In Vivo NH₂-terminal Acetylation of Sindbis Virus Proteins*

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The in vivo incorporation of exogenous radioactive acetate into two proteins of Sindbis virus, the capsid protein and PE2, is described. Under appropriate labeling conditions, 40-50% of the label in the capsid protein is found in an N-acetyl group which constitutes the NH₂-terminal modification of this blocked protein. The incorporated radiolabeled acetate was useful in the purification and analysis of peptides derived from the NH₂-terminus of the capsid protein, and from these peptides the NH₂-terminal sequence of the protein was determined to be N-acetyl-Met-Asx-Asx-Asx-Met, with the Asx group most likely asparagine. The analysis of a peptide derived from the NH₂-terminus of PE2 and containing 45% of the acetate-derived label in this protein leads us to conclude that at least a significant fraction of PE2 is also blocked by N-acetylation.

In this paper we describe the in vivo incorporation of tritiated acetate into the blocked NH₂ terminus of the capsid protein of Sindbis virus with improved specificity and report the primary sequence of its NH₂ terminus. In the course of this study, PE2 was also found to be specifically labeled with acetate. This unexpected result led us to examine this protein in more detail, and we show that a significant fraction of PE2 also has an acetylated NH₂ terminus. The results of this study may be of use in the identification of other N-acetylated proteins and the determination of their NH₂ termini.

EXPERIMENTAL PROCEDURES

Virus Strains and Cell Infection—The virus strain used in these studies was the large plaque variant of the HR strain of Sindbis virus, which was originally obtained from Dr. B. W. Burge, National Institutes of Health, Bethesda, MD, and plaque purified several times in our laboratory. The virus was grown in chick embryo fibroblasts (primary cultures, except as noted below) or BHK-21 cells (the generous gift of Dr. B. M. Sefton, Salk Institute, San Diego, CA). The preparation of chick cells and infection with Sindbis virus were as described (8).

Growth and Purification of Viral Proteins Radiolabeled with Amino Acids—When viral proteins labeled with an essential amino acid were to be prepared, the medium used for incubation postinfection and for labeling contained one-tenth the normal concentration of that amino acid, except as noted. At the end of the labeling period, usually from 7 to 8 h postinfection, monolayers were washed twice with ice-cold phosphate-buffered saline and dissolved in a small volume of 1% SDS and 10 mM Tris-Cl, pH 7.5.

Viral proteins were purified from infected cell monolayers, or from the virus released from infected cells by a high salt wash (8), by preparative polyacrylamide gel electrophoresis. Protein was precipitated by the addition of 2 volumes of ethanol, frozen on dry ice, warmed to 0 °C, and centrifuged for 15 min at 15,000 rpm at 0 °C in a Sorvall SS-34 rotor. Precipitated protein was dissolved in sample buffer and electrophoresed in 8-mm diameter polyacrylamide gels using the buffer system of Laemml (9), except that the concentration of Tris in the gels was halved, and 0.01% mercaptoethanol was included in the sample and upper electrode buffers. After electrophoresis, the gel was sliced into approximately 100 fractions, each consisting of 0.5-mm slices, and each fraction was eluted in 1 ml of 0.5% SDS, 10 mM 2-(N-morpholino)ethanesulfonic acid, 2.9 mM HCl, 1 mM dithiothreitol, and 20 μg/ml of phenylmethylsulfonyl fluoride. After shaking for 9 h at room temperature, an aliquot of each fraction was assayed for radioactivity by liquid scintillation counting. Appropriate fractions were pooled, and the volume of each pool was reduced 10-fold by lyophilization. Just before use, purified proteins were precipitated with ethanol as described above with tuna heart cytochrome c added as carrier to at least 20 μg/ml.

