Biochimica et Biophysica Acta, 557 (1979) 363—371

BBA 78545

FORMATION OF DISULFIDE BONDS BETWEEN GLUTATHIONE AND MEMBRANE SH GROUPS IN HUMAN ERYTHROCYTES

C.W.M. HAEST, D. KAMP and B. DEUTICKE

Abt. Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, D-5100 Aachen (F.R.G.)

(Received February 2nd, 1979)

Key words: Membrane protein; Glutathione; Disulfide bond; Spectrin; (Erythrocyte)

Summary

In erythrocytes treated with the SH-oxidizing agent, diamide, mixed disulfide bonds between membrane proteins and GSH are formed involving 20% of the membrane SH groups. To study the distribution of these mixed disulfides over the membrane protein fractions, intracellular GSH was labelled biosynthetically with [2-^{3}H]glycine prior to diamide treatment of the cells and the radioactivity of defined membrane peptide fractions determined. Mixed disulfides preferentially occur in the extrinsic protein, spectrin (six SH groups), in addition to the formation of peptide disulfides. Intrinsic proteins are much less reactive: only one SH group of the major intrinsic protein (band 3) reacts with GSH, which accounts for the previously observed impossibility to dimerize band 3 via disulfide bonds in intact cells. The labelling method described offers a promising strategy to label and map exposed endofacial SH groups of membrane proteins with a physiological, impermeable marker, GSH.

In ghosts treated with diamide and GSH the number of mixed disulfides formed is greater than in erythrocytes. Polymerization of spectrin via intermolecular disulfide bridges is suppressed, while intramolecular disulfides are still formed, providing a means for the analysis of spectrin structure.

The diamide-induced mixed membrane-GSH disulfides are readily reduced by GSH. This suggests, that GSH may also be able to reduce mixed disulfides formed in the erythrocyte membrane under oxidative stress in vivo. The reversible formation of mixed disulfides may serve to protect sensitive membrane structures against irreversible oxidative damage.

Introduction

The presence of disulfide bonds between intracellular glutathione (GSH) and the SH groups of cytoplasmic and membrane proteins as well as their formation

under oxidative stress have been demonstrated in cells of various tissues [1—6]. The formation of mixed disulfide bonds is readily reversible after removal of the oxidative stress and upon supply of reducing equivalents. The physiological significance of such redox cycles, however, is unknown. It has been suggested that they serve to protect more vulnerable protein components against irreversible damage by oxidation [7].

In erythrocytes, the SH-oxidizing agent diazinedicarboxylic acid bis(*N,N'*-dimethylamide) (diamide) has been shown to induce mixed disulfide bonds between hemoglobin and GSH [2]. Whether and to what extent such bonds are also formed between SH groups of membrane proteins and GSH is hitherto unknown. Recently, we obtained evidence [7] for a diamide-induced disulfide bonding of GSH to the major intrinsic membrane protein (band 3, according to Steck [8]). The aim of the present work was to study the formation of mixed disulfide bonds and their distribution over both, intrinsic and extrinsic proteins of the human erythrocyte membrane. Such studies seemed of particular interest, since we had previously shown that as much as 80% of the membrane SH groups can be oxidized by diamide to disulfide bonds within the same or between two adjacent peptide chains [7].

Methods

Erythrocytes from freshly collected, heparinized human blood or from blood incubated for 16 h at 37°C with [2-^{3}H]glycine (500 Ci/mol) to label the intracellular GSH (see below) were washed three times with 154 mM NaCl. 1 vol. of the cells was then suspended in 10 vols. of a medium containing 90 mM KCl, 45 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4 and 44 mM sucrose (medium A) and exposed to 5 mM diamide (Calbiochem), for various periods of time (37°C, pH 7.4).

Determination of cytoplasmic glutathione. Diamide-treated and control cells were washed with cold medium A and hemolysed with 7 vols. of H_2O. The GSH content of the hemolysate was measured according to [9]. Oxidized glutathione (GSSG) was quantified by determination of the increase of SH groups in an ultrafiltrate (Amicon, PM 10 filter) of the hemolysate, caused by a reductive treatment (0.1 vol. 12.5% $NaBH_4$, 30 min, 37°C) of the ultrafiltrate, followed by destruction of excess $NaBH_4$ [5] with 0.3 vol. of trichloroacetic acid (40%, w/v) and neutralisation with 4 N NaOH.

