Greer, D. H., & Laing, W. A. (1988) *Planta 174*, 159-165.
Greer, D. H., Berry, J. A., & Bjorkman, O. (1986) *Planta 168*, 253-260.
Greer, D. H., Laing, W. A., & Kipnis, T. (1988) *Planta 174*, 152-158.
Herdman, M., Deloney, S. F., & Carr, N. G. (1973) *J. Gen. Microbiol. 79*, 233-237.
Hirayama, O. (1967) *J. Biochem. 61*, 179-185.
Holton, R. W., Blecker, H. H., & Onore, M. (1964) *Phytochemistry 3*, 595-602.
Joliot, P., & Joliot, A. (1968) *Biochim. Biophys. Acta 153*, 625-634.
Kirilovsky, D., Vernotte, C., Astier, C., & Etienne, A.-L. (1988) *Biochim. Biophys. Acta 933*, 124-131.
Kyle, D. J. (1985) in *Molecular Biology of the Photosynthetic Apparatus* (Steinback, K. E., Boitz, S., Arntzen, C. J., & Bogorad, L., Eds.) pp 33-38, Cold Spring Harbor Laboratory Press, New York, NY.
Kyle, D. J., Ohad, I., & Arntzen, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4070-4074.
Kyle, D. J., Ohad, I., & Arntzen, C. J. (1987) in *Photoinhibition* (Kyle, D. J., Osmond, C. B., & Arntzen, C. J., Eds.) Chapter 9, pp 197-226, Elsevier Science Publishers BV, Amsterdam, New York, and Oxford.
Ladbrooke, B. D., & Chapman, D., (1969) *Chem. Phys. Lipids 3*, 304-356.
Lönneborg, A., Kalla, S. R., Samuelsson, G., Gustafsson, P., & Oquist, G. (1988) *FEBS Lett. 240*, 110-114.
Manodori, A., & Melis, A. (1985) *FEBS Lett. 181*, 79-82.
Murata, N., & Fork, D. C. (1975) *Plant Physiol. 56*, 791-796.
Murata, N., Troughton, J. H., & Fork, D. C. (1975) *Plant Physiol. 56*, 508-517.
Ohad, I., Kyle, D. J., & Arntzen, C. J. (1985) *J. Cell Biol. 99*, 481-485.
Ohad, I., Koike, H., Shochat, S., & Inoue, Y. (1988) *Biochim. Biophys. Acta 933*, 288-298.
Ohad, I., Adir, N., Koike, H., Kyle, D. J., & Inoue, Y. (1990) *J. Biol. Chem. 265*, 1972-1979.
Oquist, G., Greer, D. H., & Ogren, E. (1987) in *Photoinhibition* (Kyle, D. J., Osmond, C. B., & Arntzen, C. J., Eds.) Chapter 3, pp 67-87, Elsevier Science Publishers BV, Amsterdam, New York, and Oxford.
Osmond, C. B. (1981) *Biochim. Biophys. Acta 639*, 77-98.
Powles, S. B. (1984) *Annu. Rev. Plant Physiol. 35*, 15-44.
Schreiber, U., Rijgersberg, C. P., & Amesz, J. (1979) *FEBS Lett. 104*, 327-331.
Setlik, I., Allakhverdiev, S. I., Nedbal, L., Setlikova, E., & Kilmov, V. V. (1990) *Photosynth. Res. 23*, 39-48.
Styring, S., Virgin, I., Ehrenberg, A., & Andersson, B. (1990) *Biochim. Biophys. Acta 1015*, 269-278.
Theg, S. M., Filar, L. J., & Dilley, R. A. (1986) *Biochim. Biophys. Acta 849*, 104-111.
Vass, I., Mohanty, N., & Demeter, S. (1988) *Z. Naturforsch. 43C*, 871-876.
Vernotte, C., Etienne, A. L., & Briantais, J. M. (1982) *Biochim. Biophys. Acta 545*, 519-527.

Structure, Stability, and Receptor Interaction of Cholera Toxin As Studied by Fourier-Transform Infrared Spectroscopy†

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ABSTRACT: The structure and thermal stability of isolated B and A subunits of cholera toxin, as well as the interaction of the B subunit with a ganglioside G_{M1} receptor, were studied by Fourier-transform infrared spectroscopy. The B subunit of the toxin is highly folded; its secondary structure consists predominantly of β-sheets. The temperature dependence of the infrared spectrum indicates that the B subunit undergoes thermal unfolding in the temperature range between approximately 66 and 78 °C. Binding to the ganglioside G_{M1} receptor or to its oligosaccharide moiety results in only marginal, if any, change in the secondary structure of the B subunit; however, the receptor-associated subunit does show a markedly increased thermal stability. The secondary structure of the enzymatically active A subunit is less ordered and much less stable than that of the B subunit. The relatively loose folding of the A subunit is likely to be of importance for the effective membrane translocation of this subunit.

