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Interactions of Wild-Type and Mutant M Protein of Vesicular Stomatitis Virus with Viral Nucleocapsid and Envelope in Intact Virions. Evidence from [^{125}I]Iodonaphthyl Azide Labeling and Specific Cross-Linking†

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ABSTRACT: Four different temperature-sensitive M protein mutants (tsM) of vesicular stomatitis virus (VSV) were characterized with regard to the association of the mutated M protein either with nucleocapsids or with membranes in the intact virions. Virions were labeled with the photoreactive hydrophobic probe [^{125}I]iodonaphthyl azide (INA) to assess interactions between viral proteins and the lipid envelope. In wild type (wt) virions, the three major structural proteins—G, M, and N—were labeled in the ratio ca. 1.0:0.4:0.2. INA labeled only the membrane-associated peptide of G protein, both in the intact virion and in reconstituted G protein–viral lipid vesicles, demonstrating the specificity of INA for lipid bilayer regions. Labeling of tsM virions with INA resulted in a 2–3-fold greater incorporation into M protein than was found for wt virions, suggesting increased M–membrane associations in the mutant virions. Temperature-stable revertants from tsM possessed wt labeling characteristics. Interaction of the M protein with nucleocapsids was assessed from the abundance of disulfide-linked M–N complexes found after disruption of the virions by sodium dodecyl sulfate solution under nonreducing conditions. The abundance of such complexes was 30–80% less from tsM virions than from wt virions, suggesting decreased M–nucleocapsid interactions in tsM virions. Temperature-stable revertants from tsM resembled wt in the abundance of M–N complex formed. We conclude that the mutations alter M protein in such a way as simultaneously to increase its association with membrane and to decrease its affinity for nucleocapsids in the intact virion.

The vesicular stomatitis virus (VSV)[1] matrix (M) protein, a nonglycosylated polypeptide of M_r 27 000, is one of the three major structural proteins of the virion (Bishop & Smith, 1978). Recent experiments utilizing VSV temperature-sensitive (ts) mutants and viral pseudotypes have shown that M protein is essential for the budding of virions or virus-like particles from the plasma membrane of an infected cell (Schnitzer et al., 1979; Schnitzer & Lodish, 1979; Weiss & Bennett, 1980). Specific functional interactions between M protein and viral nucleocapsid on the one hand and between M protein and membrane containing the viral glycoprotein (G) on the other

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[1] Abbreviations used: VSV, vesicular stomatitis virus; wt, wild-type VSV; wtO, wild-type VSV, Orsay variant; wtG, wild-type VSV, Glasgow variant; ts, temperature sensitive; tsM, temperature-sensitive mutant of complementation group III; $NaDodSO_4$, sodium dodecyl sulfate; [^{125}I]-INA, 5-iodonaphthyl 1-azide containing ^{125}I.

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hand are implied by these findings. While the nature of these interactions remains largely unknown, studies were recently reported suggesting how these interactions are altered upon mutation of the M protein to a ts phenotype (tsM; Reidler et al., 1981; Wilson & Lenard, 1981). A decrease in the (predominantly electrostatic) interaction between M protein and nucleocapsids characterizes the tsM mutants as compared with wild type (wt), in a detergent-disrupted viral preparation (Wilson & Lenard, 1981). By use of the technique of fluorescence photobleaching recovery, a decreased mobility of G protein on the surface of tsM-infected cells was found, indicating an increased interaction of mutant M protein with G protein containing regions of infected cell membrane (Reidler et al., 1981). All the tsM mutants studied behaved similarly in each of these experiments.

In this paper we have used [^{125}I]iodonaphthyl azide ([^{125}I]INA) to characterize interactions between viral proteins and the lipid envelope in intact virions. [^{125}I]INA is a hydrophobic, photoreactive compound that partitions preferentially into hydrophobic regions, most notably the interior of lipid bilayers (Sigrist-Nelson et al., 1977; Gitler & Bercovici, 1980; Kahane & Gitler, 1978; Karlish et al., 1977; Cerletti & Schatz, 1979; Zakowski & Wagner, 1980). The M protein in tsM virions is more extensively labeled with [^{125}I]INA than is the M protein in wt virions, suggesting a more intimate M–lipid association in the tsM virions. In other experiments, the formation of stable M–N cross-links from virions upon disruption under nonreducing conditions (Dubovi & Wagner, 1977) is decreased in tsM as compared with wt virions, suggesting a decreased M–nucleocapsid association in tsM virions.