Determination of mixed disulfide bonds between membrane proteins and GSH. Diamide-treated erythrocytes were washed three times with cold medium A and ghosts prepared according to [10]. Disulfide bonds between protein and GSH were cleaved by treating 1 ml of packed white ghosts, containing 3—5 mg of protein, determined according to [11], with 0.1 ml of a 12.5% aqueous solution of $NaBH_4$ containing 5 mM EDTA. The protein was then precipitated with 0.2 ml of a 40% solution of trichloroacetic acid [5]. After centrifugation 0.5 ml of 0.5 M Na_2HPO_4 were added to 1 ml of the supernatant, the pH adjusted to 7.4 with 4 N NaOH and SH groups quantified with 0.5 ml of a 1 mM solution of 5,5'-dithio-bis(2-nitrobenzoic acid) in 1% sodium citrate [9]. After 10 min the absorbance at 412 nm was read against an appropriate blank.

Alternatively, membrane-bound GSH was estimated from the binding of labelled intracellular GSH to the membrane in the presence of diamide. GSH was labelled in vitro, following the procedure of [12], by incubation (16 h, 37°C) of blood with [2-^{3}H]glycine, which is biosynthetically incorporated into GSH. The amount of bound GSH was calculated from the radioactivity bound to the membranes, isolated from the cells after extensive washing, and the specific activity of GSH. The specific activity of GSH was calculated from the amount of GSH and the radioactivity in a lysate of the cells, corrected for the contribution of [2-^{3}H]glycine. To obtain this contribution, the distribution of the radioactivity between free [2-^{3}H]glycine and labelled GSH was determined by thin-layer chromatography. To this aim the hemolysate of cells not treated with diamide was freed of protein by the addition of trichloroacetic acid and centrifugation. The supernatant was subjected to chromatography on silica plates (Merck, Darmstadt, No. 5715) using a mixture of propanol-2 and 25% NH_3 (65 : 35, v/v). The spots containing glycine (R_F 0.14) and GSH (R_F 0.57), detected with ninhydrin, were scraped from the plate, and extracted with 0.8 ml of H_2O. The supernatant after centrifugation was mixed with 5 ml scintillator (Quickscint 212, Zinsser, Frankfurt) and counted for radioactivity.

Results and Discussion

Formation of mixed disulfide bonds between membrane proteins and GSH

During incubation of erythrocytes with 5 mM diamide, a highly permeable SH oxidant [13], intracellular GSH rapidly disappears (Fig. 1A) while oxidized glutathione, GSSG, accumulates. In addition, the sum of GSH and GSSG diminishes within 30 min to about 30% of the total amount of glutathione originally present in the cells (Fig. 1A). Concomitantly, about 3% of the GSH become bound to membrane protein. This amount is also found by measuring the amount of radioactive GSH, bound to ghosts derived from diamide-treated, [2-^{3}H]glycine-loaded cells. Due to the binding of GSH, free membrane SH groups decrease by about 15 nmol/mg protein as shown in Fig. 1B (upper curve). The total decrease of membrane SH groups (Fig. 1B, lower curve), however, exceeds the number of SH groups blocked by GSH. The difference (indicated by cross-hatched arrows) can be explained by the formation of intra- and intermolecular disulfide bonds [7] in proteins. The major part of the decrease of the sum of GSH + GSSG (about 70%) is due to binding of GSH to cytoplasmic proteins, in particular hemoglobin, as previously demonstrated by Srivastava et al. [2]. Our results agree fairly well with his data in that up to 0.25 mol of GSH/mol of hemoglobin tetramer are bound upon a treatment of erythrocytes with diamide.

In order to elucidate whether the GSH bound to the membrane is distributed evenly among all membrane proteins or exhibits selectivity of binding, we localized the mixed disulfide bonds to peptide fractions, as defined by SDS gel electrophoresis, by means of GSH labelled biosynthetically with [2-^{3}H]glycine. The number of molecules of GSH bound per copy of a peptide fraction was calculated from (1) the radioactivity and the protein content of the gel section containing a complete defined band; (2) the apparent molecular weight of the fraction [15,16], and (3) the specific activity of GSH (see Methods). From

