Binding of Rabbit Muscle Aldolase to Band 3, the Predominant Polypeptide of the Human Erythrocyte Membrane†

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ABSTRACT: Aldolase is a trace protein in isolated human red cell membrane preparations. Following total elution of the endogenous enzyme by a saline wash, the interaction of this membrane with rabbit muscle aldolase was studied. At saturation, exogenous aldolase constituted over 40% of the repleted membrane protein. Scatchard analysis revealed two classes of sites, each numbering approximately 7×10^5 per ghost. Specificity was suggested by the exclusive binding of the enzyme to the membrane's inner (cytoplasmic) surface. Furthermore, millimolar levels of fructose 1,6-bisphosphate eluted the enzyme from ghosts, while fructose 6-phosphate and NADH (a metabolite which elutes human erythrocyte glyceraldehyde-3-phosphate dehydrogenase (G3PD) from its binding site) were ineffective. Removing peripheral membrane proteins with EDTA and lithium 3,5-diiodosalicylate did not diminish the binding capacity of the membranes. An aldolase-band 3 complex, dissociable by high ionic strength or fructose 1,6-bisphosphate treatment, was demonstrated in Triton X-100 extracts of repleted membranes by rate zonal sedimentation analysis on sucrose gradients. We conclude that the association of rabbit muscle aldolase with isolated human erythrocyte membranes reflects its specific binding to band 3 at the cytoplasmic surface, as is also true of G3PD.

Aldolase has been found associated with human erythrocyte membranes (ghosts) isolated by hypotonic hemolysis (Schrier, 1963; Mitchell et al., 1965; Nilsson and Ronquist 1969; Duchon and Collier, 1971; Shin and Carraway, 1973); however, the nature of this binding was not characterized. Another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (G3PD)[1], exhibits similar behavior and its binding to isolated membranes (Shin and Carraway, 1973; Kant and Steck, 1973; McDaniel et al., 1974) and membrane proteins (Yu and Steck, 1975a,b) has been recently investigated in detail. We have undertaken an analysis of the interaction of aldolase with the human erythrocyte membrane, by studying the association of the purified components. Rabbit muscle aldolase was used since it was commercially available in purified form and resembled the human erythrocyte enzyme in its physical, catalytic, and membrane-binding properties.[2]

Experimental Procedures

Materials. α-Glycerophosphate dehydrogenase–triose phosphate isomerase mixture (type III) and rabbit muscle aldolase (Grade I) (EC 4.1.2.13) and their substrates were obtained from Sigma. Lithium 3,5-diiodosalicylate (LIS) was from Eastman. All chemicals and solvents were reagent grade or better from Fisher, Mallinckrodt, or Baker.

Membranes. Unless otherwise noted, all procedures were performed at 0–5 °C and all centrifugations were at 15 000 rpm in a Sorvall SS-34 or SM-22 rotor. *Standard, hemoglobin-free, unsealed ghosts* were prepared from freshly drawn or freshly outdated bank blood by the method of Fairbanks et al. (1971).

Saline washed ghosts devoid of aldolase and G3PD were obtained by washing standard ghosts with 150 mM NaCl–5P8 (Kant and Steck, 1973), followed by two or more washes with 5P7.

Resealed ghosts were prepared in 1 mM $MgSO_4$–5P8 according to Kant and Steck (1973) and given a final wash with 1 mM $MgSO_4$–5P7.

Depleted membranes (EDTA vesicles) were prepared as described by Steck et al. (1976) and washed twice with 5P7.

LIS vesicles were prepared according to Yu et al. (1973) at a final LIS concentration of 40 mM; they were given two additional washes with 5P7. Pellets resulting from the EDTA or LIS treatment of standard unsealed ghosts were resuspended in 5P7 to their original ghost pellet volume (equivalent to $\sim 7 \times 10^9$ ghost/ml or 4 mg of standard ghost protein/ml; Fairbanks et al., 1971).

PAGE. Electrophoresis was performed on gels containing 5% acrylamide (including 0.19% *N,N'*-methylenebis-(acrylamide)) 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 under identical conditions with Coomassie blue R. Gel scanning and densitometry followed procedures of Fairbanks et al. (1971). The major membrane polypeptides were enumerated according to their characteristic electrophoretic mobilities and staining pattern according to Fairbanks et al. (1971), as modified by Steck (1972, 1974).

