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SPIN LABELING OF HUMAN SPECTRIN

EFFECTS OF TEMPERATURE, DIVALENT CATIONS AND REASSOCIATION WITH ERYTHROCYTE MEMBRANE

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Summary

Spectrin extracted from human red blood cells has been spin labeled in its dimeric and tetrameric forms with five different nitroxide derivatives of increasing chain length between their maleimide binding group and their nitroxide reporter group. Three molecules of spin label are bound per spectrin dimer.

Electron spin resonance spectra show the simultaneous presence of strongly and weakly immobilized spin labels. Their relative proportion depends on the label length and is suddenly modified when it reaches 12 Å This indicates the presence of cavities of approximately this size in the tertiary structure of spectrin in solution at 0°C.

The conformation of spectrin varies greatly with temperature. Reversible changes occur between 0 and 35°C. At higher temperatures, partial denaturation is observed.

Divalent cations (Mg^{2+} and Ca^{2+}) stabilize spectrin in a more constrained conformation and protect it against thermal denaturation. The same behavior is observed when spin-labeled spectrin is reassociated with spectrin-depleted inside-out erythrocyte vesicles. When fatty acid spin labels are incorporated in the phospholipidic structure of these vesicles, the reassociation of spectrin does not change their electron spin resonance spectra. This result confirms the fact that spectrin interacts predominantly with proteins on erythrocyte membranes.

Introduction

Because of the role of spectrin in the properties of the erythrocyte membrane [1—6], it is particularly interesting to study the molecular aspects of the interactions of this peripheral protein with the inner surface of the membrane.

Details on the nature of these interactions cannot be properly considered without taking into account the physico-chemical properties of spectrin. The data now available on its conformational properties have been obtained by the use of different techniques, including hydrodynamic [7,8], light-scattering [9], electro-optical [10,11] and electron-microscopic measurements [12,13]. These data show that spectrin dimers are flexible and convoluted molecules of approx. 80—100 Å length. The equilibrium between purified spectrin dimers and tetramers has been recently documented [14]. Circular dichroism measurements have also given information on the thermal denaturation of spectrin [15]. However, very few data are available on the characterization and the reactivity of the residues present at the surface of the protein, except for attempts to identify some of the free thiols [16] and to map the binding sites of spectrin for the apolar heme molecule [17,18].

The absence of a specific chromophore on spectrin limits somewhat the experimental possibilities of studying this protein. The use of labeling techniques is thus appropriate, and we report in this paper the application of the well established nitroxide radical spin-labeling method [19] to the study of spectrin.

We have attached several nitroxide derivatives of maleimides to spectrin and have studied by using electron spin resonance (ESR) the mobility of the label in a large temperature range and under different ionic conditions. Information on the thermal denaturation and changes in conformation of spectrin have been obtained, as well as on the effects produced by divalent cations and recombination of spin-labeled spectrin with inside-out vesicles prepared from human erythrocytes.

Some of the results presented here have already been given in a preliminary report [20].

Materials and Methods

Preparation and spin labeling of spectrin. Freshly drawn human blood was washed six times with isotonic chloride solution. Ghosts were prepared from the packed cells by using the method of Dodge et al. [21]. Preparation of spectrin from the membranes was performed by classical methods [14,22]. The extraction was performed at low ionic strength at 35°C. This temperature was chosen in order to protect completely the spectrin from denaturation which begins to occur irreversibly above 35°C as this will be shown in Results. The extracted solution was then concentrated by ultrafiltration under vacuum, dialyzed against 10 mM Tris, 100 mM NaCl, 5 mM β-mercaptoethanol, pH 7, at 4°C and filtered in a cold room on a Sepharose 4B column equilibrated with the same buffer. The elution pattern was sensibly similar to the one given by Ungewickell and Gratzer [14]. Spectrin dimers (460 000 dalton) were collected, as well as the most aggregated form of spectrin which was eluted in the