The clinical manifestations of cholera are attributable to cholera toxin, an enterotoxic protein produced by *Vibrio cholerae* [for recent reviews of cholera toxin chemistry and biological activity, see Lai (1980), Fishman (1982), Finkelstein (1988), and Moss and Vaughan (1988)]. The toxin molecule is composed of two structurally and functionally distinct subunits, A and B (Lannroth & Holmgren, 1973; Cuatrecasas et al., 1973). The A promoter consists of two polypeptide chains, A_1 (M_r 23 000) and A_2 (M_r 5500), that are linked by a single disulfide bridge (Gill, 1976). The B subunit is believed to contain five identical polypeptide chains (M_r 11 600) arranged in a noncovalently associated pentameric ring (Gill, 1976; Lai et al, 1977; Dwyer & Bloomfield, 1982; Ludwig et al., 1986; Ribi et al., 1988). The functions served by these two subunits are complementary (Lai, 1980; Fishman, 1982; Finkelstein, 1988; Moss & Vaughan, 1988). Upon penetration through the cell membrane, the toxic A protomer enzymatically activates intracellular adenylate cyclase, leading to the increased levels of cAMP. The primary role of the B subunit is to initiate the toxin–target cell interaction by binding to specific receptor sites on the membrane surface.

The receptor for cholera toxin has been identified as monosialoganglioside G_{M1} (van Heyningen et al., 1971; Cuatre-

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casas, 1973; Fishman, 1982). The toxin–receptor association is believed to cause a conformational change in the B protomer that, in turn, may facilitate and/or mediate the insertion of the A subunit into the membrane. The notion of such a conformational change is based primarily on the results of fluorescence studies (Mullin et al., 1976; Moss et al., 1977a; Fishman et al., 1978; De Wolf et al., 1981); it has not yet been verified by other experimental approaches. Further progress in elucidating molecular aspects of toxin–receptor interactions, as well as in understanding the mechanism by which the enzymatically active A subunit penetrates the membrane barrier, seems to be critically dependent on the detailed characterization of cholera toxin at the level of its secondary and tertiary structure. Yet, data available in this respect are scarce.

In the present paper, we have used Fourier-transform infrared spectroscopy to probe the conformation, interaction with receptor, and stability of cholera toxin B and A subunits. Infrared spectra are known to report directly on the secondary structure of the protein backbone (Susi, 1969; Krimm & Bandekar, 1986). The technique is of particular value in structural studies of membrane or lipid-associated proteins (Surewicz et al., 1988; Chapman et al., 1989; Surewicz & Mantsch, 1989). In contrast to other optical methods, infrared spectroscopic measurements are essentially unaffected by light scattering on protein–lipid aggregates or membrane fragments.

Materials and Methods

Cholera toxin B subunit and cholera toxin A subunit were obtained from Sigma Chemical Co. (St. Louis, MO); they were found to be at least 95% pure by SDS–polyacrylamide gel electrophoresis and were used without further purification. Prior to use, the toxin subunits were dialyzed overnight against two changes of 20 mM ammonium bicarbonate and lyophilized. Monosialoganglioside G_{M1} was obtained from Sigma, G_{M1}-pentasaccharide (oligo-G_{M1}) was from Biocarb (Lund, Sweden), and ditetradecylphosphatidylcholine was from Dr. R. Bertchold, Biochemisches Labor, Bern. Protein solutions were obtained by dissolving 0.3 mg of toxin in 15 μL of 50 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid]/100 mM NaCl buffer prepared in deuterium oxide (99.8% purity, MSD Isotopes, St. Louis, MO) and adjusted to p^{2}H 7.5. For preparation of protein complexes with ganglioside G_{M1} or with oligo-G_{M1}, the glycolipid (or its oligosaccharide moiety) was added to the solution of B subunit in buffer to obtain a receptor to B subunit monomer molar ratio of 2:1. The mixture was then lightly vortexed and incubated for 30 min. For experiments with ganglioside G_{M1} in a phospholipid bilayer matrix, small unilamellar vesicles were prepared by sonication of ditetradecylphosphatidylcholine in the same buffer as that used for dissolving protein samples. Vesicles were then centrifugated at 15000*g* for 30 min to remove residual multilamellar liposomes. Incorporation of ganglioside into vesicular membranes was performed by mixing vesicles with G_{M1} solution and incubating the mixture for 90 min at the temperature above the lipid phase transition. This procedure allows for the asymmetric distribution of ganglioside in which it is present only in the outer monolayer of the vesicles (Felgner et al., 1981). The ganglioside to phospholipid molar ratio was 1:20.

