Characterization and Localization of CIV Polypeptides

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In order to detect the structural proteins linked with disulfide bonds, CIV was solubilized and electrophoresed under nonreducing conditions in the first dimension and then under reducing conditions in the second dimension. The viral polypeptides linked originally with disulfide bonds were separated into subunits. The complexes were trimers (P'50) or dimers (P60 and P10). The apparent molecular weights of P81, P53, and P49 changed significantly according to the composition of the lysis buffer used, suggesting that the differences in their molecular weights were due to conformational changes produced by reduction of their intramolecular disulfide bonds. Sulfhydryl-containing polypeptides (P'50-P50, P60, P100, and P33) were detected by N-[^{14}C]ethylmaleimide, and the accessibility of these residues was analyzed after successive stripping of the CIV particle. Radioiodination of external polypeptides by [^{125}I]iodosulfanilic acid shows only one intensively labeled spot corresponding to the P50 polypeptide, whereas P'50 was only slightly labeled. Six viral polypeptides P81, P60, P31, P17, P13, and P10 were revealed to possess high affinity for CIV DNA. A structural model of CIV is proposed and discussed. © 1985 Academic Press, Inc.

INTRODUCTION

Iridescent virus type 6 or CIV (Fukaya and Nasu, 1966) is a large icosahedral deoxyribovirus with an intracytoplasmic maturation site. Thin sections of virus particles show a high electron-dense, irregularly shaped core enveloped by a single unit membrane which might be of viral origin (Balange-Orange and Devauchelle, 1982). Subunits uniformly disposed on the external surface of this membrane appear to be responsible for the typical icosahedral shape of the shell (Stoltz, 1973). Biochemical studies have established its overall polypeptide (Kelly and Tinsley, 1972; Barray and Devauchelle, 1979) and lipid composition (Balange-Orange and Devauchelle, 1982). Barray and Devauchelle (1979) have shown that, after solubilization of CIV particles with SDS-β-mercaptoethanol, 16 polypeptides could be resolved with molecular weights ranging from 7000 to 120,000, with a major

polypeptide of 51,000 whereas after solubilization with SDS–urea 26 polypeptides were resolved with molecular weights ranging from 10,000 to 230,000.

The present work has extended these previous studies in establishing the characterization and localization of some structural proteins. This was achieved by (i) analysis of CIV polypeptides linked by disulfide bridges in two-dimensional gel electrophoresis, (ii) detection of sulfhydryl-containing polypeptides and analysis of accessibility of these residues after successive stripping of CIV particles, (iii) detection of external polypeptides by surface labeling, (iv) detection of DNA binding activity to identify CIV nucleoproteins.

MATERIALS AND METHODS

Virus. CIV was grown in late instar *Galleria mellonella* larvae. Larvae were infected by intrahemocoelic inoculation of a sterile suspension of CIV. Twelve days later, larvae were sacrificed and the virus was extracted and purified as described by Kelly and Tinsley (1972).

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SDS-polyacrylamide gradient gel electrophoresis. One-dimensional SDS-polyacrylamide gradient gel electrophoresis was carried out using the Laemmli (1970) discontinuous buffer system. Samples were treated with lysis buffer containing either 2% SDS, 5% β-mercaptoethanol (SDS-β-ME lysis buffer) or 2% SDS alone (SDS-lysis buffer). Samples were boiled for 5 min and analyzed on a 8-16% linear polyacrylamide gradient gel. The gels were run at 150 V for approximately 4 hr, and stained with Coomassie blue R-250.

For two-dimensional SDS-polyacrylamide gradient gel electrophoresis, samples were treated with SDS-lysis buffer. They were boiled for 5 min and analyzed in the first dimension on 8-16% linear polyacrylamide gradient gel rods (14 cm). After electrophoresis the gel rods were incubated for 1 hr in 0.05 M Tris-hydrochloride, pH 6.8, 15% glycerol, 2% SDS, and 5% β-mercaptoethanol, and layered on a 8-16% linear gradient slab gel (14 × 18 cm) for the second dimension.

N-[^{14}C]ethylmaleimide labeling of viral polypeptides. Purified viral suspension in 0.01 M sodium phosphate buffer, pH 7, was distributed into three batches. Batch 1 consisted of 100 μl of viral suspension and batches 2 and 3 of 100 μl of viral suspension + 20 μl 10% SDS.

