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Restricted Reactivity of the ε-Amino Groups of Tobacco Mosaic Virus Protein toward Trinitrobenzenesulfonic Acid*

Robert B. Scheele† and Max A. Lauffer

ABSTRACT: The reactivity of the ε-amino groups of tobacco mosaic virus protein was investigated with trinitrobenzenesulfonic acid, a reagent specific for primary amines. The kinetics of trinitrophenylation was followed spectrophotometrically at room temperature in the pH range from 7.5 to 10.4. The half-times of the trinitrobenzenesulfonic acid reaction with tobacco mosaic virus protein were compared with the half-times of the trinitrobenzenesulfonic acid reaction with bovine serum albumin, poly-L-lysine, and denatured (performic acid oxidized) tobacco mosaic virus protein, under identical conditions. Trinitrophenylation of tobacco mosaic virus protein was found to proceed many times more slowly than the reaction of trinitrobenzenesulfonic acid with the other materials. At pH 7.5 tobacco mosaic virus protein did not react at all with the trinitrobenzenesulfonic acid, while the other materials did react. The reaction proceeds to completion at the higher pH values but denatures the tobacco mosaic virus protein. Whole virus did not react with trinitrobenzenesulfonic acid at pH 8.6.

A model involving two proton-sharing associations between lysine and tyrosine side chains is proposed to explain these observations and several anomalies of the chemistry of the ε-amino groups of tobacco mosaic virus protein previously reported in the literature.

The chemistry of the two ε-amino groups of tobacco mosaic virus protein (TMVP) has been investigated by many workers and found to be very unusual. Fraenkel-Conrat and Singer (1954) investigated the reaction of TMVP with fluorodinitrobenzene and found that only one of the ε-amino groups becomes dinitrophenylated. Ramachandran (1959) tried to react *O*-methylisourea with TMVP and found no reaction except in the case of denatured TMVP in which only one of the two ε-amino groups appeared to react. On the other hand, Fraenkel-Conrat and Colloms (1967) found that these groups react readily with acetic anhydride. Fisher and Lauffer (1949), using electrophoresis, found an increase in the net negative charge of whole virus, TMV, upon reaction with formaldehyde which they interpreted as an abolishment of positive charges of the ε-amino groups. However, the present authors have found that these groups cannot be titrated and apparently remain uncharged over the pH range from neutrality to the alkaline limits of titration reversibility (pH >11) (Scheele and Lauffer, 1967). In apparent contradiction to

* From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. Publication No. 160 of the Department of Biophysics and Microbiology. *Received March 24, 1969.* This work was supported in part by a grant from the U. S. Public Health Service (GM 10403).

† Present address: Department of Biology, State University of New York at Buffalo, Buffalo, N. Y. 14214.

this result, Perham and Richards (1968) have shown that alterations in the ε-amino groups which do not permit the lysine side chains to be positively charged do not allow polymerization of the protein, while altered side chains which would be expected to be capable of becoming positively charged do allow polymerization of the protein to viruslike rods.

The present work, an extension of the investigation of the chemistry of the ε-amino groups of TMVP, employs a kinetic approach. The reaction of these groups with TNBS[1] at several different pH values was studied. This reaction is specific for and proceeds readily with free primary amino groups (Okuyama and Satake, 1960; Satake *et al.*, 1960; Goldfarb, 1966a,b) and can be followed spectrophotometrically. A high extinction coefficient of the product and low extinction coefficients of the reagent and protein at 345 mμ simplify the method by permitting the use of low protein concentrations. The TNBS reaction is especially useful with TMVP because of its specificity for primary amines and the fact that TMVP contains no amino groups other than the two ε-amino groups of lysines-53 and -68, the N-terminal α-amino group being acetylated (Anderer *et al.*, 1965). The spectrophotometric results should then be uncomplicated and quantitatively meaningful; since the only groups of TMVP that can possibly react with TNBS are the two ε-amino groups under study.

