122. Tobacco Mosaic Virus as a Carrier for Small Molecules. II. Cooperative Affinity Labeling of Membrane Vesicles with a TMV Angiotensin Conjugate[1])

Preliminary Communication

by **Verena M. Kriwaczek, Jean-Claude Bonnafous[2]), Martin Müller[3])** and **Robert Schwyzer**

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, CH-8093 Zürich

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Summary

A (3-mercapto-1-succinyl)-TMV ε-maleimidohexanoyl-[1-asparagine, 5-valine]-angiotensin II conjugate was prepared and used for cooperative affinity labeling of bovine adrenal cortex cell membrane vesicles containing angiotensin-binding sites. The labeling was demonstrated by electron microscopy and by CsCl and sucrose density gradient centrifugation. Preliminary evidence for specific binding and for the postulated cooperative affinity interaction is produced.

The idea of cooperative affinity labeling was introduced in the preceding paper [2]. There, an unexpectedly strong melanophore-stimulating activity of a TMV α-melanotropin (500)[4]) conjugate was demonstrated, for which cooperativity is a possible explanation. Here, we describe preliminary observations on a very strong physical binding of TMV loaded with the hormone angiotensin II to a bovine adrenal cortex cell membrane vesicle preparation containing angiotensin discriminators (binding sites) [3]. The evidence is by EM. and density gradient centrifugation. Our observations are supported by recent experiments with quasi-elastic light scattering[5]).

The *synthesis* of (3-mercapto-1-succinyl)-TMV ε-maleimidohexanoyl-[1-asparagine, 5-valine]-angiotensin II was carried out according to the *Scheme.* [1-Asparagine, 5-valine]-angiotensin II [4] was substituted at the free α-amino group with *p*-nitrophenyl ε-maleimidohexanoate in very good yield to produce ε-maleimidohexanoyl-[1-asparagine, 5-valine]-angiotensin II (= ANG'). Unfortunate-

[1]) This work was supported by the *Swiss National Science Foundation.* Abbreviations: TMV = tobacco mosaic virus; EM. = electron microscopy; symbols for amino-acids and peptides according to the IUPAC-IUB rules [1].

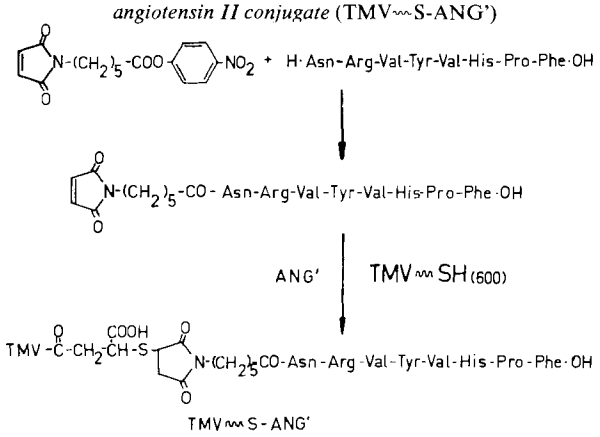
[2]) Present address: E.N.S. Chimie, 8, rue de l'Ecole Normale, F-34075 Montpellier-Cedex.

[3]) Laboratorium für Elektronenmikroskopie I, ETHZ, 8092 Zürich.

[4]) The number in brackets indicates the average number of hormone molecules covalently bound to one TMV particle (maximum: 2130).

[5]) This work was performed in the Laboratorium für Festkörperphysik der ETHZ (Prof. Dr. *W. Känzig*) by *H.-R. Haller, K. Baumann & V.M. Kriwaczek* (report in preparation).

Scheme. *Preparation of (3-mercapto-1-succinyl)-TMV (ε-maleimidocaproyl)-[1-asparagine, 5-valine]-angiotensin II conjugate* (TMV〰S-ANG')



ly, this compound is very sparingly soluble in water at pH 7. Therefore, coupling with (3-mercapto-1-succinyl)-TMV (TMV〰SH) [2] had to be done at pH 3, a compromise between TMV〰SH stability and ANG' solubility. The resulting TMV〰S-ANG' contained about 600 covalently and 500 non-covalently bound ANG' residues. Because preliminary experiments with electrodialysis removed the excess ANG' only concomitantly with the destruction of the virus particles, the crude preparation was used in the labeling experiments (the non-covalently bound ANG' would be expected to compete with 'covalent' TMV〰S-ANG' for the binding sites, but not to assist binding).

