The Aldolase-binding Site of the Human Erythrocyte Membrane Is at the NH₂ Terminus of Band 3*

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(Received for publication, March 15, 1981)

**The Journal of Biological Chemistry**

Vol. 256, No. 3, issue of November 10, 11203–11208, 1981

**Printed in U.S.A.**

Band 3 is the predominant membrane-spanning polypeptide and the mediator of anion transport in the human erythrocyte. In addition, it provides the sites of association for fructose 1,6-bisphosphate aldolase and other cytoplasmic proteins with the membrane. The aldolase-binding activity of water-soluble fragments of band 3 was measured by their inhibition of aldolase catalytic activity and by their displacement of aldolase from ghosts. At saturation, the binding of one band 3 or certain of its fragments per aldolase molecule partially inhibited the catalytic activity and band 3 binding of the unliganded subunits of the tetramer through an apparently cooperative mechanism. An NH₂-terminal 23,000-dalton fragment generated by S-cyanation of the cytoplasmic pole of band 3 was approximately 20% as avid in binding aldolase as was native band 3. Several fragments cleaved from the NH₂-terminal portion of the 23,000-dalton peptide by trypsin, mild acid hydrolysis, and cyanogen bromide digestion all bound aldolase, while fragments from the rest of the polypeptide were essentially inactive. The first 31 residues of band 3 contained 16 Asp plus Glu, no basic residues, and a blocked α-amino terminus. The highly acidic composition of this region is consistent with the strongly electrostatic character of the interaction between band 3 and aldolase, presumably at the strongly basic catalytic center of the enzyme. We conclude that the NH₂-terminal region of band 3 bears the membrane-binding site for aldolase.

Band 3 is the predominant membrane-spanning polypeptide of the human erythrocyte. This ~93,000-dalton glycoprotein mediates the exchange of anions across the membrane (see Refs. 1-5 for review). Furthermore, in vitro, the cytoplasmic domain of band 3 is the exclusive site of binding of aldolase (6, 7), gyceraldehyde-3-P dehydrogenase (8), and probably phosphofructokinase (9) and hemoglobin (10-12). The binding is reversibly raised by the ionic strength and by the presence of metabolites which interact with the respective enzymes. Both gyceraldehyde-3-P dehydrogenase (13) and aldolase (14) bind rapidly and reversibly to the membrane in the intact cell. Band 3 also associates with band 2.1 (14, 15) which, by simultaneously binding to spectrin (16, 17), can couple a subjacent filamentous reticulum (18, 19) to the cytoplasmic surface of the membrane.

The binding of glycolytic enzymes to membranes and other structural elements of the cell has been widely observed in vitro (20-22), but the specificity and physiologic significance of the phenomenon is ill defined. In the present study, a site of association for fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) was found in the NH₂-terminal region of band 3. The highly acidic amino acid composition of this region supports the hypothesis that band 3 associates electrostatically with glycolytic enzymes. A preliminary account of this research has been published (23).

**EXPERIMENTAL PROCEDURES**

**Materials**—Outdated units of blood from normal human donors were generously provided by the Blood Bank of the University of Chicago. From Sigma we obtained rabbit muscle aldolase (Grade I), the accessory components for its assay (6, 7), and 2-mercaptoethanol. We obtained L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin from Worthington and cyanogen bromide from Eastman. SP-Sephadex C-25, Sephadex G-50, and Sephadex G-10 were products of Pharmacia. Bio-gel P-10 and P-30 were obtained from Bio-Rad. Citraconic anhydride was obtained from Pierce. Chemicals were reagent grade or better from Fisher, Mallinckrodt, or Baker. 2-Nitro-5-thiocyanobenzoate was synthesized as described (24).

**Preparations**—All procedures were at 0-5 °C, and all centrifugations were performed in a Sorvall SS-34 rotor at 15,000 rpm unless otherwise indicated. Ghosts were prepared as described (25), except as whole unit preparations (26). The 23,000-dalton fragment was generated by the S-cyanation of alkali-scrapped ghosts, precipitated by cold isopropanol, and extracted into water (26, 27). The extract was concentrated by ultratropillation (Amicon PM-10) to about 10 ml and subjected to preparative gel electrophoresis (28). Fractions containing the 23,000-dalton fragment were pooled, concentrated, precipitated with isopropanol, and the protein was freeze-dried. Typically, 15-20 mg of the purified 23,000-dalton fragment were isolated per unit of blood.

