Biochimica et Biophysica Acta, 985 (1989) 355–358
Elsevier

BBAMEM 70634

BBA Report

Identification by site-directed mutagenesis of Lys-558 as the covalent attachment site of H_2DIDS in the mouse erythroid band 3 protein

Detlef Bartel, Heidrun Hans and Hermann Passow

Max-Planck-Institut für Biophysik, Frankfurt am Main (F.R.G.)

(Received 13 July 1989)

Key words: Band 3 protein; cRNA expression; Oligonucleotide-directed mutagenesis; Inhibitor binding site; (*Xenopus* oocyte)

After functional expression of mouse erythroid band 3 by cRNA microinjection into *Xenopus* oocytes, $^{36}Cl^-$ efflux is irreversibly inhibited by H_2DIDS. When a cRNA is injected that is derived from a cDNA in which the nucleotides encoding for lysine-558 were replaced by nucleotides encoding for asparagine, transport and inhibition of transport by H_2DIDS still occur. However, when measured under conditions where no intramolecular crosslinking takes place the inhibition by H_2DIDS is no longer irreversible. This indicates that thiourea bond formation between H_2DIDS and band 3 takes place at Lys-558.

The stilbene disulfonate derivative H_2DIDS is a highly specific inhibitor of band 3 protein-mediated anion exchange across the red cell membrane [1]. The reaction of the inhibitor with the protein takes place in two consecutive steps [1,2]. First, almost instantaneously non-covalent attachment takes place, which leads to inhibition. This process is fully reversible. Second, a covalent reaction of the isothiocyanate groups of the inhibitor with two adjacent lysine residues follows. This process is irreversible. The covalent bond formation takes place first at a lysine residue called Lys *a*, which is located at the chymotryptic N-terminal 65 kDa fragment of band 3. More slowly follows the reaction with the other lysine residue called Lys *b* which resides on the 35 kDa, C-terminal fragment of band 3 [3]. In the present paper we deal with an attempt to identify the lysine residue involved in covalent bond formation on the chymotryptic 65 kDa fragment.

Recently, we have shown by immunological techniques that a cRNA derived from the amended cDNA clone pMEB 3.18 of Demuth et al. [4] can be expressed in oocytes of *Xenopus laevis* and that the expressed protein accomplishes an anion exchange which does not normally occur in the oocytes and which exhibits features that are characteristic for band 3-mediated anion transport as measured in the red cell of the mouse. This opens up the possibility to identify the functions of individual amino acid residues by site-directed mutagenesis [5–7]. Indirect evidence derived from the work of many laboratories has lead to the suggestion that Lys *a* is identical to either Lys-558 or Lys-561 (using the numbering of the amino acid residues in the amino acid sequence of mouse erythroid band 3 protein as introduced by Kopito and Lodish [8]).

In the previous study [5] we were able to show that the replacement of both Lys-558 and Lys-561 by asparagine residues yields a gene product that is still capable of H_2DIDS-inhibitable anion exchange. However, when the covalent reaction was performed under conditions where a reaction with Lys *b* is minimized, the inhibition remained reversible. This suggests that indeed one of the two lysine residues is identical to Lys *a*. In the present communication we report that mutation of Lys-558 into an asparagine residue suffices to produce the same effect. This indicates that this lysine residue is Lys *a*.

Methods. The experiments were performed as follows: A vector was constructed containing the cDNA for mouse band 3 (obtained from pMEB 3.18; Demuth et al. [4]), a synthetical translation initiation sequence (5′-nontranslated region, start-codon and a coding region for the first 12 N-terminal amino acids) and RNA polymerase promoters suitable for in vitro transcription

Abbreviations: H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; BSA, bovine serum albumin.

Correspondence: H. Passow, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, 6000 Frankfurt/M.71,F.R.G.

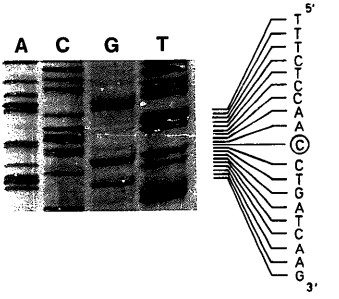

Fig. 1. Autoradiography of a polyacrylamide/urea gel showing the products of sequencing reactions using T7 DNA polymerase. The sequence pertains to pDB3/5 and shows the desired mutation of Lys-558 (AAG) into an asparagine residue (AAC) at the corresponding nucleotide site.

of cRNA. The mutant cDNA was constructed using the gapped duplex DNA method [9] for oligonucleotide-directed mutagenesis as described previously [5]. The mutagenic primer $^{5'}$ATCCTGATCAGGTTGGAGAA-AGTCTC$^{3'}$ was synthesized on an Applied Biosystems 380A synthesizer using phosphoamidite chemistry and purified by polyacrylamide gel electrophoresis. The $P_{pu}MI$ fragment (n 1430-1855; Demuth et al. [4]) of band 3 cDNA was inserted into the Xba I-site of M13mp18am phage. The procedures for single-stranded vector preparation, generation of gapped duplex DNA and enriching the synthetic marker are described in Refs. 9 and 10.

