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Effect of site-directed mutagenesis of the arginine residues 509 and 748 on mouse band 3 protein-mediated anion transport

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Abstract

Using site-directed mutagenesis, the arginine residues 509 and 748 in mouse band 3 protein were substituted by Lys, Thr, and Cys, or by Lys and Gln, respectively. After expression in *Xenopus* oocytes of the cRNAs encoding wild type band 3 or any one of the band 3 mutants, chloride equilibrium exchange was measured. When the flux measurements were performed two to three days after microinjection of the cRNAs, in contrast to the wild type, neither one of the mutants was able to accomplish transport, with the possible exception of the mutants R509K and R748K both of which showed some transport activity of doubtful significance. Immunoprecipitates revealed that the Arg 748 mutants were expressed similar to the wild type band 3 while no expression of the Arg 509 mutants could be detected. When the flux measurements were performed only 3 h after microinjection of the cRNAs, transport activity was observed in the oocytes that had received cRNAs encoding wild type band 3. In some oocytes of a population, a very slight transport activity was brought about by cRNA encoding Arg 509 mutants. No transport activity could be detected after injection of the Arg 748 mutant. Immunoprecipitation demonstrated the successful biosynthesis of wild type band 3 and of both the Arg 509 and the Arg 748 mutants. The experiments suggest that mutation of Arg 748 leads to biosynthesis of an inactive form of the band 3 protein, while that of Arg 509 results in expression of an abnormally folded, possibly functionally more or less intact form, which is proteolytically degraded within less than one day. © 1998 Elsevier Science B.V.

Keywords: Chloride transport; Anion exchanger AE1; Site-directed mutagenesis; Oocyte; Red blood cell; (*Xenopus*)

1. Introduction

In previous investigations, we have used molecular biological methods as a tool for the identification and functional characterization of specific amino acid residues involved in mouse band 3-mediated anion transport. In the course of this work we have explored the effects of deletion of the entire hydrophilic domain of the band 3 protein [1] and of the mutagenic substitution of individual lysine, histidine, glutamate and sulfhydryl groups [2–4,27]. (The earlier work has been summarized by Passow et al. [5,26].) In the present paper we report about studies on the functional consequences of site-directed mutagenesis of two specific arginine residues, Arg 509 and Arg 748.

The anion-transporting, hydrophobic domain of mouse band 3 protein (AE1) contains 18 arginines,

Abbreviations: DNDS: 4,4′-dinitro stilbene-2,2′-disulfonate; PG: phenylglyoxal; SDS: sodium dodecyl sulfate

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13 of which are conserved or conservatively substituted by lysine residues (derived from the data compiled by Wood [6]). Approximately one third of the total number of arginines can be modified by the arginine specific reagent phenylglyoxal (PG) without altering anion transport [7]. Nevertheless, amongst the remaining arginine residues there are some that are essential for the maintenance of the anion transporter in a functional state. This is suggested by the observation that, under appropriate experimental conditions, treatment of the red cell membrane with the arginine-specific reagents cyclohexane dione [8,9] or phenylglyoxal [10–15] leads to inhibition of anion equilibrium exchange. These suggestions were supported by observations on the pH dependence of anion equilibrium exchange [16] and on the effect of pH on the rate of irreversible inhibition of the transporter by PG [11]. Chloride equilibrium exchange was found to be progressively inhibited by increasing the pH to values above 11, which could be quantitatively described on the assumption that the inhibitory process was related to the deprotonation of a single, titratable site with an intrinsic pK of 11.3, i.e., the pK similar to that expected for an arginine residue [17]. Moreover, the rate constants for the time course of irreversible inactivation of the transport protein by PG showed a virtually identical pH dependence, suggesting that the rate of inactivation was facilitated by the deprotonation of the same arginine residue that controls the pH dependence of the anion transport process. A more detailed analysis of the action of pH, PG concentration, and electrolyte composition of the medium on PG binding and inhibition of anion equilibrium exchange lead to the suggestion that two to three distinct arginine residues are required for the normal execution of the transport process (summarized by Bjerrum [18,19]). This conclusion was supported by experiments in which the transport activity of the phenylglyoxalated anion exchanger was measured at a range of pH values and ion concentrations in the medium [2]. They revealed that there exist at least two distinct effects of PG: one inhibitory, and another leading to a shift of the pK value of a transport rate-controlling titratable site in the band 3 protein.

