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# Isolation by High-Performance Liquid Chromatography and Partial Characterization of a 57,000-Dalton Herpes Simplex Virus Type 1 Polypeptide

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**A Nonidet P-40 extract of HSV-1-purified virions was fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). The first peak fraction eluted at 25% organic solvent. Polyacrylamide gel electrophoresis showed that it contained a 57,000-dalton polypeptide. The polypeptide was characterized by determination of the amino acid composition and the N-terminal amino acid sequence. Adsorption of the detergent extract before RP-HPLC showed that the polypeptide reacted with monoclonal antibodies LP1 directed against herpes simplex virus polypeptide VP-16.**

Much attention has been paid to the isolation and characterization of the major glycoproteins of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively): gB, gC-1, gD, gE, gG, and an HSV-2 130,000-dalton (130K) protein (2, 3, 4, 30, 31, 35, 41, 42, 52, 55). However, in addition to these glycoproteins, HSV contains also non-glycosylated proteins which may be of importance during the infection process. Immune precipitation and immunoblot studies have indeed shown that during infection antibodies are produced primarily directed against the major glycoproteins, but in addition, that antibodies were found directed against other components of the virion with lower molecular masses of between 66K and 34K (1, 14, 18, 54).

Since HSV polypeptides which can be extracted by a mild nonionic detergent generally are located at or close to the surface of virions (44) it was argued that characterization of detergent extracts of purified virions could indicate which proteins other than the major glycoproteins are surface located and might play a role in the infection process or have some immunological relevance. One of these polypeptides is the major tegument polypeptide VP16 (8, 22, 43), or Vmw65, M65, P65, and ICSP 31 as designated by others (31, 39). The VP16 polypeptide is phosphorylated (17, 26, 28) and can be extracted together with the major glycoproteins. The protein VP16 appears to play an important role in the induction of the immediate early functions during the infection process (7).

In this study we report the isolation of a detergent-extracted 57K HSV-1 polypeptide by reversed-phase high-performance chromatography (RP-HPLC). Further characterization was achieved by amino acid analysis, determination of the N-terminal amino acid sequence in a gas-phase sequencer and by reaction with monoclonal antibodies directed against VP16 before RP-HPLC.

## MATERIALS AND METHODS

**Cells and viruses.** HSV-1 strain McIntyre was used. The propagation of this strain has been described elsewhere (50). Vero cells were cultured in roller bottles in medium 199 supplemented with 10% newborn calf serum, antibiotics (100 U of penicillin G per ml, 100 μg of streptomycin per ml), 2.5 μg of amphotericin B per ml, and 2 mM L-glutamine. Cells were infected for 18 to 20 h with HSV-1 at a multiplicity of 10 PFU per cell. Infected cells were harvested by scraping off the wall of the roller bottles and collected by low-speed centrifugation. After two wash steps with Hanks solution, the cell pellet was resuspended in the same volume of a solution containing 0.25 M sucrose, 0.05% (wt/vol) bovine albumin, 0.02 mM $MgSO_4$, $10^{-3}$ mM N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma Chemical Co., St. Louis, Mo.), and 1 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.4. The cells were then disrupted by nitrogen cavitation according to the method of Spear and Roizman (43). The nuclei were removed from the cell homogenate and virions were purified by dextran T10 gradient centrifugation (43). After centrifugation for 1 h at 50,000 × *g*, the virus band was collected, and dextran was removed by dilution in 1 mM phosphate buffer (pH 7.4) and subsequent centrifugation.

**Extraction.** Virion envelope extracts were prepared by suspending 5 to 10 mg of purified HSV-1 virions in 100 μl of 1 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.5% Nonidet P-40 (NP-40). After 15 min of incubation at 0°C, the extracted viral envelope proteins were separated from insoluble residues by centrifugation at 140,000 × *g* for 90 min.

