Anchimeric Assistance in the Intramolecular Reaction of Glucose-dehydrogenase–Polyethylene Glycol NAD Conjugate*

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Polyethylene glycol-bound derivatives of NAD(P) (PEG-NAD(P)) are water-soluble macromolecular coenzymes used in continuous enzyme reactors. These NAD(P) derivatives have good coenzyme activity for many dehydrogenases, but some enzymes such as glucose dehydrogenase (EC 1.1.1.47) from Bacillus megaterium (7) is a tetrameric enzyme that catalyzes the oxidation of D-3-glucose to D-glucono-1,5-lactone, which is spontaneously hydrolyzed to glucic acid, using NAD or NADP as coenzyme. As the equilibrium of the overall reaction lies much in favor of NAD(P)/H formation, this enzyme is useful as an NAD(P)H regenerator in enzyme reactors (8). Glucose dehydrogenase, however, shows very low activity for PEG-NAD(P); the reduction rate of PEG-NAD is only 0.08% of that of NAD (5). In the present work, we demonstrate that the activity of this enzyme for PEG-NAD is revived by preparing a covalently linked glucose-dehydrogenase–PEG-NAD conjugate.

EXPERIMENTAL PROCEDURES

Materials–Glucose dehydrogenase from B. megaterium was a generous gift from Amano Pharmaceutical Co. Ltd. and was used after purification by DEAE-Sephadex A-50 column chromatography. PEG was purchased from Nakarai Chemicals (Kyoto); MTT was from Dojin Laboratories (Kumamoto); TSK-Gel G3000SW was from Toyo Soda Co. Ltd. (Tokyo); marker proteins for gel filtration and electrophoresis were from Oriental Yeast Co. Ltd. (Tokyo) and Boehringer Mannheim, respectively. PEG-NAD was a generous gift from Kojin Co. Ltd. (Tokyo). PEG-NAD and 3,3′-[(1,6-dioxo-1,6-hexanediyl)bis-2-thiazolidinethione were prepared as described in Refs. 2 and 9, respectively.

Macromolecular derivatives of NAD, NADP, and ATP have been prepared as reusable coenzymes, but their coenzyme activities are generally lower than those of native ones (1). PEG–NAD(P) are the most active derivatives of NAD and NADP and have been used in continuous enzyme reactors (2–6). However, there are still some enzymes that can not use them as coenzymes (5, 6). PEG–NAD(P) have a simple structure in that one NAD(P) molecule is attached to a terminal of the linear, flexible, and hydrophilic chain of polyethylene glycol (M, 3000), and therefore it seems difficult to improve the structure of PEG–NAD(P). One possible way is to change the structure of the coenzyme-binding site of an enzyme by protein engineering so that the enzyme can use PEG–NAD(P) as coenzyme, but this method is applicable only to the enzymes whose structures are well known. We describe here a much simpler method for reviving the enzyme activity for PEG–NAD, i.e. covalent linking of PEG–NAD to an enzyme which has very low activity for the derivative.

Glucose dehydrogenase (EC 1.1.1.47) from Bacillus megaterium (7) is a tetrameric enzyme that catalyzes the oxidation of D-3-glucose to D-glucono-1,5-lactone, which is spontaneously hydrolyzed to glucic acid, using NAD or NADP as coenzyme. As the equilibrium of the overall reaction lies much in favor of NAD(P)/H formation, this enzyme is useful as an NAD(P)H regenerator in enzyme reactors (8). Glucose dehydrogenase, however, shows very low activity for PEG–NAD(P); the reduction rate of PEG–NAD is only 0.08% of that of NAD (5). In the present work, we demonstrate that the activity of this enzyme for PEG–NAD is revived by preparing a covalently linked glucose-dehydrogenase–PEG-NAD conjugate.

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Enzyme Assay—Enzyme reactions were measured at 30 °C with a Hitachi 220A spectrophotometer with a thermostatted cell compartment and a magnetic stirrer. The activity in the presence of exogenous NAD was assayed in 75 mM Tris/HCl buffer, pH 8.0, containing 0.1 mM D-glucose and 2.0 mM NAD, and the reactions were recorded as the increase in absorbance at 340 nm. The activity was also assayed by the tetrazolium salt method (12) in 20 mM Tris/HCl buffer, pH 8.0, containing 1 M NaCl, 0.1 mM D-glucose, 20 mM PES, and 1.7 mM MTT, and the reactions were recorded as the increase in absorbance at 570 nm due to formazan formation from MTT. The concentration of the formazan was measured using a molar absorption coefficient of 13,000 M⁻¹ cm⁻¹ at 570 nm (13).

RESULTS AND DISCUSSION

PEG-NAD was covalently linked to glucose dehydrogenase (Fig. 1) by the procedure described for the preparation of malate-dehydrogenase-PEG-NAD conjugate (9). Fig. 2 shows the DEAE-Sephadex column chromatography after the reaction of glucose dehydrogenase with the activated PEG-NAD. The first and the second peaks had no enzyme activity, and their ultraviolet spectra showed that unbound NAD derivative and 2-thiazolidinethione produced by the hydrolysis of the activated PEG-NAD were included. GlcDH-PEG-NAD was obtained from the third peak. Under the same experimental conditions, native glucose dehydrogenase was eluted at a higher ionic strength (32 ms).

GlcDH-PEG-NAD, thus prepared, has the following characteristics. The average number of NAD moieties bound per molecule of enzyme subunit (NAD content (9)) is 2.1. High performance gel filtration chromatography (TSK-Gel G3000SW equilibrated with 50 mM phosphate buffer, pH 6.5, containing 10% glycerol) shows a single peak with M₀ of 205,000, which is larger than the value of the native enzyme of 140,000. The value of the native enzyme is a little larger than the value of 118,000 obtained in 50 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl; the latter is in good agreement with those reported in Ref. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis as in Ref. 9 shows at least four bands with the apparent M₀ of 39,000, 39,100, 46,400 and 54,000; these bands seem to correspond to the subunits with 0, 1, 2, and 3 molecules of PEG-NAD, respectively. About 70% of the bound NAD moieties of GlcDH-PEG-NAD is reduced by its enzyme moiety in the presence of D-glucose. The specific activity of GlcDH-PEG-NAD in the presence of exogenous NAD is about 92% of that of the native enzyme.

These results indicate that a tetramer of GlcDH-PEG-NAD has about six molecules of active and covalently linked NAD moieties, and that the covalent linking causes only a slight loss in the activity of its enzyme moiety; similar results were obtained for malate-dehydrogenase-PEG-NAD conjugate (9). The Kᵣ value of GlcDH-PEG-NAD for exogenous NAD is 0.8 mM and the value is similar to that of the native enzyme (0.9 mM). These results indicate that the NAD(H) moiety of the conjugate does not compete with exogenous NAD for the coenzyme-binding site of the enzyme moiety. This is probably due to the fact that glucose dehydrogenase shows very low activity for NAD(P) derivatives alkylated at the 6-amino group (5, 14).

The activity of the enzyme moiety of GlcDH-PEG-NAD toward the bound NAD moiety can be measured by the coupled redox system of PES and MTT in the presence of glucose (internal activity (9)). In this reaction system, the NAD moiety is recycled by the two reactions of the enzyme moiety with glucose and PES with MTT, and the concentrations of PES and MTT are made high enough to keep more than 95% of the coenzyme moiety in the oxidized form at steady state. The concentration of glucose (0.1 mM) is much higher than the Kᵣ for glucose in a similar recycling assay system: GlcDH-PEG-NAD, 31 mM; glucose dehydrogenase + NAD, 18 mM. The concentrations of the coenzymes (<0.1 mM) are far below the Kᵣ for NAD (0.9 mM), and the Kᵣ for PEG-NAD is supposed to be much larger than for native NAD (5, 14). The internal activity of GlcDH-PEG-NAD at different concentrations of the conjugate is shown in Fig. 3 together with the activities of control systems containing native glucose dehydrogenase (GlcDH) plus NAD and GlcDH plus PEG-NAD; in the system of GlcDH + PEG-NAD, the concentrations of the enzyme and NAD are varied with a fixed ratio of 1:6.1 of [NAD]/[GlcDH], whereas in the system of GlcDH + PEG-NAD, concentration of PEG-NAD is varied and that of glucose dehydrogenase is fixed at 0.31 mM.