Several recombinant clones were sequenced by the dideoxy chain termination method of Tabor and Richardson [11] for screening the presence of the desired mutation (Fig. 1). Capped cRNAs encoding mouse band 3 protein were transcribed in vitro with SP6 RNA polymerase [12,13]. After template removal by subsequent DNase digestion, unincorporated ribonucleotides and the RNA cap structure were removed by two ethanol/ammonium acetate precipitations. Unmutated mouse band 3 cRNA was synthesized from transcription vector pSPT19Bd.3 [5], mutated cRNA from pDB3/5 vector, which contained the mutation at nucleotide 1644 G → C.

About 30 ng of the mutant or non-mutated band 3 cRNA (in a volume of 50 nl) were injected into defolliculated *Xenopus* oocytes of stage 5 or 6 [14]. After 16 h of incubation at 18 °C, the oocytes were microinjected with 75 nl $^{36}Cl^-$ solution (0.113 mCi/ml) and placed into the hair loop of a perfusion chamber, the bottom of which consisted of the mica window of a Geiger Müller tube. The chamber was then perfused with Barth's solution (a modified amphibian Ringer's solution, for composition see Ref. 15). This solution carried away the radioactivity escaping from the $^{36}Cl^-$-microinjected oocyte into the medium. The Geiger Müller tube was connected to a rate meter and a paper recorder which recorded the time course of washout of the radioactivity. From this time course, rate constants can be calculated, using the expression

$$^{o}k_{Cl} = (0.693/\Delta t)\ \ln\ y_1/y_2$$

where Δt is the time period of observation during which the radioactivity in the oocyte changes from y_1 to y_2 [15,16].

Results. There is little if any $^{36}Cl^-$ efflux from control oocytes that had not received cRNA (not shown). Fig. 2(A) shows the protocol of a washout experiment, with a single oocyte that had received cRNA derived from *unmutated* mouse band 3-encoding cDNA. During a first perfusion period, the perfusate (i.e., Barth's solution) contained the reversibly binding stilbene-

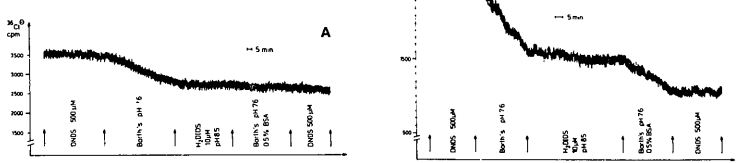

Fig. 2. Oocytes were microinjected with (A) unmutated band 3-encoding cRNA or (B) mutated cRNA and incubated over night in Barth's medium at 18 °C. After microinjection with $^{36}Cl^-$, the oocyte was placed on the mica window of a Geiger-Müller tube which formed the bottom of a perfusion chamber [15,16]. The counting rate is continously determined by a rate meter and recorded by a chart recorder during perfusions (see text).

disulfonate DNDS at a concentration that should suffice to block virtually completely mouse band 3-mediated anion transport. The figure shows that little if any $^{36}Cl^-$ is released from the oocyte. This indicates that the oocyte behaves like a *Xenopus* oocyte that had not been microinjected with band 3-encoding cRNA (see Refs. 5, 15, 16). When the perfusion medium is changed to Barth's solution without DNDS, rapid Cl^- efflux takes place. The rate constant is $0.41 \cdot 10^{-2}$ min^{-1}. When the perfusion medium is now changed to Barth's solution containing 10 μM H_2DIDS at pH 8.5, inhibition occurs. After exposure to H_2DIDS for about 1 hour, return to perfusion at pH 7.6 in the absence of H_2DIDS (i.e., to normal Barth's solution) does not lead to the reappearance of $^{36}Cl^-$ efflux. The efflux does not reappear even when the perfusate contains bovine serum albumine, which is known to bind free H_2DIDS with high avidity. This indicates that H_2DIDS binding to the mouse band 3 protein was irreversible, as one would expect after covalent bond formation with Lys *a*.