**RP-HPLC.** The HSV-envelope extract was subjected to RP-HPLC. An HPLC system equipped with a Rheodyne 7125 injector, a Pye Unicam LC-UV detector, and a Waters 6000A delivery system was used. The column (300 by 4.6 mm) was packed with Nucleosil 10 C-18. The elution was performed with a two-solvent gradient system. Solvent A consisted of 87% water, 3% *n*-butanol, 10% 2-methoxyethanol, and 0.1% trifluoroacetic acid (TFA). Solvent B contained 70% ethanol, 20% *n*-butanol, 10% 2-methoxyethanol, and 0.025% TFA. A gradient was generated by low-pressure mixing, using a homemade gradient control system consisting of an Acorn computer and a solenoid valve (47). The following gradient was used for elution: 7 min with isocratic solvent A and 0 to 10% solvent B over 3 min, followed by 10 to 60% solvent B over 40 min and finally 60% solvent B isocratically for another 6 min. Protein in the eluate was

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detected by monitoring the effluent at 215 nm. Pyridine was added to neutralize the collected fractions to a final concentration of 0.1%. The organic solvent was removed by evaporation in a Speed Vac model SVC-100H centrifuge (Savant Instruments, Hicksville, N.Y.). The remaining aqueous solution was lyophilized.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the buffer system of Laemmli (27). Samples of the collected peak fractions were analyzed on 12.5% SDS gels cross-linked with *N,N'*-diallyltartardiamide (20). Proteins were visualized by the silver staining method of Wray et al. (51).

**Amino acid analysis.** Amino acid analysis was performed with a Kontron Liquimat amino acid analyzer. Samples of 600 pmol were hydrolyzed for 18 h in 0.4 ml of 6 M HCl at 110°C in evacuated sealed glass tubes. The amounts of cysteine and tryptophan were not determined.

**Adsorption of NP-40 envelope extract with monoclonal antibodies LP1.** The monoclonal antibodies LP1 were kindly provided by A. C. Minson (Cambridge University). The properties of the monoclonal antibodies, which are directed against VP16, have been described elsewhere (33). Monoclonal antibodies (150 μl) were adsorbed to 40 mg of washed protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden). The beads with the adsorbed LP1 antibodies were washed three times with phosphate-buffered saline, followed by another four washes with the extraction buffer, 1 mM phosphate buffer (pH 7.4) containing 0.15 m NaCl and 0.5% NP-40. The HSV-1 envelope extract (400 μl) was added to the immobilized LP1 antibodies and incubated for 90 min at 0°C. After incubation, the beads were removed by centrifugation, and the supernatant, designated as LP1-adsorbed HSV-1 envelope extract, was applied to RP-HPLC.

**Amino acid sequencing.** Microsequencing was carried out in the Applied Biosystems gas-phase sequencer (Foster City, Mich.), using the standard RUNTFA program with 25% TFA as the conversion reagent (21). The phenylthiohydantoin amino acid derivatives (PTHs) were identified and quantified by HPLC on a 5 μm IBM cyanopropyl column of (250 by 4.6 mm; Biotech, Hertford, U.K.), using gradient elution as described by Hunkapiller and Hood (23). The gradient program described before (23) was adapted to our equipment configuration, which consisted of the Waters Intellink System with two 6000A pumps, the 720 system controller, the 730 data module, and the WISP autosampler 710B. The PTHs were detected both at 269 and at 323 nm, respectively, with a Perkin Elmer LC 75 and a Pye Unicam LC-UV detector.

## RESULTS AND DISCUSSION

**Isolation of the 57K HSV-1 polypeptide.** Initial studies showed that, depending on the amount of the HSV-1 envelope extract subjected to RP-HPLC, the elution time of particular polypeptides varied. Most consistent elution patterns were observed upon chromatography of at least 200 μl of extract originating from 10 to 20 mg of purified virions. The HPLC elution profile is shown in Fig. 1a. The peaks were collected, and samples were analyzed by SDS-PAGE. The proteins were visualized by silver staining (Fig. 1b), using a relatively long development time of 20 to 25 min.

