Interaction of the Aldolase and the Membrane of Human Erythrocytes†

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ABSTRACT: Up to 80% of cellular aldolase (EC 4.1.2.13) was retained in the membrane fraction isolated following hemolysis of human erythrocytes under appropriate conditions. Binding was reversed by increasing the pH and ionic strength. Millimolar levels of the substrate, fructose 1,6-bisphosphate, selectively eluted aldolase from the membrane, while related metabolites did not. Using the membrane as a high affinity adsorbant, electrophoretically pure aldolase of high specific activity was prepared in high yield. The reassociation of pure aldolase and membranes was characterized. The sole site of human erythrocyte aldolase binding was shown to be the cytoplasmic surface domain of band 3, the predominant membrane-spanning polypeptide. One aldolase molecule was bound per band 3 polypeptide. Upon binding to either whole membranes, solubilized band 3, or proteolytic fragments from the cytoplasmic surface pole of band 3, aldolase underwent a profound loss of catalytic activity, reversed by raising the substrate concentration.

Glycolytic enzymes have been identified on hemoglobin-free plasma membranes (ghosts) obtained by hemolysis of erythrocytes in low ionic strength buffers (Schrier, 1963; Mitchell et al., 1965; Green et al., 1965; Nilsson and Ronquist, 1969; Duchon and Collier, 1971), but the physiological significance of this binding has remained obscure. The reversible association of glyceraldehyde-3-phosphate dehydrogenase (G3PD)[1] with human erythrocyte membranes has been examined in detail (Shin and Carraway, 1973; Kant and Steck, 1973; McDaniel et al., 1974). It has recently been found that the sole site of G3PD binding in vitro is the cytoplasmic surface domain of band 3, the predominant membrane-spanning polypeptide (Yu and Steck, 1975a,b), believed to facilitate the diffusion of anions across this membrane (Cabantchik and Rothstein, 1972; Ho and Guidotti, 1975; Zaki et al., 1975).

A preliminary study of the binding of rabbit muscle aldolase to ghosts has been published (Strapazon and Steck, 1976). A systematic investigation of human erythrocyte aldolase binding has awaited a satisfactory method for the isolation of this enzyme in sufficient quantity and purity. We now report on the interaction of the aldolase and membranes from human erythrocytes and describe an efficient method for aldolase purification, exploiting the high affinity and specificity of this association. A brief account of this work has been reported (Strapazon, 1976a).

Experimental Procedures

Materials. All biochemicals and enzymes were obtained from Sigma Chemical Co., except trypsin, which was purchased from Worthington. Other chemicals were reagent grade or better from Baker, Fisher, or Mallinckrodt.

Membranes. For analytical experiments, standard, hemoglobin-free, unsealed ghosts were prepared from freshly drawn or freshly outdated bank blood in 5 mM sodium phosphate (pH 7.0 or 8.0) by the method of Fairbanks et al. (1971). In some experiments, membranes were stripped of endogenous, saline-elutable glycolytic enzymes by washing with 0.15 M NaCl in 5 mM sodium phosphate (pH 8.0) (Fairbanks et al., 1971; Kant and Steck, 1973).

Assays. Spectrophotometric assays for human erythrocyte hexokinase, phosphoglycerate kinase, pyruvate kinase, and lactic dehydrogenase were performed by coupling these reactions to NADH oxidation or NAD reduction as described by Beutler (1975). G3PD was assayed as described by Steck and Kant (1974). Aldolase (EC 4.1.2.13) activity under standard conditions was measured using a modification of the method of Wu and Racker (1959). Assays were performed at ambient temperature (~23 °C) in 0.1 M Tris-HCl (pH 7.5) containing 0.2 mM NADH, 50 μg/mL of an α-glycerophosphate dehydrogenase-triosephosphate isomerase mixture, 3 mM fructose 1,6-bisphosphate, and the sample. Rates were obtained from continuous recordings of the changes in optical absorbance at 340 nm.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on gels containing 5% acrylamide (including 0.19% *N,N'*-methylenebisacrylamide) and 0.2% sodium dodecyl sulfate following the procedure of Fairbanks et al. (1971) as modified by Steck and Yu (1973). All gels in a given experiment were stained and scanned under identical conditions (Fairbanks et al., 1971). The major polypeptides were enumerated according to their characteristic electrophoretic mobilities and staining pattern (cf. Steck, 1974).