Fig. 2(B) shows a similar experiment in which an oocyte had been used that had been microinjected with cRNA derived from the *mutated* cDNA, in which Lys-558 had been replaced by an asparagine residue. As in the control, during perfusion with DNDS-containing Barth's solution there is little if any $^{36}Cl^-$ efflux. Efflux is seen, however, when the perfusate is changed to Barth's solution without DNDS. This indicates that the presence of Lys-558 is neither essential for the execution of anion transport nor for the inhibition by the reversibly binding stilbenedisulfonate, although a more detailed study will still be necessary to exclude a modifying influence on transport. When the perfusion is continued with H_2DIDS, inhibition occurs. However, even though the time and pH of exposure were the same as in the oocyte containing band 3 from unmutated cRNA, inhibition remains largely reversible. Washout with albumine-containing Barth's medium reestablishes $^{36}Cl^-$ efflux. The rate constant before and after treatment with H_2DIDS as calculated by the logarithmic expression above are $1.32 \cdot 10^{-2}$ min^{-1}, and $0.84 \cdot 10^{-2}$ min^{-1}, respectively; i.e., an inhibition of 36% persists. This is the highest residual inhibition observed in a series of similar experiments (Table I). On the average, after wash-out of the reversibly bound H_2DIDS, the residual inhibition amounted to about 12%. This residual inhibition is not unexpected since H_2DIDS-binding to Lys *b*, which had not been mutated, cannot be prevented entirely under realistic experimental conditions. These conditions (pH 8.5, room temperature) represent a compromise to achieve a sufficiently high reaction rate with Lys *a* ($pK \approx 7.5$) and to avoid reaction with Lys *b* ($pK \approx 10$, see Ref. 20). When the perfusion is now continued with DNDS, inhibition occurs promptly. This rules out that the reestablishment of $^{36}Cl^-$ efflux after washout with albumine-containing Barth's solution is due to leakage.

It should be noted that efflux experiments with single oocytes as represented in Figs. 2(A) and (B) are far superior to influx studies, in which continuous recording of the uptake of $^{36}Cl^-$ is not feasible. In the efflux experiments the effects of perfusion with the inhibitor can be expressed as a percent of the efflux observed *in the same oocyte* after perfusion in the absence of the inhibitor. In other words, each oocyte serves as its own control and hence yields an unequivocal result. Variations in the absolute values of efflux observed in different oocytes do not affect this conclusion. They simply represent differences of the amounts of band 3 expressed in the individual cells. This also applies to the

TABLE I

Effect of H_2DIDS on anion transport mediated by unmutated band 3 and by band 3 in which Lys-558 had been replaced by an asparagine residue

Same experimental arrangement as in Fig. 2. In the experiments with the mutated band 3 we attribute the residual inhibition after washout of H_2DIDS to covalent bond formation of some of the previously bound H_2DIDS with Lys *b*, which is impossible to avoid completely (see Ref. 20). Efflux measured at room temperature (20–25 °C).

Microinjection of:	Non-mutated band 3			Mutated band 3		
Expt. No.	efflux rate constants in the controls [min^{-1}] (= 100% efflux)	inhibition by H_2DIDS (%)	residual inhibition after perfusion with Barth's BSA (%)	efflux rate constants in the absence of H_2DIDS [min^{-1}] (= 100% efflux)	inhibition by H_2DIDS (%)	residual inhibition after perfusion with Barth's BSA (%)
1	$0.53 \cdot 10^{-2}$	96	89	$0.59 \cdot 10^{-2}$	87	5
2	$0.66 \cdot 10^{-2}$	97	70	$1.32 \cdot 10^{-2}$	92	36
3	$0.82 \cdot 10^{-2}$	98	82	$1.11 \cdot 10^{-2}$	82	0
4	$0.46 \cdot 10^{-2}$	85	85	$0.74 \cdot 10^{-2}$	92	0
5	$0.41 \cdot 10^{-2}$	88	82	$1.75 \cdot 10^{-2}$	96	7
6				$1.45 \cdot 10^{-2}$	96	11
7				$1.59 \cdot 10^{-2}$	96	23
Mean values	$0.58 \cdot 10^{-2}$ ($\pm 0.17 \cdot 10^{-2}$)	93 ± 6	82 ± 7	$1.22 \cdot 10^{-2}$ ($\pm 0.43 \cdot 10^{-2}$)	92 ± 5	12 ± 13

differences observed in the absolute values of transport seen in oocytes microinjected with wild type or mutated band 3 cRNA. In a larger number of additional experiments performed to study other effects of mutation of Lys-558 on transport we found that the averages of the fluxes are rather similar and within the limits of statistical variation.