Materials and Methods

Virus and Protein Preparation. TMV was prepared by a standard method which was essentially the same as that employed by Boedtker and Simmons (1958). TMVP was prepared according to the method described by Scheele and Lauffer (1967). The chemically determined molecular weight (17.53×10^3) reported by Anderer (1963) was used in all calculations. Virus and protein concentrations were determined spectrophotometrically using the exctinction coefficients determined by Fraenkel-Conrat and Williams (1955) after correction for light scattering according to Englander and Epstein (1957). For TMV $\epsilon_{1\%}^{1\,cm}$ is 2.6 at 260 mμ, and for TMVP, $\epsilon_{1\%}^{1\,cm}$ is 1.3 at 280 mμ.

Buffers and Reagent. Buffers of 0.1 μ for trinitrophenylation media were prepared at four different pH values as follows: potassium phosphate, pH 7.5; sodium borate, pH 8.6; and sodium carbonate, pH 9.5 and 10.4.

TNBS reagent was prepared at 0.1% (w/v) concentration in 100-ml quantities in the appropriate buffer immediately before an experiment was begun.

Standard Reactants. Trinitrophenylation reactions were carried out on three substances in addition to TMV and TMVP. These substances were BSA, poly-L-lysine, and $TMVP_{ox}$.

BSA was obtained from Sigma Chemical Co. in recrystallized, lyophilized form and prepared for trinitrophenylation by dialysis against 0.1 M KCl with several changes of solvent The concentrations of BSA solution were determined spectrophotometrically. The extinction coefficient reported by Tanford and Roberts (1952), $\epsilon_{1\%}^{1\,cm}$ 6.60 at 280 mμ, was used. The poly-L-lysine was a product of General Biochemicals, Chagrin Falls, Ohio. Preparation for trinitrophenylation was carried out in the same manner as for BSA. The molecular weight reported by the manufacturer was 19×10^4. The concentration of poly-L-lysine was determined by the biuret method.

$TMVP_{ox}$ was prepared by performic acid oxidation in formic acid according to the scheme of Sanger (1949): (a) lyophilize *ca.* 200 mg of protein and dissolve in less than 10 ml of HCOOH (97%) at 0°; (b) add dropwise 10 ml of supercooled HCOOOH. Prepare this reagent by combining 9.5 ml of HCOOH and 0.15 ml of 30% H_2O_2. Allow the HCOOH to stand at room temperature for 2 hr before cooling to 0°; (c) leave the protein–HCOOOH mixture at 0° for 2 hr; (d) add 200 ml of H_2O to stop the reaction; (e) dialyze against cold H_2O (5 l. in two steps); (f) precipitate the protein with a small amount of 0.1 M KOH; (g) wash the precipitate centrifugally several times in H_2O; and (h) dissolve the $TMVP_{ox}$ in 0.1 M KCl with the addition of a few drops of 0.1 M KOH.

The spectra of $TMVP_{ox}$ solutions were variable, apparently because of uncontrollable side reactions, including the breakage of the indole ring of tryptophan (Hirs, 1967). Therefore, the concentrations of $TMVP_{ox}$ solutions used in reaction with TNBS were estimated from the extent of the reaction. It was assumed that the extinction coefficient of the product was 1.2×10^4 optical density units $mole^{-1}$ l. cm^{-1} per amine at 345 mμ (Goldfarb, 1966a), and that the reaction of $TMVP_{ox}$ with TNBS was complete.

Amine Concentration. The stock solutions of the reactants in 0.1 M KCl were diluted with the appropriate buffer for trinitrophenylation such that the final amine concentration in the reaction mixtures would be of the order of 0.5×10^{-4} M. The concentration of amino groups was calculated from the concentration determinations of the standard reactants and their respective chemical analyses. The amino acid analysis of TMVP was that of Anderer *et al.* (1965). The amino acid analysis of BSA (Stein and Moore, 1949) and the known structure of poly-L-lysine were used for the other two reactants. The amine contents of the various reactants are listed in Table I.

Experimental Steps. The method of Goldfarb (1966b) was used in the reaction procedure. Limitations of this method have been discussed by Satake *et al.* (1960) and by Scheele (1968).

