Biochimica et Biophysica Acta, 649 (1981) 310–316
Elsevier/North-Holland Biomedical Press

BBA 79515

PROTOPORPHYRIN-INDUCED PHOTODYNAMIC EFFECTS ON BAND 3 PROTEIN OF HUMAN ERYTHROCYTE MEMBRANES

T M.A.R. DUBBELMAN, A F.P.M DE GOEIJ, K. CHRISTIANSE and J. VAN STEVENINCK

Sylvius Laboratories, Department of Medical Biochemistry, Wassenaarseweg 72, 2333 AL Leiden (The Netherlands)

(Received July 13th, 1981)

Key words Photodynamic effect, Photo-oxidation, Cross-linking, Protoporphyrin, Band 3 protein, (Human erythrocyte)

In previous studies it has been shown that protoporphyrin-induced photodynamic effects on red blood cells are caused by photooxidation of amino acid residues in membrane proteins and by the subsequent covalent cross-linking of these proteins. Band 3, the anion transport protein of the red blood cell membrane, has a relatively low sensitivity to photodynamic cross-linking. This cannot be attributed to sterical factors inherent in the specific localization of band 3 in the membrane structure. Solubilized band 3, for instance, showed a similar low sensitivity to cross-linking. By extracellular chymotrypsin cleavage of band 3 into fragments of 60 000 and 35 000 daltons it could be shown that both fragments were about equally sensitive to photodynamic cross-linking. The 17 000 dalton transmembrane segment, on the other hand, was completely insensitive. Inhibition of band 3-mediated sulfate transport proceeded much faster than band 3 interpeptide cross-linking, presumably indicating that the inhibition of transport is caused by photooxidation of essential amino acid residues or intrapeptide cross-linking. A close parallel was observed between photodynamic inhibition of anion transport and decreased binding of 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS), suggesting that a photooxidation in the immediate vicinity of the H_2DIDS binding site may be responsible for transport inhibition.

Introduction

During illumination of human red blood cells or ghosts in the presence of a sensitizer extensive photodynamic membrane damage occurs. This is reflected by deterioration of many membrane functions [1], mutilation of ultrastructure as shown by electron microscopic studies [2] and extensive covalent cross-linking of membrane proteins to high molecular weight complexes [2–5]. It has been shown that cross-linking is a secondary, light-independent process, caused by reactions between photooxidized amino acid residues and reactive groups in the protein molecules [6].

In the present investigations the photodynamic effects on band 3, the anion transport protein of the red blood cell, were studied in more detail. As shown previously, band 3 cross-linking in red blood cell membranes proceeds much more slowly than, for example, spectrin cross-linking [7]. It seemed conceivable that this relatively low sensitivity to cross-linking could be caused by steric factors, inherent in the specific localization in the membrane structure. Therefore cross-linking was studied both on intact membranes, on band 3 vesicles and on solubilized band 3 protein.

In previous studies on cross-linking the localization of the cross-link site(s) in the protein molecule was not considered. Band 3 protein seemed to be an appropriate model for preliminary studies on cross-link localization, as it consists of an extracellular, a transmembrane and a cytoplasmic fragment. In the present study the problem of cross-link localization was approached via fragmentation of band 3 by con-

Abbreviations: H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate, SDS, sodium dodecyl sulfate.

trolled peptic digestion. Disturbed function of membrane proteins may be caused either by photooxidation of essential amino acid residues or by the secondary cross-linking. A prerequisite for discrimination between these possibilities is an adequate knowledge of the functional site of the protein. Although the exact alignment of the functional region of band 3 is not yet known, many studies have indicated that H_2DIDS is an excellent probe for the transport controlling site [8–9]. Therefore simultaneous measurements of photodynamic anion transport inhibition, band 3 interpeptide cross-linking and H_2DIDS binding capacity were performed. The results of these studies are discussed in the present communication.

Materials and Methods

Illumination with visible light in the presence of protoporphyrin or deuteroporphyrin 2,4-disulfonic acid as sensitizer was carried out as described before [1]

Hemoglobin-free ghosts were prepared from washed erythrocytes by the osmotic lysis method of Weed et al. [10]. Preparation of band 3-containing vesicles and solubilization of band 3 protein was done according to the method of Wolosin et al. [11]. Triton X-100 was removed by treatment with biobeads SM-2 as described by Holloway [12]. Proteolytic fragmentation of band 3 was performed according to the methods of Steck et al. [13] and Cabantchik and Rothstein [14]. Preparation of spectrin-depleted ghosts was performed by the procedure described by Bennett and Branton [15].

