A Spectral Study of Cobalt(II)-Substituted *Bacillus cereus* Phospholipase C†

Roy Bicknell,* Graeme R. Hanson,‡ and Barton Holmquist
Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115
Clive Little
Institute of Medical Biology, University of Tromso, N-9000 Tromso, Norway
Received January 30, 1986; Revised Manuscript Received April 3, 1986

ABSTRACT: The coordination sphere of both the structural and catalytic zinc ions of *Bacillus cereus* phospholipase C has been probed by substitution of cobalt(II) for zinc and investigation of the resultant derivatives by a variety of spectroscopic techniques. The electronic absorption, circular dichroic, magnetic circular dichroic, and electron paramagnetic resonance spectra were found to be strikingly similar when cobalt(II) was substituted into either site and are consistent with a distorted octahedral environment for the metal ion in both sites. Octahedral coordination appears comparatively rare in zinc metalloenzymes but has been suggested for glyoxalase I [Sellin, S., Eriksson, L. E. G., Aronsson, A.-C., & Mannervik, B. (1983) *J. Biol. Chem. 258*, 2091–2093; Garcia-Iniguez, L., Powers, L., Chance, B., Sellin, S., Mannervik, B., & Mildvan, A. S. (1984) *Biochemistry 23*, 685–689], transcarboxylase [Fung, C.-H., Mildvan, A. S., & Leigh, J. S. (1974) *Biochemistry 13*, 1160–1169], and the regulatory binding site of *Aeromonas* aminopeptidase [Prescott, J. M., Wagner, F. W., Holmquist, B., & Vallee, B. L. (1985) *Biochemistry 24*, 5350–5356]. Phospholipase C is so far unique in having two such sites.

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* contains two zinc ions (Little & Otnaess, 1975). Studies have shown that one of these is exchange-labile and catalytically essential while the other is more tightly bound and appears to have a structural role (Little & Otnaess, 1975; Little, 1981). Several metal ions, including the spectroscopic probe cobalt(II), can be substituted for zinc to give enzymes of reduced activity and altered substrate specificity (Little & Otnaess, 1975; Little et al., 1982). However, the structural characteristics of the metal binding sites have not been previously investigated, although the zinc ions are known from X-ray crystallography to be separated by around 6 Å (Aalmo et al., 1984). This paper presents a detailed spectral study of the cobalt(II)-substituted enzyme. The spectra suggest that Co(II) ions—and by inference zinc ions—occupy octahedral coordination sites and that the coordination environments of the two ion sites are remarkably similar.

Materials and Methods

Materials. Phospholipase C was isolated from cultures of *Bacillus cereus* as described by Myrnes and Little (1980). Phospholipase C concentrations were determined from A_{280} where $\epsilon = 51\,000$ M^{-1} cm^{-1} (Little, 1977). [Co,Zn(PLC)][1] was prepared by extensive dialysis of [Zn,Zn(PLC)] against solutions of cobalt(II) chloride (2×10^{-3} M) at pH 7.3 (Little, 1971). [–,Co(PLC)] and [Co,Co(PLC)] were prepared by first making apoenzyme (Little, 1977) and then either adding 0.9 mol of Co(II)/mol of apoenzyme (for [–,Co(PLC)]) or dialyzing apoenzyme against solutions of cobalt(II) chloride (2×10^{-3} M). Enzyme samples were concentrated by ultrafiltration, free Co(II) being removed by repeated concentration of enzyme followed by dilution in 0.1 M sodium acetate (pH 6.0). Free Co(II) represented <2% of the total Co(II) in the enzyme sample.

Methods. Electronic absorption spectra were recorded with a Cary 219 spectrophotometer interfaced to an Apple IIe microcomputer. CD and MCD measurements were performed at 25 °C on a Cary 61 circular dichroic spectropolarimeter equipped with a Varian 4145 superconducting magnet and a Varian 4106 supreconducting power supply. The Cary 61 is interfaced to an Apple IIe microcomputer allowing signal averaging and data manipulation procedures to be routinely performed.

