Biochimica et Biophysica Acta, 527 (1978) 182—192
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BBA 68580

USE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE-DEPLETED HUMAN ERYTHROCYTE GHOSTS AS SPECIFIC HIGH AFFINITY ADSORBENTS FOR THE PURIFICATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM VARIOUS TISSUES

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(Received May 2nd, 1978)

Summary

Human erythrocyte ghosts depleted of glyceraldehyde-3-phosphate dehydrogenase are used as specific high-affinity adsorbents for the purification of glyceraldehyde-3-phosphate dehydrogenase from mouse muscle, liver, kidney and brain. On incubation with the crude tissue homogenates, the depleted ghosts bind glyceraldehyde-3-phosphate dehydrogenase, aldolase, and a few other proteins. Washing the incubated ghosts several times with 5 mM phosphate buffer (pH 8.0) removed several of the non specifically bound proteins. Aldolase can be eliminated from the membrane by incubating the ghosts for 30 min in 5 mM phosphate buffer (pH 8.0)/2 mM fructose 1,6-biphosphate, and then washing with the same solution. Glyceraldehyde-3-phosphate dehydrogenase can then be specifically eluted from the ghosts by incubating them with 2 mM NADH in 5 mM phosphate buffer (pH 8.0). Although the enzyme from brain appears to bind less strongly to the ghosts it was possible, using this procedure, to purify glyceraldehyde-3-phosphate dehydrogenase from all the tissues investigated. The purified enzyme exhibits high specific activity and migrates as a single band (during SDS polyacrylamide gel electrophoresis) which corresponds to a protomer molecular weight of 37 000.

Introduction

Human erythrocyte ghosts prepared by osmotic hemolysis retain a large fraction of total erythrocyte glyceraldehyde-3-phosphate dehydrogenase (D-glyce-

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raldehyde-3-phosphate : NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) and the membrane-associated enzyme constitutes 5—7% of the total ghost membrane protein [1,2]. Although the in vivo association of the enzyme with the red blood cell membrane has been questioned [3—5], the enzyme has a rather specific and strong affinity for the membrane at low ionic strengths; the binding sites are located on the cytoplasmic side of the band 3 polypeptide [1,6]. In addition to the enzyme from human erythrocytes, glyceraldehyde-3-phosphate dehydrogenase purified from pig and rabbit muscle also binds strongly to human erythrocyte ghosts [7], presumably due to their almost identical amino acids sequences. Amino acid composition and sequence data from a number of species indicates that, relative to other enzymes, the primary structure of glyceraldehyde-3-phosphate dehydrogenase is highly conserved during evolution [8].

Glyceraldehyde-3-phosphate dehydrogenase can be purified from the muscle of several animals, where it constitutes 5—10% of the total proteins, by simple $(NH_4)_2SO_4$ fractionation [9,10]. However, in other tissues, the concentration of the enzyme is much lower and its purification requires several fractionation steps [11—13]. In the present communication, we describe a single step procedure for the purification of the enzyme from muscle, kidney and brain. This procedure is based upon the affinity of glyceraldehyde-3-phosphate dehydrogenase for glyceraldehyde-3-phosphate dehydrogenase-depleted human erythrocyte ghosts and the ability of NADH to elute specifically the enzyme from the ghost membrane. It was possible, using this procedure, to obtain preparations of the enzyme which exhibited high specific activity and appeared homogeneous on SDS polyacrylamide gel electrophoresis.

Materials and Methods

Fresh blood obtained from apparently healthy donors or recently outdated blood was used with results that were not affected by the blood group. DL-glyceraldehyde 3-phosphate was obtained from Serva (G.F.R.) as the diethyl acetal barium salt which was deionized on Dowex-50 and heated for 3 min to obtain the free aldehyde. NAD^+, NADH and fructose 1,6-biphosphate were also obtained from Serva. Marker proteins for gel electrophoresis were the products of Boehringer Mannheim.

