Biochimica et Biophysica Acta, 692 (1982) 339–344
Elsevier Biomedical Press

BBA 71381

MICELLAR GANGLIOSIDES MEDIATE THE LIPID INSERTION OF CHOLERA TOXIN PROTOMER A

M. TOMASI [a], G. D'AGNOLO [a] and C. MONTECUCCO [b]

[a] *Laboratorio di Biologia Cellulare e Immunologia, Istituto superiore di sanità, Viale Regina Elena 299, 00161 Roma and* [b] *Centro C.N.R. per la Fisiologia dei Mitocondri e Laboratorio di Biologia e Patologia Molecolare, Istituto di Patologia Generale, Università di Padova, Via Loredan 16, Padova (Italy)*

(Received June 18th, 1982)

Key words: Cholera toxin; Ganglioside; Micelle; Detergent; Photolabeling

The topology of the interaction of cholera toxin with ganglioside and detergent micelles was studied with the technique of hydrophobic photolabelling. Cholera toxin α and γ polypeptide chains appear to penetrate into the hydrophobic core of ganglioside micelles. Micelles of SDS cause the labelling also of the β polypeptide chains, while Triton X-100 micelles have little ability to mediate the labelling of the toxin. The specific reduction of the α-γ disulfide bond allows the penetration of the α polypeptide chain into Triton X-100 micelles, but does not affect the interaction of cholera toxin with either ganglioside or SDS micelles. Thus, ganglioside micelles appear to cause a conformational change of the native toxin, such as to induce the penetration of the α chain into the micelle hydrophobic core.

Introduction

Several intoxication phenomena involve the crossing of the plasma membrane by a protein toxic subunit [1]. The trigger of the process is considered to be the binding of the toxin to the saccharide component, either of a glycoprotein or of a glycolipid, on the external surface of the cell membrane. However, the molecular steps which allow the water-soluble toxin to cross the lipid barrier are poorly understood.

On the basis of nuclear magnetic resonance and electron microscopy studies, Cullis and De Kruijff [2] have suggested that membrane processes such as fusion and transport may be mediated by a transient destabilization of the lipid bilayer structure. Freeze-facture experiments have shown that within a membrane there are microenvironments organized not in a bilayer but in a micellar-like structure [3]. Among natural lipids the gangliosides appear to be good candidates for the formation of non-bilayer structures because of their large hydrophilic moiety [4,5]. This is reflected by the formation in water solutions of micelles of 250–450 kDa at concentrations lower than 10^{-6} M [6,7]. Furthermore, even though the two hydrocarbon chains of gangliosides allow their insertion into the membrane, ganglioside concentrations higher than 12–16% with respect to total lipids are incompatible with a bilayer organization [8]. Thus, if gangliosides reach in selected regions of a biological membrane a critical concentration the bilayer may be destabilized. In fact, it has been shown that a modification of membrane fluidity is detectable when gangliosides are somehow segre-

Abbreviations: G_{M1}, galactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide; azidoglycolipid, 12-amino-*N*(2-nitro-4-azidophenyl)dodecanoyl[6-^{3}H]glucosamine; SDS, sodium dodecyl sulfate.

gated in the membrane [9]. In this respect, Kohn et al. [10] have advanced the idea that gangliosides, acting as emulsifying agents, could allow specific water-soluble proteins to interact with the lipid bilayer.

As a matter of fact, gangliosides do show emulsifying properties when they interact in micellar physical form with bovine serum albumin [7,11]. As other charged amphiphiles [12], they induce a drastic conformational change in the albumin structure. However, a major difference was found between gangliosides and other amphiphiles, i.e., the interaction with bovine serum albumin appears to occur via a direct fusion of one molecule of protein with one micelle of ganglioside as such, containing 340–350 monomers [6]. Thus gangliosides, if present in micellar-like form on the membrane, may provide a suitable mean for the entrapment and transport of water-soluble proteins across the lipid bilayer.

At present a wealth of information is available on the specificity and the dynamics of the interaction of a water-soluble protein like cholera toxin and its membrane receptor, the ganglioside G_{M1} [13–16]. It is generally accepted that the oligosaccharide moiety of the glycolipid contains the determinants for toxin binding. However, while most of the attention has been focused on the carbohydrate moiety of G_{M1}, some authors [17,18] have also suggested a role for the lipid portion of the ganglioside in mediating the membrane events necessary to the action of cholera toxin. Therefore, it seemed of interest to investigate a simple model made up of ganglioside micelles and cholera toxin in order to verify if ganglioside micelles can exert a hydrophobic effect on the protein, thus modulating the membrane-toxin interactions. For this purpose, we have taken advantage of the hydrophobic photolabelling technique, which consists in the specific radioactive labelling of those polypeptide chains exposed to the lipid hydrophobic milieu [19,20].

