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Biochimica et Biophysica Acta 1194 (1994) 341–344

BBA Biochimica et Biophysica Acta

Anion transport function of mouse erythroid band 3 protein (AE1) does not require acylation of cysteine residue 861

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Received 10 March 1994

Abstract

Cys-861 of mouse band 3 is equivalent to Cys-843 of human band 3, the only acylated cysteine residue in the anion exchanger AE1 of the red blood cell (Hamasaki et al. (1992) Progress Cell Res. 2, 65–71). Mutation of Cys-861 to serine or methionine caused no significant changes of band 3-mediated anion exchange as measured after expression of the appropriate cRNAs in *Xenopus* oocytes. Susceptibility to inhibition of transport by 4,4′-dinitrostilbene-2,2′-disulfonate and PCMBS was not affected. We conclude that palmitoylation is not an absolute requirement for the successful execution the anion transport function by the hydrophobic domain of band 3 in the plasma membrane.

Key words: Chloride transport; Palmitoylation; Site-directed mutagenesis; Oocyte; (*Xenopus*)

1. Introduction

Human red blood cells extracted with organic solvents contain fatty acids that are covalently bound to membrane proteins [6,7,10]. Seventy percent of the acids are linked to protein by O-ester bonds, the remainder by thioester bonds. About one half of the latter are constituents of the band 3 protein, each of which carries one of the fatty acid molecules [12]. The binding site is Cys-843 [4,8]. Amongst the fatty acids bound to band 3, stearate and palmitate predominate. Monoethenoic acid derivatives represent only a minor fraction. In the mature erythrocyte, the bound fatty acids of the acylated proteins undergo a fairly rapid turnover, except in band 3 where the exchange is barely detectable.

In a number of instances, reviewed by Shultz et al. [9], the functional significance of acylation for the anchoring of membrane bound proteins to the lipid bilayer has been demonstrated. In other cases no such function was apparent. Hamasaki and his colleagues [3] raised the question whether or not acylation of Cys-843 is of functional significance for band 3. The present results show that acylation of Cys-861 in mouse band 3, which is homologous to Cys-843 in human band 3, is neither required for the attachment or proper folding of mouse band 3 in the lipid bilayers of the ER or the plasma membrane, nor for the execution of anion transport, the most prominent function of the hydrophobic C-terminal domain of band 3 where this residue is located.

2. Experimental procedures

2.1. Measurement of mouse band 3-mediated anion transport in Xenopus oocytes

All experiments were performed after expression of mouse band 3- encoding cRNA in *Xenopus* oocytes. Measurements of $^{36}Cl^-$ efflux were performed in single oocytes as described previously [1,2]. Briefly, about 25 ng of wild type or mutant band 3- encoding cRNA in 50 nl water were injected in *Xenopus* oocytes of stage 5 or 6. After 48 h of incubation at 18°C in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 5 mM Hepes-NaOH (pH 7.6), about 75 nl of a solution

Abbreviations: DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; PCMBS, *p*-chloromercuribenzenesulfonate.

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SSDI 0005-2736(94)00124-8

containing about 0.4 kBq of $^{36}Cl^-$ were injected. An oocyte was then placed into a perfusion chamber, the bottom of which was formed by the mica window of a Geiger Müller tube. The radioactivity escaping from the oocyte was continuously washed away while the remaining radioactivity was recorded as a function of time. The rate constants of efflux (k, min^{-1}) were determined after electronic digitalization and fitting a single exponential to the data by a nonlinear curve-fitting procedure.

2.2. Preparation of mutants C861S and C861M

The plasmid pSPT 19.bd3.5x mut containing wild type mouse band 3- encoding cDNA (see [1], modified by Lepke and Passow) was treated with the restriction enzymes *Bam*HI and *Hin*dIII to produce two fragments of band 3 cDNA one of which remains attached to a flanking sequence of the plasmid. These fragments consisted of 5566 bp and 571 bp. The latter fragment is cloned into the polylinker of another vector, pSPT 19, at a site that is cut out with *Bam*HI and *Hin*dIII. This yields pSPT 19/1, consisting of 3678 bp. This construct is restricted by *Bam*HI and *Pfl*MI to yield one fragment of the plasmid with one flanking sequence of band 3 cDNA (3434 bp) and another fragment of band 3 of 244 bp. The first of the two fragments is ligated with an oligonucleotide containing the mutation and obtained by PCR. This yields pSPT 19/2, with 3678 bp. The construct is restricted with *Bam*HI and *Hin*dIII. This results in plasmid DNA and band 3- encoding DNA from nucleotide 2459 up to nucleotide 3030 (= 571 bp). The latter contains the mutation. It is cloned into the plasmid obtained by the first restriction of pSPT 19.bd3.5x mut.

The PCR reaction was preceeded by cutting out of pSPT 19.bd3.5x mut a 1042 bp fragment, using *Xma*I and *Hin*dIII for restriction. This was subjected to the PCR reaction [5], using the following primers:

A1 = 5′-GGTCACTTTGGCATCATCACC-3′
A2 = 5′-TACATGGGAGTCACATCCCTC-3′
Is1 = 5′-CAGATCATCTCCCTGGCTGTG-3″
Is2 = 5′-CACAGCCAGGGAGATGATCTG-3′
I11 = 5′-CAGATCATCATGCTGGCTGTG-3
I12 = 5′-CACAGCCAGCATGATGATCTG-3

Restriction of the PCR products with *Bam*HI and *Pfl*M1 yielded the oligonucleotide described above for cloning into pSPT 19/1. The success of the procedures was verified by DNA sequence analysis with standard techniques.

2.3. Palmitoylation of band 3 expressed in oocytes

Two times sixty oocytes were injected with either 25 ng cRNA, encoding wild type mouse band 3 or the band 3 mutant C861S. Sixty control oocytes remained uninjected. The oocytes were incubated for 72 h at 18°C in 0.6 ml Barth's solution containing 3.7 MBq/ml [9,10-$^3H_2$]palmitic acid (NEN) and 20 MBq/ml [^{35}S]-methionine (Amersham). The palmitic acid was concentrated 2-fold under a stream of nitrogen prior to use. At the end of the incubation period, the oocytes were homogenized in 0.3 ml of 5 mM Na-phosphate buffer, pH 8 at 4°C. After centrifugation, the pellets were solubilized with 0.3 ml of solubilizing buffer, containing 50 mM Tris-HCl, 50 mM NaCl, 4% sodium dodecyl sulfonate (SDS), 5 mM EDTA and 10 units/ml aprotinin (pH 7.4). After centrifugation for 3 min at 4 degrees C, 250-μl aliquots of the extracts were heated at 100°C for 4 min. They were then diluted first with an equal volume of water, and subsequently with 8 volumes of dilution buffer containing 60 mM Tris-HCl, 190 mM NaCl, 2.5% Triton X-100, 6 mM EDTA and 10 units/ml approtini (pH 7.4). After addition of 180 μl of an antiserum containing antibodies against the chymotryptic 67 kDa peptide of mouse band 3, the immunocomplex was bound to protein-A Sepharose (Sigma) (100 μl of an 80% suspension in dilution buffer) by end-over-end mixing at 4°C for 4 h. The bound protein was eluted with SDS-PAGE sample buffer containing 1% β-mercaptoethanol. After boiling at 100°C for 5 min, the samples were applied to a preparative SDS-PAGE using a 3% stacking gel and a 9% separating gel. The gels were cut into 2-mm slices. Each slice was placed into a separate counting vial containing 1 ml of Biolute-S (Zinsser Analytic) and incubated at 50°C for 3 h. After cooling to room temperature, each vial received 10 ml Quickscint 701 (Zinsser Analytic) with 0.5% acetic acid and was counted.

3. Results and discussion

Figs. 1 and 2 show the $^{36}Cl^-$ content as a function of time of single oocytes containing either wild type band 3 or the band 3 mutant C861S. The oocytes are fixed in a hair loop close to the mica window of the Geiger Müller Tube that forms the bottom of a perfusion chamber continuously flushed with Barths solution. During the initial perfusion period, the solution contains a reversibly binding, band 3 specific inhibitor. This prevents band 3-mediated efflux, indicating that on the time scale of the experiments, the oocytes are impermeable for chloride. After establishing this base line, perfusion is continued with Barths solution without inhibitor. This leads to band 3-mediated $^{36}Cl^-$ efflux, which manifests itself by a continuous decrease of the radioactivity in the oocytes. Evidently, the band 3 protein is capable of executing its transport function, regardless of whether or not an acylatable cysteine residue is present or not.