The trinitrophenylation reactions were carried out at room temperature in quartz spectrophotometer cells with 1-cm path lengths. Samples of dialysate and reactant were diluted to the same extent with reaction buffer without TNBS, and the ultraviolet spectrum was measured to determine the initial optical density, OD_0. Then a volume of each solution was combined with an equal volume of 0.1% TNBS solution in the reaction buffer in a test tube and transferred to a Cary Model 14 M spectrophotometer where the cells had already been placed

TABLE I

	moles of ϵ-NH_2	moles of α-NH_2
TMVP	$2/17.53 \times 10^3$ g	0
BSA	$60.5/69 \times 10^3$ g	$1/69 \times 10^3$ g
Poly-L-Lys	1/146 g	Negligible

[1] Abbreviations used are: TNBS, trinitrobenzenesulfonic acid; $TMVP_{ox}$, performic acid oxidized TMVP; BSA and HSA, bovine and human serum albumin, respectively.

and blanked at 345 mμ. The control solution (solvent) was prepared and placed in the reference chamber before the preparation of the sample solution. A stopwatch was started at the time the stream of reagent from the pipet first hit the reactant solution already in the tube. Recording of the optical density at a constant wavelength of 345 mμ was begun and the time was noted, usually 60 sec. The speed of the recording chart was selected to be appropriate to the rate of the reaction. Automatic slit control was used at the narrowest possible slit widths with the dynode tap on the first setting. All experiments were carried out at room temperature.

Whenever practical, the reaction was followed to completion, although recordings of the optical density were seldom made beyond 3 hr after the start, except to determine the final optical density. The optical density at time t, OD_t, was translated to absorbance by subtraction of the initial optical density, OD_0, assumed to be totally due to light scattering. The initial optical density was calculated from the spectrum of the reactant, measured prior to the reaction, and the subsequent dilution factors.

Occasionally an experiment would last so long that it was impractical to leave the cells in the spectrophotometer until the reaction was complete. In these cases the control solution was prepared in double the usual volume. In addition to the reference control, a second control cell was put in a second position of the sample chamber. The optical density of the second control solution sample could then be compared with the optical density of this. With this system no errors due to changes in the spectrophotometer base line could occur. Capped cells were always used whenever the reaction required more than a few hours. Cells removed from the spectrophotometer during a reaction were kept in darkness until recording of optical density was resumed.

In calculations of results a correction of the final absorbance value was made to account for the disappearance of an amount of TNBS equal to the initial concentration of amine in the solution. The excinction coefficient of TNBS at 345 mμ was determined in each of the four buffers used for reaction, at the initial concentration of TNBS in the reaction mixture (0.05%). The average value was 5.22×10^2 optical density units $mole^{-1}$ l. cm^{-1}.

Results

In Figure 1 the results of the trinitrophenylation of BSA are plotted with the assumption that pseudo-unimolecular reaction conditions prevail (log α *vs.* time, where $\alpha = (OD_\infty - OD_t)/(OD_\infty - OD_0)$. It is seen that the data do not fit a straight line over the whole time range, but that the initial slopes are greater than the later slopes. This observation can be qualitatively compared with the results of Goldfarb (1966b), who found that human serum albumin (HSA) has ε-amino groups of several classes of reactivity toward TNBS. But in contrast to the results with HSA which showed that only about 31% of the amino groups will react with TNBS, the results of the present study give clear indication that the reaction with BSA can be followed to completion. The extent of the reaction can be estimated from the third column of Table II where the final apparent extinction coefficients per mole of trinitrophenylated amine, calculated on the basis of total amine content of the reactants, are given. In each case the expected value is 1.2×10^4 optical density units $mole^{-1}$ l. cm^{-1} (Goldfarb, 1966a). The first three columns of Table II give, from left to right, the pH of the reaction medium, the half-time of the reaction, $t_{1/2}$, and the apparent molar extinction coefficient, ϵ_p, of the product. As a convenience for comparison of the results of trinitrophenylation of the different reactants, the last column of Table II gives "reactivity factors" relative to the reactivity of native TMVP under the same conditions of pH. These factors are the reciprocals of the ratios of the half-times of the respective reactions to the half-time of the reaction with TMVP under the same conditions.

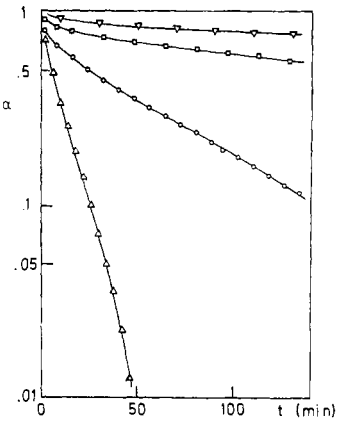

FIGURE 1: Reaction of BSA with TNBS. pH values: 7.5 (▽), 8.6 (□), 9.5 (○), and 10.4 (△). Amine concentration range, $0.61–0.65 \times 10^{-4}$ M.