Samples for infrared spectroscopy were placed between two CaF_2 windows separated by a 50-μm-thick Teflon spacer. Infrared spectra were recorded by using a Digilab FTS-60 instrument. For each spectrum, 200 interferograms were co-added and Fourier-transformed to give a resolution of 2 cm^{-1}. Temperature was controlled by the computer and, during data acquisition, was stable to within 0.5 °C. The spectra in the 1500–1800 cm^{-1} region were corrected for the weak absorption of the buffer. To eliminate spectral contributions of atmospheric water vapor, the instrument was continually purged with dry nitrogen. Overlapping infrared bands were resolved by using Fourier self-deconvolution procedures (Kauppinen et al., 1981). Fractional areas of these bands were estimated by using standard curve-fitting procedures (Fraser & Suzuki, 1966).

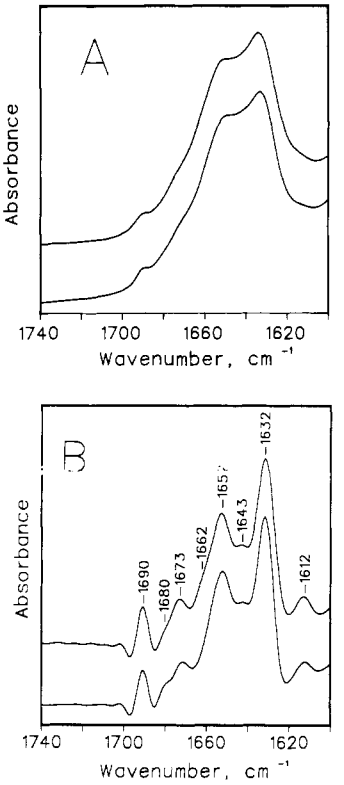

FIGURE 1: (A) Infrared spectra of cholera toxin B subunit free in aqueous solution (bottom spectrum) and in the presence of ganglioside G_{M1} at the glycolipid to the B-subunit monomer molar ratio of 2:1 (top spectrum). (B) Same spectra after band narrowing by Fourier self-deconvolution using a Lorentzian line shape of 15 cm^{-1} half-width and a resolution enhancement factor of 2 (Kauppinen et al., 1981).

Results

The B Subunit. The bottom trace in Figure 1A represents the conformationally sensitive amide I region of the infrared spectrum of cholera toxin B subunit in ^2H_2O buffer. The broad amide I band contour exhibits a maximum at 1633 cm^{-1} and two well-defined shoulders around 1650 and 1690 cm^{-1}. While the position of the band maximum is typical of proteins containing a high content of β-sheet structure (Susi, 1969; Krimm & Bandekar, 1986), further details are obscured by the overlapping of the amide I component bands that represent different elements of protein secondary structure. Some of these components can be resolved by the computational procedure of band narrowing by Fourier self-deconvolution (Byler & Susi, 1986; Surewicz & Mantsch, 1988). Indeed, the deconvolved spectrum of the B subunit (bottom trace in Figure 1B) reveals the presence of seven bands in the amide I region. The major band at 1632 cm^{-1} represents the β-structure, while the bands at 1643, 1652, and 1662 cm^{-1} can be assigned to an unordered structure (with deuterium-exchanged NH groups of the polypeptide backbone), α-helices, and turns, respectively (Byler & Susi, 1986; Surewicz & Mantsch, 1988). The features at 1673, 1680, and 1690 cm^{-1} are in the spectral region characteristic of the "in phase" vibrations of amide groups involved in β-sheets, although the relatively weak bands at

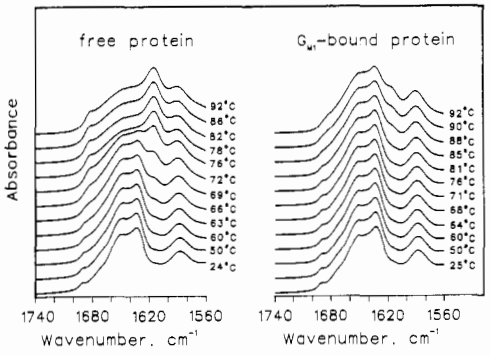

FIGURE 2: Temperature dependence of infrared spectra of cholera toxin B subunit free in aqueous solution (left panel) and in the presence of ganglioside G_{M1} at the glycolipid to the B subunit monomer molar ratio of 2:1 (right panel). Spectra were recorded during the heating cycle at a heating rate of approximately 20 °C/h.

1680 and 1690 cm^{-1} may in fact originate from turns (Byler & Susi, 1986; Krimm & Bandekar, 1986; Surewicz & Mantsch, 1988). The weak band at 1612 cm^{-1} represents largely side chain vibrations [tyrosine and guanidine group of arginine (Chirgadze et al., 1975)].