Up to the present, neither the locations of these residues in the band 3 amino acid sequence nor their functions in anion transport have been established. Zaki [20] and Julien et al. [21] postulated that Arg 490 in human band 3 would be the most important site of transport inhibition by PG. They suggested that this residue is an essential constituent of the substrate binding site (the so-called transfer site) and hence directly involved in the transport process. Zaki [9] provided an elaborate hypothesis on its functional significance under the designation ‘the cascade model’, which involved fairly explicit assumptions about the role of the arginine residue in anion binding and translocation. We felt it useful, therefore, to include in our work on the effects of site-directed mutagenesis on band-3-mediated anion transport a study of the consequences of the substitution of Zaki’s arginine residue by other amino acids. Well aware of the speculative character of her cascade model, Zaki [20] thought it possible that instead of Arg 490, some other arginine residue could constitute the target site for PG and she suggested Arg 730 as another possible candidate.

Independent of this latter suggestion, it seemed desirable to elucidate the role of Arg 730. Recent work of Müller-Berger et al. [3,4] lead us to suspect that the positive charge of Arg 748 in mouse band 3, which is homologous to Arg 730 in man, is essential for anion transport. We suggested that this residue increases the dissociation constant of a neighboring histidine residue (H752) that, together with a glutamate residue (E699), is responsible for the pH dependence of band 3-mediated chloride equilibrium exchange. We expected that substitution of Arg 748 by an uncharged amino acid residue should result in an inhibition of transport.

The present paper shows that in contrast to the expectation, Arg 509 in mouse band 3, which is homologous to Arg 490 in human band 3, does not seem to be required for the execution of the transport function. However, in agreement with expectation, Arg 748 in mouse band 3 is essential for band 3-mediated chloride equilibrium exchange, although it does not necessarily represent a constituent of the substrate binding site or the site of the inhibitory action of PG.

2. Materials and methods

All mutants used for expression in the *Xenopus* oocytes were prepared from an expression plasmid

Table 1
Sequences of the oligonucleotides used in the polymerase chain reaction for mutagenesis

Primer	Sequence	Strand
	+1372 +1379	
A1	5′-CAGCTGCTCATCTCCACAGCA-3′	plus
	+1636 +1616	
A2	5′-GATGAAGATGAGGGAGATGAG-3′	minus
	+1517 +1537	
R509K I1	5′-GATCCACGCTTTGCCCACGAT-3′	minus
R509T I1	5′-GATCCACGCGGTGCCCACGAT-3′	minus
R509C I1	5′-GATCCACGCGCAGCCCACGAT-3′	minus
	+1537 +1517	
R509K I2	5′-ATCGTGGGCAAAGCGTGGATC-3′	plus
R509T I2	5′-ATCGTGGGCACCGCGTGGATC-3′	plus
R509C I2	5′-ATCGTGGGCTGCGCGTGGATC-3′	plus

(pSPT19-Bd3) containing wild type mouse band 3-encoding cDNA [22] or a derivative of this cDNA containing 5 silent mutations introduced to create five additional restriction sites (Lepke and Passow, unpublished).

For the construction of the mutants R748K and R748Q, the *Xma*I/*Hin*dIII fragment of wild type band 3 cDNA was subcloned in pSPT19 to obtain single *Sty*I/*Mlu*I restriction sites. In this subclone the 43 bp *Sty*I/*Mlu*I fragment was substituted by a

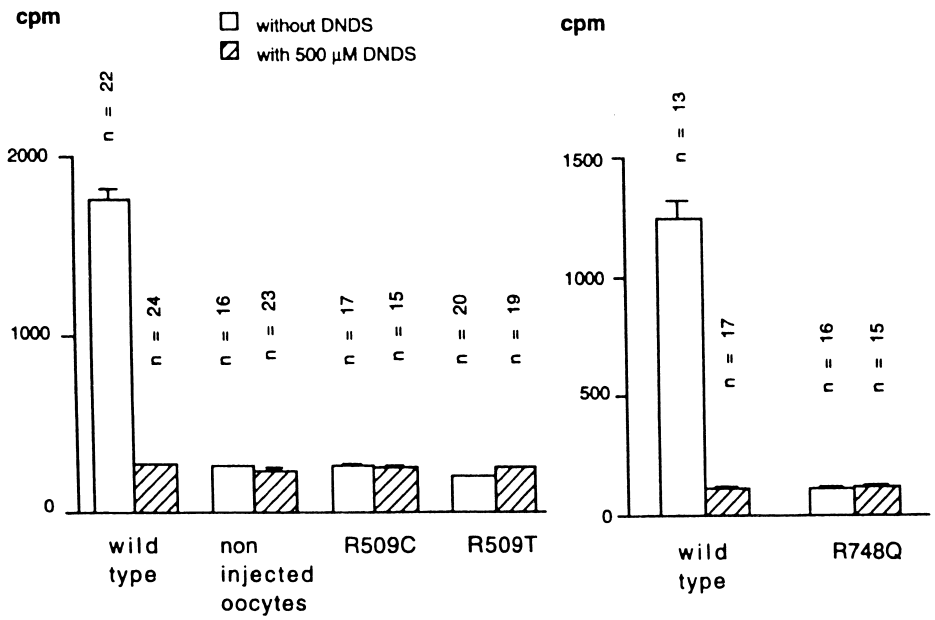

Fig. 1. Determinations of ^{36}Cl uptake by oocytes two days after microinjection of the cRNAs encoding wild type band 3 and the band 3 mutants indicated in the figure. The oocytes were incubated in Barth's medium, pH 7.6, containing ^{36}Cl, at 23°C for 90 min, either in the presence or absence of 500 μM DNDS, a specific, reversibly binding inhibitor of band 3-mediated anion transport. The oocytes were subsequently washed free of external radioactivity in Barth's medium pH 7.6 containing 500 μM DNDS, solubilized in SDS and individually counted in a liquid scintillation counter (see Morgan et al. [24]). Ordinate: cpm per oocyte at the end of the incubation period. In this figure, in Fig. 2 and in Fig. 7 the error bars represent standard errors of the means.

cassette containing in place of the codon for R748 (AGA) the codons for K748 (AAA) or Q748 (CAA). The insertion of the mutations was verified by didesoxy sequencing. Subsequently, the *Xma*I/*Hin*dIII fragment of the subclone was excised and inserted into the original *Xma*I/*Hin*dIII-restricted plasmid. The correct insertion of the mutations was again verified by sequencing.

The mutants R509K, R509C and R509T were prepared by the PCR method. Single *Bgl*II/*Bsm*I sites were created by excising the *Xma*I/*Hin*cII fragment of band 3 cDNA and subcloning in pSPT19. For mutagenesis PCR reactions were carried out with the plus strand primer A1 and the minus strand primer I1, or with the minus strand primer A2 and the plus strand primer I2 (for primer sequences and positions see Table 1). Using the *Xma*I/*Hin*cII fragment as template, in the oligonucleotides the R509-encoding triplet (CGC) and its complementary sequence (GCG) is mutated to the codons underlined in Table 1. The products derived from these PCR reactions were combined and another PCR reaction was carried out using the primers A1 and A2. The resulting PCR fragment was restricted with *Bsm*I and *Bgl*II and ligated to the subcloned band 3 fragment which has also been treated with *Bsm*I and *Bgl*II. After transformation the clones were screened by didesoxy sequencing. From clones containing the mutations the *Xma*I/*Hin*cII fragment was excised to insert the mutation in wild type cDNA pretreated with the same restriction enzymes.

cRNA was produced as described elsewhere [22]. It was shown, that the appropriate translation products were obtained in cell free reticulocyte lysates. The appearance of band 3 and its mutants in the oocytes was determined by immunoprecipitation and subsequent SDS polyacrylamide gel electrophoresis of the precipitated protein as described in detail in previous publications from this laboratory [23,24].