void volume of the column. Both fractions were concentrated by ultrafiltration up to 15 mg/ml, dialyzed against 0.7 mM sodium phosphate, 20 mM KCl, pH 7.6, and centrifuged for 30 min at 145 000 × *g*. We discarded the gel of copolymerized spectrin, actin and protein 4.1 which was present at the bottom of the tubes when the more aggregated fraction was prepared. In this case, the supernatant was analyzed by sodium dodecyl sulface (SDS) polyacrylamide gel electrophoresis and by analytical ultracentrifugation and was shown to be pure spectrin in a tetrameric form. It was not possible to detect the presence of rapidly sedimenting material within the time necessary for the rotor to reach normal speed. Spectrin dimers and tetramers were stored at 2°C and used in the week following their preparation. Electrophoresis was frequently performed during storage in order to insure that degradation of the protein did not occur. In the experiments performed at above 30°C, spectrin tetramers dissociate partially into dimers [14]. In this case, they are quoted in the text as 'tetramers' to indicate the origin of the compound used without inference to the state of polymerization. Generally, before the EPR experiments, the samples were run for 30 min at 145 000 × *g* in a preparative ultracentrifuge.

Spectrin concentrations were measured spectrophotometrically on a Cary 118 spectrophotometer, using the value $A^{\%}_{280\ nm} = 10.1$ [23]. The molar concentrations were calculated as the amounts of dimers.

Phosphorus analysis was performed according to the method of Ames and Dubin [24].

Spin labeling of spectrin was performed in 50 mM phosphate buffer, pH 7, at 2°C, by incubating the protein 18 h with a 15-fold molar excess of the different spin labels. Unreacted maleimides were eliminated by an exhaustive dialysis against 0.7 mM sodium phosphate, 20 mM KCl, pH 7.6. The five spin labels used were purchased from Syva. Their formulae are shown in Fig. 1. Analytical ultracentrifugation of spin-labeled spectrin did not reveal any modification in its sedimentation coefficient.

Preparation of right-side-out and spectrin-depleted inside-out vesicles. Right-side-out vesicles were prepared from freshly washed and packed human erythrocytes according to the method of Steck [25]. The quality of the preparation was determined by measuring the NADH-cytochrome *c* oxidoreductase activity with and without saponin: the ratio of activities was greater than 50 [25].

Spectrin-depleted inside-out vesicles were prepared according to the method of Bennett and Branton [26]. The absence of spectrin was confirmed by SDS-polyacrylamide gel electrophoresis. The amount of membrane proteins was assayed by using the method of Lowry et al. [27]. Inside-out erythrocyte vesicles (8 mg protein per ml) were labeled with 5-doxyl and 16-doxyl stearic acids (Syva) by adding 1% (v/v) of a $5 \cdot 10^{-3}$ M stock solution of spin label in ethanol.

Reassociation of spectrin with erythrocyte membranes. Spectrin was reassociated with inside-out vesicles following the procedure of Bennett and Branton [26]. The mixture (300 μg spectrin per mg membrane protein) was incubated at 2°C at least 2 h in 0.7 mM sodium phosphate, 20 mM KCl, pH 7.6. It was then centrifuged (30 min at 30 000 × *g*) and washed twice with a large excess of the same buffer. The reassociation of spectrin to the membrane was con-

trolled by SDS-polyacrylamide gel electrophoresis. When spin-labeled spectrin was used in the recombination step, a further control of the quality of binding was made by measuring the ESR spectra of the reconstituted material.

Recording of ESR spectra. Spin-labeled spectrin and membrane samples were put in 100 μl quartz cells. The temperature was varied between 0 and 50°C and controlled to ±0.2°C with a laboratory-built regulation device. ESR spectra were recorded with a Varian E 3 spectrometer.

The number of spin labels bound on spectrin was measured as follows: labeled spectrin was denaturated in 6 M urea at 50°C for 30 min. The spectra were then recorded under overmodulation conditions (10 G). Known concentrations of maleimide nitroxides were analyzed under the same conditions. Double integration of both series of spectra was performed on an Intertechnique Multi 20 computer.