Cooperative affinity labeling. The experiments are listed in the *Table;* most of them were reproduced repeatedly with different vesicle and TMV〰S-ANG' preparations. It was verified that the membrane vesicle preparations had the same binding capacity for angiotensin as reported earlier [3]; this capacity was reduced by 25% in the presence of 27% aqueous CsCl-solution (d = 1.25, corresponding roughly to the position of the vesicle/TMV〰S-ANG' band in the density gradient).

The labeling experiments 2.1 and 2.2 produced the results schematically shown in *Figure 1.* EM. inspection showed that, indeed, an aggregation between a part of the vesicle population and TMV〰S-ANG' had occurred. On density gradient centrifugation in CsCl-solution these aggregates (C3) were nicely separated and were intermediate between the membrane (C2) and the virus particle bands (C4)

Table. *Cooperative affinity labeling of bovine adrenal cortex membrane vesicle angiotensin binding sites with (3-mercapto-1-succinyl)-TMV ε-maleimidohexanoyl-[1-asparagine, 5-valine]-angiotensin II conjugate*

1. Controls: 1.1. TMV + membranes; sucrose *(Fig. 3);* 1.2. TMV〰S-MAC + membranes; sucrose *(Fig. 4);* 1.3. TMV〰S-ANG' + heated membranes; CsCl.

2. Labeling: 2.1. TMV〰S-ANG' + membranes; sucrose *(Fig. 2);* 2.2. TMV〰S-ANG' + membranes; CsCl.

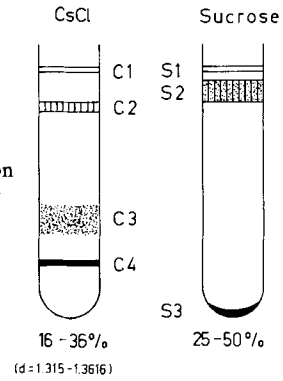
3. Reversibility: 3.1. TMV〰S-ANG' + membranes + [1-asparagine, 5-valine]-angiotensin II; CsCl; 3.2. TMV〰S-ANG' + membranes + [1-asparagine, 3-valine]-angiotensin II; sucrose; 3.3. TMV〰S-ANG' + membranes, sucrose, then angiotensin + CH_3COOH; CsCl.

(an example of affinity density perturbation [5]). They remained associated with the membrane band (S2) in sucrose, where the virus particles (S3) sedimented to the bottom *(Fig. 1* and *2)*.

The control experiments were characterized by the lack of a pronounced vesicle/virus aggregation as shown by an almost completely suppressed band C3 in experiment 1.3 and EM. inspection of experiments 1.1–1.3 (for examples see *Fig. 3* and *4*).

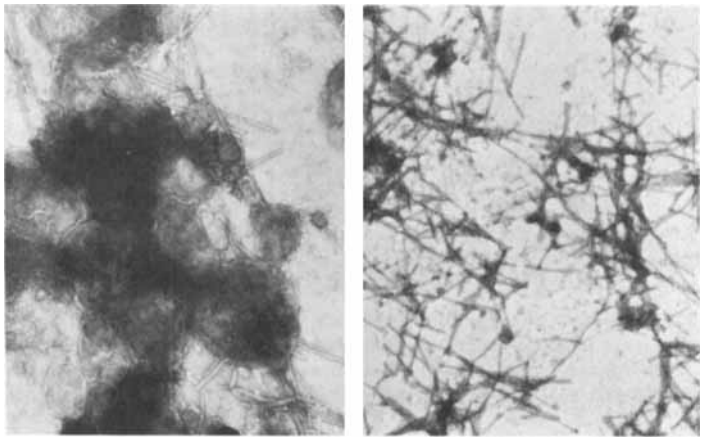
A *reversibility* or *competitive inhibition* of cooperative affinity labeling in the presence of 0.2–1 mmol [1-asparagine, 5-valine]-angiotensin II could not be demonstrated in the experiments 3.1 and 3.2. Only if the vesicle/TMV〰S-ANG' band S1 of experiment 2.1 was isolated and treated with 0.33 mmol [1-asparagine, 5-valine]-angiotensin II for 90 min at 4° it was decomposed into a vesicle band and a virus particle band. These experiments indicate that the overall association of the hormone-loaded virus to the vesicles is much stronger than that of the hormone