Rate Zonal Sedimentation Analysis. Methods for the preparation of Triton X-100 membrane extracts and rate zonal sedimentation analysis were, with slight modifications, those of Yu et al. (1973, 1975b). Linear 5–20% (w/v) sucrose gradients (5 ml) were overlaid with 0.25 ml of pro-

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[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; 5P8, 5 mM sodium phosphate, pH 8.0; 5P7, 5 mM sodium phosphate, pH 7.0; G3PD, glyceraldehyde-3-phosphate dehydrogenase; LIS, lithium 3,5-diiodosalicylate; PAS 1-3, the principal periodic acid–Schiff stained glycoproteins demonstrated by PAGE.

[2] E. Strapazon and T. L. Steck, unpublished data.

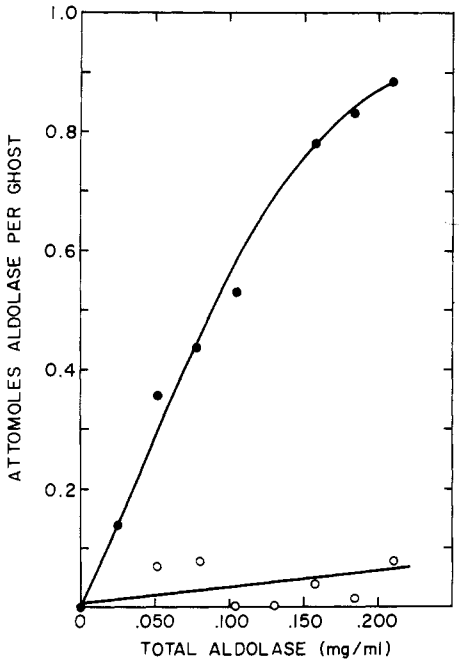

FIGURE 1: Aldolase binding to unsealed and Mg^{2+}-resealed ghosts. Aldolase solutions (0.9 ml) of increasing concentrations were mixed with 0.1 ml of either Mg^{2+}-resealed or saline-washed (unsealed) ghosts in 1 mM $MgSO_4$-5P7. Following a 2-h incubation on ice, the membranes were pelleted and the supernatant aldolase assayed. Membrane-bound aldolase was estimated by subtracting the supernatant activity from the total activity in the suspension before centrifugation.

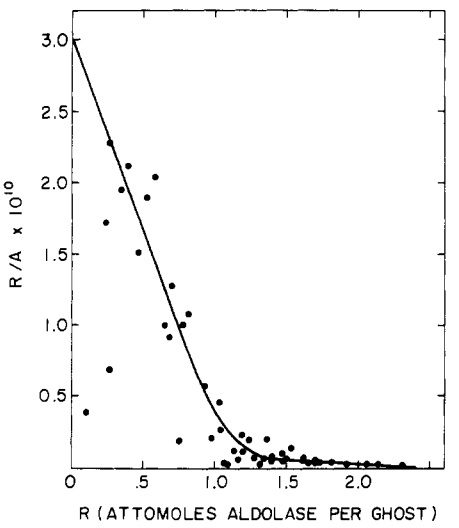

FIGURE 2: A Scatchard plot of aldolase binding to unsealed ghosts. One milliliter aliquots of aldolase solutions of increasing concentration were combined with 0.1 ml of saline-washed ghosts. Following a 2-h incubation on ice, the suspensions were sampled, the membranes were pelleted, and the supernatant fluid was recovered. Membrane-bound activity was taken as the difference between total activity before centrifugation and supernatant activity afterward. The binding data were plotted according to Scatchard's equation (Scatchard, 1949), $R/A = nK - RK$, where R is attomoles (10^{-18}mol) of aldolase bound per ghost; A is the molar concentration of free aldolase; n is the number of binding sites per ghost, and K is the affinity constant. The data points are pooled from three experiments.

tein solution and centrifuged for 6.0 h at 50 000 rpm in a Spinco SW 50.1 rotor without braking. Gradient fractions (0.20 ml) were displaced with a Micrometric syringe buret through a puncture at the tube bottom, combined with 0.050 ml of "PAGE concentrate" (Steck et al., 1976) and analyzed by PAGE.

Analytical Procedures. Aldolase activity 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 of a α-glycerophosphate dehydrogenase-triosephosphate isomerase mixture, 3 mM fructose 1,6-bisphosphate, and sample in a final volume of 1 ml. Aldolase concentrations were determined using an $E^{1\%}_{280nm}$ value of 9.38 (Donovan, 1964) or by amino acid analysis. Reaction rates were converted to concentrations using the enzyme's measured specific activity and a molecular weight of 156 000 daltons (Castellino and Barker, 1968). Ghosts were counted in a Model ZB Coulter Counter (100-μm aperture) and corrected for coincidence. Protein was determined by the method of Lowry et al. (1951), absorbance at 280 nm, and amino acid analysis.