**Acetylation (29)**—Samples were dispersed in 100 μl of water, and an equal volume of a saturated solution of sodium acetate was added. The sample was cooled and stirred at 0 °C while 5 aliquots of acetic anhydride were added in the course of an hour to equal the weight of the peptide. The reaction mixture was dialyzed by gel filtration on Sephadex G-10.

**Peptides**—Typically, the 23K² fragment obtained from one unit of blood was dissolved in 5 ml of 0.1 M Tris-acetate buffer, pH 8.5. Solid guanidine hydrochloride was added to make a 6 M solution. Three hundred μl of citraconic anhydride were added in 20-μl aliquots over the course of an hour; the pH was maintained between 8.2 and 8.6 by the addition of 1 M sodium hydroxide (30). The solution was dialyzed against 6 M guanidine hydrochloride.
against 0.2 mM ammonium bicarbonate. The citraconylated protein was incubated overnight at 37 °C in the same buffer with trypsin (1% of its weight). The digest was chromatographed on a column of Bio-Gel P-30 (3 × 42 cm) in 30% formic acid. The peptides were identified by absorbance at 280 nm and by ninhydrin reaction after alkaline hydrolysis. The largest tryptic peptide, TCI, emerged from the column at 85–105 ml volume in pure form, as determined by sodium dodecyl sulfate-urea and basic urea gel electrophoresis.

The TCI tryptic peptide was acid cleaved in 70% formic acid at 37 °C for 3 days. The cleavage products were resolved by chromatography on a Bio-Gel P-10 column (1.5 × 45 cm) in 30% formic acid and then on an SP-Sephadex column (1.5 × 10 cm) in 10% acetic acid.

Cytochrome c digestion was carried out overnight in 70% formic acid for 14–16 h at 37 °C. The CNBr reaction mixture was diluted with water and freeze-dried. The products were resolved by gel filtration on a Bio-Gel P-10 column (1.5 × 45 cm) equilibrated with 0.05 M ammonium bicarbonate or a Sephadex G-50 column (9 × 50 cm) in 10% acetic acid.

Amino acid analysis was performed following 20-h hydrolysis at 115 °C with 5.7 M hydrochloric acid containing 0.05% 2-mercaptoethanol and 1 mM phenol on a Beckman Model 121 M amino acid analyzer coupled to a System AA computing integrator (28).

Enzyme Assays and Analysis of Inhibitor Binding—Maximal aldolase activity (i.e., in the presence of sufficient fructose 1,6-bisphosphate to overcome the inhibitory effects of any band 3 fragments present) was measured using minor modifications (6, 7) of the method of Wu and Racker (31), as described for individual experiments. Inhibition of aldolase activity by membrane peptides was measured under conditions which favored enzyme-band 3 association (50 μM substrate in 10 mM imidazole acetate, pH 7.0, etc.), as described in Ref. 7 and Figs. 1–3 below. Kᵢ values and the percentage of maximal inhibition were determined by fitting the data to a simple partial inhibition model, using a measured Kᵢ of 1.55 μM to represent the dissociation constant of the substrate-aldolase complex and an estimated 1.2 × 10⁶ band 3-aldolase binding sites per ghost (1, 3, 7, 8). The best fits were obtained when the number of band 3 molecules bound per aldolase tetramer was taken as unity, the stoichiometry found for aldolase binding to band 3 both in intact ghosts and in Triton X-100 solution (6, 7). The standard deviation of the fractional inhibition observed ranged from 8% for band 3 to 22% for TCI A in Fig. 3; the Kᵢ values obtained are probably reliable only to within a factor of two (Table 1).

Enzyme Displacement Assay—Ghosts were freed of endogenous glycolytic enzymes by washing in 0.15 M ammonium bicarbonate (6–8) followed by washing in 10 mM imidazole acetate (pH 7.0). Rabbit muscle aldolase freshly dialyzed in the same buffer was added to the ghosts so that it exceeded their band 3 content by 20% (mole/mole). Band 3 was estimated from coincidence-corrected Coulter counts of ghosts, assuming 1.2 × 10⁶ band 3 binding sites/ghost (3, 6, 7). Aldolase concentration was calculated by assuming an absorbance at 280 nm of 9.24 for a 1% solution and Mᵢ = 160,000 (32). After a 30–60 min equilibration, the aldolase-loaded ghosts were washed twice in 10 mM

Fig. 1. Aldolase inhibition by water-soluble band 3 fragments. a, human erythrocyte aldolase (2.6 μg) was mixed with 0.1 mM NADH and 20 μg of an α-glycerophosphate dehydrogenase-triosephosphate isomerase mixture in 0.925 ml of 10 mM imidazole-HCl buffer (pH 7.0). Absorbance at 340 nm was recorded. At the times indicated these additional components were added in the same buffer: b, 0.05 ml of 1 mM fructose 1,6-bisphosphate (FDP); c, 0.02 ml of a 1.25 mg/ml solution (i.e. 25 μg) of cytoplasmic pole of band 3 cleaved from ghosts by trypsin (Refs. 26 and 27); d, 0.1 ml of 3 M NaCl; and e, 0.1 ml of 72 mM fructose 1,6-bisphosphate.