Materials and Methods

Cells and Viruses. All virus samples were grown in BHK 21-F cells and purified as described previously (Wilson & Lenard, 1981; Miller & Lenard, 1980).

Synthesis of [^{125}I]INA. INA was synthesized by method three of Bercovici & Gitler (1978) or method two of Cerletti & Schatz (1979). Both procedures were modified in the following manner. Iodination of the diazonium salt from 5-aminonaphthyl 1-azide was carried out directly in the vial containing 5 mCi of carrier-free ^{125}NaI (Amersham) after the addition of cold NaI. When the reaction was complete, a small amount of NaOH was added, and [^{125}I]INA was extracted 5 times with *n*-hexane. These were combined on a single 2.5 × 10 cm silica plate (GHLF uniplate, Analtech, Newark, DE), while a sixth extraction was placed on a duplicate plate. Both were developed simultaneously in *n*-hexane and dried under a hood. The plate containing the sixth extraction was visualized under UV, and a single spot (R_f 0.58) which turned brown in the light was identified. The corresponding region was scraped from the first plate and extracted in ether. The ether solution was divided into aliquots and evaporated, and the compound was stored in the dark at −70 °C in ethanol. A fresh aliquot was used for each experiment. The yield of [^{125}I]INA obtained was 11–20% by using the method of Cerletti & Schatz (1979), which was found to be the more convenient of the two methods.

[^{125}I]INA Labeling of VSV. Two milliliters (ca. 1 mg) of purified VSV stored <3 days in phosphate-buffered saline (PBS), pH 7.2, was added to the tube containing [^{125}I]INA (0.2–15 μg) in 10–20 μL of ethanol. Covalent attachment of [^{125}I]INA to VSV was carried out by irradiation with a UV lamp as described by Bercovici & Gitler (1978) after a 5-min preincubation at 37 °C. The labeled virions were then repurified on a 5–40% potassium tartrate gradient spun 60 min at 17 000 rpm in a Beckman SW27 rotor. This procedure removed most of the noncovalently bound [^{125}I]INA, as well as any viral particles that may have been disrupted during photolysis. Viral bands were then collected and immediately pelleted and washed twice. All manipulations up to this point were done in subdued light. Virions were then either precipitated with 9 volumes of acetone at −20 °C and pelleted or resuspended directly in $NaDodSO_4$ buffer solution for gel electrophoresis.

Protease Treatment. Spikeless particles were prepared according to published procedures (Schloemer & Wagner, 1975), labeled with [^{125}I]INA before or after digestion, and repurified as described above. A protein to enzyme ratio of 5:1 was used in the proteolytic digestions with chymotrypsin, trypsin, and Pronase. After an incubation of 30 min at 37 °C, the reaction was terminated by pelleting the virus. Labeled polypeptides were analyzed on 15% polyacrylamide gels in $NaDodSO_4$. One-millimeter slices were counted in a Beckman γ counter.

Reconstituted G protein–viral lipid vesicles were prepared from an octyl glucoside extract of VSV by dialysis as described by Miller et al. (1980). [^{125}I]INA labeling of vesicles was performed under conditions identical with those used to label VSV.

Gel Electrophoresis and Radioautography. Viral samples were analyzed on $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) for quantitation of [^{125}I]INA/protein ratios. The gels were fixed, stained with Coomassie Brilliant Blue R-250, destained, dried, and radioautographed with Kodak SB-5 X-ray film. The autoradiograms were scanned with a Joyce-Loebl 3CS microdensitometer. Protein concentrations were determined by scanning either stained gels with an ISCA gel scanner before drying or the negatives of stained dried gels by using the microdensitometer. Standards were run by using Coomassie stained viral protein, and the concentration of virus analyzed were always chosen so as to be within the linear range. Specific activities were determined by performing radioautography and protein determination on the same gel.