X-band EPR spectra were obtained with a Varian E-109 EPR spectrometer. An Air Products Heli-Tran liquid helium transfer line was employed to obtain sample temperatures of 14 K. The magnetic field was calibrated with a Radiopan NMR magnetometer, and the microwave frequency was measured with an Autonet Model 331 microwave frequency counter. Atomic absorption analyses were performed in triplicate with a Perkin-Elmer 5000 graphite furnace spectrophotometer.

Results

Metal Content. Three forms of Co(II)-substituted phospholipase C have been prepared in which Co(II) is substituted into (i) the catalytic metal binding site, [Co,Zn(PLC)], (ii) the structural metal binding site, [–,Co(PLC)], and (iii) both sites, [Co,Co(PLC)]. Atomic absorption analysis showed their cobalt content to be 1.2, 1.05, and 1.8 mol of Co/mol of protein

† This work was supported by funds for Research and Teaching in the CBBSM. R.B. holds an SERC/NATO British Overseas Postdoctoral Fellowship. G.R.H. was supported during the EPR study by National Institutes of Health Grant RR-01008 awarded to the National Biomedical ESR Center, University of Wisconsin, Milwaukee, WI.
* Author to whom correspondence should be addressed.
‡ Present address: Department of Physics, Monash University, Clayton, Victoria, Australia.

[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; PLC, phospholipase C; [X,Y(PLC)], phospholipase C in which X is the metal bound in the exchange-labile catalytic site and Y is the metal bound in the tighter structural site; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/86/0425-4219$01.50/0

Table I: Electronic Absorption, CD, and MCD Spectral Properties of Co(II)-Substituted Phospholipase C[a]

	electronic absorption		CD		MCD	
enzyme	λ_{max}	ϵ	$\lambda_{extrema}$	$\Delta\epsilon \times 10^3$	$\lambda_{extrema}$	$\Delta\epsilon_M \times 10^3$
[Co,Zn(PLC)]	481 (s)	17	481 (s)	−27		
	507 (s)	20	500 (s)	−38	493 (s)	−30
					516	−38
	534	21	529	−44	572 (s)	−36
			580 (s)	19		
	~590 (s)	6	593	25		
[−,Co(PLC)]	478 (s)	28	478 (s)	−27		
	500	32	504 (s)	−38	500 (s)	−35
	540	35	532	−40	526	−51
	~585 (s)	29	584	57	572	−45
[Co,Co(PLC)]	475 (s)	34	482 (s)	−44		
	500	40	500 (s)	−50	498 (s)	−49
	540	45	527	−65	530	−74
	~585 (s)	33	585	80	566	−64

[a] Units for absorption (ϵ), CD ($\Delta\epsilon$), and MCD ($\Delta\epsilon_M$) are M^{-1} cm^{-1}, M^{-1} cm^{-1}, and M^{-1} cm^{-1} T^{-1}, respectively, and are calculated on the basis of the cobalt(II) concentration of each sample determined by atomic absorption analysis; (s) refers to a shoulder.

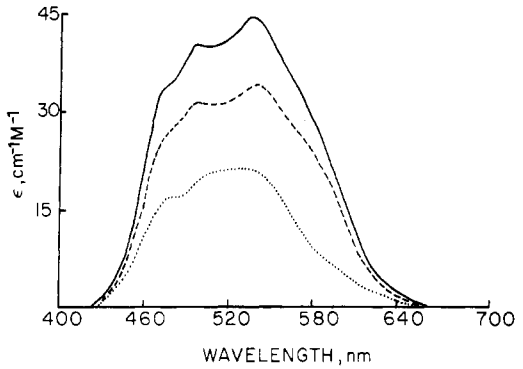

FIGURE 1: Electronic absorption spectra of Co(II)-substituted phospholipase C: [Co,Co(PLC)] (—), [−,Co(PLC)] (−−), and [Co,Zn(PLC)] (···). Spectra were recorded in 0.1 M sodium acetate/acetic acid at pH 6.0. [Zn,Zn(PLC)] was used as reference to correct for the tailing absorption from the tryptophan residues.