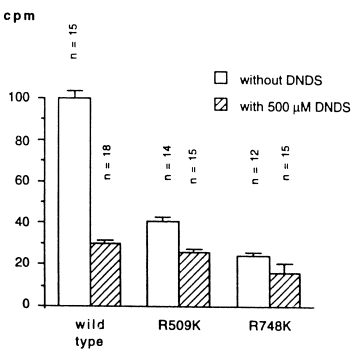

Fig. 2. Same experimental arrangement as in Fig. 1, except that the oocytes had been microinjected with cRNAs encoding the mutants R509K and R748K.

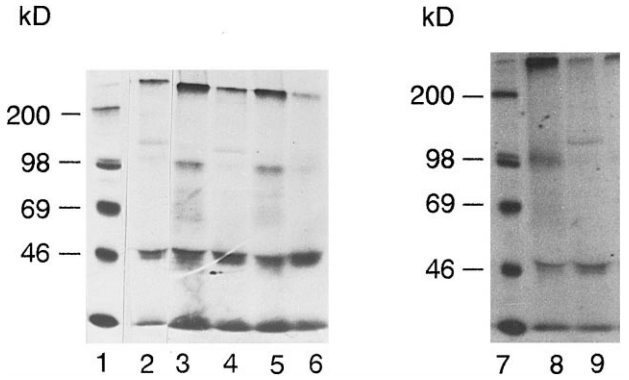

Fig. 3. Immunoprecipitates of ^{35}S-methionine-labeled band 3 after expression of the band 3-encoding cRNAs of wild type and the R509 mutants indicated in the figure. The bands at 98 kD on the SDS polyacrylamide gel electropherograms (7.5%) represent band 3. For details of the experimental procedure see Grygorczyk et al. [23]. Lanes 1 and 7: molecular weight markers; lane 2: uninjected oocytes; lanes 3 and 4: mutant R509C after 3 h and 48 h incubation, respectively; lanes 5 and 6: mutant R509T after 3 h and 48 h of incubation, respectively; lanes 8 and 9: Double mutant R509K/R748K after 3 h and 48 h of incubation, respectively.

Expression of cRNAs encoding wild type band 3 or its mutants in *Xenopus* oocytes and the subsequent measurements of chloride fluxes were performed as described previously [22,24].

The observation of chloride fluxes was taken as evidence for the presence of the expressed band 3 protein in the plasma membrane of the oocytes. In cases where mutagenesis leads to a loss of transport activity, we followed our ‘two days’ rule: In previous work, in which the band 3 distribution in microscopic slices of frozen *Xenopus* oocytes was determined by means of fluorescent antibodies, it was observed that band 3 mutants not exported to the plasma membrane and retained in the ER or cytosol were proteolytically degraded within a few hours after microinjection of the mutant-encoding cRNA and protein biosynthesis (Staub, Thesis, Frankfurt, 1994; see also Ref. [3]). The present paper illustrates in addition an interesting case, where a mutant, partially exported to the mem-

brane, seems to be still able to accomplish some transport. However, the transport activity survives only for a few hours until the mutated protein is degraded, suggesting inappropriate folding that renders the transporter susceptible to proteolytic attack, even after insertion into the plasma membrane. In view of these observations, if not expressly stated otherwise, the microinjected oocytes were incubated for at least 2 days before using them for immunoprecipitation and flux measurements. In the case of the mutant R748Q, which exhibits no sign of transport activity, we demonstrated that part of the synthesized band 3 (as determined by immunoprecipitation) can be split by external chymotrypsin (5 mg/ml at 4°C for 2 h) to yield the expected 63-kDa fragment of mouse band 3. This is a clear indication of the successful export to the plasma membrane of at least a fraction of the mutated band 3 protein (summarized by Tanner et al. [25]). In contrast to the R509 mutants, the R748 mutants survived in the oocytes for at least two days.