Results and Discussion

Retention of Aldolase by Ghosts. Initially we surveyed the extent of binding of several glycolytic enzymes by ghosts prepared at 0–5 °C by the method of Fairbanks et al. (1971). Less than 2% of the total cellular activity of hexokinase, triosephosphate isomerase, phosphoglycerate kinase, pyruvate kinase, and lactate dehydrogenase was recovered with the membrane fraction. On the other hand, 15–30% of the aldolase and 60–70% of the G3PD activity was retained by the ghosts, in accord with previous studies performed under other condi-

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‡ Supported by United States Public Health Service Training Grant No. GM 424. The data are taken in part from a dissertation submitted to the University of Chicago by E.S. in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] The abbreviation used is: G3PD, glyceraldehyde-3-P dehydrogenase.

tions (Schrier, 1963; Mitchell et al., 1965; Green et al., 1965; Nilsson and Ronquist, 1969; Duchon and Collier, 1971). Preparing membranes at 30–37 °C by prewarming the buffer, centrifuge, and rotor increased the membrane bound aldolase to ~70%, but did not significantly alter the retention of the other enzymes. A 30-min incubation of the initial hemolysate at 37 °C prior to centrifugation further enhanced aldolase retention to 80–90%.

Effect of Ionic Milieu on Aldolase Retention. The association of some glycolytic enzymes with ghosts is known to be sensitive to pH and ionic strength (Mitchell et al., 1965; Green et al., 1965; Duchon and Collier, 1971), but the influence of these variables on the binding of aldolase to ghosts had not previously been examined systematically. As shown in Figure 1A, the release of bound aldolase increased sigmoidally with ionic strength. Increasing the pH shifted the elution curve to the left, potentiating the dissociation. These data suggested an electrostatic component in the binding reaction.

Effect of Metabolites on Aldolase Retention. Certain metabolites eluted aldolase from ghosts (Figure 1B). The substrate for this enzyme, fructose 1,6-bisphosphate, was the most potent compound tested, followed by 2,3-bisphosphoglycerate and ATP. (The effective concentration of fructose 1,6-bisphosphate was presumably less than that stated, however, since the enzyme will have cleaved some of the substrate during the prolonged incubation period.) Neither of the reaction products, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, eluted aldolase at millimolar levels. (These assays were performed by polyacrylamide gel electrophoresis since these metabolites interferred with the assays of the catalytic activity of aldolase.) Fructose 1-P and fructose 6-P failed to elute aldolase, as did NADH, which readily releases G3PD from this membrane (Kant and Steck, 1973; McDaniel and Kirtley, 1975). We note that the effectiveness of the various metabolites in eluting aldolase parallels their potency as substrates and competitive inhibitors of its catalytic activity (Fornaini, 1968; Srivastava and Beutler, 1972; Beutler, 1974), suggesting that the active site of the enzyme may be involved in its membrane attachment.

Purification of Aldolase. The above findings suggested the following approach to the isolation of human erythrocyte aldolase.

(*a*) *Preparation of Membranes.* One unit of outdated bank blood (~450 mL) was diluted to 1 L in 150 mM NaCl in 5 mM sodium phosphate (pH 7.0) and the suspension centrifuged at 4500 rpm for 5 min in the HS-4 rotor of a Sorvall RC-3 centrifuge. The supernatant fluid and buffy coat were carefully removed (Fairbanks et al., 1971). Three more wash cycles were performed with the same buffer.

Because attempts at preparing membranes from whole units of blood in one batch reduced the recovery of aldolase, 42-mL aliquots of packed red cells were hemolyzed by dilution to 1700 mL in 5 mM sodium phosphate (pH 7.0), prewarmed to 37 °C. After incubation for 30 min at 37 °C, the suspension was centrifuged at 13 000 rpm for 20 min at 37 °C in a Sorvall RC-5 centrifuge and GSA rotor. The supernatants and buttons of debris were aspirated away (Fairbanks et al., 1971). The pellets were resuspended in the same buffer at 37 °C and centrifuged as before. The resultant red membrane pellets were stored on ice, while the same procedure was performed on the remaining erythrocytes. The pellets could be kept at this stage for several days at 0–5 °C with no loss of enzyme activity.