Results

ESR spectra of spin-labeled spectrin at 0° C

Dimers and tetramers of spectrin are easily labeled at 2°C with maleimide nitroxides and the number of spin labels bound (per dimer), determined by

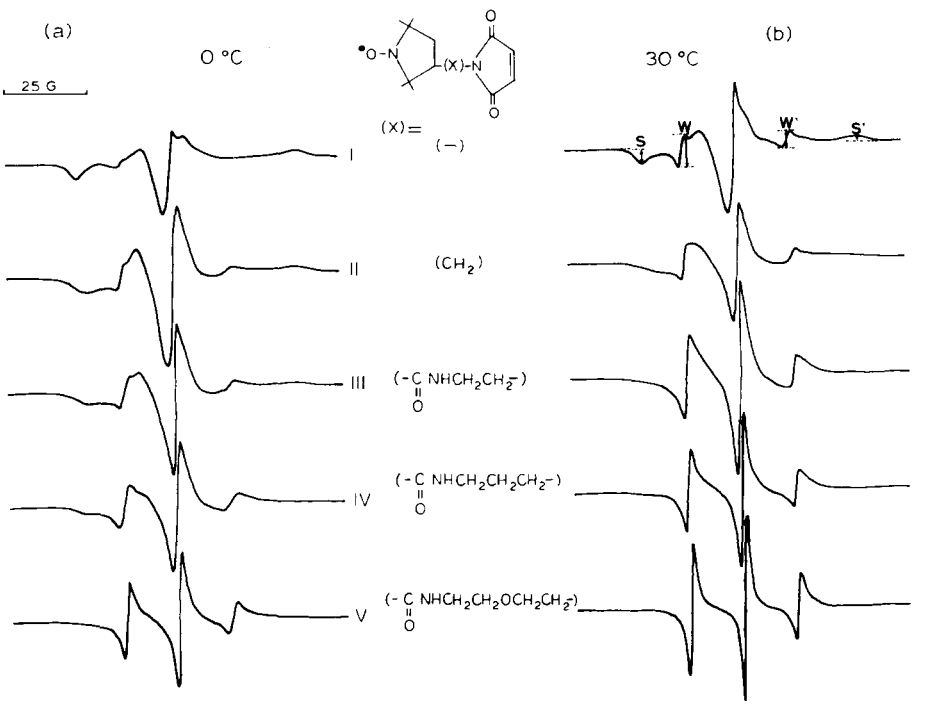

Fig. 1. ESR spectra recorded at 0 and 30° C of spectrin labeled with five nitroxide derivatives of maleimides of increasing length (compounds I to V). For clarity in the figure, the sizes of the spectra have been either increased or reduced. If the amplitude of the central line of spin label I spectrum at 0° C is taken as 1, the values for the other spectra are as follows. At 0° C, label II and III 1.2, label IV 3, label V 3.5; at 30° C, label I 1.1, label II 1.7, label III 2.4, label IV 5. label V 5.7.

double integration of the ESR spectra, was found to be 3 ± 0.3 in both forms, for each maleimide nitroxide derivative.

Fig. 1a shows the spectra obtained at 0°C with the five spin labels used. Their shapes vary considerably as a function of the spin label length. With spin label I, a characteristic spectrum of immobilized nitroxides is recorded, showing mainly broad lines (S and S′) separated by 66.7 G. With labels II and III, the broad lines are always the principal signal but one also observes sharper low-intensity lines (W and W′) with a coupling constant of 15.8 G. The definitions of these lines are given in Fig. 1b. With label IV, a large change in the shape of the spectrum is observed; the hyperfine constant of the S and S′ lines is measurable no more and the W and the W′ lines are now well resolved. Finally, with label V, the S lines are only observable as a slight deviatin of the baseline, and the W lines predominate, indicating that the largest part of the labels is weakly immobilized.

In order to characterize quantitatively each spectrum, we have measured the ratios of the amplitudes of the low-field broad line (S) and sharp line (W) (*W/S* ratio) and of the low- and high-field sharp lines (*W/W′* ratio) when possible. Table I summarizes the results of these measurements obtained at several temperatures with the five spin labels used.

Effects of temperature

Fig. 1b shows the same series of spectra recorded at 30°C. The W and W′ lines are now present on the spin label I spectrum and their amplitude is further increased with the other labels. The temperature effects on the spectra

TABLE I

VARIATIONS IN THE MAXIMAL HYPERFINE SPLITTING (SS′), THE RATIOS *W/S* AND *W/W′* OF SPIN-LABELED SPECTRIN, AS A FUNCTION OF TEMPERATURE AND OF THE LENGTH OF THE LABEL

n.m., not measurable.

