Biochimica et Biophysica Acta, 942 (1988) 333–340
Elsevier

BBA 74042

Direct involvement of spectrin thiols in maintaining erythrocyte membrane thermal stability and spectrin dimer self-association

Sara Streichman, Ety Hertz and Ilana Tatarsky

Department of Hematology, Rambam Medical Center and Faculty of Medicine, Technion-Israel Institute of Technology, Haifa (Israel)

(Received 22 December 1987)
(Revised manuscript received 8 April 1988)

Key words: Erythrocyte membrane; Thermal stability; Spectrin thiol; (Human blood)

Human erythrocytes vesiculate upon exposure to temperatures of 49 °C and above. Pretreatment of the cells with the thiol-alkylating agent *N*-ethylmaleimide (NEM) lowers the temperature needed to produce the same effect. Concomitant with the cells' heat susceptibility, skeletal mechanical instability and an increase in spectrin dissociation have been reported (Smith and Palek (1983) Blood 62, 1190). In the present study, similar results were achieved by preincubation of the cells with diamide, which could be reversed by reduction with dithiothreitol. Another oxidative agent, sodium tetrathionate, could only induce the temperature susceptibility, with little effect on spectrin dissociation. Incubation of spectrin solutions with NEM or diamide caused decreased association of spectrin dimers and increased dissociation of spectrin tetramers. Estimation of membrane and spectrin thiols in the treated cells showed that NEM was effective while blocking less than 20% of the thiols. Diamide and tetrathionate blocked more than 50% of the thiols, but were less effective than NEM. It is suggested that some very defined population of thiols is essential for spectrin self-association and for membrane thermal stability. They are more available to NEM than to diamide and less so to tetrathionate. Other thiols participate in maintaining the membrane thermal stability only.

Introduction

The major components of the erythrocyte skeleton are the spectrin heterodimers, associated head to head to form tetramers and higher oligomers. The result spectrin network, in combination with other proteins, comprises the erythrocyte skeleton, which seems to maintain the mechanical stability of the membrane and to control cell shape and deformability [1,2].

Any modification in one of the protein components may greatly perturb the mutual interactions of the membrane elements and lead to interference with its normal functions. One of the modifications frequently studied is the oxidation of the membrane thiols by different agents.

It has been shown that the oxidation induced by agents such as diamide and sodium tetrathionate is associated with the formation of disulfide bonds that cross link spectrin and other membrane proteins [3]. Consequently, these agents lead to membrane leakiness [4], membrane stiffness [5] and the abolishment of the phospholipid

Abbreviations: NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

Correspondence: S. Streichman, Department of Hematology, Rambam Medical Center, P.O. Box 9602, Haifa 31096, Israel.

asymmetry that normally exists in the erythrocyte membrane [6]. Since these effects could be prevented by prior blockage of the thiols with *N*-ethylmaleimide (NEM), it is concluded that it is not the diminishment of SH that accounts for the above phenomenon, but rather a protein cross-linking formation.

Recently it has been shown that some events do occur solely a a result of the blocking of SH groups: thermal instability of the treated red cells, skeletal mechanical fragility and the appearance of an increased percentage of spectrin dimers upon hypotonic extraction have all been reported to occur in human red cells pretreated with NEM [7]. A direct relationship between skeletal stability and percentage of spectrin dimers has already been established by Liu et al. [8]. However, it is less clear whether the increased susceptibility of the cells to thermal damage also depends on the extent of spectrin self-association. The involvement of spectrin thiols in skeletal interactions was further demonstrated recently by Becker et al. [9], who showed that mild oxidation of spectrin by diamide, in vitro, reduced its ability to bind actin via protein 4.1.

In the present study we investigated the participation of thiol groups in skeletal interactions by using different thiol reagents. It seemed to enable differentiating between spectrin thiols essential for self-association and others which are involved in membrane resistance to thermal damage.