In Fig. 1b the extract (E) and the peak fractions (lanes 1 to 10) are compared by SDS-PAGE. A considerable degree of purification was obtained. Especially the fraction at a retention time of 33 min contained predominantly a ca. 57K polypeptide. Occasionally, a possible multimer of this polypeptide was observed in the gels. Of interest also is that

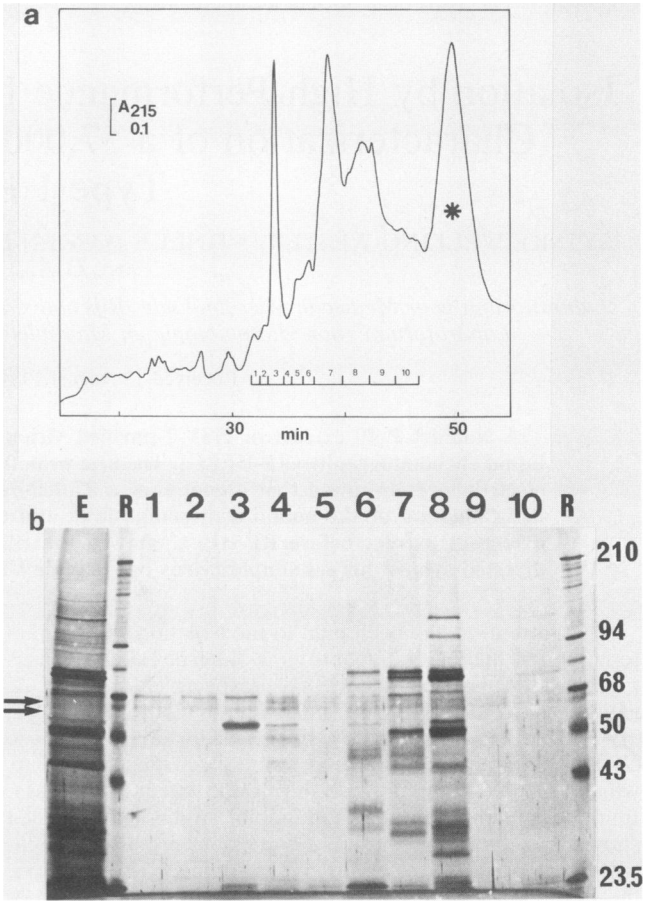


FIG. 1. (a) HPLC elution profile of a NP-40 detergent extract of purified HSV-1 virions. The absorbed proteins were eluted at room temperature from a reversed-phase Nucleosil 10 C-18 column (300 by 4.6 mm) at a flow rate of 1.0 ml/min. The absorbance was monitored at 215 nm ($A_{215}$). A two-solvent gradient was used. Solvent A contained 87% water, 3% *n*-butanol, 10% 2-methoxyethanol, and 0.1% TFA. Solvent B contained 70% ethanol, 20% *n*-butanol, 10% 2-methoxyethanol, and 0.025% TFA. The gradient used was as follows: 7 min with isocratic solvent A, 0 to 10% solvent B over 3 min, 10 to 60% solvent B over 40 min, and 60% solvent B isocratically. The fractions were collected as indicated. *, NP-40 micelles. (b) SDS-PAGE analysis of the collected peak fractions of the RP-HPLC. The fractions were made 0.1% in pyridine. The organic solvent was removed by centrifugation in a Speed Vac centrifuge and the remaining solution was lyophilized. The freeze-dried fractions were dissolved in water and samples of 15 μg of protein were prepared for electrophoresis on SDS gels. After electrophoresis gels were silver stained. The lane numbers correspond to the fraction numbers in (a). R, Molecular mass markers myosin (210K), phosphorylase b (94K), bovine serum albumin (68K), immunoglobulin heavy chain (50K), ovalbumin (43K), and chymotrypsinogen (23.5K); *, NP-40 micelles; arrows, artifact bands due to the silver staining procedure (32, 45).

during silver staining of this polypeptide, a negative image developed, which slowly turned into a red-brownish band. The 57K protein could be easily identified in the gels by its red-brownish color; the other HSV polypeptides showed a dark brownish color upon silver staining.

