Protection of Satellite Tobacco Necrosis Virus Ribonucleic Acid by Wheat Germ 40S and 80S Ribosomes†

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ABSTRACT: Wheat germ ribosomes combine with the AUG codon at positions 30–32 from the 5′ terminus of in vitro radioiodinated satellite tobacco necrosis virus (STNV) ribonucleic acid (RNA) to form initiation complexes that protect specific regions of the RNA from attack by ribonucleases. Wheat germ 80S ribosomes convey partial protection to a region 20–52 nucleotides from the 5′ terminus of STNV RNA and convey protection against high levels of ribonucleases to a region 20–44 nucleotides from the 5′ terminus of STNV RNA. Wheat germ 40S ribosomes convey partial protection to a region 3–52 nucleotides from the 5′ terminus of STNV RNA and convey protection against high levels of ribonucleases to a region 10–47 nucleotides from the 5′ terminus of STNV RNA. Characterization of these levels of protection against attack by specific ribonucleases establishes that wheat germ 40S and 80S ribosomes form initiation complexes with a linear conformation of STNV RNA lacking the 5′-terminal stem and loop anticipated by Leung and co-workers [Leung, D. W., Browning, K. S., Heckman, J. E., RajBhandary, U. L., & Clark, J. M., Jr. (1979) *Biochemistry 18*, 1361–1366] for the 5′ terminus of STNV RNA.

Satellite tobacco necrosis virus (STNV)[1] ribonucleic acid (RNA) provides a unique mRNA for investigation of features that govern the correct initiation of mRNA translation. STNV RNA can be obtained in milligram quantities by convenient virus growth and RNA isolation procedures (Clark & Klein, 1974). STNV RNA is correctly translated in both procaryotic and eucaryotic systems to yield a single and characterized protein product (Klein et al., 1972; Lundquist et al., 1972; Klein & Clark, 1973; Leung et al., 1976). STNV RNA also lacks the $m^7G(5')ppp(5')Np$ capping group characteristic of most eucaryotic mRNA (Leung et al., 1976). Thus, STNV RNA can be derivatized with, and assayed for the effect of, an added 5′-terminal capping group (Smith & Clark, 1979).

The 5′-terminal nucleotide sequence of STNV RNA has been characterized to reveal an energetically favorable 5′-terminal stem and loop secondary structure (Leung et al., 1979). Similar 5′-terminal stem and loop structures have been implicated in the formation of eucaryotic 40S ribosome initiation complexes with reovirus mRNAs (Kozak & Shatkin, 1977a,b). One should therefore ask whether eucaryotic ribosomes utilize or bind to the potential 5′-terminal stem and loop structure of STNV RNA during the formation of 40S and 80S translation–initiation complexes. Such recognition of the 5′-terminal stem and loop structure of STNV RNA would imply a functional role for this structure and direct further experiments on STNV RNA structure and function.

Ribosome protection assays with uniformly radiolabeled mRNAs can characterize regions of an mRNA that bind with ribosome forms during the formation of translation–initiation complexes (Steitz, 1969). STNV [^{125}I]RNA, containing ^{125}I label only in 5-[^{125}I]iodocytidine as a result of Commerford's (1971) essentially cytidine specific radioiodination procedure, forms specific 80S translation–initiation complexes with wheat germ ribosomes (Leung et al., 1976). This suggests that one can use ribosome protection analyses with the nonuniformly labeled STNV [^{125}I]RNA and then relate the results of these assays to the known 5′-terminal nucleotide sequence of STNV RNA to establish regions of STNV [^{125}I]RNA that associate with wheat germ 40S and 80S ribosomes in initiation complexes. This paper utilizes this approach to achieve a characterization of the protection of STNV RNA from specific ribonucleases by wheat germ 40S and 80S translation–initiation complexes.

Experimental Procedures

Materials. Cell free, 30000*g* supernatant (S-30) extracts of wheat germ were prepared by the method of Roberts & Paterson (1973) and then stored (without previous incubation) at −80 °C until used. STNV RNA was prepared from germinating mung beans infected with the B strain of TNV and the SV1 strain of STNV (Clark & Klein, 1974). RNase T_1, RNase T_2, and RNase U_2 were from Calbiochem-Behring Corp. RNase A was from Worthington Biochemical Corp. Sparsomycin was from the Upjohn Co. Guanosine 5′-(β,γ-methylene)triphosphate was from Miles Laboratories, Inc. Dr. O. C. Uhlenbeck generously provided specific oligonucleotides for use as specific size standards in gel electrophoretic analyses, e.g., 33 and 37 nucleotide long oligonucleotides derived from yeast $tRNA^{Phe}$ by cleavage at the Y base, by partial digestion with RNase A (Philippsen et al., 1968; RajBhandary et al., 1967), and by partial digestion with [5′-^{32}P]poly$(A)_{27}$ and [5′-^{32}P]poly$(A)_{12}$ prepared by phosphomonoesterase treatment of a partial digest of poly(adenylic acid) followed by resolution of individual oligonucleotides by high-pressure liquid chromatography and final incubation of the resolved oligonucleotides with [γ-^{32}P]ATP and polynucleotide kinase. