PREPARATION OF POLYETHYLENE GLYCOL-BOUND NAD AND ITS APPLICATION IN A MODEL ENZYME REACTOR

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1. Introduction

Recently many kinds of water-soluble macromolecular NAD derivatives have been prepared and their coenzymatic properties and applications in biochemical reactors have been reported [1–4]. However, there have been no reports of systematic investigations about the structure and cofactor activity of macromolecular NAD derivatives.

We have synthesized a NAD derivative carrying a vinyl group [5]. The NAD derivative was copolymerized with acrylamide or methacrylamide and various kinds of water-soluble macromolecular NAD derivatives (polymeric NAD derivatives) were obtained [5–7]. Based on the results of the investigation into the coenzymatic properties of the polymeric NAD derivatives, it was suggested that an NAD derivative with a smaller molecular size and a lower NAD content in the polymer chain would have higher cofactor activity. Indeed, a polymeric NAD derivative prepared by copolymerization with methacrylamide had good cofactor activity [6], but the polymeric NAD derivative was not suitable for application in enzyme reactors because of its low NAD content in the total polymer and the wide distribution of its molecular size. Therefore, we planned to make a new water-soluble macromolecular NAD derivative with a fixed NAD content and an appropriate molecular size.

From among soluble polymers suitable for binding of NAD, we used polyethylene glycol, which was commercially available in a variety of molecular weights (1500–20 000) and had only two functional groups at its ends. Here, polyethylene glycol-bound NAD (PEG-NAD) was prepared by coupling of N^6-(2-carboxyethyl)-NAD to monoaminopolyethylene glycol (M_r 3000–3700) with water-soluble carbodiimide. PEG-NAD had as high cofactor activity as N^6-(2-carboxyethyl)-NAD with various dehydrogenases and was used in a continuous enzyme reactor containing rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase.

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2. Materials and methods

2.1. *Materials*

NAD was purchased from Oriental Yeast (Tokyo); horse liver alcohol dehydrogenase (1.85 U/mg), yeast alcohol dehydrogenase (368 U/mg), rabbit muscle lactate dehydrogenase (635 U/mg), chicken heart lactate dehydrogenase (740 U/mg), pig heart mitochondrial malate dehydrogenase (1,160 U/mg), and crystalline bovine serum albumin were from Sigma (St Louis, MO); polyethylene glycol (4000, M_r 3000–3700), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide–HCl, lithium D,L-lactate, sodium D,L-malate, and semicarbazide hydrochloride were from Nakarai Chemicals (Kyoto); silica gel G (type 60) and silica gel HF_{254} (type 60) were from Merck (Darmstadt); Sephadex G-50, SP-Sephadex C-25 and DEAE-Sephadex A-25 were from Pharmacia (Uppsala). N^6-(2-carboxyethyl)-NAD was prepared as in [5].

2.2. *Analytical procedures*

Thin-layer chromatography was carried out on silica gel G in methanol/dichloromethane (1/5, v/v, system I) or on silica gel HF_{254} in isobutyric acid/water/28% aqueous NH_3 (66/33/1.7, v/v, system II). Aliphatic amines were detected by ninhydrin spray, NAD and its derivatives by ultraviolet light, and poly-

ethylene glycol and its derivatives by I_2 vapor. Ultraviolet spectra were obtained with a Hitachi 200-20 spectrophotometer. Infrared spectra were obtained with a Hitachi 215 infrared spectrophotometer. The concentration of the amino groups was determined using 2,4,6-trinitrobenzene sulfonic acid by the method in [8] assuming a molar absorption coefficient of 20 000 $M^{-1} \cdot cm^{-1}$ at 420 nm for the trinitrophenylated amino group. The nucleotide concentration was determined spectrophotometrically assuming molar absorption coefficients of 18 000 $M^{-1} \cdot cm^{-1}$ at 260 nm for NAD, 18 600 $M^{-1} \cdot cm^{-1}$ at 266 nm for N^6-(2-carboxyethyl)-NAD [5], and 23 500 $M^{-1} \cdot cm^{-1}$ at 266 nm for polyethylene glycol bound NAD, which was determined as in [5].

