Voltammetry of Tobacco Mosaic Virus and Its Isolated Protein at the Graphite Electrode

G. Ruttkay-Nedecký[1] and V. Brabec[2]

1 *Institute of Virology, Slovak Academy of Sciences, Mlynská dolina 1, 817 03 Bratislava, Czechoslovakia*
2 *Institute of Biophysics, Czechoslovak Academy of Sciences, Královopolská 135, 612 65 Brno, Czechoslovakia*

Abstract. The electrochemical behaviour of tobacco mosaic virus (TMV) and its isolated protein was studied using differential pulse (DP) voltammetry at a graphite electrode and by direct current (DC) polarography in Brdička solution. TMV and its isolated protein were found to be electrooxidized at the graphite electrode in the adsorbed state. Both species yielded two oxidation peaks on DP voltammograms. The first, more negative peak, corresponded to electrooxidation of tyrosine residues, whereas the other, more positive, peak corresponded to electrooxidation of tryptophan residues. DC polarography was used to detect degradation of TMV and denaturation of TMV-protein induced by an increased pH and by the addition of urea, respectively. These structural transformations resulted in increased DP voltammetric oxidation currents as recorded using a graphite working electrode. It has been suggested that the higher oxidation currents were due to an increase in the number of tyrosine and tryptophan residues accessible to the reaction at the graphite electrode. The results of these electrochemical investigations were in a good agreement with the estimation of the accessibility of tyrosine and tryptophan residues based on the well-explored three-dimensional structure of TMV and its isolated protein.

Key words: Tobacco mosaic virus — Voltammetry — Electrooxidation — Graphite electrode — Classical polarography

Introduction

TMV is currently used as a suitable model for the study of in vitro assembly of simple biological systems (Fraenkel-Conrat 1981). The rod-shaped particle of TMV is composed of 2130 helically arranged identical protein structure units, each formed of a single polypeptide chain having a molecular mass of 17530, (the

capsid), and a single RNA strand (molecular mass 2×10^6). Treatment of the virus with mild alkali results in the degradation of viral rods and the release of oligomers of structure units with preserved native conformation of the polypeptide chain. This native conformation can be disordered (denatured) by treatment of the isolated TMV-protein or of the viral rods with 8 mol/l urea or some other suitable denaturing agent (e. g. water saturated phenol). By lowering pH and ionic strength of a solution of disordered polypeptide chains using dialysis against suitable buffers, the original ordered structure of the TMV capsid can be restored. If isolated viral RNA is added during this process, the infectious virus can be reconstituted (for reviews, see Anderer 1963 and Caspar 1963). The process itself proceeds in two main steps: first, re-formation of the specific conformation of the TMV polypeptide chain (renaturation) and second, assembly of the structure units and the viral RNA in the TMV rod (virus assembly).

The virus assembly process has so far been studied using electron microscopy or some other methods based on size-dependent physico-chemical properties (e. g. sedimentation coefficient). The study of the conformational change accompanying the renaturation step is beyond the capabilities of these methods. Up to now, the only physico-chemical method used for the specific detection of the renaturation step has been DC polarography (Ruttkay-Nedecký and Bezúch 1971a). This method is essentially based on the polarographic catalytic activity of cysteine-containing proteins in the presence of cobalt and ammonium ions, called the Brdička current (Brdička 1933; for reviews, see Březina and Zuman 1958; Paleček 1983 and Müller 1963). Alkaline degradation of TMV with the concomitant release of the depolymerized (native) TMV-protein is accompanied by multiple increase of the polarographic catalytic maximum B generated by the specifically masked single cysteinyl residue present in the TMV-polypeptide (cysteinyl-27). The denaturation of the TMV-protein results in the appearance and rise of the polarographic catalytic maximum C, caused by the unmasking of the —SH group of cysteinyl-27. This change is reversible: in the course of TMV-protein renaturation the —SH group remasks and the maximum C disappears from the polarographic curve (Ruttkay-Nedecký and Bezúch 1971a, b; Ruttkay-Nedecký et al. 1977). The variation of the Brdička current in the course of alkaline degradation of TMV and its denaturation by 8 mol/l urea have also been studied by DP polarography (Ruttkay-Nedecký and Paleček 1980). Because of an improved resolution of catalytic polarographic maxima A and B by this method, DP polarography has made it possible to study variations of maximum A in the course of TMV degradation.

Recently it has been shown that proteins can yield oxidation currents at graphite electrodes and that the occurrence of these currents is determined by the accessibility of tyrosine and tryptophan residues in the protein for the interaction with the graphite electrode (Brabec 1980; Brabec and Mornstein 1980a, b;

Reynaud et al. 1980). It appears reasonable to assume that, as due to alkaline degradation of TMV and denaturation of its protein, the accessibility of amino acids in TMV-protein for reaction with the environment, and thus with the electrode as well, becomes changed. The present paper is, therefore, aimed at demonstrating that degradation of TMV and denaturation of its protein influence the accessibility of tyrosine and tryptophan residues of the TMV-protein for reaction with the graphite electrode. Both these structural transformations of TMV were also monitored by an independent method, namely by the DC polarographic technique.

Materials and Methods

The strain TMV vulgare was kindly supplied by Prof. G. Melchers, Tübingen, F. R. G. The virus was propagated in tobacco plants and purified by means of ammonium sulfate precipitation and differential centrifugations. After the final step of purification the virus was dissolved in distilled water. Native TMV protein was prepared in the same way as the samples used in the study of TMV-protein conformation by means of X-ray diffraction (Mandelkow et al. 1976): the virus was treated with mild alkali (pH 10.5) and the released protein was then precipitated by ammonium sulfate. After the final step of preparation, the virus protein was dialysed at 4 °C against 0.02 mol/l sodium carbonate ($Na_2CO_3/NaHCO_3$), pH 10.5 for 20 hours and clarified by centrifugation at $80{,}000 \times g$ for 90 minutes. Details of the methods of TMV purification and TMV-protein preparation used have been described earlier (Ruttkay-Nedecký et al. 1977). The desired concentrations of the virus protein were adjusted by means of the interferometric device of an Antweiler's microelectrophoretic apparatus (BOSKAMP Geräte-Bau K. G. Hersel, F. R. G.).

