Exploration of the functional significance of the stilbene disulfonate binding site in mouse band 3 by site-directed mutagenesis

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In 1985, it was shown that microinjection into *Xenopus* oocytes of mRNA prepared from the spleens of anemic mice leads to biosynthesis of band 3 protein (as shown by immunoprecipitation) and induction of a Cl^- flux, which is not normally seen in the oocytes (Morgan et al., 1985). This flux can be inhibited by typical inhibitors of band 3-mediated anion exchange, including 4,4′-dinitro stilbene-2,2′-disulfonate (DNDS), dipyridamole, phloretin and H^+. These results encouraged us to microinject band 3 (AE1)-encoding cRNA derived from mouse band 3 cDNA (Hanke-Baier et al., 1988).

After biosynthesis of band 3, we microinjected $^{36}Cl^-$ into oocytes and placed them individually into a perfusion chamber the bottom of which is formed by the mica window of a Geiger Müller tube. Perfusion of the chamber with a modified Amphibian Ringer solution removes $^{36}Cl^-$ escaping from the oocyte. The radioactivity inside the oocyte is recorded as a function of time. On a semilog scale, a plot of radioactivity vs. time yields a straight line relationship which persists until ~80% of the initial radioactivity has left the oocyte. The slope of the straight line yields the rate constant for Cl^- efflux (Grygorczyk et al., 1987, 1989; Kietz et al., 1991*a*). This efflux is inhibited when the Cl^- of the Ringers solution is replaced by slowly penetrating SO_4^{2-} ions or nonpenetrating gluconate ions, indicating that, as in the red cell, the $^{36}Cl^-$ efflux can only take place by anion exchange. The K_I values for H_2DIDS and DNDS are of the same order of magnitude as in the red cell (Kietz et al., 1991*a*; Morgan et al., 1985). Moreover, again as in the red cell, the hydrophobic domain suffices for the mediation of anion exchange after expression in the oocyte and remains susceptible to inhibition by H_2DIDS or DNDS (Lepke et al., 1991).

The agreement of important features of mouse band 3-mediated anion transport in red cells and oocytes encouraged us to use oligonucleotide-directed mutagenesis of a number of (conserved) lysine residues, Lys 449,558,649,869, to localize the sites of action of a range of covalently binding, inhibitory amino group reactive agents in the known primary structure of band 3 (see Fig 1). Regardless of the mutation, anion exchange is still accomplished. Thus, none of these residues is directly involved in anion binding and translocation. However, the susceptibility to inhibition by covalently and noncovalently binding inhibitors is drastically altered.

Substitution by Asn of Lys 558 and Lys 561 (Bartel et al., 1989*b*), or of Lys 558 alone (Bartel, 1989*a*), does not prevent reversible inhibition of Cl^-/Cl^- exchange by H_2DIDS, but prevents the subsequent irreversible reaction. Each of the two isothiocynate (NCS) groups of H_2DIDS is able to react with a different lysine residue in wild type band 3, to form an intramolecular cross-link. Because the NCS groups only react with the deprotonated forms of the lysine NH_2 group, the reaction rate depends on pH. One of the NCS groups reacts with a lysine residue with pK ≈ 8.2, the other with a residue with pK ≈ 10.8 (20°C). In the wild type, the rate of covalent H_2DIDS binding varies with pH in a manner which suggests reaction with the lysine residue with the lower pK value. After the mutation K558N, the pH dependence changes to a pattern compatible with a reaction with the lysine residue with the higher pK value (Kietz, 1991*b*).

Pyridoxal phosphate (P-5P; 5 mM, pH 7.6) produces irreversible inhibition in the wild type (KK) and in the mutant in which Lys 558 (NK) or Lys 869 (KM) had been replaced by asparagine (N) or methionine (M), respectively. However, when both residues are replaced (NM), irreversible inhibition can no longer be achieved. This shows that P5-P is capable of producing inhibition with either one of the lysine residues 558 or 869 (Wood et al., 1991; Kawano et al., 1988).

Inhibition by DNDS changes dramatically upon mutation. The K_{iapp} increases from 6.0 μM in the wild type (KK) to 23 μM in the mutant NK, to 73 μM in the mutant KM, and to 473 μM in the mutant NM. The K_m value for activation of the transport system is ~11 mM both in the wild type (KK) and in the mutant (NM) if measured by isosmotic substitution of Cl^- by gluconate, which is not a substrate of band 3 (Passow et al., 1992). The results show that both Lys 558 and Lys 869 are involved in the maintenance of the structure of the overlapping binding sites for stilbene disulfonates and the substrate Cl^- and that at last one of the two is allosterically linked to the substrate binding site.