Cholera toxin is a multisubunit protein composed of three polypeptide chains, α, β and γ, with molecular formula $\alpha\gamma\beta_5$. Responsible for the binding to the cells are the five identical β polypeptide chains, forming protomer B. After the binding, a series of unknown events lead the α chain to penetrate the lipid bilayer and to modify enzymatically a regulatory protein of the adenylate cyclase system [21]. In the native toxin the α chain is linked through a disulfide bridge to the γ chain, thus forming protomer A. Protometers A and B are non-covalently bound and can be dissociated only under denaturing conditions. In the present paper we show that micellar gangliosides are able to mediate the hydrophobic labelling of the α and γ polypeptide chains. This specific labelling of definite regions of cholera toxin is due to the ability of the gangliosides to interact as micelles with water-soluble proteins.

Materials and Methods

Cholera toxin was a generous gift of Dr. J.L. Tayot (Institute Merieux, France); for some experiments it was also isolated as described by Tomasi et al. [22]. Gangliosides G_{M1} and G_{D1a}, purified according to Tettamanti et al. [23], had the physicochemical characteristics described by Corti et al. [6] and were a generous gift of Prof. G. Tettamanti, Istituto di Chimica Biologica, Università di Milano. The photoreactive probe 12-amino-*N*-2-nitro-4-azidophenyl) dodecanoyl-[6-^{3}H]glucosamine, with a specific radioactivity of 38 Ci/mmol, was prepared as described by Bramhall et al. [24]. All operations involving the photoreactive glycolipid were performed under a red safety light.

Preparation of tagged micelles of amphiphiles. Micelles tagged with the azidoglycolipid were prepared by mixing in chloroform/methanol (2:1, v/v) 150 μg ganglioside and 1 μl of an ethanol solution containing 1.2 μCi of the tritium-labelled azidoglycolipid. The solutions were taken to dryness and the tagged micelles were formed by resuspending the lipid mixture in 0.1 ml Tris-HCl buffer (pH 8.2)/0.002 M EDTA. The tagged micelles of gangliosides were allowed to stand at 20°C for at least 1 day before use. Tagged micelles of SDS and Triton X-100 were prepared by mixing 2 mg SDS or 1 mg Triton X-100, respectively, with 1 μl of the azidoglycolipid in ethanol. After drying, the tagged micelles were formed as described above.

Binding of cholera toxin to micelles of amphiphiles. Cholera toxin (0.24 nmol) was incubated for 1 h at 37°C unless otherwise indicated with 0.1 ml suspension of the tagged micelles.

When necessary, aliquots were withdrawn, at the end of the incubation, and reduced by adding 2 μmol of reduced glutathione and further incubating at 37°C for 30 min. The reduced and unreduced samples were then dialyzed overnight against 0.1 M Tris-HCl buffer (pH 8.2)/0.002 M EDTA. After dialysis, the samples were irradiated with a 100 W ultraviolet lamp as described [20]. The protein, precipitated by the addition of 0.7 ml of cold acetone, was collected by centrifugation in a microfuge. The pellets were washed twice with cold chloroform/methanol (2:1, v/v) in order to remove the gangliosides and, after drying under a nitrogen stream, were analyzed by SDS-polyacrylamide disc gel electrophoresis according to Swank and Munkres [25]. The gels were stained and analyzed as previously reported [20].

Results

Cholera toxin labelling with ganglioside micelles

Fig. 1A shows the densitometric profile and Fig. 1B the labelling pattern of cholera toxin incubated for 1 h at 37°C with tagged micelles of ganglioside G_{M1}. The radioactivity appears mainly incorporated into the α and γ polypeptide chains which compose the protomer A. This hydrophobic labelling of protomer A can be mediated either by the specific interaction of protomer B with the oligosaccharide portion of G_{M1} or by an intrinsic ability of ganglioside micelles to induce the penetration of the α and γ chains in the lipid core. Therefore, we tested a different ganglioside, G_{D1a}, which possesses a carbohydrate moiety unable to interact with cholera toxin [26]. Fig. 1C shows the pattern of labelling obtained with micelles of G_{D1a}. Also in this case protomer A incorporates all the radioactivity associated with the toxin, thus indicating that G_{D1a} micelles are at least as efficient as G_{M1} micelles in mediating hydrophobic interactions with protomer A.