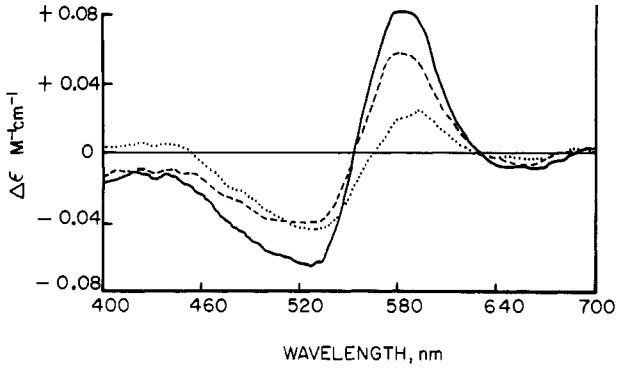

FIGURE 2: CD spectra of Co(II)-substituted phospholipase C: [Co,Co(PLC)] (—), [−,Co(PLC)] (−−), and [Co,Zn(PLC)] (···). Spectra were recorded in 0.1 M sodium acetate/acetic acid at pH 6.0 and room temperature.

for [−,Co(PLC)], [Co,Zn(PLC)], and [Co,Co(PLC)], respectively. The samples were also analyzed for Zn, Cu, Fe, and Mn. The Zn content of [Co,Zn(PLC)] was 0.95 mol/mol. In all other cases the metal content was below the detectable limit, i.e., Zn < 0.005, Cu < 0.02, Fe < 0.03, and Mn < 0.01 mol/mol. The greater affinity of apoenzyme for Zn(II) relative to Co(II), together with significantly tighter binding at the structural site (Little & Otnaess, 1975; Little, 1981), precluded preparation of [Zn,Co(PLC)].

Electronic Absorption Spectra. The electronic absorption spectra of phospholipase C in which Co(II) has been substituted into (i) the catalytic site, [Co,Zn(PLC)], (ii) the structural site, [−,Co(PLC)], and (iii) both sites, [Co,Co(PLC)], are shown in Figure 1. The wavelength maxima for the major bands and their molar absorptivities are summarized in Table I. The spectra of the two mono-Co(II) forms did not change with time (up to 4 weeks), indicating that the Co(II) ions do not exchange between the two sites. The striking feature of the absorption spectra is their low molar absorptivities, especially that of [Co,Zn(PLC)]. The low ϵ and the comparatively low wavelength of λ_{max} both suggest octahedral coordination sites (Holmquist et al., 1975). The absorption spectra of the mono-Co(II)-substituted species, apart from showing similarity to each other with regard to the band positions, also show close similarity to that of Co(II)-substituted glyoxylase I (Sellin et al., 1983) in which the metal ion has been shown to occupy a distorted octahedral site by EXAFS spectroscopy (Garcia-Iniguez et al., 1984). The molar absorptivities of the substituted phospholipases are somewhat larger than those of symmetric octahedral Co(II) complexes [e.g., $Co(H_2O)_6^{2+}$ (Sutton, 1968)], indicative of distortion of the coordination environment, a common occurrence in zinc metalloenzymes (Vallee & Galdes, 1984).

Circular Dichroic and Magnetic Circular Dichroic Spectra. The CD and MCD spectra of the three cobalt-substituted phospholipase samples are shown in Figures 2 and 3, respectively, and the spectral data are summarized in Table I. Four bands seen in each CD spectrum correspond closely to transitions observed in the visible spectra. The three bands of lowest wavelength exhibit negative ellipticity. In contrast, the highest wavelength band, which is the weakest in the visible absorption spectrum, shows a strong positive Cotton effect. The differences in the CD spectra of the two monocobalt forms indicate that the two metal binding sites are not identical.

A clear differentiation of the cobalt atoms when in different sites is also apparent in the MCD. The negative extremum near 570 nm, present in [Co,Co(PLC)] and [−,Co(PLC)], is absent in [Co,Zn(PLC)]. The relatively low wavelength and low intensity of the MCD spectra suggest octahedral cobalt coordination and clearly contrast with the MCD of cobalt-substituted enzymes known from X-ray crystallography to have four- or five-coordinate geometries, e.g., carboxypeptidase A (Figure 3b). In fact, the MCD spectra of the cobalt in both metal binding sites of phospholipase C are more similar to model six-coordinate complexes (Holmquist et al., 1975) than has previously been observed for any zinc metalloenzyme.