Preparation of ghosts and elution of glyceraldehyde-3-phosphate dehydrogenase

Erythrocytes were washed twice with several volumes of isotonic NaCl solution. Ghosts were prepared by the procedure described by Steck et al. [14], which is based on the principle of hypotonic hemolysis defined by Dodge et al. [15]. The washed erythrocytes were hemolyzed with 30—40 vols. cold buffer 5P8 (5 mM phosphate buffer at pH 8.0) and the suspension centrifuged at 15 000 $\times g$ for 30 min. The cells were washed twice more with 40 vols. 5P8 to remove the hemoglobin. For elution of glyceraldehyde-3-phosphate dehydrogenase, the washed ghosts were suspended in 20—30 vols. cold 5P8/2% NaCl and the suspension was gently stirred for 1 h. The ghosts were then centrifuged and washed twice with the same solution followed by one or two washes with

5P8. The procedure is essentially that of Kant and Steck [1] and results in complete removal of glyceraldehyde-3-phosphate dehydrogenase from the ghosts.

Preparation of tissue homogenates

2 g fresh tissue was homogenized in an Ultra Turrax homogenizer (Janke and Kunkel, Staufen, G.F.R.) for 30 s followed by homogenization for 2 min in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle in 5P8/10 mM β-mercaptoethanol. The final volume of the homogenate was adjusted to 20 ml unless stated otherwise and the homogenate was stirred in the cold for 1 h. The homogenate was centrifuged at 15 000 × *g* for 45 min. The supernatant obtained was filtered through a millipore filter (HA 0.45 μm), and the filtrate is described as the crude extract.

Enzyme assays

For the assay of glyceraldehyde-3-phosphate dehydrogenase the procedure described by Tanner and Gray [3], which is a modifcation of that of Velick [16], was used. The assay mixture consisted of the following in a total volume of 3 ml: 1.66 mM NAD^+, 8 mM sodium arsenate, 0.13 mM EDTA, 0.13 M triethanolamine-HCl buffer (pH 8.7), 0.1% Triton X-100, and 0.07% β-mercaptoethanol. The reaction was initiated by the addition of glyceraldehyde 3-phosphate. The absorbance change at 340 nm between 15 and 45 s was determined on a Beckman model Acta MVI recording spectrophotometer at 25°C, in a cuvette with 1-cm light path. Aldolase activity was determined by the procedure of Sibley and Lehninger [17].

Protein estimation

Ghost protein concentration was determined according to the procedure of Lowry et al. [18] after dissolving the ghosts in 0.2% SDS. NADH gives an intense color with Lowry's reagent; therefore, for calculating the specific activity of glyceraldehyde-3-phosphate dehydrogenase the protein concentration was measured by the dye binding procedure of Bradford [19]. NADH gives a slight increase in the absorbance at 595 nm with the color reagent used in this procedure. This was corrected by including appropriate amounts of NADH in the blank.

Polyacrylamide gel electrophoresis

Solubilization of the ghosts, SDS-polyacrylamide gel electrophoresis, staining and destaining were carried out as described by Fairbanks et al. [20]. The molecular weight of the glyceraldehyde-3-phosphate dehydrogenase protomer was determined using *Escherichia coli* RNA polymerase, bovine serum albumin and soya bean trypsin inhibitor as marker proteins [21,22].

Results and Discussion

Binding of glyceraldehyde-3-phosphate dehydrogenase from mouse tissue homogenates to glyceraldehyde-3-phosphate dehydrogenase depleted human erythrocyte ghosts

Incubation of glyceraldehyde-3-phosphate dehydrogenase-depleted human

erythrocyte ghosts with the crude homogenates of mouse liver, muscle, kidney and brain resulted in significant binding of glyceraldehyde-3-phosphate dehydrogenase from the homogenates to the ghosts. Fig. 1 shows the result of incubation of glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts with 3 vols. 5% crude tissue homogenates. Significant amounts of glyceraldehyde-3-phosphate dehydrogenase were associated with the ghosts, although the enzyme bound from the liver, kidney and especially the brain homogenate constituted only a small fraction of the total ghost proteins. Considering the fact that only about 60 μg ghost proteins have been applied on the gels, significant amounts of enzyme can be bound and purified using a few mg of the ghosts. The maximum amount of binding achieved corresponded to 6—7 units/mg ghost protein. But to achieve this level of binding with tissue homogenates other than muscle it was essential to incubate the glyceraldehyde-3-phosphate