(*b*) *Elution of Aldolase.* Membrane pellets from 1 unit of cells were pooled, resuspended to 480 mL in 5 mM sodium phosphate (pH 7.0) and mixed with 480 mL of 2 mM fructose

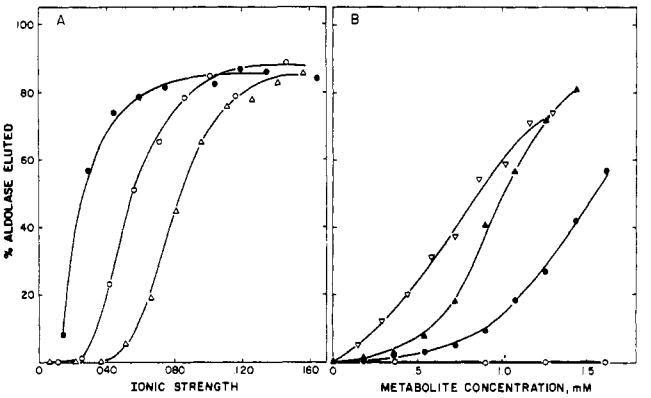

FIGURE 1: The membrane association of aldolase. (A) The effect of ionic strength and pH. Unsealed ghosts (0.1 mL) were incubated for 2 h on ice with 1.9 mL of 5 mM sodium phosphate buffer, pH 6–8, adjusted with NaCl to the indicated ionic strength. Following centrifugation, the supernatant fractions were analyzed for aldolase activity. (●) pH 8.0; (○) pH 7.0; (△) pH 6.0. (B) The effect of metabolites. Unsealed ghosts were incubated as in A with the indicated metabolites in 5 mM sodium phosphate buffer adjusted with NaOH or HCl to pH 7.0. (▽) Fructose 1,6-bisphosphate; (▲) 2,3-bisphosphoglycerate; (●) ATP; (○) NADH, fructose 1-phosphate, and fructose 6-phosphate.

1,6-bisphosphate in water. The suspension was brought to pH 7.8 with 1 N NaOH and stirred in an ice-water bath for 1 h before centrifugation at 13 000 rpm for 1 h at 0 °C. The supernatant solution was carefully collected by aspiration and recentrifuged to remove the last traces of membrane material. We found that these fragments would otherwise rebind the enzyme after the subsequent acidification step and thereby compromise aldolase purification and recovery. (It can be calculated from the data presented below that the high affinity membrane binding sites are in 50-fold excess over the aldolase present in the cell, so that even a few percent contamination is detrimental.)

The aldolase-free membrane residue recovered at this step is a rich and convenient source for the purification of G3PD.

(*c*) *Ion-Exchange Chromatography.* The aldolase-rich eluate was adjusted to pH 6.5 with 10% acetic acid and passed through a 1.2 × 12 cm column of carboxymethylcellulose (CM-22 from Whatman) at a maximum flow rate (12–16 mL per min). (This column had been prewashed with 1.5 M NaCl, then 100 mM sodium phosphate, pH 6.5, and finally 5 mM sodium phosphate, pH 6.5.) While the column was kept at room temperature, both the input and effluent solutions were maintained in ice-water baths. This column adsorption step removed the hemoglobin contaminant, often sizable, without binding aldolase.

The effluent from this column was adjusted to pH 5.5 with 10% acetic acid and applied to a second CM-22 column prepared as above, except at pH 5.5. The loaded column was washed with 25 mL of 5 mM sodium phosphate, pH 5.5, and then developed with a 30-mL gradient of 0–300 mM NaCl in the same buffer. One-milliliter fractions were collected. A single aldolase peak was detected by its absorbance at 280 nm and its enzymic activity. Peak fractions were pooled, and concentrated and desalted in an Amicon Diaflo cell with a PM-30 membrane. Alternatively, the preparation was stored in a solution of 3 M ammonium sulfate at 5 °C.