CsCl density gradient: C1 = buffer layer of about 100–300 µl incubation fluid; C2 = membrane vesicle layer without TMV or TMV〰S-ANG' particles; C3 = membrane vesicle/TMV〰S-ANG' aggregates (missing in incubation of vesicles with native TMV); C4 = TMV or TMV〰S-ANG' particles.



Sucrose density gradient: S1 = corresponds to C1; S2 = mixture of membrane vesicle/TMV〰S-ANG' aggregates with membrane vesicles (practically no virus particles with TMV or TMV〰S-MAC); S3 = TMV, TMV〰S-MAC, or TMV〰S-ANG' particles.

Fig. 1. *Density gradient centrifugation* (schematic representation).



a) b)

Fig. 2. *Labeling experiment 2.1 with TMV〰S-ANG'* (see *Table*). EM. views of sucrose density gradient centrifugation *(Fig. 1)*. a) Band S2 showing TMV〰S-ANG'/vesicle aggregates; magnification × 50,000. b) Band S3 showing TMV〰S-ANG' particles plus debris (no clear-cut vesicles); magnification × 27,000

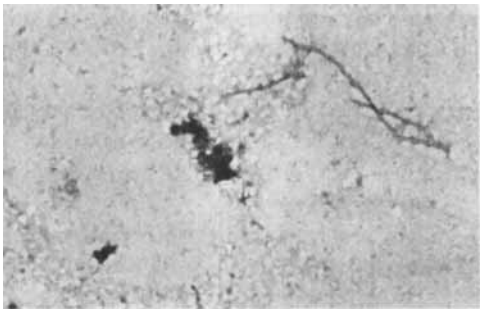
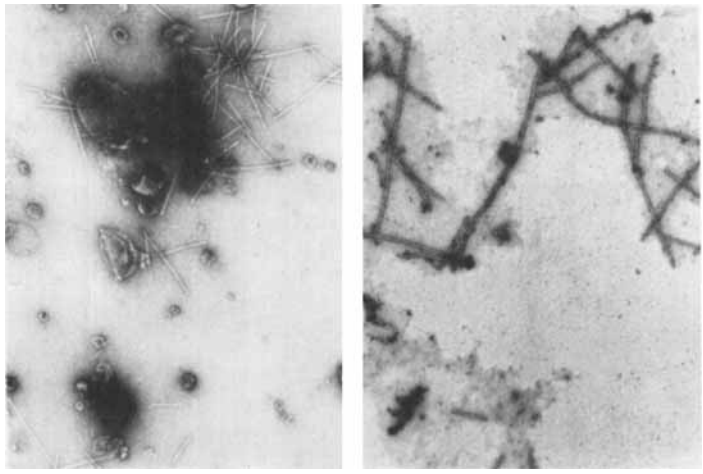


Fig. 3. *Control experiment 1.1 with native TMV* (see *Table*). EM. view of sucrose density gradient centrifugation *(Fig. 1)*: band S2 shows a few native TMV particles and membrane vesicles; some vesicles form aggregates, but not with TMV; magnification × 50,000

alone (in the incubation mixture, the overall concentration of covalently bound hormone was about 0.01 mmol/l, that of the competing 'free' hormone around 1 mmol/l). A 100- to 1000-fold increase of the association constant is readily explained by a simultaneous interaction of the hormone 'side-chains' of one virus particle with only very few binding sites of one and the same vesicle.

