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STUDY ON THE PROPERTIES OF DEXTRAN-LINKED ADENINE NUCLEOTIDE DERIVATIVES

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A simple method is described for the preparation of dextran-linked coenzyme derivatives. Several different 8-(6-aminohexyl)amino-adenine nucleotide coenzymes and their derivatives were covalently attached to dextran by incubation with bromohydroxypropyl derivatives of dextran at room temperature in an alkaline medium. The polymer-linked adenine nucleotide derivatives were separated from the free coenzyme derivatives by a Sephadex G-50 column. The prepared dextran derivatives have ligand densities ranging from 20 to 100 μmol/g of dextran derivatives depending on the conditions of coupling and derivatives. NMR studies revealed that proton resonances of the polymer-linked coenzymes exhibit short transverse relaxation times (T_2) but long longitudinal relaxation times (T_1). This phenomenon was interpreted in terms of the anisotropic motions of the dextran-bound coenzyme derivatives in which the fast axial motions and slow restricted transverse motions of the bound coenzyme derivatives are postulated. These observations could properly explain why the polymer-linked coenzymes exhibit lower biological activity, but similar binding affinity to most enzymes.

INTRODUCTION

The application of immobilized enzymes has become increasingly important in industry as well as in clinical and biochemical research (1). The polymer-linked coenzyme derivatives are usually introduced in the enzyme reactors requiring coenzymes, since they are able to be retained upon ultrafiltration or dialysis and are recyclable.

Another important aspect for the application of polymer-linked coenzyme derivatives is in the field of affinity partitioning (2–4). With biospecific affinity ligands on either dextran or polyethylene–glycol (PEG) phase, the cell particles or proteins can be separated based not only on their surface properties but also on their biospecific affinity in either phase.

Many reports are available regarding the preparation of polymer-linked coenzyme derivatives (5–7). The dextran-bound NAD^+ derivatives prepared by the CNBr activation method have been shown to have the serious problem of leakage, because of the instability of an isourea bond between the coenzyme derivatives and dextran (6). The polyglutamate or

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polylysine derivatives of coenzymes (7) have the disadvantage of the electrostatic interactions between the multicharged polymers and enzymes possibly interfering with their biological activity and the efficiency of affinity partitioning (2). In this communication, we present a simple method for the preparation of stable polymer-linked coenzymes and their derivatives as well as some physical and biochemical characterization in an attempt to broaden the scope of their applications.

MATERIALS AND METHODS

NAD^+ and $NADP^+$ were obtained from P-L Biochemicals, Inc. (Milwaukee, Wisconsin). AMP and ATP were from Sigma Chemical Company (St. Louis, Missouri). Bromohydroxypropyl-dextran (BHP-dextran, mol. wt. 70,000) was a gift from Professor Albertson in Sweden and is now commercially available from Pharmacia Fine Chemicals (Sweden). Lactate dehydrogenase from mouse muscle was prepared according to the procedure of Bachman and Lee (8). Hexokinase and glucose-6-phosphate dehydrogenase were from Sigma Chemical Company.

A steady state kinetic study of various enzymatic systems with the prepared coenzyme derivatives was performed on a Beckman Acta spectrophotometer. NMR spectra were recorded on an HR 220 high resolution NMR spectrometer equipped with Fouriertransform facilities.

RESULTS AND DISCUSSION

Preparation of 8-(6-Aminohexyl)amino-Adenine Nucleotide Coenzymes and Derivatives

All the 8-(6-aminohexyl)amino-adenine nucleotide derivatives have been shown to be good coenzymes or inhibitors of many cofactor-dependent enzymes (9, 10). When immobilized on Sepharose, these derivatives were also shown to be good general ligands in affinity chromatography and were widely employed for the purification of as many as 60 different enzymes (11) including dehydrogenases, kinases (12), aminoacyl-*t*-RNA synthetases (12), and CoA-dependent enzymes (14). It is expected that similar applications can be extended to either affinity partitioning or the immobilized enzyme reactors when these cofactor derivatives are covalently linked to polymers such as dextran or PEG. 8-(6-Aminohexyl)amino-5′-AMP and -ATP were prepared according to the reported procedure (10–13). 8-(6-Aminohexyl)amino-NAD^+ and -$NADP^+$ and their corresponding reduced coenzyme derivatives were prepared according to the modified procedure

(15). 8-(6-Aminohexyl)amino-2',5'-ADP and -3',5'-ADP were prepared according to the procedure of Lee and Johansson (14).