Besides the 57K polypeptide, other HSV-1 proteins eluted in subsequent fractions. Fractions 4 through 8 (Fig. 1a) contained proteins of 110K, 80K, 75K, 56K, 53K, 42K, and 30K, of which the 80 to 75K and 56 to 53K proteins were the most abundant. We identified the 56 to 53K polypeptide as

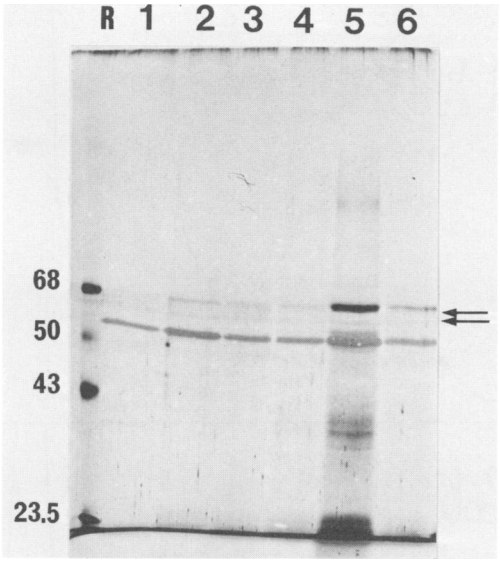


FIG. 2. SDS-PAGE analysis of RP-HPLC runs of different HSV-1 envelope extract preparations. Only the 57K peak fractions from six different runs were analyzed (lanes 1 to 6). The light grey of the 57K band reflects its red-brownish color upon silver staining in contrast to the dark color of the other bands. R, Molecular mass markers bovine serum albumin (68K), immunoglobulin G heavy chain (50K), ovalbumin (43K), and chymotrypsinogen (23.5K). Arrows indicate artifact bands due to the silver staining procedure (32, 45).

gD by reaction with monoclonal antibodies against gD. It has been reported (36, 37, 53) that HSV polypeptides in Vero cells are subject to proteolysis. We added $10^{-3}$ mM TLCK during the extraction procedure, but this was not sufficient since we found lower molecular masses for the proteins in the extract of purified virions than those reported earlier (42) for HSV glycoproteins in HEp-2 cells. Despite this proteolysis, the infectivity of the purified virions was not affected. On the basis of their molecular weights only it is difficult to designate the proteins in the different HPLC fractions. Additional studies with monoclonal antibodies against the individual glycoproteins are needed to indicate which glycoproteins are present in the different fractions.

**Amino acid composition and N-terminal amino acid sequence of the 57K polypeptide.** The elution profile of different HSV-1 envelope extract preparations showed that the procedure for the isolation of the 57K polypeptide was very reproducible. The analysis of the fractions containing this polypeptide from six different RP-HPLC runs of different envelope extracts is shown in Fig. 2. Occasionally, fractions (Fig. 2, lane 5) were contaminated by other polypeptides. The fractions were pooled, and the 57K polypeptide was further purified by RP-HPLC under the same conditions. The peak was collected as two different fractions (Fig. 3). The left part contained, in addition to the 57K polypeptide, contaminating proteins, whereas the right part contained only the 57K polypeptide.

The elution position of the 57K polypeptide suggests that it is a relatively hydrophilic polypeptide (46). Generally, proteins are denatured by the low pH and the high concentration of the organic modifier in the solvent. The amino acid composition of the 57K polypeptide (Table 1) reflected the hydrophilic/hydrophobic properties of the protein. In addition, posttranslational modification may affect these properties. The 57K polypeptide contained 44 serine residues which may be phosphorylated.