Addition of ganglioside G_{M1} to the protein solution, at the concentration corresponding to a 2-fold molar excess of glycolipid over the B-subunit monomer, results in an increased turbidity of the sample. This reflects binding of the pentameric B subunit to the micellar ganglioside G_{M1}, concomitant with a cross-linking of adjacent micelles (Dalziel et al., 1984). The protein–receptor association appears to have very little effect on the infrared spectrum on the B subunit. The original amide I band contour of the ganglioside G_{M1}-bound protein (top trace in Figure 1A), as well as the spectrum after Fourier self-deconvolution (top trace in Figure 1B), shows characteristics very similar to those found in the corresponding spectra of the free B subunit.[1] Spectra essentially identical with those represented by the top traces in Figure 1A,B were also obtained for the B subunit associated with the isolated oligosaccharide moiety of the ganglioside G_{M1} and for the protein bound to the intact ganglioside G_{M1} receptor incorporated into the membrane of unilamellar ditetradecylphosphatidylcholine vesicles.

A visual comparison of the infrared spectra of a free and ganglioside G_{M1}-bound B subunit clearly indicates the absence of major changes in the protein secondary structure upon its association with the receptor. A more quantitative insight into the degree of this structural similarity has been obtained from a curve-fitting analysis of amide I band contours (Byler & Susi, 1986). For a free B subunit, the component band representing α-helices accounts for 25% of the total area of the amide I band contour, and the integrated fractional area of the two bands (at 1632 and 1673 cm^{-1}) assigned to β-sheet structure amounts to 42%.[2] The respective numbers derived from the analysis of the spectrum of the receptor-bound protein are 21% for the α-helical band and 44% for bands representing the β-structure.

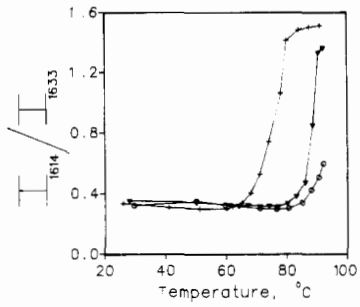

FIGURE 3: Ratio of cholera toxin B-subunit amide I band intensity at 1614 cm^{-1} to that at 1633 cm^{-1} as a function of temperature. Free B subunit in aqueous solution (+) and B subunit in the presence of oligo-G_{M1} (▲) or intact ganglioside G_{M1} (○). The molar ratio of ganglioside G_{M1} or oligo-G_{M1} to the B-subunit monomer is 2:1

Temperature Dependence of Infrared Spectra of the B Subunit. Studies of the temperature dependence of amide I bands can provide useful information pertaining to the thermal stability of proteins, as well as to the mechanism of protein unfolding (Surewicz et al., 1987; Arrondo et al., 1988). As shown in Figure 2 (left), the amide I band contour of the free B subunit remains virtually unchanged upon temperature increase from 20 °C up to approximately 66 °C, indicating that in this temperature range the protein retains its "native" secondary structure. A further increase in temperature results in a serious modification of the amide I band contour. These spectral changes, which occur over a relatively narrow temperature range between 66 and 78 °C, reflect the cooperative unfolding of the B subunit. The well-defined peaks representing β-structure and α-helices in the spectrum of the native protein are replaced by a strong band around 1614 cm^{-1}, a weaker band at 1683 cm^{-1}, and a broad band centered around 1644 cm^{-1}. While the latter feature is characteristic of unordered structures (Byler & Susi, 1986; Surewicz & Mantsch, 1988), the conformational assignment of the dominant band at 1614 cm^{-1} is not fully clear. This low-wavenumber band, seen also in the spectra of other thermally denatured proteins (unpublished data from this laboratory), may represent a special kind of extended structures which are formed upon aggregation of the thermally unfolded protein. Such aggregation is likely to be induced by the exposure to water of originally "buried" hydrophobic segments of the protein.

Binding to ganglioside G_{M1} results in a marked increase in the thermal stability of the B subunit. As demonstrated in Figure 2 (right), the spectrum of the B subunit in the presence of ganglioside G_{M1} (at the 2-fold molar excess of glycolipid over the protein monomer) remains essentially unaltered up to approximately 87 °C. An increase in the intensity of the band at 1614 cm^{-1} seen above 87 °C indicates the beginning of a thermotropic transition. However, a comparison of the spectra in the left and right sides of Figure 2 suggests that the denaturation process of ganglioside G_{M1}-bound B subunit is not completed even at 92 °C, which is the highest temperature attainable in our measurements.

Experiments performed in the presence of oligo-G_{M1} indicate a qualitatively similar stabilization of the B subunit, although the increase in denaturation temperature caused by the intact ganglioside is somewhat larger than that observed in the presence of an equivalent concentration of oligo-G_{M1}. In the latter case, the denaturation process starts at approximately 82 °C and appears to be completed at 92 °C, as indicated by

[1] It should be noted that the infrared spectrum of ganglioside G_{M1} shows a broad band which overlaps the amide I band of the protein backbone. However, under the conditions of our experiments, the contribution of ganglioside absorption is very weak and does not exceed a few percent of the total area of the protein amide I band. In order to further minimize this contribution, spectra of receptor–protein complexes were corrected by subtracting the spectra of free G_{M1} at the equivalent concentration. This subtraction was found to have a very small effect on the overall shape of the amide I band contours and did not affect the positions of bands identified in the deconvolved spectra.