Preparation of Dextran-Bound Cofactor Derivatives

All the prepared 8-substituted adenine nucleotide coenzyme derivatives carry a six-carbon unit of spacer arm with a terminal aliphatic amino group ready for the nucleophilic substitution. They can be covalently attached to bromohydroxypropyl-dextran derivatives by replacement reaction under mild alkaline conditions. A general scheme for the preparation of these dextran-bound cofactor derivatives is shown in Fig. 1.

In general, 100 mg of 8-(6-aminohexyl)amino-adenine nucleotide derivatives (concentration 0.1 M) was mixed with the BHP-dextran (100 mg) in 0.1 M $NaHCO_3$ buffer at pH 10.0 for 24 h at 25°C. Because of the instability of the oxidized pyridine coenzyme derivatives at alkaline pH, 8-(6-aminohexyl)amino-NADH and -NADPH were employed for the covalent coupling to the dextran derivative. Less than 10% of decomposition of the coenzyme derivatives was observed during the coupling reaction. After 24 h, 1 M ethanolamine at pH 10.0 was added to the solution mixture for another 10 h at room temperature.

After the reaction, the solution mixture was then passed through a G-50 Sephadex column (2.5 × 80 cm) which had been equilibrated with

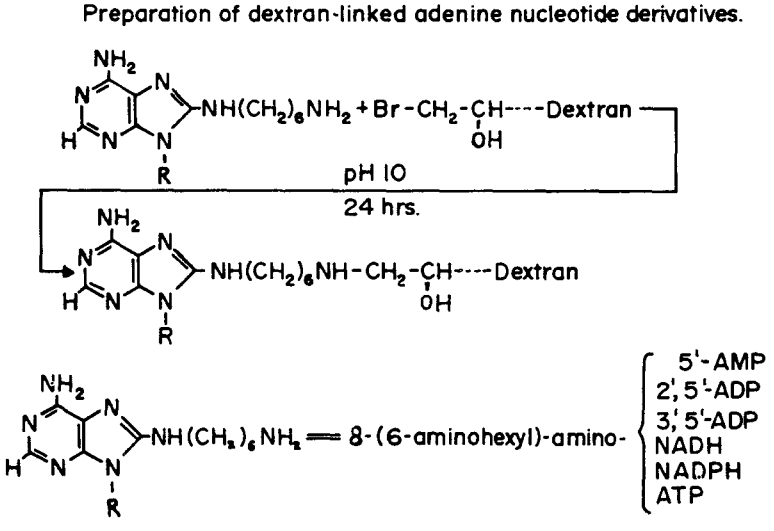

FIG. 1. A general scheme for the preparation of dextran-linked 8-(6-aminohexyl)amino-adenine nucleotide derivatives.

0.1 M$(NH_4)_2CO_3$ at pH 9.0 at room temperature. The elution was made with the same buffer and the polymer-linked coenzyme derivatives appeared in the void volume, whereas the unreacted free coenzyme derivatives were exclusively retarded. Proper fractions were collected and lyophilized to dryness. The prepared dextran-bound cofactor derivatives have ligand densities ranging from 20 to 100 μmol/g of dry dextran derivatives under the experimental conditions just described.

The prepared dextran-linked NADH and NADPH derivatives were further oxidized enzymatically to the corresponding NAD^+ and $NADP^+$ derivatives. One hundred milligrams of the dextran-bound NADH derivative was dissolved in 2 ml of 0.1 M ammonium bicarbonate buffer at pH 7.8 containing 10 U of yeast alcohol dehydrogenase and 1% acetaldehyde. After complete oxidation, the solution was acidified to pH 1.0 and the precipitation was removed by centrifugation. Dextran-bound NAD^+ was then precipitated by 3 volumes of cold ethanol and lyophilized to dryness. In the case of the dextran-bound NADPH derivative, a system of glutathione reductase and oxidized glutathione was employed for the preparation of the oxidized coenzyme derivative.

Structure Studies

High resolution HR 220 NMR spectroscopy was employed for the comparative structure analysis of the free and the dextran-bound coenzyme derivatives. Typical free and dextran-bound NAD^+ derivatives are given in Fig. 2. It was observed that the dextran-bound NAD^+ showed significant broadening of coenzyme resonances as compared to those of the free coenzyme resonances. However, no differences were observed regarding the chemical shifts of free and dextran-bound NAD^+ proton resonances. From the linewidth of the observed proton resonances of the free and dextran-bound NAD^+ derivatives, the transverse relaxation times (T_2) of the dextran-linked NAD^+ proton resonances were estimated. They are presented in Table 1. It can be shown that the T_2 values are on the order of 10 msec for various proton resonances of the dextran-linked NAD^+ derivatives. The estimated T_2 values are at least one order of magnitude shorter than those of free 8-hexyl-NAD^+. The observed T_2 levels of the dextran-bound NAD^+ derivative were found to increase with increasing temperature from 22° to 37°C.