Fig. 2. Double reciprocal analysis of the inhibition by band 3 of aldolase. 1.5-ml ghosts loaded with aldolase were incubated in 28.5 ml of 0.075% Triton X-100 in 10 mM imidazole-HCl (pH 7.0) for 20 min on ice. Following a 30-min centrifugation the supernatant fluid was collected; a portion was adjusted to 0.3 M NaCl by the addition of the solid. After a 1-h incubation on ice, aldolase activity was measured as a function of substrate concentration in the presence (O) and absence (●) of 0.3 M NaCl. In this experiment, 0.3 M NaCl shifted the apparent Kᵢ from 650 μM to 50 μM. That this effect is attributed to the dissociation of the enzyme-band 3 complex is shown by the fact that, in the absence of membranes, 0.3 M NaCl increased the Kᵢ from 2 to 50 μM. Thus, the inhibitory potency of the membranes is even more profound than suggested in this experiment. FDP, fructose 1,6-bisphosphate.

Fig. 3. Inhibition of aldolase activity by band 3 fragments. Various soluble fragments of band 3 were prepared as described in the text and mixed with rabbit muscle aldolase (11 pmol) in 10 mM imidazole acetate, pH 7.0, containing 0.004% Triton X-100 (final). Aldolase activity was then assayed with 50 μM fructose 1,6-bisphosphate. The curves were generated from the values given in Table 1 (representative experiment). A, ●—● ghosts. (We assumed 1.2 × 10⁶ band 3 polypeptides per ghost (1, 3, 7, 8).) C1-C12, acetylated TCIT1CN2. B, □—□, TC1A. ▲—▲, TC1A. ▲—▲, CN1. An alignment of the peptides is shown in Fig. 4.
The dependence of aldolase inhibition on band 3 concentration in preparations of ghosts freshly dissolved in Triton X-100 is shown in the top curve of Fig. 3A (○—○). The data were well fitted to a hyperbola by assuming a simple binding isotherm, a 1:1 stoichiometry (6, 7), and the parameters shown in Table 1 (namely, a, of 1 × 10−10 M and 90% inhibition at saturation).

**An Aldolase-Inhibitory 23,000-dalton Fragment from Band 3**—A water-soluble 23K fragment was cleaved by S-cyanolation from the NH2-terminal end of band 3 and purified (26, 27). Its inhibitory effect on aldolase activity varied somewhat among several preparations; representative results are summarized in Fig. 3A (○—○) and in Table 1. In this representative experiment, the avidity of this fragment for aldolase was approximately one-fifth that of band 3; like band 3, aldolase activity was never completely inhibited when saturated with the fragment.

**Tryptic Subfragments from 23,000-dalton Fragment**—The 23K fragment was cyanolyzed, digested with trypsin, and the cleavage products resolved by gel filtration on Bio-Gel P-30. The largest subfragment, TC1, was recovered from column chromatography in pure form (see under "Experimental Procedures"). The electrophoretic mobility of the peptide in a basic urea gel system was found to be nearly that of the tracking dye, bromphenol blue. The acidic nature of TC1 was confirmed by its amino acid composition; 34 of its 75 residues were Asx or Glx (Table II).

Table 1

| Peptide          | Aldolase inhibition assay | Displacement assay |
|------------------|---------------------------|--------------------|
|                  | 10⁻⁵ M | % | 10⁻⁴ M |
| Ghost band 3     | 1     | 90 | 85     |
| 23,000 dalton     | 5     | 85 | 87     |
| TC1              | 6     | 77 | 17     |
| TC1T1            | 6     | 79 | 9      |
| TC1A1            | 10    | 39 | 16     |
| TC1A2            | 33    | 39 | 33     |
| CNI              | infinity |     |        |
| Acetylated TC1T1CN2 | 200   | 33 |        |
| Acetylated TC1T1CN2 | 50    | 85 |        |

The affinity of the band 3 in ghosts for aldolase was determined to be Kᵢ = 1.8 × 10⁻¹⁰ M, against which the displacement assays were compared.