Experimental Part

(3-Mercapto-1-succinyl)-TMV ε-maleimidohexanoyl-[1-asparagine,5-valine]-angiotensin conjugate (TMV〰S-ANG'). 1 mmol of [1-asparagine,5-valine]-angiotensin II [4] and 5 mmol of *p*-nitrophenyl 6-maleimidohexanoate[6]) were dissolved in 6 ml of pyridine/dimethylformamide 1:2. After 4 h at 20° the pyridine was removed in a rotary still in vacuum. The product was precipitated with ether and washed repeatedly with fresh ether. Yield: 90% of apparently pure ANG'. TLC. (silica gel): one spot of



a) b)

Fig. 4. *Control experiment 1.2 with TMV〰S-MAC* (see *Table*). EM. view of sucrose density gradient centrifugation *(Fig. 1)*: a) Band S2 (after incomplete separation of vesicles and virus) showing separate membrane and TMV〰S-MAC particles with no apparent virus/membrane aggregation; magnification × 45,000. b) Band S3 with TMV〰S-MAC particles plus debris as in *Figure 2b;* magnification × 38,000

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[6]) Prepared from ε-maleimidohexanoic acid [6], *p*-nitrophenol, and dicyclohexyl-carbodiimide in chloroform in 95% yield (after crystallization from 2-propanol), m.p. 76–77°, Rf 0.805 on silica gel with chloroform/methanol 1:1 (*v/v*). ^{1}H-NMR. in $CDCl_3$ indicated both purity and identity.

Rf 0.495 (ethyl acetate/pyridine/water, 20:10:11 (*v/v*)), 0.327 (1-butanol/pyridine/acetic acid/water, 42:24:4:30 (*v/v*)), no traces of starting material or of their hydrolysis products.

With [^{3}H]angiotensin II [3] a tritium-labelled ANG' with a specific activity of 20 mCi/mmol was prepared by the same procedure.

(3-Mercapto-1-succinyl)-TMV (TMV〰SH) was synthesized as described [2] and the number of introduced mercapto groups assayed by the *Ellman* DTNB reagent [7] as about 600 per TMV particle. A ten-fold excess of ANG' over TMV〰SH was dissolved in a small volume of glacial acetic acid/methanol and its apparent pH adjusted to 3.0 with dilute NaOH-solution. The solution of TMV〰SH in was adjusted to the same pH value with dilute acetic acid. The two solutions were combined and slowly rotated in a flask for 1 h at 20°. The TMV〰S-ANG' was purified by precipitation with NaCl and polyethyleneglycol *(Carbowax)*, 4 g/100 ml each [8], and centrifugation for 10 min at 10,000 rpm. The pellet was dissolved in a buffer at pH 7.0. Measurements of radioactivity indicated a content of 1140 ANG' molecules per TMV particle. Gel-electrophoresis at pH 6.8 showed that a certain amount of non-covalently bound ANG' was associated with the TMV. Electrodialysis [2] at pH 4.5 removed the excess ANG': 635 ANG' molecules remained (covalently) bound to the virus; however, the TMV particles were strongly damaged and degraded to RNA and capsomers as shown by EM. and by CsCl gradient centrifugation.

(3-Mercapto-1-succinyl)-TMV ε-maleimidohexanoic acid conjugate (TMV〰S-MAC). TMV〰SH in phosphate buffer of pH 6.8 was treated with a 10-fold excess of ε-maleimido-hexanoic acid in the same solvent for 1 h at 20°. The substituted virus was isolated as described for TMV〰S-ANG'. With *Ellman's* reagent [7], no mercapto groups were detectable.

Caesium chloride gradient centrifugation. Linear gradients of 16–36% CsCl in water (d = 1.1315 to 1.3616) were employed. Centrifugation was carried out in a *Beckman Spinco* Rotor SW 39 at 40,000 rpm and 4° for 75 min. After this time equilibrium had been reached by both the membranes and the virus particles. The bands containing membranes and TMV were visible (about 0.1–0.6 mg of membranes and TMV were applied). The density of TMV was found to be 1.33, that of the membrane vesicles about 1.17–1.20.

Sucrose gradient centrifugation was carried out in the same manner using a linear gradient of 25–50% sucrose in water at 10,000–40,000 rpm for 30 min. The membrane band was found near the top of the gradient, the virus particles sedimented to a pellet at the bottom of the tube *(Fig. 1)*.

Cooperative affinity labeling and controls. About 400 µg of purified bovine adrenal cortex cell membrane vesicles (containing angiotensin binding sites) [3] were incubated with about 600 µg of TMV (unsubstituted or substituted) in a total volume of about 300 µl at 20° for 10 min and then layered on top of the density gradient. The experiments outlined in the *Table* were performed and all of the bands examined by EM.

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