APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 6, 85-90 (1981)

Immobilization of *myo*-Inositol-1-phosphate Synthase Containing Active, Self-Regenerating Coenzyme (NAD^+) on the Same Matrix

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Received July 9, 1980; Accepted July 20, 1980

Abstract

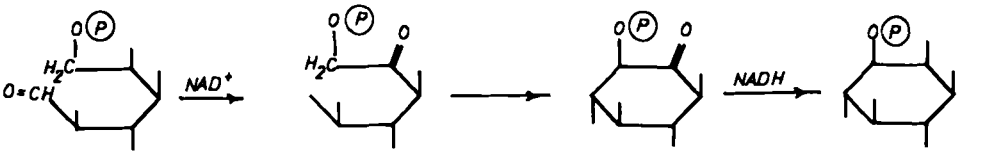
myo-Inositol-1-phosphate synthase (EC 5.5.1.4) from rat testes, an NAD^+-containing enzyme, which converts D-glucose 6-phosphate to 1L-*myo*-inositol 1-phosphate, could be immobilized together with its cofactor and bovine serum albumin by crosslinking with glutaraldehyde at pH 4.5. The enzyme bound to the gel showed a specific activity of 5.6% of that of the native enzyme, but the activity could be increased to 21% by pretreatment with urea.

Index Entries: Glutaraldehyde crosslinking; immobilized inositol phosphate synthase; bound NAD; synthase, immobilized inositol phosphate; NAD, immobilization of bound.

Introduction

During the last few years, several attempts were made to immobilize enzymes together with their cofactors. Some examples are given in the references *1–5*. From the point of view of economics, this can be of great importance because cofactors are in most cases rather expensive. Immobilization therefore may provide a feasible method for their retention and regeneration. Unfortunately, nearly all of these enzymes (especially pyridine nucleotides), when immobilized with their cofactors must be combined with external regenerating systems to

0273-2289/81/0600-0085$02.00



SCHEME 1. The reaction sequence catalyzed by *myo*-inositol-1-phosphate synthase. NAD^+ is converted to NADH during the first reaction step, reforming NAD^+ by reducing the keto group of the last intermediate at the end of the sequence. **1,** D-glucose 6-phosphate; **2,** D-xylo-hexos-5-ulose; **3,** 2 L-2-phospho-1,2,5/3,4-pentahydroxycyclohexanone; **4,** 1 L-myo-inositol-1-phosphate.

prevent the reaction from stopping after only a short time (i.e., when all the cofactor present is converted to either its reduced or oxidized form). Another disadvantage in most cases is that the enzyme and its cofactor are usually immobilized on different carriers. For an investigation of the behavior of an immobilized enzyme co-immobilized with its cofactor under specified conditions (e.g., for the influence of organic solvents, urea, temperature, etc.), it is advantageous to choose a system in which no external regeneration is needed. The *myo*-inositol-1-phosphate synthase system is well-suited to such investigations. This enzyme converts D-glucose 6-phosphate to 1L-*myo*-inositol 1-phosphate in the presence of the cofactor NAD^+. The reaction steps are shown in scheme 1. As can be seen from this scheme NAD^+ is reduced during the first reaction step; the NADH formed, then reduces the keto group during the final reaction step, and NAD^+ is again formed. In this paper, immobilized *myo*-inositol-1-phosphate synthase is therefore studied as a convenient model of an enzyme with a self-regenerating coenzyme, both of which are immobilized on the same matrix.

Materials

All buffer substances, acetic acid, ninhydrin, and glutaraldehyde were obtained from Merck, Darmstadt. Bovine serum albumin, NAD^+, D-glucose 6-phosphate, and dithiothreitol were from Sigma, St. Louis.

Methods

myo-Inositol-1-phosphate synthase from rat testes was purified as published in previous papers (*6, 7*); partially purified enzyme prepared according to the method of Barnett et al. (*8*) has been used as well.

The assay of the native enzyme was carried out according to the protocols of Barnett et al. (*8*).

The assay of immobilized enzyme was made by incubating 100 mg of wet gel in 2.5 mL of 50 m*M* Tris-acetate buffer, pH 7.7, which was 2 m*M* with respect

to glucose 6-phosphate. The reaction mixture was kept in a tightly stoppered tube at 37°C on a thermostated shaker for several hours. The reaction was stopped by removal of the gel by centrifugation. Aliquots of the solution containing the product were treated according to the assay procedure mentioned above.

To compare the activity of the polyprotein gels to that of the native enzyme, the specific activity of the protein gel was measured by the following procedure: Wet gel (100–200 mg) was assayed for activity as described above. Then the wet gel was dehydrated by lyophylization and the dry weight determined on a microbalance. Dry polyprotein (75 mg) could be obtained from 1 g of wet gel. The protein concentrations of the mixture of native enzyme with bovine serum albumin before the polymerization with glutaraldehyde were determined according to Jacobs (*10*).

Immobilization of the enzyme was carried out according to Mosbach (*9*), with certain modifications: The purified *myo*-inositol-1-phosphate synthase was dialyzed for several hours against a 1*N* acetate buffer, pH 4.5. Small amounts of denatured protein were removed by centrifugation. Enzyme ($1.6–2.0 \times 10^{-3}$ μkat) and bovine serum albumin (650 mg) were dissolved in 30 mL 1*N* acetate buffer, pH 4.5, containing 0.1 mmol glucose 6-phosphate. The mixture was kept at room temperature for 10 min. Then 6 mL of 2.5% aqueous glutaraldehyde solution were added dropwise under stirring. The mixture was kept without shaking at room temperature for 3 h and then stored overnight at +1°C. The gel formed was cut into pieces, suspended in a 0.15 *M* KCl solution that contained 50 mmol of lysine. The homogenized gel was washed with the same solution, and then with a 0.15 *M* KCl solution without lysine until the filtrate did not react with ninhydrin. The gel could be stored under the same solution in the refrigerator.