Growth and Purification of Viral Proteins Radiolabeled with Tritiated Acetate—Two days before use, cell monolayers were harvested by trypsinization and resuspended in the following medium. To Eagle's minimal essential medium (10) without NaCl, it was added l-aspartic acid to 2 mM (from 0.1 M stock in 0.2 M HCl). l-glutamic acid-HCl to 15 mM, l-proline to 30 mM, NaCl to 33 mM, and NaOH to 36 mM. The amounts of NaCl and NaOH added were calculated to preserve neutrality and isotonicity. Fetal calf serum was added to this mixture to a concentration of 10% (v/v). Chicken embryo fibroblasts did not grow well in this medium and so were not split during the transfer, while BHK-21 cells were split 2-fold. Virus infection and postinfection

* The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.
incubation were carried out in the above medium with 10% dialyzed fetal calf serum in place of fetal calf serum and containing 1 pg/ml of actinomycin D. At about 7 h postinfection, cells were labeled for 20 min by the inclusion of 1 mcI/ml of \[^{3}H\]acetate (2 Ci/mmol) in the medium and harvested as described above.

Preparative polyacrylamide gel electrophoresis of acetate-labeled proteins was preceded by an additional purification step of affinity chromatography which will be described in detail elsewhere. Briefly, molecules harvested with SDS were diluted with a buffer containing Triton X-100 and passed over a column containing covalently coupled antibodies (anti-C and anti-E2) produced in rabbits in response to purified SDS-denatured proteins. (Anti-E2 also binds PE2.) Bound protein was eluted with SDS and purified by preparative polyacrylamide gel electrophoresis as above.

**Staphylococcal Protease Digestion**—Ethanol-precipitated radiolabeled capsid protein plus cytochrome c carrier was resuspended in 67 μl of 0.1% SDS, 0.05 M NaPO₄, pH 7.8, and 1 mm ethylenediaminetetraacetic acid containing Staphylococcus aureus V8 protease to give a carrier protein concentration of 0.8 mg/ml and a protease concentration of 0.1 mg/ml. In this buffer, the protease cleaves peptide bonds involving the carboxyl groups of aspartic acid and glutamic acid (11). After incubation for 4 h at 37 °C, the sample was lyophilized and analyzed by electrophoresis on cylindrical polyacrylamide gels containing SDS, urea, and a high concentration of acrylamide (12). After electrophoresis, the gel was sliced into 1-mm fractions and counted by liquid scillation. Identical results were found when the digestion was extended an additional 4 h.

**Chymotrypsin Digestion and Dowex 50W Chromatography**—Ethanol-precipitated radiolabeled capsid protein or PE2 was suspended in 0.05 M CO₃, pH 7.8, usually 0.5 ml, to give a protein concentration (carrier or chemically pure capsid protein or PE2) of 120 μg/ml. One-tenth volume of chymotrypsin at 2 mg/ml was added, and the mixture was incubated for 12 h at room temperature. An additional one-tenth volume of chymotrypsin at 2 mg/ml was added, and the incubation continued an additional 12 h, at which time the sample was lyophilized, dissolved in a small volume of distilled water, and lyophilized again. The sample was dissolved in 200 μl of 0.5% β-mercaptoethanol and applied to a column (0.7 × 4 cm) of AG 50W-X2 (H⁺ form), 200–400 mesh (purified Dowex resin from Bio-Rad Laboratories), in distilled water. The column was run at a flow rate of 1.35 ml/h at 4 °C, and 1-min fractions were collected. The column was washed with distilled water for 8 min, and then bount peptides were eluted with 1 μl NaOH. Fractions containing base were neutralized with HCl, and an aliquot of each fraction was assayed for radioactivity by liquid scintillation counting.

Chymotrypsin digestion and Dowex 50W chromatography were performed at room temperature and 4 °C, respectively, to minimize cyclization of newly exposed NH₂-terminal glutamine or glutamic acid residues to pyridoline carboxyl groups (13).