The slope of the logarithmic plot shown in Fig. 3 gives the value of 118,000 obtained in 50 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl; the latter is in good agreement with those reported in Ref. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis as in Ref. 9 shows at least four bands with the apparent M₀ of 39,000, 39,100, 46,400 and 54,000; these bands seem to correspond to the subunits with 0, 1, 2, and 3 molecules of PEG-NAD, respectively. About 70% of the bound NAD moieties of GlcDH-PEG-NAD is reduced by its enzyme moiety in the presence of D-glucose. The specific activity of GlcDH-PEG-NAD in the presence of exogenous NAD is about 92% of that of the native enzyme. This low value of Kᵣ for glucose in a similar recycling assay system: GlcDH-PEG-NAD, 31 mM; glucose dehydrogenase + NAD, 18 mM. The concentrations of the coenzymes (<0.1 mM) are far below the Kᵣ for NAD (0.9 mM), and the Kᵣ for PEG-NAD is supposed to be much larger than for native NAD (5, 14).
Glucose-dehydrogenase–NAD Conjugate

As the order of these reactions is different, the reaction rate of GlcDH–PEG–NAD can not be compared directly with those of GlcDH + NAD and GlcDH + PEG–NAD; the ratio of the reaction rates of GlcDH–PEG–NAD and GlcDH + NAD (or PEG–NAD) increases with the decrease in the concentration used for the assay. However, it is apparent that the rate of GlcDH–PEG–NAD is much higher than that of GlcDH + PEG–NAD, and is even higher than that of GlcDH + NAD under the conditions shown in Fig. 3. Namely, the low reaction rate of GlcDH + PEG–NAD is greatly enhanced just by covalently linking the enzyme and PEG–NAD. This effect of the covalent linking is a kind of “anchimeric assistance” (16), and the magnitude of the effect can be estimated using the ratio of \( \frac{K_{\text{comp}}}{K_{\text{PEG-NAD}}} \), which is known as the effective concentration (16). The effective concentration of the NAD moiety of GlcDH–PEG–NAD is 4.2 mM; this means that the enzyme moiety of GlcDH–PEG–NAD acts as if it were in a solution containing 4.2 mM PEG–NAD irrespective of the actual concentration of the conjugate, assuming the proportionality of the reaction rate of GlcDH + PEG–NAD to the concentration of PEG–NAD up to 4.2 mM (in other words, \( K_a \) for PEG–NAD is much higher than 4.2 mM). The ratio of the effective and the actual concentrations corresponds to the ratio of the reaction rates of GlcDH–PEG–NAD and GlcDH + PEG–NAD at the same actual concentration. For example, the rate of GlcDH–PEG–NAD at its concentration of 0.31 \( \mu \)M is estimated to be 6,500-fold the rate of GlcDH (0.31 \( \mu \)M) + PEG–NAD (0.65 \( \mu \)M); this value increases to 10,000-fold if account is taken of the fact that the specific activity of the enzyme moiety of the conjugate is 92% of that of the native enzyme, and that of the enzymically reducible NAD moiety of the conjugate is 70%.

The dramatic improvement of the activity of glucose dehydrogenase for PEG–NAD by this simple method of covalent linking has significant implications for enzyme technology, for it indicates that a much wider range of enzymes and coenzyme derivatives can be made applicable in the analytical and industrial fields.

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