[2] Due to the uncertainty in their conformational assignment (see text), the minor bands at 1680 and 1690 cm^{-1} were not included in the estimation of a β-sheet structure. These bands account for 1–2% (1980 cm^{-1}) and 6% (1690 cm^{-1}) of the total area of the amide I band contour, both for the free and for the receptor-bound protein.

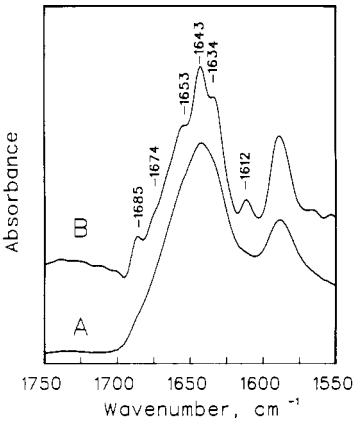

FIGURE 4: Infrared spectrum of cholera toxin A subunit in aqueous solution (A) and same spectrum after band narrowing by Fourier self-deconvolution using a Lorentzian line shape of 15 cm^{-1} half-width and a resolution enhancement factor of 2 (Kauppinen et al., 1981).

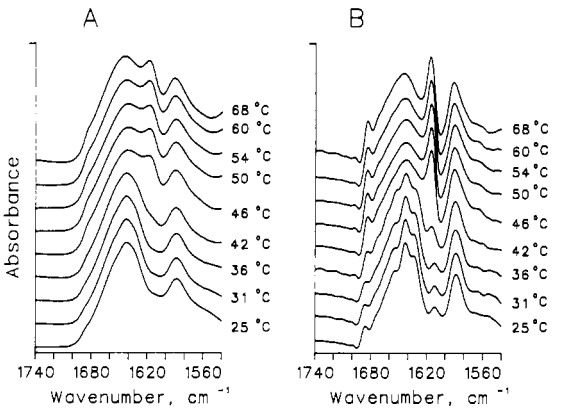

FIGURE 5: Temperature dependence of infrared spectrum of cholera toxin A subunit. (A) Original spectra; (B) same spectra after band narrowing by Fourier self-deconvolution. Spectra were recorded during the heating cycle at a heating rate of approximately 20 °C/h.

an infrared spectrum which is essentially identical with that of a denatured free protein (spectra not shown for brevity).

A convenient empirical parameter for following the thermal denaturation of the B subunit is the intensity ratio of the amide I band at 1614 and 1633 cm^{-1} (Figure 3). The midpoint transition temperatures identified by this parameter are 74, 89, and 93–94 °C for the free protein and for protein in the presence of 2-fold molar excess of oligo-G_{M1} or intact ganglioside G_{M1}, respectively (the last value being an estimate from the extrapolation of data in Figure 3).

The A Subunit. The amide I band contour of the A subunit of cholera toxin exhibits a maximum at 1643 cm^{-1} (Figure 4). The Fourier self-deconvolved spectrum reveals a major component band at 1643 cm^{-1}, along with weaker bands representing β-sheets (1634 cm^{-1}), α-helices (1653 cm^{-1}), and turns and/or β-sheets (1674 and 1685 cm^{-1}). Both the position of the maximum of the nondeconvoluted amide I band contour and also the dominance of the 1643 cm^{-1} band in the deconvolved spectrum point to a relatively high proportion of poorely defined or "nonordered" structure in the A subunit. This is in clear contrast to the B subunit, which gives rise to only a weak component band in the spectral region characteristic of an unordered structure.

The temperature dependence of the infrared spectrum of the A subunit (Figure 5) indicates a relatively low thermal stability of this subunit. The infrared bands characteristic of the native conformation gradually disappear in the temperature range between approximately 40 and 46 °C; they are replaced by the broad band at 1643 cm^{-1} and two sharper bands at 1616 and 1683 cm^{-1}.

Discussion

The entry of cholera toxin into susceptible cells begins with the binding of the pentameric B subunit to ganglioside G_{M1} receptor. The binding triggers the membrane insertion and translocation of the A subunit which, eventually, leads to the enzymatic activation of adenylate cyclase (Lai, 1980; Fishman, 1982; Finkelstein, 1988; Moss & Vaughan, 1988). However, the detailed nature of these events, particularly at the molecular level, remains poorly understood.