**Analysis of Dowex 50W Unbound Peptides**—Pooled fractions from Dowex 50W chromatography were dried under nitrogen at 50 °C, dissolved in 10 μl of 1% β-mercaptoethanol, and analyzed in one of two ways. Silica gel chromatography utilized ITLC SA "instant thin layer chromatography" sheets (Gelman Instrument Co.). Ascending chromatography was performed with the solvent 1-butanol/acetone/acetic acid/water (4:1:1). The dried chromatogram was cut into 3-mm fractions which were counted directly in Aquasol (New England Nuclear) containing 6% water. Alternatively, samples were analyzed by reversed phase HPLC on columns (4.6 mm × 25 cm) (DuPont Instruments) at 1 ml/min at room temperature. Elution was with a gradient from buffer A (0.1% trifluoroacetic acid) to buffer B (0.1% trifluoroacetic acid, 33% methanol, 33% acetonitrile, and 33% water). For peptide analysis, a 7-h postinfection sample from PE2, a Zorbax CN column was used, and a 30-min linear gradient from buffer A to 25% buffer B (v/v in buffer A) was applied 10 min after injection, followed by a 30-min linear gradient to 100% buffer B and continued elution with buffer B. Fractions of 1 ml were collected, and an aliquot of each fraction was analyzed for radioactivity. A Zorbax octadecylsilyl column was used for the analysis of peptides derived from the capsid protein, and in this case a 30-min linear gradient from buffer A to 20% buffer B was applied starting at 10 min after injection, and 0.7-min fractions were collected. Pooled fractions from HPLC were dried under nitrogen at 50 °C, hydrolyzed in evacuated tubes with 6 N HCl at 145 °C for 4 h, washed with distilled water for 8 min, and then bound peptides were analyzed on a Durrum D-500 mark II amino acid analyzer. Chromatographic markers were prepared by acetylation of \[^{3}S\]methionine, containing a small amount of \[^{35}S\]methionine sulfoxide, with acetic anhydride in glacial acetic acid (14).

### RESULTS

**Radiolabeling with \[^{3}H\]Acetate**—In preliminary experiments, when infected cells were incubated with tritiated acetate, harvested, and analyzed by polyacrylamide gel electrophoresis, it was found that E1 (which is not blocked in the virion) and is not known to be otherwise acetylated) contained a significant amount of tritium label (Fig. 1A). Since acetate occupies a central position in the intermediary metabolism of the host cell, one would expect to find the radioactivity derived from exogenous acetate in a variety of chemical forms including long chain fatty acids and the amino acids synthesized from citric acid cycle intermediates. Therefore, viral proteins were purified from infected cells which had been labeled with \[^{3}H\]acetate in a common growth medium (Eagle's minimal essential medium (10) plus 2% fetal calf serum), and the proteins were analyzed to determine the fate of the radioactive metabolite. After HCl hydrolysis, greater than 60% of the tritium label was found in the amino acids proline (about three-fourths of the total amino acid label), glutamic acid, and, to a minor extent, aspartic acid (data not shown). The Sindbis glycoproteins also contain covalently coupled long chain fatty acids (16). These fatty acids were released from the purified proteins by base hydrolysis in methanol, extracted with organic solvents, and the O-methyl esters of long chain fatty acids were identified by adsorption chromatography on silica gel (results not shown). Radioactively labeled Sindbis-infected cells. Infected BHK-21 monolayers were labeled with tritiated acetate at 1 μCi/ml (2 Ci/mmol), harvested as described under "Experimental Procedures," and an aliquot analyzed by polyacrylamide gel electrophoresis with the buffer system of Laemmli (9). After electrophoresis, the gel was sliced into 1-mm fractions and analyzed for radioactivity by liquid scintillation counting. Electrophoresis was from left to right, and the positions of the viral proteins are identified in the figure, A, the cells were labeled for 1 h in minimal essential medium (10) containing one-twentieth the usual concentration of methionine, 2% dialyzed fetal calf serum, 1 μg/ml of actinomycin D, and 3 μCi/ml of \[^{35}S\]methionine (1.2 Ci/mmol). The dye marker (bromphenol blue) was located at fraction 87. Note the corresponding breaks in the tritium and \(^{14}C\) scales in the capsid protein peak. B, the cells were labeled as described for acetate labeling under "Experimental Procedures" with medium containing, in addition, 0.17 μCi/ml of \[^{14}C\]leucine (0.4 μCi/mmol final). The dye marker was located at fraction 85.