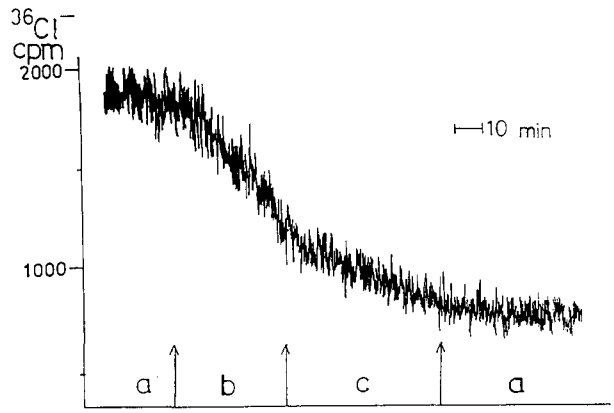

Fig. 1. $^{36}Cl^-$ efflux from single oocyte mediated by the band 3 mutant C861S. The oocyte rests on the mica window of a Geiger Müller tube which forms the bottom of a chamber that is perfused with Barth's solution to remove the radioactivity that left the oocyte. The ordinate indicates the radioactivity inside the oocyte as a function of time (abscissa), as measured by means of a rate meter. During the initial time period (designated a), the Barth's solution contained 500 μM DNDS. During the time period b, perfusion was continued without DNDS in the solution. Finally, in the time period c, the Barth's solution contained $x = 10$ μM DNDS. At the end of the experiment, perfusion with Barth's solution containing 500 μM DNDS (a) was resumed. After digitizing the data (see Fig. 2), rate constants were calculated as described in Materials and methods for the various time intervals pertaining to the various experimental conditions. When these constants are expressed as a fraction of the rate constant measured in the absence of the inhibitor, one obtains the fractional inhibition seen in this particular oocyte. Using this protocol at a range of different DNDS concentrations x, curves relating inhibition to inhibitor concentration could be constructed and apparent K_i values could be calculated (see Fig. 3).

The results shown in the Fig. 1 were confirmed by the additional experiments summarized in Table 1.

In parallel experiments, the palmitoylation of band 3 wild type and mutant C861S was determined. SDS-PAGE of the isolated oocyte membranes showed that during biosynthesis neither the wild type nor the mutant incorporated any ^{3}H-labeled palmitic acid. The fatty acid was, however, incorporated into the phospholipids, indicating that the oocytes possess the metabolic capabilities to use palmitic acid in the metabolism of constituents of the plasma membrane (not shown). It is evident, therefore, that mouse band 3 in the oocyte plasma membrane does not require palmitoylation as a prerequisite for the execution of its transport function.

This conclusion prompted us to explore whether or not anion transport mediated by the wild type would be susceptible to the action of SH reagents. In the wild type, where the cysteine residue is presumably protected by palmitoylation, iodoacetate or iodoacetamide were unable to induce changes of Cl^- transport (not shown). With PCMBS, however, a partial inhibition was observed (Fig. 2B, Table 1). The same degree of inhibition was also found in the mutant. This suggests