It should be noted that in the case of BSA at pH 7.5 the re-

TABLE II: Summary of Trinitrophenylation Results.

	pH	$t_{1/2}$ (min)	$\epsilon_p \times 10^4$ (optical density units $mole^{-1}$ l. cm^{-1})	Reactivity Factor
(a) BSA	10.4	6	1.2	1.7
	9.5	24	1.2	6.0–7.3
	8.6	180	1.3	2.8
	7.5	~900		∞
(b) Poly-L-Lys	10.4	0.4	0.73	25
	9.5	0.4	0.69	360–440
	8.6	0.6	0.68	>800
	7.5	11–15	0.46	∞
(c) TMVP	10.4	10	1.4	1
	9.5	145–175	1.5	1
	8.6	>500		1
	7.5	∞		0
(d) TMV	8.6	∞		0
(e) $TMVP_{ox}$	10.4 (I)	0.8		12
	10.4 (II)	2		5
	9.5 (I)	3		48–58
	9.5 (II)	9		16–19
	8.6 (II)	40		>12
	7.5 (II)	~160		∞
(f) BSA_{ox}	10.4	2.5		4.0

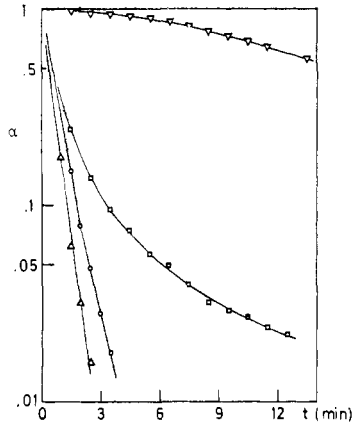

FIGURE 2: Reaction of poly-L-lysine with TNBS. pH values: 7.5 (▽), 8.6 (□), 9.5 (○), and 10.4 (△). Amine concentration, 0.65×10^{-4} M.

action was not followed to completion. For these experiments the data for the first few hours were used with the assumption that the value of the final absorbance could be estimated from the total amine concentration and the average final apparent extinction for all of the BSA experiments conducted at higher pH values.

A general feature of these experiments is shown clearly in both Figure 1 and Table IIa, namely, that high pH values enhance the rate of the reaction. This result indicates that the uncharged form of the amine is the more reactive.

Figure 2 shows first-order plots of the reaction of TNBS with poly-L-lysine. As can be seen, the reaction of TNBS with poly-L-lysine was much faster than was the reaction with BSA. This result would suggest that at least some of the ϵ-amino groups of BSA are partially masked, a finding in accord with the results of Goldfarb (1966b) for HSA. It is clear that the data do not fit very well with the assumption that the kinetics of the reaction are first order with respect to the amine concentration. But the log α *vs.* time plots are still convenient for visualizing the relative rates of reaction. Table IIb summarizes the results of the poly-L-lysine experiments.

It should be noted that the measured extinction coefficients for the trinitrophenylated poly-L-lysine range from 40 to 60% of the expected value of 1.2×10^4. This result suggests either incomplete reaction of the ϵ-amine groups with the TNBS or hypochromism of the product. It must be pointed out that hypochromicity of the product of the reaction of TNBS with poly-L-lysine is to be expected and therefore should not affect the validity of any results of the reaction with TMVP. Hypochromicity of poly-ϵ-trinitrophenyllysine is to be expected since it is uncharged and therefore likely to have a helical secondary structure. Such a structure would result in stacking of the side-chain rings and a tightening of the helix due to the well-known interaction of nitro-substituted rings. The resulting hypochromicity is not expected of trinitrophenylated TMVP, however, since TMVP has only two ϵ-amino groups and such a stacking arrangement is extremely unlikely.