Spin label	Temperature (°C)			
	0	20	30	40
Width between S and S′ lines (G)				
I	66.7	64.2	63.4	62
II	61	56.7	50.7	n.m.
III	59.5	n.m.	n.m.	n.m.
W/S				
I	0	1	1.5	5.2
II	1.9	2.9	3.4	6.4
III	3.7	8.7	14.2	18.5
IV	11.5	24.4	24.5	n.m.
V	n.m.	n.m.	n.m.	n.m.
W/W′				
I	n.m.	2.3	1.7	1.9
II	3.2	3	2.7	2.1
III	4.1	2.9	2.5	2.3
IV	2.8	2.4	2.2	1.9
V	2.5	2.1	1.9	1.7

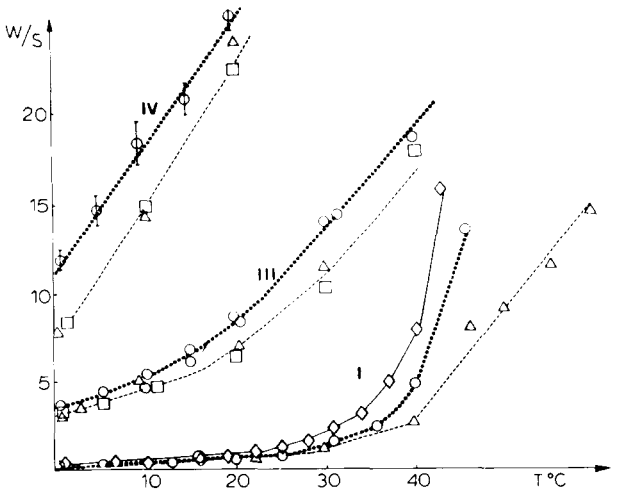

Fig. 2. Variations in *W/S* of spectrin labeled with compounds I, III and IV, as a function of temperature. Effect of Ca^{2+} and Mg^{2+}. ◇, spectrin dimers; ○, spectrin tetramers; □, spectrin in the presence of 4 mM Ca^{2+}; △, spectrin in the presence of 4 mM Mg^{2+}.

are represented in Fig. 2 where we have plotted *W/S* vs. temperature. With label I, this ratio increases linearly and weakly between 0 and 30°C, and above 40°C it rises very sharply. Similar behavior is observed with label II (curve not shown). For label III, the variations show an inflection near 20°C, for label IV, *W/S* is only measurable between 0 and 20°C and it is not measurable even at 0°C with label V. Slightly larger values of *W/S* are obtained above 37°C with label I for the spectrin dimers as compared to the tetramers.

The reversibility in the dependence upon temperature of the shape of the ESR spectra of spectrin was studied as follows. The spectrum of a spin-labeled sample of the protein was measured before and after it was allowed to stand for a given time at higher temperature. Between 0 and 35°C, the reversibility is complete for all the labels studied. Above 35°C it is only partial. When the temperature is decreased, the *W/S* values measured on label I are always higher than the ones measured initially for the native sample (Fig. 3a). This difference varies as a function of the time and temperature the protein has been left to stand. With label IV the reversibility, expressed by the variation of *W/W'*, seems to be obtained up to a slightly higher temperature (38°C) than with label I (Fig. 3b).

Effects of calcium and magnesium

Ca^{2+} and Mg^{2+} produce identical effects on the ESR spectra of spin-labeled spectrin. At a concentration of 4 mM, they decrease *W/S* in a way which depends on the label and on the temperature (Fig. 2). This is observable only above 30°C with spin label I and is considerable for the higher temperatures studied, since *W/S* is yet measurable at 60°C whereas in the absence of ions, above 45°C, the spectra show only sharp W and W' lines.

When spectrin is labeled with labels III and IV, the decrease in *W/S* induced by divalent cations begins to be observed at 10 and 0°C, respectively (Fig. 2).

