THE PREPARATION AND CHARACTERISATION OF A WATER-SOLUBLE COENZYMICALLY ACTIVE DEXTRAN-NAD$^+$

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1. Introduction

Matrix-bound nucleotides have recently attracted considerable interest mainly due to their excellent properties as adsorbents for affinity chromatography [1–5]. The application of immobilised nucleotides as active cofactors for dehydrogenases is another aspect that is also rapidly gaining momentum. Matrix-bound nicotinamide nucleotides could be repeatedly used and would thus be attractive components in enzyme reactors and enzyme electrodes. It has been shown that NAD$^+$ coupled to agarose [6] or glass [7] is indeed active as a cofactor, but compared with free NAD$^+$ the efficiency is rather limited, probably due to steric problems. Considerably more favourable results are likely to be achieved with soluble carriers as polyethyleneimine [8] and dextran which allow a more unhindered interaction between the cofactor and enzyme.

The present report describes the preparation and characterisation of a soluble high molecular weight derivative of NAD$^+$, dextran–NAD$^+$, obtained by coupling NAD$^+$-N^6-[*N*-(6-aminohexyl)-acetamide] to cyanogen bromide activated dextran, mol. wt. 40 000. It is coenzymically active with yeast alcohol dehydrogenase (ADH) and rabbit muscle lactate dehydrogenase (LDH) and can also recycle in a system containing both enzymes.

2. Materials and methods

Dextran T 40 (mol. wt. = 40 000) and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Yeast alcohol dehydrogenase (335 U/mg) and rabbit muscle lactate dehydrogenase (675 U/mg) were purchased from Sigma (St. Louis, Mo., U.S.A.). NAD$^+$-N^6-[*N*-(6-aminohexyl)-acetamide] was prepared essentially as described earlier [5] and the reduced analogue was obtained after reduction with yeast ADH [9]. Sepharose-NAD$^+$ was prepared by coupling NAD$^+$-N^6-[*N*-(6-aminohexyl)-acetamide] to cyanogen bromide activated Sepharose 4B [5,10]. To minimise crosslinking of the gel, a rather low cyanogen bromide concentration (10 mg/g wet gel) was employed. The resulting Sepharose-NAD$^+$ contained 25 μmoles nucleotide/g wet gel. Chemical reduction with sodium dithionite yielded Sepharose-NADH [5].

2.1. *Preparation of dextran-NAD$^+$*

Dextran T 40 (5.0 g) was dissolved in 50 ml water (20°C), a solution of 0.25 g cyanogen bromide in 5 ml of water was added and pH maintained at 10.8 by continuous addition of 1 M NaOH. After 5 min the consumption of NaOH ceased and the activation was judged complete. The pH was lowered to 8.5 with 0.1 M HCl and 0.90 mmol of NAD$^+$-N^6-[*N*-(6-aminohexyl)-acetamide] dissolved in 5 ml water was added. The coupling was allowed to proceed at pH 8.5 for 12 hr at room temperature whence any residual active groups were quenched by treatment with 0.2 M ethanolamine-HCl buffer pH 8.0 for 1 hr at room temperature. The reaction mixture was diluted to 500 ml with 0.1 M LiCl, adjusted to pH 6.8 with HCl and applied to a Sephadex G-50 column (85 × 5 cm). The dilution lowered the viscosity enough to prevent anomalous gel filtration behaviour. Elution was performed with 0.1 M LiCl and the effluent collected as 20 ml

fractions. Fractions 25–55 contained dextran–NAD^+ and fractions 65–100 uncoupled analogue. The dextran derivative was concentrated on a rotary evaporator (30°C) to approximately 100 ml and then pipetted into 1.5 l of vigorously stirred ethanol. The precipitate was filtered off, washed with ethanol and ether and dried in a vacuum yielding 5.1 g of a white powder. Ultraviolet measurements indicated a nucleotide content of 65 μmoles/g and a coupling yield of 36% (assuming a molar absorption coefficient of 21 700 M^{-1} cm^{-1} [5]). The same procedure was followed when 'black dextran' was prepared. In this case the NAD^+ analogue was replaced by 0.90 mmol of *n*-butylamine. Chemical reduction with sodium dithionite gave dextran-NADH (11). The product contained 65 μmoles/g according to measurements at 340 nm, assuming a molar absorption coefficient of 6200 M^{-1} cm^{-1}.

2.2. *Enzyme assays*

Cofactor reduction rates were determined with yeast ADH, cofactor oxidation rates with rabbit muscle LDH and cofactor cycling rates with both enzymes present in the assay medium. All determinations were carried out at 25°C and at pH 8.0 in an assay medium of the following composition: 100 mM ethanol, 10 mM pyruvate, 1 mM glutathione, 50 mM Tris–HCl and 0.10 mM oxidised or reduced cofactor. In some assays 15.5 mg/ml of blank dextran was added (see table 1).

The reduction and oxidation rates were calculated from direct spectrophotometric measurements at 340 nm, and the reactions were initiated by adding 0.30 μg/ml ADH or 0.06 μg/ml LDH. Assays with Sepharose-NAD(H) were carried out in a cell equipped with magnetic stirring to prevent settling of the gel, and to obtain reasonable conversion rates enzyme concentrations also higher than those given above were used (1–20 times).