3. Results

3.1. Mutation of Arg 509

Two days after microinjection into the oocytes of the cRNA's encoding wild type band 3 or band 3 mutants (R509K, R509C, R509T), chloride influx [24] or efflux [22] measurements were carried out. Regardless of whether influx (Fig. 1) or efflux (Fig. 4) was determined, we were unable to discover any transport activity. Only in the mutant R509K was found a slight transport activity of doubtful significance (Fig. 2). Immunoprecipitates revealed that under the described conditions none of the mutants was present at detectable concentrations. Thus it seemed

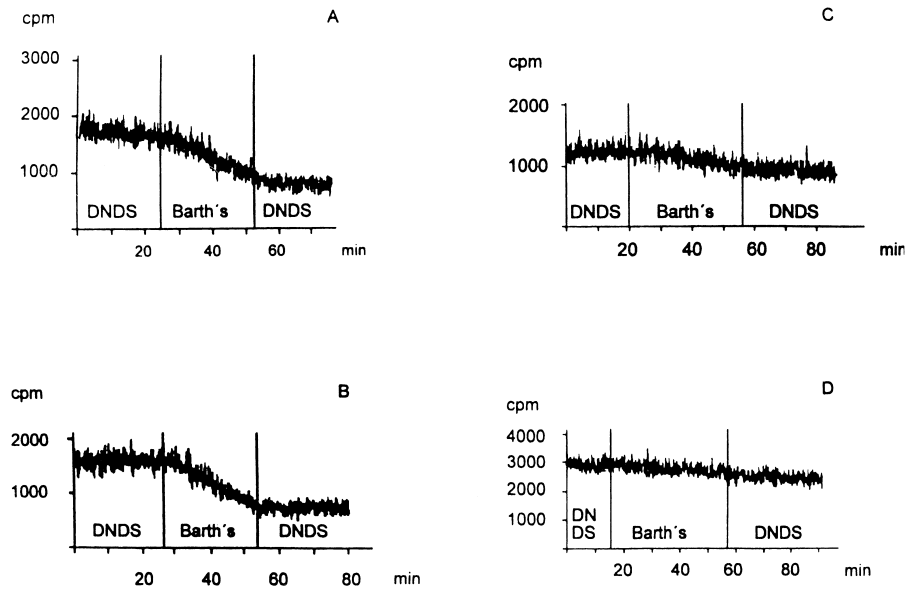

Fig. 4. ^{36}Cl efflux from oocytes 3 h or 48 h after microinjection of cRNAs encoding wild type band 3 or the band 3 mutants listed below. The measurements were performed after microinjection of ^{36}Cl into single oocytes, placing them individually into the hair loop of a perfusion chamber the bottom of which is formed by the mica window of a Geiger–Müller tube. The radioactivity leaving the oocyte is washed away while the radioactivity remaining in the oocyte is continuously counted with a rate meter (see Refs. [22,23]). During the initial time period (designated 'DNDS') the perfusion medium (Barth's solution, pH 7.6) contained 50 μM DNDS to demonstrate the absence of ^{36}Cl leakage via pathways other than band 3. During the subsequent perfusion period without DNDS (designated Barth's), the band 3-mediated efflux takes place. Finally, perfusion with DNDS-containing Barth's medium is resumed to demonstrate that the oocyte membrane retained its impermeability to ^{36}Cl. (A), (B) Wild type: 3 h after injection of the cRNA, $k = 0.024$; 48 h after injection, $k = 0.029$. (C), (D) Mutant R509T, 3 h after injection: $k = 0.0083$; 48 h after injection $k = 0.0008$. The rate constants k are in units of min^{-1}. Ordinates: cpms in the oocyte; abscissa: time in minutes. The curves represent originals *with different scales*.