Materials and Methods

Incubations. Erythrocytes (freshly drawn or 1-day refrigerated as whole blood: from healthy volunteers were washed three times in 10 mM phosphate-buffered saline (pH 7.4), and resuspended in the same medium to give a final 10% hematocrit. NEM, diamide or sodium tetrathionate at the indicated concentrations was added, and the erythrocyte suspensions were then incubated either for 1 h at room temperature (NEM, diamide) or for 90 min at 37 °C (tetrathionate). After incubation, the cells were washed. In some experiments, the cells were subsequently incubated for 1 h with 20 mM dithiothreitol at room temperature and washed again. The cells from the final wash were tested for thermal stability and subjected to spectrin analysis.

Heat treatment. The washed pretreated cells and controls were suspended in phosphate-buffered saline to 50–70% hematocrit, and aliquots of 100 µl were heated to 44–50 °C for 10 min. After 10 min at any of the above temperatures, the erythrocyte suspensions were cooled to 0 °C, and an equal volume of 1% glutaraldehyde in phosphate-buffered saline was added. The suspensions were gently mixed and incubated at room temperature for 15–20 min. Blood films were made, air dried, stained with Wright's stain and examined microscopically under oil immersion (magnification: 1000 ×).

Spectrin extraction. White ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate buffer (pH 8.0) including 0.12 mM phenylmethylsulfonyl fluoride (PMSF), according to the procedure of Dodge et al. [9]. Spectrin was extracted from these ghosts, either by overnight incubation at 0 °C with an equal volume of 0.1 mM sodium phosphate (pH 8.0) containing 0.1 mM EDTA and 0.12 mM PMSF or for 30 min at 37 °C with the same medium, following the procedure of Liu et al. [10], which yields spectrin tetramers and dimers, respectively. Calculation of spectrin extraction was based on the ratio of absorbance at 280 nm of the extracted spectrin to that of the relevant membranes.

Electrophoresis. Nondenaturing electrophoresis of the spectrin extracts was performed on 2% agarose according to Evans et al. [11], replacing Tris-Bicin as a running buffer with Tris-glycine.

Reassociation experiments. Reassociation of spectrin dimers to tetramers was performed according to Dhermy et al. [12], with some modifications. Briefly, 1 ml of spectrin dimers (about 500 µg/ml) extracted from untreated cells at 37 °C was dialyzed overnight at 0 °C against phosphate-buffered saline containing 0.1 mM EDTA and 0.12 mM PMSF. 200-µl aliquots were then incubated for 3 h at 30 °C in the absence or presence of 2 mM NEM or 10 mM diamide. Samples were then analyzed by agarose nondenaturing electrophoresis.

In vitro dissociation. Hypotonic solutions (about 500 µg/ml) of spectrin tetramers (extracted at 0 °C from untreated cells) were incubated for up

to 24 h at 0 °C in the presence of NEM or diamide. Samples were taken at recorded time intervals and analyzed by nondenaturing agarose electrophoresis.

Densitometry. Percentages of spectrin dimers and tetramers were estimated by densitometry (Profile Acrane, Sebia, France) of the nondenaturing agarose electrophoresis gels, taking the sum of dimers and tetramers as 100%.

Determination of thiols. The thiol groups of the membrane and the extracted crude spectrin tetramers were determined according to the method of Habeeb [13], using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as the thiol reagent. Usually 0.1 ml of ghosts dissolved in sodium dodecyl sulfate (SDS) or 0.5 ml of native spectrin was taken for analysis.

Results

To study the involvement of spectrin thiols in membrane thermal susceptibility and spectrin self-association, we compared the action of diamide and sodium tetrathionate, which act on thiols via oxidative mechanisms, to the reported action of NEM [7], which acts via alkylation of thiol groups.

Fig. 1 shows typical vesiculation and budding observed after 10 min at 46 °C of cells previously treated with either 2 mM NEM or 10 mM diamide (concentrations used in most experiments). As can be seen, NEM and diamide affected the cells similarly. Untreated erythrocytes showed the same pattern only when heated to 49 °C, behavior typical of normal erythrocytes.