In the mutant NM, the K_I value for reversible H_2DIDS binding is considerably increased. Nevertheless, at alka-

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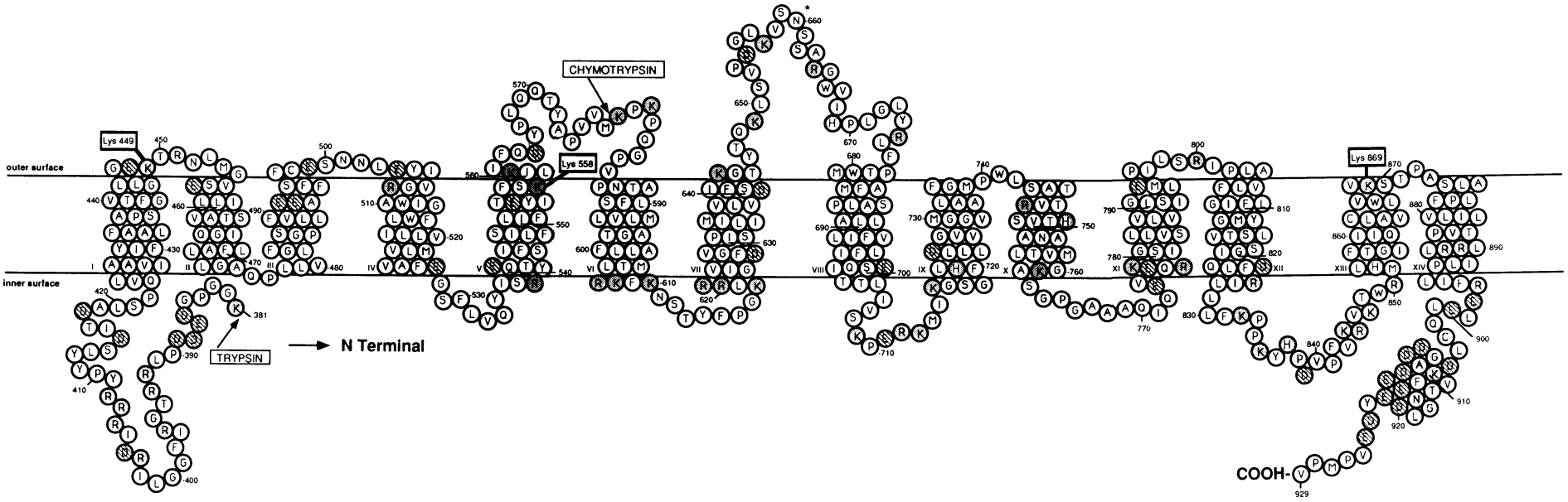
outer surface
inner surface
Lys 449
Lys 556
Lys 869
CHYMOTRYPSIN
TRYPSIN
N Terminal
COOH-

FIGURE 1

line pH, some irreversible inhibition can still be observed. Thus, it remains undecided whether or not Lys 869 is the lysine residue which in addition to Lys 558 is involved in the well-known intramolecular cross-linking by H_2DIDS (Wood et al., 1991). An alternative candidate would be Lys 649.

The mutation K449S has no effect on reversible or irreversible inhibition by H_2DIDS. Reversible inhibition by eosine 5-maleimide (E5-M) is still possible. However, inhibition no longer becomes irreversible. In the mutatant K558N, E5-M still acts irreversibly. These observations confirm (Cob and Beth, 1990) that Lys 449 is the site of covalent action of E5-M (Gehring, H., H. Appelhans, and H. Passow, unpublished data). The susceptibility of the mutant K649S to reaction with inhibitors is still under investigation.

We suspect that in band 3 in situ the lysine residues 449, 558, and 869 (and possibly Lys 649) are located in close juxtaposition and form the entrance of an access channel which leads from the external medium to the substrate binding site. At least, one of these amino acid residues (probably Lys 558 with its abnormally low pK value) is allosterically linked to the latter site such that its susceptibility to chemical modification depends on the occupancy of the transfer site.

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