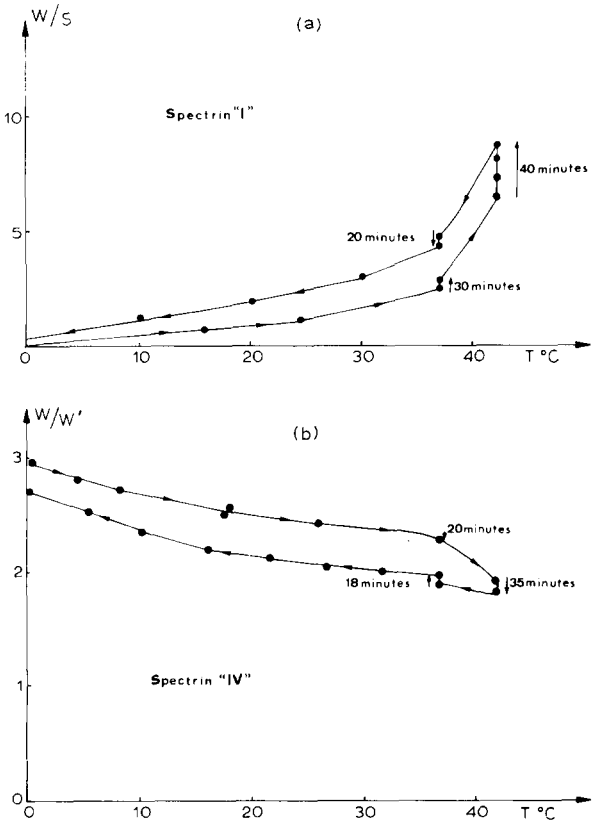

Fig. 3. Studies on the thermal denaturation of spectrin. Variations as a function of temperature and time, in the ratios W/S and W/W' of spectrin labeled, respectively, with compounds I and IV. The stability and reversibility in the shape of the EPR spectra were complete between 0 and 35°C. At 37°C, the labeled spectrin was allowed to stand for 30 min (a) or 20 min (b). The temperature was then increased up to 43°C and maintained constant for 40 min. The evolution of ratios W/S or W/W' is indicated in the figure. Thereafter, the temperature was decreased with the same time schedule. Spectra taken 24 h after return to 0°C showed no further variation.

Effects of the reassociation of spin-labeled spectrin with spectrin-depleted inside-out vesicles

Labeling spectrin with maleimide nitroxide derivatives does not prevent its reassociation with spectrin-depleted inside-out vesicles. This is clearly observable by means of SDS-polyacrylamide gel electrophoresis and the ESR spectra. As a matter of fact, exhaustively washed vesicles, after incubation with spin-labeled spectrin, give an ESR signal that corresponds to an amount of membrane-bound spectrin similar to the expected quantity estimated from the work of Bennett and Branton [26] with ^{32}P-labeled spectrin. The fact that this reassociation occurs exclusively with inside-out vesicles was demonstrated by verifying that no ESR signal was recorded with right-side-out vesicles incubated under similar conditions. The effects on the ESR spectra produced by the reassociation of spin-labeled spectrin with membranes are seen in Fig. 4. They are quite similar to the ones obtained with divalent ions and are the most

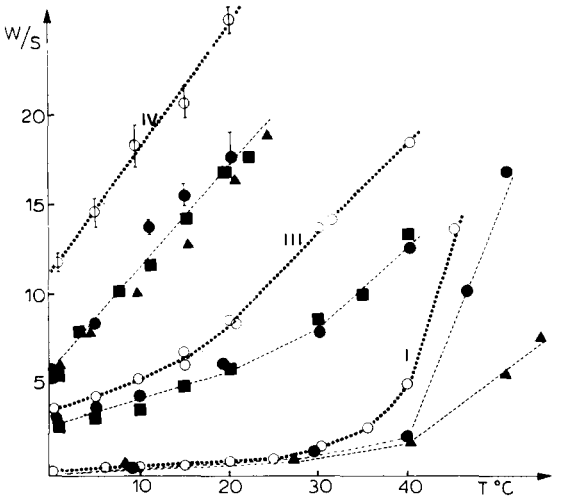

Fig. 4. Variations in the ratio *W/S* of spectrin labeled with compounds I, III and IV, as a function of temperature. Effect of spectrin reassociation with inside-out erythrocyte vesicles in the presence or absence of Ca^{2+} or Mg^{2+}. ○, spectrin tetramers in solution; ●, spectrin reassociated with inverted vesicles; ■, spectrin reassociated with inverted vesicles in the presence of 4 mM Ca^{2+}; ▲, spectrin reassociated with inverted vesicles in the presence of 4 mM Mg^{2+}.

clearly observed with label IV, since at 0 and 20°C the decrease in the *W/S* value is, respectively, 50 and 40%.