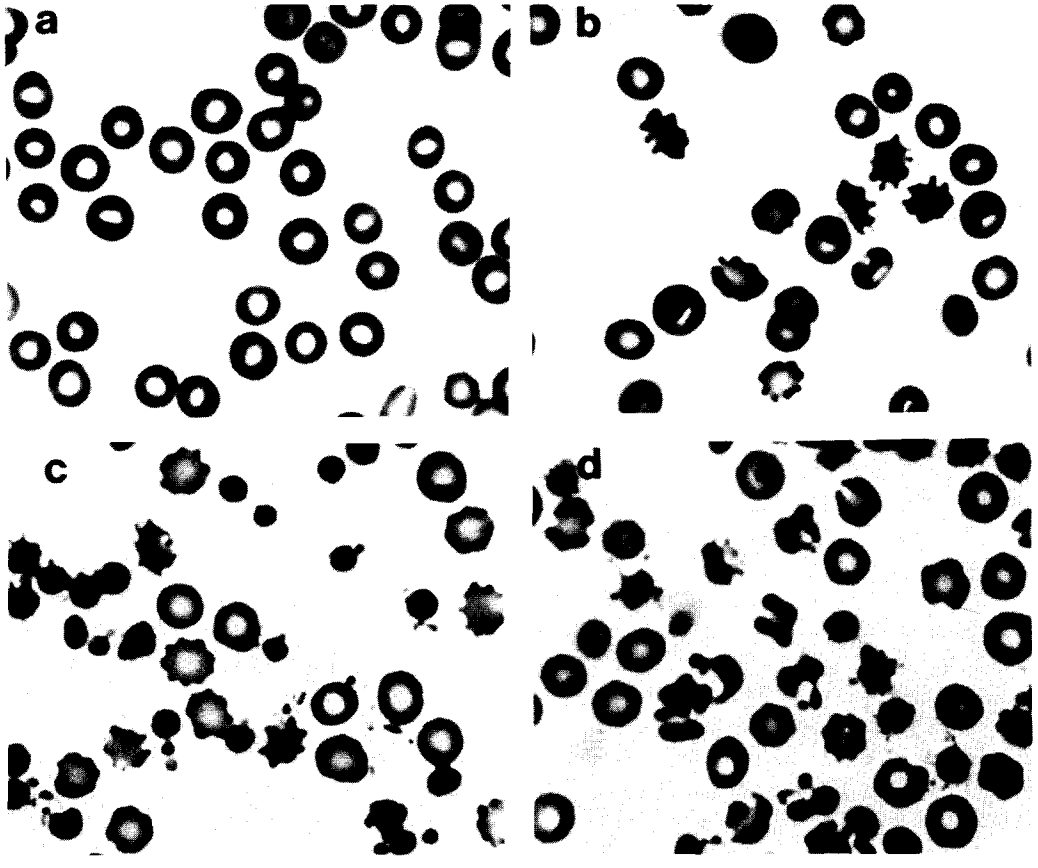

Fig. 1. Heat damage (10 min at 46 °C) caused by preincubation of cells (3 h at 0 °C) in the presence of no added reagents (a), 2 mM NEM (b), 10 mM diamide (c), and as in (a) but heated to 49 °C instead of 46 °C (d).