Results

myo-Inositol-1-phosphate synthase could be immobilized together with its cofactor NAD^+ by aggregation with bovine serum albumin and the crosslinking reagent glutaraldehyde according to the procedure described above. The polymerization was best obtained at pH 4.5; with neutral or slightly basic conditions, no insolubilization took place. To investigate whether the NAD^+ concentration used during the immobilization step influences the activity of the insolubilized enzyme, the NAD^+ concentration in different batches of incubation mixture (see methods section) was varied from 0.001 to 0.025 mmol/mL. The gels formed were homogenized, washed, and assayed as described above. Concentrations of 0.005 mmol NAD^+/mL produced the most active gels. The specific activity of the *myo*-inositol-1-phosphate synthase immobilized together with its cofactor NAD^+ under optimum conditions was 1.4×10^{-5} μkat/100 mg protein, resembling 5.6% activity of the mixture before polymerization, which had an activity of 25×10^{-5} μkat/100 mg protein. But the specific activity of this gel could be increased 3.8-fold (to

5.32×10^{-5} μkat/100 mg) when kept for 24 h in a 4 *M* urea solution in 50 m*M* tris-acetate buffer, pH 7.7, that was 2 m*M* with respect to glucose 6-phosphate, and when subsequently washed with 0.15 *M* KCl solution. In that case 21.3% of the activity of the native enzyme mixture could be obtained. When larger amounts of NAD^+ were used in the polymerization mixture, the gels were less active, the specific activity decreasing to less than 50% of the activity at optimal conditions, when the NAD^+ concentration was raised to values of 0.025 mmol/mL polymerization mixture. The same happened when less than the optimal NAD^+ concentration was used.

Addition of the soluble NAD^+ to gels that were not polymerized with sufficient NAD^+ improved the activity to that of the gel crosslinked at optimal conditions. Gels polymerized with NAD^+ concentrations higher than 0.005 mmol/mL showed no increase of activity with the addition of soluble NAD^+. Moreover, concentrations of more than 0.01 mmol soluble NAD^+/mL assay solution (containing the usual amount of substrate) led to a decrease in the specific activity to 57% of that obtained in the absence of soluble NAD^+. The microenvironment of the gel did not change the pH optimum of the enzyme action, which is 7.7 in the native as well as in the immobilized enzyme. When stored in the cold at 4° C, the gel was stable for months. It is possible to store it for prolonged periods without significant loss of activity at −15° C.

Discussion

It is already well known that in most cases immobilized enzymes behave quite differently from the native ones with respect to the influence of organic solvents, urea, temperature, etc. It would be of great interest too, to gain a better knowledge of the behavior of such systems where both enzyme and cofactor are immobilized. In most systems published to date, enzyme and cofactor are bound to different carriers. M. D. Legoy et al. (*4*) bound an enzyme and NAD^+ to the same matrix, but it is common to all of the systems using NAD^+ as a cofactor that they need an additional regenerating system to prevent the reaction from stopping when all the cofactor is used up. These systems are not convenient for such studies as those mentioned at the beginning of this section because it is difficult to distinguish between the contributions of the different compartments of such systems to the action of organic solvents, temperature, etc. Therefore, *myo*-inositol-1-phosphate synthase, bound with its self-regenerating cofactor on the same matrix, is a good model for such studies. This immobilized system is very stable. The activity can be significantly increased when the gel is treated with urea. One possible explanation might be that treatment with urea results in the loosening of bonds within the macromolecules, giving bound enzyme a chance to rearrange to a more active conformation. Otherwise, the enzyme, which consists of subunits in its native form (*6*), is more active when immobilized to a matrix and split into its constituent subunits by urea.

It could be shown that there is an optimal concentration of NAD^+ for co-immobilization, larger amounts leading to a decrease in activity. The reason might be that too much NAD^+ co-immobilized near the active site makes it less accessible for the substrate. Part of this effect might owe to the fact that too much NAD^+ in the reaction mixture causes a reaction of glutaraldehyde with two molecules of NAD^+ rather than its coupling to the protein matrix. The deepening of the color of such polymerization mixtures where too much NAD^+ was present, and the looser network of such gels may help account for that fact. It must be pointed out that the presence of the substrate D-glucose 6-phosphate during polymerization was necessary to protect the active site, which contained lysine (*11*), against an attack of glutaraldehyde.

The immobilized enzyme has the same pH-optimum (7.7) as the native one, indicating that its microenvironment is probably similar to that of the native enzyme. The reason is that *myo*-inositol-1-phosphate synthase and bovine serum albumin have their isoelectric points in the same range, providing electric charge environment for the active site similar to that of the native enzyme.

It must also be pointed out that there are some other enzyme–cofactor systems besides *myo*-inositol-1-phosphate synthase in which the cofactor is regenerated during the reaction steps, for example: some NAD^+-requiring epimerases; furylfuramide isomerase, which requires NADH; and mutases, which require cobamide. It should be possible to immobilize them together with their cofactors by the same procedure, and to use them as models for studies on the influence of organic solvents, urea, temperature, etc. that, in the case of *myo*-inositol-1-phosphate synthase, are already in progress and will be published soon.

Acknowledgments

The author thanks Prof. Dr. O. Hoffmann-Ostenhof for critically reviewing the manuscript. This investigation was supported by grants of the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich, project 3389.

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