By this procedure, aldolase was purified approximately 10 000-fold and recovered at 80% yield (Table I and Figure 2). There was negligible contamination by G3PD (<0.1% of input). Gel electrophoresis at pH 8.0 in the absence of deter-

TABLE I: Purification of Human Erythrocyte Aldolase.[a]

Purification step	Vol (mL)	Protein (mg)	Aldolase activity: Total (μmol min^{-1})	Aldolase activity: Specific [μmol min^{-1} (mg of protein^{-1})]	Recovery (%)	Purification
A. Hemolysate	10200	77500	89	0.0011	100	1
B. Membranes	960	1400	79	0.056	89	51
C. Fructose 1,6-bisphosphate eluate	890	83.7	70	0.836	76	760
D. pH 6.5 eluate	880	71.2	68	0.955	76	868
E. Purified product (pH 5.5 column peak)	20	6.8	71	10.4	80	9455

[a] One unit of blood (236 mL of packed cells) was processed as described in the text. Aliquots were taken at steps A–E for biochemical and electrophoretic analysis (see Figure 3).

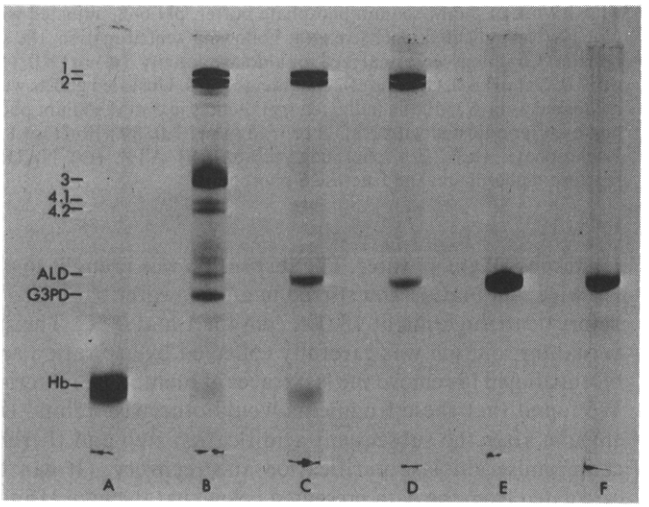

FIGURE 2: The purification of human erythrocyte aldolase. Aliquots taken at the stages designated in the text and in Table I were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Bands 1, 2, 3, 4.1, and 4.2 are principal polypeptides of the red cell membrane (Steck, 1974); ALD is aldolase, G3PD is glyceraldehyde 3-P dehydrogenase, and Hb is the globin chain of hemoglobin (Steck, 1974). (A) The unfractionated hemolysate. (B) The washed membranes. (C) The fructose 1,6-bisphosphate eluate. (D) The effluent from the first carboxymethylcellulose column (pH 6.5). (E) The pooled, peak fractions from the second carboxymethylcellulose column (pH 5.5): pure aldolase. (F) A rabbit muscle aldolase standard.

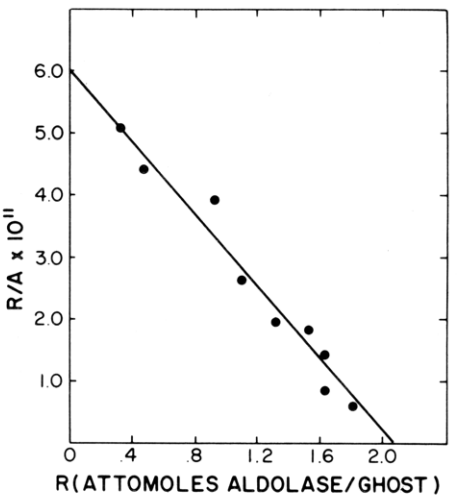

FIGURE 3: Scatchard analysis of aldolase binding to ghosts. Unsealed ghosts were washed with 0.15 M NaCl–5 mM sodium phosphate, pH 8.0 (to remove endogenous enzyme), and then with 5 mM sodium phosphate, pH 7.0. Aldolase solutions in this buffer (1 mL) were incubated with packed ghosts (0.05 mL) for 2 h on ice. Each mixture was sampled for total aldolase activity; the membranes were pelleted and the supernatant fluids sampled for determination of the activity of free aldolase. Membrane-bound aldolase was taken as the difference. Aldolase activities were converted to molar concentrations using the specific activity of the enzyme stock and an estimated molecular weight of 160 000. Ghost numbers were estimated in the Coulter Counter. The data were plotted according to Scatchard (1949) as $R/A = nK - RK$, where R is attomoles (10^{-18} mol) of aldolase bound per ghost, A is the concentration of free aldolase (M), n is the number of binding sites per ghost, and K is the association constant.

gent revealed one band, migrating anodally; at pH 6, a single component with a very low cathodal mobility was detected (not shown). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed a single band whose electrophoretic mobility coincided with that of rabbit muscle aldolase (Figure 2, gels E and F), indicating protomers of molecular weight 40 000 (Kawahara and Tanford, 1966; Sia and Horecker, 1968). Similarly, the sedimentation velocity of the intact molecule on 5–20% sucrose gradients did not detectably differ from that of purified rabbit muscle aldolase.