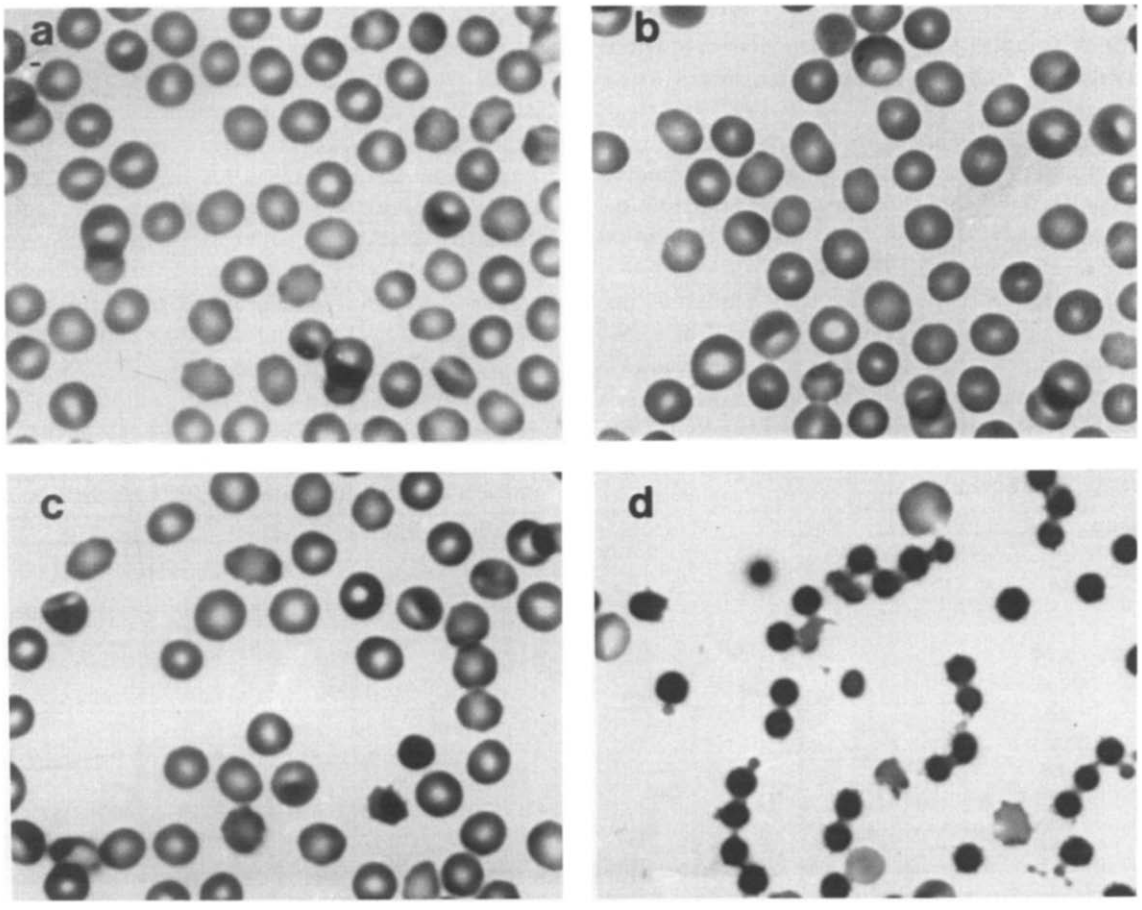

Fig. 2. Reversibility of the effect of diamide. Erythrocytes were preincubated at room temperature in the presence of 10 mM diamide for different periods of time, washed, further incubated in the presence of 20 mM dithiothreitol for 15 min at 37 °C, washed again and finally heated for 10 min at 46 °C. (a) Preincubation for 30 min; (b) for 90 min; (c) for 3 h; (d) as in (a) but without addition of dithiothreitol.

The induced changes caused by diamide were completely reversible by application of dithiothreitol to the treated cells (dithiothreitol did not reverse the NEM effect) before heating. The reversibility of the diamide effect was the same whether the incubation period with the oxidant was 30 min or 3 h (Fig. 2).