Conclusions. In conclusion we may state that the single mutation Lys-558 → Asn-558 removes the capacity for irreversible inhibition by H_2DIDS without abolishing the capacity for reversible inhibition by H_2DIDS or DNDS. This suggests that Lys-558 is involved in covalent bond formation with H_2DIDS. The continuation of transport in the mutant indicates that Lys-558 is not essential for the transport function, although it cannot be excluded that it plays a modifying role. None of these conclusions comes as a surprise. The localisation of Lys *a* was predicted on the basis of a comparison of the amino acid sequences of the erythroid band 3 proteins from several species which indicated that only Lys-558 existed in all of these species but not Lys-561 [17]. However, it was shown previously by studying band 3-mediated transport in human red blood cells [18] as well as by measuring $^{35}Cl^-$ binding to band 3 by means of the NMR technique [19], that Lys-558 is not essential for substrate binding to the transfer site. It is gratifying to know that the results obtained by site-directed mutagenesis of mouse band 3-encoding cDNA, expression and flux measurements in oocytes of *Xenopus* lead to results that fit into current views on band 3-mediated transport and the mode of action of transport inhibitors. It suggests that mutations of other amino acid residues may be helpful for a further elucidation of the relationship between molecular structure and transport function of the transport protein.

We thank Mrs. W. Ritz for her valuable collaboration. We are obliged to Prof. Engels, Frankfurt, for synthesizing the oligonucleotides. We are also most grateful to Dr. H. Appelhans who accompanied the various stages of our work with advice and encouragement. The cDNA clone pMEB 3.18 was kindly supplied by Dr. P. Curtis, The Wistar Institute, Philadelphia and amended in collaboration with Mrs. S. Lepke. We thank Dr. P. Wood for reading the manuscript.

References

1 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membr. Biol. 15, 207–226.
2 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membr. Biol. 29, 147–177.
3 Jennings, M.L. and Passow, H. (1979) Biochim. Biophys. Acta 554, 498–519.
4 Demuth, D.R., Showe, L.C., Ballantine, M., Palumbo, A., Fraser, P.J., Cioe, L., Rovera, G. and Curtis, P.J. (1986) EMBO J. 5, 1205–1214.
5 Bartel, D., Lepke, S., Layh-Schmitt, G., Legrum, B. and Passow, H. (1989) EMBO J., in press.
6 Passow, H., Bartel, D., Lepke, S., Layh-Schmitt, G., Raida, M., Wendel, J., Legrum, B. and Furuto-Kato, S. (1989) in Proceedings of the Symposium 'Recent Advances in Molecular Mechanics of Anion Transport' (Hamasaki, N. and Jennings, M., eds.), in press.
7 Passow, H., Raida, M., Wendel, J., Legrum, B., Bartel, D., Lepke, S. and Furuto-Kato, S. (1989) Biochem. Soc. Transactions, 812–815.
8 Kopito, R.R. and Lodish, H.F. (1985) Nature 316, 234–238.
9 Fritz, H.-J. (1986) in DNA Cloning, A Practical Approach (Glover, D.M., ed.), Vol. 1, pp. 151–164, IRL Press, Oxford, Washington.
10 Kramer, W. and Fritz, H.-J. (1987) Meth. Enzymol., in press.
11 Tabor, S. and Richardson, C. (1987) Proc. Natl. Acad. Sci. USA 84, 4767.
12 Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucl. Acids Res. 12, 7035–7056.
13 Drummond, D.R., Armstrong, J. and Colman, A. (1985) Nucl. Acids Res. 13, 7375–7394.
14 Colman, A. (1984) in Transcription and Translation, A Practical Approach (Hames, B.D., Higins, S.J., eds.), pp. 271–302, IRL Press, Oxford, Washington.
15 Grygorczyk, R., Schwarz, W. and Passow, H. (1987) J. Membr. Biol. 99, 127–136.
16 Grygorczyk, R., Hanke-Baier, P., Schwarz, W. and Passow, H. (1989) Meth. Enzymol. 173, 453–466.
17 Tanner, M., Martin, P.G. and High, S. (1988) Biochem. J. 256, 703–712.
18 Passow, H., Fasold, H., Gärtner, E.M., Legrum, B., Ruffing, W. and Zaki, L. (1980) Ann. N.Y. Acad. Sci. 341, 361–383.
19 Falke, J.J. and Chan, S.I. (1986) Biochemistry 25, 7888–7894.
20 Kampmann, L., Lepke, S., Fasold, H., Fritzsch, G. and Passow, H. (1982) J. Membr. Biol. 70, 199–216.