicotinamide mononucleotide and dinicotinamide-dinucleotide. A further coenzymically active fraction, probably trifluoracetyl-amino-alkyl-NAD^+ (representing 30% of the total NAD^+ formed) was eluted by a 0 to 1 M gradient of formic acid. N^6-(6-Aminohexyl)-NAD^+ was separated from other nicotinamide-containing products by paper electrophoresis at pH 1.9. The desired product was eluted with water following location by enzymatic analysis of ultraviolet absorbing bands. The yield of N^6-(6-Aminohexyl)-NAD^+ was 20–25% based on the initial N^6-(6-Aminohexyl)-5'-AMP concentration; the product was assayed in the yeast alcohol dehydrogenase system: 0.1 M sodium pyrophosphate, pH 10.0, 0.5 M ethanol.

No significant difference in the yield of N^6-(6-Aminohexyl)-NAD^+ was apparent when dinicotinamide-dinucleotide was used as the starting material in preference to nicotinamide mononucleotide. Likewise nicotinamide adenine dinucleotide can be used directly in the synthesis although some considerable difficulty was experienced in separating the reaction products.

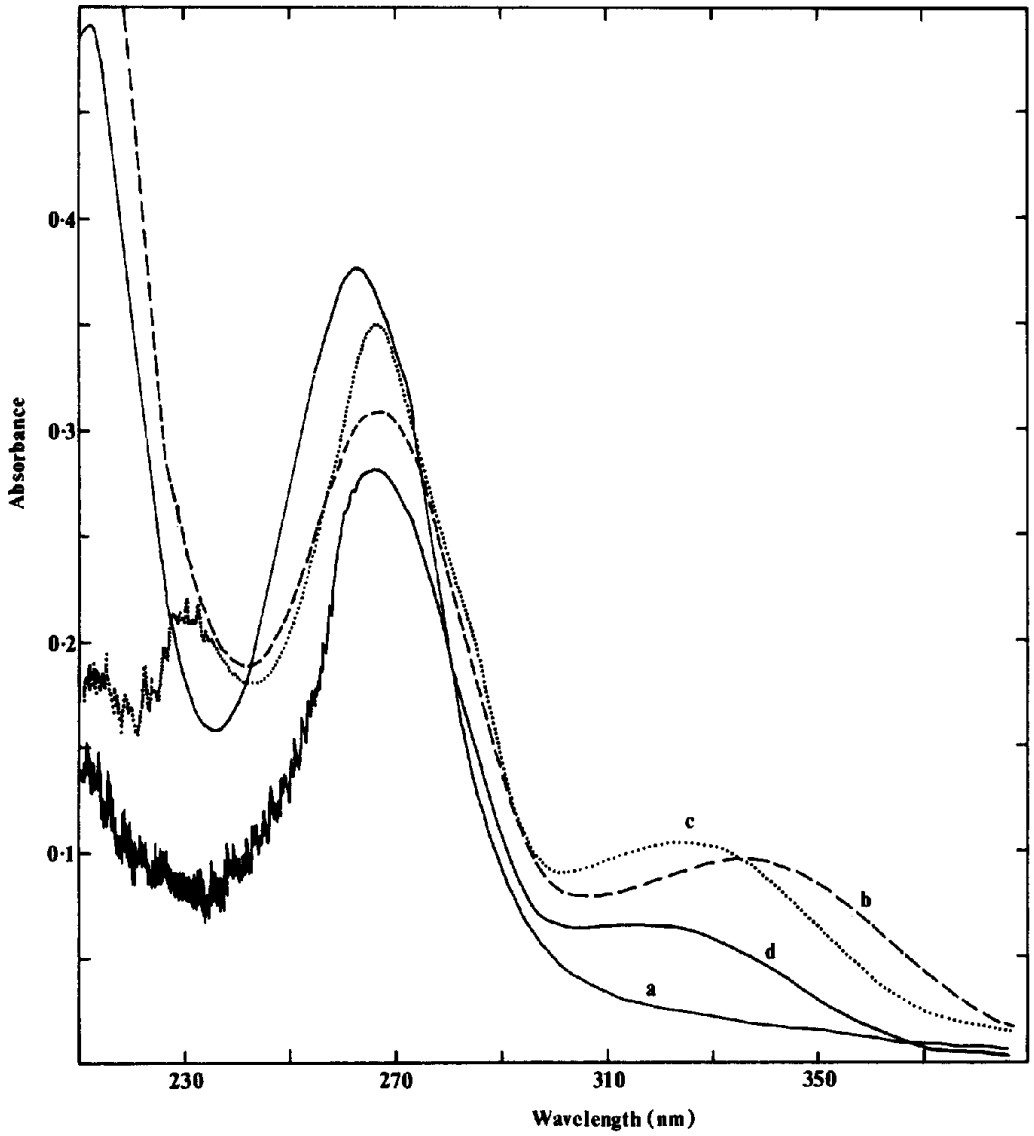

Fig. 1. Absorption spectra; (a) (——) N^6-(6-Aminohexyl)-NAD$^+$, pH 7.0; (b) (·----) N^6-(6-Aminohexyl)-NADH, enzymic reduction; (c) (. . . .) N^6-(6-Aminohexyl)-NAD-cyanide adduct; (d) (——) N^6-(6-Aminohexyl)-NAD-sulphite adduct.