Nondenaturing electrophoresis of spectrin (Fig. 3) extracted from ghosts derived from the treated cells showed the expected dissociation to dimers induced by NEM [7] and a similar pattern caused by treatment with diamide. This pattern can be considered as representative of the in vivo situation, since the extent of extraction fo spectrin was normal and even enhanced in the NEM-treated

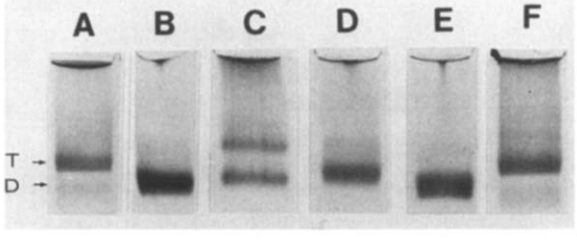

Fig. 3. Nondenaturing electrophoresis of spectrin extracted at 0 °C from the following pretreated (1 h at room temperature) erythrocytes: (A) untreated control; (B) with 2 mM NEM; (C) with 5 mM diamide; (D) with 10 mM diamide; (E) control extracted at 37 °C; (F) as in (D) but with 20 mM dithiothreitol following the diamide.

cells (unlike the results reported in Ref. 7) and 70–80% of the normal amounts in the case of diamide treatment (see Table III). The spectrin bands extracted from the diamide-treated cells were displaced from their normal position, probably because of the formation of intramolecular disulfide bonds, which may affect the Stokes' radius of the molecules [14]. The dissociation of spectrin to dimers originated mainly in the tetramers, while the amount of higher complexes of the spectrin remained relatively constant. That was already shown in the NEM-induced dissociation by Smith and Palek [7]. In our agarose nondenaturing electrophoresis method, the high-molecular-weight fraction of crude spectrin remains at the starting position of the agarose, and we did not quantitate it. Nevertheless, we could draw a similar conclusion, also, for the action of diamide, since the intensities of the tetramer and dimer bands of the spectrin, derived from the differently treated cells, were close to each other if equal amounts of spectrin were applied. As with the diamide-induced susceptibility to thermal damage, the dissociation of spectrin to its dimeric forms induced by diamide was also reversible if dithiothreitol was subsequently added to the diamide-treated cells. Nonreducing SDS-polyacrylamide gel electrophoresis of the spectrin extracted from the NEM-treated cells showed the normal alpha and beta bands. These bands appeared sharper than the control, possibly because of blockage of the thiols, which may have protected them from spontaneous oxidations. The spectrin derived from the diamide-treated cells appeared as diffuse staining of the gel and a significant reduction in the alpha and beta bands, indicating the production of interchain cross-linking (not shown).

TABLE I

THERMAL DAMAGE AND SPECTRIN DIMERS OF RED CELLS PRETREATED WITH SODIUM TETRATHIONATE

Sodium tetrathionate (mM)	Thermal damage			Spectrin dimers (%)
	25 °C	46 °C	47 °C	
0	–	–	–	6–8
2.5	–	+	+ + +	n.d.
6.5	–	+ + + [a]	+ + + +	n.d.
20.0	±	+ + + + [b]	+ + + +	12–13 [c]

[a] Vesiculation and budding equivalent to Figs. 1c and 1d.
[b] Vesiculation and budding equivalent to Fig. 2d.
[c] The extraction of crude spectrin from the tetrathionate-treated cells was about half the usual amount.

TABLE II

REASSOCIATION OF SPECTRIN DIMERS IN THE PRESENCE OF NEM OR DIAMIDE DURING IN VITRO INCUBATION FOR THREE HOURS AT 30 °C IN ISOTONIC SOLUTION

Spectrin	Before incubation (%)	After incubation (%)		
		Control	NEM (2 mmol/l)	Diamide (10 mmol/l)
Dimers	97	67	96	78
Tetramers	3	33	4	22

Another oxidative agent that was used, sodium tetrathionate, induced thermal susceptibility similar to diamide, but failed to induce significant spectrin dissociation, even at concentrations as high as five times the concentration needed to induce the increased sensitivity to heat (Table I). As noted in Table I, the extractability of spectrin was greatly inhibited by the tetrathionate pretreatment. Nevertheless, these results, which are relevant only to the extractable spectrin fraction, still indicate the prevalence of tetramers in the extracted spectrin.