TMV was degraded by dialysis of 0.2% aqueous virus solution at 4 °C for 20 hours against 0.02 mol/l sodium carbonate, pH 10.5. The residual non-degraded virus was removed from the solution by centrifugation at $80{,}000 \times g$ for 90 minutes. Denaturation of TMV and TMV-protein was performed by dilution of TMV or TMV-protein solution with urea (final concentration 8 mol/l) in Britton-Robinson buffer, pH 8.3 or in 0.02 mol/l sodium carbonate, pH 10.5.

Lysozyme (of hen egg white) was obtained from Nutritional Biochemicals (Ohio).

DP voltammetric measurements at the WISGE were performed with a prototype pulse polarograph PA 3 (Laboratory Instruments, Prague) at following settings: sweep rate 5 mV s^{-1}, pulse amplitude 50 mV, drop time control at 1.0 s, the time constant of the analogue memories at 100 ms. WISGE was prepared and used in the same way as described earlier (Brabec 1979). It had a geometric area of 7 mm^2. Voltammograms of TMV and TMV-protein were obtained in a 2 ml capacity thermostated cell. A three electrode system was used, including a graphite electrode, Pt-counter-electrode and a saturated calomel reference electrode. The procedure for TMV and TMV-protein voltammetry was not very different from that used for DNA voltammetry (Brabec and Dryhurst 1978; Brabec 1979). WISGE was inserted into the tested solution contained in the electrochemical cell, and allowed to stand for ca 10 s with no potential applied. Then, an initial potential of 0.2 V was applied for the next 120 s, with subsequent voltammetric sweep. Unless stated otherwise, measurements were carried out with the electrochemical cell maintained at 25 °C. The heights of the DP voltammetric oxidation peaks were measured as shown in Fig. 1*b*. The possibility that real peak height values are slightly different from those reported in this paper cannot be ruled out. A small difference might be due to the somewhat inappropriate manner of measuring the height of peaks. For instance, the voltammetric peak height is often measured from the background electrolyte curve. This perhaps more suitable way could not, however, be employed in our work. Before the start of each DP voltammogram the WISGE had to be

resurfaced, which did not always result in the same residual current. Owing to this phenomenon the way of the peak height measurement as shown in Fig. 1*b* was prefered in this work. The precision of the measurement of the DP voltammetric peak heights carried out as shown in Fig. 1*b* was determined on the basis of measurements of ten identical samples of TMV-protein at a concentration of 100 μg/ml each. In Britton-Robinson buffer, pH 8.3, and in 0.02 mol/l sodium carbonate, pH 10.5 precisions of 2.1 and 1.6% were obtained, respectively. Thus each peak height value reported in this paper was estimated as an average of three measurements carried out with the same TMV or TMV-protein sample but always with a freshly resurfaced WISGE. Moreover, some DP voltammetric measurements were carried out in the presence of urea. It has been shown (Schindlerová 1981) that the presence of urea in the test solution at concentrations as used in this work does not interfere with the electrode reactions during which proteins are electrooxidized at the WISGE. All potentials reported in this paper have been given against the saturated calomel electrode.

DC polarographic measurements were carried out on a Polarograph LP 7 (Laboratory Instruments, Prague) using a two-electrode system with DME and saturated calomel electrode. DME had, at a mercury column height $h = 60$ cm, a flow rate of 3.2 mg s^{-1} and a drop time of 2.4 s (in 1 mol/l KCl and electrodes short circuited). The polarographic curves were all recorded from -0.8 V, using negative scan range of 0 to -4 V and a scan rate of 200 mV min^{-1}. The temperature of the analysed solution was 0 °C, the background electrolyte contained 10^{-3} mol/l Co $(NH_3)_6Cl_3$; 10^{-1} mol/l NH_4Cl; 10^{-1} mol/l NH_3; pH 9.4 (measured at 25 °C) (Brdička solution). These conditions of the polarographic analysis are further referred to as „standard conditions". For details of the polarographic analysis at 0 °C see (Ruttkay-Nedecký et al. 1977). The time interval between the contact of virus or virus protein with the background electrolyte and the start of the recording of the polarographic curve was 2 min. Before mixing with the background electrolyte, the virus was dissolved in water, degraded TMV and isolated native TMV-protein in 0.02 mol/l sodium carbonate, pH 10.5, and denatured TMV or TMV-protein in 8 mol/l urea with 0.02 mol/l sodium carbonate, pH 10.5. As confirmed in preliminary experiments, the presence of urea or sodium carbonate at the concentrations used, in the background electrolyte did not appreciably influence the shapes of the polarographic curves or the heights of the polarographic catalytic maxima of degraded virus or virus protein. The heights of the polarographic catalytic maxima B and C on the polarograms were measured starting on the cobalt reduction wave.

Results

DP voltammetry of TMV at the WISGE. Effect of alkaline degradation and urea treatment of the virus. DP voltammograms of TMV at the WISGE were measured at 25 °C in two media: Britton-Robinson buffer, pH 8.3, and 0.02 mol/l sodium carbonate, pH 10.5. Freshly prepared solutions of TMV (measured within 10 minutes after mixing of virus with the background solution) yielded two peaks in both media in the potential range 0.45—0.7 V (Figs. 1*a* and 2*a*). Better developed peaks were obtained at pH 10.5; but they appeared at potentials more negative (by ca 0.1 V) as compared with the peaks recorded at pH 8.3. The dependence of DP voltammetric peak heights on the concentration of TMV tended towards limiting values (Fig. 3). In the concentration range from 2 to 300 μg/ml the ratio of the height of the more negative peak to that of the more positive one varied between 1.2 and 1.4 at pH 8.3 and between 1.1 and 1.2 at pH 10.5. In the same concentration range both peaks recorded at pH 10.5 were higher than those recorded at pH 8.3: the more positive peak was ca 1.5—1.8 times higher