All batches received 40 μl (12.5 μCi) of *N*-[^{14}C]ethylmaleimide (NEM) (2-10 mCi/mmol Amersham) and were incubated at 37°. Four hours later, the reaction was stopped in batches 1 and 2 by the addition of β-mercaptoethanol (final concentration 5%). Batch 3 was boiled for 5 min prior to the addition of β-mercaptoethanol. The three-labeled viral extracts were treated with SDS-β-ME lysis buffer and analyzed by one-dimensional polyacrylamide gel electrophoresis. One sample from batch 3 was analyzed by two-dimensional electrophoresis. Slab gels were stained, dried, and subjected to autoradiography using Fuji X-ray films.

Surface labeling of CIV particles. Surface viral polypeptides were labeled with ^{125}I using an iodosulfanilic acid-labeling kit (New England Nuclear). Prior to coupling with proteins, [^{125}I]iodosulfanilic acid was converted to the diazonium salt by addition of 5 μl of 0.05 M NaNO$_2$ and 5 μl of 0.1 M HCl. Five minutes later, the reaction mixture was neutralized by the addition of 0.05 M phosphate buffer, pH 7.5.

The purified viral suspension was distributed into two identical batches. Batch 1 consisted of 2 ml of viral suspension treated for 3 hr with SDS (final concentration 2%). Batch 2 consisted of 2 ml of native CIV. Twenty-five microliters of the neutralized diazonium salt were added to batches 1 and 2 and the reaction mixtures were gently stirred in an ice bath. Fifteen minutes later, unreacted diazonium salt was discarded from the native viral suspension (batch 2) by sedimenting the virus through a 20-40% (w/v) sucrose gradient. The sample was centrifuged at 13,000 rpm in a Beckman SW27 rotor for 30 min. Purified labeled viral suspension was then pelleted at 14,000 rpm in a Sorvall SS-34 rotor for 1 hr. The pelleted virus was resuspended in SDS-lysis buffer. The labeled viral suspension of batch 1 was directly mixed with SDS-lysis buffer. Both viral preparations were analyzed by two-dimensional polyacrylamide gel electrophoresis. Slab gels were stained, dried, and subjected to autoradiography.

DMSO treatment of viral particles. A purified viral suspension was pelleted at 15,000 rpm in a Sorvall SS-34 rotor for 30 min and resuspended in 140 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, 5 mM glucose, 20 mM HEPES, pH 7.05, DMSO 10%. After standing overnight at 4°, the viral suspension was layered on 10% sucrose and centrifuged at 36,000 rpm in a Beckman T40 rotor for 90 min. The supernatant was extensively dialyzed at 4° against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl.

Detection of DNA-binding proteins. DNA-binding activity of CIV structural polypeptides was detected by the method reported by Bowen *et al.* (1980). Viral polypeptides were electrophoreses in one- or two-dimensional polyacrylamide gradient gel slabs, and the proteins were transferred onto nitrocellulose sheets (Schleicher and Schüll BA85, 0.45 μm). The blots were preincubated for 30 min at room temperature in 1 mM EDTA, 10

mM Tris–hydrochloride, pH 7.0, 50 mM NaCl, 0.02% bovine serum albumin fraction V, 0.02% Ficoll 400, and 0.02% polyvinylpyrrolidone 360. The blots were then incubated at 37° with ^{32}P-labeled viral DNA at a concentration of 5 μg/ml (10^4 cpm/ml). After 6 hr incubation, blots were rinsed four times with 2× SSC for 30 min at room temperature, dried, and autoradiographed.

CIV viral DNA was isolated from ^{32}P-labeled virus by treatment with pronase (500 μg/ml) in the presence of 1% SDS, followed by extraction with phenol–chloroform. Purified viral DNA was used without heat denaturation.