The assay of aldolase inhibition—Membranes from erythrocytes (7) and other tissues (22) reversibly inhibit the catalytic activity of mammalian aldolases. Band 3 provides the exclusive binding site for this enzyme in red cell membranes (6, 7). The inhibition of aldolase activity thus affords an assay for the identification of its binding site among fragments of band 3.

Unsealed ghosts, Triton X-100 extracts of ghosts, and the cytoplasmic pole of band 3 all inhibited aldolase activity immediately upon addition to the assay cuvette (Ref. 33 and Fig. 1). This effect was rapidly reversed by conditions which elute the enzyme from the membrane, millimolar levels of substrate or high ionic strength (Fig. 1). The form of the inhibition appeared superficially to be competitive, in that a mutual antagonism between substrate and ghosts, reversed at high ionic strength, was manifest (Fig. 2). However, we shall demonstrate below that a more complex mechanism is likely.

**RESULTS**

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ment of the binding of the various band 3 fragments to aldolase was provided by their ability to displace the enzyme from ghosts. Unsealed ghosts were freed of their endogenous glycolytic enzymes and loaded to near saturation with freshly dialyzed rabbit muscle aldolase (6). Varied amounts of water-soluble fragments of band 3 were then equilibrated with the ghosts on ice overnight. Alternatively, the band 3 fragments were first added to depleted ghosts followed by a subsaturating dose of rabbit muscle aldolase. A critical test of the method was that in both procedures, a similar plateau of aldolase displacement was reached for a given equilibrium mixture. The data were treated as in other competitive displacement assays except that we solved for the potency of fragments relative to that of ghost band 3 rather than the amount of the competitor.

Included in Table I is a comparison of the binding potency of various band 3 fragments determined by the aldolase inhibition and displacement techniques described. The validity of both methods is affirmed by the close agreement between them for the several peptide species; the average difference between pairs of values was 18%.

**Discussion**

This study has delineated two features of the interaction of aldolase with the membrane; the inhibition of its catalytic activity and its locus of binding to band 3.

Previous studies on the binding of aldolase to intact or dissolved ghosts revealed a stoichiometry of one enzyme molecule per band 3 polypeptide (6, 7). This result signifies that, by binding to one of its protomers, a band 3 polypeptide can inhibit an entire aldolase tetramer. Since aldolase contains four identical, catalytically active subunits per tetramer (35), its nearly complete inhibition upon binding to a single band 3 polypeptide is incompatible with a simple competitive mechanism. The observed stoichiometry, furthermore, argues against a mechanism of inhibition involving dissociation of the tetramer into inactive subunits, since this would require multiple band 3 ligands per tetramer. We also discount the possibility that a single 40,000-dalton cytoplasmic pole of band 3 (3) could bind to all four 40,000-dalton subunits of aldolase, which are arranged with 2-fold symmetry around three mutually perpendicular axes (36).

Simple competition is also incompatible with the fact that the presence of up to 1200 pmol of band 3 never completely inhibited 11 pmol of aldolase. This effect is even more dramatic in the case of certain small peptide fragments where inhibition at saturation was less than 40% (Table I and Fig. 3B). Thus, although band 3 and the substrate displace one another from aldolase (Fig. 2) and may both bind to the same (active) site, the data suggest the formation of partially catalytically active ternary complexes of inhibitor, enzyme, and substrate. That such ternary complexes exist was previously inferred from the observation that the residual aldolase activity found in the presence of saturating levels of ghosts sedimented with the membranes even while the aldolase reaction was in progress (7). (This finding rules out, furthermore, the possibility that some aldolase molecules resist inhibition by failing to bind band 3.)

That the saturation of the aldolase with band 3 or its fragments caused less than full inhibition also signifies that the binding of multiple band 3 polypeptides to a single aldolase tetramer is curtailed; otherwise the inhibition would approach completion as all four subunits became occupied. (It seems unlikely that band 3 binding to the first of four identical aldolase subunits is inhibitory while its binding to the others is not.) The binding of a single band 3 polypeptide to one subunit thus appears to reduce the affinity of the remaining protomers for band 3, independent of their catalytic activity. This behavior suggests a high degree of negative cooperativity among aldolase subunits. Mammalian aldolases have not been found to exhibit cooperative kinetics (36). However, Penhoet and Rutter (37) showed that specific noncross-reacting antibodies directed against homomeric aldolases Aα, Bα or C, were capable of inhibiting entirely the activity of heteromeric hybrids containing only one antibody-sensitive protomer, this finding suggests an interaction among subunits similar to that observed here.