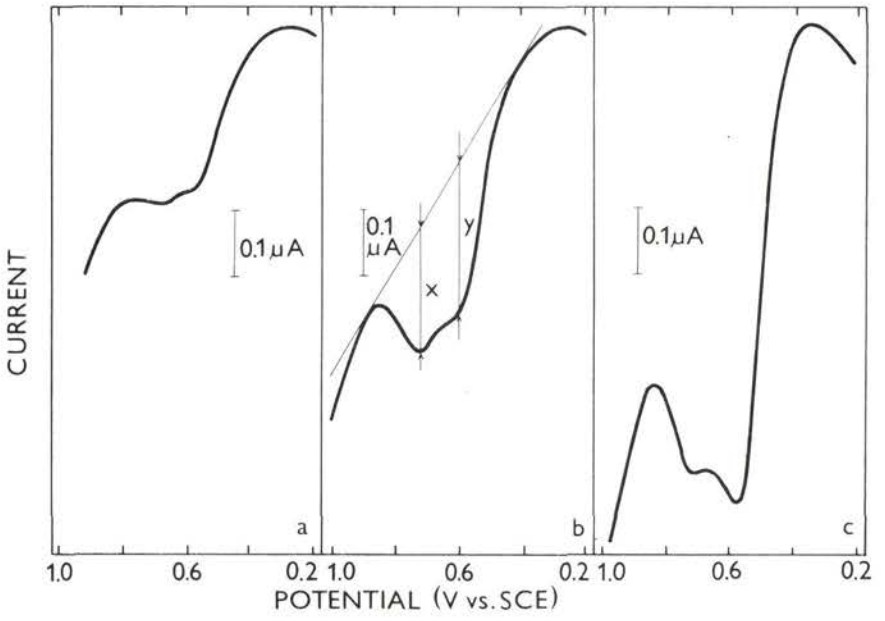

Fig. 1. Differential pulse voltammograms at the WISGE in Britton-Robinson buffer, pH 8.3. (*a*) TMV vulgare, (*b*) TMV vulgare degraded at pH 10.5, (*c*) TMV vulgare denatured by 8 mol/l urea; TMV concentration: 100 μg ml^{-1}. The values x and y were taken to represent the heights of the more positive and the more negative peaks, respectively.

(depending on the concentration), and the more negative by ca 1.3—1.6 times higher.

If the voltammetric curves were recorded at pH 8.3, preceding alkaline degradation of the virus (by dialysis at pH 10.5, see Materials and Methods) resulted in an increase of both voltammetric peaks (cf. Figs. 1*a* and 1*b*). For instance, at a virus concentration of 100 μg/ml both peaks doubled. Nevertheless, the DP voltammetric curve of previously degraded TMV measured at pH 10.5 was almost identical to that obtained for untreated TMV mixed with 0.02 mol/l sodium carbonate, pH 10.5 immediately before the electrochemical measurement (cf. Figs. 2*a* and 2*b*).

Treatment of TMV by 8 mol/l urea led to a significant increase of DP voltammetric peaks in both media used (Figs. 1*c* and 2*c*): if the analysis was performed at a virus concentration of 100 μg/ml and at pH 8.3, the more negative peak increased approx. fourfold and the more positive peak approx. threefold; at pH 10.5 the more negative peak increased approximately 2.3 times and the more positive peak 1.5 times.

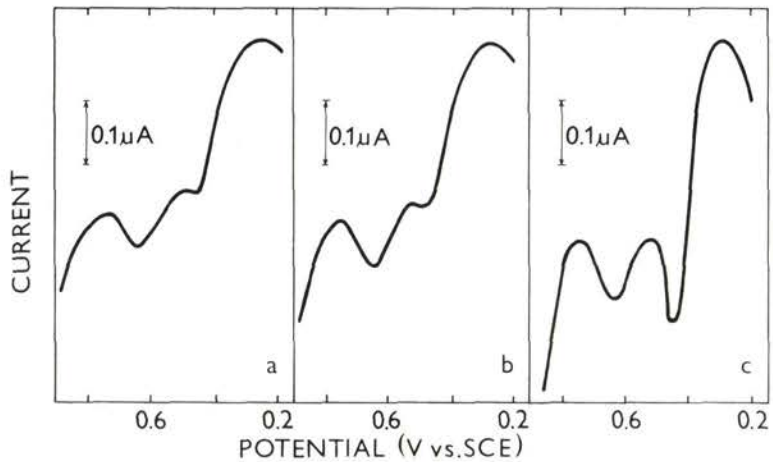

Fig. 2. Differential pulse voltammograms at the WISGE in 0.02 mol/l sodium carbonate, pH 10.5: (*a*) TMV vulgare, (*b*) TMV vulgare degraded at pH 10.5, (*c*) TMV vulgare denatured by 8 mol/l urea; TMV concentration: 100 μg ml^{-1}.

DP voltammetry of isolated TMV-protein at the WISGE. Effect of protein denaturation by 8 mol/l urea. In both media used for the DP voltammetric analysis of the virus, the isolated TMV-protein also showed two DP voltammetric peaks in the same potential range as did TMV (Figs. 4 and 5). As in the case of TMV, DP voltammetric peaks of the isolated protein approached limiting heights with the increasing protein concentration (cf. Figs. 3 and 6). At pH 8.3 the DP voltammetric peaks of isolated (native) TMV-protein were significantly higher than those yielded by untreated virus (cf. Figs. 1*a* and 4*a*): At concentrations ranging from 10 to 100 μg/ml the heights of both voltammetric peaks were roughly twice those yielded by the untreated virus; only at very low concentrations (2—5 μg/ml) the peaks of the isolated protein were about three times higher. However, virus degraded (by dialysis at pH 10.5) prior to the electrochemical analysis, yielded DP voltammetric curves almost identical to those yielded by the isolated (native) protein (cf. Figs. 1*b* and 4*a*). If the electrochemical measurements were performed at pH 10.5, TMV-protein yielded both peaks with heights almost identical with those yielded by untreated virus; only at very low concentrations (2—5 μg/ml) the TMV-protein yielded somewhat higher peaks (ca. 1.5 times).