RESULTS

Analysis of CIV Polypeptides

We have compared the electrophoretic behavior of CIV polypeptides under reducing and nonreducing conditions. CIV samples were treated either with SDS (SDS–PAGE) or with SDS + β-ME (SDS–β-ME–PAGE) lysis buffer. Electrophoretic patterns (Fig. 1) show very significant differences, in particular the presence in SDS–PAGE, of a second major polypeptide with a high-molecular-weight (SDS 6 polypeptide, 150K MW). The relationship between these two different profiles was established by two-dimensional polyacrylamide gradient gel electrophoresis as described under Materials and Methods. Figure 2A shows that some of the viral polypeptides have a particular behavior. Mercaptoethanol nonsensitive polypeptides migrated in the same manner in the two dimensions and were lined up as a diagonal in accordance with their molecular sizes. Some polypeptides migrated as much smaller molecules in the second dimension; for example, the major polypeptide SDS 6 migrated in the first dimension at 150K MW and corresponded to a trimeric form of a 50K MW polypeptide (P′50). In the same manner, polypeptides SDS 7 (120K MW) and SDS 30 (20K MW) corresponded respectively to a dimeric form of P60 and P10. Three spots (P81, P53, and P49) appeared above the
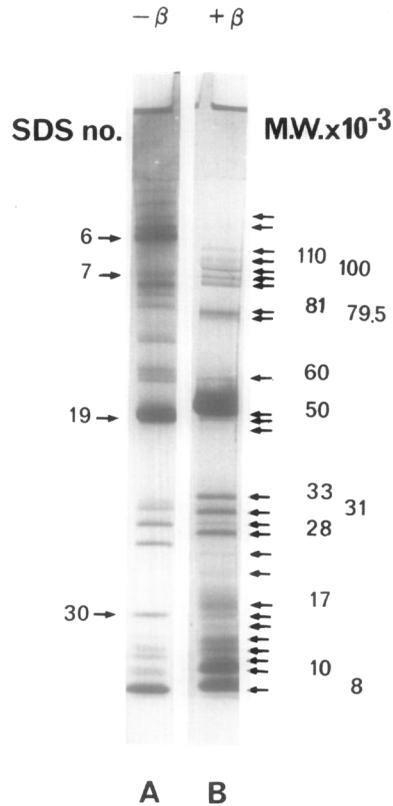
FIG. 1. Comparison of the electrophoretic behavior of CIV polypeptides under reducing and nonreducing conditions. Lane A, SDS–PAGE of CIV disrupted by SDS-lysis buffer. Lane B, SDS–PAGE of CIV disrupted by SDS + β-ME-lysis buffer.

diagonal line of polypeptides. These polypeptides migrated with a lower molecular size in the first dimension and behaved as much larger molecules in the second dimension. This shift indicates a conformational change of the molecule probably due to an intramolecular disulfide link(s). Addition of 8 M urea in the lysis buffer and in the first-dimension gel did not modify the profile of the two-dimensional electrophoresis (Data not shown.). Figure 2B shows a two-dimensional SDS–β-ME–PAGE profile since the polypeptides were solubilized at each of the two electrophoretic steps by the SDS–β-ME lysis buffer. All of the polypeptides migrated to a diagonal line. The reduced state was maintained during two successive electrophoreses.
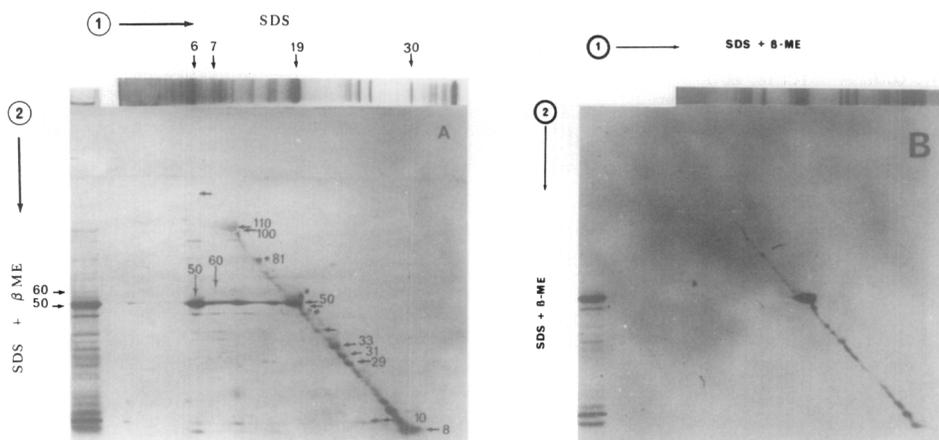
FIG. 2. Two-dimensional SDS–PAGE analysis of proteins and their disulfide complexes in CIV. CIV was fractionated in the first dimension in nonreducing (A) or reducing (B) SDS–PAGE and in the second dimension in reducing SDS–PAGE. Reference SDS–PAGE profiles are indicated along the upper end and the left sides of the two-dimensional profiles.