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ity was found in these long chain fatty acids under the above
labeling conditions. Surprisingly, no radioactivity was found
in the glycoproteins, which contain N-acetyl glucosamine and
sialic acid, generated from the Sindbis glycoproteins by pro-
nase digestion and analyzed as described (17).

In view of these findings, in all subsequent experiments we
used a modified labeling procedure, as described under “Ex-
perimental Procedures,” which consisted of preincubation of
the cells as well as labeling in a medium containing higher
than normal concentrations of serum and large amounts of
aspartic acid, proline, and glutamic acid. It was also necessary
to use short labeling periods, and we found 20 min to be
optimum (data not shown). The effects of this modification of
the labeling procedure can be seen in Fig. 1, which shows the
SDS-polyacrylamide gel patterns of BHK-21 cells labeled
with [3H]acetate and [35S]methionine or [14C]leucine. The
results in Fig. 1A were obtained during preliminary experi-
ments with no attempt to suppress metabolic conversion of
acetate, while the infected cells of Fig. 1B were labeled under
the modified labeling protocol. The ratio of tritium to
[3H]acetate is similar in all of the structural proteins of
the virus in Fig. 1A, but in Fig. 1B E1 is not extensively
labeled with tritium, while the capsid protein has still incor-
porated significant amounts of the label. Interestingly, even
with the modified labeling procedure, PE2 is labeled with
tritium to nearly the same extent as the capsid protein.
The amount of tritium incorporated into either PE2 or the capsid
protein is quite low, and there is a very large tritium peak
near the dye front.

A similar result is found when infected chick cells are
labeled in this manner. The ratio of tritium to [14C] in PE2 and
in the capsid protein is similar to that seen in BHK-21 cells
when labeling medium containing the same ratio of [3H]acetate
and [14C]leucine is used. However, in chick cells a large
unidentified tritium peak, which we have also observed in
uninfected cells, obscures the E1 peak and seems to be more
prominent in the gel patterns of short labeling periods. A small
amount of this unidentified host-derived material can also
be seen in BHK-21 cells (fraction 35 in Fig. 1B).

In order to examine the distribution of acetate-derived
tritium label along the polypeptide chain of the capsid protein,
capsid protein from chick cells was digested with S. aureus
V8 protease, and the digest was analyzed by SDS-polyacryl-
amide gel electrophoresis. As shown in Fig. 2, about 70% of
the tritium label was found in one polypeptide fragment,
which was also labeled extensively with methionine. The

![Fig. 2. S. Aureus V8 peptides of acetate-labeled capsid pro-
tein. The peptides derived from capsid protein, which had been
labeled with tritiated acetate and [3H]methionine and purified from
infected chick embryo fibroblasts, were analyzed by SDS-polyacyr-
amide gel electrophoresis. Electrophoresis was from left to right.
Arrows indicate the approximate molecular mass in thousands of
daltons at various points on the gel.](image)

acetate and [14C] labels in this experiment were derived from
separate preparations of the capsid protein, and the major
peaks migrate at slightly different rates because one or more
methionine residues in the acetate-labeled protein became
oxidized during purification. A similar difference in migration
rate during SDS-gel electrophoresis due to oxidation is also
seen in the intact capsid protein.

**Acetate-labeled Peptides of the Capsid Protein—Having
developed conditions for specifically labeling the blocked cap-
sid protein with acetate and having shown that the label was
incorporated in this protein in a nonrandom manner, we next
purified small acetate-labeled peptides from proteolytic di-
gests of the capsid protein. We used chromatography of an
exhaustive chymotrypsin digest on a strong cation exchanger
(Dowex 50W, H+ form). Under the chromatographic condi-
tions used, a blocked peptide with no lysine or arginine, i.e., a
peptide with no cationic groups, should not be bound to the
resin and should be found in the column wash through (5).