Results and Discussion

Our preparations of hemoglobin-free ghosts retained approximately 15% of total cellular aldolase. Under special preparative conditions, the bulk of the enzyme can be recovered on the membrane.[2] Treatment with 150 mM NaCl-5P8 completely eluted the endogenous aldolase from the membranes along with G3PD (Kant and Steck, 1973), freeing all liganding sites for repletion studies. The binding of rabbit muscle aldolase to these ghosts reached equilibrium within 120 min at 0 °C. The enzyme was rapidly eluted at high ionic strength.

Specificity of Aldolase Binding. 1. Sidedness. The association of aldolase with unsealed ghosts obeyed a simple saturation isotherm, as shown in Figure 1. We estimate that 2.4 amol (attomoles) of enzyme was bound per ghost at saturation, amounting to 45% of the total membrane protein (Figure 2). Mg^{2+}-resealed ghosts, which present only their outer surface to the external medium (Kant and Steck, 1973), bound only a few percent at best of the aldolase associated with unsealed ghosts (Figure 1), and even this could represent a small contamination (~3%) by unsealed ghosts. These data indicate that all the aldolase binding sites were confined to the membrane's inner surface.

2. Substrate Elution. At pH 7.0, 1 mM fructose 1,6-bisphosphate eluted 61% of the bound enzyme from aldolase enriched ghosts. Fructose 6-phosphate and NADH, a metabolite which effectively removes membrane-bound human erythrocyte G3PD (Kant and Steck, 1973), eluted less than 8%.

Characterization of Aldolase Binding. The capacity and affinity for the ghost-aldolase interaction were estimated by plotting equilibrium binding data according to Scatchard (1949). A curve of concave shape was obtained (Figure 2), indicative of two classes of binding sites: 0.67×10^6 aldolase sites per ghost were of high affinity ($K_A = 27 \times 10^7$ M^{-1}), and 0.77×10^6 per ghost were of low affinity ($K_A = 0.7 \times 10^7$ M^{-1}). Figure 2 suggests the presence of either two major classes of binding sites of approximately equal prevalence or negative cooperativity in the binding of aldolase to a single molecular species with two (or more) liganding sites. (Two data points in Figure 2 suggest the possibility of a downward deflection of the curve at low binding levels; i.e., positive cooperativity. Since such a pattern has been observed in this system for G3PD (Yu and Steck, 1975b), we evaluated this issue in a separate study of aldolase binding. The isotherm in the low R region gave no indication of sigmoidicity or positive cooperativity; hence the