Accessibility of Sulfhydryl Residues in the CIV Particle

Sulfhydryl-containing polypeptides of CIV were detected in one- and two-dimensional polyacrylamide gel electrophoresis profiles after reaction with N-[^{14}C]ethyl-2,3-maleimide labeling. As described under Materials and Methods [^{14}C]NEM labeling was performed in three different CIV batches. One-dimensional autoradiogram (Fig. 3A) of native CIV (batch 1) only shows a weak labeling of (P50-P'50). The intensity of (P50-P'50) labeling was significantly increased by a 2% SDS treatment of the CIV particle (batch 2). In addition we could observe labeling of P60. In experiment 3 (batch 3), where CIV was boiled for 5 min with 2% SDS, the labeling intensity of (P50-P'50) was increasing, whereas labeling of P60 seemed unchanged. In addition we could notice the appearance of two new highly labeled polypeptides P100 and P33. Two-dimensional analysis of the latter sample (Fig. 3B) allowed further detection of all of the sulfhydryl-containing polypeptides exposed after boiling with 2% SDS. This experiment shows a very significant difference of labeling intensity between P'50 and P50.

Identification of Surface Viral Polypeptides

Surface viral polypeptides were labeled with ^{125}I by using an iodosulfanilic acid-labeling kit (New England Nuclear), and analyzed by two-dimensional polyacrylamide gel electrophoresis. Iodosulfanilic acid reacts with amino groups of tyrosine and histidine and with sulfhydryl groups of cell surface proteins (Carraway, 1975). It has been shown that diazotized sulfanilic and iodosulfanilic acids do not enter the cells and only the exposed membranes were labeled (Tinberg et al., 1974). Figure 4 shows autoradiograms obtained after ^{125}I labeling of either native CIV particles (panel B) or 2% SDS-treated CIV particles (panel A). In autoradiogram A, all the polypeptides were labeled and their labeling densities were quite similar to the Coomassie blue staining intensity.

Autoradiogram B shows only one intensively labeled spot which corresponds to P50. P'50 was only slightly labeled. After these experiments of specific labeling of surface polypeptides of the CIV particle, we have selectively solubilized the P50 polypeptide by incubating the CIV suspension with DMSO buffer. After overnight treatment at 4°, the viral suspension was layered on 10% sucrose and centri-
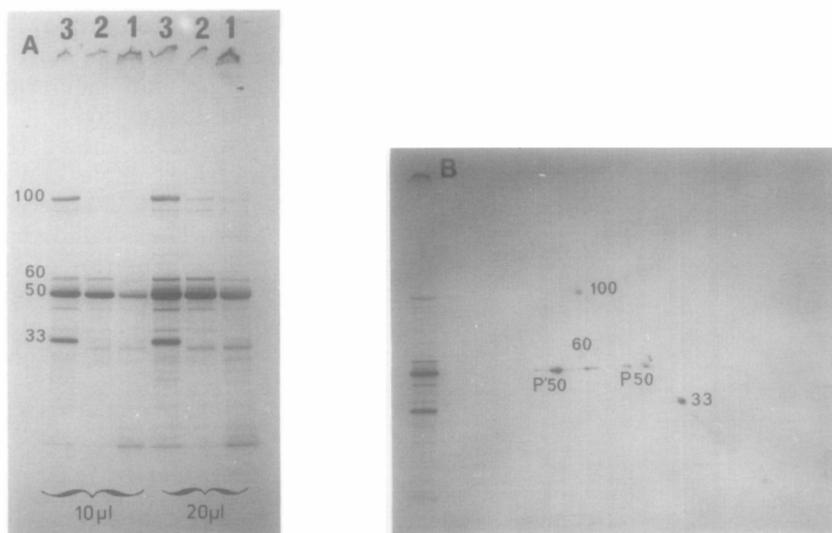
FIG. 3. Sulfhydryl-containing polypeptides of CIV. (A) One-dimensional SDS–PAGE profile of [^{14}C]NEM-labeled virus. The viral suspension was distributed into three batches. Batch 1 consisted of 100 μl of viral suspension, batches 2 and 3 of 100 μl of viral suspension + 20 μl of 10% SDS. All the batches received 40 μl of [^{14}C]NEM and were incubated at 37°. Four hours later, the reaction was stopped in batches 1 and 2 by the addition of β-mercaptoethanol (final concentration 5%). Batch 3 was boiled for 5 min prior to the addition of β-mercaptoethanol. (1) Native CIV (batch 1); (2) CIV disrupted by 2% SDS (batch 2); (3) CIV disrupted by 2% SDS and boiled for 5 min (batch 3); (B) two-dimensional SDS–PAGE profile of [^{14}C]NEM-labeled virus. An aliquot from batch 3 was analyzed by a two-dimensional SDS–PAGE with β-ME included in the second dimension. The polypeptides were revealed by autoradiography.