Transport of sulfate in intact red blood cells was measured as described by Knauf and Rothstein [16]. Histidine was determined according to the method of Sokolovsky and Vallee [17], tryptophan as described by Spies and Chambers [18] and tyrosine according to Uehara et al. [19]. Free NH_2 groups were assayed with the fluorescamine method described by Udenfriend et al. [20]. H_2DIDS binding to band 3 protein in intact red blood cells and chemical cross-linking of the 60 000 and 35 000 dalton fragments of band 3 by H_2DIDS were measured as described by Jenning and Passow [21].

For gel electrophoresis samples were dissolved in a solution containing 10 mM Tris (pH 8.0)/1 mM EDTA/40 mM dithiothreitol/1% SDS. This solution was incubated for 30 min at 37°C, prior to electrophoresis. In most experiments SDS-polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. [22]. In studies on the 17 000 dalton transmembrane fragment of band 3, urea-SDS gel electrophoresis was done according to the method of Swank and Monkries [23]. Analysis of the 60 000 and 35 000 fragments was done on gradient slab gels as described by Jennings and Passow [21].

Proteins were stained with Coomassie Brilliant Blue G 250. Scans of stained gels were recorded on a Zeiss PMQ II spectrophotometer with scanning device The disappearance of Coomassie Brilliant Blue bands from their normal positions in the electrophoretogram was taken as a measure of protein cross-linking. Control experiments showed that this is justified, as photooxidation did not reduce Coomassie Brilliant Blue staining in these experiments. The total amount of dye bound to monomeric and cross-linked band 3, for instance, appeared to be constant over the experimental period. Enzymes were obtained from Boehringer, Mannheim, protoporphyrin and deuteroporphyrin 2,4-disulfonic acid from Porphyrin Products, Utah. H_2DIDS and $^3H_2$DIDS were a gift of Prof. H. Passow, Max-Planck Institute, Frankfurt am Main.

Results

Illumination of human red blood cell membranes in the presence of protoporphyrin resulted in extensive cross-linking of membrane proteins (Fig. 1). Spectrin and band 6 especially were readily cross-linked to a high molecular weight complex, whereas band 3 reacted much more slowly.

To investigate the possible influence of other membrane proteins and membrane structure on the band 3 cross-linking pattern, the band 3 environment was modified in a series of experiments. It appeared that neither extraction of about 95% of the spectrin according to the method of Bennett and Branton [15] nor removal of virtually all other membrane proteins according to the method of Wolosin et al. [11] had any influence on the cross-linking velocity of band 3 protein. Solubilized band 3 also showed about the same sensitivity to cross-linking as band 3 anchored in the membrane structure (Fig. 2) With solubilized band 3 two cross-linking products

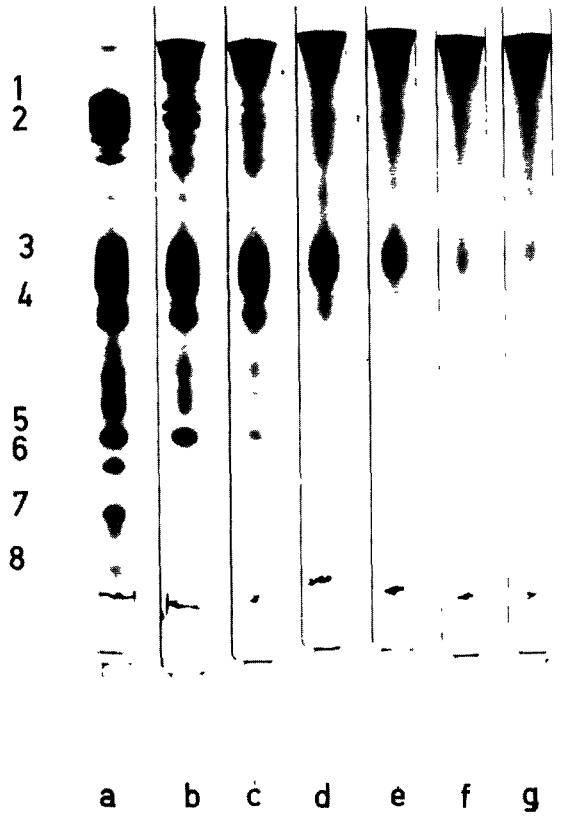

Fig. 1. Protoporphyrin-induced cross-linking of red cell membrane proteins Ghosts were illuminated in the presence of 25 μM protoporphyrin during 0 (a), 5 (b), 10 (c), 20 (d), 40 (e), 60 (f) and 90 (g) min, at a protein concentration of 1 mg/ml at pH 8 6

