Biochimica et Biophysica Acta, 601 (1980) 220—227

BBA 78909

TEMPERATURE DEPENDENCE OF PHOTODYNAMIC RED CELL MEMBRANE DAMAGE

T.M.A.R. DUBBELMAN, C. HAASNOOT and J. VAN STEVENINCK

Laboratory for Medical Chemistry, Wassenaarseweg 72, Leiden (The Netherlands)

(Received March 7th, 1980)

Key words: Amino acid; Cation permeability; Membrane damage; Photo-oxidation; Protein cross-linking; Spectrin; (Red blood cell)

Summary

1. The protoporphyrin-sensitized photo-oxidation of free amino acids, amino acid residues in solubilized spectrin and amino acid residues in red blood cell membranes appeared to be virtually independent of temperature over the range 0—37°C. The photodynamically produced increase in cation permeability in intact cells was also almost temperature independent.

2. The photodynamic cross-linking of the membrane proteins, on the other hand, was clearly temperature dependent, both when the proteins were present in the membrane structure and when isolated and purified.

3. With red cell membranes, illuminated in the presence of protoporphyrin at 0°C, it could be shown that during subsequent incubation in the dark at 37°C the protein cross-linking increased considerably.

4. The results indicate that cross-linking of membrane proteins is a secondary reaction in which rather stable photo-oxidation products of susceptible amino acid residues are involved. Furthermore, these experiments strongly suggest that the deterioration of membrane function, leading to increased cation permeability, is caused by photo-oxidation of amino acid residues rather than by cross-linking of membrane proteins.

Introduction

Photoporphyrin-sensitized photo-oxidation of many compounds has been described and is believed to be caused by the formation of singlet oxygen via the light-excited dye molecule [1,2]. The photodynamic effect of protoporphyrin on the membrane of human red blood cells has many structural and functional consequences, like photo-oxidation of amino acid and unsaturated

fatty acid residues [3], inhibition of membrane-bound enzymes [4,5], mutilation of the ultrastructure as visualized by electron-microscopic studies [6], changes in cell deformability [7], inhibition of carrier-mediated transport [8], covalent cross-linking of membrane proteins [5,9,10] and increased passive permeability to cations, leading to photohemolysis [3]. The causal relationships between these phenomena have only been partially elucidated in recent studies. It could be shown that neither protein cross-linking nor photohemolysis were caused by lipid peroxidation [11]. Furthermore, there was no correlation between inhibition of several membrane-bound enzymes and photohemolysis [4]. The decrease in cell deformability could, most likely, be attributed to the photodynamic cross-linking of spectrin [7], whereas inhibition of anion transport and of membrane-bound glyceraldehyde-3-phosphate dehydrogenase could be ascribed to photo-oxidation of essential amino acid residues in band 3 and in band 6 protein, respectively, rather than to cross-linking of these proteins [5,8]. Finally, it is highly probable that photodynamic protein cross-linking is caused by a secondary reaction between photo-oxidized histidine residues and free amino groups [12].

As described previously, increased passive permeability to cations, leading to colloid-osmotic photohemolysis occurring after illumination of intact red blood cells in the presence of protoporphyrin, is caused by photodynamic effects on the membrane proteins [3]. It is not clear, however, whether this is the consequence of photo-oxidation of essential amino acid residues, or of the subsequent cross-linking of membrane proteins. An attempt was made to discriminate between these two possibilities by studying the temperature effects on K^+ leakage, photo-oxidation and cross-linking. The results of these studies are presented in this paper.

Methods

Erythrocytes were washed three times with phosphate-buffered isotonic NaCl. Ghosts were prepared by using the method of Weed et al. [13]. Spectrin was extracted as described by Bennett and Branton [14], purified by precipitation at pH 5.1 and dissolved in 50 mM phosphate buffer, pH 8.0.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. [15], after solubilization of the proteins in a solution containing 1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 40 mM dithiothreitol. The gels were always loaded with the same amount of protein. Enlarged photographs of the gels were scanned on a Chromoscan. Photography was performed in the linear part of the film-response curve. This curve was constructed by scanning photographs of a mask, taken at different exposure times.