The central question related to the mode of action of cholera toxin (and also some other bacterial toxins) is the mechanism by which the active A subunit inserts into and translocates across the hydrophobic barrier of a cell membrane. Several reports have considered the possibility of conformational change in the B subunit upon its binding to the receptor. Such conformational change in the binding subunit could provide the necessary driving force for the exposure and subsequent penetration of the A subunit into the membrane. One model even suggests that binding to the receptors causes a major refolding of the B subunit, allowing it to enter the lipid bilayer and to form a hydrophobic transmembrane channel through which the A component can diffuse (Gill, 1976). The experimental evidence for conformational change in the B subunit has been derived mainly from fluorescence studies and was corroborated, to some extent, by circular dichroism and ultraviolet absorption experiments (Mullin et al., 1976; Moss et al., 1977a; Fishman et al., 1978; De Wolf et al., 1981; Tomasi et al., 1984). However, the direct information provided by most of these spectroscopic studies pertains, strictly speaking, to protein perturbations at the level of individual side chains. The circular dichroism data of Fishman et al. (1978) suggest also that receptor binding may affect the secondary structure of the B subunit. However, the oligo-G_{M1}-induced changes in the far-ultraviolet circular dichroism spectrum of the protein are very small, and, furthermore, the structural interpretation of these changes is complicated by the fact that they occur largely in the 230-nm region of the spectrum, i.e., the region which may contain significant contributions from aromatic side chains (Khan et al., 1989). It remains thus unclear whether the receptor-induced perturbations of cholera toxin represent mostly local effects or whether they reflect more extensive changes in the folding of the polypeptide backbone.

Infrared spectroscopy reports directly on the nature of hydrogen bonding involving C=O and NH moieties and has proven sensitive in detecting even subtle changes in the secondary structure of proteins (Susi, 1969; Surewicz & Mantsch, 1988). Detailed analysis of the amide I band indicates a highly ordered secondary structure of cholera toxin B subunit, with a high proportion of β-sheets. Importantly, only marginal changes in the amide I band of the B subunit could be detected in the presence of ganglioside G_{M1} or the isolated oligosaccharide moiety of the glycolipid. A quantitative analysis of the spectra allowed us to estimate that no greater than 3–4% change in β-sheet or α-helix content could occur in the presence of the receptor. Previous fluorescence and absorption spectroscopy titration experiments with oligo-G_{M1} (Fishman et al., 1978; Tomasi et al., 1984), as well as the estimated toxin–receptor binding constants (Sattler et al., 1977, 1978; Fishman et al., 1978), strongly indicate that under the experimental conditions of this study (receptor to B subunit monomer molar ratio of 2:1) virtually all the protein present in solution is

associated with the receptor. The absence of unbound protein in our samples of subunit B mixtures with ganglioside G_{M1} or oligo-G_{M1} is further corroborated by the studies of temperature dependence of infrared spectra (Figures 2 and 3), which demonstrate a complete elimination of a thermotropic transition characteristic of the free protein. The present data thus argue against any extensive change in the backbone conformation of the B subunit caused by binding to the surface receptor. In view of this, the previously reported perturbations of the protein (see above) are likely to be of a highly localized nature. Particularly, the changes in fluorescence spectra may reflect a direct involvement of a single tryptophan residue of each B monomer in the toxin–receptor association. The direct participation of tryptophan in the receptor binding event has been previously suggested by De Wolf et al. (1981) and by Ludwig et al. (1985).

The apparent insensitivity of the backbone conformation of the B subunit to receptor binding may be related to the high stabiity of this subunit, as indicated by recent differential scanning calorimetry experiments (Dalziel et al., 1984; Goins & Freire, 1988) and confirmed in this study by the analysis of temperature dependence of infrared spectra. The thermal stability of the B subunit is further increased considerably upon binding to ganglioside G_{M1} receptor. A qualitatively similar although somewhat less pronounced increase in unfolding temperature also occurs in the presence of the isolated oligosaccharide portion of the glycolipid (Figure 3). Therefore, the stabilization of the B subunit does not seem to be largely due to possible specific surface or interfacial effects (which may arise from the localization of the receptor in a membrane environment), but rather due to the effects similar to those observed for other proteins interacting with water-soluble ligands (Sturtevant, 1987).

With respect to the A subunit, the infrared data demonstrate a less ordered secondary structure of this subunit than that of the B component. Furthermore, the A subunit is also considerably less stable, as indicated by an almost complete loss of its original secondary structure at the temperature as low as 46 °C. This apparently "loose" folding may be instrumental in allowing the A chain to adapt to the different conditions and requirements imposed by the diverse environments of aqueous medium, water–membrane interface, and a hydrophobic interior of the lipid bilayer. It is thus likely to be of direct relevance to the mechanism by which the A subunit inserts into and translocates across the hydrophobic barrier of cell membranes.

In conclusion, our infrared spectroscopic experiments argue against any substantial refolding or other extensive changes in the backbone conformation of cholera toxin B subunit upon its binding to ganglioside G_{M1} receptor. The structural properties of subunits B and A are generally consistent with the view (Goins & Freire, 1988) that the main role of the B subunit is to provide a water-soluble carrier to the A subunit and to place it in close proximity of the membrane surface. The actual mechanism of membrane translocation of the toxic A subunit is likely to take advantage of the intrinsic properties of this subunit, such as its hydrophobicity (Moss et al., 1977b; Goins & Freire, 1985; De Wolf et al., 1987) and a relatively loose folding.

Registry No. Ganglioside G_{M1}, 37758-47-7.

REFERENCES

Arrondo, J. L. R., Young, N. M., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 261–268.

Byler, M., & Susi, H. (1986) *Biopolymers 25*, 469–487.

Chapman, D., Jackson, M., & Haris, P. I. (1989) *Biochem. Soc. Trans. 17*, 617–619.

Chirgadze, Y. N., Fedorov, O. V., & Trushina, N. P. (1975) *Biopolymers 14*, 679–694.

Cuatrecasas, P. (1973) *Biochemistry 12*, 3558–3566.

Cuatrecasas, P., Parikh, I., & Hollenberg, M. D. (1973) *Biochemistry 12*, 4253–4264.

Dalziel, A. W., Lipka, G., Chowdhry, B. Z., Sturtevant, J. M., & Schafer, D. E. (1984) *Mol. Cell. Biochem. 63*, 83–91.

De Wolf, M. J. S., Fridkin, M., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 5489–5496.

De Wolf, M. J. S., Van Dessel, G. A. F., Lagrou A. R., Hilderson, H. J. J., & Dierick, W. S. H. (1987) *Biochemistry 26*, 3799–3806.

Dwyer, J. D., & Bloomfield, V. A. (1982) *Biochemistry 21*, 3227–3231.

Felgner, P. L., Freire, E., Barenholz, Y., & Thompson, T. E. (1981) *Biochemistry 20*, 2168–2172.

Finkelstein, R. A. (1988) in *Handbook of Natural Toxins, Bacterial Toxins* (Hardegree, M. C., & Tu, A. T., Eds.) Vol. 4, pp 1–38, Marcel Dekker, New York.

Fishman, P. H. (1982) *J. Membr. Biol. 69*, 85–97.

Fishman, P. H., Moss, J., & Osborne, J. C. (1978) *Biochemistry 17*, 711–716.

Fraser, R. D. B., & Suzuki, E. (1966) *Anal. Chem. 38*, 1770–1773.

Gill, D. M. (1976) *Biochemistry 15*, 1242–1248.

Goins, B., & Freire, E. (1985) *Biochemistry 24*, 1791–1797.

Goins, B., & Freire, E. (1988) *Biochemistry 27*, 2046–2052.

Kauppinen, J. K., Moffatt, D. J., Mantsch, H. H., & Cameron, D. C. (1981) *Appl. Spectrosc. 35*, 271–277.

Khan, M. Y., Villanueva, G., & Newman, S. A. (1989) *J. Biol. Chem. 264*, 2139–2142.

Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 181–364.

Lai, C. Y. (1980) *CRC Crit. Rev. Biochem. 9*, 171–206.

Lai, C. Y., Mendez, E., Chang, D., & Wang, M. (1977) *Biochem. Biophys. Res. Commun. 74*, 215–222.

Lonnroth, I., & Holmgren, J. (1973) *J. Gen. Microbiol. 76*, 417–427.

Ludwig, D. S., Holmes, R. K., & Schoolnik, G. K. (1985) *J. Biol. Chem. 23*, 12528–12534.

Ludwig, D. S., Ribi, H. O., Schoolnik, G. K., & Kornberg, R. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8585–8588.

Moss, J., & Vaughan, M. (1988) in *Handbook of Natural Toxins, Bacterial Toxins* (Hardegree, M. C., & Tu, A. T., Eds.) Vol. 4, pp 39–87, Marcel Dekker, New York.

Moss, J., Osborne, J. C., Fishman, P. H., Brewer, H. B., Vaughan, M., & Brady, R. O. (1977a) *Proc. Natl. Acad. U.S.A. 74*, 74–78.

Moss, J., Richards, R. L., Alving, C. R., & Fishman, P. H. (1977b) *J. Biol. Chem. 252*, 797–798.

Mullin, B. R., Aloj, S. M., Fishman, P. H., Lee, G., Kohn, L. D., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1679–1683.

Ribi, H. O., Ludwig, D. S., Mercer, K. L., Schoolnik, G. K., & Kornberg, R. D. (1988) *Science 239*, 1272–1276.

Sattler, J., Schwarzmann, G., Staerk, J., Ziegler, W., & Wiegandt, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 159–163.

Sattler, J., Schwarzmann, G., Kneck, I., Rohm, K. H., & Wiegandt, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 719–723.