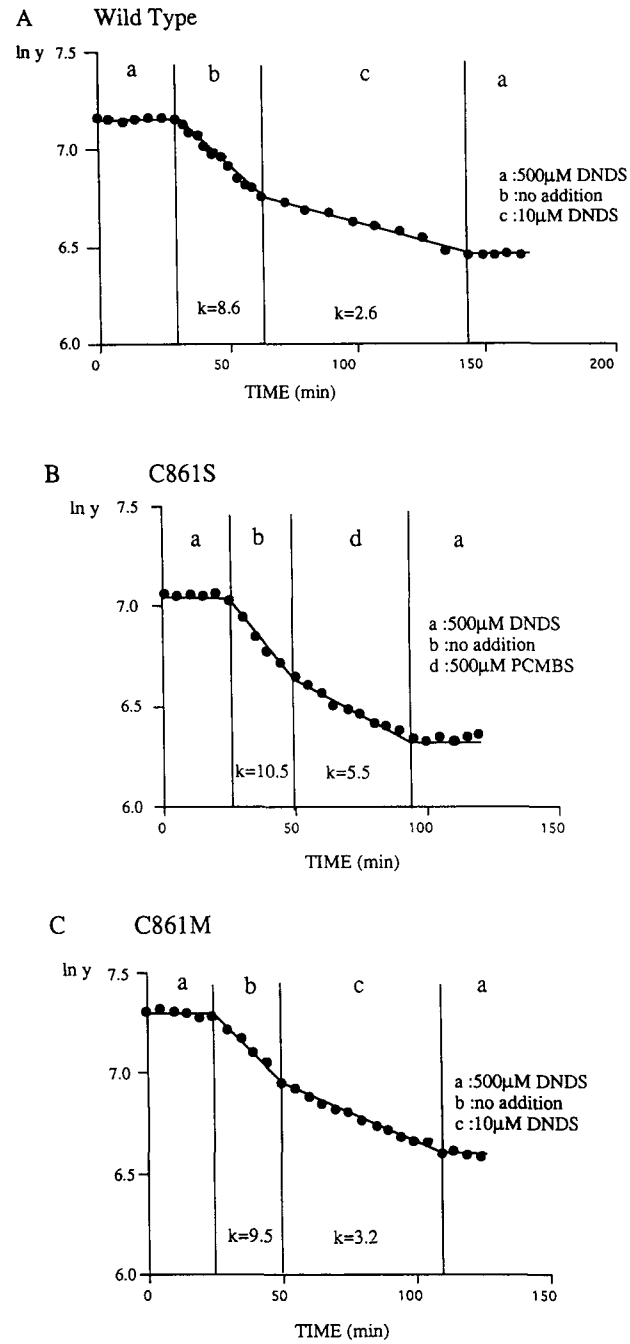

Fig. 2. Effects of DNDS and PCMBS on band 3-mediated $^{36}Cl^-$ efflux from single oocytes. (A) Oocyte microinjected with wild type cRNA, (B) and (C) with mutated cRNA, C861S or C861M, respectively. Same experimental procedure as in Fig. 1. However, the readings of the rate meter were digitized, averaged over 5-min intervals, and replotted on a semilog scale. The slopes k represent rate constants pertaining to the perfusion with Barth's medium containing the additions indicated next to the various panels.

that the SH group of Cys-861 is either not susceptible to reaction with PCMBS, or that its modification by the agent is without effect on transport.

The absence of an effect of palmitoylation of Cys 861 is remarkable since this residue is located in the

Table 1
Effect of mutation of Cys 861 on band 3-mediated 36-Cl efflux from *Xenopus* oocytes

	efflux rate constant ($min^{-1} \times 1000$)	per cent inhibition	
		10 μM DNDS	500 μM PCMBS
WT	7.2 ± 1.3 (12)	72.4 ± 3.3 (9)	37.5 ± 10.4 (10)
C861S	8.2 ± 3.7 (14)	67.5 ± 5.4 (16)	45.5 ± 10.7 (14)
C861M	6.6 ± 3.1 (14)	67.9 ± 7.8 (13)	–

The effluxes were measured and rate constants calculated as exemplified in Figs. 1 and 2. Each value in the table represents the mean value of all measurements with a particular band 3 species, the standard deviation, and the number of oocytes used. -: not determined. WT: wild type band 3

putative helix 13 that is well known to be intimately associated with the transport function. The hydropathy plot of band 3 suggests that the cysteine residue is situated inside the membrane, fairly close to Lys-869, which is located near the outer membrane surface [1,3,8]. This residue is known to play a decisive role in the reversible binding of stilbene disulfonates, like the highly band 3 specific DNDS, and the reversible, as well as irreversible binding of pyridoxal phosphate. Moreover, the inward-facing loop that interconnects helix 12 with helix 13 also contains lysines, prolines and a histidine that are essential for the transport function [9]. We conclude that the maintenance of the quaternary structure of the anion transporter in this functionally important region does not necessarily require an acylation of Cys-861. This conclusion is not only based on the observation of the continuation of DNDS-sensitive (i.e., band 3-mediated) anion transport in the mutant, but also on the finding that the absolute magnitude of the K_i value for inhibition of anion transport by DNDS remains virtually unaffected by the substitution of Cys-861 by Ser (c.f., Fig. 3). Similarly, although not measured with the same degree of accuracy, it is clear that the sensitivity to inhibition by PCMBS also remains unaffected (c.f., Table 1).

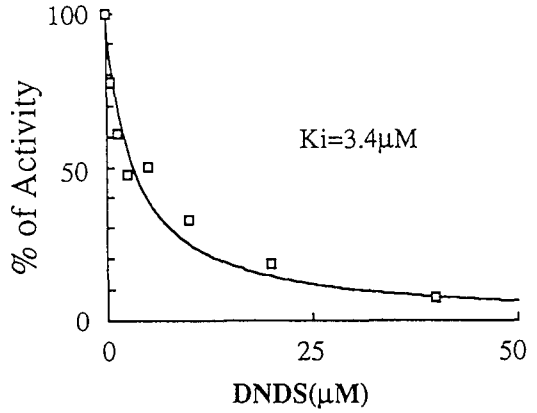

Fig 3. Concentration dependence of the effect of DNDS on $^{36}Cl^-$ efflux mediated by the band 3 mutant C861S. Ordinate: Percentage of residual activity (corresponding to 1-fractional inhibition) measured (as exemplified in Fig. 1 for a DNDS concentration of $x = 10$ μM) at the DNDS concentrations indicated on the abscissa. The drawn line was fitted to the data using the equation: per cent activity $= 100K_i/(K_i + [DNDS])$, with $K_i = 3.4$ μM. The K_i value for wild type band 3 is 5.8 μM (not shown).

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