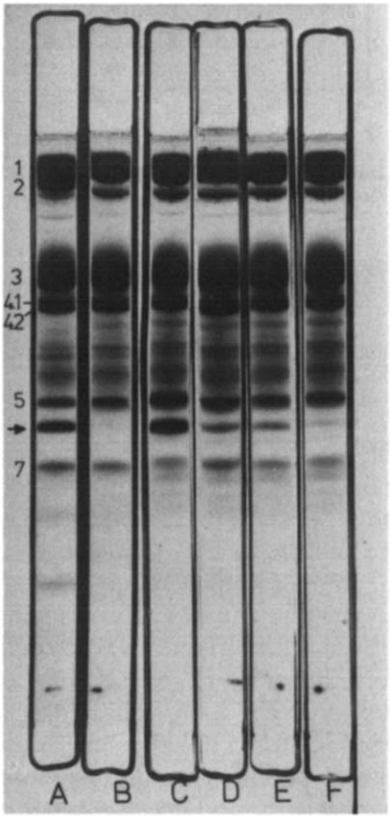

Fig. 1. Binding of glyceraldehyde-3-phosphate dehydrogenase from mouse tissue homogenates to glyceraldehyde-3-phosphate dehydrogenase-depleted human erythrocyte ghosts. Ghosts were prepared and depleted of dehydrogenase as described in the text. 1 vol packed glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts (4.5 mg protein/ml) was incubated with 3 vols. 5% homogenates at 0°C for 2 h. After the incubation the suspensions were centrifuged at 15 000 × g for 30 min. The sedimented ghosts were washed 4 times with 20 vols. 5P8 and dissolved in SDS solution [20]. About 60 μg protein was applied to each gel. A. Osmotic ghosts; B. Glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts; C,D, E and F: glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts incubated respectively with homogenates from muscle, liver, kidney and brain. The arrow indicates the glyceraldehyde-3-phosphate dehydrogenase protomer. The bands are numbered according to the system of Fairbanks et al. [20].

dehydrogenase depleted ghost with 20—25% (w/v) crude homogenates. Under these conditions a large number of polypeptides appear to be associated with the ghosts, some arising presumably by proteolysis, and specific elution of the enzyme in homogeneous form becomes extremely difficult. Although the amount of enzyme bound to the ghosts was dependent on the concentration of the enzyme in the homogenates, there appear to exist quantitative differences in the extent of binding of glyceraldehyde-3-phosphate dehydrogenase from different tissues; maximum binding was observed with the muscle and minimum binding with the brain homogenates. We do not know at this stage if the apparent differences in affinity are related to differences in the structure of glyceraldehyde-3-phosphate dehydrogenase from different tissues or are due to the presence of interfering protein or non-protein factors in the homogenates. Experiments involving dialysis of the homogenates (unpublished data) indicated that small molecular weight substances may not contribute to the observed differences in binding. Moreover, the possible existence of an inhibitor of the enzyme in the homogenates could be eliminated as the activities of added rabbit muscle glyceraldehyde-3-phosphate dehydrogenase could be quantitatively determined from the homogenates of all the tissues. As evident from Fig. 1, few proteins other then glyceraldehyde-3-phosphate dehydrogenase bind to the membrane during incubation with the homogenates. The principal one of these, a polypeptide of about 40 000 daltons, is evident as a darkening of band 5. We have observed a certain correlation between this band and the aldolase activity of the incubated ghosts, which together with the observations of Strapazon and Steck [23,24] suggest this to be aldolase. The dark-staining region below band 2, which can be seen in the glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts even before incubation with the homogenates, appears to be a product of endogenous proteolysis [25]. The binding of the dehydrogenase from the homogenates to the ghosts is a slow process (Fig. 2). To achieve maximum binding of the dehydrogenase, it was necessary to incubate the ghosts for over 1 h with muscle and liver homogenates and for 2 h with kidney and brain homogenates. This slow binding of the enzyme to the membrane is related to interference by other factors in the homogenates. This is shown by experiments with purified enzymes in which much shorter times are required to saturate the membrane with the enzyme [7]. The association between the ghosts and the dehydrogenase from all the tissues was strong. Repeated washing resulted in the loss of not more than 4% of the enzyme and it was possible to bind more than 90% of the glyceraldehyde-3-phosphate dehydrogenase from the liver, muscle and kidney homogenates to the ghosts by taking appropriate amounts of ghosts. However, the glyceraldehyde-3-phosphate dehydrogenase activity of the brain homogenates was bound to a markedly smaller extent. Thus, for equal units of enzyme, an amount of ghosts that bound more than 95% of the activity of enzyme derived from muscle, liver and kidney bound only about 65% of the enzyme from the brain homogenates (Fig. 3). This is surprising in view of the results of Kochman and Rutter [13], which indicate that the rabbit brain dehydrogenase is almost indistinguishable from the muscle enzyme in physical and catalytic properties. We are also investigating whether the apparent low binding of the enzyme from mouse brain homogenates represents the presence of a structurally different enzyme with low