EPR Spectra. The EPR spectra of the three Co(II)-substituted species are shown in Figure 4, and the effective g values are listed in Table II. The disappearance of the EPR signals from each cobalt derivative at temperatures greater than 20 K and the observed g anisotropy (Table II) indicate

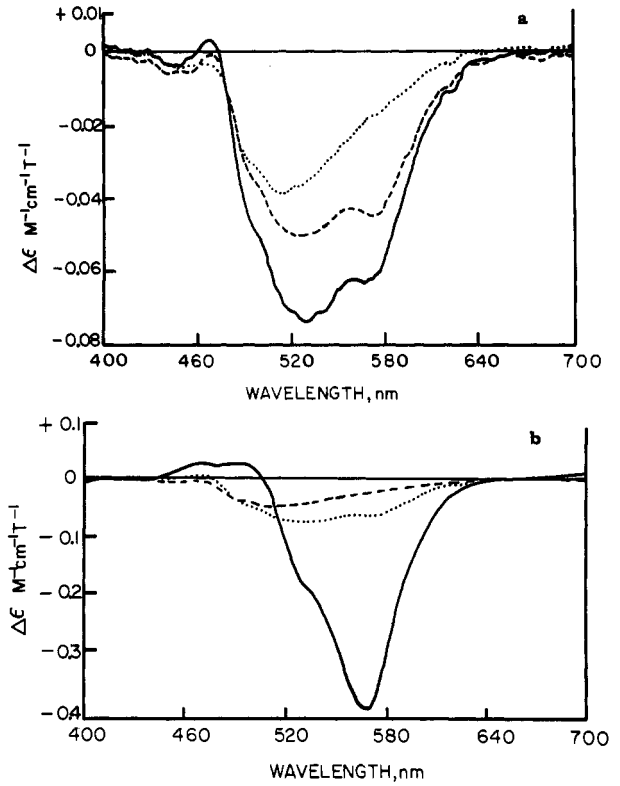

FIGURE 3: (a) MCD spectra of Co(II)-substituted phospholipase C: [Co,Co(PLC)] (—), [–,Co(PLC)] (– –), and [Co,Zn(PLC)] (···). Spectra were recorded in 0.1 M sodium acetate/acetic acid, pH 6.0, at room temperature. (b) Comparison of the MCD spectra of Co(II) carboxypeptidase A (—) with those of Co(II)-substituted phospholipase C, [Co,Zn(PLC)] (– –) and [Co,Co(PLC)] (···). Spectra were recorded as in (a) except that Co(II) carboxypeptidase A was prepared in 0.05 M Hepes/1 M sodium chloride, pH 7.0.

Table II: EPR Parameters for Co(II)-Substituted Phospholipase C

enzyme	A_1 (cm^{-1})	g_1	g_2	g_3	g_{iso}[a]
[Co,Zn(PLC)]	0.025	6.88	2.73	1.99	3.87
[–,Co(PLC)]	0.026	6.87	2.72	1.99	3.86
[Co,Co(PLC)]	0.026	6.87	*b*	2.0	*b*

[a] $g_{iso} = (g_1 + g_2 + g_3)/3$. [b] Unable to measure.

the presence of high-spin Co(II) ($S = {}^3/_2$) in sites of orthorhombic or lower symmetry. Crystal field analysis of high-spin Co(II) in an octahedral environment gives rise to a single Kramers doublet ^2E for the ground state. The isotropic g value expected for transitions within this doublet, calculated from the Landē g formula, is 4.3 (Abragam & Bleaney, 1970). The difference between this value and those listed in Table II for the phospholipase samples can be reconciled by either increased covalent bonding or a distortion away from ideal octahedral geometry, as suggested by the MCD spectra.

Coupling of the electron and nuclear spins [$J = {}^1/_2$ and $I = {}^7/_2$ for octahedral Co(II)] gives rise to eight transitions observed on the g_1 resonance in the EPR spectrum of [Co,Zn(PLC)] with a hyperfine coupling constant of $A_1 = 0.025$ cm^{-1}. The hyperfine coupling is poorly resolved in the EPR spectrum of [–,Co(PLC)] and almost completely absent from the EPR spectrum of [Co,Co(PLC)]. The loss of hyperfine in [Co,Co(PLC)] may be attributed to line broadening arising from interactions between the two paramagnetic centers. Hyperfine coupling of the order of 0.02–0.04 cm^{-1} in the $g = 4$–7 region is indicative of octahedral or square-pyramidal coordination to Co(II) (Bencini et al., 1981) and is observed in the EPR spectra of Co(II)-substituted glyoxalase I (Sellin et al., 1983) and Co(II)-substituted transcarboxylase (Fung et al., 1974) and when Co(II) is substituted into the magnesium binding sites of enolase (Rose et al., 1984) and alkaline phosphatase (Anderson et al., 1972). Furthermore, the effective g values for the substituted phospholipases are close to those reported for the aforementioned Co(II) enzymes and are again consistent with a distorted octahedral geometry [see Kennedy et al. (1972)].