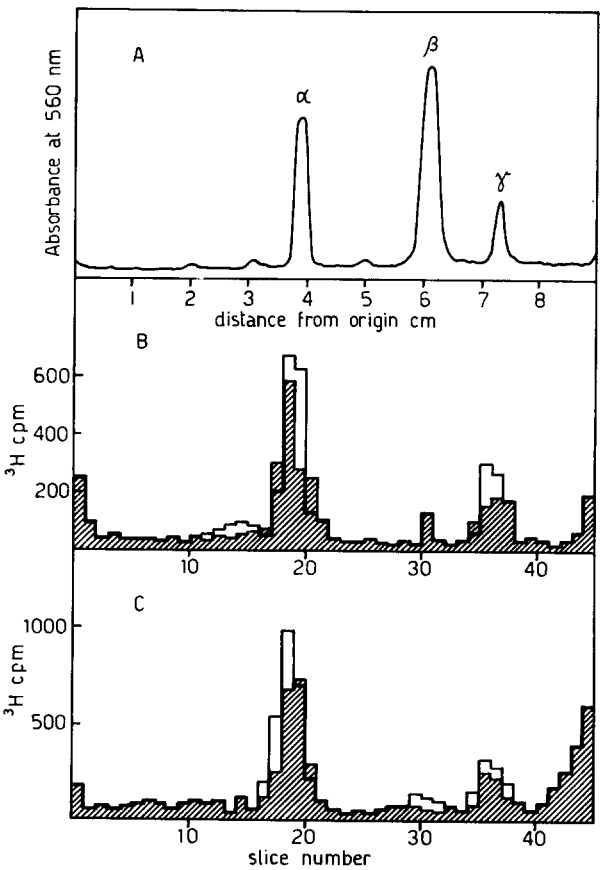

Fig. 1. Labelling of cholera toxin bound to ganglioside micelles tagged with the azidoglycolipid. (A) Densitometric scan of a Coomassie blue-stained gel of the toxin after labelling and electrophoresis. (B) and (C) Histograms of the radioactivity associated with cholera toxin subunits after incubation with G_{M1} and G_{D1a} micelles, respectively. Experimental details are indicated in the text. The data relative to the unreduced and reduced (shaded area) samples are shown.

Temperature effect on cholera toxin labelling

Fig. 2 shows that the extent of the interaction between protomer A and the micelle hydrophobic core is temperature dependent. The sigmoidal pattern of the curve indicates that this interaction is ruled by the laws governing the formation of protein-surfactant complexes [12]. Therefore, the ability to expose cholera toxin hydrophobic surfaces should not be a unique property of micellar gangliosides but also of other micelle-forming amphiphiles.

Cholera toxin labelling with SDS and Triton X-100 micelles

Fig. 3A shows that both protomer A and protomer B are strongly labelled when cholera toxin is incubated with SDS-tagged micelles. The labelling of the β polypeptide chains may be explained by the well-known structural rearrangement induced by SDS, which will allow the reaction of the

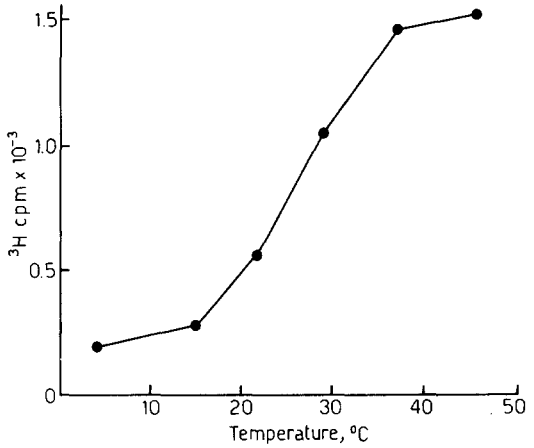

Fig. 2. Temperature effect on the labelling of cholera toxin after incubation with G_{M1} micelles tagged with the azidoglycolipid. Cholera toxin (0.24 nmol) was incubated for 1 h at the temperatures indicated, with 0.1 ml suspension of G_{M1} micelles tagged with the azidoglycolipid. At the end of the incubation, the samples were illuminated and analyzed as described under Materials and Methods. Results are expressed as the total amount of radioactivity incorporated into cholera toxin polypeptide chains, as determined after SDS-polyacrylamide gel electrophoresis.