Measurement of M–N Complex. Freshly purified virus was pelleted and resuspended in 50 mM sodium phosphate containing 2% $NaDodSO_4$. Phosphate buffers with different pHs were prepared by mixing 50 mM NaH_2PO_4 and 50 mM Na_2HPO_4 in varying proportions. Samples were boiled for 3 min before being applied to gels. For quantitation of the M–N complex, $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on a 5–16% linear gradient slab formed with the LKB Ultrograd electronic gradient mixer and topped with a 5% stacking gel. Samples were run in the unreduced state (without addition of 2-mercaptoethanol). These gels were stained, scanned, and quantitated as described above. A two-dimensional electrophoresis system as described by Markwell & Fox (1980) was used to identify the components of the M–N complex. Protein bands were identified after staining with Coomassie Blue.

Results

Labeling of wt VSV and Reconstituted G Protein–Lipid Vesicles with [^{125}I]INA. VSV was reacted with [^{125}I]INA in order to characterize the interaction of each of the viral proteins with the lipid envelope. In studies using wt VSV, it was found that incorporation of INA into viral protein did not increase after 6–8 min of irradiation, at which time the virus remained fully infectious as measured by plaque assay. Ten to thirty percent of added label was incorporated into virus under these conditions, of which 70% was associated with lipid, as judged by its extractability into organic solvents. Negligible incorporation occurred in an unirradiated sample.

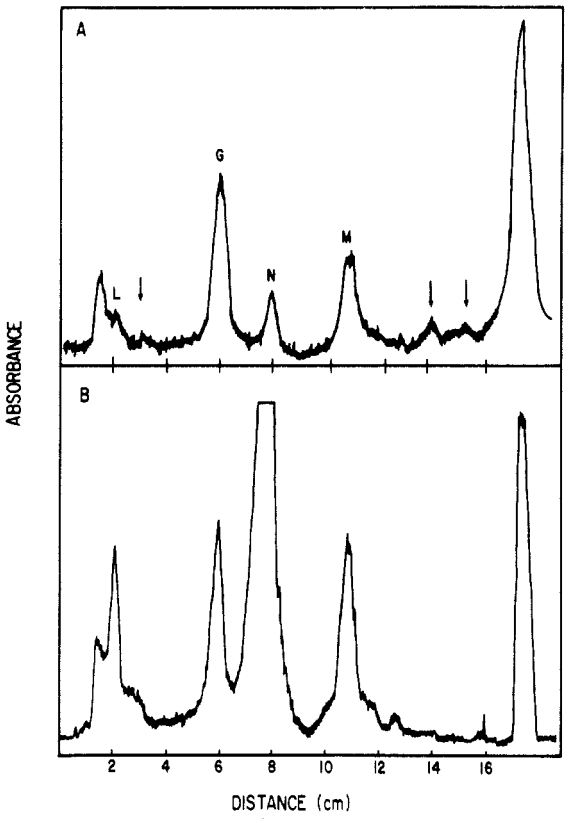

FIGURE 1: Densitometer scans of radioautograms of $NaDodSO_4$–polyacrylamide gel electrophoresis (10% gel) obtained after labeling with [^{125}I]INA. (A) Intact virions; (B) virion disrupted with $NaDodSO_4$ prior to labeling. [^{125}I]INA specific activity = 1.87 Ci/mmol.

All of the major proteins of VSV were labeled with INA in the intact virion (Figure 1A). The G protein was most heavily labeled, and the M protein was also substantially labeled, followed by lower and variable amounts of label in the N and L proteins. Additional labeled bands, which did not correspond to viral proteins, were also consistently seen (arrows, Figure 1A). These probably arise from small amounts of host cell membrane proteins incorporated into budding VS virions (Lodish & Porter, 1980). Varying the concentration of [^{125}I]INA over an ca. 100-fold range did not significantly affect the distribution of label in each of the viral proteins (data not shown).