The purified human enzyme showed a broad pH optimum between pH 6 and 8 and a K_m of 1.8×10^{-5} M under our standard assay conditions. Based on a protein determination according to Lowry et al. (1951), a maximum velocity of 10.2 $\pm$ 0.6 (SD) mol min^{-1} (mg of protein^{-1}) was found in five preparations. We conclude that this isolate compares favorably in all respects with the only previously described preparation of human erythrocyte aldolase (Fornaini, 1968).

Quantitative Analysis of the Aldolase–Membrane Interaction. The binding of purified human erythrocyte aldolase to unsealed ghosts yielded a linear Scatchard plot, suggesting a single class of homogeneous, noninteracting binding sites (Figure 3). The binding capacity was 2.2×10^{-18} mol of aldolase (1.2×10^6 aldolase tertramers) per ghost and the affinity was $K_A = 2.7 \times 10^7$ M^{-1} in 5 mM sodium phosphate, pH 7.0.

Membrane Sidedness of Human Aldolase Binding Sites. The binding of aldolase to either unsealed ghosts, where both membrane surfaces were exposed, or Mg^{2+}-resealed ghosts, which present only their outer surface to the bathing medium, was determined. A negligible amount of aldolase bound to resealed ghosts compared with the avid and extensive binding of the enzyme to unsealed ghosts. These data demonstrate that the high affinity binding sites for human aldolase were confined to the membrane's cytoplasmic surface, just as was previously shown for rabbit muscle aldolase (Strapazon and Steck, 1976).

Aldolase Binds Exclusively to Band 3. We prepared three membrane isolates from which the principal peripheral poly-

TABLE II: Stoichiometry of Aldolase Binding to Band 3.[a]

Membrane Preparations	Aldolase:band 3	
	Stain intensity ratio	Molar ratio
A. Membranes minus band 6	2.01	0.88
B. Membranes minus bands 1, 2, 5, 6	2.47	1.09
C. Membranes minus bands 1, 2, 4.1, 4.2, 5, 6	2.10	0.92
D. Sucrose gradient purified complex	2.20	0.97
Average ± SD	2.20 ± 0.20	0.97 ± 0.09

[a] Unsealed ghosts were prepared as in Experimental Procedures and then stripped of various peripheral proteins and analyzed according to Strapazon and Steck (1976). Sample A ghosts were washed with 150 mM NaCl–5 mM sodium phosphate (pH 8.0) to remove endogenous glycolytic enzymes, principally band 6 (Kant and Steck, 1973). Membranes in sample B were treated as in A and then washed twice with 5 mM sodium phosphate (pH 8.0) and stripped of bands 1, 2, and 5 by incubation at 37 °C for 15 min in 0.1 mM EDTA (pH 8.0) (Fairbanks et al., 1971; see also the depleted membranes of Steck et al., 1976). Sample C contained membranes stripped of all peripheral proteins by incubation with 40 mM lithium 3,5-diiodosalicylate (Yu et al., 1973). Membranes A–C were finally washed with 5 mM sodium phosphate (pH 7.0) and then incubated in this buffer for 2 h on ice with a calculated 2.7-fold molar excess (over band 3) of human erythrocyte aldolase. The membranes were pelleted and washed once in the same buffer. Sample D was a peak fraction from the band 3–aldolase complex region of a rate zonal sedimentation analysis in a sucrose density gradient containing Triton X-100 (as in Strapazon and Steck, 1976). Aliquots of samples A–D were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The gels were scanned and the absorbance of the band 3 and aldolase peaks was integrated. The ratios of these staining intensities are entered in the left-hand column. Molar ratios were estimated as described in the text (see also Yu and Steck (1975b) and Strapazon and Steck (1976)), using molecular weight values of 160 000 for aldolase and 91 000 for band 3 (Steck et al., 1976).

peptides (bands 1, 2, 4.1, 4.2, 5, and 6) were extracted. The membranes were saturated with aldolase, washed with 5 mM sodium phosphate (pH 7.0) to remove the unbound enzyme, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Strapazon, 1976b). Desitometric quantitation (Table II, left-hand column) revealed that the staining intensity of aldolase remained constant, relative to that of band 3, despite the loss of the major peripheral polypeptides (comprising approximately half of the mass of protein in standard ghosts). Since band 3 is quantitatively retained in these stripped membranes (Steck and Yu, 1973), we infer that the peripheral membrane proteins did not provide sites for aldolase binding.