likely that the absence of transport activity was due to an unsuccessful translation of the mutated cRNAs or to the degradation of the synthesized proteins prior to the flux measurements. To explore this possibility, immunoprecipitates and flux measurements were also made after only 3 h of incubation after microinjection of the cRNAs encoding the mutants R509T and R509C. After this shorter period of time the presence of immunoprecipitates (Fig. 3) could be demonstrated and in a number of the oocytes of the population, DNDS inhibitable transport could be detected (Fig. 4). In these oocytes transport activity was much lower than the activity seen after expression of wild type encoding cRNA for the same length of time. The interpretation of this observation is difficult. We suspect, however, that in the mutant the degradation of the protein begins immediately after its biosynthesis and hence, at all times, the steady state concentration in the membrane must be smaller than that of the wild type.

3.2. Mutation of Arg 748

The results obtained with the mutants of R748 were immediately apparent. Two days after microinjection of the corresponding cRNAs the immunoprecipitates were easily detectable and yielded bands on the SDS-PAGE which were rather similar to those of the wild type after this length of incubation time (Fig. 5). Nevertheless, neither one of the mutants was able to accomplish anion movements (Figs. 1 and 2). Moreover, exposure to external chymotrypsin yielded the expected 63-kDa fragment of mouse band 3 (Fig. 6; see Ref. [25]). We assume, therefore, that the mutated band 3 protein is properly inserted into the plasma membrane (see Section 2) and conclude that R748 is essential for band-3-mediated anion transport.

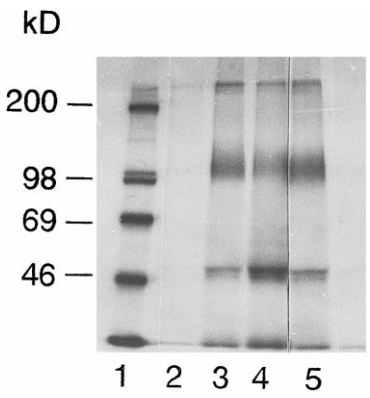

Fig. 5. Immunoprecipitates of band 3 two days after microinjection of wild type band 3-encoding cRNA and cRNA's encoding R748 mutants. Lane 1: molecular weight markers; lane 2: uninjected oocytes; lane 3: wild type; lane 4: mutant R748K; lane 5: double mutant K558N/R748Q.

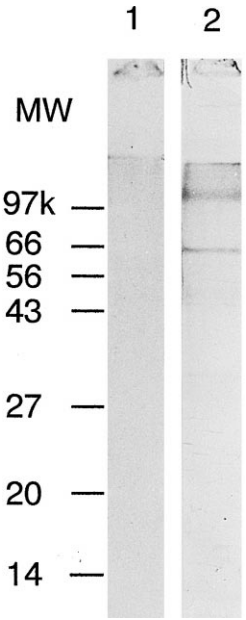

Fig. 6. SDS polyacrylamide gel electropherogram of the immunoprecipitate of ^{35}S-methionine labeled mutant R748 Q. The precipitate was prepared two days after microinjection of the cRNA and subsequent exposure to 5 mg/ml chymotrypsin for 2 h at 4°C. Left lane: uninjected oocytes. Right lane: cRNA-injected oocytes. The upper band represents intact band 3, the lower band represents its *c*-terminal chymotryptic fragment, indicating the cleavage of a fraction of band 3 by external chymotrypsin.

In previous work from our laboratory it was suggested that there exist allosterical relationships between histidine residues in the hydrophobic, anion-transporting domain and Lys 558 in Helix 5 [3], one of the sites of covalent binding of the anion transport inhibitor H_2DiDS [22]. We wanted to know therefore, whether or not the second site mutation K558N/R748K would lead to the modification of the inhibition seen in the single-site mutation R748K. No significant change could be detected. We have also prepared the double mutant R509K/R748K. Two days after microinjection of the corresponding cRNA,