The distribution of label in the VSV proteins is drastically altered if the viral particle is first disrupted with $NaDodSO_4$. While G is predominantly labeled in the intact virion, N protein is predominantly labeled after disruption (Figure 1). The labeling pattern in the intact virion thus reflects the structure of the virion and is grossly altered upon disruption by $NaDodSO_4$.

In order to assess the specificity of [^{125}I]INA labeling, we determined which part of the G polypeptide chain was labeled in intact virions and in reconstituted vesicles. Intact virions were digested with proteases, repurified, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. As expected, the band at ca. 67 000 daltons corresponding to intact G protein disappeared after this treatment. The new 5000–7000-dalton peptide that appeared on the gel corresponds to the membrane-associated peptide of the G protein (Mudd, 1974; Schloemer & Wagner, 1975). This peptide contained virtually all of the label previously associated with undigested G protein (Figure 2; Table I). Thus, labeling of G protein is essentially restricted to the membrane-associated portion of the polypeptide chain. These results confirm those previously reported by Zakowski & Wagner (1980).

Table I: Distribution of [^{125}I]INA in VSV Proteins after Labeling of Intact Virons. Effect of Protease Digestion

	N/G	M/G
undigested	0.38[a]	0.63[a]
after digestion by		
chymotrypsin	0.37[b]	0.56[b]
thermolysin	0.40[b]	0.60[b]
Pronase	0.26[b]	0.52[b]
trypsin	0.38[b]	0.47[b]

[a] G protein (ca. M_r 67 000). [b] G peptide (M_r 5000–7000).

Table II: G/Lipid Ratio of [^{125}I]INA in VSV and Reconstituted Vesicles[a]

VSV	0.26
G vesicles	0.26
G vesicles after thermolysin treatment	0.23[b] (0.26)[c]

[a] Determined after separation on 15% $NaDodSO_4$–polyacrylamide gel electrophoresis. [b] 5000–7000 region only. [c] 5000–7000 + 67 000 region.

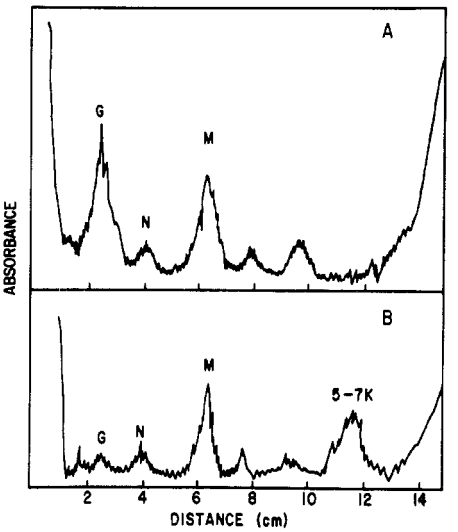

FIGURE 2: Densitometer scans of radioautograms of $NaDodSO_4$–polyacrylamide gel electrophoresis (15% gel) obtained from [^{125}I]-INA-labeled intact virions (A) before and (B) after digestion with thermolysin. [^{125}I]INA specific activity = 8.35 Ci/mmol.

If labeling was carried out on virions after proteolytic digestion and repurification of the spikeless particles, the distribution of radioactivity between M and N proteins and the 5000–7000 peptide of G was identical with that found if the intact virion was labeled and then digested. This indicates that the presence of the external portion of the G protein does not influence the distribution of label between the viral proteins.

Reconstituted G protein–viral lipid vesicles were prepared from an extract containing virtually all the G protein and lipid of the extracted virions (Miller et al., 1980). They should therefore have a G protein to lipid ratio essentially identical with that of the intact virion. After being labeled with [^{125}I]INA, the ratio (label in G protein/label in viral lipid) was identical in vesicles and in intact virions (Table II). Further, digestion of the vesicles with thermolysin followed by repurification of the vesicles yielded a peptide of M_r 5000–7000 that contained virtually all the label originally present in G protein (Table II). This provides additional evidence that G protein in reconstituted vesicles is oriented in the lipid bilayer in a manner that closely resembles that in the intact virion [cf. Miller et al. (1980)] and that in this orientation it is labeled in its membrane-associated region by [^{125}I]INA.