fuged at 36,000 rpm for 90 min. The dialyzed supernatant and pellet were analyzed by one- and two-dimensional electrophoresis. Figure 5 shows that whereas P50 was solubilized by the DMSO treatment, the trimeric form of P'50 was still found associated with the pellet.

Viral DNA-Binding Proteins

To identify polypeptides with a DNA-binding capacity we used the method previously described by Bowen *et al.* (1980). Purified virus was electrophoresed in one- or two-dimensional polyacrylamide gradient gel slabs and the polypeptides were transferred onto nitrocellulose sheets. The nitrocellulose sheets were incubated at 37° in a solution containing ^{32}P-labeled CIV DNA. After 6 hr incubation, the sheets were washed with 2× SSC. Adsorbed DNA was revealed by autoradiography. Figure 6 shows that six polypeptides P81, P60, P31, P17, P13, and P10 bound CIV DNA. All these DNA–protein associations were very stable, the adsorbed DNA could not be released by repeatingly washing the blots with 2× SSC.

DISCUSSION

Several authors have reported the presence of disulfide-linked proteins in a number of unrelated viruses, such as orthomyxoviruses (Selimova *et al.*, 1982), paramyxoviruses (Smith and Hightower, 1981; Sugawara *et al.*, 1982), coronaviruses (King and Brian, 1982), togaviruses (Waxham and Wolinsky, 1983), oncornaviruses (Yoshinaka *et al.*, 1984), herpesviruses (Sarmiento and Spear, 1979; Britt, 1984), and poxviruses (Ichihashi *et al.*, 1984). The importance of disulfide-linked proteins for biological activities, structure, and morphogenesis of viral particles is well known. Neurath *et al.* (1973) have shown that
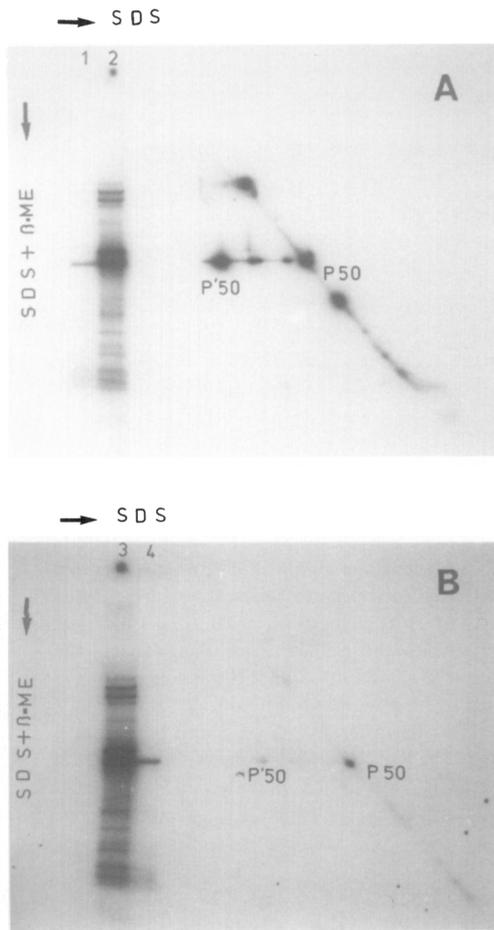
FIG. 4. Identification of surface viral polypeptides. Surface viral polypeptides were labeled with [125]I (New England Nuclear, iodosulfanilic acid labeling kit) and analyzed by two-dimensional SDS–PAGE as described under Materials and Methods. (A) Autoradiogram of two-dimensional analysis of CIV disrupted by 2% SDS and labeled with [125I]-iodosulfanilic acid. (B) Autoradiogram of two-dimensional analysis of native CIV labeled with [125I]iodosulfanilic acid. Reference profiles of monodimensional electrophoresis are indicated along the left sides of the two-dimensional profiles. Lanes 1 and 4 are native CIV; lanes 2 and 3, CIV disrupted by 2% SDS.

of HSV. This glycoprotein plays a role in viral penetration into the host cell and may mediate or promote membrane fusion (Sarmiento and Spear, 1979).