The amino acid composition of the 57K polypeptide was compared with the average composition of 314 proteins of different families (10) and with the composition of HSV gD (without signal sequence) (15, 48), HSV gC (13, 16), and HSV gB (6), both without the proposed signal sequences. The percentage of hydrophobic residues (Leu, Ile, Val, Phe, and Met) in the 57K polypeptide was 21.0%, while it is 25.0 in the average composition. In HSV glycoproteins gB, gC, and gD, it is 25.0, 21.4, and 25.8, respectively. The percentages of hydrophilic residues (Asx, Glx, Lys, Arg, and His) were 41.4, 34.8, 34.7, 26.7, and 29.8 for the 57K polypeptide, the average composition, HSV gB, gC, and gD, respectively. This shows that the 57K polypeptide was relatively hydrophilic. If only the percentage of basic amino acids is compared for these proteins, the 57K polypeptide was still the most hydrophilic. It has been shown that HSV gD and gC contain relatively much proline, 12.1 and 13.2%, respectively. The proline content of the 57K polypeptide was almost similar to that of the average protein, but it was characterized by its relatively high content (4.4%) of histidine, of which HSV gB, gC, gD, and the average protein contained 2.7, 2.6, 2.5, and 2.1%, respectively.

Automatic Edman degradation in the Applied Biosystems gas-phase sequencer was performed to determine the N-terminal amino acid sequence, using 8 and 11 μg of the 57K polypeptide. The first run yielded the sequence given in Fig. 4. The N-terminal residue was shown to be alanine. At position 2, an increase of both proline and valine occurred by factors of 1.4 and 1.1, respectively. At the next position there was a further 1.8-fold increase of PTH-proline but an 8.0-fold increase of PTH-tyrosine. The data can be explained by assuming that the sequence is Pro-Tyr with an incomplete cleavage reaction at proline 2. In spite of background PTHs, residues 3 to 20 could easily be assigned (Fig. 4). At position

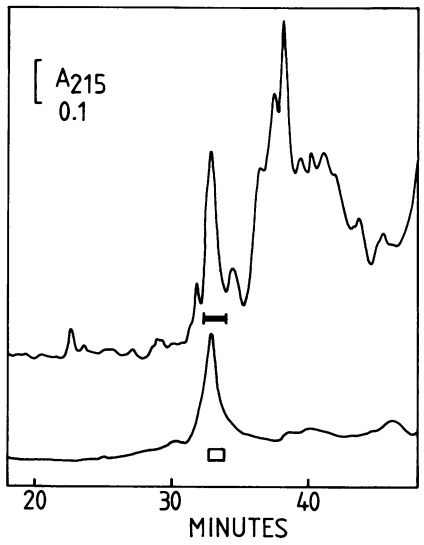


FIG. 3. Purification of the 57K polypeptide from the NP-40 detergent extract of purified HSV-1 virions. The upper profile shows the elution pattern for the NP-40 detergent extract applied to a Nucleosil 10 C-18 column. Lyophilized pools of the fractions containing the 57K polypeptide (▬) were injected for the second time on the same column (lower profile). Elution conditions were identical. The left part of the peak in the lower profile contained contaminating proteins, and the right part (□) contained the purified 57K polypeptide. This fraction was used for determination of the amino acid composition and for the second run on the sequencer.

TABLE 1. Amino acid composition of the 57K HSV-1 polypeptide in comparison with the average amino acid composition of a protein and of HSV-1 glycoproteins gB, gC, and gD[a]