The noneluted (integral) proteins were analyzed for complex formation with aldolase, as in Strapazon and Steck (1976). Saline-stripped ghosts were replenished with pure aldolase and extracted with Triton X-100. The aldolase was found to be selectively solubilized along with the integral proteins (Yu et al., 1973). Rate zonal sedimentation analysis of these extracts on sucrose density gradients showed that band 3 and aldolase cosedimented more rapidly than did either pure component. That the proteins in the cosedimenting peak comprised a specific aldolase–band 3 complex was demonstrated by its dissociation to the normally sedimenting forms in the presence of 0.3 M NaCl or 2 mM fructose 1,6-bisphosphate, which elute aldolase from its site on the membrane.

We measured the stoichiometry of aldolase bound to band

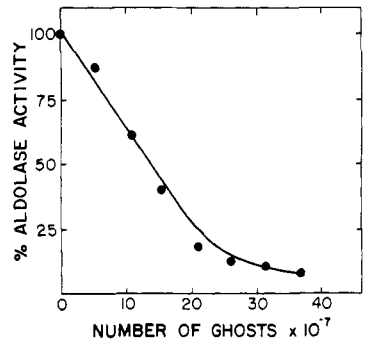

FIGURE 4: The inhibition of aldolase activity by ghosts. Unsealed ghosts were washed with 0.15 M NaCl–5 mM sodium phosphate (pH 8.0) to remove endogenous enzyme and then washed thoroughly with 10 mM imidazole hydrochloride (pH 7.0). Aldolase (3 μg) was incubated with increasing numbers of ghosts in 1 mL of 10 mM imidazole hydrochloride (pH 7.0) for 2 h at 23 °C. The mixture was directly assayed for aldolase activity using 50 μM fructose 1,6-bisphosphate according to the modification described in the text.

3 in membranes and in soluble complexes as follows. Aliquots of pure aldolase and band 3 (the generous gift of Susanna Rudofsky and James Koziarz) containing 1–12 μg of protein (as calibrated by amino acid analysis) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and their staining intensities integrated. The specific staining of aldolase was determined to be 1.34 times that of band 3. The staining intensities of aldolase and band 3 in gels from the various membrane preparations saturated with aldolase were then integrated and the molar ratios of these two species estimated (Table II, right-hand column). The specific aldolase binding capacity of the various membranes did not differ significantly from that of pure band 3. Therefore, band 3 can account for all the aldolase binding activity in the membrane. The average molar ratio of 0.97 indicates that one band 3 polypeptide binds one aldolase tetramer.

The Catalytic Activity of Bound Aldolase. Aldolase is quantitatively eluted from the membrane at the ionic strength, pH, and fructose 1,6-bisphosphate level used in our standard aldolase assay mixture. To measure the activity of aldolase while on the membrane, we changed the buffer to 10 mM imidazole hydrochloride (pH 7.0) and reduced the substrate level to 50 μM. Under these conditions, aldolase retention on membranes was nearly quantitative (see Figures 1 and 2 and below). This low level of substrate was still ten times greater than the K_m of the free enzyme, so that linear reaction rates and Michaelis–Menton kinetics were obtained for free aldolase under these conditions. Control experiments assured that the auxiliary enzymes (α-glycerophosphate dehydrogenase and triosephosphate isomerase) were not rate-limiting nor membrane bound under the new assay conditions (Strapazon, 1976b).

Figure 4 demonstrates that ghosts inhibited aldolase catalytic activity. This effect was evident within 10 s of ghost addition and was rapidly reversed by the addition of millimolar fructose 1,6-bisphosphate or by raising the ionic strength to 0.15, treatments which elute aldolase from the membrane.