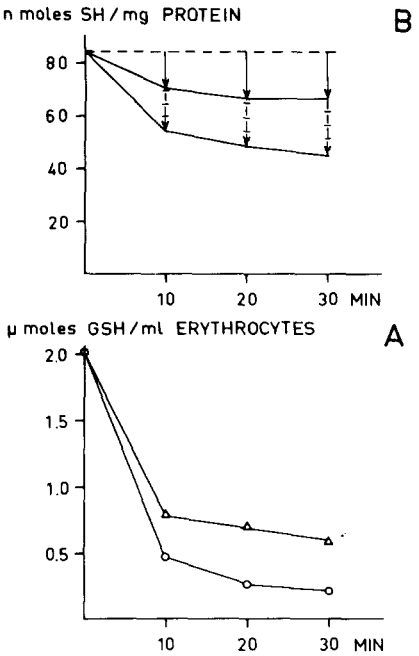

Fig. 1. Time dependency of the oxidation of GSH to GSSG (A) and of membrane SH groups (B). Erythrocytes were treated with diamide for various times, washed and hemolysed. GSH, GSSG and free membrane SH groups were quantified as described in Methods. Mixed disulfide bonds between membrane proteins and GSH were quantified by measuring the release of GSH after reductive treatment of ghosts with $NaBH_4$ (see Methods). (A) ○, GSH; △, GSH + GSSG. (B) The arrows represent the decrease of membrane SH groups due to the formation of disulfide bonds between peptide chains (cross-hatched) and due to mixed disulfide formation with GSH (continuous arrows).

Table I it becomes evident that in the intact erythrocyte GSH is bound predominantly to bands 1 and 2, i.e. monomeric spectrin and to band $(1 + 2)_n$, the polymer of spectrin formed by intermolecular cross-linking via S-S bonds in the presence of diamide [7]. As much as six molecules of GSH (24 nmol GSH/mg spectrin) react with one spectrin monomer, the major extrinsic membrane protein, while only one GSH becomes bound per monomer of band 3, the major intrinsic membrane protein. Even less is bound to other protein fractions. It can be concluded therefore from these calculations that in intact erythrocytes 30% of the 21 SH groups present per spectrin monomer [17] readily react with GSH. In contrast, only 15%, (one out of six [20]) of the SH groups in the intrinsic protein band 3, less than 4% (0.1 out of three [21]) in the extrinsic protein band 6 and 4% (0.2 out of five [22]) in the extrinsic protein band 5 (actin), are reactive. In this latter calculation it is assumed that erythrocyte actin and muscle actin have the same number of SH groups. In view of the impermeability of the membrane towards GSH, the one SH group of band 3 accessible to GSH can be assumed to be located at the cytoplasmic surface of the membrane.

The formation of a mixed disulfide bond between GSH and band 3 confirms our previous suggestion [7] that binding of GSH to band 3 prevents the dimerization of band 3 via disulfide bonds in diamide-treated erythrocytes.

TABLE I

DISTRIBUTION OF RADIOACTIVITY OF ^{3}H-LABELLED GSH AMONG THE MEMBRANE PROTEINS OF ERYTHROCYTES TREATED WITH DIAMIDE

The intracellular GSH of erythrocytes was labelled with [2-^{3}H]glycine (500 Ci/mol) and the cells treated with diamide (5 mM, 30 min, 37°C, pH 7.4). Ghosts prepared from the cells were solubilized in 2% SDS and subjected to gel electrophoresis [14]. Radioactivity of separate bands was determined by slicing of the gel according to the Coomassie blue staining profile as described previously [14]. The results of one typical experiment out of a series of 3 are shown below. Percent of total radioactivity are given in parentheses.

Band number	Radioactivity (cpm of band)	Number of labelled SH groups/protein molecule
$(1+2)_n$ } $1+2$	854 (59.7)	6
3	285 (19.9)	0.9
4.1	48 (3.4)	0.5
4.2	78 (5.5)	0.6
4.5	96 (6.7)	0.4
5	44 (3.1)	0.2
6	19 (1.3)	<0.1
7	6 (0.4)	<0.1

This suggestion was based on the observation that, in contrast to fresh erythrocytes, band 3 can be dimerized in erythrocytes after blockage of GSH by iodoacetate, and in ghosts. In complementation of these previous results we could now demonstrate that in leaky ghosts the dimerization of band 3 and even the polymerization of spectrin by diamide (20 mM) are almost completely inhibited by 0.5 mM GSH (Fig. 2). This finding is readily explained by the formation of mixed disulfide bonds between GSH and SH groups of spectrin, which prevents the involvement of these SH groups in cross-linking of spectrin via disulfide bonds. The reason for this different behavior of intact cells and ghosts, in spite of the presence of GSH in both systems, is not clear. The absence of hemoglobin, competing with membrane proteins for GSH, can only in part be responsible, since it only binds 70% of the cellular GSH, leaving a residual concentration of 0.6 mM GSH, which is essentially the concentration present in the ghost system. Alternatively, a structural rearrangement of spectrin in the ghost membrane, exposing SH groups to GSH, might be considered.