Table III: Specific Labeling of VSV wt, tsM, and tsM Revertants by [^{125}I]INA

virus	complementation group (protein)	ratio of [^{125}I]INA/protein[a] N	M
wtG		0.12	0.37
tsG 31	III (M)	0.14	0.70
tsG 33	III (M)	0.16	0.74
tsG 31R4		0.14	0.32
tsG 33R5		0.21	0.47
wtO		0.35	0.40
tsO 23	III (M)	0.37	1.19
tsO 89	III (M)	0.28	1.03
tsO 23R7		0.35	0.40
tsO 89R5		0.40	0.56
tsO 45	V (G)	0.23	0.49
tsO 110	V (G)	0.37	0.52
tsO 125	IV (N)	0.24	0.50
tsO 194	IV (N)	0.35	0.62

[a] Normalized to G = 1.0.

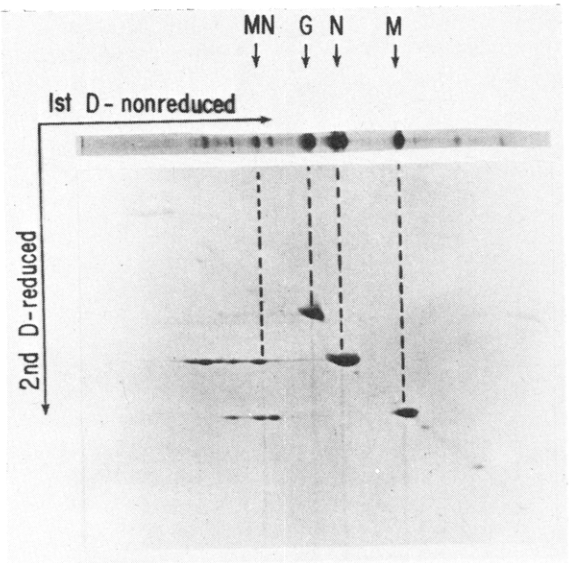

FIGURE 3: Identification of the M–N complex by two-dimensional gel electrophoresis.

Labeling of tsM Mutants and Revertants with [^{125}I]INA. Specific labeling of each of the viral proteins in wt, tsM, and tsM revertant virions was measured to determine whether changes in lipid–protein interactions resulted from mutations. Results are shown in Table III. The specific labeling of M protein in each of the mutants was enhanced relative to that of wt by 2–3-fold. The M protein of revertants was labeled to a similar extent as wt, indicating that the enhanced labeling in the mutants is associated with the ts phenotype. The apparent differences in N protein labeling between Orsay and Glasgow variants (Table III) arose because the variants were studied in separate experiments and this was not a reproducible finding. The enhancement of labeling of mutant M protein, on the other hand, was consistently seen although precise values varied from experiment to experiment. This enhancement was not observed when ts mutants of group IV (N protein) or group V (G protein) were labeled with [^{125}I]INA (Table III). No significant differences were found in the phospholipid compositions of wt and tsM virions, suggesting that altered lipid composition did not cause the changes in M protein labeling (data not shown).

M–N Complexes in wt VSV. When wt VSV was disrupted in $NaDodSO_4$ and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions, a prominent new band arose corresponding to about 87 000 daltons. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis in the second dimension, using a reducing agent, showed this band to consist of M and N proteins, presumably in equimolar amounts (Figure 3; Dubovi & Wagner, 1977). We observed that the amount of the 87 000-dalton band depended upon the pH of the $NaDodSO_4$ solution used for viral disruption. Much lower quantities were found if the virions were disrupted at acidic pH (Figure 4). This result would be expected if the M–N complex arose from a process of disulfide exchange, which would be enhanced by elevated concentrations of $R–S^-$ anion. Additional evidence that the M and N proteins in this complex are joined by disulfide bonds came from the observations of Dubovi & Wagner (1977), who showed that the reducing agent 2-mercaptoethanol and the oxidizing agent hydrogen peroxide both abolished the complex. Peroxide destroys cystine disulfide bonds by oxidation to cysteic acid residues (Toennis & Homiller, 1942).