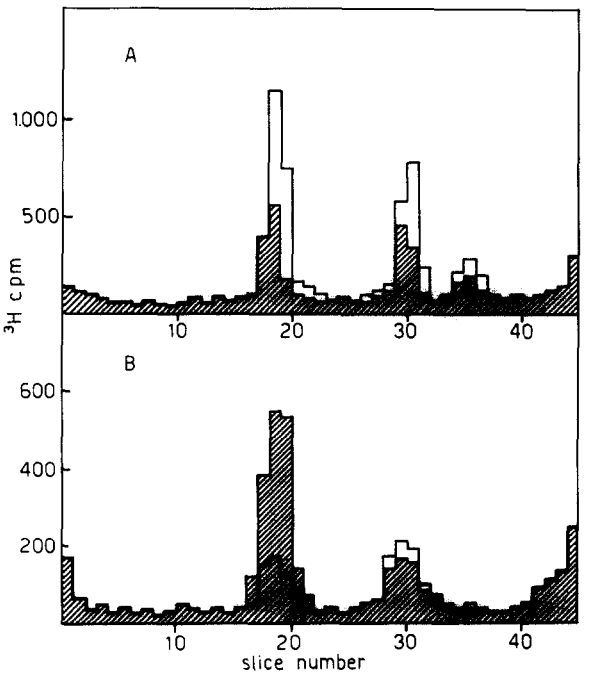

Fig. 3. Histograms of the radioactivity covalently incorporated into cholera toxin subunits after incubation with SDS- (A) or Triton X-100- (B) tagged micelles. Experimental details are indicated in the text. The data relative to the unreduced and reduced (shaded area) samples are shown.

hydrophobic probe with the previously folded non-accessible regions of the toxin. Nevertheless, the α chain incorporates 4-times as much radioactivity per mol protein than do the β and γ chains. These results suggest that the α chain possesses lipophylic peptide segments, in agreement with the recent findings of Lai et al. [27], while in this respect the β and γ chains show a prevalently hydrophilic behaviour in agreement with the reported amino acid sequences [28–30].

Fig. 3B shows the labelling pattern of cholera toxin in the presence of micelles of Triton X-100 containing the protoreactive azidoglycolipid. It is evident that the interaction of cholera toxin with Triton X-100 produces a low labelling. Furthermore, since the distribution of radioactivity is roughly proportional to the protein subunit stoichiometry, the micelles of Triton X-100 do not appear to be able to recognize specific hydrophobic areas. These data indicate that micellar gangliosides, behaving differently from other amphiphiles, are able to recognize within an oligomeric protein its hydrophobic domains. However, an important point to be clarified is to what extent a conformational change is produced during the formation of micellar ganglioside-protein complexes and whether this structural rearrangement is involved in the unmasking of the hydrophobic peptides.

Effect of reduction of the α-γ disulfide bridge on cholera toxin labelling

An event that seems to unmask hydrophobic regions in cholera toxin is the splitting of the disulfide bridge which holds together the two functional regions α and $\gamma\beta_5$ [22,31,32]. The reduction of this disulfide bridge leads to a slow dissociation of the α chain from the $\gamma\beta_5$ region with the formation of water-insoluble aggregates. Moreover the reduction of the disulfide bridge, which is a prerequisite for the activation of the enzymic α subunit [33], is a structural event which permits the lipid insertion of the active α chain [31]. Therefore, we investigated the possible relationship between the area disclosed by the splitting of the disulfide

bridge and the area recognized by micellar gangliosides, performing experiments of hydrophobic photolabelling on reduced cholera toxin. These experiments were based on the assumption that if the reduction unmasks a new lipophylic area, an increase of labelling should be measured. The fact that no significant differences of labelling are visible using either ganglioside G_{M1} or G_{D1a} (Fig. 1, shaded area), is indicative of a similar size of the areas unmasked either by the reducing agents or by the micelles of ganglioside. Conversely, the treatment with reducing agents produces a strong labelling of only the α subunit in the presence of Triton X-100 (Fig. 3A). This result confirms that the splitting of the disulfide bridge induces the exposure of hydrophobic areas on the α chain surface.

Discussion

This paper presents information on the hydrophobic interaction of cholera toxin with micelles of gangliosides and of ionic or neutral detergents such as SDS and Triton X-100.