Illuminations in the presence of protoporphyrin as sensitizer were carried out as described before [6]. Unless otherwise stated, the protoporphyrin concentration was 0.05 mM, at a ghost protein concentration of 2 mg/ml or a spectrin concentration of 1 mg/ml. Protein concentrations were measured according to the method of Lowry et al. [16]. K^+ leakage during illumination was measured in the medium with a flame photometer, after centrifugation of the cell suspension.

Histidine was determined according to the method of Sokolovsky and Vallee [17] sulfhydryl groups by using the method of Sedlak and Lindslay [18]. Lipid peroxidation was measured with the thiobarbituric acid method as described by Ottolenghi [19].

Oxygen consumption was measured manometrically, with differential respirometer units or with a Clark-type electrode, connected to a recording YSI oxygen monitor.

Cholesterol depletion of erythrocytes was accomplished by using the method described by Cooper et al. [20]. The cholesterol concentration in the membranes was measured as described by Zlatkis et al. [21], after extraction according to the method of Rose and Oklander [22]. Diamide treatment of ghosts was performed as described by Haest et al. [23].

Results

Illumination of a red blood cell suspension in the presence of protoporphyrin results in K^+ leakage and covalent cross-linking of membrane proteins. The temperature dependence of K^+ leakage is shown in Fig. 1. An apparent energy of activation of 2.5 kcal/mol was calculated from the experimental data.

The velocity of cross-linking of membrane proteins appeared to be clearly temperature dependent. Fig. 2 shows the cross-linking after an illumination period of 15 min at different temperatures, whereas Fig. 3 shows the decline of spectrin during illumination at different temperatures as measured by scanning of gel photographs. Band 3 protein is cross-linked with a lower velocity, but with a similar temperature dependence. From an Arrhenius plot of the experimental results an energy of activation of 11.3 kcal/mol could be calculated. The same results were obtained with purified, solubilized spectrin.

When ghosts were illuminated at 0°C and subsequently incubated in the dark at 37°C, protein cross-linking continued in the dark (Fig. 4).

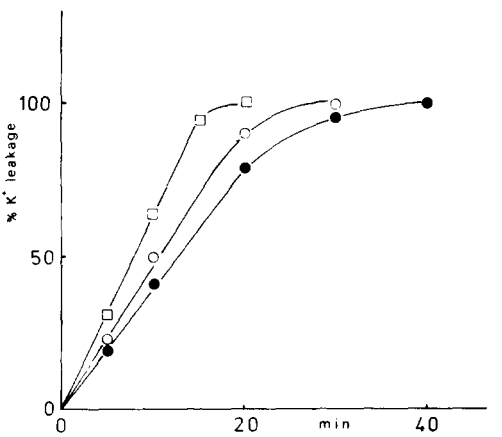

Fig. 1. K^+ leakage induced by illumination of erythrocytes with protoporphyrin at different temperatures. ●———●, 5°C; ○———○, 20°C; □———□, 37°C. Experimental conditions: 2% erythrocyte suspension; protoporphyrin concentration 1 μM.