An intact membrane was not required for this effect, in that Triton X-100 extracts of ghosts and purified band 3 showed the same reversible inhibitory behavior. Water-soluble fragments of the cytoplasmic pole of band 3, released by digestion of inside-out vesicles with trypsin (Steck et al., 1976), also inhibited aldolase activity rapidly and reversibly (Strapazon, 1976b). Identical inhibitory effects were also observed with rabbit muscle aldolase.

The inhibition of aldolase by ghosts and their Triton X-100 extracts was strictly competitive in form. A K_I of 10^{-7} M was estimated in 5 mM sodium phosphate (pH 7.0), assuming that there are 1.2×10^6 aldolase binding sites per ghost. This value resembled the $K_D = 0.4 \times 10^{-7}$ M, determined by Scatchard analysis (Figure 3).

Membrane-bound aldolase was not totally inactive. As implied in Figure 4, aldolase activity dropped smoothly to about 15% of maximum, but fell no further when membranes were present in vast excess. Rapid centrifugation of such mixtures, while the reaction was in progress (using 50 μM substrate and low ionic strength buffer), demonstrated that most of the residual activity was sedimentable (hence, membrane bound).

Conclusions

Human erythrocyte aldolase resembled rabbit muscle aldolase (and G3PD from these two sources) in its binding to isolated human red cell membranes (cf. Kant and Steck, 1973; McDaniel et al., 1974; Yu and Steck, 1975b; Strapazon and Steck, 1976). In all these cases, the reaction was of high affinity ($>10^7$ M^{-1}) at low ionic strength but was weakened as the electrolyte concentration and pH increased. Liganding of both the aldolases and G3PD occurred only to the cytoplasmic pole of band 3, the major membrane polypeptide. In all cases, a binding stoichiometry of one tetramer per band 3 polypeptide was inferred both from quantitative densitometry of gels of enzyme-saturated membranes (Table II) and from the binding capacity inferred from Scatchard analysis (Figure 3), since there are $\sim 1 \times 10^6$ band 3 polypeptides per ghost (Fairbanks et al., 1971; Steck, 1974).

The binding of human erythrocyte G3PD to ghosts showed a positive cooperativity (Yu and Steck, 1975b) and that of rabbit muscle aldolase, a negative cooperativity (Strapazon and Steck, 1976); human erythrocyte aldolase, however, showed a simple saturation isotherm (Figure 3). Band 3 is a dimer (Yu and Steck, 1975b) which could undergo different conformational changes upon liganding each of these enzymes. A simpler hypothesis, however, is that the first glycolytic enzyme bound to the band 3 dimer can promote or weaken the binding of a second enzyme molecule by direct interactions between the ligands. Likewise, the observation that G3PD can block aldolase binding (Strapazon, 1976b) may reflect only steric hindrance and not competition for a single binding site on or a conformational change in the band 3 molecule.

Because the liganding of both G3PD and aldolase is reversed by relevant phosphorylated metabolites, we supposed that their site of association with band 3 might also be phosphorylated. However, we have found by chemical analysis that the P_i content of band 3 is less than 0.3 mol per mol of protomer and, hence, is insufficient to account for the 1:1 binding stoichiometry observed (T. L. Steck, unpublished data).

Other workers have observed that aldolase can associate in vitro with various subcellular structures, producing phenomena similar to those observed here (e.g., a loss of catalytic activity reversed by the elution of the enzyme by its substrate). These systems include F-actin (Arnold and Pette, 1970), membrane fragments from rat brain homogenates (Clarke and Masters, 1972), rat liver microsomes (Foemmel et al., 1975; T. L. Weiss and I. A. Bernstein, personal communication), and human erythrocyte membranes (Solti and Friedrich, 1976). If aldolase associates with the red cell membrane in vivo, its reversible inhibition upon binding to the membrane might have physiologic importance. However, neither the specific binding of aldolase (and G3PD) to band 3 in the isolated red cell membrane nor their ready elution provides adequate grounds for inferring their disposition in vivo. In situ studies on intact cells are needed to answer this question.

Acknowledgments

The authors thank Heinz Köhler for performing the amino acid analysis and Mrs. Benita Ramos for her technical assistance.