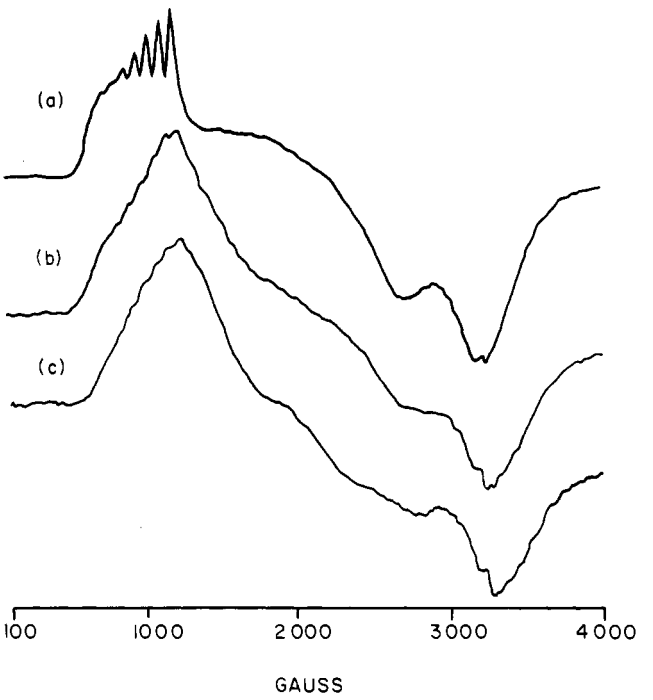

FIGURE 4: EPR spectra of Co(II)-substituted phospholipase C: (a) [Co,Zn(PLC)], (b) [–,Co(PLC)], and (c) [Co,Co(PLC)]. Spectra were recorded in 0.1 M sodium acetate/acetic acid, pH 6.0, at 14 K. Spectrometer settings were as follows: microwave frequency, (a) 9.3024, (b) 9.3009, and (c) 9.302 GHz; Microwave power, 200 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz. Twenty scans were averaged to obtain a final spectrum.

Discussion

The spectra presented here indicate that Co(II), when substituted into the strucutral or catalytic binding site of phospholipase C, occupies distorted octahedral coordination sites. Further support for octahedral coordination comes from recent EXAFS spectroscopy of phospholipase C, which indicates average ligand–metal bond lengths that are readily reconciled with distorted octahedral coordination (M. C. Feiters, S. S. Hasnain, and C. Little, unpublished work). This is unusual for a zinc metalloenzyme; four- or five-coordination is generally the rule (Vallee & Galdes, 1984). However, octahedral coordination has been suggested for glyoxalase I (Sellin et al., 1983; Garcia-Iniguez et al., 1984), transcarboxylase (Fung et al., 1976), and the regulatory binding site of *Aeromonas* aminopeptidase (Prescott et al., 1985). When Co(II) is substituted for magnesium, as in alkaline phosphatase and enolase, the spectroscopic properties of the Co(II) are also consistent with octahedral coordination (Anderson et al., 1976; Rose et al., 1984). Magnesium shows a strong preference for octahedral coordination, and in contrast to other zinc metalloenzymes, both apoglyoxalase I and [–,Zn(PLC)] bind magnesium in the catalytic site to give substituted enzymes with significant activity (Sellin et al., 1980; Little & Otnaess, 1975).