2.3. *Preparation of aminopolyethylene glycol*

The terminal hydroxylic groups of polyethylene glycol were converted to amino groups as in [9] with some modifications. Polyethylene glycol (3 g) was dissolved in CH_2Cl_2 (50 ml), and pyridine (2 ml) and tosylchloride (3.8 g) were added to the solution. After refluxing for 22 h, the mixture was evaporated under reduced pressure. The residue was dissolved in water, filtered to remove the remaining precipitate, and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in CH_2Cl_2 (5 ml), precipitated by addition of ether (200 ml), harvested by filtration, and dried. Yield: 3 g; IR (film): 2880, 1640, 1480, 1200, 1190, 1120 cm^{-1}. The tosylated polyethylene glycol (3 g) was dissolved in dimethylformamide (30 ml), and potassium phthalimide (1 g) was added to the solution. The reaction mixture was stirred under N_2 at 120°C for 3 days, and the polymer product was purified as above. Yield: 3 g; IR (film): 2880, 1780, 1720, 1480, 1120 cm^{-1}. The phthalimide derivative of the polyethylene glycol (3 g) was dissolved in a mixture of CH_2Cl_2 (10 ml) and hydrazine hydrate (100%, 50 ml), and kept for 3 days at 70°C [10]. The polymer product was purified as above. Yield: 2.7 g. The infrared spectrum of the product showed no absorption band for the carbonyl group due to the phthalimide moiety. The polymer product was a mixture of polyethylene glycol derivatives containing 0–2 amino groups/1 polymer molecule, and was chromatographed on a SP-Sephadex C-25 (H^+) column (4 × 60 cm) with an NaCl gradient of 0–30 mM. Two peaks containing amino groups were obtained and were separately extracted with CH_2Cl_2. The organic layers were dried over anhydrous Na_2SO_4, evaporated to remove the solvent, and the content of the amino groups and the dry weight of each residue were measured. Monoaminopolyethylene glycol was obtained from the first peak of the chromatography and its purity was estimated by thin-layer chromatography (1.2 g, R_F in system I = 0.66). Diaminopolyethylene glycol was obtained from the second peak of the chromatography and its purity was estimated by thin-layer chromatography (0.5 g, R_F in system I = 0.54).

2.4. *Preparation of polyethylene glycol-bound NAD*

The monoaminopolyethylene glycol (5 g) was dissolved in water (5 ml), the pH was adjusted to 4.5 with 1 N HCl, and N^6-(2-carboxyethyl)-NAD (0.6 g) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide–HCl (5 g) were added to the solution. The reaction mixture was stirred for 20 h at room temperature keeping pH at 4.5, concentrated by evaporation, and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to remove the solvent. The residue was dissolved in water and passed through a SP-Sephadex C-25 (H^+) column (4 × 60 cm, twice), a DEAE-Sephadex A-25 (Cl^-) column (1.5 × 20 cm, twice), and then a SP-Sephadex C-25 column. The passed fractions were combined, concentrated, extracted with CH_2Cl_2, and the organic layer was evaporated to yield polyethylene glycol bound NAD (PEG-NAD, 0.9 g). The PEG-NAD preparation did not contain other NAD derivatives; this was checked by thin-layer chromatography (R_F of PEG-NAD in system II = 0.16). However, the preparation contained a small amount (~60 mg) of monoaminopolyethylene glycol; this was checked by reaction with 2,4,6-trinitrobenzenesulfonic acid. Sephadex G-50 column chromatography showed that PEG-NAD had larger molecular size than monoaminopolyethylene glycol.

2.5. *Enzyme assays*

The reduction of the NAD and its derivatives was followed at 340 nm with a Hitachi 356 spectrophotometer with a mixing plunger at 30°C. The reactions were initiated by adding a chilled enzyme solution (10 μl) to the prewarmed substrate solution (3 ml). The components of the reaction mixtures are given in the legend to table 1. The enzymes were dissolved in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.005% bovine serum albumin.

2.6. *Continuous enzyme reaction*

A reaction mixture (1 ml) containing 10 mM pyruvate, 0.5 M ethanol, 3 mM PEG-NAD, 0.3% bovine serum albumin and 30 mM sodium phosphate buffer (pH 7.5) was put in a Model 8MC ultrafiltration apparatus (Amicon Corp., Lexington, MA) fitted with a UM 2 ultrafiltration membrane placed in a temperature-controlled chamber (Tabai LN-110) at 30°C. The reaction was initiated by adding lactate dehydrogenase (rabbit muscle, 20 μg) and alcohol dehydrogenase (yeast, 60 μg), and 30 min later, continuous ultrafiltration was done with a substrate solution containing 10 mM pyruvate, 0.5 M ethanol, 50 μg streptomycin/ml and 30 mM sodium phosphate buffer (pH 7.5) as eluent, under 3 kg/cm^2 N_2 pressure, keeping the volume of the reaction mixture constant at 1 ml. The filtrate was collected in fractions at 0.5 h intervals, and the L-lactate concentration and volume of each fraction were measured. L-Lactate was determined with lactate dehydrogenase and NAD, as in [11].

3. Results and discussion

3.1. *Properties of polyethylene glycol-bound NAD*

Polyethylene glycol-bound NAD (PEG-NAD) had an ultraviolet spectrum in water with an absorption maximum at 266 nm (ϵ = 23 500 $M^{-1} \cdot cm^{-1}$).

Table 1 shows the initial rate of reduction of N^6-(2-carboxyethyl)-NAD and PEG-NAD with various dehydrogenases relative to the reduction rate of native NAD under identical conditions. PEG-NAD showed as high cofactor activity as N^6-(2-carboxyethyl)-NAD. As a control experiment, the reduction rate of NAD was measured in the presence or absence of 50 μM monoaminopolyethylene glycol and there was no difference in the rate, whether the glycol was present or not.