When Mg^{2+} or Ca^{2+} (4 mM) are added to inside-out vesicles reconstituted with labeled spectrin, no further decrease in *W/S* is observed with labels III and IV. With label I, however, above 40°C, the slope of the *W/S* vs. temperature plot is considerably decreased.

Fatty acid spin labeling of inside-out erythrocyte vesicles

ESR spectra of spectrin-depleted inside-out erythrocyte vesicles labeled with fatty acid spin probes were recorded as a function of temperature between 0 and 45°C before and after their reassociation with unlabeled spectrin. No significant difference was observed in the spectral parameters measured ($2T_{//}$ hyperfine splitting and order parameter with 5-doxyl stearate or rotation frequency of the label with 16-doxyl stearate).

Discussion

Number of spin labels bound on spectrin

Haest et al. [16] have previously demonstrated that three thiol groups of spectrin were labeled by radioactive *N*-ethylmaleimide. With the nitroxide maleimide we have used, we found the same number. It represents only a part of the 15 to 25 cysteine residues determined by the amino acid analysis of spectrin [28,29]. We have found the same number of spin labels bound by the 460 000 dalton unit of spectrin for the dimer as well as for the tetramer. This result agrees well with the electron-microscopic study of Shotton et al. [13] which showed that spectrin tetramers are formed by the 'head to head' association of two dimers. If tetramers in solution were to possess a more globular

structure, the number of reactive sulfhydryl groups could be decreased, some of them being buried in the area of association between both dimers.

Conformation of the spectrin molecule around the label binding sites

On the spectrum recorded with label I at 0°C, only wide lateral lines with a high coupling constant are observed, showing that the labels are strongly immobilized. This indicates that the three titratable sulfhydryl goups of spectrin are not exposed at the surface of the molecule, but buried inside folds of its tertiary structure. When the length of the label is increased (compounds II and III) the SS′ coupling constant decreases, whereas the sharp W and W′ lines appear and increase slightly. This shows that at least one of the labels begins to rotate in a less sterically hindered environment. As spin label III is changed for spin label IV, a drastic spectral modification is observed at 0°C. The SS′ coupling constant is no longer measurable, and the amplitude of the W line (Fig. 1a) as well as *W/S* increase greatly (Table I). Finally, these modifications are still more pronounced with label V.

The ratio *W/S* is an empirical means of evaluating the modifications of such composite spectra obtained on proteins [30–32]. A slight change of the S line amplitude implies a large variation in the amount of immobilized labels, since the double integration of this very wide line would show that it corresponds to a large amount of nitroxides. Furthermore, its shape varies considerably with the rotation correlation time of the label [19]. Thus, the important modifications of the spectral parameters observed when one exchanges label III for label IV, differing in length by only one $-CH_2-$ group (Fig. 1), are an indication of the existence of folding of the protein structure constituting some 'cavities' in which the labels are buried. The maximum depth of these cavities can be evaluated as being approx. 12 Å at 0°C, the length of spin label III based on a molecular model, but more probably is less due to the random configurations of the label structure.

Effect of temperature on spectrin conformation

When spectrin is labeled with maleimides I to IV, the W and W′ lines of the spectra, which are characteristic of mobile components, appear and increase reversibly when one raises the temperature from 0 to 35°C, (Fig. 2 and Table I). This effect stresses the peculiar behavior of spectrin of changing conformation in the vicinity of its thiol groups as a function of temperature. As a comparison, such a phenomenon does not exist with globular proteins such as hemoglobin or human serum albumin [33,34], which exhibit only very small changes in their ESR spectra when they are labeled with compound I. One can suggest that the cavities where the three maleimide spin labels are bound correspond more to weak foldings of the peptidic chain than to ordered and rigid structures.

Above 35°C spectrin becomes unstable. The strong increase in *W/S* observed with the short label I (Fig. 3) and the decrease in *W/W′* observed with the longer labels, both variations being partially irreversible (Fig. 3), indicate an unfolding of the protein, at least in the vicinity of its titrable thiol groups.

Our results are in agreement with the work of Brandts et al. [15] who observed, by means of circular dichroism, changes in the secondary structure

of spectrin with temperature. It is interesting to correlate the thermal denaturation of spectrin with the drastic and irreversible changes in the shape, elastic properties and deformability of red blood cells which have been observed after their heating in the same range of temperature [35].