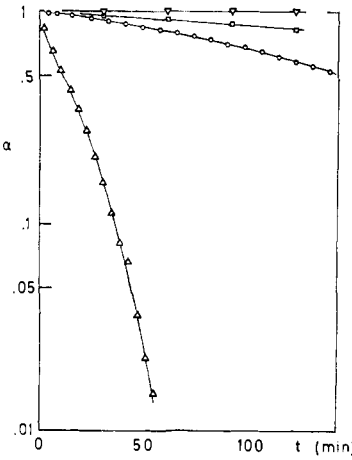

FIGURE 3: Reaction of TMVP with TNBS. pH values: 7.5 (▽), 8.6 (□), 9.5 (○), and 10.4 (△). Amine concentration range, 0.45–0.63 × 10^{-4} M.

The results of trinitrophenylation of TMVP are shown in Figure 3 and Table IIc. Although the data are plotted as first-order kinetics, it can be shown that the data at pH 9.5 fit an assumption that the reaction is zero order. The data at pH 8.6 do not permit an unambiguous choice between zero-order and first-order kinetics. Whatever the order of the kinetics, however, the important points to note here are that trinitrophenylation is substantially slower with TMVP than with either BSA or poly-L-lysine, and also that no reaction occurs at all in the first few hours with TMVP at pH 7.5. In the case of TMVP at pH 8.6 the value of OD_∞ was not measured but was estimated from the average ϵ_p measured in the experiments with TMVP at higher pH. The validity of this procedure depends upon the assumption that both of the ϵ-amino groups of TMVP react with TNBS at pH 8.6. It is clear that at the higher pH values both of the groups react, since the final optical density is close to that expected for two reacting groups (Goldfarb, 1966a,b; Okuyama and Satake, 1960). The alternative assumption that only one of the ϵ-amino groups of TMVP reacts with TNBS at pH 8.6 is not excluded by any of the experimental results. However, this alternative appears to be unfounded since there are no results to suggest that the reactivity of either group is more pH dependent than that of the other group.

A sample of TMVP reacted with TNBS at pH 10.4 was dialyzed against 0.1 M KCl, and the pH was lowered to below 6. Electron microscopy of this sample showed nonspecific aggregation, but no polymerization to viruslike rods was evident.

The results of the attempt to react whole virus with TNBS at pH 8.6 showed no increase in optical density at 345 mμ. This observation is expressed in Table IId as an infinite half-time for the reaction.

Figure 4 and Table IIe show the results of the experiments with oxidized TMVP. Although the difficulty of controlling the oxidation procedure is reflected in the inhomogeneity of reactivity of the two different preparations toward TNBS, the important feature to note here is that the reactivity of the ϵ-amino groups of TMVP is enhanced 5–50-fold by the oxidation procedure.

In order to determine the effect of the oxidation procedure on the ϵ-amino groups of BSA, a sample of BSA was subjected to the performic acid procedure and reacted with TNBS at pH 10.4. These results are shown in Table IIf. It can be seen that the half-time has been reduced to the same order of magnitude as the half-time for $TMVP_{ox}$. This de-

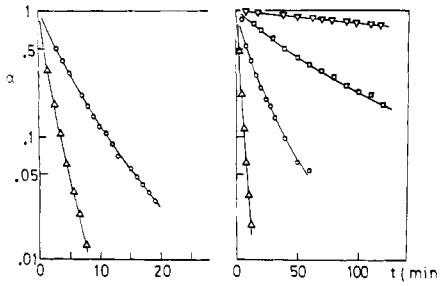

FIGURE 4: Reaction of $TMVP_{ox}$ with TNBS. Left graph, preparation I, amine concentration 0.33×10^{-4} M; right graph, preparation II, amine concentration 0.19×10^{-4} M; pH values: 7.5 (▽), 8.6 (□), 9.5 (○), and 10.4 (△).

crease represents only about a threefold enhancement of rate for the BSA reaction. However, this result should not be surprising since unoxidized BSA has already been shown to be about two to four times as reactive as TMVP.