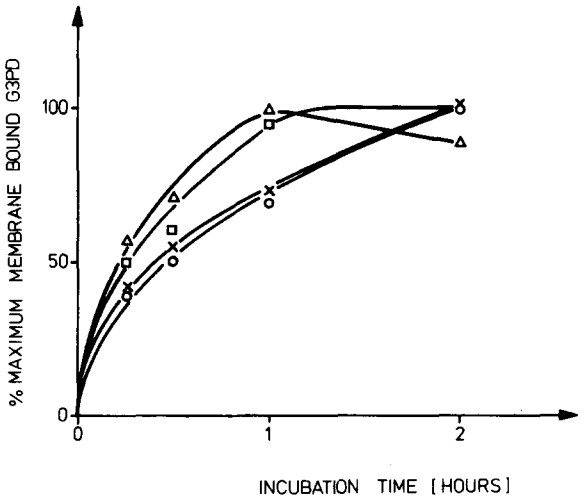

Fig. 2. Time course of binding of glyceraldehyde-3-phosphate dehydrogenase (G3PD) from mouse tissue homogenates on glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts. One vol. packed glyceraldehyde-3-phosphate-depleted ghosts (4.5 mg/ml) was incubated at 0°C with 3 vols. 5% homogenates from muscle (△), liver (□), kidney (X) and brain (○). At the appropriate times the suspension was diluted with 10 vols. of 5P8 and immediately centrifuged. The ghosts were washed 4 times with 20 vols. 5P8 and the activity of the dehydrogenase in the ghosts was determined. Each value represents the average result of at least 3 independent experiments.

affinity or the existence of more than one form of enzyme with differing affinities. The later possibility is less likely as isoenzymes of glyceraldehyde-3-phosphate dehydrogenase have not been detected in mammalian systems. Little proteolysis (except the endogenous proteolysis mentioned above) or fragmentation

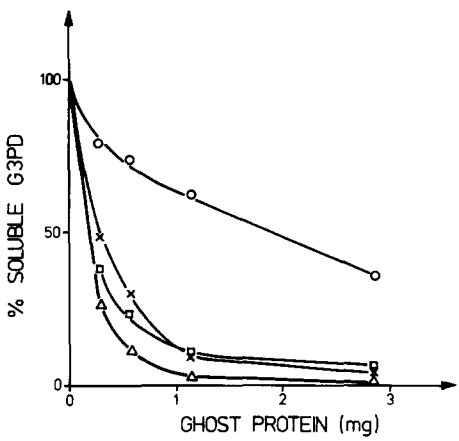

Fig. 3. Quantitative binding of glyceraldehyde-3-phosphate dehydrogenase (G3PD) from the mouse tissue homogenates by glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts. In a total volume of 2 ml the glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts were incubated at 0°C for 2 h with amounts of tissue homogenates from muscle (△), liver (□), kidney (X) and brain (○) corresponding to 5 units of glyceraldehyde-3-phosphate dehydrogenase. The suspensions were then centrifuged and the remaining dehydrogenase activity in the supernatants was determined. A good correlation was obtained between the loss of dehydrogenase activity from the supernatant and increase in the activity in the ghosts. Each value represents the average of results obtained from at least three independent experiments.

of the ghosts could be detected during the incubation with the tissue homogenates.

Elution of the ghost-bound glyceraldehyde-3-phosphate dehydrogenase from the ghosts