Since mixed disulfide bonds, which prevent cross-linking of spectrin, are formed in ghosts in the presence of GSH, but not in erythrocytes, it would seem likely that in ghosts the number of diamide-induced mixed disulfide bonds between membrane SH groups and GSH exceeds that formed in erythrocytes. Indeed, in the presence of 0.5 mM GSH the number of mixed disulfide bonds between membrane SH groups and GSH in ghosts treated with diamide amounts to 27.5 ± 1.9 (S.D.; $n = 4$) nmol/mg protein. This value is significantly higher than the number of membrane mixed disulfides formed in the native cells under comparable conditions (13.9 ± 1.9 (S.D.; $n = 8$), cf. also Fig. 1). Expressed as percent of the total number of SH groups, these values correspond to 30 and 15%, respectively. Increasing the concentration of GSH to 2 mM GSH only slightly enhances the percentage of ghost membrane SH

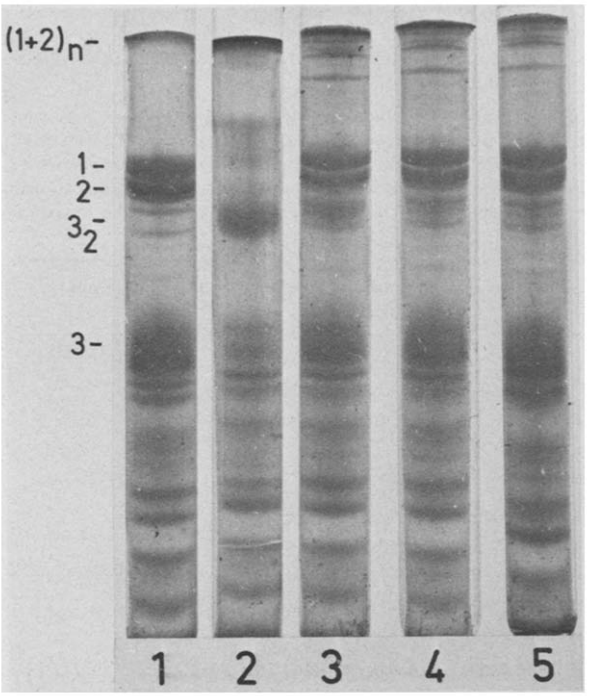

Fig. 2. Protein profiles of ghosts incubated with diamide in the presence of GSH. Ghosts were incubated with 20 mM diamide (37°C, pH 7.4) for 15 min in the absence or presence of 0.5—2 mM GSH, washed three times, solubilized in SDS (2% final concentration) and subjected to gel electrophoresis [14]. 1, control; 2, diamide; 3, GSH (0.5 mM) + diamide; 4, GSH (1.0 mM) + diamide; 5, GSH (2.0 mM) + diamide. 1, 2 and $(1 + 2)_n$, spectrin monomer and oligomer respectively; 3, and 3_2, band 3 monomer and dimer respectively.

groups that bind GSH (Table II), while at high levels of GSH (up to 10 mM) much higher values (up to 60%) were obtained. No clearcut correlation, however, between the concentrations of GSH and the number of mixed disulfides could be established at these high concentrations, for reasons unclear as yet. Interestingly, cysteine is much less potent in building up mixed disulfide bonds (Table II).

Considering that 20% of the membrane SH groups are not reactive or accessible to oxidative agents at all [7] one can conclude from these findings that in ghosts at 0.5 mM GSH only 50% (100% — 20% — 30%) of the SH groups are engaged in disulfide bonds between peptide chains, in contrast to 65% (100% — 20% — 15%) in whole cells under our experimental conditions. Interestingly, formation of intermolecular disulfide bonds in spectrin is suppressed in these ghosts as indicated by a lack of cross-linking revealed by gel electrophoresis (cf. Fig. 2). According to preliminary results, the total number of oxidized SH groups in spectrin, however, exceeds the number of mixed disulfides formed. The difference must be due to formation of intramolecular disulfide bonds between cysteinyl residues within the same peptide chain.