| Amino acid | 57K polypeptide | | Avg protein[b] (%) | HSV gB[c] (%) | HSV gC[d] (%) | HSV gD[e] (%) |
|---|---|---|---|---|---|---|
| | Amino acid | % | | | | |
| Ala | 39 | 7.2 | 9.0 | 10.1 | 8.3 | 10.2 |
| Arg | 22 | 4.0 | 5.1 | 7.7 | 6.8 | 6.2 |
| Asn | — | — | 4.5 | 4.7 | 2.8 | 3.6 |
| Asp | — | — | 5.8 | 5.9 | 4.1 | 4.8 |
| Asx | 63 | 11.7 | 10.3 | 10.6 | 6.8 | 8.4 |
| Cys | nd | nd | ni | ni | ni | ni |
| Gln | — | — | 4.1 | 2.7 | 3.6 | 4.5 |
| Glu | — | — | 6.1 | 6.4 | 4.9 | 4.8 |
| Glx | 78 | 14.5 | 10.4 | 9.1 | 8.6 | 9.3 |
| Gly | 45 | 8.3 | 10.5 | 7.0 | 7.7 | 5.6 |
| His | 24 | 4.4 | 2.1 | 2.7 | 2.6 | 2.5 |
| Ile | 20 | 3.7 | 4.7 | 3.2 | 3.6 | 6.2 |
| Leu | 39 | 7.2 | 7.8 | 6.8 | 5.1 | 9.5 |
| Lys | 38 | 7.0 | 6.9 | 4.6 | 1.9 | 3.4 |
| Met | 11 | 2.0 | 1.8 | 2.8 | 1.7 | 2.0 |
| Phe | 18 | 3.3 | 3.8 | 4.6 | 2.8 | 2.5 |
| Pro | 32 | 6.0 | 5.5 | 5.9 | 13.7 | 12.1 |
| Ser | 44 | 8.1 | 7.3 | 5.2 | 7.3 | 6.2 |
| Thr | 27 | 5.0 | 6.4 | 8.0 | 12.0 | 5.6 |
| Trp | nd | nd | ni | ni | ni | ni |
| Tyr | 13 | 2.4 | 3.6 | 4.4 | 3.0 | 4.8 |
| Val | 26 | 4.8 | 6.9 | 7.6 | 8.1 | 5.6 |
| Hydrophobic[f] | | 21.0 | 25.0 | 25.0 | 21.4 | 25.8 |
| Hydrophilic[g] | | 41.6 | 34.8 | 34.7 | 26.7 | 29.8 |
| Basic[h] | | 15.4 | 14.1 | 15.0 | 11.3 | 12.1 |

[a] Determination on 600 pmol of 57K polypeptide, 20-h acid hydrolysis. nd, Not determined; ni, not included.
[b] Average amino acid composition of 314 proteins from different families (8).
[c] Composition of HSV-1 gB (6) corrected for the proposed signal peptide.
[d] Composition of HSV-1 gC (13, 16) corrected for the proposed signal peptide.
[e] Composition of HSV-1 gD (48) corrected for the signal peptide (15).
[f] Leu, Val, Ile, Phe, and Met.
[g] Asx, Glx, Arg, Lys, and His. To allow comparison of the 57K polypeptide with HSV-1 glycoproteins gB, gC, and gD and with the average composition, the values of asparagine and aspartic acid and of glutamine and glutamic acid were combined.
[h] Arg, Lys, and His.