Comparison between ESR spectra obtained with label I on spectrin dimers and tetramers in solution deserves some comments. Ungewickell and Gratzer [14] showed that the tetramer dissociates into dimers above 37°C and that the proportions of the two forms are governed by a simple thermodynamic equilibrium. Thus, one would expect to obtain similar EPR spectra for the two forms above this temperature. In fact, we observe that *W/S* of the tetramers is slightly but significantly lower than that of the dimers (Fig. 2), indicating that the two compounds are somewhat different. In this respect, we have found that if the dimer contains four phosphorus atoms per molecule, the tetramer always gives three to four more phosphorus atoms which can be extracted as phospholipids (Cassoly, R. and Wolf, C., unpublished results). This result could be a preliminary indication concerning the role of phospholipids in the conformation and the thermal stability of the spectrin molecule.

Effects of divalent cations

Between 0 and 35°C, the shorter labels I and II, which are deeply buried in the tertiary structure of the protein, are not responsive to the presence of the ions. On the contrary, the mobility of the longer labels is hindered (decrease in *W/S*, Fig. 2). As these labels, particularly label IV, are long enough to allow their nitroxide moiety to rotate relatively freely in the vicinity of the protein surface, one can infer that bridging of negatively charged amino acids by the divalent ions constrains the tertiary structure of the molecule. In relation to this, it must be recalled that spectrin contains a large amount of aspartic and glutamic acids [28,29].

Divalent cations protect spectrin against thermal denaturation as demonstrated with label I, which remains strongly immobilized up to 40°C. Furthermore, *W/S* increases much more slowly at higher temperatures than when cations are absent.

At the concentrations we have used (4 mM), no difference was observed between the effects of Ca^{2+} and Mg^{2+}. However, the observed effects are related to the divalent character of these ions, since no modification of the spectra of spin-labeled spectrin has been recorded when the cationic concentration of the solution was increased up to 150 mM with K^+ or Na^+. Observations have been made of multiple effects of divalent ions on the properties of the red blood cell membranes [2,25,36,37]. Our results suggest that these ions could have also a direct action on the spectrin conformation.

The effects exerted by divalent cations on the EPR spectra of spin-labeled spectrin are not related to protein aggregation, which has been shown to occur in low ionic strength crude extracts of ghosts in presence of Ca^{2+} [38]. In fact, it has been shown that these aggregating effects are not very reproducible and are even absent in highly purified spectrin [39]. Furthermore, we have taken care to incubate Ca^{2+} and spectrin just before the experiment and to subject the samples to high-speed centrifugation (see Materials and Methods).

In the case of Ca^{2+}, we have to be aware, however, that the normal intra-

cellular concentration is considerably smaller than that used in this work, but local microcompartimentalization of Ca^{2+} on the cytoplasmic side of the membrane cannot be excluded.

Effects of spectrin reassociation with erythrocyte membranes

The same spectral modifications as those previously discussed in the case of Ca^{2+} and Mg^{2+} are observed when spin-labeled spectrin is reassociated with spectrin-depleted inside-out erythrocyte vesicles. The same type of stabilizing effect is thus produced by this reassociation.

It has been shown that the structure of artificial phospholipid vesicles is modified in the presence of spectrin [40—43]. We were unable to confirm these findings when spectrin was reassociated with erythrocyte membranes labeled with spin label fatty acids. This shows that on the natural membrane, interactions between spectrin and phospholipids are not preponderant, and that spectrin is more specifically bound on membrane proteins, as indicated by numerous biochemical studies [26,44—49].

Furthermore, our results suggest an important role of divalent cations in this binding mechanism as can be shown by the further increase in protection against thermal denaturation they induce (Fig. 4). This finding can be correlated with the fact that Ca^{2+} and Mg^{2+} inhibit the dissociation of spectrin from ghosts even at low concentration [26,50].

In conclusion, the ESR measurements on spin-labeled spectrin presented in this work allow a qualitative description of the protein structure in the vicinity of the three thiol groups which are reactive toward maleimides. They give an insight on the reversible and irreversible changes in conformation of spectrin induced by temperature, divalent cations and reassociation with the innner surface of the erythrocyte membrane. They emphasize the preponderance of spectrin-protein interactions and the role of divalent cations in this association.

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