Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 689-695

UNIQUE MODIFICATION OF HUMAN HEART GLYCEROL 3-PHOSPHATE DEHYDROGENASE BY BLUE AGAROSE

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Received September 16, 1983

SUMMARY: The major form of glycerol phosphate dehydrogenase in human heart (GPDH-1) is a minor form (<15%) in brain and other tissues and is extremely labile. After GPDH-1 was eluted from an agarose column to which Cibacron blue F3GA had been covalently linked, (a) it was no longer labile (t 1/2 @ 40°C changed from 1.6 min to >180 min); (b) it could now be stained for activity on native gels following electro-phoresis; and (c) it now migrated with the bromphenol blue dye front. The results suggest that this stabilized form of GPDH-1 is due to the covalent binding of charged ligands from the column and that this technique may be useful for studying the molecular structure and/or the active site of GPHD-1 and possibly of other enzymes which bind to blue agarose.

INTRODUCTION: Glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) is a soluble cytoplasmic enzyme which catalyzes the reversible oxidation of alpha glycerol phosphate to dihydroxyacetone phosphate while simultaneously reducing NAD to NADH. Multiple molecular forms of this enzyme have been demonstrated in a variety of species including rat (1,2,3), chicken (4), mouse (5), and rabbit (6). Biochemically and immunologically distinct forms have been demonstrated in heart tissue from rat (7), rabbit (6) and human (8). We recently demonstrated (8) that within the human organs tested, heart was the major source of the most labile form of GPDH. Using DEAE cellulose it was possible to separate three forms of human heart (GPDH). Attempts to further purify and characterize GPDH-1 have been hampered by its extreme lability. Therefore, a number of different affinity resins have been tested, including Cibacron

0006-291X/83 $1.50

blue F3GA covalently attached to agarose (9). However, following its elution from blue agarose, GPDH-1 was no longer heat labile, could now be stained for activity on native gels following electrophoresis and had a relative mobility of one (i.e., it migrated with the bromphenol blue dye front). The results presented in this report suggest that this stabilized form of GPDH is probably due to the covalent binding of charged ligands from the column and that this may represent an important analytical procedure for studying the molecular structure and/or the active site (8) of GPHD-1 and possibly of other enzymes which bind to blue agarose.

METHODS: All chemicals were reagent grade and were obtained from commercial sources. Human heart was obtained from autopsy material and stored frozen at -70°. The tissue was treated as described previously (8,10). All buffers used contained 2 mM EDTA and 1 mM dithioerythritol. In brief, following homogenation and centrifugation, the supernatant was put through an affinity column synthesized by coupling trinitrobenzene sulfonic acid to agarose using a modification (10) of the method of Kornbluth et al. (11). Following NADH elution, ammonium sulfate (70%) precipitation, desalting on a G-25 Sephadex column, the sample was applied to a Whatman DE 52 column (2.5 x 40 cm) equilibrated with 2 mM sodium phosphate buffer, pH 7.5. The column was eluted with a linear gradient (500 ml) of phosphate buffer from 2 to 100 mM. The peaks of GPDH activity peaks were labeled 1, 2 and 3 in the order of elution and were concentrated with Amicon ultrafiltration and the buffer exchanged for 50 mM Tris HCl pH 8.3. Aliquots of the separated forms of GPDH were applied to columns of blue agarose and subsequently eluted with 0.2 mM NADH in the same buffer. The enzymes were either used immediately or stored at 4°C as a precipitate in 70% ammonium sulfate. Native polyacrylamide gel electrophoresis was performed by the method of Davis (12), as described previously (13), except it was run on a vertical slab gel using a water jacketed apparatus maintained at 10°C. GPDH activity in the gels was visualized using the method described (13). Blue agarose was synthesized by the method of Travis et al. (9) using Sepharose 4B from Pharmacia and Cibacron blue F3GA from Polysciences, Inc.

Glycerol phosphate dehydrogenase activity was assayed by following the loss of NADH as described previously (14). One unit of activity in that amount of enzyme which catalyzes the oxidation of one umole of NADH per min under conditions of the assay. Protein was assayed by the method of Lowry (15) using crystallized bovine serum albumin as a standard. Thermal stability experiments were performed as described (14).