![Fig. 3. Dowex 50W chromatography of chymotryptic di-
gests of Sindbis proteins purified from infected chick embryo
fibroblasts. A, peptides derived from tritiated acetate and [3H]
methionine-labeled capsid protein. B, peptides derived from tritiated
acetate and [14C]alanine-labeled PE2. In both A and B, the first one-
half of the [14C] curve is also plotted in the upper portion of the panel
with an expanded scale in order to clearly show the details of the peaks.
The large peaks at fraction 22 are the peptides eluted from the resin with NaOH.
](image)

We next analyzed the peptides contained in the flow
through fraction of the Dowex 50W column by silica gel
chromatography and reversed phase HPLC. A silica gel chro-
matogram of the Dowex 50W flow through of Fig. 3A is shown
in Fig. 4A, and, as can be seen, the [3H]acetate and
[14C]methionine labels comigrate as three peaks. Peak III was
identified by co-chromatography with synthetic markers as
N-acetyl methylamine (results not shown), and hence it must be
the modified NH2-terminal amino acid of the protein. Peak
I was found in varying amounts, usually much smaller than in the
chromatogram shown in Fig. 4A. In addition, while peak II
migrates between methionine and N-acetyl methionine, in
other experiments, peak I was found to migrate between the oxidized (sulfoxide) forms of these two species. We, therefore,
believe that peak I is an oxidation product of peak II, contain-
ing a methionine sulfoxide residue.

An HPLC chromatogram of these acetate-labeled peptides
is shown in Fig. 4B. In this experiment, [3H]acetate-labeled
capsid protein which became oxidized during purification was
digested with chymotrypsin, chromatographed on Dowex
50W, and the flow through mixed with [35S]-labeled synthetic

![Fig. 3A shows the chromatogram obtained when the sample
applied to the column contained [3H]methionine-labeled cap-
sid protein in addition to the [3H]acetate-labeled protein.
Thirty percent of the [3H]acetate-derived label is found in the
column flow through, while a small but significant amount
(6%) of the methionine label is also unbound.

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The large peaks at fraction 22 are the peptides eluted from the resin with NaOH.
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is also seen. The peak at fraction markers and subjected to HPLC chromatography. The major tritium-labeled peak comigrates with N-acetyl methionine labeled capsid protein which had been purified from infected mination of the NH2-terminus of the capsid protein in virus particles. Comparable results (40% of the tritium label in the N-acetyl group) are obtained by a similar analysis of the amounts of the two labels in the N-acetyl methionine peak of the silica gel chromatogram of Fig. 4A.

Acetate-labeled Peptides of PE2—Previous results have shown that sequence data can be obtained by Edman degradaton of PE2 in Sindbis-infected cells (20), indicating that at least a fraction of this protein is not N-acetylated. However, we found a significant amount of acetate-derived label in PE2, comparable to that in the capsid protein, although we have shown that 40–50% of the acetate-derived label in the capsid protein was incorporated into the NH2-terminal acetyl group. Therefore, in order to fully characterize the fate of the exogenously supplied acetate in infected cells, it was also necessary to determine the chemical form of the acetate-derived label in PE2. The results of Dowex 50W chromatography of the chymotrypsin digestion products of PE2 labeled with tritiated acetate and [14C]alanine are shown in Fig. 3B, in which two unbound peaks are partially resolved. A large fraction of the tritium label and about 5% of the [14C]alanine label is found in a flow through peak for the digestion of PE2. This material was pooled and analyzed by HPLC and silica gel chromatography (see below). The large alanine-containing peak at fraction 6 probably contains peptides which are either large or exist in solution as aggregates so that they cannot enter the pores of the resin, as this is the expected position of the excluded volume of the column. We have also found that the peptides in this early peak do not move from the origin in silica gel chromatography (data not shown).