Although the two metal binding sites in phospholipase C are very similar, they are not identical. Differences were first noted in ^{113}Cd NMR studies on [Cd,Cd(PLC)] (Aalmo et al.,

1984) and are apparent in the electronic absorption as well as the CD and MCD spectra. In the CD, the positive band in the region of 590 nm is much less intense when cobalt is in the catalytic site ($\Delta\epsilon = 25 \times 10^{-3}$ M^{-1} cm^{-1}) than when in the structural site ($\Delta\epsilon = 57 \times 10^{-3}$ M^{-1} cm^{-1}), whereas in the MCD spectra the 572-nm band appears to signal the cobalt atom at the structural site. The CD spectra are remarkably similar for different binding sites, especially in view of the fact that the catalytic metal is exchange-labile while the structural metal is so tightly bound that it is only removed by prolonged dialysis against EDTA (Little & Otnaess, 1975).

In contrast to tetrahedral Co(II) complexes in which distortion from perfect ligand symmetry decreases the molar absorptivity, distortion in octahedral Co(II) complexes enhances this parameter (Ballhausen, 1960). The larger molar absorptivity of Co(II)-substituted PLC relative to those of model octahedral complexes is consistent with asymmetry arising from both (i) a combination of nitrogen and oxygen ligands and (ii) a slight distortion from six- toward four/five-coordination. Distortion is a characteristic feature of the metal binding sites in zinc metalloenzymes (Vallee & Galdes, 1984) and is thought to be of importance in their catalytic mechanism. This premise forms the basis of the entatic-state hypothesis of Vallee and Williams (1968). Recent cryospectrokinetic studies of catalysis by carboxypeptidase A (Auld et al., 1984) and β-lactamase II (Bicknell & Waley, 1985) have lent support to those postulates.

The spectra of [Co,Co(PLC)] are not simply the sum of the spectra of the two monocobalt-substituted enzymes, indicating that metal binding in one site promotes a conformational change in the ligand environment (and hence spectra) of the metal ion in the second site. This is not surprising in view of the 6-Å separation of the metal sites revealed by X-ray crystallography (Aalmo et al., 1984). Interacting metal binding sites have been previously characterized in *Aeromonas* aminopeptidase (Prescott et al., 1985).

Active Site of Phospholipase C. The natural substrates of phospholipase C are large water-insoluble phospholipid structures, for example, cell membranes. In addition, the enzyme has been found to catalyze the hydrolysis of monomolecularly dispersed short-chain synthetic lecithins, e.g., dihexanoyl-, diheptanoyl-, and dioctanoyllecithins, at rates that are much faster when these artificial substrates are present as micelles (Little, 1977a; El-Sayeed et al., 1985). These observations suggest a large and extended active site with many enzyme/substrate interactions. An extended active site is supported by chemical modification studies, which have shown that, in addition to the active site zinc atom and its (unidentified) ligands, at least five additional residues have been implicated in catalysis, namely, two lysines (Aurebekk & Little, 1977a), a carboxylate (Little & Aurebekk, 1977), a histidine (Little, 1977b), and an arginine that binds substrate (Aurebekk & Little, 1977b).

Two octahedral metal coordination spheres imply 12 metal ligands. ^{133}Cd NMR of the cadmium-substituted enzyme (Aalmo et al., 1984) and recent EXAFS data on [Zn,Zn(PLC)] (M. C. Feiters, S. S. Hasnain, and C. Little, unpublished work) are consistent with a combination of nitrogen and oxygen ligands to each metal. There are no detectable sulfhydryl groups in the denatured enzyme (Otnaess et al., 1977), suggesting that cysteine is not a metal ligand. This has been confirmed here by the absence of characteristic sulfur–cobalt(II) charge-transfer transitions in the visible, CD, and MCD spectra. Four histidine metal ligands have been identified by chemical modification (Little, 1977b); however, it is not known how the four are distributed between the two metal binding sites. The remaining eight ligands are at present unidentified, although near-ultraviolet CD studies have shown that metal removal causes extensive perturbation of tyrosine residues that could be involved in metal ligation (Little, 1978). By analogy to other zinc metalloenzymes (Vallee & Galdes, 1984), the other unidentified ligands are probably a combination of enzyme carboxylate residues and displaceable solvent-derived water molecules. Further studies are required to enable identification of all the metal ligands.

ACKNOWLEDGMENTS

We thank Dr. Bert L. Vallee for advice, support, and critical reading of the manuscript.