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Dentin Phosphoprotein: An Extracellular Calcium-Binding Protein†

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ABSTRACT: The (ethylenedinitrilo)tetraacetic acid extracted phosphoprotein of bovine dentin contains 37.6 residue % of aspartic acid and 41.8 residue % serine, most of which is phosphoserine. The phosphoprotein binds calcium ions to both high- and low-affinity sites, with association constants 3.6×10^4 and 5.1×10^2 mol^{-1}, respectively. Alkaline-hydrolysis studies showed that γ-carboxyglutamic acid was absent from this calcium-binding protein. The binding interactions are nonideal, showing a dependence of the number of high-affinity sites per milligram of protein on the protein concentration. This appears to be the result of a calcium-ion mediated concentration-dependent aggregation of the phosphoprotein. Conformational studies, using circular dichroism, show the protein to have a random chain conformation in aqueous solution. The addition of either methanol (to 50%, v/v) or Ca ion caused a change in the circular dichroism spectra, suggesting a transition to a more ordered extended chain conformation. ^{31}P nuclear magnetic resonance spectra of the phosphoprotein showed resonances corresponding only to the presence of orthophosphates. Spectra determined during the titration of an alkaline solution of the sodium ion salt of the phosphoprotein showed the presence of a single set of acidic groups with a pK_a of 6.8, also characteristic of orthophosphates. The addition of calcium ions caused a significant signal broadening of the orthophosphate resonance, indicating a specific calcium ion–orthophosphate interaction. A calcium–phosphoprotein complex precipitates upon standing at neutral pH at room temperature. Infrared spectra of this precipitate showed the presence of bands at 500–600 cm^{-1}, characteristic of calcium–orthophosphate interaction. Examination of the infrared bands in the carboxylate region also established the presence of calcium–carboxylate interactions in the calcium–phosphoprotein complex. Both carboxylate and orthophosphate groups thus appear to be involved in providing high-affinity binding sites for calcium ions. In the trans-extended chain conformations suggested by the circular dichroism data, sheet-like arrays would present high-affinity calcium-binding faces that could initiate crystal phase growth.

A variety of noncollagenous proteins is present in the organic extracellular matrix of dentin (Veis et al., 1972; Leaver and Shuttleworth, 1966; Jones and Leaver, 1974; Butler et al., 1972). A major component of these noncollagenous proteins of human and animal dentin is a group of acidic, phosphorus-containing proteins, referred to as dentin phosphoproteins. The phosphoprotein isolated from unerupted bovine molars contains large amounts of serine, phosphoserine, and aspartic acid. These residues comprise at least 70% of the total amino acid composition of the bovine dentin phosphoprotein.

The interaction between the dentin phosphoprotein and calcium ion is of interest in connection with the mechanism of mineralization of the collagenous matrices of bone and dentin. Mineralization involves the deposition of calcium phosphate as amorphous calcium phosphate or crystalline calcium hydroxylapatite in and around the collagen fibers (Posner, 1973; Posner and Betts, 1975; Termine and Posner, 1965). The newly synthesized dentin phosphoprotein in the developing rat incisor is secreted and appears rapidly at the mineralizing front (Weinstock and Leblond, 1973). Moreover, some of the phosphoprotein binds directly to the collagen matrix (Dickson et al., 1975; Carmichael et al., 1971). These considerations all point to a calcium ion–organic matrix interaction as a key element in the nucleation of calcium phosphate deposition in dentin. Nawrot et al. (1976) have recently shown that the addition of dentin phosphoprotein to a metastable solution of calcium phosphate catalyzes the formation of hydroxylapatite crystals. The matrix–mineral phase interaction in bone may be of similar nature, since bone contains strongly polyanionic noncollagenous proteins which also interact strongly with collagen (Shuttleworth and Veis, 1972) and with divalent metal ions (Peacocke and Williams, 1966).

Since phosphate and carboxylate groups are known to chelate divalent cations (Chaberek and Martell, 1959), it was suggested that a possible biological function of the phosphoprotein was in the initiation of calcification of dentin. It was proposed that phosphoproteins could initiate calcification by acting as a nucleation site for the initial epitactic localization of calcium within the collagen-fibril network (Veis et al., 1969). Alternatively, it was suggested that the phosphoproteins could inhibit calcification by complexing calcium ions, thereby preventing nucleation within the collagen-fibril network (Veis et al., 1969). In either case, the same questions arise: What is the affinity of the dentin phosphoprotein for calcium ions, and how does the phosphoprotein relate to the precipitation of calcium phosphates? The experiments summarized in this

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