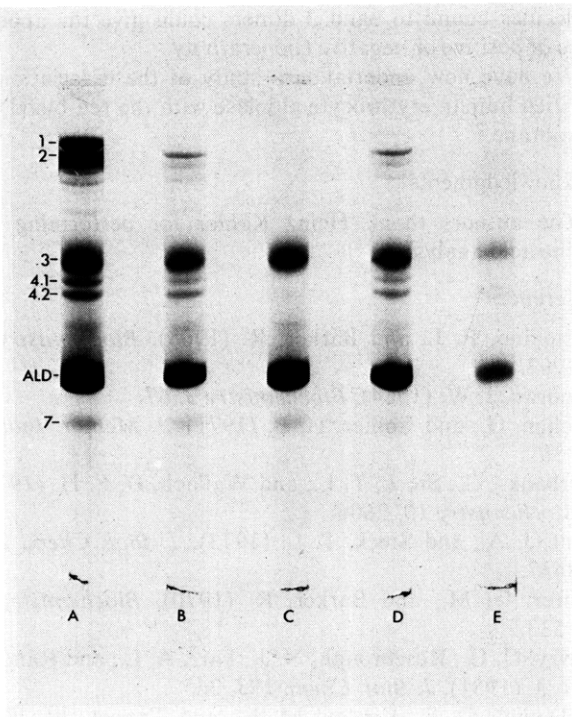

FIGURE 3: Aldolase binding to protein-depleted membranes. Saline-washed ghosts, depleted vesicles, and LIS vesicles were prepared as in Experimental Procedures and diluted fourfold in 5P7. One milliliter of each preparation was combined with 9 ml of 5P7 containing 0.25 mg ml^{-1} of aldolase and incubated for 2 h on ice. Following a 30-min centrifugation, the supernatants were removed and 0.25 ml of 2% Triton X-100 in 5P7 added to each pellet which was then resuspended to 0.5 ml with 5P7. A 0.05-ml aliquot of each of the following preparations was taken for sodium dodecyl sulfate–PAGE analysis: (A) saline-washed ghosts; (B) depleted vesicles; and (C) LIS vesicles. The pellet of (B) (the aldolase-saturated, depleted vesicles) was resuspended in Triton X-100 and incubated on ice for 20 min, and then centrifuged for 30 min. This supernatant (0.050 ml), (D), was removed for PAGE–sodium dodecyl sulfate analysis. Another 0.30 ml of D was centrifuged on a standard sucrose gradient, and the fractions were analyzed by sodium dodecyl sulfate–PAGE. Gel E was taken from a representative fraction from the complex region of the gradient (see text for details).

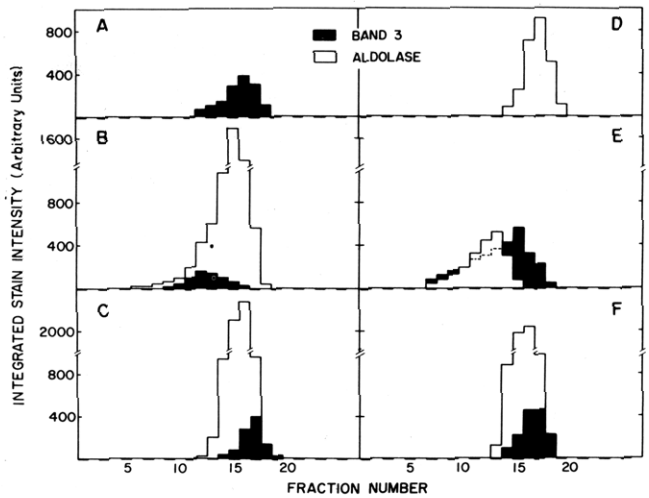

FIGURE 4: Aldolase binding to band **3**. Saline-washed ghosts were incubated for 2 h with an equal volume of 5P7 containing the following amounts of rabbit muscle aldolase: (A) none; (B) 2.70 mg ml^{-1}; (C) and (F) 2.5 mg ml^{-1}; and (E) 0.43 mg ml^{-1}. The membranes were pelleted, combined with an equal volume of 2% Triton X-100 in 5P7, incubated for 20 min on ice, and then centrifuged for 30 min. Aliquots of the supernatants from (A), (B), and (E) along with a sample of pure aldolase (D) were centrifuged on 5–20% sucrose gradients in 5P7 containing 1% Triton X-100. For the dissociation experiments, aliquots of the supernatants were incubated for 30 min on ice in 5P7 made 150 mM in NaCl (C) or 2 mM in fructose 1,6-bisphosphate (F) prior to ultracentrifugation on gradients containing the corresponding solutes.

two points at issue in Figure 2 can be attributed to experimental error and the sensitivity of the plotting form.)

Aldolase Binding to Depleted Membranes. To identify the site(s) of aldolase attachment, these three membrane preparations differing in their peripheral protein content were tested for binding: (1) saline-washed unsealed ghosts, lacking aldolase and G3PD (band **6**; Fairbanks et al., 1971); (2) "depleted membranes", the EDTA vesicles of Fairbanks et al. (1971), lacking bands **1, 2, 5,** and **6** (Steck et al., 1976); and (3) LIS vesicles, which lack all peripheral proteins and in which band **3** is the predominant Coomassie blue-stained component (Steck and Yu, 1973). Excess aldolase was reacted with each preparation; binding of aldolase was assessed by gel electrophoresis of the membrane fractions (Figure 3, A through C). We found that aldolase binding does not decline when some (gel B) or all (gel C) of the peripheral membrane proteins are removed (see below).