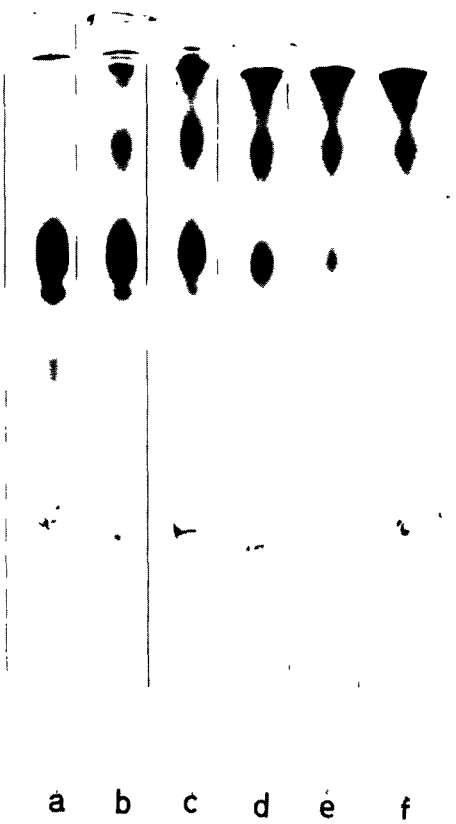

Fig. 2. Protoporphyrin-induced cross-linking of solubilized band 3 protein, at pH 8.6 Protoporphyrin, 25 μM, protein, 1 mg/ml. Illumination period 0 (a), 5 (b), 10 (c), 30 (d), 60 (e) and 90 (f) min.

are visible; a high molecular weight complex on top of the gels, as in the membrane preparations, and a band 3 dimer. Despite treatment of the band 3 solution with biobeads SM-2, a residual Triton X-100 concentration of 0 002–0.005% was present in the final solution, in accordance with the findings of Wolosin et al. [11]. Therefore, in control experiments, the influence of this detergent on photodynamic cross-linking was checked. It appeared that Triton X-100, in concentrations up to 0.5%, had no influence on protein cross-linking. Fig. 3 shows the photooxidation of histidine, tryptophan and tyrosine and the concomitant decrease of free NH_2-groups in solubilized band 3 protein during illumination in the presence of protoporphyrin.

In experiments designed to localize the site(s)

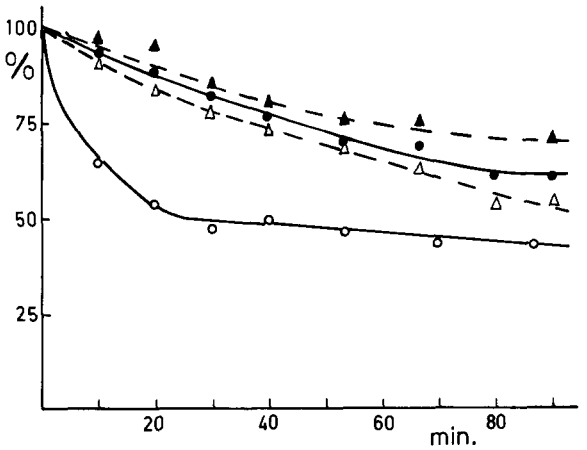

Fig 3. Photooxidation of histidine (○), tryptophan (△) and tyrosine (▲) in solubilized band 3 protein. Also shown is the loss of NH_2-groups (●) as assayed with fluorescamine Experimental conditions as in Fig. 2.