Since the capsid protein of virus particles is readily purified (15), we were able to obtain, for further characterization, chemically pure (as opposed to radiochemically pure) preparations of the peptide called peak II above by HPLC of the Dowex 50W unbound fraction of the chymotrypsin digestion products of chemically pure virion capsid protein. HCL hydrolysis followed by amino acid analysis established that this peptide contained only aspartic acid or asparagine in addition to the methionine detected by radiolabeling techniques. (Tryptophan would not have been detected). Although we have not directly determined the structure of this peptide, it almost certainly is N-acetyl-Met-Asn, also derived from the NH2 terminus of the protein. It contains the same amount of tritium label per methionine residue as the N-acetyl methionine, and it was isolated by Dowex 50W chromatography, selective for blocked peptides. The presence of asparagine rather than aspartic acid is strongly suggested by the specificity of chymotrypsin. Although the enzyme preferentially hydrolyzes peptide bonds COOH-terminal to aromatic amino acids, it is also known to cleave, at a slower rate, methionyl and asparaginyl bonds (18), the latter particularly when followed by a basic amino acid (19) (see also under “Discussion”). In addition, we note that peak II is not generated by cleavage of the capsid protein with S. aureus V8 protease under conditions in which the peptide bonds COOH-terminal to aspartic acid and glutamic acid are cleaved (see Fig. 2, and unpublished data).

Assuming that peak II is indeed N-acetyl-Met-Asn derived from the NH2-terminal end of the capsid protein, we can estimate the fraction of the acetate-derived tritium label which is in the NH2-terminal acetyl group of the capsid protein from the fractions of the input tritium and [14C] labels recovered in the Dowex 50W unbound fraction. Since 6% of the applied [14C]methionine is found in the Dowex 50W unbound fraction, and there are 10 methionines in the capsid protein (15), the yield of acetylated NH2-terminal methionine in Fig. 3A is 60%. The remainder of the NH2 terminus is presumably in larger peptides containing basic amino acids, and hence is retained on Dowex 50W. The recovery of 30% of the tritium label in the unbound fraction implies that about 50% of the tritium label is in the NH2-terminal acetyl group in the intact protein. Comparable results (40% of the tritium label in the N-acetyl group) are obtained by a similar analysis of the amounts of the two labels in the N-acetyl methionine peak of the silica gel chromatogram of Fig. 4A.

![Fig. 4. Analysis of chymotryptic peptides not bound to Dowex 50W. A, silica gel chromatography of tritiated acetate and [14C]methionine-labeled peptides derived from the capsid protein. The origin is located at fraction 0, and migration is from left to right, with the solvent front at fraction 38. B, reversed phase HPLC of tritiated acetate-labeled peptides derived from the capsid protein and [35S]labeled markers indicated in the figure as mso (methionine sulfoxide), N-Ac-mso (N-acetyl methionine sulfoxide), met (methionine), and N-Ac-met (N-acetyl methionine). C and D, reversed phase HPLC of peptides derived from PE2 and labeled with (C) [3H]proline and [14C]alanine; (D) tritiated acetate.](https://example.com/fig4.png)
phoresis. From this material we purified 80 pmol of the major acetate-labeled peptide by reverse phase HPLC from the Dowex 50W unbound fraction of a chymotryptic digest. The peptide was found to have the amino acid composition (Ala, Leu, Pro, Ser), without correcting for the decomposition of serine during HCl hydrolysis. Since there are about 40 alanine residues in PE2, if we assume this peptide contains two alanines, it is obtained from chymotrypsin digestion and Dowex 50W chromatography in nearly 100% yield, and it contains 45% of the total tritium label in PE2. We also found that this peptide is not labeled if derived from PE2 label in vitro with tritiated palmitate (which labels the covalently coupled lipid of the protein) or tritiated glucosamine (results not shown).