Thus, in erythrocyte membranes three classes of membrane SH groups can be defined: one class that in the presence of diamide builds up mixed disulfides

TABLE II

FORMATION BY DIAMIDE OF MIXED DISULFIDE BONDS BETWEEN MEMBRANE PROTEINS AND GSH IN GHOSTS

Ghosts (10) were treated with diamide (20 mM, pH 7.4, 37°C) and GSH or cysteine for 15 min, washed three times and the mixed disulfide bonds quantified (see Methods) either directly or after solubilization in 2% SDS and three ultrafiltrations (Amicon, filter PM 10) in order to remove residual non-covalently bound GSH. Results of one typical experiment out of a series of 2—4 are shown below. Membrane SH groups were determined as described before [14].

Thiol	Concentration (mM)	Exposure (min)	Mixed disulfide bonds (nmol/mg protein)		Membrane SH groups remaining (nmol/mg protein; % of control in parentheses)
			Before ultra-filtration	After ultra-filtration	
None	0	0	0	—	88.7 (100)
None	0	15	0	—	15.8 (18)
GSH	0.5	15	29	27	18.3 (21)
GSH	2	15	32	35	17.4 (20)
Cysteine	5	30	8	—	—
Cysteine	10	30	10	—	—

with GSH, a second class forming inter- and intrapeptide disulfide bonds and a third one not reacting at all, even upon an extensive treatment with diamide [7]. The SH groups in the third class amount to about 20% of the membrane SH groups [7]. These SH groups may be assumed to be buried deeply within the hydrophobic core of the membrane. Accordingly, preliminary data show that these SH groups are located in intrinsic proteins.

The ratio of the number of SH groups in the first and the second class is variable and depends on the nature and concentration of the non-protein thiol as well as on the presence or absence of hemoglobin. Those SH groups which build up mixed disulfide bonds already in the presence of very low concentrations of a hydrophilic thiol are probably located in extrinsic proteins (cf. Table I) or in hydrophilic domains of intrinsic proteins, e.g. band 3.

The formation of mixed disulfide bonds between GSH and membrane proteins offers a promising method to label and map exposed SH groups of membrane proteins with a physiological marker, GSH. Furthermore, it can be helpful to localize SH groups within the protein structure by studying their reactivity towards non-protein thiols varying in charge and molecular size. The possibility to suppress intermolecular disulfide formation in spectrin (in ghosts treated with diamide in the presence of GSH), while maintaining intramolecular disulfide formation may become useful in studies on the folding of the peptide chains in this major extrinsic protein of the erythrocyte membrane.

Reduction of mixed disulfide bonds

In a further series of experiments we studied the reduction of the mixed disulfide bonds. From Table III it becomes evident that these bonds cannot be reduced by glutathione reductase even in the presence of catalytic amounts of GSH or GSSG. The mixed disulfide bonds are reduced, however, by sulfite and GSH (2 mM). This finding agrees with the observations [18,19] that GSH can

TABLE III

REDUCTIVE CLEAVAGE OF MIXED DISULFIDE BONDS BETWEEN MEMBRANE PROTEINS AND GSH

Erythrocytes, whose intracellular GSH was labelled with [2-^{3}H]glycine, were treated with diamide (5 mM, 30 min, 37°C, pH 7.4). After three washings, ghosts were prepared from the cells and incubated with 10 vols. of buffer containing one of the reducing agents listed below (60 min, 37°C, pH 7.4) and the radioactivity released into the supernatant measured by liquid scintillation counting. The total number of mixed disulfide bonds amounted to 12 nmol/mg protein. The results of one typical experiments out of a series of 3 are shown below.