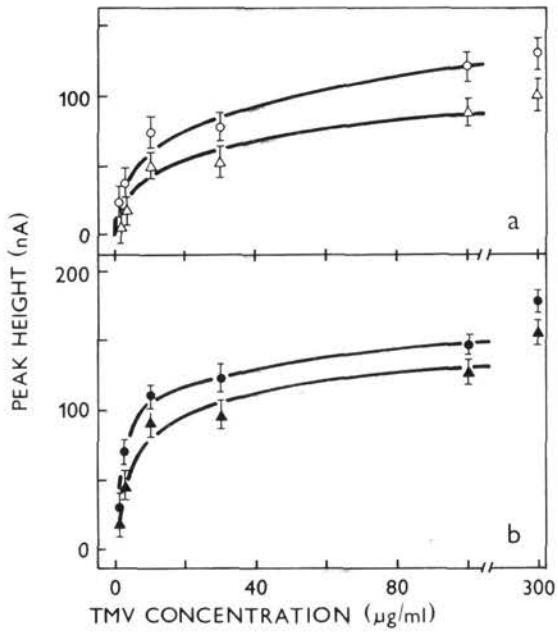

Fig. 3. Changes in differential pulse voltammetric peak heights with changing concentrations of TMV vulgare at the WISGE in (*a*) Britton-Robinson buffer, pH 8.3, (*b*) 0.02 mol/l sodium carbonate, pH 10.5. (○, ●) the more negative peak, (△, ▲) the more positive peak. Estimated maximum experimental error is indicated.

Also DP voltammetric curves for lysozyme at the WISGE were recorded under the conditions of DP voltammetric measurements performed with TMV and its isolated protein. In both media used throughout this work this protein also yielded two peaks at potentials at which DP voltammetric peaks of TMV and its isolated protein appeared (cf. Figs. 4*a* and 5*a*).

Denaturation of the isolated TMV-protein by 8 mol/l urea (see Materials and Methods) resulted in a well-pronounced increase of DP voltammetric peaks of this protein when the electroanalysis was carried out at 25 °C both at pH 8.3 and 10.5. For instance, at a protein concentration of 100 μg/ml, at pH 8.3 the more negative peak doubled and the more positive peak was 1.5 times higher while at pH 10.5 the more negative peak doubled and the more positive peak was 1.3 times higher. The DP voltammetric curves of the denatured TMV-protein were almost identical to those yielded by TMV following its treatment with 8 mol/l urea (cf. Figs. 1*c*, 4*b* and 2*c* and 5*b*). DP voltammetry was also used to monitor the kinetics of the denaturation of TMV-protein and TMV by 8 mol/l urea, in media used throughout this work. These measurements revealed that the denaturation was completed below 3 minutes.

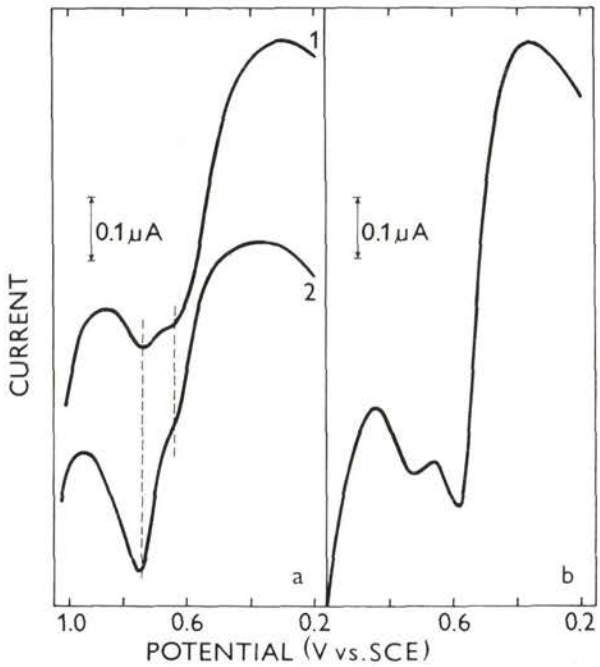

Fig. 4. Differential pulse voltammograms at the WISGE in Britton-Robinson buffer, pH 8.3: (*a*) TMV vulgare-protein (curve 1) and lysozyme (curve 2), (*b*) TMV vulgare-protein denatured by 8 mol/l urea; concentration of proteins: 100 μg ml^{-1}.

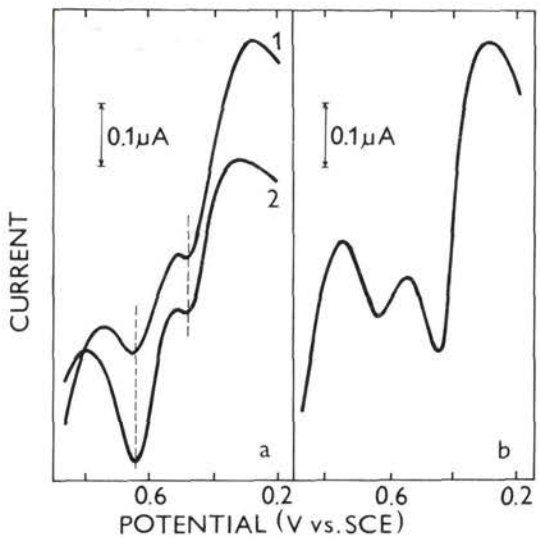

Fig. 5. Differential pulse voltammograms at the WISGE in 0.02 mol/l sodium carbonate, pH 10.5: (*a*) TMV vulgare-protein (curve 1) and lysozyme (curve 2), (*b*) TMV vulgare-protein denatured by 8 mol/l urea; concentration of proteins: 100 μg ml^{-1}.