Recent studies have shown that some vaccinia polypeptide complexes were found to exist as high-molecular-weight multiplicated proteins. The disulfide bonds may participate in the formation of virus substructures by intramolecular and intermolecular binding. The abundance of such proteins in vaccinia virus suggests that virus membrane and core morphogenesis is ascribable to self assembly (Ichihashi et al., 1984).

Different polypeptide patterns have been published for members of the invertebrate iridovirus group (Kelly and Tinsley, 1972; Krell and Lee, 1974; Wagner et al., 1974). These studies demonstrated the
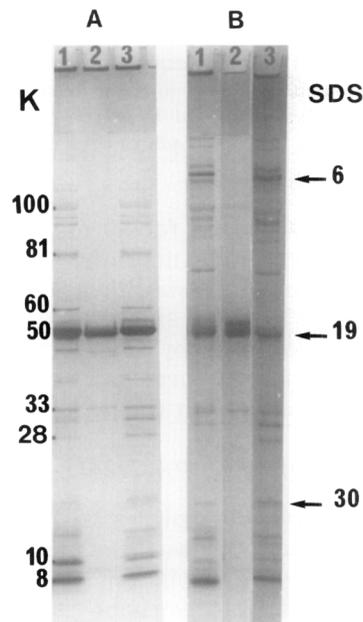
FIG. 5. Selective solubilization of P50 polypeptide by DMSO buffer. After treatment with DMSO buffer (Materials and Methods) the viral suspension was layered on 10% sucrose and centrifuged at 36,000 rpm for 90 min. The dialyzed supernatant and pellet were analyzed by one-dimensional electrophoresis under reducing (A) and nonreducing (B) conditions. Lane 1, Pellet; lane 2, supernatant; lane 3, native CIV.

Sendai virus treated with dithiothreitol lost hemagglutinin-neuraminidase and hemolysin activities. One report demonstrated the presence of disulfide-linked glycoproteins (VP$_7$B$_2$) within the envelope
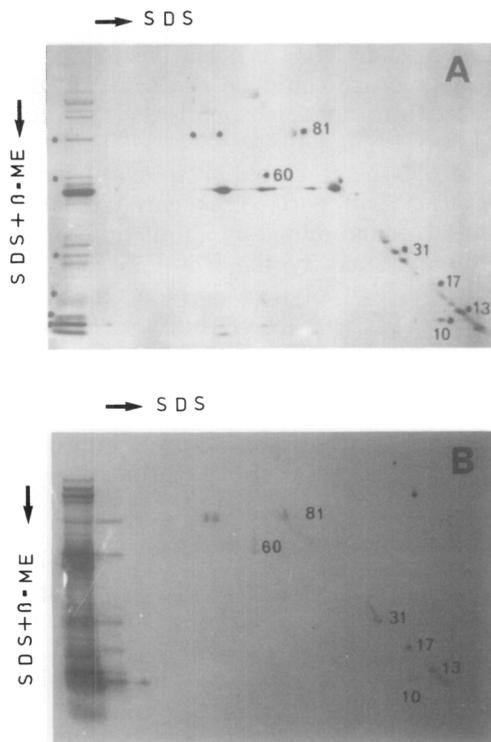
FIG. 6. CIV DNA-binding proteins. Nitrocellulose sheets blotted with viral proteins were immersed 6 hr in ^{32}P-labeled CIV DNA solution (10^4 cpm/ml). Bound DNA was detected by autoradiography. (A) Two-dimensional SDS-PAGE profile of control virus stained with Coomassie brillant blue. (B) Autoradiogram of a nitrocellulose sheet processed as described in the text and soaked in ^{32}P-labeled CIV DNA.

high complexity of the polypeptide content of these viruses but no observations were reported on the possible presence of disulfide-linked protein complexes.

The presence of trimeric subunits in the surface capsomers of FV$_3$, a vertebrate iridovirus, has been observed. However, there was no evidence for disulfide-linked proteins (F. Darcy, personal communication).