In order to elute the dehydrogenase as a homogeneous protein, prior elimination of other adsorbed proteins was essential. Aldolase was particularly troublesome in this regard and unless this was carefully removed significant amounts of this enzyme co-eluted with glyceraldehyde-3-phosphate dehydrogenase. The following pretreatment was found to be adequate for the elution of homogeneous glyceraldehyde-3-phosphate dehydrogenase. Ghosts which had been incubated with the various homogenates were washed 2 or 3 times with 5P8 and suspended in about 20 vols. cold 5P8/15 mM NaCl/2 mM fructose 1,6-bisphosphate (pH 8.0). The ghosts were incubated in an ice bath for 30 min with mild shaking. Subsequently the ghosts were centrifuged and washed once or if required twice with the same solution before a final wash with 5P8. Fructose 1,6-bisphosphate has been shown to elute aldolase almost quantitatively from human erythrocyte ghosts [24] and from phosphocellulose [26]. Moreover, the ionic strength of the solution used for aldolase elution appears to be sufficient to elute most of the aldolase from the human erythrocyte membrane without resulting in significant elution of glyceraldehyde-3-phosphate dehydrogenase [1]. This process of washing removed not more than 5% of the membrane-associated glyceraldehyde-3-phosphate dehydrogenase. The elution of the enzyme was performed with NADH [1]. Although a number of other metabolites have been shown to elute human erythrocyte membrane-associated glyceraldehyde-3-phosphate dehydrogenase, NADH was chosen due to its ability to elute the enzyme effectively at low concentrations and its lack of any adverse effect on the enzyme activity [1]. NAD^+ has also been used for elution of the dehydrogenase from affinity columns containing insolubilized nucleotides and their derivatives [27]. In agreement with the observations of Kant and Steck [1], we have found that about 90% of the membrane-associated glyceraldehyde-3-phosphate dehydrogenase can be eluted by incubating the ghosts in 5P8 containing 2 mM NADH for 90 min. A further 5—6% could be recovered by centrifuging and resuspending the ghosts in a fresh solution of

TABLE I

PURIFICATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM MOUSE TISSUES BY BINDING TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE-DEPLETED HUMAN ERYTHROCYTE GHOSTS AND ELUTION WITH NADH.

Tissue	Specific activity (μmol NAD/min per mg protein)		Purification (fold)
	Crude extract	Erythrocyte membrane binding and elution	
Muscle	7.4	153	24
Liver	1.1	90	82
Kidney	1.0	146	146
Brain	3.3	144	44

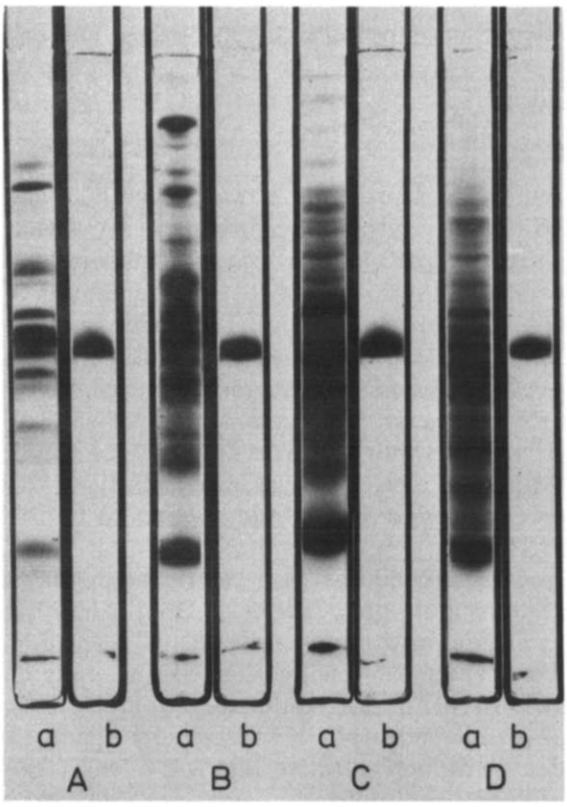

Fig. 4. Purification of glyceraldehyde-3-phosphate dehydrogenase from mouse muscle (A), liver (B), kidney (C) and brain (D); SDS gel electrophoresis before (a) and after (b) binding to glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts and elution with NADH. About 15 μg of pure enzyme were applied to the gels.

NADH. Most of the NADH could be removed by dialysing the solution with an excess of 5P8 at 0°C. In all the cases the total recovery of the enzyme amounted to 85—95% of the bound enzyme. As evident from the Table I, the eluted enzyme exhibited an activity of about 150 units/mg protein except in the case of the liver enzyme which was found to exhibit significantly lower activity. It is difficult to conclude if this low activity is an characteristic of the enzyme from the mouse liver or if it represents an artifactual inactivation during the purification procedure. Due to the short duration, the present procedure is not likely to lead to enzyme inactivation, however, the possibility of the liver enzyme being more labile can not be excluded. In fact several differences in the structural as well as in the catalytic properties of liver enzymes as compared to the muscle enzyme have been demonstrated [28,29]. As evident from the Fig. 4, glyceraldehyde-3-phosphate dehydrogenase purified from various tissues migrated essentially as a single band on SDS polyacrylamide gels. The migration corresponded to an apparent mol. wt. of 37 000 which is in good agreement with the protomer molecular weight of the enzyme from several sources. The specific activities are in good agreement with those of the purified preparation for rabbit [30] and rat muscle [31]. The eluted ghosts

retain their ability to bind glyceraldehyde-3-phosphate dehydrogenase and can be reused.