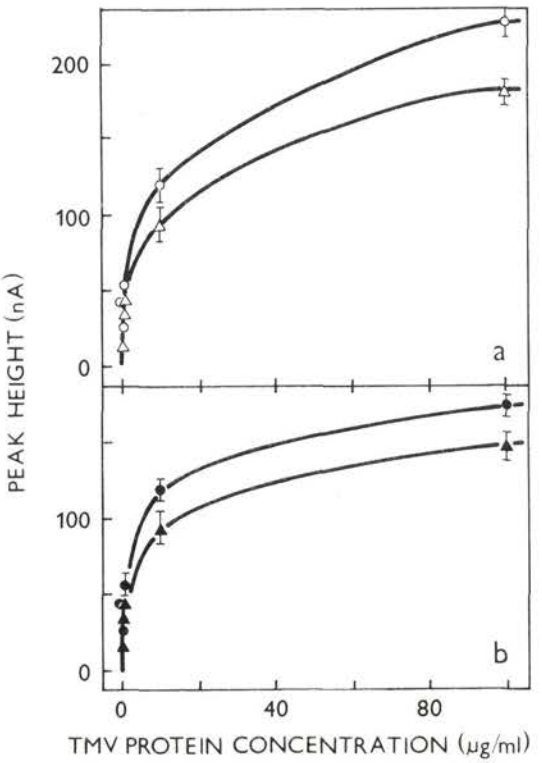

Fig. 6. Changes in differential pulse voltammetric peak heights with changing concentrations of TMV vulgare-protein at the WISGE in (*a*) Britton-Robinson buffer, pH 8.3, (*b*) 0.02 mol/l sodium carbonate, pH 10.5. (○, ●) the more negative peak, (△, ▲) the more positive peak. Estimated maximum experimental error is indicated.

DP voltammograms of both the isolated TMV-protein and TMV were also recorded at 0 °C in both media used in this study. A decrease in temperature from 25 °C to 0 °C resulted in a shift of the peaks towards more positive potentials (by 0.03—0.06 V). Moreover, the background discharge curve was shifted by ca. 0.1 V towards more positive potentials, so that particularly the more positive voltammetric peak became better developed. The heights of the peaks were only slightly influenced by a temperature decrease (within 10%) except for the peaks yielded by TMV in the medium of pH 8.3 (pH measured at 25 °C). In the latter case both peaks were higher at 0 °C as compared with those recorded at 25 °C: the more negative peak increased by ca. 40%, the more positive one by 80%.

Detection of TMV degradation and TMV-protein denaturation by means of DC polarography. All the samples analysed by DP voltammetry at the WISGE were

tested in parallel using DC polarography (at DME) at 0 °C, using the catalytic Brdička current under standard conditions (see Materials and Methods). Degradation of TMV (by dialysis at pH 10.5) was reflected by a multiple increase of the catalytic polarographic maximum B (cf. Figs. 7*a*—*c* and 8*a*, *b*). Thus 100 μg/ml of degraded TMV generated a maximum B 2.5 times higher than 1200 μg/ml of untreated TMV (cf. Figs. 7*a* and 8*a*). The splitting of the TMV particle into its subunits by alkaline degradation also resulted in a suppression of the non-catalytic maximum (NM) of cobalt (cf. Figs. 7*b*, *c* and 8*a*, *b*). Treatment of TMV with 8 mol/l urea resulted in the appearance of a very high polarographic maximum C (Figs. 8*c*, *d*), which did not appear on the polarographic curves of untreated or degraded TMV (this maximum is characteristic for the unfolding of the TMV-protein (Ruttkay-Nedecký et al. 1977)). The DC polarographic curves of the degraded TMV and those of the isolated (native) TMV-protein were essentially identical, showing maxima B of comparable heights and no maximum C. The denaturation of the isolated TMV-protein by 8 mol/l urea was expressed on the DC polarogram in the same way as TMV treatment with 8 mol/l urea, namely by the appearance of a maximum C having similar heights at comparable protein concentrations.

Discussion

The nature of anodic currents yielded by TMV-protein at the WISGE. At the graphite electrode TMV-protein generated two oxidation currents at the same potentials as does lysozyme (Figs. 4*a* and 5*a*). For lysozyme it has been shown (Brabec and Mornstein 1980a) that the more negative anodic current is due to the electrooxidation of tyrosine residues and the more positive one to the electrooxidation of tryptophan residues. Therefore, it can be suggested that the two voltammetric peaks of TMV-protein originate from similar electrochemical processes. The observed shift of these two voltammetric peaks towards more negative potentials when 0.02 mol/l sodium carbonate, pH 10.5 instead of the Britton-Robinson buffer, pH 8.3 is used for electroanalysis is in accordance with previous works dealing with the electrooxidation of tyrosine and tryptophan residues contained in other proteins (Brabec 1980; Brabec and Mornstein 1980a, b; Reynaud et al. 1980). It has been shown that the peak-potentials of both oxidation currents of lysozyme, like those of the free amino acids tyrosine and tryptophan, are shifted towards more negative values with increasing pH. Since this shift is larger for tyrosine as compared to tryptophan, the resolution of the two voltammetric peaks of TMV-protein appeared to be better at pH 10.5 as compared to those recorded at pH 8.3.