Table 2 shows the kinetic constants for the reduction of NAD, N^6-(2-carboxyethyl)-NAD and PEG-NAD by rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase. The K_m value for NAD decreased due to the carboxyethylation and then increased due to the coupling to polyethylene glycol. The V_{max} value for PEG-NAD was similar to that for N^6-(2-carboxyethyl)-NAD and was ~40% of that for NAD.

These results indicate that the effect of the binding of N^6-(2-carboxyethyl)-NAD to polyethylene glycol on the cofactor activity was small and that the polyethylene glycol was a good carrier for the macromolecularization of NAD. This supports our suggestion [6] that an NAD derivative with a smaller molec-

Table 1
Relative initial rates of reduction of N^6-(2-carboxyethyl)-NAD and PEG-NAD with different dehydrogenases

Enzyme	Relative reduction rate (NAD = 100)	
	N^6-(2-carboxyethyl)-NAD	PEG-NAD
Lactate dehydrogenase (rabbit muscle)	77	77
Lactate dehydrogenase (chicken heart)	24	20
Alcohol dehydrogenase (yeast)	64	50
Alcohol dehydrogenase (horse liver)	51	61
Malate dehydrogenase (pig heart)	63	64

The reaction mixture (3.0 ml) contained 0.05 mM coenzyme, 40 mM sodium phosphate buffer (pH 7.5), 10 mM semicarbazide–HCl (pH 7.5), 0.05% bovine serum albumin, and the following components: (a) Lactate dehydrogenase, 0.1 M lithium D,L-lactate and 1.0 μg enzyme; (b) alcohol dehydrogenase, 0.1 M ethanol and 0.3 or 30 μg enzyme from yeast or horse liver, respectively; (c) malate dehydrogenase, 0.1 M sodium D,L-malate and 0.55 μg enzyme

Table 2
Kinetic constants for the reduction of NAD and its derivatives with rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase

Enzyme	Coenzyme	K_m (μM)	V_{max} (%)
Lactate dehydrogenase (rabbit muscle)	NAD	120	100
	N^6-(2-carboxyethyl)-NAD	39	35
	PEG-NAD	67	42
Alcohol dehydrogenase (yeast)	NAD	130	100
	N^6-(2-carboxyethyl)-NAD	86	45
	PEG-NAD	200	44

The reduction mixture contained the following components. For lactate dehydrogenase, 0.2 M lithium D,L-lactate and 0.05–0.3 mM coenzyme; for alcohol dehydrogenase, 0.3 M ethanol and 0.1–1.0 mM coenzyme. Components other than the substrate and coenzyme were the same as those in the legend to table 1

ular size and a lower NAD content in the polymer chain has higher cofactor activity, because the molecular weight of the polyethylene glycol was much smaller than other polymers (M_r 40 000–500 000) used in the macromolecular NAD derivatives [1–5] and PEG-NAD has only 1 NAD moiety/molecule.

3.2. *Continuous enzyme reaction*

To demonstrate the usefulness of PEG-NAD for enzyme reactors, continuous production of L-lactate from pyruvate was performed in a reactor containing PEG-NAD, rabbit muscle lactate dehydrogenase and yeast alcohol dehydrogenase. Fig.1 shows the time course of the continuous production of L-lactate. After 5 h from the start of the reaction, the concentration of L-lactate became constant at ~9 mM. At the steady state, the production rate of L-lactate was 90%. The steady state was maintained for 20 h and then the concentration of L-lactate decreased gradually. The L-lactate concentration became nearly zero at 185 h but it could recover to 8 mM by the addition of the initial amount of both dehydrogenases. Therefore, the decrease in the L-lactate concentration was mainly due to the loss of enzyme activity and PEG-NAD seemed to be active and remained in the reactor up to 200 h.

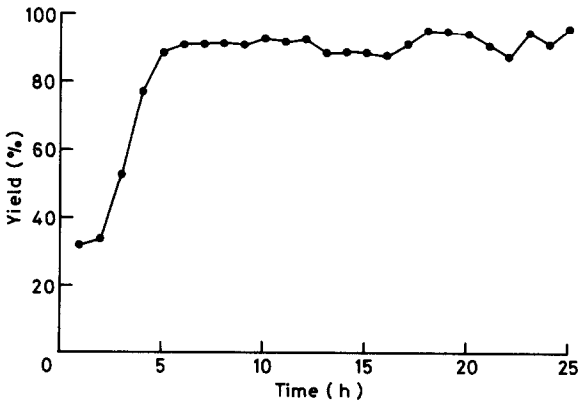

Fig.1. Continuous conversion of pyruvate to L-lactate in an enzyme reactor containing lactate dehydrogenase (rabbit muscle), alcohol dehydrogenase (yeast) and PEG-NAD with a flow rate of 2.8 ml/h. The operational conditions are in section 2.

These results indicate that PEG-NAD is useful for enzyme reactors. The conversion ratio was much higher than the reported value with a similar system [12] because the operational conditions were set to give a higher conversion ratio. Kinetic analysis of operational conditions for a continuous enzyme reactor is in progress.

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