Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 423-427

ROLE OF LYSINE RESIDUES IN THE BINDING OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE TO HUMAN ERYTHROCYTE MEMBRANES

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Received September 9, 1983

SUMMARY: Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) binds reversibly to human erythrocyte membranes. Several specific amino acid residues involved in the enzyme-membrane contact region have already been identified. These include tyrosine 46 and threonine 150. Covalent modification of lysines 212 and 191 with pyridoxal phosphate results in a decreased affinity of the enzyme for erythrocyte membranes if the enzyme-linked pyridoxal phosphate is not reduced prior to binding. Reduction of the pyridoxal phosphate-lysine complex completely inhibits the binding of the enzyme to erythrocyte membranes. These results suggest a role for lysines 212 and 191 in the interaction of glyceraldehyde-3-phosphate with human erythrocyte membranes.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating); EC 1.2.1.12) binds reversibly to the erythrocyte membrane (1). The stoichiometry of binding of the dehydrogenase to the cytoplasmic surface of the erythrocyte membrane has been determined (1,2), as have the binding constants for the enzyme-membrane interactions. Kliman and Steck (3) have provided evidence that the dehydrogenase is bound to the membrane *in vivo*. The site in the red blood cell membrane to which the enzyme binds is the intrinsic membrane protein, band 3, which forms the principal anionic channel of this membrane (4). It is not yet known how the binding site and the bound protein interact with each other.

We have previously identified several specific amino acid residues as having possible involvement in the glyceraldehyde-3-phosphate dehydrogenase-human erythrocyte membrane contact region (5). These include tyrosine 46 and threonine

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0006-291X/83 $1.50

150. Covalent modification of lysines 212 and 191 with pyridoxal phosphate results in a slightly reduced affinity of the enzyme for the erythrocyte membrane. These results were evidenced by SDS gel electrophoresis and they are the same for pig muscle glyceraldehyde-3-phosphate dehydrogenase, whose amino acid sequence is known, as they are for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

In this communication we report that the reduction of glyceraldehyde-3-phosphate dehydrogenase linked pyridoxal phosphate-lysine complexes with sodium borohydride completely inhibits the ability of the enzyme to bind to human erythrocyte membranes.

MATERIALS AND METHODS

Glyceraldehyde-3-phosphate from rabbit muscle was from Boehringer Mannheim. Its specific activity was 50-60 units/mg. Glyceraldehyde-3-phosphate dehydrogenase was isolated from pig muscle by the method of Elodi and Szarenyi (6). Its specific activity was 60-70 units/mg. DL-glyceraldehyde-3-phosphate diethyl barium salt, from Sigma, was deionized on Dowex 50W (hydrogen form) and the resulting solution heated at 100°C for 3 min to obtain the free aldehyde. NAD^+, dithiothreitol, and pyridoxal-5'-phosphate were all from Sigma. All other chemicals (reagent grade) were from Fisher or Baker.

Enzyme Assay. Glyceraldehyde-3-phosphate dehydrogenase activity was measured as described elsewhere (7) using a Gilford spectrophotometer Model 240. The specific activity of the enzyme was expressed as units per milligram of protein, where a unit represents one micromole of NAD^+ reduced per minute. Protein concentrations were determined by the method of Lowry et al. (8).

Enzyme Membrane Preparation. Unsealed human erythrocyte membranes were prepared as described by McDaniel et al. (1) from outdated packed erythrocytes within one week following the expiration date. Erythrocytes were washed with 5 mM sodium phosphate and 0.15 M NaCl, pH 7.5, and subsequently hemolyzed in 9 volumes of 5 mM phosphate, mM EDTA, mM dithiothreitol, pH 7.5. The membranes were pelleted by centrifugation at 37,000 g for 20 min, and were given three or four additional washes with phosphate buffer. All operations were carried out at 4° C.

Binding of Glyceraldehyde-3-phosphate dehydrogenase to Membranes. Enzyme was added to aliquots of the enzyme depleted, washed membranes, incubated at 4° C for 15 min, and pelleted by centrifugation at 37,000 g for 30 min. The supernatant was assayed for dehydrogenase activity. The precipitated membranes were resuspended in phosphate buffer, washed several times, and then assayed for glyceraldehyde-3-phosphate dehydrogenase. In all binding experiments, 0.5 mg of enzyme was incubated with about 2.5 mg of erythrocyte membrane protein.

Electrophoresis. Bound glyceraldehyde-3-phosphate dehydrogenase and membrane proteins were detected by electrophoresis on a 7.5% polyacrylamide gel containing 0.1% sodium dodecylsulfate. Protein was stained in a solution containing 0.25% Coomassie brilliant blue, 45% ethyl alcohol, and 10% glacial acetic acid.

Modification of Enzyme Lysyl Residues With Pyridoxal Phosphate. The enzyme was treated with pyridoxal-5'-phosphate according to the procedure of Ronchi et

al. (9). Enzyme was diluted to a concentration of 7.0 micromolar and incubated with 1.0 mM pyridoxal phosphate for 15 min at 25° C in 45 mM pyrophosphate, mM EDTA buffer, pH 8.4. Quantitative determination of the enzyme-linked pyridoxal phosphate was carried out after the reduction of pyridoxal phosphate with sodium borohydride as described by Ronchi et al. (9) by measuring the absorption at 325 nm of the reduced pyridoxal-5' -phosphate-enzyme complex assuming a molar extinction coefficient of 9,710 for the ε-pyridoxal-lysine.