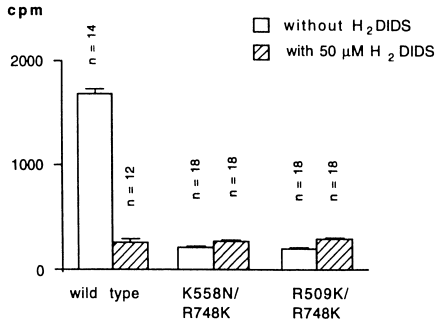

Fig. 7. Determination of ^{36}Cl uptake by oocytes 2 days after microinjection of the cRNA's encoding band 3 and the double mutants R509K/R748Q and K558N/R748K. Same experimental arrangement as in Fig. 1.

the expression of the mutant could no longer be confirmed by immunoprecipitation (Fig. 3) and no transport activity could be observed (Fig. 7).

4. Discussion

The experiments described above served to explore the possible participation of two arginine residues—R509 and R748—in band-3-mediated anion transport. One of these residues, Arg 509, had been proposed by Zaki (review, Ref. [20]) to be involved in the inhibition of anion transport by PG and in anion binding at the transfer site of the anion exchanger. The participation of Arg 748 was suggested as an alternative in case Arg 509 should turn out to be the wrong guess. Zaki's proposal was purely speculative and so far not substantiated by direct experimental observations. The present experiments demostrate that one of these residues is in fact of functional significance but the relationship to Zaki's speculations remains obscure.

So far as R509 is concerned it seems likely that this residue is not necessary for the normal functioning of the transport protein. It seems to be clear, however, that it is required for the protection of the protein against proteolysis on its way to the plasma membrane and, apparently, even after its insertion into that membrane. When Arg 509 is replaced by Cys, Thr or Lys, the protein is synthesized but—in contrast to the wild type—destroyed by proteolysis within less than 2 days.

Interestingly enough, in a number of oocytes of the population, the rate of protein biosynthesis seems to exceed sufficiently the rate of concomitant proteolytic degradation to permit the successful export of a small number of band 3 molecules to the plasma membrane where they execute the barely detectable anion transport shown in Fig. 4. It should be emphasized, however, that after 3 h of expression not all of the oocytes injected with mutant-encoding cRNA show a measurable anion flux. Thus, although our findings do not definitely demonstrate the survival of the transport activity of the synthesized mutant, they provide no support for the assumption that Arg 509 in mouse band 3 (which corresponds to Arg 490 in human band 3) plays a mandatory role in anion transport, either as a constituent of the transfer site or in some other capacity.

In contrast to Arg 509, Arg 748 is certainly essential for anion transport. The protein derived from the mutated cRNA shows no degradation and survives equally well as the wild type band 3, suggesting proper folding and export to the plasma membrane. Arg 748 is located in the amino acid sequence close to His 752 which, together with Glu 699 seems to be responsible for the pH dependence of anion equilibrium exchange [4]. We suspect, therefore, that the proximity of the positive charge of the arginine residue is responsible for the unusually low pK value of His 752 ($pK \sim 5.9$) that governs, directly or allosterically, the pH dependence of anion equilibrium exchange in wild type band 3. It remains open whether the inhibition caused by the mutation of this arginine residue is the indirect consequence of its effect on the dissociation of the histidine residue or if the abolishment of additional functions, for example, as a constituent of the substrate binding site also plays a role.

So far as Arg 748 is concerned, our experiments confirm Zaki's alternative guess [20] that this residue may play a role in anion transport. They do not permit, however, to decide, whether or not the modification of this residue is responsible for the inhibition of anion transport by PG as believed by Zaki (review, Ref. [20]). Phenylglyoxalation causes several different effects on anion transport. At least two types of inhibitory effects have been attributed to reaction with outside- and inside-facing arginines [18,19]. The phenylglyoxalation of a third arginine seems to shift the pH dependence of anion transport such that over

the pH range below 7, the anion flux is enhanced [2]. On the basis of the published evidence it seems impossible to tell which one of the different residues is identical to Arg 748. So far, it is not even clear beyond doubt whether or not Arg 748 becomes phenylglyoxalated at all. This question could only be answered by microsequencing of the PG-labeled band 3 protein.

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