The cycling experiment was initiated by the addition of 3.0 μg/ml ADH and 0.60 μg/ml LDH. At intervals, samples of 0.50 ml were withdrawn and pipetted into 0.50 ml of 0.25% H_2O_2 and the mixture heated at 100°C for 20 min. The lactate concentrations were then determined enzymically by mixing the peroxide treated sample (0.050–0.200 ml) with 0.95–0.80 ml assay medium containing 100 μmoles glycine–NaOH buffer, pH 9.6, 200 μmoles hydrazine and 2.0 μmoles NAD^+. LDH (50 μg) was added and after 2 hr the change in absorbance at 340 nm was measured and the lactate concentration estimated by comparison with a standard curve.

3. Results and discussion

The NAD^+ analogue NAD^+-[*N*-(6-aminohexyl)-acetamide] was coupled via its terminal amino group to a soluble dextran giving dextran-NAD^+ with a nucleotide content of 65 μmoles/g, which corresponds to about three cofactor molecules per dextran molecule. A procedure with a very low cyanogen bromide/dextran ratio (1 molecule of CN Br per 15 glucose residues) was adopted in order to minimise crosslinking of the polymer and also to ensure a medium practically devoid of unreacted cyanogen bromide when the NAD^+ analogue was added.

The ultraviolet spectrum of dextran-NAD^+ in Tris buffer (fig. 1) had a maximum at 266 nm, characteristic of N^6 substituted adenosine derivatives, and in 1 M KCN the spectrum showed an additional peak at 325 nm, typical of N^1 substituted nicotinamide. The ratio A_{325}/A_{266} for dextran-NAD^+ was calculated as 0.34 and the same ratio for the free NAD^+ analogue as 0.35, indicating that only neglible loss of nicotinamide had occurred during coupling. This was also supported by spectra obtained from dextran–NAD^+ before and after reduction with dithionite. Reduction with yeast ADH at pH 9.5 also afforded the expected peak at 340 nm, and the amount of enzymically reducible nucleotide was calculated as approximately 80%. The remaining 20% was probably sterically unavailable to the enzyme due to crosslinking of the polymer. The derivative was readily soluble in water, but yielded solutions more viscous than the original Dextran T 40 did, suggesting that some crosslinking actually had occurred during the activation and coupling steps. In line with this observation it was found that a 5-fold increase in cyanogen bromide concentration gave a very good coupling yield, but the derivative was difficult to dissolve in water and could be reduced enzymically only to the extent of 25%. The possibility that the coenzymically inactive portion consisted of analogues bound to dextran via some other group than the terminal aliphatic amino group is considered unlikely based on a separate experiment, where activated dextran was allowed to react with unsubstituted NAD^+. Only

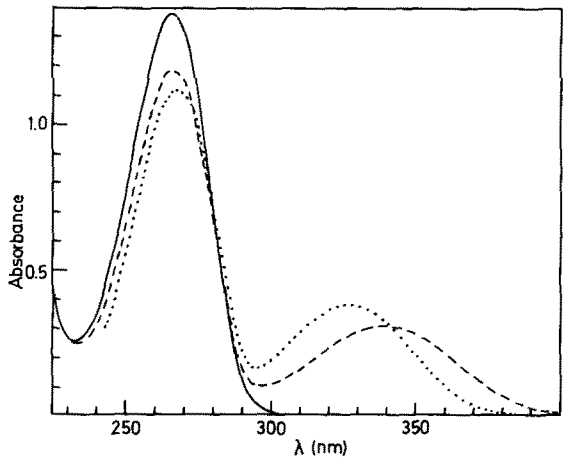

Fig. 1. Ultraviolet spectra of dextran-bound NAD^+ derivatives. The spectra were recorded on a Beckman Acta III spectrophotometer using a cell with a path length of 1.00 cm. The concentration of dextran–NAD^+ was 1.00 mg/ml. Spectrum in 0.10 M Tris–HCl buffer, pH 7.5 (———). Spectrum in 1.0 M KCN, (.....). Spectrum obtained after reduction for 15 min with yeast ADH (25 μg/ml) in 0.33 M ethanol and 0.10 M glycine–NaOH buffer, pH 9.5 (- - - - - -). The reference cells contained the same ingredients as the sample cells except that dextran–NAD^+ was replaced by blank dextran.

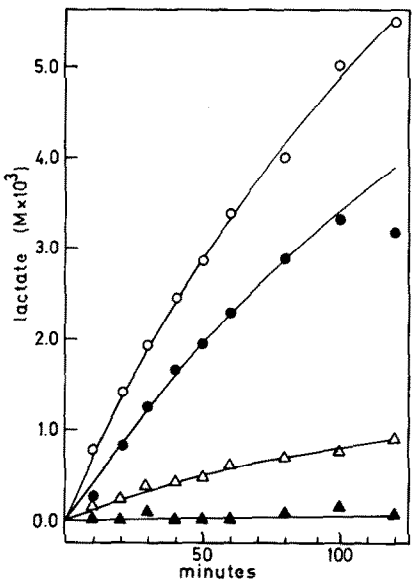

Fig. 2. Recycling of free and polymer-bound nicotinamide nucleotides by the two-enzyme system alcohol dehydrogenase and lactate dehydrogenase. The cycling was monitored by determinations of lactate in samples withdrawn from the incubation mixtures. Details are given in Methods. ○ = NAD^+. ● = NAD^+-N^6-[*N*-(6-aminohexyl)-acetamide]. △ = dextran–NAD^+. ▲ = Sepharose–NAD^+.

a very low coupling yield was however observed (1 μmole/g).