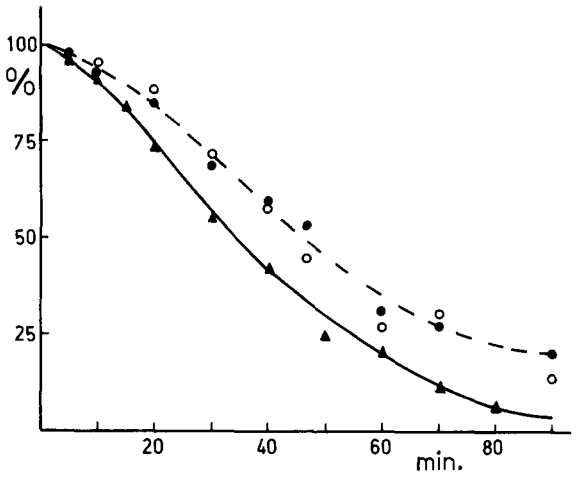

Fig. 4. Protoporphyrin-induced cross-linking of band 3 protein (▲), the 60 kdalton fragment (●) and the 35 kdalton fragment (○). Experimental conditions as in Fig. 1.

of cross-linking in the band 3 polypeptide chain, band 3 was split into 60 and 35 kdalton fragments by chymotrypsin treatment of intact red cells. Ghosts, prepared from these cells, were treated with NaOH to remove most of the peripheral proteins [13] and subsequently illuminated in the presence of protoporphyrin. As shown in Fig. 4, the 60 and 35 kdalton fragments were cross-linked with a velocity only slightly lower than that of intact band 3.

Extensive proteolytic digestion of ghosts yielded a membrane preparation containing only the 17 kdalton transmembrane fragment of band 3 [13]. This 17 kdalton fragment appeared to be insensitive to photodynamic cross-linking (Fig. 5). In other experiments normal ghosts were illuminated in the presence of protoporphyrin till all membrane proteins were completely cross-linked. Subsequently these ghosts were treated with NaOH and subjected to proteolytic digestion. Gel electrophoresis of these membranes yielded results identical to those shown in Fig. 5. Again only the 17 kdalton fragment of band 3 was visible and even after prolonged illumination periods no decrease of this fragment was found.

During illumination of intact red blood cells in the presence of protoporphyrin the transmembrane sulphate transport velocity decreased dramatically. This decrease was much faster than the concomitant band 3 cross-linking (Fig. 6). This discrepancy was even

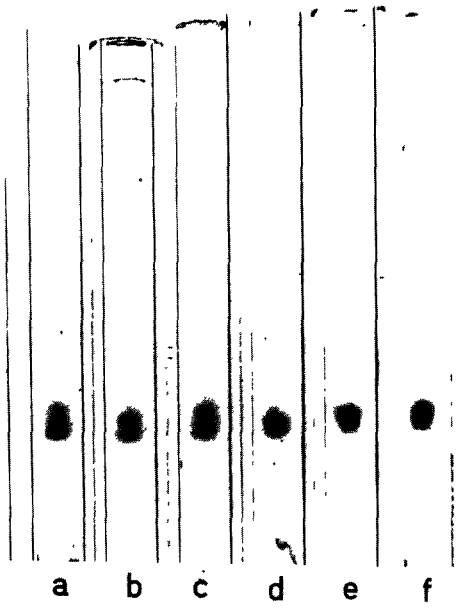

Fig. 5. The 17 kdalton fragment of band 3 after illumination in the presence of 25 μM protoporphyrin at pH 8.6, during 0 (a), 5 (b), 10 (c), 30 (d), 60 (e) and 90 (f) min

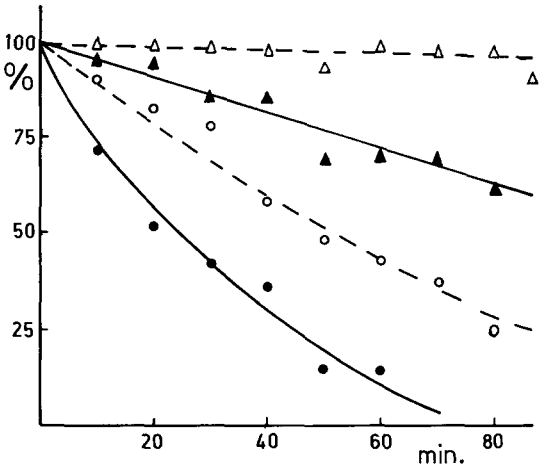

Fig. 6. Photodynamic cross-linking of band 3 and inhibition of sulfate transport in intact red cells (10% suspension) at pH 7.9. Illumination was carried out in the presence of 0.2 mM protoporphyrin or 0.8 mM deuteroporphyrin 2,4-disulfonic acid. With protoporphyrin ●———●, sulfate transport, ▲———▲, remaining band 3 With deuteroporphyrin 2,4-disulfonic acid. ○- - - - -○, sulfate transport; △- - - - -△, remaining band 3. Sulfate transport and band 3 cross-linking were measured after illumination during the indicated period.