The most parsimonious hypothesis consistent with these findings is that a band 3 peptide can bind to any of four identical sites on the aldolase tetramer and thereby reduce both the catalytic activity and the affinity of the remaining three subunits for the inhibitor.

We have provisionally ordered the fragments capable of inhibiting aldolase activity as shown in Fig. 4. It is evident from a comparison of this figure with Table I that the N-terminal sequence of band 3 contains the membrane-binding site for aldolase. While the 23K fragment exhibited only one-fifth of the affinity for aldolase of whole band 3, this decrease

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**Table II**

Amino acid composition of inhibitory peptides

|   | 23K | TCI | TCI1 | TCI1A | TCI1C | TCI1C1 | TCI1C1N1 | TCI1C1N2 | TCI1C1N3 | CN1 |
|---|-----|-----|------|-------|-------|-------|----------|---------|---------|-----|
| Asp | 18  | 10  | 5    | 8     | 3     | 3     | 2        | 8       |         |     |
| Thr | 12  | 6   | 6    | 1     | 5     | 6     | 1        | 8       |         |     |
| Ser | 10  | 2   | 2    | 1     | 1     | 1     | 1        | 8       |         |     |
| Glx | 47  | 24  | 12   | 12    | 17    | 5     | 9        | 23      |         |     |
| Pro | 10  | 4   | 4    | 4     | 1     | 1     | 1        | 9       |         |     |
| Gly | 11  | 1   | 1    | 1     | 4     | 1     | 1        | 9       |         |     |
| Ala | 13  | 4   | 4    | 4     | 1     | 1     | 1        | 9       |         |     |
| Val | 10  | 3   | 3    | 3     | 1     | 1     | 1        | 9       |         |     |
| Met | 5   | 4   | 2    | 2     | 3     | 1     | 1        | 2       |         |     |
| Ile | 3   | 1   | 1    | 1     | 1     | 1     | 1        | 2       |         |     |
| Leu | 30  | 5   | 2    | 3     | 2     | 1     | 1        | 25      |         |     |
| Tyr | 4   | 4   | 2    | 2     | 3     | 1     | 1        | 2       |         |     |
| Phe | 5   | 5   | 5    | 5     | 5     | 5     | 5        | 5       |         |     |
| His | 9   | 4   | 4    | 4     | 4     | 4     | 4        | 5       |         |     |
| Lys | 5   | 2   | 2    | 2     | 1     | 1     | 1        | 4       |         |     |
| Arg | 10  | 1   | 1    | 1     | 1     | 1     | 1        | 9       |         |     |
| Trp | 3   | 3   | 3    | 3     | 3     | 3     | 3        | 3       |         |     |
| Cys | 1   | 1   | 1    | 1     | 1     | 1     | 1        | 1       |         |     |
| Total | 296 | 75  | 23   | 52    | 56    | 11    | 19       | 25      | 125     |     |

Values are in residues per mol of protein (rounded to nearest integer). Met was determined as homoserine in the CNBr peptides. Trp and Cys were determined only in the 23K fragment.
Band 3-binding Site for Aldolase

![Diagram of Band 3-binding Site for Aldolase]

The enzyme, such as band 3, is bound to an anionic site within the cytoplasmic pole of band 3 (39). Pyridine nucleotides rather than fructose 1,6-bisphosphate should be used for soluble glycolytic enzymes.

Two forms of negative cooperativity were seen in the interaction of band 3 with aldolase, an enzyme generally considered to lack interprotomer interactions. These were manifested in the inhibition of binding of multiple band 3 peptides and in the inhibition of the catalytic activity of the unliganded enzyme subunits. These effects speak against a simple or non-specific interaction. It is conceivable that it would be highly detrimental for band 3 molecules to aggregate in the membrane. The observed negative cooperativity could serve to suppress the cross-linking of multivalent band 3 particles (presumably dimers (3, 8, 26, 40) or tetramers (41, 42)) by tetraferrable enzymes. The physiologic significance of the inhibition of catalytic activity upon membrane binding, however, remains obscure.

Acknowledgments—We thank Donald P. Madden for his excellent technical assistance and Ernest Strapazon for providing the data presented in Figs. 1 and 2 from his thesis (Ref. 35).

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