The longitudinal relaxation times (T_1) were also measured for the proton resonances of both free and dextran-linked NAD^+ derivatives from Fourier-transformed partially relaxed NMR spectra. They are presented in Table 2. In contrast to T_2 values, both the free and dextran-linked NAD^+ derivatives exhibit relatively long T_1 ranges, which are on the order of

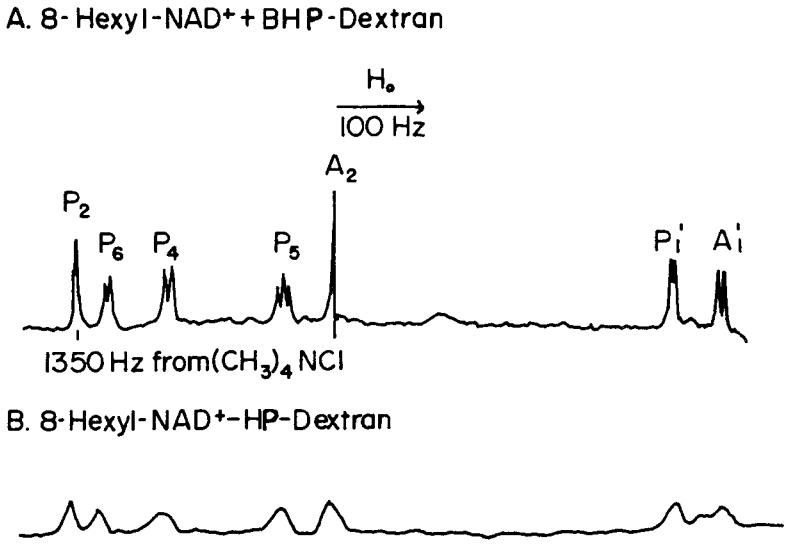

FIG. 2. NMR spectra of some (A) free and (B) dextran-linked NAD^+ proton resonances. In A the spectrum was recorded in a sample containing 5 mg/ml of 8-(6-aminohexyl)amino-NAD^+ and 45 mg/ml of BHP-dextran in 0.1 M phosphate–D_2O buffer at pD 7.0. In B the spectrum was recorded in a sample containing 50 mg/ml of dextran-bound NAD^+ derivatives in the same buffer.

TABLE 1. **Transverse Relaxation Times (T_2) and Observed Line Width ($\Delta\nu$) of Proton Resonances of Free and Dextran-Linked NAD^+**

Compound	Temperature (°C)		T_2 (sec) and $\Delta\nu$ (Hz) of proton resonances						
			P_2	P_6	P_4	P_5	A_2	P'_1	A'_1
Dextran-NAD^{+a}	22	$\Delta\nu^b$	11	11	12	—	20	9	15
		T_2	0.04	0.04	0.03	—	0.02	0.05	0.02
8-Hexyl-NAD^{+c}	22	$\Delta\nu$	4	1.4	1.6	1.2	1.2	1.4	1.4
Dextran-NAD^{+a}	37	$\Delta\nu$	8	8	9.5	8.5	16	7.5	12
		T_2	0.06	0.05	0.05	0.05	0.03	0.05	0.03
8-Hexyl-NAD^{+c}	37	$\Delta\nu$	3.5	1.3	1.4	1.2	1.2	1.2	1.2

[a] NAD^+ was covalently linked to dextran (mol. wt. 70,000) through the 8 position of the adenine moiety of NAD^+ (sample concentration 50 mg/ml, containing 5 mg of bound NAD^+).

[b] $\Delta\nu$ is related to T_2 by $\Delta\nu = \Delta\nu_0 + 1/\pi T_2$ where $\Delta\nu$ is the observed line width in hertz, $\Delta\nu_0$ is the line width contributed by field inhomogeneity, and T_2 is expressed in seconds.

[c] Sample containing 45 mg/ml of BHP-dextran and 5 mg/ml of 8-(6-aminohexyl)amino-NAD^+ (8-hexyl-NAD^+) as a control.