2.5. *Coupling of N^6-(6-Aminohexyl)-NAD$^+$ to Sepharose 4B*

Cyanogen bromide-activated-Sepharose 4B (3 g moist weight) was suspended in a solution of 0.1 M $NaHCO_3$, pH 10.0, containing 6.0 μmoles of N^6-(6-Aminohexyl)-NAD$^+$. The suspension was rotated overnight (16 hr) on a Coulter mixer at 4°C. The matrix was subjected to successive washings in water, 1 M KCl and water prior to storage in 0.02% sodium azide solution. Spectral analysis (267 nm) of these washings indicated a quantitative coupling on the assumption that E_{260nm} N^6-(6-Aminohexyl)-NAD$^+$ was 20.2×10^6.

Table 1
A comparison of the binding of various enzymes to immobilised nucleotide affinity columns.

EC Number	Enzyme	Binding (β)		
		I	II	III
1.1.1.27	Lactate dehydrogenase (pig heart)	0.35[a]	> 1M[b]	325
	Lactate dehydrogenase (rabbit muscle)	0.80[a]	> 1M[b]	500
1.1.1.1.	Alcohol dehydrogenase (yeast)	700	400	415
	Alcohol dehydrogenase (horse liver)	290	450[d]	130
1.1.1.49	D-Glucose 6-phosphate dehydrogenase	140	0	125
1.1.1.37	L-Malate dehydrogenase	300	65	45
1.1.1.42	Isocitrate dehydrogenase	0	0	220
1.1.1.30	D-3-Hydroxybutyrate dehydrogenase	185	250[d]	250
1.2.1.12	D-Glyceraldehyde 3-phospahte dehydrogenase	1.0[a]	0	> 1M[b]
1.4.1.3	L-Glutamate dehydrogenase	430	c	c
1.6.4.2.	Glutathione reductase	75	RT	120
2.7.1.1.	Hexokinase	0	0	100
2.7.1.30	Glycerokinase	RT	122	NT
2.7.1.40	Pyruvic kinase	100	100	0
2.7.2.3.	3-Phosphoglycerate kinase	NT	70	NT
2.7.3.2.	Creatine kinase	NT	0	0
2.7.4.3.	Myokinase	50	0	RT

I N^6-(6-Aminohexyl)-NAD^+-Sepharose.
II N^6-(6-Aminohexyl)-5'-AMP-Sepharose.
III ϵ-Aminohexanoyl-NAD^+-Sepharose.

'Binding (β)' refers to the measure of the strength of the enzyme-immobilised nucleotide interaction and is the concentration (mM) of KCl at the centre of the enzyme peak when the enzyme was eluted with a linear gradient of KCl. The immobilised ligand concentration was 2.0 μmoles nucleotide/ml for each polymer.

[a] Elution could not be effected by 1M KCl, enzyme eluted in 0–5 mM NADH gradient (10 ml total volume).
[b] Elution was effected by a 200 μl pulse of 5×10^{-3} M NADH.
[c] No enzyme recovered.
[d] β Determined on a polymer containing 16 μmoles 5'-AMP/ml.
RT Enzyme retarded by the polymer and eluted behind the void volume as determined by bovine serum albumin.
NT Not tested

3. Results and discussion

3.1. *Physical and chemical properties of N^6-(6-Aminohexyl)-NAD^+*

The ultraviolet spectra of N^6-(6-Aminohexyl)-NAD^+ in the oxidised and reduced state, and in the presence of cyanide and sulphite, are illustrated in fig. 1. The absorption maximum of N^6-(6-Aminohexyl)-NAD^+ was observed at 263 nm, thus being similar to the maxima observed for N^6-(6-Aminohexyl)-5'-AMP [9]. This maximum shifted slightly upon enzymic reduction and the maximum of the reduced form was located at 338 nm. The near ultraviolet bands of the cyanide and sulphite adducts of N^6-(6-Aminohexyl)-NAD^+ have maxima of 325 nm and 318 nm respectively and compare favourably with the known maxima of NAD^+ in the presence of these compounds.

The R_f values for N^6-(6-Aminohexyl)-NAD^+ after thin-layer chromatography in solvents I to VII are compared with those of NAD^+, NMN, dinicotinamide-dinucleotide and N^6-(6-Aminohexyl)-5'-AMP in table 1. The single ultraviolet absorbing spot associated with N^6-(6-Aminohexyl)-NAD^+ reacted positively with the nicotinamide ribosyl detecting reagent, ethyl methyl ketone:0.880 NH_4OH (1:1) [16]. Bound phosphate located by the Hanes–Isherwood reagent [17] was

coincident with this spot; no inorganic phosphate could be detected. Ninhydrin and bromocresol green reagents gave a positive reaction for an amino group. Likewise the quantitative coupling observed on reaction with cyanogen bromide-activated-Sepharose 4B indicated the involvement of the terminal primary amino group of N^6-(6-aminohexyl)-NAD^+ in the immobilisation of this nucleotide to the Sepharose matrix.

3.2. *Enzyme studies with N^6-(6-Aminohexyl)-NAD^+-Sepharose*

The interaction of several dehydrogenases and kinases with N^6-(6-Aminohexyl)-NAD^+-Sepharose 4B is documented in table 2 and compared with the binding data obtained with N^6-(6-Aminohexyl)-5′-AMP-Sepharose [9] and ϵ-aminohexanoyl-NAD^+-Sepharose [18] prepared according to the procedure of Larsson and Mosbach [19]. In general it was observed that the interaction as measured by β, of NAD^+-dependent dehydrogenases increased with the defined NAD^+-polymer over that observed not only with the undefined NAD^+-polymer but also N^6-immobilised 5′-AMP. This was particularly apparent with yeast alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase.

In summary, the affinity of kinases and $NADP^+$-dependent dehydrogenases for N^6-immobilised NAD^+ is decreased or unchanged. This indicates that the specificity of the affinity matrix is increased for NAD^+-linked dehydrogenases upon immobilisation of their natural cofactor.

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