Conclusions

The data presented in this communication demonstrates that glyceraldehyde-3-phosphate dehydrogenase-depleted human erythrocyte ghosts can be used as specific high affinity adsorbents for the purification of glyceraldehyde-3-phosphate dehydrogenase from several tissues of the mouse. Glyceraldehyde-3-phosphate dehydrogenase from rat tissues can also be similarly purified (Saleemuddin, M., unpublished data). The observations together with the binding studies of Solti and Friedrich [7] with purified pig and rabbit muscle dehydrogenase and the fact that the dehydrogenase represents a class of enzyme with a high degree of homology [8] suggests that the procedure may be useful to purify the dehydrogenase from other sources. However, the affinity of the yeast dehydrogenase for the human erythrocyte membrane appears to be too weak to be utilized for purification [2].

A number of affinity chromatographic procedures have been developed for the purification of glyceraldehyde-3-phosphate dehydrogenase from a number of sources utilizing immobilized nucleotides as the general ligands [27,32—38]. These procedures usually require prior partial purification of the enzyme [36,37] or yield preparations which may not be completely homogeneous [27]. The principle disadvantage of affinity adsorbents with general ligands is co-binding of several dehydrogenases. Although selective removal of dehydrogenases from affinity columns containing insolubilized nucleotides appears to be possible in some instances [39], the binding of a particular dehydrogenase might be greatly influenced by the concentrations of other dehydrogenases in the homogenates and/or of their affinities for the immobilized ligand. In this respect the present procedure appears to offer advantage in that the glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts act as highly specific adsorbents of the enzyme (although aldolase also binds, it can be selectively eluted due to its low affinity).

Considerable information is available regarding the glyceraldehyde-3-phosphate dehydrogenase isolated from muscles of several animal species [9], but much less is known about the properties of the enzyme from other tissues. Glyceraldehyde-3-phosphate dehydrogenase from liver and brain appears to resemble the muscle enzyme in several respects [12,13]. However, differences have been demonstrated between the liver and muscle enzyme in NAD^+ binding, number of sulfhydryl groups, allosteric properties, regulatory effects of inorganic phosphate [11] as well as in the primary sequence near the amino-terminal [29]. Further studies on the tissue-specific nature of the enzyme are interesting in view of the correlation of the properties of the liver enzyme with the gluconeogenic function of this tissue [28]. Our present procedure appears to be particularly suited to such studies although differences appear to exist in the affinities of glyceraldehyde-3-phosphate dehydrogenase binding from the crude homogenates to the glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts.

The procedure is convenient and relatively inexpensive in view of the useful-

ness of the outdated blood for the ghost preparation. Also the procedure requires a relatively short time particularly if one starts with the enzyme-depleted ghosts. Ghosts prepared by the procedure used in this communication can, under suitable conditions, be stored for several days [1] and this appears to be true with the glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts. In this regard our preliminary studies indicate that crosslinking of the enzyme depleted ghosts with glutaraldehyde enhances their storage stability and resistance to proteolysis without significantly altering their affinity for glyceraldehyde-3-phosphate dehydrogenase. The possibility of immobilizing the ghosts to overcome the repeated centrifugation required in the present procedure is being investigated. Elegant methods are available for immobilization of microorganisms [40—43].

On the basis of these observations we are tempted to believe that it should be possible, using this principle, to purify other enzymes by binding to membranes if the membrane possesses the enzyme and if it can be selectively and reversibly eluted under mild conditions. Indeed other examples of such enzymes are available [44,45]. Modification of the membrane composition and structure or membrane surface charge may be an alternative way of changing the selectivity of enzyme binding in the future. Thus, it would be possible to use cellular systems as a general tool in the purification of diverse enzymes in a single step procedure.

Acknowledgement

This work was supported by a grant to M.S. from the DAAD, Bonn-Bad Godesberg and a grant No. BCT 112 from the BMFT, Bonn, to U.Z.

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