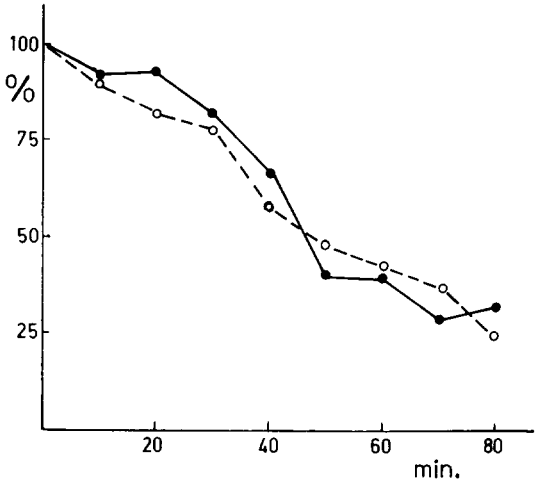

Fig 7 Inhibition of sulfate transport (○- - - - -○) and H_2DIDS binding (●——●) after illumination of a 10% red blood cell suspension at pH 7 9 in the presence of 0 8 mM deuteroporphyrin 2,4-disulfonic acid

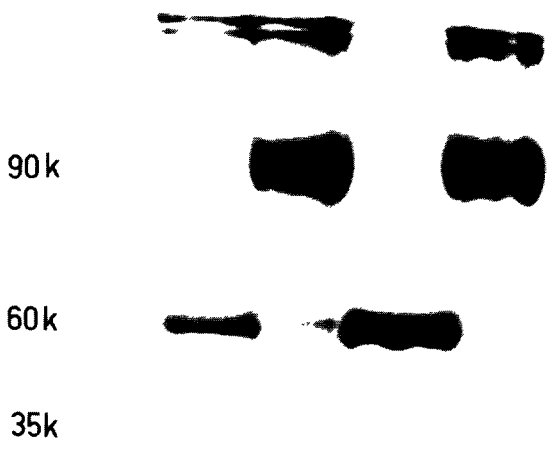

Fig 8 a NaOH-stripped ghosts from red blood cells, illuminated for 15 min in the presence of deuteroporphyrin 2,4-disulfonic acid and subsequently labeled with H_2DIDS and chymotrypsinized. b as a, but incubated at pH 9 5 for 1 h after chymotrypsin treatment. c. as a, but not illuminated. d as b, but not illuminated

more pronounced with deuteroporphyrin 2,4-disulfonic acid as sensitizer. To obtain comparable inhibition of sulphate transport this sensitizer was added at a final concentration of 0.8 mM, as compared to 0.2 mM for protoporphyrin. Band 3 cross-linking was virtually absent under these experimental conditions (Fig. 6). H_2DIDS is a strong inhibitor of anion transport that binds in a covalent reaction. When added to intact red blood cells H_2DIDS binding is highly localized in band 3 protein [9,21]. After illumination of intact red blood cells in the presence of a sensitizer H_2DIDS binding to band 3 protein appeared to be diminished. In parallel experiments it appeared that anion transport and H_2DIDS binding decreased in close parallel by illumination in the presence of deuteroporphyrin 2,4-disulfonic acid (Fig 7).

After chymotrypsin treatment of intact red blood cells, yielding the 60 and 35 kdalton fragments of band 3, H_2DIDS binding at neutral pH to the intact cells is localized in the 60 kdalton fragment [14] Subsequent incubation of the cells at pH 9.5 leads to virtually complete cross-linking of the pairs of chymotryptic fragments and thus to reconstitution of band 3. This cross-linking is caused by a covalent reaction of the bifunctional 60 kdalton fragment-bound H_2DIDS with a susceptible group of the 35 kdalton fragment at pH 9.5 [21]. To investigate photodynamic effects on H_2DIDS-induced cross-linking of the 60 and 35 kdalton fragments, red cells were illuminated for 15 min in the presence of 0.8 mM deuteroporphyrin 2,4-disulfonic acid. Subsequently the cells were chymotrypsinized, exposed to H_2DIDS at neutral pH and finally inducated at pH 9.5, according to the procedure described by Jennings and Passow [21]. After illumination H_2DIDS binding to band 3 was about 15% reduced. In accordance 10–20% of the proteolytic fragments could not be cross-linked by H_2DIDS (Fig. 8).