RESULTS AND DISCUSSION

Covalent modification of lysine residues of glyceraldehyde-3-phosphate dehyrogenase results in a slightly decreased affinity of the pig muscle enzyme for human erythrocyte membranes. This effect is observed only when 10.2 moles of lysine are modified per mole of enzyme monomer, and only in experiments with the pig enzyme (Table I).

When the pyridoxal-lysine residues are reduced with sodium borohydride to stabilize the adduct prior to binding inhibition of binding to the erythrocyte membranes is observed. Figure 1 shows unmodified glyceraldehyde-3-phosphate dehydrogenase in the first channel of the gel. The second channel contains membranes with sodium borohydride modified enzyme bound. The third channel contains enzyme depleted erythrocyte membranes. Channel 4,5 and 6 show that

Table 1. Inhibition of Binding of Glyceraldehyde-3-phosphate Dehydrogeanse to Erythrocyte Membranes by Modification of Lysine Residues.

Modifying Agent	Moles Lysine Modified / Mole enzyme Monomer	% Inhibition of Enzyme Activity		Binding to Membrane	
		Rabbit	Pig	Rabbit	Pig
Pyridoxal phosphate + $NaBH_4$		2.0	20	22	--
	5.2	70	68	-	-
	8.8	100	100	-	-
	11.0	100	100	-	-
$NaBH_4$	0	17	23	+	+
Pyridoxal phosphate	2.2	19	18	+	+
	4.9	56	59	+	+
	10.2	76	79	+	±

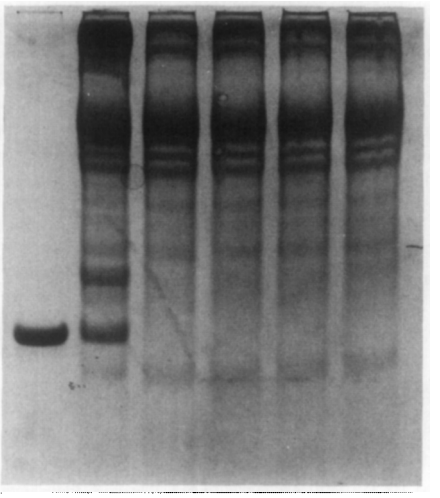

Figure 1. SDS Gel Electrophoresis. Inhibition of Binding of Pyridoxal Phosphate + $NaBH_4$ Modified Glyceraldehdye-3-phosphate Dehydrogenase to Erythrocyte Membranes.

Channels from left to right: (1) Unmodified enzyme (2) Membranes + $NaBH_4$ modified enzyme (3) Enzyme depleted membranes (4) Membranes + enzyme modified with pyridoxal phosphate and $NaBH_4$, 2 moles of lysine modified/mole of enzyme monomer (5) Same as (4) but with 5 moles of lysine modified/mole enzyme monomer (6) Same as (4) but with 10 moles of lysine modified/mole of enzyme monomer.

enzyme modified with pyridoxal phosphate and sodium borohydride fails to erythrocyte membranes. This inhibition is observed at concentrations as low as 2.0 moles of pyridoxal-lysine residues per mole of enzyme monomer. The results are the same for the pig muscle enzyme, whose amino acid sequence is known, as they are for rabbit muscle enzyme.

Forcina et al. (10) have shown that native glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle may be preferentially labeled at lysine 191 and lysine 212 with a concomitant loss of catalytic activity. It is known that lysine 212 and lysine 191 are residues in the catalytic domain of glcyeraldehyde-3-phosphate dehydrogenase (11).

The results of these studies suggest a role for lysine 212 and lysine 191, both in the catalytic domain, in the interaction of glyceraldehyde-3-phosphate dehydrogenase with erythrocyte membranes.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant GM-19394.

REFERENCES

1. McDaniel, C.F., Kirtley, M.E. and Tanner, M.J.A. (1974) J. Biol. Chem. 249, 6478-64-85.
2. Yu, J. and Steck, T.L. (1980) J. Biol. Chem. 250, 9176-9184.
3. Kliman, H.J. and Steck, T.L. (1980) J. Biol. Chem. 255, 6314-6321.
4. Steck, T.L. (1978) J. Supramol. Struct. 8, 311-324.
5. Eby, D. and Kirtley, M.E. (1982) Fed. Proc. 41, #5297.
6. Elodi, P. and Szorenyi, E. (1956) Acta Physiol. 9, 339-350.
7. Eby, D. and Kirtley, M.E. (1976) Biochem. 15, 2168-2171.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Ronchi, S., Zapponi, M.C. and Ferri, G. (1969) Eur. J. Biochem. 8, 325-331.
10. Forcina, B.C., Ferri, G., Zapponi, M.C. and Ronchi, S. (1971) Eur. J. Biochem. 20, 535-540.
11. Moras, D., Olsen, K.W., Sabeson, M.N., Ford, G.C. and Rossman, M.G. (1975) J. Biol. Chem. 250, 9137-9162.