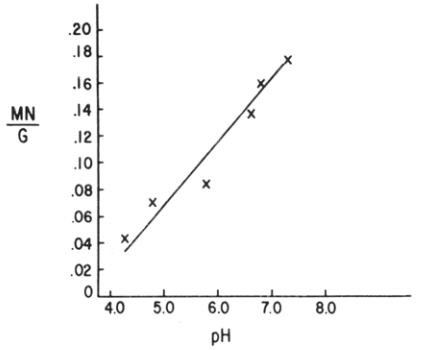

FIGURE 4: Dependence of abundance of the M–N complex on the pH of the $NaDodSO_4$ dissociation buffer.

Table IV: Abundance of M–N Complexes after Nonreductive Dissocation of Virions by $NaDodSO_4$, pH 7.0

	MN/G expt 1[a]	expt 2[b]	expt 3[b]		MN/G expt 4[a]	expt 5[b]	expt 6[b]
wtG	0.49	0.51	0.23	wt 0	0.78	0.38	0.52
tsG 31	0.35	0.10	0.10	ts 023	0.49	0.26	0.35
tsG 33	0.34	0.10	0.14	ts 089	0.59	0.20	0.42
tsG 31R4	0.52	0.54	0.24	ts 023R7	0.77	0.39	0.53
tsG 33R5	0.49	0.59	0.23	ts 089R7	1.10	0.47	0.57

[a] Determined by counting gel slices after $NaDodSO_4$–polyacrylamide gel electrophoresis of the [^{35}S]Met-labeled virus. [b] Determined by densitometry after staining the gels with Coomassie Blue.

M–N Complexes in tsM Mutants and Revertants. The abundance of M–N complexes produced by disruption of wt, tsM, and tsM revertant virions by $NaDodSO_4$ at pH 7.5 was measured as an indication of the proximity of M protein with nucleocapsid in the undisrupted virion. Results are given in Table IV . The amount of M–N complex was reduced in all the mutants examined but was found in amounts similar to those of wt for all the revertants. The total amounts of M and N proteins did not differ in wt and tsM virions, as estimated from $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of reducing agents (not shown). Thus, a decreased ability to form M–N complexes is characteristic of ts M protein mutants and is related to the ts phenotype.

Discussion

Experiments performed on several different membrane systems have demonstrated that [^{125}I]INA is a reliable probe for the labeling of membrane-associated proteins (Kahane &

Gitler, 1978; Karlish et al., 1977; Cerletti & Schatz, 1979; Zakowski & Wagner, 1980). The observation that labeling is restricted to the membrane-associated fragment of VSV G protein in the intact virion (Table I; Zakowski & Wagner, 1980) provides good evidence that labeling is limited to lipid-associated regions of membrane proteins.

The reconstituted vesicles prepared by dialysis of octyl glucoside extracts of VSV have previously been described (Miller et al., 1980). These vesicles consist of all the G protein and all the lipid originally present in the virions and have been shown to be predominantly unilamellar and asymmetric, with the G protein oriented outward (Miller et al., 1980). Results of [^{125}I]INA labeling of these vesicles show that a peptide of identical molecular weight (5000–7000) to the membrane-associated peptide in the intact virion contains virtually all of the G protein label. In addition, the ratio of label in G protein to that in lipid is identical in virions and in reconstituted vesicles (Table II). This provides very strong evidence that the associations between lipids and G protein in intact virions and in reconstituted vesicles are virtually identical.

The specific labeling of M protein by [^{125}I]INA has been used in this paper to indicate the extent of association between M protein and the viral lipid envelope. While the possibility that INA might label highly hydrophobic, nonlipid regions of some proteins cannot be ruled out, the experiments described above and in other systems make this possibility unlikely. The increase in specific labeling of mutant M protein as compared with that of wt (Table III) is most readily interpreted as indicating that the tsM protein is more intimately associated with the hydrophobic interior of its viral envelope bilayer than is the wt M protein. Several previous reports have shown that M protein is associated with membranes in infected cells (David, 1973; Hunt & Summers, 1976; Atkinson et al., 1976; Morrison & McQuain, 1977; Knipe et al., 1977).