From its chromatographic behavior of Dowex 50W and the extent of labeling with tritiated acetate, we conclude that this PE2-derived peptide is N-acetylated, and thus derived from the NH2 terminus of PE2. This in turn implies that at least a significant fraction of PE2 is blocked in the infected cell.

**DISCUSSION**

Before analyzing the incorporation of tritiated acetate into the structural proteins of Sindbis virus, we considered it desirable to suppress the metabolic conversion of exogenously supplied acetate into other chemical forms. We estimate the specificity of the acetate labeling, the fraction of the label not metabolically converted to other chemical forms in the viral proteins, to be 40-50% in chick cells labeled with the procedure which we developed. This is based on the amount of label recovered in two overlapping N-acetylated peptides derived from the capsid protein, and the amount of label recovered in an N-acetylated peptide derived from PE2. We also recovered 70% of the acetate-derived label in chick-grown capsid protein in one 11,000-dalton fragment, which must, therefore, contain the acetylated (NH2-terminal) residues of this protein. The additional tritium label in the large fragment is due to the fact that it contains 70% of the proline and 50% of the glutamic acid plus glutamine of the intact protein, and these are two of the amino acids into which metabolic conversion of acetate was found. We note that the specificity of the label is such that it can be used to identify large proteolytic fragments containing the NH2 terminus of the capsid protein as well as small fragments of one or two amino acids. We have not directly examined the distribution of label in BHK-21-grown, acetate-labeled Sindbis proteins. However, the extent of the difference in the labeling of E1, compared with PE2 and the capsid protein, suggests that the specificity of the acetate label may be even greater in this system.

Chromatography on Dowex 50W-X2 of proteolytic digestion products was first purified to determine the NH2-terminal peptide of a blocked protein in studies on the tobacco mosaic virus coat protein. Narita (21), in his classic studies, reasoned that a blocked peptide, if it contained no lysine or arginine, could not be bound to a strong cation exchanger, while all other peptides in the digest should contain at least the NH2-terminal amino group and should have an affinity for the resin. Since that time, a number of authors have used this technique (5, 22). The use of radiolabel, in particular radioactive acetate in the case of an acetylated NH2-terminus, has allowed us to extend this technique to situations in which it is difficult to obtain sufficient material for the usual biochemical analyses. In the case of Sindbis virus-infected cells, it is easy to obtain radiochemically pure viral proteins and their precursors since the virus shuts down host cell protein synthesis (see, for example, Fig. 1). It has also been found that most of the contaminating peptides which are present in the Dowex 50W-X2 unbound fraction are relatively large, as they are also not retained by the anion exchanger Dowex 1-X2 (23). The use of radiolabeled acetate has allowed us to extend the discrimination of the Dowex 50W chromatographic separation, for we can distinguish and partially separate those small peptides which enter the pores of the resin and are not bound, from peptides which are not retained on the column simply because they are excluded from the pores of the ion exchanger.

Based on an analysis of two peptides isolated by these procedures from the capsid protein of Sindbis virus, we have determined that the NH2 terminus of this protein, both in the virion and inside the infected cell, is an N-acetylated methionine residue, and the results also imply that the next residue is most likely asparagine. This sequence is in complete agreement with the results of several other studies. The capsid protein is known to be blocked in the virion (3), and methionine is the NH2-terminal amino acid of this protein when it is synthesized in vitro in the presence of an enzymatic system which removes acetyl-CoA from the reaction mixture (20). In addition, a blocked peptide with the amino acid composition (Asx, Met, Arg) has been isolated from trypptic digests of the capsid protein (24). Finally, our protein sequence is consistent with the sequence of the viral mRNA coding for this protein (28), in which the deduced primary transcript begins Met-Asn-Arg-. This allows the unambiguous identification of the second residue in the protein sequence as asparagine, rather than aspartic acid, and shows that the NH2 terminus of the capsid protein, although blocked by N-acetylation, is not processed by proteolytic cleavage and retains the initiating methionine. It is also clear that no NH2-terminal peptides with more than two amino acid residues could have been detected by our techniques, since they all would contain arginine and hence be retained on Dowex 50W.