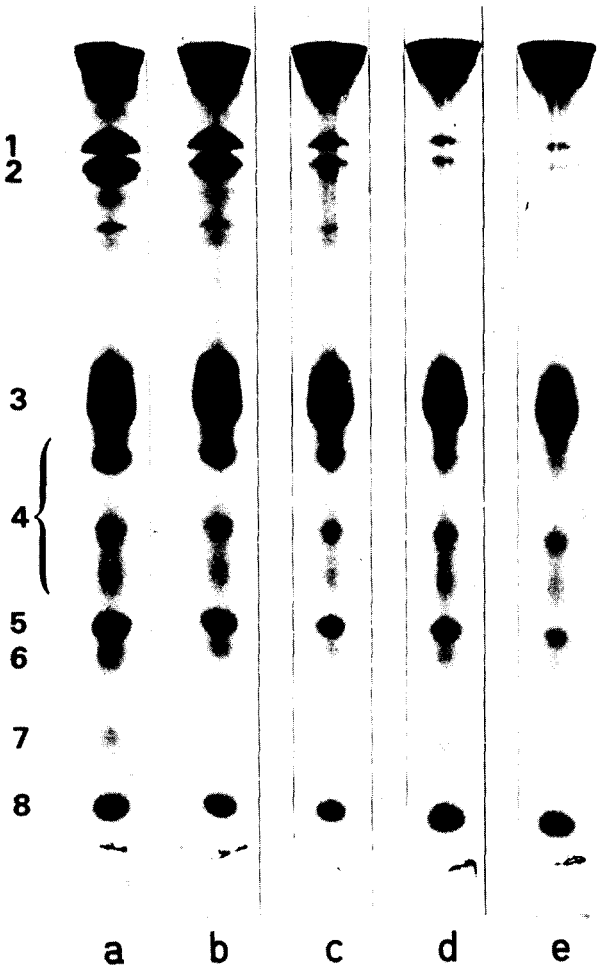

Fig. 2. Temperature dependence of photodynamic cross-linking of membrane proteins. Polyacrylamide gel electrophoresis of solubilized ghosts after 15 min illumination at: a, 0°C; b, 10°C; c, 20°C; d, 30°C; e, 37°C. For experimental conditions see Methods.

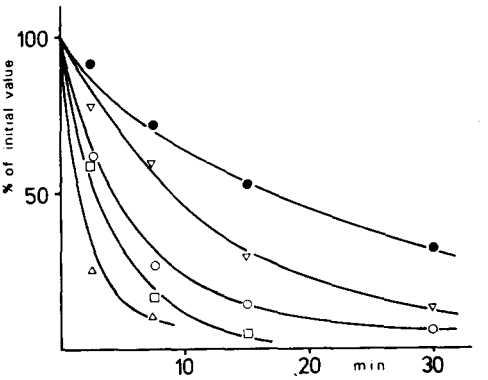

Fig. 3. Decrease of spectrin (bands 1 and 2) during illumination of ghosts in the presence of protoporphyrin at: ●———●, 0°C; ▽———▽, 10°C; ○———○, 20°C; □———□, 30°C; △———△, 37°C. For experimental conditions see Methods.

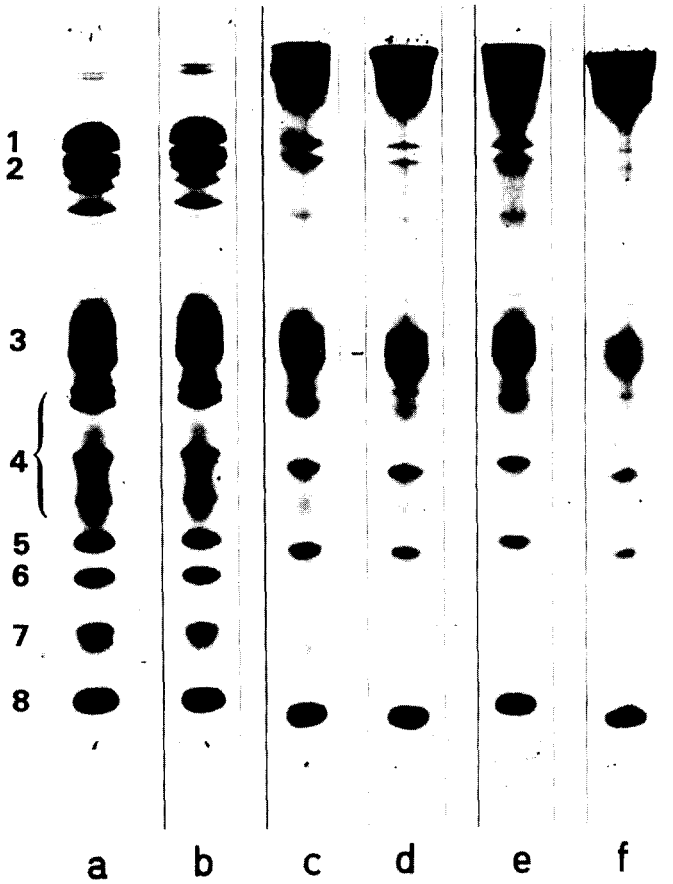

Fig. 4. Cross-linking of membrane proteins after illumination of ghosts in the presence of protoporphyrin. a, not illuminated, directly solubilized; b, not illuminated, solubilized after 20 min at 37°C in the dark; c, solubilized directly after 10 min of illumination at 0°C; d, illuminated as in c, solubilized after an additional 20 min at 37°C in the dark; e, solubilized directly after 20 min of illumination at 0°C; f, illuminated as in e, solubilized after an additional 20 min at 37°C in the dark. For experimental conditions see Methods.