The amount of disulfide-linked M–N complex present in gels after $NaDodSO_4$ disruption of tsM virions is reduced as compared with that of wt (Table IV). The fact that the amount of complex found depends upon the pH of the $NaDodSO_4$ disruption medium (Figure 4) indicates that the complex may be formed and/or dissociated during disruption and might be simply an artifact of the disruption process. While its existence as a major band on the gel does not provide evidence for its presence in the undisrupted virion, its relative abundance under carefully controlled disruption conditions may be considered to indicate the relative proximity of the M and N proteins in the virion.[2] The decreased abundance of M–N complexes in gels from tsM virions as compared with those from wt virions thus indicates a closer proximity of M to nucleocapsids in the wt virions than in the tsM virions. Further, the reversion to wt abundance of the M–N complex in temperature-stable revertants indicates that this, like the [^{125}I]INA-labeling profile, reflects a necessary concomitant of the temperature-sensitive phenotype.

The [^{125}I]INA-labeling experiments and the M–N cross-linking experiments, taken together, provide a direct demonstration that the coordinate changes in affinity for nucleocapsid and membrane inferred previously from studies of infected cells and in disrupted virions (Reidler et al., 1981; Wilson & Lenard, 1981) actually characterize the intact, infectious virion as well. Wilson & Lenard (1981) used salt dependence of the M protein mediated inhibition of viral RNA polymerase activity in detergent-disrupted viral preparations to demonstrate a decreased electrostatic interaction between nucleocapsids and mutant M protein, as compared with wt M protein. Reidler et al. (1981) used the fluorescent photobleaching recovery technique to demonstrate that G protein on the surface of tsM-infected cells was less mobile than G protein on the surface of wt-infected cells. This was attributed to an increased association of mutant M protein with the cell surface. It is noteworthy that these striking alterations in the affinity of M for other viral components are compatible with normal viral function.

The simplest explanation of how two such coordinate effects could arise in common in all the tsM mutants is to postulate two independent binding sites on the M protein, one mediating attachment to membrane and the other to nucleocapsid. A mutation leading to a decreased affinity for nucleocapsid could then increase the association with membrane purely by mass action:

$$\text{M–nucleocapsid} \rightleftharpoons \text{M}_{\text{soluble}} \rightleftharpoons \text{M–membrane}$$

Other possibilities are that the two binding sites are not completely independent or that the mutation causes a conformational change that affects two different regions in a coordinate manner, e.g., by altering the phosphorylation state of the protein (Clinton et al., 1978). Further insights into how ts mutations in M protein can cause such coordinate effects may arise from knowledge of the sequences of the wt protein, the ts mutant proteins, and the temperature-stable revertants isolated from them.

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[2] This interpretation assumes that the half-cystine residues themselves are not altered by the mutation. Such a specific alteration induced by the mutation seems unlikely, however, since the change in abundance of the M–N complex was a common feature of all the tsM mutants examined.

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S-Adenosylhomocysteinase from Yellow Lupin Seeds: Stoichiometry and Reactions of the Enzyme·Adenosine Complex†

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ABSTRACT: Plant (*Lupinus luteus*) *S*-adenosylhomocysteinase, an α_2 dimer, forms a 1:2 enzyme·adenosine complex. The binding sites for adenosine are not equivalent. Binding of the first molecule of adenosine is fast ($k > 7 \times 10^5$ M^{-1} s^{-1}), whereas the second molecule of adenosine binds in a slow process with a half-life of 5 min. Adenosine in the 1:1 and 1:2 enzyme–substrate complexes reacts slowly ($k = 0.05$ min^{-1}) to give finally free enzyme, adenine, and ribose. The enzyme does not lose its ability to catalyze the synthesis of *S*-adenosylhomocysteine during the reactions. The relevance of the data to the catalytic functioning of the plant *S*-adenosylhomocysteinase is discussed.