Discussion

The described photodynamic effects on band 3 protein comprise photooxidation of sensitive amino acid residues (Fig. 3), covalent cross-linking of both the extracellular and intracellular fragment (Fig 2

and 4), deterioration of function, as reflected by inhibition of sulfate transport (Fig 6), and decreased H_2DIDS binding (Fig. 7). As shown previously, reactions of photooxidized histidine, tryptophan and tyrosine with NH_2 groups appear to be a significant source of covalent cross-links [24,25], although experimental evidence presented by Girotti [26] indicates that other cross-link mechanisms are also operative. The decrease of (photooxidation-insensitive) NH_2 groups during illumination of band 3 in the presence of protoporphyrin is in accordance with this type of secondary reaction (Fig. 3).

After an illumination period of 30 min about 50% of the histidine, 22% of the tryptophan and 14% of the tyrosine residues are photooxidized (Fig. 3). Considering the amino acid composition of band 3 [13,27], this corresponds to about 104, 23 and 32 nmol/mg protein, respectively. It is interesting to compare these data with those on photooxidation of solubilized spectrin. As shown previously, under identical experimental conditions of pH, sensitizer and protein concentration, photooxidation of spectrin after an illumination period of 30 min amounted to 30, 16 and 2% of the histidine, tryptophan and tyrosine residues, corresponding to 74, 24 and 4 nmol/mg protein, respectively [25]. This demonstrates that band 3 protein is certainly not less sensitive to photooxidation. Yet, as judged from the electrophoretograms, spectrin is completely cross-linked within 10 min under these conditions [25], whereas cross-linking of band 3 is not complete, even after 90 min (Fig. 2). Apparently the lower sensitivity of band 3 protein to photodynamic cross-linking cannot be attributed to a slower rate of photooxidation. Presumably differences in conformation of various proteins play an important role in this connection.

As shown previously, the velocity of band 6 cross-linking is considerably higher in red cell membranes than in solubilized band 6 [7]. It was tentatively suggested that this may indicate a higher probability of, for example, a spectrin-band 6 than of a band 6-band 6 cross-link. The band 3 cross-linking pattern is quite different in this respect The rate of band 3 cross-linking was about equal in red cell membranes, spectrin-depleted membranes, band 3 vesicles and solubilized band 3. Apparently the presence of other membrane proteins in red cell membranes has no, or only a minor, influence on the cross-linking velocity of band 3 protein.

Both the 35 and the 60 kdalton fragment of band 3 participate in interpeptide cross-linking (Fig. 4). Further proteolytic cleavage of the 60 kdalton fragment into the intracellular and 17 kdalton transmembrane fragment demonstrated that the 17 kdalton fragment is not involved in interpeptide cross-linking (Fig. 5). Thus both the extracellular and the intracellular fragments of band 3 are sensitive to photodynamic cross-linking. The insensitivity of the 17 kdalton fragment is presumably not caused by the absence of reactive groups. In fact the amino acid composition of the 17 kdalton fragment is not grossly different from the amino acid composition of the complete band 3 protein [13]. More likely the fixed position of band 3 in the lipid bilayer impedes the required juxtaposition of reactive groups.

The results described above indicate that photodynamic inhibition of anion transport is caused by photooxidation or intrapeptide cross-linking and not by interpeptide cross-linking. Decrease of anion transport during illumination in the presence of protoporphyrin proceeded much faster than interpeptide cross-linking of band 3 (Fig. 6). Even at a 4-times higher concentration than protoporphyrin, deuteroporphyrin 2,4-disulfonic acid is less effective in photoinactivation of anion transport. This is in accordance with observations of Sandberg and Romslo [28], showing that more hydrophilic porphyrins are less effective against membrane-associated components. The fact that interpeptide cross-linking is virtually absent under these conditions (Fig. 6) affirms that there is no causal connection between these two phenomena. On the other hand, there is a close parallel between photodynamic inhibition of anion transport and decreased H_2DIDS binding to band 3 protein (Fig. 7). As H_2DIDS is a well-known probe for the transport site of band 3 [8,9,21], this observation strongly suggests that the photodynamic inhibition of anion transport should be attributed to photooxidation at or near the transport site.