Reductive treatment	% of mixed disulfides cleaved
No addition	0
GSH (2 mM)	27
GSH (10 mM)	78
NADPH (2 mM)	0
NADPH + glutathione reductase (10—100 I.U./ml)	0 *
Na_2SO_3 (20 mM)	85

* In the absence or presence of 0.2 μM GSH or GSSG.

also reduce mixed disulfide bonds between GSH and the SH group β-93 of hemoglobin, and indicates that the equilibrium of the reaction: protein-SH + GSSG ⇌ protein-S-SG + GSH is in favor of the reduced form of the protein SH groups. This assumption is also supported by experiments showing that at low concentrations of GSSG a minor reaction with membrane SH groups occurs detectable only by the formation of GSH. At higher concentrations of GSSG (50 mM) a measurable decrease of membrane SH groups is observed. This decrease corresponds to the increase of GSH in the medium, as has to be expected from the equilibrium shown above (Table IV).

Our data support the concept that intracellular GSH may protect sensitive structures against irreversible damage by oxidative stress via the formation of mixed disulfides between proteins and GSH. Upon removal of the oxidative stress these mixed disulfide bonds are readily reduced by intracellular GSH, maintained at high levels by glutathione reductase in the metabolizing erythrocyte. This protective mechanism of a formation of mixed disulfide bonds is

TABLE IV

REACTION OF GSSG WITH SH GROUPS OF MEMBRANE PROTEINS

1 vol. of ghosts (10) were incubated with 1 vol. of 12.5 mM phosphate buffer containing GSSG (60 min, pH 7.4, 37°C). The reaction of GSSG with membrane SH groups was quantified in two different ways: (a) Measurement of the amount of GSH [9] in the supernatant after centrifugation of the ghosts. (b) Determination of membrane SH groups [14] after three washings of the ghosts. Mean values (± S.D.) of three experiments.

Concentration of GSSG (mM)	GSH formed (nmol/mg membrane protein)	Change of membrane SH groups (nmol/mg membrane protein)
0	0	0
4	4.3 ± 0.3	+1.4 ± 0.7
8	5.9 ± 0.3	—2.0 ± 2.9
50	9.4 ± 2.6	—6.8 ± 0.2

probably operative in addition to the formation of intra- and intermolecular disulfides in membrane proteins, since these latter types of disulfide bonds are also reversible by GSH (unpublished results).

Acknowledgements

We wish to thank Mrs. M. Brendt and Mrs. R. Schäfer for secretarial help in preparing the manuscript and Mr. F.J. Kaiser for drawing the figures. This study was supported by the Deutsche Forschungsgemeinschaft (SFB 160).

References

1 Allen, D.W. and Jandl, J.H. (1961) J. Clin. Invest. 40, 454—475
2 Srivastava, S.K., Awasthi, Y.C. and Beutler, E. (1974) Biochem. J. 139, 289—295
3 Harrap, K.R., Jackson, R.C., Riches, P.G., Smith, C.A. and Hill, B.T. (1973) Biochim. Biophys. Acta 310, 104—110
4 Modig, H. (1968) Biochem. Pharmacol. 17, 177—186
5 Delucia, A.J., Mustafa, M.G., Hussain, M.Z. and Cross, C.E. (1975) J. Clin. Invest. 55, 794—802
6 Isaacs, J. and Binkley, F. (1977) Biochim. Biophys. Acta 497, 192—204
7 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226—230
8 Steck, T.L. (1972) J. Mol. Biol. 66, 295—305
9 Beutler, E., Duron, D. and Mikus Kelly, B. (1963) J. Lab. Clin. Med. 61, 882—888
10 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Hochberg, A., Rigbi, M. and Dimant, E. (1964) Biochim. Biophys. Acta 90, 464—471
13 Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969) Biochem. Biophys. Res. Commun. 37, 593—596
14 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21—32
15 Knüfermann, H., Bhakdi, S. and Wallach, D.F.H. (1975) Biochim. Biophys. Acta 389, 464—476
16 Steck, T.L. (1974) J. Cell Biol. 62, 1—19
17 Fuller, G.M., Boughter, J.M. and Movazzani, M. (1974) Biochemistry 13, 3036—3041
18 Birchmeier, W., Tuchschmid, P.E. and Winterhalter, K.H. (1973) Biochemistry 12, 3667—3672
19 Kosower, N.S., Kosower, E.M. and Koppel, R.L. (1977) Eur. J. Biochem. 77, 529—534
20 Steck, T.L., Koziarz, J.J., Singh, M.K., Reddy, G. and Köhler, H. (1978) Biochemistry 17, 1216—1222
21 Carraway, K.L. and Shin, B.C. (1972) J. Biol. Chem. 247, 2102—2108
22 Elzinga, M. (1970) Biochemistry 9, 1365—1374