The direct action of diamide and NEM on soluble spectrin in vitro was studied by incubation of the spectrin dimers (extracted at 37 °C from untreated cells) in isotonic medium at 30 °C. Usually these conditions induce reassociation of the dimers to tetramers. The inclusion of 2 mM NEM or 10 mM diamide in the medium (Table II) suppressed this ability, completely (NEM) or partially (diamide). The interaction of these two agents with sites which are essential for spectrin self-association is further demonstrated in Fig. 4. As can be seen, both diamide and NEM in the medium enhanced the dissociation rate of spectrin tetramers in a concentration- and time-dependent manner. While the only observed action of NEM in these experiments was the prevention of reassociation of dimers or induction of dissociation of tetramers, diamide also caused nonspecific di-

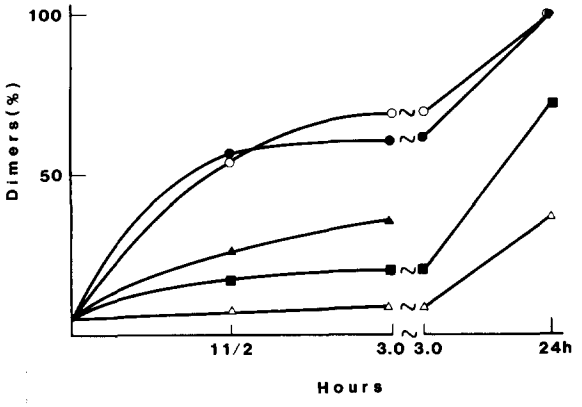

Fig. 4. Effect of NEM and diamide on spectrin dissociation during in vitro incubation at 0 °C. Key: △, control; ■, 5 mM diamide; ▲, 10 mM diamide; ●, 0.5 mM NEM, ○, 2 mM NEM.

sulfide cross-linkages in the spectrin, which appeared as diffuse staining of the agarose. When dithiothreitol was present in the medium, the action of diamide was prevented, including the diffuse staining. When 24 h incubation in the presence of 10 mM diamide was done, a considerable amount of the spectrin did not appear in the agarose, probably as a result of the formation of cross-linked higher complexes of spectrin.

The different behaviors obtained by the three thiol reagents, regarding their effect on thermal instability and spectrin self-association, suggested that they may act with different thiol populations. A preliminary attempt to differentiate between spectrin thiols essential for self-association and for thermal stability is shown in Table III. As seen, alkylation by NEM of 20%or less of the membrane and the spectrin thiols led to complete dissociation of the extractable spectrin concomitant with increased susceptibility of the cells to heat. The other two agents, which blocked 50% or more of the membrane and the spectrin SH, caused partial or insignificant spectrin dissociation. Our determination of native spectrin thiol number resembles that of others [7,9], but our values of thiol groups found in the membrane were consistently lower than previously reported [15,16]. Nevertheless, it is still reasonable to assume that these comparative analyses suggest the presence of at least two different thiol populations.

The low ionic extraction at 0 °C is believed to preserve the intracellular associations of spectrin [17], and since most of the normal amounts of spectrin were extracted following NEM and diamide treatments, we might assume that the above results reflect the in vivo consequences of the

TABLE III

BLOCKAGE OF MEMBRANE AND SPECTRIN THIOLS BY DIFFERENT REAGENTS

Agent (mM)	Thermal damage	Spectrin extractability (%)	Spectrin dimers (%)	Thiol blockage (%)	
				membrane	native spectrin
None	–	100	5–8	0 [a]	0 [b]
NEM					
0.5	+	96–100	100	5–20 [c]	2–22
1.0	+ + +	100–105	100	35–45	60–70
2.0	+ + + +	120	100	35	60
Diamide					
2.0	+	75–80	n.d.	55	55
5.0	+ + + +	73–78	58	45–50	65–75
10.0	+ + + +	67	100	50–60	60–75
Tetrathionate					
3.0	+	38–48	n.d.	35–40	30
6.5	+ + +	n.d.	n.d.	50	n.d.
20.0	+ + + +	40	13	50	n.d.