TABLE 2. **Longitudinal Relaxation Times (T_1) of Proton Resonances of Free and Dextran-Linked NAD^+**

Compound	Temperature (°C)	T_1 (sec) of NAD^+ proton resonances						
		P_2	P_6	P_4	P_5	A_2	P_1'	A_1'
Dextran-NAD^+[a]	22	0.52	0.47	0.66	0.60	0.47	0.50	0.50
8-Hexyl-NAD^+[b]	22	0.60	0.54	0.75	0.64	0.81	0.80	0.45
Dextran-NAD^+[a]	37	0.70	0.62	0.91	0.80	0.65	0.60	0.60
8-Hexyl-NAD^+[b]	37	0.80	0.59	0.90	0.78	1.03	0.48	0.84

[a] NAD^+ was covalently linked to dextran (mol. wt. 70,000) through the 8-position of the adenine moiety of NAD^+ (sample concentration 50 mg/ml, containing 10% by weight of NAD^+).
[b] Sample containing 45 mg/ml of BHP-dextran and 5 mg/ml of 8-(6-aminohexyl)amino-NAD^+ (8-hexyl-NAD^+) as a control.

0.1–1 sec. Furthermore, the proton resonances of both free and dextran-linked NAD^+ exhibit almost the same T_1. The observed T_1 levels were also found to increase with increasing temperatures from 22° to 37°C.

The fact that both T_1 and T_2 values of the dextran-linked NAD^+ derivative are not equal and both increase with increasing temperatures suggests that molecular motions of dextran-bound NAD^+ derivatives are anisotropic in nature (16–18). Because of the covalent linkage to dextran, the molecular motions of the bound NAD^+ along the hydrocarbon chain axis are relatively fast as compared to its perpendicular motions. The correlation time ($\tau_\parallel$) for the molecular motions along the chain axis could be as short as 1×10^{-10} sec (18). This is comparable to that of the isotropic motions of the free NAD^+ derivative. On the contrary, the correlation time ($\tau_\perp$) for the restricted perpendicular motions of the bound NAD^+ is on the order of 1×10^{-9} to 1×10^{-8} sec (18). These anisotropic motions result in the observed long T_1 and short T_2 for the proton resonances of the dextran-bound NAD^+ derivative. Judging from the temperature dependence of the observed T_1 and T_2 values of the dextran-linked NAD^+ derivative, our experimental observations cannot be interpreted in terms of the slow isotropic motions with $\tau_c\omega_0 \gg 1$ where ω_0 is the resonance frequency. The characteristics of the molecular motions of the dextran-linked coenzymes are similar to those of the lipid bilayers described by Horwitz et al. (18).

Biochemical Studies

Comparative biochemical studies were performed for both free and dextran-bound NAD^+ derivatives. In the case of lactate dehydrogenase from mouse muscle, free and dextran-bound NAD^+ derivatives exhibit K_m values of 3×10^{-4} and 4.5×10^{-4} M, respectively, for the oxidation of lactate

at pH 8.5 in 0.1 M Tris-HCl buffer. However, V_{max} of the dextran-linked NAD^+ derivative is only 8% of that of the free 8-(6-aminohexyl)amino-NAD^+ under the same experimental conditions. Similarly, in the case of yeast hexokinase, the dextran-linked ATP derivative gave only 12% of the activity as compared to free 8-(6-aminohexyl)amino-ATP. In the case of the dextran-bound AMP derivative, it exhibits the same degree of inhibition as the free 8-(6-aminohexyl)amino-AMP with an apparent K_i of 8×10^{-4} M when lactate dehydrogenase from mouse muscle was employed for inhibition studies.

It is evident that the kinetic constants obtained with the polymer-linked coenzymes depend greatly on the mechanism of actions of individual enzymes as well as the nature of polymers (19–21). If the covalent attachment of the coenzyme derivatives to polymers does not change their interactions to the enzyme surface, one should not expect significant differences in enzyme affinity between the free and the polymer-linked coenzyme analogs. If the dissociation of coenzyme from the enzyme surface is a rate-determining step during the enzyme-catalyzed reactions, the relative mobility of the polymer-linked coenzyme and the turnover number of the enzymes should become critical factors in determining its relative activities. It has been shown by our previous studies that the polymer-linked NAD^+ exhibits relatively better activity for the slow turnover dehydrogenases than for the fast turnover enzymes (21). Although there are few reported cases in which the polymer-linked coenzymes showed better activity than the free coenzyme analogs (19), lower enzymatic activity was observed in all the enzymes that we have examined. Our findings seem consistent with the NMR structural analysis. The restricted motions of the polymer-linked coenzymes may be one of the reasons to account for their low biological activity in many enzymatic systems. Further studies regarding the application of the dextran-linked coenzyme derivatives in the immobilized enzyme reactors and in the affinity partitioning are now in progress.

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