In model experiments, photo-oxidation of histidine in solution was studied both by measuring oxygen consumption and histidine destruction. With both methods it appeared that photodynamic destruction of histidine in aqueous solution was temperature independent. On measuring SH- and histidine oxidation in ghost suspensions it appeared that SH-group oxidation was temperature independent, whereas photo-oxidation of histidine residues exhibited a very slight temperature dependence, with an energy of activation of about 2.0 kcal/mol.

During illumination of ghosts some lipid peroxidation occurred. This lipid peroxidation appeared to temperature dependent, with an energy of activation of about 4.8 kcal/mol (Fig. 5).

In order to estimate a possible effect of the lipid bilayer structure on membrane protein cross-linking, erythrocytes were depleted with respect to cholesterol. In a typical experiment 40% of the cholesterol was extracted, but the

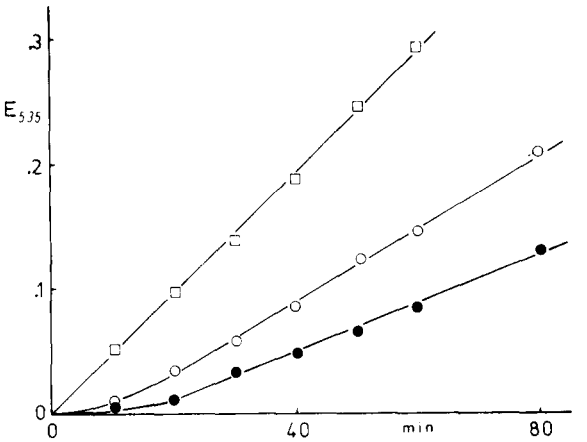

Fig. 5. Formation of malonaldehyde, expressed in E_{535} units, during photodynamic action of protoporphyrin upon ghosts at: ●———●, 0°C; ○———○, 20°C; □———□, 37°C. For experimental conditions see Methods.

sensitivity of the membrane proteins to cross-linking was not changed.

When protein mobility was restrained via S-S bridge formation by diamide treatment, photodynamic cross-linking was again unchanged.

Discussion

In photodynamic effects on biomembranes different steps can be distinguished. First the sensitizer (protoporphyrin) is excited by light. Subsequently, molecular oxygen reacts with the sensitizer in the excited state, yielding singlet oxygen and ground-state sensitizer. The singlet oxygen can react with target molecules in the membrane, leading to photo-oxidation of unsaturated fatty acids, cholesterol and amino acid residues and to protein cross-linking.

In general, the results presented are in accordance with the fact that photo-oxidation per se is temperature independent. The very limited influence of temperature on histidine oxidation in membrane proteins can be easily explained by temperature-induced conformational changes in the membrane proteins, rendering some histidine residues more accessible to singlet oxygen. It is well known that the conformation of, e.g., spectrin is very sensitive to temperature [24]. Also, the slight temperature dependence of peroxidation of unsaturated fatty acid residues in the lipid bilayer does not necessarily violate the general rule. With increasing temperature the lipid bilayer changes from a rigid gel into a fluid state, as reflected, e.g., by a strong decrease of the microviscosity of the membrane [25]. Therefore, it seems probable that the accessibility of unsaturated fatty acid residues in the lipid bilayer to singlet oxygen will increase with increasing temperature.

As shown in Fig. 4, the cross-linking reaction continued after cessation of photo-oxidation by placing the incubation mixture in the dark. Furthermore, the two processes exhibited quite different apparent energies of activation (approx. 11.3 kcal/mol and 0—2.0 kcal/mol, respectively). These results support our previous conclusion that cross-linking is not a primary photodynamic

reaction, but a secondary reaction between photo-oxidized amino acid residues and reactive groups in the membrane proteins [12].