[a] Total membrane SH = 70.4 ± 4.6 nmol SH/mg protein ($n = 5$).

[b] Total crude spectrin SH = 51.5 ± 4.2 nmol SH/mg protein ($n = 5$), equivalent to 22.7 ± 1.8 SH/spectrin dimer.

[c] Most determinations were done two to three times.

action of the thiol agents. The remaining 20–30% unextracted spectrin in the diamide pretreatment and 60% in the tetrathionate treatment probably consist of higher cross-linked spectrin products, as was previously described when diamide was applied to human [3,5] or dog [18] red cells.

Discussion

The involvement of spectrin thiol groups in maintaining membrane resistance to thermal damage and in controlling self-association of dimers was suggested by Smith and Palek [7]. Their experiments were based on blocking the spectrin SH in the intact cells by the alkylating agent NEM. In the present study we were able to obtain similar results by applying an oxidative agent rather than an alkylating one. As can be seen in Fig. 1, both diamide, which was previously shown to induce thermal instability [19], and NEM caused enhanced vesiculation and budding of red cells upon heating. These two agents also induced spectrin dissociation (Fig. 3). The use of diamide enabled us to test the reversibility of the above effects: indeed, we could show that both the dissociation and the thermal susceptibility were completely reversible (Figs. 2 and 3). In the heat-treatment experiments, it became evident that the reversibility of the diamide action was independent of the time of exposure to this agent, provided a reduction with dithiothreitol followed. It seems, therefore, that the major event leading to the observed modification resides in the free thiols themselves, and any secondary conformational change that follows this event should be totally reversible and probably minor. Tetrathionate, which has been reported to oxidize erythrocyte thiols and to change permeability similarly to diamide [3,4], induced increased sensitivity of the cells to thermal damage, but failed to induce significant spectrin dissociation (Tables I and III).

Direct evidence for the involvement of the free thiols in the spectrin dimer self-association arises from our in vitro experiments. As shown in Table II, NEM entirely prevented and diamide partially suppressed the reassociation of spectrin dimers when added to the isotonic incubation medium at 30 °C. Moreover, these two agents enhanced the dissociation rate of spectrin tetramers when included in the hypotonic incubation solution of spectrin tetramers at 0 °C (Fig. 4). The action of diamide is probably more complex than that of NEM; it seems to involve the production of disulfide bonds, mainly within the spectrin dimers, either intra- or interchains. Intermolecular disulfide cross-linking of spectrin dimers in solution has been shown to depend both on high protein concentration and on high diamide concentration [9]. These results differ from those of Smith and Palek [7], who could not get dissociation of the solubilized spectrin tetramers by NEM. Becker et al. [9], who applied diamide to spectrin in solution, were also unable to get alteration in the spectrin self-association, but they used very low concentrations of the reagent (up to 0.1 mM) and found that only two to four thiols per molecule of spectrin were modified. We did not estimate the thiols in these experiments; but since we used much higher concentrations of diamide (5–10 mM), we probably blocked many more thiols in the spectrin molecule, including those which are essential for self-association.

As noted by Smith and Palek [7], the thermal sensitivity, the skeletal mechanical stability and spectrin dissociation all had the same NEM concentration dependence, indicating a causal relationship between all three phenomena. (A direct relationship between skeletal stability and spectrin dissociation was previously shown by Liu and Palek [20].) From our results, it seems more likely that the dissociation of spectrin to dimers and the change in the membrane thermal sensitivity are not directly related, since, unlike with NEM, the use of oxidizers of thiol groups (diamide and tetrathionate) showed that they could induce thermal instability to red cells with lower concentrations than needed to cause spectrin dissociation. Indeed, Ravindranath and Johnson [21] reported a case of congenital hemolytic anemia with altered spectrin association and membrane fragility without abnormal spectrin thermal sensitivity.