Although the capsid protein was previously known to be blocked, Bonatti and Blobel (20) obtained a partial amino acid sequence from radiolabeled PE2 by Edman degradation. We have recently purified chemically pure PE2 from Sindbis virus-infected cells and examined its NH2 terminus in detail. These results confirmed and extended the previously determined sequence, but they also showed that 90-95% of our PE2 preparation was blocked and that it was unlikely that this NH2-terminal modification occurred during purification. In the presence of a modified NH2-terminus in a large fraction of PE2 is the observation that a comparable fraction of the glycoprotein E3 is blocked. This small glycoprotein, consisting of the NH2-terminal 62-64 amino acids of PE2 (25), is produced by proteolytic cleavage of PE2 during its maturation to the virion glycoprotein E2 (2), and during Sindbis virus infection is secreted into the culture fluid (26). Other workers have reported that the E3 of the closely related Semliki Forest virus (which in this case is retained in the virion) also appears to be blocked (27).

The results of the acetate labeling of PE2 reported here provide additional, independent evidence that a large fraction of PE2 is blocked, allow the identification of the NH2-terminal modification as an acetyl group, and have facilitated the purification of an NH2-terminal acetylated peptide from this protein. The amino acid composition of this peptide is the same as that of the NH2-terminal pentapeptide of unblocked PE2, whose NH2-terminal sequence is Ser-Ala-Ala-Pro-Leu. We, therefore, conclude that the blocked fraction of PE2 is derived from the unblocked fraction by N-acetylation. In addition, the observation that E3, produced at a late stage in the maturation of PE2 (2), is also blocked indicates that

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* J. R. Bell, unpublished data.

\(^3\) J. T. Mayne and M. W. Hurkapiller, personal communication.
the large blocked fraction of PE2 is biologically significant, rather than being the product of a dead end pathway.

One feature of the list of proteins which are known to be blocked (5, 24) is that these proteins are often ones which the cell would not be expected to turn over and resynthesize extensively. Some examples are actin and cytochrome c, both from a variety of sources, and keratin from sheep wool and emu feather. The nature of these blocked proteins suggests that the lack of a free N-terminus may have some role in the protection of these proteins from proteolytic digestion, presumably initiated by exopeptidases (4, 28). One might then expect viral proteins to be often blocked, since it is obviously advantageous to a virus that its structural proteins not be degraded. In fact, blocked structural proteins have been found in tobacco mosaic virus and other plant viruses (5, 28), Mengo virus (29), Sendai virus (30), influenza virus (22), murine leukemia virus (31), avian myeloblastosis virus (32), and Rous sarcoma virus (32), and in the last case the blocking group is known to be present in the polypeptide precursor of the blocked virion protein (33). Even more dramatic examples are provided by adenovirus and reovirus structural proteins. In adenovirus, of the five structural proteins of known NH2-terminal sequence, four are blocked (6, 34, 35), and the other is produced by proteolytic cleavage from a blocked precursor (36). In reovirus, six of the seven virion proteins are blocked, the unblocked protein also being derived from a blocked protein by proteolytic cleavage (37). Thus the determination of the NH2-termini of acetylated proteins may be a problem encountered particularly often in the sequencing of viral proteins, and in vivo labeling of such proteins with radioactive acetate may frequently be useful in the elucidation of their primary structures.

Acknowledgments—We are grateful to Dr. L. E. Hood and his colleagues, in particular Dr. M. W. Hunkapiller, for the use of their equipment and expertise in the separation of peptides by HPLC and the determination of amino acid compositions, to C. M. Rice for his scintillating discussions during the course of this work, and to E. M. Lenches for expert technical assistance and conscientiously growing large amounts of unlabeled virus.

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