The mechanism of decreased H_2DIDS-binding is not yet clear. H_2DIDS binding to band 3 comprises a primary, reversible interaction and a subsequent irreversible binding [29]. Each of these two processes might be inhibited by photodynamic perturbation of the local conformation at the H_2DIDS binding site. On the other hand, Ramjeesingh et al. [30] have shown in a recent paper that the H_2DIDS binding

site is a lysine residue. It is tempting to speculate that this NH_2 group may be involved in intrapeptide cross-linking. Further experiments will be needed to elucidate the exact mechanism.

Acknowledgements

This work was supported by the Netherlands Foundation for Fundamental Medical Research (FUNGO, grant 13-39-28) and the Netherlands Foundation for Biophysics (12-25-05).

References

1 Schothorst, A.A., Van Steveninck, J , Went, L N. and Suurmond, D (1971) Clin Chim. Acta 33, 207–213
2 De Goeij, A F.P.M , Ververgaert, P H.J.Th and Van Steveninck, J. (1975) Clin Chim. Acta 62, 287–292
3 De Goeij, A F P.M., Van Straalen, R.J.C. and Van Steveninck, J. (1976) Clin. Chim Acta 71, 485–494
4 Girotti, A W (1975) Biochemistry 14, 3377–3383
5 Girotti, A W (1979) Biochemistry 18, 4403–4411
6 Dubbelman, T.M.A.R., Haasnoot, C and Van Steveninck, J (1980) Biochim Biophys. Acta 601, 220–227
7 Dubbelman, T M A.R., De Goeij, A.F.P.M and Van Steveninck, J (1978) Photochem. Photobiol. 28, 197–204
8 Lepke, S , Fasold, H., Pring, M. and Passow, H (1976) J. Membrane Biol 29, 147–177
9 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1980) Biochim. Biophys. Acta 599, 127–139
10 Weed, R.I , Reed, C.F. and Berg, G. (1963) J. Clin Invest 42, 581–588
11 Wolosin, J.M , Ginsburg, H. and Cabantchik, Z.I (1977) J Biol. Chem. 252, 2419–2427
12 Holloway, P.W (1973) Anal. Biochem. 53, 304–308
13 Steck, T.L , Kozlarz, J.J., Singh, M K., Reddy, G and Kohler, H (1978) Biochemistry 17, 1216–1222
14 Cabantchik, Z I. and Rothstein, A. (1974) J Membrane Biol 15, 227–248
15 Bennett, V. and Branton, D. (1977) J. Biol Chem. 252, 2753–2763
16 Knauf, P.A. and Rothstein, A. (1971) J. Gen Physiol. 58, 190–210
17 Sokolovsky, M. and Vallee, B.L (1966) Biochemistry 5, 3574–3581
18 Spies, J R and Chambers, D.C. (1949) Anal. Chem 21, 1249–1266
19 Uehara, K , Mannen, S. and Kishida, K (1970) J. Biochem 68, 119–124
20 Udenfriend, S., Stein, S., Bohlen, P., Dairman, W , Leimgruber, W and Weigele, M (1972) Science 178, 871–872
21 Jennings, M L and Passow, H (1979) Biochim. Biophys. Acta 554, 498–519
22 Fairbanks, G Steck, T L. and Wallach, D F.H (1971) Biochemistry, 10, 2606–2617
23 Swank, R T and Monkries, K D. (1971) Anal. Biochem 39, 462–477
24 Dubbelman, T M A R., De Goeij, A.F P.M. and Van Steveninck, J. (1978) Biochim. Biophys Acta 511, 141–151
25 Verweij, H., Dubbelman, T M.A R and Van Steveninck, J (1981) Biochim. Biophys. Acta 647, 87–94
26 Girotti, A W (1980) Biochim. Biophys Acta 602, 45–56
27 Yu, J and Steck, T L (1975) J. Biol Chem. 250, 9170–9175
28 Sandberg, S. and Romslo, I. (1981) Clin Chim. Acta 109, 193–201
29 Cabantchik, Z.I , Knauf, P.A and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
30 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1981) Biochim. Biophys Acta 641, 173–182