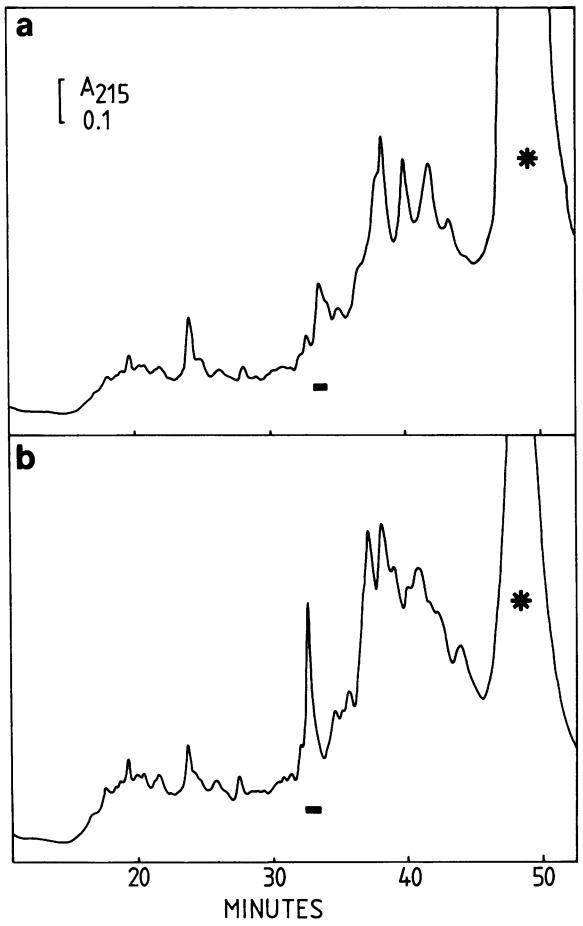


FIG. 5. The elution profiles of a NP-40 detergent extract of HSV-1 virions on RP-HPLC before and after adsorption with monoclonal antibodies LP1. Elution conditions were as indicated in the legend to Fig. 1a. (a) Detergent extract was adsorbed with immobilized monoclonal antibodies LP1 prior to RP-HPLC. Adsorption was carried out with 400 μl of NP-40 extract and 40 mg of protein A-Sepharose C1-4B, to which 150 μl of monoclonal antibodies was adsorbed. (b) Without adsorption with immobilized LP1. Symbols: —, elution position 57K polypeptide; *, NP-40 micelles.

18, no PTH amino acid could be identified, although there was evidence for two further Edman steps. Several explanations may be possible. This position may be occupied by cysteine or methionine, which may not be detected by the method used for the PTH identification (23). Residue 18 may also be a posttranslationally modified amino acid. The second sequence run confirmed residues 1 to 13 but did not yield any further sequence information beyond position 14.

**Effect of LP1 adsorption of envelope extract on the RP-HPLC elution profile.** Since the 57K polypeptide was extracted from the purified virions under the same conditions as the HSV glycoproteins, and also the molecular mass indicated a possible relationship with HSV polypeptide VP16, we investigated the reaction of monoclonal antibodies LP1, which are directed against VP16, with the detergent extract of purified HSV-1 virions. SDS-PAGE of the detergent extracts before and after reaction with monoclonal antibodies LP1 against VP16 showed that only the red-brownish polypeptide band of the 57K polypeptide could be eliminated from the extract.

RP-HPLC is performed in high concentrations of organic solvents and usually results in conformational change and loss of activity of proteins (29, 49). Rather than doing immunoprecipitation studies with the purified 57K polypeptide, we subjected the extract to RP-HPLC before and after reaction with immobilized monoclonal antibodies LP1. The elution patterns are shown in Fig. 5. Although the 57K peak

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Ala-Pro/Val-Iyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-Trp-Thr-Asn-Asp- X -Ile-Asp-

FIG. 4. N-terminal amino acid sequence of the 57K HSV-1 polypeptide. The amino acid sequence given was obtained after two separate runs in a gas-phase sequencer, using 140 and 200 pmol of the protein. The underlined residues were identified in both runs, whereas the evidence for the other residues was obtained from the first run only. X is an unidentified residue at position 18. The PTHs were analyzed by HPLC on an IBM cyano column, using the protocol of Hunkapiller and Hood (23) with some modifications.

was not entirely eliminated from the elution pattern (Fig. 5a), a significant decrease in absorbance was observed. This was confirmed by SDS-PAGE of the eluent fractions.

The reaction between the LP1 monoclonal antibodies and the 57K polypeptide strongly suggests that the 57K protein was related to VP16. Others have estimated for VP16 a molecular mass of approximately 65K (8, 22, 31, 33, 39, 43). Recent studies, however, report lower molecular masses of 63K and 60K (7, 25). On the other hand, the possibility that the 57K polypeptide is a major fragment of VP16 derived by proteolytic cleavage may not be excluded.

In conclusion, RP-HPLC was used for the isolation of a 57K HSV-1 polypeptide. In this particular case, 50 mg of purified virions were extracted, resulting after one RP-HPLC run in ca. 45 μg of the 57K polypeptide, which is 774 pmol.

It has been shown that HPLC is an excellent technique for the separation of many compounds (12). Now methods are available for HPLC of proteins (40). Reports on the separation of viral proteins by HPLC are scarce (5, 9, 11, 19, 34, 38, 49) and reflect the problems which are encountered when proteins with aggregating properties have to be purified. New elution systems had to be developed which imply detergents in the solvents or high organic solvent concentrations to avoid aggregation. Generally only small amounts of virus proteins are available, therefore the combination of HPLC and microsequencing supplement each other. Partial amino acid sequences from virus proteins obtained in this way will be a useful addition to amino acid sequences deduced from nucleotide sequences data of virus DNA (24).

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