RESULTS: Three molecular forms of GPDH had previously been demonstrated in homogenates of human heart tissue (8). These

forms were separable on DEAE cellulose (DE52) and were shown to react differentially to heating at 48°C. Form 1 was the most labile with a half life of about 1.5 min, whereas forms 2 and 3 had half lives of approximately 25 and 70 minutes respectively. Attempts to purify the major heart form of GPDH were hampered by its extreme lability. It was found that Cibacron blue F3GA which had been coupled to agarose bound GPDH. However, the GPDH-1 eluted was found to be stable at 48°C. The results of such an experiment are shown in Fig. 1 where the effect of passage through the blue agarose column is dramatically obvious. The control sample exhibited a half life at 48°C of about 1.6 min, whereas that portion of the sample which was applied to the column was completely stable even after 180 minutes. The control sample of GPDH 1 was passed through an agarose column without cibacron blue F3GA; NADH was added to give a final concentration of 0.2 mM and both treated and untreated samples were put through a Sephadex G25 column to remove the NADH prior to testing for heat lability. The

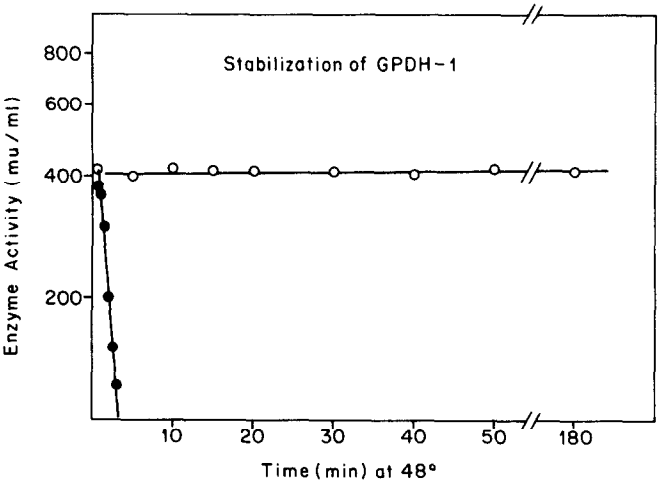

Fig. 1. Stabilization of GPDH-1 after binding to blue agarose. The three forms of GPDH from human heart were partially purified and separated on a DE-52 column, and GPDH-1 was applied to and eluted from a blue agarose column as described in Methods. The percent of enzyme activity remaining after heating at 48°C was determined for the control sample (●) and for the sample which had been eluted from blue agarose (○).

other forms of GPDH (2 and 3) were completely unaffected by passage through the blue agarose.

Prior to separation on DE52, the three different forms of GPDH could be visualized after native gel electrophoresis by staining for enzyme activity (Fig. 2). However, after separation, GPDH-1 and GPDH-2 stained only faintly or not at all even when up to 300 U units of activity were applied whereas GPDH-3 stained prominently with as little 3 U. After passage through blue agarose, GPDH-1 is now easily seen, but now it runs with the bromphenol blue dye front marker giving it an R.F. value of 1.0. As with heat stability, the electrophoretic mobility of GPDH-2 and GPDH-3 is unaffected by their transit through blue agarose. Both the unstable and the stabilized forms of GPDH-1 exhibited the same