Moreover, direct estimation of thiols in the membranes of the treated cells and in the extractable spectrin derived from these cells showed that NEM could induce both thermal instability and spectrin dissociation at a concentration that blocked less than 20% of the total SH, while diamide and tetrathionate at concentrations that

were effective for thermal instability blocked more than 50% of the thiols in the membrane and in the extractable spectrin, but still caused only partial spectrin dissociation (Table III).

We may assume, thus, that the association of spectrin heterodimers depends on a defined group of thiols that react with decreasing affinities towards NEM, diamide and tetrathionate. In parallel, we also assume that the induction of thermal sensitivity of the red cells by the above reagents also depends on the abolishment of free thiols, but may be less specific regarding the population of the involved thiol groups. Alternatively, we could assume that the induction of thermal sensitivity by the above agents goes through different mechanisms: the oxidants form certain S-S bonds which lead to the increased thermal sensitivity, while the action of NEM depends on the blockage of certain thiols, those which are essential for spectrin dimer self-association. These two possibilities would explain the differences in the concentrations of diamide needed to cause spectrin dissociation or cell thermal sensitivity and also the action of sodium tetrathionate, which caused thermal sensitivity of the red cells, but did not induce significant spectrin dissociation. According to the two interpretations, tetrathionate fails to react with certain spectrin thiols which are directly involved in the association of spectrin dimers, but does act with other thiols whose abolishment or cross-linkage with adjacent thiols leads to the increased temperature sensitivity of the cells.

Understanding the role of thiols in the mechanism of erythrocyte thermal susceptibility may contribute to our understanding of the basis of the spontaneous molecular events leading to thermal fragmentation in cases of hereditary pyropoikilocytosis and some cases of hereditary elliptocytosis.

Acknowledgments

This work was supported by grants from the Mitchell-Copp Research Fund and the Technion V.P.R. Fund – L. Levin-Mandell Leukemia Research Fund. We thank Miss Ruth Singer for typing the manuscript.

References

1 Marchesi, V.T. (1979) J. Membr. Biol. 51, 101–131.
2 Mohandas, N., Chasis, J.A. and Shohet, S.B. (1983) Semin. Hematol. 20, 225–242.
3 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230.
4 Deuticke, B., Poster, B., Lutkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210.
5 Fischer, T.M., Haest, C.W.M., Stohr, M., Kamp, O. and Deuticke, B. (1978) Biochim. Biophys. Acta 510, 270–282.
6 Bergmann, W.L., Bressler, V., Heast, C.W.M., Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398.
7 Smith, D.K. and Palek, J. (1983) Blood 62, 1190–1196.
8 Liu, S.C., Palek, J., Prchal, J. and Castleberry, R.P. (1981) J. Clin. Invest. 68, 597–605.
9 Becker, P.S., Cohen, C.M. and Lux, S.E. (1986) J. Biol. Chem. 261, 4620–4628.
10 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
11 Evans, J.P.M., Baines, A.J., Hann, I.M., Al-Haim, I., Knowles, S.M. and Hoffbrand, A.V. (1983) Br. J. Haematol. 54, 163–172.
12 Dhermy, D., Lecomte, M.C., Garbarz, M., Bounier, O., Galand, C., Gautero, H., Feo, C., Alloisio, N., Delaunay, J. and Boivin, P. (1982) J. Clin. Invest. 70, 707–715.
13 Habeeb, A.F.S.A. (1972) Methods Enzymol. 25, 457–464.
14 Steck, T. (1972) J. Mol. Biol. 66, 295–305.
15 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
16 Haest, C.W.M., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326.
17 Liu, S.C., Windisch, P., Kim, S. and Palek, J. (1984) Cell 37, 587–594.
18 Johnson, G.J., Allen, D.W., Flynn, T.P., Finkel, B. and White, J.G. (1980) 66, 955–959.
19 Zarkowsky, H.S. (1982) Br. J. Haematol. 50, 361–365.
20 Liu, S.C. and Palek, J. (1980) Nature 285, 586–588.
21 Ravindranath, Y. and Johnson, R.M. (1985) Am. J. Hematol. 20, 53–65.