Protomer A alone is labelled by the lipid hydrophobic probe when cholera toxin is incubated with ganglioside micelles. Our results show the occurrence of a particular type of interaction between the proteins and the gangliosides in micellar form. In fact, identical results were obtained both with the specific ligand of cholera toxin, ganglioside G_{M1}, and ganglioside G_{D1a}, which is unable to interact with the binding region protomer B [26]. These results suggest that the forces driving the insertion of protomer A into the lipid milieu, rather than the specific interaction of the β chains with ganglioside G_{M1}, are the surfactant properties of the ganglioside micelles. In micellar physical form the gangliosides do show these properties in other experimental systems [7,11]. In addition, the experiment of Fig. 2 shows that the thermodynamics of the formation of cholera-toxin micellar ganglioside complexes appear to be similar to the formation of protein-detergent complexes [12].

The differences observed between the labelling patterns obtained with gangliosides and SDS, or Triton X-100, may arise from the dissimilar way in which the gangliosides and the two detergents interact with proteins. While SDS, producing a drastic conformational change in the protein structure, mediates the hydrophobic labelling of the entire toxin, Triton X-100, which surrounds the lipohilic domains without affecting the integrity of the protein structure [34,35], appears to be less effective than G_{M1} or G_{D1a}. In contrast, both gangliosides in micellar form have the property of forming stable stoichiometric complexes with water-soluble proteins (Ref. 11 and unpublished results). Such a stability seems to be achieved as a consequence of conformational changes wherein the hydrophobic side-chains of the protein become inserted into the lipophilic milieu of the ganglioside micelle.

In the model system described in this paper, of the entire toxin only protomer A shows a marked preference for being in a hydrophobic environment. Therefore, local rearrangements of the protein structure should occur as a part of the association process, in order to expose on protomer A sufficiently large hydrophobic areas to constitute binding sites for the amphiphilic ganglioside. This hypothesis is supported by the following evidence: (a) a mild detergent such as Triton X-100, which is known to preserve the protein structure, is not able to expose hydrophobic areas on the toxin; (b) only a covalent modification, as the reduction of the α-γ disulfide bond, allows the hydrophobic insertion of the active α chain into lipid bilayers [31] and into Triton X-100 micelles as well. Thus, the gangliosides appear to interact with cholera toxin in two different modes. The first mode concerns the well known highly specific association of protomer B to the oligosaccharide moiety of ganglioside G_{M1}, without any substantial conformational change of the toxin structure [13–16]. In the second mode the ganglioside micelle, behaving as a common detergent, binds to a grossly altered structure of the toxin, rearrangement of which is induced by the association with the micelle itself.

At present only a hypothesis can be advanced on the biological role of the latter mode of interaction. However, the results reported here are surprisingly similar to those obtained by Wisnieski and Bramhall [36] on the hydrophobic labelling of cholera toxin protomer A with Newcastle disease virus. In contrast in another model system, i.e., liposomes containing G_{M1}, the mere binding of cholera toxin to G_{M1} is not sufficient for inserting

the enzymatically active α chain into the membrane [31]. The specific reduction of the α-γ disulfide bond on the external surface of the membrane appears to be necessary. The common denominator of these and other results [13–18,31] seems to be that the binding of the toxin to its membrane receptor should be followed by a series of membrane events necessary for the penetration of the α component of the toxin. Although the presence of ganglioside micellar microdomains on the plasma membrane has not yet been demonstrated, their formation can be induced by the binding of cholera toxin to the membrane receptor. There is evidence that the binding of cholera toxin to the ganglioside G_{M1} produces a clustering of the ganglioside on the membrane [37]. In these clusters, which may involve other gangliosides and membrane proteins as well, the lipid portion of the ganglioside may modulate the toxin-membrane interactions necessary for the penetration of the α chain. In other words, if the ganglioside clusters can assume micellar-like structures, our results show that this ganglioside configuration, capable of specifically recognizing the hydrophobic segments of the protein, may act as biotransducer of protomer A into the membrane.

Acknowledgement

We thank Mr. M. Santato for help in some experiments.