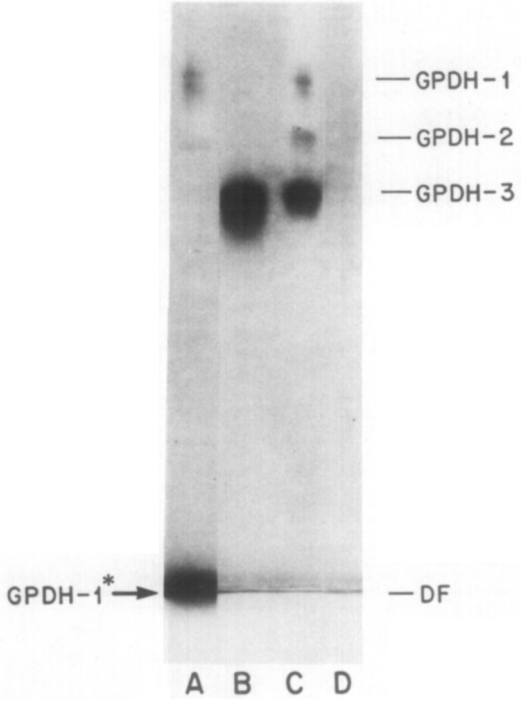

Fig. 2. Visualization and increased mobility of stabilized GPDH-1. Following electrophoresis, the gel was stained for GPDH activity. Electrophoretic mobility of GPDH formed prior to separation on DE-52 is shown in lane C. After DE-52, but before blue agarose, GPDH-1 lane (D) could not be detected. However, after passage through blue agarose GPDH-1 activity was easily detected (lane A) but it now ran with the dye front (DF). GPDH-3 mobility was unaffected (lane B) by passage through blue agarose.

molecular weight (about 75000 daltons) on Sephadex G-200. Passage of the sample through blue agarose before DE-52 does not produce the stabilized form. Direct addition of the dye itself to solutions of GPDH completely inactivated all forms of GPDH, and neither dialysis nor chromatography on Sephadex G-25 nor on DE52 resulted in recovery of activity.

DISCUSSION: The data demonstrate that passage of a partially purified fraction of GPDH-1 through blue agarose results in a dramatic increase in thermal stability of GPDH-1. Neither of the other two forms (2 and 3) is affected by similar treatment, suggesting that something unique to GPDH-1 enables this stabilization to occur. The stabilized form of GPDH-1 was also shown to be readily detected by an activity stain following native polyacrylamide gel electrophoresis, and it now had a relative mobility of 1.0.

The lack of effect of blue agarose on GPDH-2 raises an interesting point. GPDH is known in a number of species to be a dimer composed of identical subunits (16). However, either both subunits of GPDH-1 are required for the stabilization binding to occur or GPDH-2 represents a post-translational modification of GPDH-3 and has only one type of subunit (i.e., GPDH-3) present. This may be resolved with antibodies which react with GPDH-1 but not GPDH-3 and/or by purification and amino acid sequence analysis of all three forms.

The ability of a wide range of kinases and dehydrogenases to bind to this dye is believed to be due to the presence of the "dinucleotide fold" in the structure of these enzymes (16,17,18), although many other types of enzymes can bind as well. The stabilization of activity is in contrast to the hyperanodic forms of glucose-6-phosphate dehydrogenase which form in solution by an enzyme-catalyzed covalent coupling of an NADP derivative which

results in loss of dehydrogenase activity (19). Since the conversion of GPDH-1 to the stabilized form occurs within minutes at 4°C, the modification is probably not enzyme catalyzed. This, combined with the retention of the same molecular weight and the acquisition of a high net charge, suggests that some small ligand(s) is being attached to the enzyme molecule. It has been shown by Weber et al. (20) that commercial preparations of cibacron blue F3GA are contaminated with at least eight other components. They also showed that at least one of the components covalently bound to and irreversibly inactivated both phosphoglycerate kinase and isoleucyl tRNA synthetase. The fact that direct addition of the uncoupled dye to enzyme solutions resulted in complete and irreversible inactivation of GPDH-1 is directly analogous to the studies of Weber et al. (20) that were done with the dye in solution. Collectively the data suggest that GPDH-1 binds to blue agarose through its NAD binding site and that this binding site becomes protected while some other site becomes covalently linked with the contaminant. Binding under these conditions results in stabilization of the entire enzyme molecule. This could be a very useful tool for examining the active site of GPDH-1 and its relationship to the rest of the molecule. Whether this phenomenon occurs with other enzymes remains to be determined, but the fact that neither GPDH-2 nor GPDH-3 undergoes this type of modification suggests that GPDH-1 has a unique site not possessed by them or possibly by most other enzymes which bind to blue agarose.

ACKNOWLEDGMENTS. I thank Patria Comiso for excellent technical assistance. This study was supported by USPHS grants HL-18567 and HD-05615.

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