Aldolase Binds to Band 3. Since the data in Figure 3 show that aldolase did not bind to the peripheral proteins, we selectively solubilized the integral ghost membrane proteins in Triton X-100 (Yu et al., 1973) and analyzed for complex formation by rate zonal sedimentation. When the Triton X-100 extract of unrepleted saline-washed ghosts was incubated with aldolase, no alteration in the sedimentation profile of the proteins was observed. When membranes repleted with aldolase were extracted instead, the enzyme was eluted along with the integral glycoproteins (Figure 3, gel D). Its complexation was made evident by sucrose density gradient centrifugation (Figure 4). Rabbit muscle aldolase alone sedimented as a peak centered between fractions 15 and 18 (Figure 4D), compatible with an $s_{20,w}^0$ of 7.4–7.8 (Lehrer and Barker, 1970). Aldolase derived from solubilized ghosts was shifted to a symmetrical peak between fractions 11 and 14 (Figure 4E). Band **3** alone formed a zone between fractions 14 and 17 (Figure 4A), corresponding to an apparent $s_{20,w}^0$ of 7.6 (Yu and Steck, 1975b). In the presence of excess aldolase, the position of band **3** was shifted to fractions 11–14 (Figure 4B). These results demonstrate the formation of an aldolase–band **3** complex which sediments faster than either component alone. That the complex was the same as that observed in the binding of aldolase to intact ghosts was demonstrated by its dissociation in the presence of 150 mM NaCl (Figure 4C) or 2 mM fructose 1,6-bisphosphate (Figure 4F). None of the sialoproteins (i.e., PAS 1, 2, 3; Fairbanks et al., 1971) were shifted from their typical position at fractions 20–22.

Aldolase Binds Exclusively to Band 3 with 1:1 Stoichiometry. We integrated the stain intensity of aldolase and band **3** in the gels shown in Figure 3 and calculated their molar ratios (Table I). These values did not differ significantly among the various preparations. The average molar ratio of 1.15 indicates that one band **3** polypeptide binds one aldolase tetramer, or two aldolase molecules per band **3** dimer.

The constancy of aldolase bound per unit band **3** in the various preparations in Table I demonstrates that no significant binding sites are lost as band **3** is isolated from the intact ghost. Therefore, band **3** is the sole membrane-binding site for aldolase.

Table I: Stoichiometry of Aldolase Binding to Band 3.[a]

Sample	Aldolase:Band 3	
	Stain Intensity Ratio	Molar Ratio
Aldolase saturated saline-washed ghosts (Figure 3A)	2.52	1.16
Aldolase saturated depleted membranes (Figure 3B)	2.35	1.08
Aldolase saturated LIS vesicles (Figure 3C)	2.61	1.20
Triton X-100 supernatant (Figure 3D)	2.69	1.24
Complex region of sucrose gradient (average of 4 fractions)	2.26	1.06
Average ± SD	2.49 ± 0.18	1.15 ± 0.08

[a] The gels shown in Figure 3, along with those of the rapidly sedimenting fractions (i.e., the complex region) of the sucrose gradient described therein, were scanned at 520 nm. The aldolase and band 3 peaks were integrated and their stain intensity ratio entered in the left-hand column. The relative staining efficiency of the two proteins was ascertained by correlating the stain intensity of 1–13 μg aliquots of each pure protein with their mass (determined by amino acid analysis), as described by Yu and Steck (1975b). This value was 1.24 (units of stain per μg of aldolase/units of stain per μg of band 3). Molar ratios were calculated from the stain intensity ratios, using the relative staining efficiency quotient and these molecular weights: aldolase, 156 000 (Castellino and Barker, 1968); band 3, 91 000 (Steck et al., 1976). Values are averages of two determinations.

Independent confirmation of this premise comes from Scatchard analysis (Figure 2). The 1.4×10^6 total binding sites per ghost, measured by enzyme activity, are taken to be equivalent to the $\sim 1 \times 10^6$ band 3 polypeptides per ghost determined by densitometry (Steck, 1974).

Conclusions

We conclude that the binding of rabbit muscle aldolase to isolated human red cell membranes involves specific and exclusive interactions with the cytoplasmic pole of band 3. This is also true of human erythrocyte and rabbit muscle G3PD (Yu and Steck, 1975b), the next enzyme in the glycolytic pathway. The number of G3PD binding sites per ghost estimated in that study was 1.3×10^6; that of aldolase is 1.4×10^6 (Figure 2). G3PD binding manifested positive cooperativity (with a Hill coefficient of 1.7); aldolase bound to approximately equal numbers of high and low affinity sites (0.67×10^6 and 0.77×10^6, respectively), suggestive of negative cooperativity. Both types of binding behavior could reflect conformational changes within band 3, a noncovalent dimer (Yu and Steck, 1975b). Alternatively, attractive or repulsive interactions between pairs of enzyme molecules bound to band 3 dimers could give the appearance of positive or negative cooperativity.

We have now undertaken a study of the association of purified human erythrocyte aldolase with the red blood cell membrane.[2]

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