Discussion

The extreme slowness of the reaction of TMVP with TNBS indicates that the ε-amino groups of TMVP are drastically restricted from behaving as normal free amines. Several other cases of anomalous chemical behavior of the ε-amino groups of TMVP have been previously reported. The titration results of Scheele and Lauffer (1967) show that the two ε-amino groups in the nucleic acid free protein of TMV do not appear to engage in hydrogen ion equilibrium with the solvent and that, in the range from pH 7.5 to the alkaline limit of reversibility, the net charge on the protein is two units per monomer more negative than would be expected on the basis of amino acid content alone. These two observations were interpreted to mean that the ε-amino groups were stabilized in the uncharged state. The hypothesis that the ε-amino groups of TMVP are uncharged at pH values far below their usual pK because they are "buried" in a region of low dielectric constant serves to explain several of the observations of the amino group chemistry of TMVP. The lack of reaction with *O*-methylisourea reported by Ramachandran (1959), the limited reaction with fluorodinitrobenzene (Fraenkel-Conrat and Singer, 1954), the apparent failure to engage in hydrogen ion equilibrium with the solvent (Scheele and Lauffer, 1967), and the slowness of reaction with TNBS reported here can all be rationalized by this hypothesis.

However, there are results which suggest that the ε-amino groups lie on or near the surface of TMVP. The increase in negative electrophoretic mobility upon reaction of whole virus with formaldehyde observed by Fisher and Lauffer (1949) supports this suggestion. Fraenkel-Conrat and Colloms (1967) have shown that these groups react with acetic anhydride. The finding of von Sengbusch (1965) that amino acid positions 65 and 66 are serologically distinguishable argues against the hypothesis that lysine-68 is completely inaccessible to the solvent. In addition, while their findings do not single out lysine-68, Perham and Richards (1968) have shown that, in order for polymerization to occur, both lysine positions cannot be without positively chargeable groups.

While auxiliary hypotheses can reconcile these results with the view that amine groups are at least partially buried, an alternative hypothesis that the ε-amino groups are involved in some interaction which makes them relatively unavailable for reaction with specific reagents can reconcile many of these observations. However, if this interaction takes place on or near the surface of the protein, it could permit or even require the amino groups to be positively charged. A likely candidate as a group with which a positively charged lysine-68 could interact might seem to be aspartic acid residue 66. But such an interaction would be of the type $COO^- \cdots {}^+NH_3$ and would not permit either component to be more negatively charged than expected at pH values above neutrality as required by the titration results.

The only groups in TMVP that could possibly have a net (positive) charge less than expected above neutrality are the lysine, cysteine, and tyrosine side chains. But the single cysteine side chain appears to titrate normally (Scheele and Lauffer, 1967), as does one of the four tyrosine residues (Chien *et al.*, 1965). Therefore, one of three situations must obtain in TMVP at pH values above 7.5: (a) two lysine residues are stabilized in their neutral state; or (b) two tyrosine residues are stabilized in their negatively charged state; or (c) some combination of a and b.

Situation a could exist and still explain most of the chemical and serological observations noted here if both lysine residues were only partially buried such that they were in a region of low enough dielectric constant to prevent their ionization. Situation a does not, however, explain the observation of Perham and Richards (1968) that preventing the ε-amino groups from being positively chargeable prevents the protein from assuming its native form. Situation b alone would explain none of the unusual chemistry of the ε-amino groups. A general mechanism that fulfills the requirements of situation c is a proton-sharing complex between a tyrosine phenolic group and a lysine ε-amino group. Two such associations are proposed to exist in TMVP. This mechanism would explain nearly all of the chemistry of the ε-amino groups of TMV and TMVP.

While there is no direct evidence to support the existence of proton-sharing complexes in TMVP, such interactions have been demonstrated in model compounds. Scott *et al.* (1968) and Scott and Vinogradov (1969) have demonstrated that equilibria of the type $RC_6H_5OH \cdots NR' \rightleftharpoons RC_6H_5O^- \cdots HN^+R'$ occur in aqueous and nonaqueous media and that the right-hand side of the equilibrium is favored, within wide limits, by higher dielectric constant. Hudson *et al.* (1969) have expanded these studies to include *N*-acetyltyrosine ethyl ester ($pK_a = 9.8$) and *N*-acetyltyrosinamide ($pK_a = 9.9$) as proton donors with triethylamine ($pK_a = 10.65$) and *N*-butylamine ($pK_a = 10.59$) as proton acceptors. They have shown that such proton-sharing complexes have negative free energies of formation as large as 4 kcal/mole in aqueous mixtures of dioxane containing 70% water.