The dextran-NAD^+ preparation was stable when kept as a dry powder. No release of free nucleotide or loss of cofactor activity could be detected after three months storage at 4°C. The stability in aqueous media was also quite good, although somewhat lower than would be expected of a derivative prepared by the cyanogen bromide coupling procedure [12]. After four days at 25°C in 0.1 M Tris–HCl buffer, pH 8.0, separation on a Sephadex G-50 column showed that 90% of the nucleotide remained attached to the dextran and was still reducible to an extent of 80% with yeast ADH.

3.1. *Cofactor activity*

The cofactor activity of native NAD^+ with yeast ADH and rabbit muscle LDH was compared with that of the free analogue, NAD^+-N^6-[*N*-(6-aminohexyl)-acetamide] and the dextran- and Sepharose-bound analogues (fig. 2, table 1). As expected, NAD^+ proved to be the most efficient cofactor in the systems tested. Substitution in position N^6 reduced the activity to 75–60%, attachment to dextran further decreased the activity to about 15% and binding to Sepharose, finally, yielded a derivative 2–3 orders of magnitude less efficient than NAD^+. It is conceivable that the decreased coenzymic activity of the immobilised nucleotides is the result of shielding by the polymer lattice and of restrictions imposed on the nucleotide mobilities. Thus, it is not surprising that 20% of dextran-bound nucleotide and about 70% of Sepharose-bound nucleotide could not be reduced at all by yeast ADH, even after extended incubation with high enzyme concentrations. The dextran polymer itself at the concentrations used seemed however not to affect the conversion rates to any appreciable extent. The table shows that addition of blank dextran to free cofactors changed the rates less than 10%. Another factor that would be especially pronounced for Sepharose–NAD^+, is the trapping of enzyme molecules in complexes with preferentially reduced polymer-bound nucleotides. Such complexes could have a considerable life-time in the absence of interacting free nucleotides, and would thus lower the

Table 1
Free and polymer-bound nicotinamide nucleotides as cofactors to alcohol dehydrogenase and lactate dehydrogenase

Cofactor	Reduction rate with ADH		Oxidation rate with LDH		Cycling rate with ADH + LDH		
	(μM/min)	(%)	(μM/min)	(%)	(μM/min)	(cycles/hr)	(%)
NAD (H)	28	100	16	100	69	41	100
NAD(H) + blank dextran	29	104	15	95	–	–	–
N^6-R-NAD(H)	17	61	12	76	42	25	61
N^6-R-NAD(H) blank dextran	16	57	11	70	–	–	–
dextran–NAD(H)	4.3	16	2.3	14	12	7.4	18
Sepharose–NAD(H)	0.2	0.7	0.02	0.1	0.3	0.2	0.5

The cofactor reduction and oxidation rates were determined at 340 nm with yeast ADH (0.30 μg/ml) or with rabbit muscle LDH (0.06 μg/ml). The cycling experiment was carried out with a 10-fold higher concentration of both enzymes, and the cycling rates are expressed as the initial rates of formation of lactate (derived from fig. 2) or as the number of redox cycles of the cofactor per hour. All rates are also given relative to free NAD(H) (NAD(H) = 100%). Further details are given in Methods. N^6-R-NAD(H) = NAD(H)-N^6-[*N*-(6-aminohexyl)-acetamide].

concentration of the catalytically operational enzyme.

The data in table 1 and the curves in fig. 2 clearly show that dextran–NAD^+ is a far more efficient cofactor than Sepharose–NAD^+. The fact that the actual numerical values with Sepharose–NAD^+ are not very accurate and that fair comparisons are difficult to achieve, does not obscure this conclusion.

4. Conclusion

The comparatively facile immobilisation technique for dextran–NAD^+ described here, is obviously applicable to other cofactors provided with aliphatic amino groups. The fact that NAD^+ is bound to the matrix through an alkyl linkage offers in our opinion an advantage over a previously described polyethyleneimine-bound NAD^+ preparation [8] involving a more labile acyl linkage. Thus the dextran–NAD^+ has excellent storage stability and satisfactory operational stability and should hence constitute a valuable tool in applications where the possibility of recycling the dextran–NAD^+ could be advantageously exploited. The high molecular weight dextran-NAD^+ can be spatially confined by porous membranes with one or more enzymes or entrapped in, for example, crosslinked polyacrylamide pellets to form complete self-contained catalytic systems. Results from such applications using dextran–NAD^+ in an enzyme electrode and in model enzyme reactors look promising (Davies, P. and Mosbach, K., to be published).

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