Sturtevant, J. M. (1987) *Annu. Rev. Phys. Chem. 38*, 463–488.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115–130.
Surewicz, W. K., & Mantsch, H. H. (1990) in *Protein Engineering—Approaches to the Manipulation of Protein Folding* (Narang, S., Ed.) pp 131–157, Butterworth, Stoneham, MA.
Surewicz, W. K., Szabo, A. G., & Mantsch, H. H. (1987) *Eur. J. Biochem. 167*, 519–523.
Surewicz, W. K., Stepanik, T. M., Szabo, A. G., & Mantsch, H. H. (1988) *J. Biol. Chem. 263*, 786–790.
Susi, H. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G., Eds.) pp 575–663, Marcel Dekker, New York.
Tomasi, M., Battistini, A., Cardelli, M., Sonnino, S., & D'Agnolo, G. (1984) *Biochemistry 23*, 2520–2526.
van Heyningen, W. E., Carpenter, C. C. J., Pierce, N. F., & Greenough, W. (1971) *J. Infect. Dis. 124*, 415–418.

^{1}H NMR Assignment and Secondary Structure of the Ca^{2+}-Free Form of the Amino-Terminal Epidermal Growth Factor like Domain in Coagulation Factor X†

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ABSTRACT: Blood coagulation factor X is composed of discrete domains, two of which are homologous to the epidermal growth factor (EGF). The N-terminal EGF like domain in factor X (fX-EGF_N), residues 45–86 of the intact protein, contains a β-hydroxylated aspartic acid and has one Ca^{2+}-binding site. Using 2D NMR techniques, we have made a full assignment of the 500-MHz ^{1}H NMR spectrum of Ca^{2+}-free fX-EGF_N. On the basis of this assignment and complementary NOESY experiments, we have also determined the secondary structure of Ca^{2+}-free fX-EGF_N in water solution. Residues 45–49 are comparatively mobile, whereas residues 50–56 are constrained by two disulfide bonds to one side of an antiparallel β-sheet involving residues 59–64 and 67–72. Another antiparallel β-sheet involves residues 76–77 and 83–84. A small, parallel β-sheet connects residues 80–81 and 55–56 and thereby orients the two antiparallel β-sheets relative to each other. Four β-turns are identified, involving residues 50–53, 56–59, 64–67, and 73–76. Residues 78–82 adopt an extended bend structure. On the basis of secondary structure and the location of the three disulfide bonds, we find that Asp 46, Asp 48, and Hya 63 are sufficiently close to each other to form a Ca^{2+}-binding site. However, the amino terminus of the Ca^{2+}-free form of fX-EGF_N is not part of a triple-stranded β-sheet as in other EGF like peptides. Differences and similarities between fX-EFG_N and murine EGF with respect to secondary structure and conformational shifts are discussed.

During the past years, a large number of extracellular and membrane proteins have been found to contain domains homologous to the epidermal growth factor (EGF)[1] (Appella et al., 1988). Among these are proteins involved in blood coagulation, i.e., factors VII, IX, X, and XII and protein C (Furie & Furie, 1988), and in fibrinolysis, such as urokinase and the tissue-type plasminogen activator (Patthy, 1985). In particular, factors IX and X and protein C have two tandemly arranged EGF like domains, where the N-terminal one contains a hydroxylated aspartic acid residue (*erythro*-β-hydroxyaspartic acid, Hya) formed by postribosomal hydroxylation of aspartic acid (Drakenberg et al., 1983; McMullen et al., 1983). The Hya residue is always found in the consensus sequence Cys-X-Hya-X-X-X-X-Tyr/Phe-X-Cys-X-Cys (Stenflo et al., 1987), and the EGF like, Hya-containing domains usually also have two Asp/Glu N-terminal to the first Cys residue (Figure 1).

It has recently been demonstrated that the Hya-containing EGF like domains in factor IX (Huang et al., 1989; Handford et al., 1990), factor X (Persson et al., 1989), and protein C (Öhlin et al., 1988) bind Ca^{2+}. A Ca^{2+}-dependent interaction between a monoclonal antibody and the isolated EGF like region of protein C suggests that binding of Ca^{2+} imposes structural changes in the domain (Öhlin & Stenflo, 1987). This local change also seems to cause a global structural change in the protein, since binding of Ca^{2+} to the EGF like domain has a dramatic effect on the rate of activation of protein C by thrombin–thrombomodulin (Esmon et al., 1983).

Coagulation factor X, the subject of the study, is a vitamin K dependent zymogen of a serine protease. In its active form

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[1] Abbreviations: NMR, nuclear magnetic resonance; COSY, *J*-correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; R-COSY, relayed COSY; DR-COSY, double-relayed COSY; 2QF-COSY, double-quantum-filtered COSY; 2Q, double-quantum; 2D, two-dimensional; EGF, epidermal growth factor; TGFα, transforming growth factor α; Hya, *erythro*-β-hydroxyaspartic acid; Hyn, *erythro*-β-hydroxyasparagine; Gla, γ-carboxyglutamic acid.

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