Below pH 7.5 the polymerization of TMVP is accompanied by the binding of protons. One proton per chemical subunit is bound during temperature-dependent polymerization at pH 6.5. This observation was interpreted by Ansevin *et al.* (1964) as a forced neutralization accompanying the burying of carboxylate ions in regions of low dielectric constant between polymerizing faces. But the data of Scheele and Lauffer (1967) show that the effect of lowering pH below 6.5 polymerizes the protein still more than can be effected by an increase in temperature at pH 6.5. The argument is as follows. Take

TMVP at pH 6.5 and 4° (depolymerized). If the pH is lowered to the point where one more proton is bound per monomer, the sample is brought along the low-temperature titration curve to a point where the titration curve is sharpest, but where the pH must be lowered still further in order to effect the convergence of the low-temperature titration curve and the high-temperature titration curve (polymerized protein). This convergence occurs with the binding of one more proton per monomer. Therefore, while it is true that only one proton per monomer is bound during the temperature-mediated polymerization of TMVP at pH 6.5, two protons per monomer are bound if lowering of pH is also used to bring about polymerization.

It can be shown that the pK_a of an amino group involved in an interaction with a free energy of formation of -4 kcal/mole would be reduced by about three pH units. Although a duction of the pK_a of lysine by three pH units does not directly explain the proton binding by TMVP between pH 6 and 7, it is possible that in a real protein system the pK_a reduction is somewhat greater than that implied by the free-energy changes observed in the model systems.

Therefore, the simplest hypothesis to explain the titration behavior of TMVP near neutrality appears to be one which proposes that the uptake of two protons per monomer during polymerization represents the titration of two lysine ϵ-amino groups which have had their pK_a shifted by involvement in proton-sharing complexes with two tyrosine phenolate side chains. Thus, the single hypothesis invoking the proton-sharing complex, which has been shown to be plausible, can be made to explain the failure of these groups to titrate normally and can serve as an alternate to the hypothesis that carboxyl groups are buried between polymerizing faces of TMVP.

Although direct confirmation of the existence of the phenolate–amine proton-sharing complexes in TMVP is lacking, the circumstantial evidence for their existence permits the substitution of a single hypothesis which accounts for nearly all the data in place of two similarly unconfirmed hypotheses.

If the hypothesis that the uptake of protons during polymerization of TMVP represents the titration of ϵ-amino groups with down-shifted pK_a values is correct it is evident that the proton-sharing complex must be regarded as a mechanism for stabilizing the depolymerized (high pH) form of TMVP. Then as the temperature of a TMVP solution at pH 6.5 is raised, the formation of entropic unions (hydrophobic bonds) brings about polymerization of the protein and a disruption of the proton-sharing complexes causing the ϵ-amino groups to bind protons.

It must be pointed out that there is one observation of this study which is not explained by the simplest form of the hypothesis of proton-sharing complexes. Whole TMV would be expected to be similar in many respects to polymerized TMVP. That is, if the tyrosine–lysine associations are broken and the amino groups are protonated in polymerized TMVP, the amino groups of whole virus should also be free of association with tyrosine and should behave normally toward amino-specific reagents and in titration. The fact that TNBS does not react at all with whole TMV is not accounted for in the proton-sharing hypothesis as it has been presented above. But the possibility that the titrated amino groups might be associated with negatively charged carboxyl groups in polymerized TMVP or in whole virus could explain the failure of TMV to react with TNBS. The sharp decrease in reactivity of the ϵ-amino groups toward acetic anhydride upon polymerization of TMVP reported by Fraenkel-Conrat and Colloms (1967) can also be explained on this basis. Two carboxylate–amine proton-sharing complexes or salt bonds also provide satisfactory and apparently necessary explanation of the titration data of Scheele and Lauffer (1967) below pH 6 if the hypothesis that the ϵ-amino groups become protonated upon polymerization is correct. The two carboxyl groups thus stabilized in the negatively charged state would also account for the apparent failure of some of these groups to titrate in TMV and TMVP and would explain the position of the isoionic points.

Caspar (1963) observed that lead binding by TMV appears to be competitive with proton binding in the neutral pH range. Also, Shalaby *et al.* (1968) have found that up to two calcium or two potassium ions bind to TMV over the pH range 6–9 with half-saturation at about pH 7. The complexing theory presented here fails in its simplest form to account for these results. However, the assumption made in the preceding paragraph could account for the binding one ion per monomer if it is noted that, as the pH is raised, a pair of carboxylate groups released from complexing with amino groups could be able to bind a positive ion as proposed by Caspar (1963).