As in the case of other proteins (Brabec and Schindlerová 1981), the dependence of DP voltammetric peaks of TMV and TMV-protein on their

concentration tended towards limiting values (Figs. 3, 6). Considering the fact that proteins are oxidized on the graphite electrode in the adsorbed state (Brabec and Schindlerová 1981) it seems reasonable to assume that at protein concentrations of 100 μg/ml and higher, the electrode surface was practically fully covered with TMV-particles or TMV-protein molecules. Thus it means that the difference in the transport rates to the electrode surface (due to virus degradation or virus-protein denaturation prior to the electroanalysis) could not have influenced the values of the oxidation currents observed at TMV or TMV-protein concentrations of 100 μg/ml and higher. Moreover, it can be assumed that during the electrooxidation of tyrosine and tryptophan residues at the graphite electrode the same number of electrons is always transferred between the amino acid and electrode (Brabec and Mornstein 1980a, b; Reynaud et al. 1980). Consequently, variations in the heights of DP voltammetric peaks observed during virus degradation or virus-protein denaturation would quantitatively reflect a change in the accessibility of tyrosine and/or tryptophan residues for their electrochemical reaction at the electrode.

Confirmation of TMV degradation and TMV-protein denaturation by means of DC polarography. The well established fact that TMV-vulgare degraded by a mild alkali (pH 10.5) preserves the native conformation of its protein structure units was confirmed for the samples analysed in this work, by means of DC polarography. On DC polarograms of the degraded virus the catalytic maximum C, typical of the unfolded TMV-protein (Ruttkay-Nedecký and Bezúch 1971a), was absent in spite of the multiple increase of the polarographic catalytic maximum B accompanying the degradation of TMV (Figs. 7 and 8*a*, *b*). The DC polarographic curves of the degraded TMV were almost identical to those of the isolated native TMV-protein. The unfolding of the polypeptide chain of TMV following the treatment of TMV or TMV-protein with 8 mol/l urea was confirmed by the appearance of extremely high and dominating maxima C on the DC polarographic curves (Figs. 8*c*, *d*). After denaturation by such a treatment, both TMV and the isolated TMV-protein exhibited maxima C of equal heights.

The effect of TMV degradation and TMV-protein denaturation on the oxidation currents yielded by TMV-protein at the WISGE in a medium with pH 8.3. The alkaline degradation of TMV, performed in 0.02 mol/l sodium carbonate, pH 10.5, led to an increase of both DP voltammetric peaks by roughly a factor of two, if Britton-Robinson buffer, pH 8.3 was used for the electroanalysis (Figs. 1*a* and 1*b*). The TMV-vulgare protein molecule contains 4 tyrosine and 3 tryptophan residues per polypeptide chain (Anderer 1963). Thus, it is apparent that in the undegraded virus either one tyrosine and one tryptophan, or two tyrosines and one tryptophan

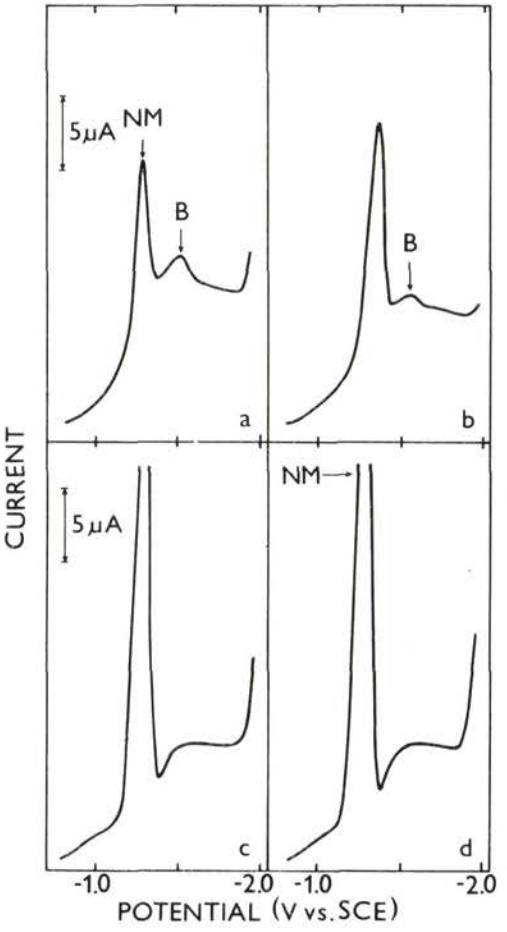

Fig. 7. DC polarograms of TMV vulgare in standard Brdička buffer, recorded at 0 °C (standard conditions). Concentrations of the virus in μg/ml: (*a*) 1200, (*b*) 100, (*c*) 10, (*d*) 0 (background solution). Arrows marked B and NM indicate the catalytic maximum B of the protein, and the non-catalytic maximum (NM) of cobalt (present in the background solution), respectively.

(per polypeptide chain) could be available for the electrode reaction. Nevertheless, in the latter case, alkaline degradation would cause the unmasking of all tyrosines and, therefore, following denaturation, only the peak due to the electrooxidation of tryptophan, i. e. the more positive peak, should increase. Since this was not the case (following denaturation of the TMV-protein the peak due to the electrooxidation of tyrosine residue also markedly increased), the former alternative appears more probable: in undegraded TMV, only one tyrosine and one tryptophan (per polypeptide chain) were accessible for the electrode reaction. This alternative has

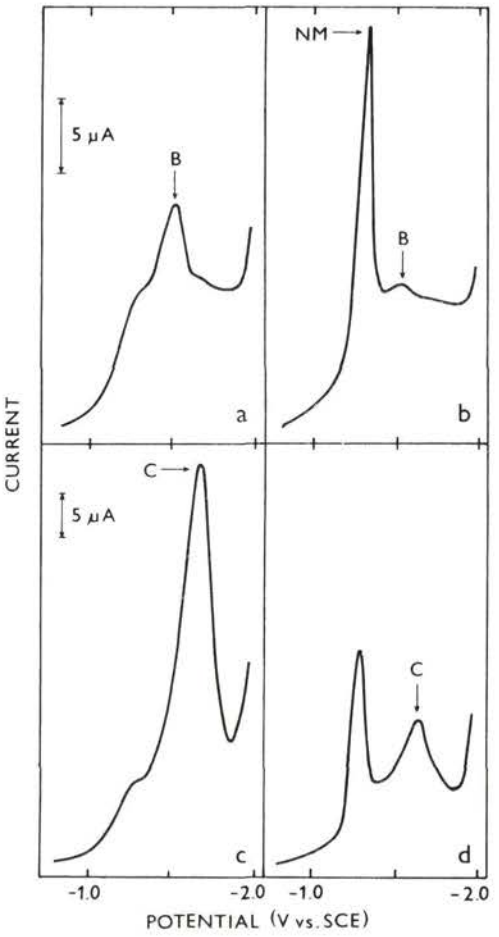

Fig. 8. DC polarograms recorded under standard conditions (see legend to Fig. 7): (*a*, *b*) TMV vulgare degraded at pH 10.5, (*c*, *d*) TMV vulgare denatured by 8 mol/l urea. Concentrations of the virus in μg/ml: (*a*, *c*) 100, (*b*, *d*) 10. Arrows marked B, C and NM indicate the catalytic maxima B and C of the protein, and the non-catalytic maximum (NM) of cobalt, respectively.