S-Adenosylhomocysteinase (EC 3.3.1.1) catalyzes the following reaction (De la Haba & Cantoni, 1959):

S-adenosylhomocysteine + H_2O ⇌ adenosine + homocysteine

This enzyme has not been found in procaryotes but occurs in all eucaryotes examined (Waler & Duerre, 1975) and has been purified to homogeneity from plant (Guranowski & Pawelkiewicz, 1977) and animal (Richards et al., 1978; Saebø & Ueland, 1978; Schatz et al., 1979; Palmer & Abeles, 1979) tissues.

Recently it has been shown that plant (*Lupinus luteus*) *S*-adenosylhomocysteinase, an α_2 dimer, forms isolatable 1:1 complex with one of its substrates, adenosine (Jakubowski & Guranowski, 1978). In this report, the evidence is presented for the existence of a 1:2 *S*-adenosylhomocysteinase·adenosine complex. Reactions of both 1:1 and 1:2 enzyme·adenosine complexes leading finally to free fully active enzyme, adenine, and ribose are also described.

Materials and Methods

Materials. Radiochemicals were purchased from The Radiochemical Centre (Amersham, England). Sephadex G-50 (fine) was from Pharmacia (Uppsala, Sweden). Nitrocellulose filters were from Schleicher & Schüll (Dassel, West Germany). Thin-layer chromatographic plates, aniline phthalate spray and ribose were obtained from Merck (Darmstadt, West Germany). Adenine, adenosine, dithiothreitol, Bicine[1] buffer, and *S*-adenosylhomocysteine were from Calbiochem (Los Angeles, CA). DL-Homocysteine was from Nutritional Biochemicals (Cleveland, OH).

Enzymes. Homogeneous *S*-adenosylhomocysteinase was prepared from yellow lupid seeds as described previously (Guranowski & Pawełkiewicz, 1977) and was stored at −20 °C as a 21 μM stock solution in 10 mM potassium phosphate buffer (pH 6.8) containing 5 mM 2-mercaptoethanol and 10% glycerol. Adenosine nucleosidase was purified from barley leaves as described previously (Guranowski & Schneider, 1977). Adenosine deaminase (specific activity 235 units/mg) was purchased from Sigma (St. Louis, MO).

Unless otherwise indicated, all experiments were performed at 25 °C in a medium (pH 8.3) containing 50 mM K_2HPO_4 and 2.5 mM 2-mercaptoethanol.

Nitrocellulose Filter Assay. Indicated concentrations of [^{14}C]adenosine (559 Ci/mol, 1 Ci = 3.7×10^{10} Bq) and lupin *S*-adenosylhomocysteinase were mixed in the medium, and after appropriate time intervals, aliquots of the reaction mixture were applied on nitrocellulose disks presoaked in the medium. The filters were then washed 4 times with 1-mL portions of ice-cold medium, oven-dried, and counted in a scintillation counter. Blanks (without enzyme) were subtracted from all experimental values. Morever, since it has been demonstrated that nitrocellulose filters adsorb adenine (Schneider & Guranowski, 1975), blanks with adenine were also run, and the results were corrected accordingly. The reproducibility of the assay was ±10%.

Gel Filtration. Gel filtration was carried out at 0–1 °C. Aliquots (20 μL) of reaction mixtures were applied onto a small Sephadex G-50 (fine) column (0.4 × 6 cm) equilibrated with the medium. Fractions (50 μL) were collected and assayed for radioactivity by scintillation counting.

Measurement of Adenine Formation. Aliquots (5 μL) of the reaction mixtures containing *S*-adenosylhomocysteinase and [^{14}C]adenosine in the medium were spotted on the origin line of silica gel plates. The plates were developed with 2-

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[1] Abbreviations used: E, *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1); Ado, adenosine; Ade, adenine; Rib, ribose; Bicine, *N*,*N*′-bis(2-hydroxyethyl)glycine.

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