Two factors could be involved in the temperature dependence of protein cross-linking: the activation energy of the chemical reaction and the restrained mobility of the proteins in the membrane structure, limiting the collision frequency of reactive groups. In this context the effect of increasing temperature could be ascribed to fluidization of the lipid bilayer, leading to enhanced mobility of the membrane proteins. This is contradicted, however, by the lack of effect of both cholesterol depletion (also leading to fluidization of the membrane [20]) and of reversible chemical cross-linking by diamide (restraining protein mobility). Moreover, the observation that the temperature effects on cross-linking of solubilized spectrin and of spectrin present in the membrane structure are equal also indicated that the temperature effect reflects the energy of activation of the chemical cross-linking reaction rather than the anchoring of the proteins in the membrane structure.

In previous papers it has been shown that the increased passive cation permeability of the membrane can not be attributed to peroxidation of unsaturated fatty acids or cholesterol, but must be ascribed to photodynamic effects on membrane proteins [11]. The energies of activation of K^+ leakage (2.5 kcal/mol) and of photo-oxidation of amino acid residues in membrane proteins (0—2.0 kcal/mol) are very close, whereas the activation energy of protein cross-linking is much higher (11.3 kcal/mol). This strongly suggests that the increased passive cation permeability is caused by photo-oxidation of certain essential amino acid residues in membrane proteins rather than by membrane protein cross-linking.

Acknowledgements

The investigations were supported by the Netherlands Foundation for Biophysics and the Foundation for Medical Research FUNGO, subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Spikes, J.D. and Livingston, R. (1969) Adv. Radiat. Biol. 3, 29—121
2 Spikes, J.D. (1975) Ann. N.Y. Acad. Sci. 244, 496—508
3 Schothorst, A.A., van Steveninck, J., Went, L.N. and Suurmond, D. (1972) Clin. Chim. Acta 39, 161—170
4 Schothorst, A.A., van Steveninck, J., Went, L.N. and Suurmond, D. (1971) Clin. Chim. Acta 33, 207—213
5 Dubbelman, T.M.A.R., de Goeij, A.F.P.M. and van Steveninck, J. (1978) Photochem. Photobiol. 28, 197—204
6 De Goeij, A.F.P.M., Ververgaert, P.H.J.T. and van Steveninck, J. (1975) Clin. Chim. Acta 62, 287—292
7 Dubbelman, T.M.A.R., de Bruijne, A.W. and van Steveninck, J. (1977) Biochem. Biophys. Res. Commun. 77, 811—817
8 Dubbelman, T.M.A.R., de Goeij, A.F.P.M. and van Steveninck, J. (1980) Biochim. Biophys. Acta 595, 133—139
9 De Goeij, A.F.P.M., van Straalen, R.J.C. and van Steveninck, J. (1976) Clin. Chim. Acta 71, 485—494
10 Girotti, A.W. (1979) Biochemistry 18, 4403—4411
11 De Goeij, A.F.P.M. and van Steveninck, J. (1976) Clin. Chim. Acta 68, 115—122
12 Dubbelman, T.M.A.R., de Goeij, A.F.P.M. and van Steveninck, J. (1978) Biochim. Biophys. Acta 511, 141—151

13 Weed, R.I., Reed, C.F. and Berg, G. (1963) J. Clin. Invest. 42, 581—588
14 Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753—2763
15 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Sokolovsky, M. and Vallee, B.L. (1966) Biochemistry 5, 3574—3581
18 Sedlak, J. and Lindsay, R.H. (1968) Anal. Biochem. 25, 192—205
19 Ottolenghi, A. (1959) Arch. Biochem. Biophys. 79, 355—363
20 Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M. and Shattil, S.J. (1978) Biochemistry 17, 327—331
21 Zlatkis, A., Zak, B. and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486—492
22 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428—431
23 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21—32
24 Ralston, G.B. and Dunbar, J.C. (1979) Biochim. Biophys. Acta 579, 20—30
25 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133—149