also been supported by the finding that the heights of the two DP voltammetric peaks of undegraded TMV did not differ appreciably from each other. In the alternative case (two tyrosine and one tryptophan residues per polypeptide chain in undegraded TMV available for electrooxidation), the height of the more negative peak should be twice that of the more positive peak.

The unfolding of the polypeptide chain of TMV-protein after denaturation with 8 mol/l urea, also confirmed by electroanalysis of denatured TMV-protein by means of DC polarography, resulted in an increase of the more negative DP

voltammetric tyrosine peak by a factor of two, and of the more positive tryptophan peak by a factor of 1.5. In relation to the number of tyrosine and tryptophan residues in the TMV-polypeptide (4 and 3, respectively), this result can be interpreted by the assumption that 2 tyrosine and 2 tryptophan residues were accessible for the electrode reaction in the native (isolated) TMV-protein. The conclusion that in undegraded TMV only one tyrosine and one tryptophan residue per polypeptide were available for the reaction at the graphite electrode is also consistent with the finding that after the treatment of TMV with 8 mol/l urea, the more negative tyrosine peak of TMV increased fourfold and the more positive tryptophan peak threefold.

In analogy to the results of DC polarographic analysis the DP voltammetric curves of degraded TMV and isolated native TMV-protein were almost indistinguishable from each other (Figs. 1*b* and 4*a*). This indicates that in the native protein the same number of tyrosine and tryptophan residues per polypeptide, presumably 2 of each, were available for the electrode reaction as compared with the degraded TMV. Similarily, both the TMV and the isolated TMV-protein gave, on denaturation with 8 mol/l urea, almost identical DC polarographic as well as identical DP voltammetric curves (Figs. 1*c* and 4*b*). Obviously, on the unfolding of the polypeptides in the case of TMV and its isolated protein, the same number of electrooxidizable groups, namely all the 4 tyrosine and all the 3 tryptophan residues, became exposed.

The effect of structural transformations of TMV-protein on its oxidation currents at the WISGE in a medium with pH 10.5. In contrast to the results obtained with electroanalysis at pH 8.3, the sample of untreated TMV electroanalysed in 0.02 mol/l sodium carbonate, pH 10.5, exhibited DP voltammetric peaks of almost identical heights as compared with the degraded virus (Figs. 2*a*, *b*). Obviously at a high pH value of the buffer the virus had already been degraded (in the bulk solution or at the WISGE) before the start of the DP voltammetric curve recording. On the other hand, if degraded TMV, urea-treated TMV, isolated (native) TMV-protein and denatured TMV-protein, respectively were electroanalyzed at pH 10.5, similar relationships between the heights of the corresponding DP voltammetric peaks were observed as with electroanalysis at pH 8.3.

An attempt to correlate DP voltammetric peaks of the various forms of TMV-protein tested with the locations of tyrosine and tryptophan residues in the TMV structure. The location of both the N- and the C-terminal ends of the TMV-polypeptide at the surface of the rod-like TMV-particle is a characteristic feature of the TMV structure (Bloomer et al. 1978). Of the four tyrosine and three tryptophan residues present in the polypeptide of TMV-vulgare, only tyrosinyl-2 and tryptophanyl-152 are sited near the N-terminal and C-terminal ends, respec-

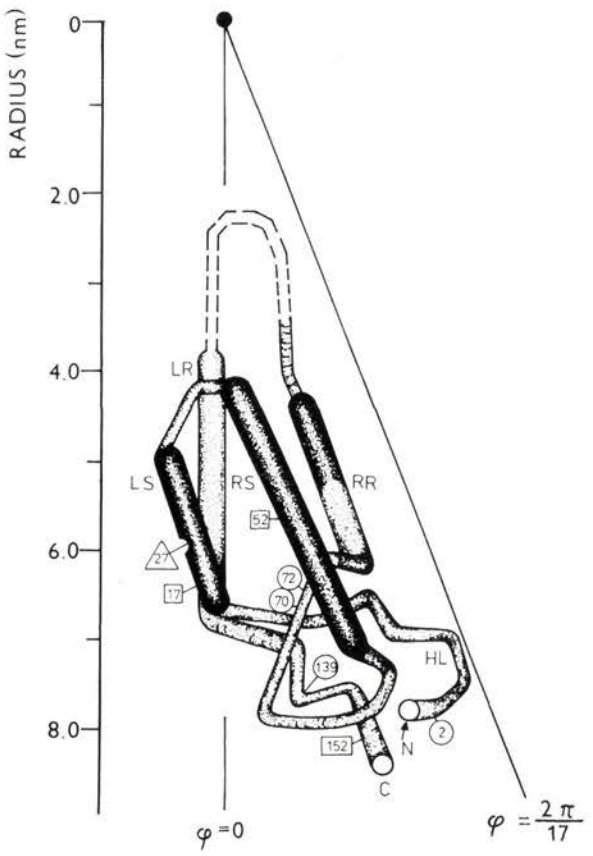

Fig. 9. A diagram of the folding of the polypeptide chain in one TMV-protein structure unit, based on the electron density map of the protein disk of TMV at 0.5 nm resolution. The point at the top indicates the projection of the TMV-particle axis. The C-terminal and N-terminal ends of the polypeptide are marked C and N, respectively. Numbered circles, squares and a triangle indicate the location of tyrosine, tryptophan and cysteine residues, respectively. The alpha-helical rods are marked: LS (left slewed helix), RS (right slewed helix), LR (left radial helix) and RR (right radial helix). LR and RR are joined by a flexible loop indicated by dashed lines. The "hanging loop" close to the N-terminus is marked HL. This figure is a modified version of that published by Bloomer et al. (1981). (Permission from the copyright owner (C) 1981, Balaban International Science Services.)