In a previous study (Barray and Devauchelle, 1979), it was shown that after solubilization of CIV with SDS-β-ME, 16 polypeptides were resolved with molecular weights ranging from 7K to 120K, with a major polypeptide of 51K. This major polypeptide represents about 40% of the total viral protein. When we compared the electrophoretic behavior of CIV polypeptides under reducing (SDS-β-ME-PAGE) and nonreducing conditions (SDS-PAGE), we could notice very significant differences, in particular the presence, in SDS-PAGE, of a second high-molecular-weight major polypeptide (SDS 6, 150K MW).

The relationship between these two different profiles was established by two-dimensional polyacrylamide gradient gel electrophoresis. This analysis showed the presence of three oligomeric proteins containing subunits linked by disulfide bonds. (i) A trimeric protein (SDS 6), composed of subunits of a 50K polypeptide (P'50) and (ii) two dimeric proteins SDS 7 and SDS 30, respectively, composed of subunits of 60K and 10K polypeptides.

Three polypeptides P81, P53, and P49 migrated as molecular species of lower size in the second dimension and were probably packed by intramolecular disulfide link(s). Trailing of the polypeptides in Fig. 2A suggested an incomplete solubilization, after boiling in 2% SDS-lysis buffer, of strongly adsorbed polypeptide complexes resulting from natural interactions in the viral particle.

Labeling with [^{14}C]NEM allowed the detection of sulfhydryl-containing polypeptides and analysis of the accessibility of these residues after successive stripping of the CIV particle. Among the four polypeptides (P50-P'50), P60, P100, and P33 which showed a high content of free sulfhydryl groups (P50-P'50) and P60 were rapidly labeled whereas labeling of polypeptides P100 and P33 required a more important alteration of the virion structure.

These results clearly show that, in contrast with previous reports (Barray and Devauchelle, 1979; Moore and Kelly, 1980) the major protein is in fact constituted by two polypeptides: (i) a 150K protein composed of three identical disulfide-linked subunits of 50K (P'50), and (ii) a 50K protein (P50). These two polypeptides showed identical migration in SDS-β-ME-PAGE. However, they differed by some

properties: (i) the accessibility of their sulfhydryl residues was quite different; (ii) radioiodination of the external polypeptides using [^{125}I]iodosulfanilic acid showed only one intensively labeled spot corresponding to P50. (iii) P50 could be selectively solubilized by a DMSO treatment. However, tryptic peptide analysis shows that P50 and P'50 have very similar peptide compositions (work in progress).

These observations indicate that the P'50 polypeptide is not directly exposed at the surface of the viral particle, in addition it does not show any affinity for viral DNA.

Six viral polypeptides P81, P60, P31, P17, P13, and P10 were revealed to possess affinity for CIV DNA. It is interesting to note that among these DNA-binding proteins, P81 contained one or more intramolecular disulfide links and that P60 and P10 polypeptides were present as dimers composed of two identical disulfide-linked subunits. In addition, P81 is phosphorylated by the endogenous viral protein kinase *in vitro* (Monnier and Devauchelle, 1980). In the same manner, six DNA-binding proteins have been identified in FV3 (Goorha, 1981). Three of these DNA-binding proteins were phosphorylated in the mature virions (Aubertin *et al.*, 1980).

We have previously described (Cerutti and Devauchelle, 1982) that treatment of the CIV particle with octylglucoside leads to a selective solubilization of the viral membrane and probably of its intrinsic polypeptides. Under these conditions, polypeptides P50 and P'50 remained strongly bound to the core fraction indicating the existence of preferential interactions between capsid polypeptides and core polypeptides. It is interesting to note that numerous biological activities were bound to this membrane fraction: cell fusion activity, inhibitory activity of cellular macromolecular syntheses (Cerutti and Devauchelle, 1982), and enzymatic activities such as nucleotide phosphohydrolase and protein kinase (unpublished data).

Our findings clearly show that different types of interactions and organization are involved in the structure and integrity of the CIV particle. A tentative model for CIV structure could be the central DNA packed into a nucleosomal-like structure (Klump *et al.*, 1983) with at least six major DNA-associated proteins constituting the viral core. This core structure would be surrounded by a unit lipid membrane spanned by the P'50-P50 complex, with the P50 polypeptide being more exposed at the surface of the viral particle. Preferential interactions between the capsid polypeptide complex and the core polypeptides might be responsible for stability of the whole particle, leading to the typical icosahedral shape of the shell.

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