References

1 Cuatrecasas, P. (1977) in The Specificity and Action of Animal, Bacterial and Plant Toxins (Cuatrecasas, P. ed.), pp. 3–66, Chapman and Hall, London
2 Cullis, P.R. and De Kruijff, (1979) Biochim. Biophys. Acta 559, 399–420
3 Hui, S.W., Stewart, T.P., Boni, L.T. and Yeagle, P.L. (1981) Science 212, 921–922
4 Klenk, E. (1942) Hoppe-Seyler's Z. Physiol. Chem. 273, 76–86
5 Brady, R.O. and Fishman, P.H. (1979) Adv. Enzymol. 50, 303–323
6 Corti, M., DeGiorgio, V., Ghidoni, R., Sonnino, S. and Tettamanti, G. (1980) Chem. Phys. Lipids 26, 225–238
7 Formisano, S., Johnson, M.L., Lee, G., Aloj, S.M. and Edeloch, H. (1979) Biochemistry 18, 1119–1124
8 Cestaro, B., Barenholz, Y. and Gatt, S. (1980) Biochemistry 19, 615–619
9 Tosteson, M.T. and Tosteson, D.C. (1978) Nature 275, 142–144
10 Kohn, L.D., Consiglio, E., DeWolf, M.J., Grollman, E.F., Ledley, F.D., Lee, G. and Morris, N.P. (1980) Adv. Exp. Med. Biol. 125, 487–503
11 Tomasi, M., Roda, L.G., Ausiello, C., D'Agnolo, G., Venerando, B., Ghidoni, R., Sonnino, S. and Tettamanti, G. (1980) Eur. J. Biochem. 111, 315–324
12 Tanford, C. (1980) The hydrophobic Effect, 2nd Edn., John Wiley & Sons, New York
13 Mullin, B.R., Aloj, S.M., Fishman, P.H., Lee, G., Kohn, L.D. and Brady, R.O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1679–1683
14 Sillerud, L.O., Prestegard, J.H., Yu, R.K., Konigsberg, W.H. and Schafer, D.E. (1981) J. Biol. Chem. 256, 1094–1097
15 De Wolf, M.J.S., Fridkin, M., Kohn, L.D. (1981) J. Biol. Chem. 256, 5489–5496
16 Van Heyningen, S. (1982) Eur. J. Biochem. 122, 333–337
17 Wiegandt, H., Ziegler, W., Staerk, J., Kranz, T., Ronneberger, H.J., Zilg, H., Karlsson, K.-A. and Samuelson, B.E. (1976) Hoope Seyler's Z. Physiol. Chem. 357, 1637–1646
18 Fishman, P.H., Pacuszka, T., Hom, B. and Moss, J. (1980) J. Biol. Chem. 255, 7657–7664
19 Bisson, R. and Montecucco, C. (1981) Biochem. J. 193, 757–763
20 Montecucco, C., Bisson, R., Gache, C. and Johansson, A. (1981) FEBS Lett. 128, 17–21
21 Gill, D.M. (1977) Adv. Cyclic Nucleotide Res. 8, 85–118
22 Tomasi, M., Battistini, A., Araco, A., Roda, L.G. and D'Agnolo, G. (1979) Eur. J. Biochem. 93, 621–627
23 Tettamanti, G., Bonali, F., Marchesini, F. and Zambotti, V. (1973) Biochim. Biophys. Acta 296, 160–170
24 Bramhall, J.S., Shiflett, M.A. and Wisnieski, B.J. (1979) Biochem. J. 177, 765–768
25 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477
26 Sattler, J., Schwarzmann, G., Staerk, J., Ziegler, W. and Wiegandt, H. (1977) Hoppe Seyelr's Z. Physiol. Chem. 358, 159–163
27 Lai, C.Y., Cancedda, F. and Duffy, L.K. (1981) Biochem. Biophys. Res. Commun. 102, 1021–1027
28 Lai, C.Y. (1977) J. Biol. Chem. 252, 7249–7256
29 Kurosky, A., Markel, D.E. and Peterson, J.W. (1977) J. Biol. Chem. 252, 7257–7264
30 Duffy, L.K., Peterson, J.W. and Kurosky, A. (1981) J. Biol. Chem. 256, 12252–12256
31 Tomasi, M. and Montecucco, C. (1981) J. Biol. Chem. 256, 11177–11181
32 Tomasi, M., Battistini, A., Ausiello, C. Roda, L.G. and D'Agnolo, G. (1978) FEBS Lett. 94, 253–256
33 Wodnar-Filipowicz, A. and Lai, C.Y. (1976) Arch. Biochem. Biophys. 176, 465–471
34 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
35 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133–170
36 Wisnieski, B.J. and Bramhall, J.S. (1981) Nature 289, 319–321
37 Bennett, V., Craig, S., Hollenberg, M.D., O'Keefe, E., Sahyoun, N., Cuatrecasas, P. (1976) J. Supramol. Struct. 4, 99–120