tively (Figs. 9 and 10), and apparently only these can be exposed in the undegraded TMV to the surface of the electrode. The location of only one tyrosine and one tryptophan residue per polypeptide at the surface of the TMV-particle is in good agreement with the results of the present DP voltammetric study of TMV, according to which only one tyrosine and one tryptophan residue per polypeptide are electrooxidizable at the WISGE in the undegraded TMV-vulgare. Thus it can

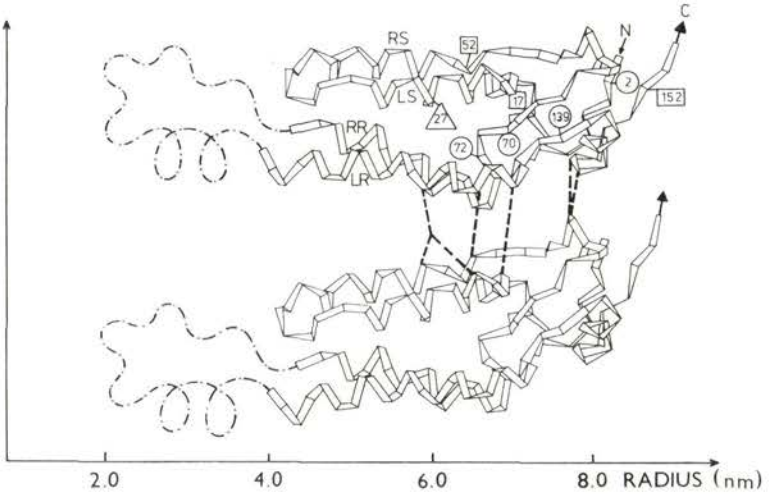

Fig. 10. Side view of a sector through the protein disk of TMV showing the folding of the polypeptide chain in two structure units, and the axial contact between them. The drawing is based on the electron density map of the disk at 0.28 nm resolution. Dashed lines indicate the network of hydrogen bonds and the salt-bridge system between the two structure units, one from the upper and the other from the lower layer of the disk. (– · – · – · – · –) represents schematically the low radius region of the polypeptide chain, which has no ordered structure in the absence of RNA. Circles, squares and the triangle have the same meaning as in Fig. 9. This figure is a modified version of that published by Bloomer et al. (1978). (Permission from the copyright owner (C) 1978 Macmillan Journals Limited.)

be concluded that the DP voltammetric peaks yielded by the undegraded TMV-vulgare in the Britton-Robinson buffer, pH 8.3, were due to tyrosinyl-2 (the more negative peak) and tryptophanyl-152 (the more positive peak).

A pair of very closely sited tyrosine residues, namely tyrosinyl-70 and tyrosinyl-72, are located in the innermost part of the TMV-protein structure unit (Figs. 9 and 10), and apparently they can be exposed to the electrode surface only after unfolding of the polypeptide chain. Most probably these two tyrosine residues become accessible to electrooxidation at the WISGE only after denaturation of the TMV-protein by 8 mol/l urea or direct urea-treatment of TMV. The fourth tyrosine residue present in the TMV-polypeptide, tyrosinyl-139, is located at the surface of the protein structure unit, but not at the surface of the TMV-rod. Therefore it is apparent that this tyrosine residue becomes accessible to electrooxidation at the WISGE after — but not before — the alkaline degradation of TMV. Since the available X-ray data (Fig. 10) indicate that tryptophanyl-52 is exposed to the surface of the TMV-protein structure unit and tryptophanyl-17 is not, it is reasonable to assume that the former becomes accessible to electrooxidation at the WISGE after alkaline degradation of TMV and the latter only after denaturation of the TMV-protein. Accordingly, the DP voltammetric peaks yielded by the isolated

(native) TMV-protein (or degraded TMV) correspond to electrooxidation of tyrosinyl-2 and tyrosinyl-139 (the more negative peak), and tryptophanyl-52 and tryptophanyl-152 (the more positive peak). Evidently, the voltammetric peaks of denatured TMV-protein (or urea-treated TMV) correspond to electrooxidation of all tyrosine and tryptophan residues present in the TMV-polypeptide, which should all be exposed to the electrode surface after the unfolding of the TMV-polypeptide. Thus in this case the tyrosine residues at positions 2, 70, 72, and 139, give rise to the more negative peak, and the tryptophan residues at positions 17, 52, and 152, give rise to the more positive peak.

Conclusion

Until now, polarography has been the only electroanalytical method used to study structural transformations of the TMV-protein. This method is based on changes in the accessibility of the —SH group of cysteinyl-27 for a catalytic reaction at the surface of a dropping mercury electrode. The present study shows that voltammetry in combination with the graphite electrode is also suitable to study these structural transformations. The latter approach is, however, based on changes in accessibilities of tyrosine and tryptophan residues to electrooxidation at the graphite electrode. The well-explored three-dimensional structure of TMV permits identification of those electroactive amino acid residues in the protein molecule which are responsible for variations in the respective electrochemical signals accompanying structural transformations.

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Abbreviations

DC	direct current
DME	dropping mercury electrode
DNA	deoxyribonucleic acid
DP	differential pulse
RNA	ribonucleic acid
TMV	tobacco mosaic virus
WISGE	paraffin wax-impregnated spectroscopic graphite electrode

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