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Interactions of Hormonal Steroids with Nucleic Acids. II. Structural and Thermodynamic Aspects of Binding*

Paul Cohen,† Ruei-Chen Chin, and Chev Kidson

ABSTRACT: Structural attributes governing associations of estradiol, progesterone, and testosterone with polyguanylic acid and denatured deoxyribonucleic acid in aqueous buffer have been determined using equilibrium dialysis as an assay method. Structural substitution of the steroids indicates that in each case two functional groups are necessary for binding to guanine residues in polyguanylic acid: the 3- and 20-keto groups of progesterone, the 3- and 17-hydroxyl groups of estradiol, and the 3-keto and 17-hydroxyl groups of testosterone. These observations, the fact that these functional groups can act as proton donors or acceptors, and the minimal effect of slightly protic solvents on the binding of the parent steroids to polynucleotides suggest that two hydrogen bonds are formed in each case between functional groups of steroid and purine. Values for free energy of binding of 7–10 kcal/mole of steriod bound, derived from binding constants corresponding to the maximum slopes of binding isotherms, are consistent with the formation of two hydrogen bonds. While the relevant functional groups of guanine residues are not defined in all cases, studies with polyinosinic acid and hydroxymethylation of the 2-amino group indicate that this group is essential to the binding of progesterone and testosterone but not of estradiol. Alteration of binding properties by factors affecting polynucleotide conformation and the finding that the number of binding sites corresponding to maximum binding constants is less than 1/10,000 nucleotide residues demonstrate the critical role of polymer geometry in the provision of optimal binding sites. Construction of space-filling molecular models indicates that these hormonal steriods may interact with short, single-stranded nucleotide sequences.

As shown by equilibrium dialysis in aqueous buffer, the hormonal steriods progesterone, estradiol, testosterone, and corticosterone bind to denatured but not to native DNA (Cohen and Kidson, 1969b). Analysis of steroid binding to synthetic polynucleotides revealed a specific requirement for guanine residues and, in accord with their binding to DNA, a preferred affinity for single-stranded regions. Although these steroids share a common specificity for guanine, only estradiol was found to bind to poly I, suggesting that different functional groups of the nucleotide base are involved in these associations. Conformation of the polynucleotides appeared to influence binding: conditions associated with increased base stacking (high salt) enhanced the binding of progesterone and testosterone to denatured DNA but did not appreciably affect the binding of estradiol.

Binding was also found to depend upon steroid structure: a 17α-OH substituent reduced binding; steroids such as hydrocortisone and ecdysone, bearing several hydroxyl groups, did not bind measurably. These data suggest that the nature of the functional groups and net hydrophobicity of the steroids may influence interactions with polynucleotides.

In the present studies we examine further some of the structural requirements for associations between three steroids (estradiol, testosterone, and progesterone) and polynucleotides. Evidence is presented which suggests that these associations involve the formation of hydrogen bonds between given groups of steroid and guanine residues in the polynucleotide.

Materials and Methods

DNA. Pseudomonas aeruginosa DNA was isolated and denatured as described previously (Cohen and Kidson, 1969b). DNA concentration was determined spectrophotometrically on the native form in HMP buffer (Ts'o and Lu, 1964) on the basis $E^{1\%}_{1\,cm}$ 200 at 260 mμ.

Ribopolymers. Poly G and poly I were obtained from Miles Laboratories, Inc. Their concentrations were determined spectrophotometrically on the basis of the following ϵ_{max} poly G (ϵ_{252} 9.10^3) and poly I (ϵ_{248} 10^4).

Radiochemicals. The following radioactive steroids of high specific activity were obtained from the New England Nuclear Corp.: [1,2-^{3}H]testosterone (50 Ci/mmole), [6,7-^{3}H]estradiol-

* From the Department of Molecular Genetics, Institute of Hormone Biology, Syntex Research Center, Palo Alto, California 94304. *Received April 9, 1969.* A brief report of this work was presented at the meetings of the Federation of American Societies for Experimental Biology held in Atlantic City, April 1969 (Cohen and Kidson, 1969a).

† Permanent address: Institut de Biochimie, Faculté des Sciences, Université de Paris, 91 Orsay, France.