REFERENCES

Aalmo, K. H., Hansen, L., Hough, E., Jynge, K., Krane, J., Little, C., & Storm, C. B. (1984) *Biochem. Int. 8*, 27–33.

Abragam, A., & Bleaney, B. (1970) *Electron Paramagnetic Resonance of Transition Metal Ions*, pp 446–449, Clarendon Press, Oxford.

Anderson, R. A., Kennedy, F. S., & Vallee, B. L. (1976) *Biochemistry 15*, 3710–3716.

Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041–5045.

Aurebekk, B., & Little, C. (1977a) *Biochem. J. 161*, 159–165.

Aurebekk, B., & Little, C. (1977b) *Int. J. Biochem. 8*, 757–762.

Ballhausen, C. J. (1960) *Prog. Inorg. Chem. 2*, 251.

Bencini, A., Bertini, I., Canti, G., Gatteschi, D., & Luchinat, C. (1981) *J. Inorg. Biochem. 14*, 81–93.

Bicknell, R., & Waley, S. G. (1985) *Biochemistry 24*, 6876–6887.

El-Sayeed, M., DeBose, C. D., Coury, L. A., & Roberts, M. F. (1985) *Biochem. Biophys. Acta 837*, 325–335.

Fung, C.-H., Mildvan, A. S., & Leigh, J. S. (1974) *Biochemistry 13*, 1160–1169.

Garcia-Iniguez, L., Powers, L., Chance, B., Sellin, S., Mannervik, B., & Mildvan, A. S. (1984) *Biochemistry 23*, 685–689.

Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454–1461.

Kennedy, F. S., Hill, H. A. O., Kaden, T. A., & Vallee, B. L. (1972) *Biochem. Biophys. Res. Commun. 48*, 1533.

Little, C. (1977a) *Acta. Chem. Scand., Ser. B B31*, 267–272.

Little, C. (1977b) *Biochem. J. 167*, 399–404.

Little, C. (1978) *Biochem. J. 175*, 977–986.

Little, C. (1981) *Acta. Chem. Scand., Ser. B B35*, 39–44.

Little, C., & Otnaess, A.-B. (1975) *Biochim. Biophys. Acta 391*, 326–333.

Little, C., & Aurebekk, B. (1977) *Acta. Chem. Scand., Ser. B B31*, 273–277.

Little, C., Aakre, S.-E., Rumsby, M. G., & Gwarsha, K. (1982) *Biochem. J. 207*, 117–121.

Myrnes, B., & Little, C. (1980) *Acta Chem. Scand., Ser. B. B34*, 375–377.

Otnaess, A.-B., Little, C., Sletten, K., Wallin, R., Johnson, S., Flengsrud, R., & Pyrdz, H. (1977) *Eur. J. Biochem. 79*, 459–468.

Prescott, J. M., Wagner, F. W., Holmquist, B., & Vallee, B. L. (1985) *Biochemistry 24*, 5350–5356.

Rose, S. I., Dickinson, L. C., & Westhead, E. W. (1984) *J. Biol. Chem. 259*, 4405–4413.

Sellin, S., Aronsson, A.-C., & Mannervik, B. (1980) *Acta. Chem. Scand., Ser. B B34*, 541–543.

Sellin, S., Eriksson, L. E. G., Aronsson, A.-C., & Mannervik, B. (1983) *J. Biol. Chem. 258*, 2091–2093.
Sutton, D. (1968) *Electronic Spectra of Transition Metal Complexes*, McGraw-Hill, New York.
Vallee, B. L., & Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498–505.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–430.

Novel Preparation of Functional Sindbis Virosomes†

Ronald K. Scheule
Department of Pharmacology, The University of Texas Medical School, Houston, Texas 77225
Received December 10, 1985; Revised Manuscript Received February 10, 1986

ABSTRACT: Lipid and protein factors important for the preparation and stability of reconstituted membranes prepared by the insertion of detergent-solubilized Sindbis virus glycoproteins into preformed lipid vesicles have been defined. It was found that both the state of aggregation of the membrane protein and the phase of the lipid are critical for the insertion of proteins into preformed lipid vesicles. The membranes prepared with the insertion technique were characterized in terms of residual detergent, protein orientation, and whether or not they were sealed. Binding and fusion experiments have been carried out with the insertion membranes and virus. It was found that BHK-21 cells at 4 °C bound one-fifth to one-tenth the number of insertion membranes as intact virus, and binding was saturable in both cases. Variation of the lipid/protein ratio did not result in significant differences in binding. The insertion membranes were found to fuse to a model lipid bilayer at least as well as the virus. These results are discussed in terms of structural factors important for the biological functionalities of the viral spike glycoproteins.

Sindbis virus is an enveloped virus approximately 700 Å in diameter (Harrison et al., 1971), composed of equimolar copies (presumably 240 per virion) of two glycoproteins embedded in its lipid coat and a third protein associated with the encapsulated viral RNA. Although the amino acid sequences and stoichiometries of the individual viral proteins are known, the molecular factors that are necessary for viral binding and fusion are understood only poorly. These two aspects of viral functionality are thought to have their origins in the "spike" glycoproteins of the viral envelope.

Sindbis virus, like many other enveloped viruses (Marsh et al., 1982, 1983a), attaches to a receptor on the host cell surface and is taken up by endocytosis. The low pH of the endosome is thought to trigger a fusion between the viral envelope and the endosomal membrane, releasing the viral RNA into the cell cytoplasm. The cell-surface receptor of Sindbis virus is presumed to be a protein, and two different candidates have been identified. A 90000-dalton cell-surface protein has been identified, by cross-linking, as the receptor for Sindbis virus on JY and Daudi cells (Maassen & Terhorst, 1981), while a 45000-dalton protein has been implicated as the viral receptor on BHK cells (Duda & Berencsi, 1980). Although another togavirus, Semliki Forest virus, appears to bind to HLA-A and HLA-B antigens (Helenius et al., 1978), it can also infect cells lacking these antigens (Oldstone et al., 1980).

Thus, while some progress has been made in identifying the cell-surface receptors of these enveloped viruses, relatively little is known about the requirements for the receptor–virus binding interaction. For example, to what extent are the binding and fusion functions of the spike proteins dependent on the specific arrangement of the spikes in the viral envelope? Also, one may ask whether or not these biological functions are indeed properties of the individual components of the viral spike or whether the two envelope proteins act in a concerted fashion to accomplish a given biological function.

The structural and compositional similarity of Semliki Forest and Sindbis viruses suggests that results of reconstitution with envelope proteins of the former virus are likely to be applicable to those of the latter. Two forms of the spike proteins of Semliki Forest virus have been used previously to examine binding to cells, namely, protein micelles (Fries & Helenius, 1979) and reconstituted membranes (virosomes) prepared by cosolubilization (Marsh et al., 1983b). Lipid-free octamers comprised of the Semliki envelope proteins can be formed by detergent disruption of the virus, sedimentation, and dialysis to remove detergent (Helenius & von Bonsdorff, 1976; Helenius et al., 1977; Simons et al., 1978). The apparent binding constants to cells for the octameric complexes were 100–1000 times lower than those for intact virus; the number of particles bound was the basis for comparison (Fries & Helenius, 1979). Although the binding of SFV virosomes to cells was not studied at physiological pH, binding at pH values below 7.0 was generally much less than that of the virus (Marsh et al., 1983b).

Both Sindbis virus and Semliki Forest virus have been shown to interact with lipid bilayers at low pH (Mooney et al., 1975; White & Helenius, 1980). The SFV[1] interaction with target liposomes has been determined to result in actual membrane fusion (White & Helenius, 1980). Thus, the viral ability to fuse membranes is not dependent on the interaction of the virus with a receptor. The fact that polykaryon formation can be induced in cells expressing the envelope glycoproteins of SFV

† This investigation was supported by Biomedical Research Support Grant 201-2-2526 from The University of Texas and Grant PCM 8303948 from the National Science Foundation.

[1] Abbreviations: TX-100, Triton X-100; BSA, bovine serum albumin; SFV, Semliki Forest virus; DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; EPE, egg phosphatidylethanolamine; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; MES, 2-(*N*-morpholino)ethanesulfonic acid; R-18, octadecylrhodamine; TLC, thin-layer chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NP-40, Nonidet P-40; DBED, dibenzylethylenediamine.

0006-2960/86/0425-4223$01.50/0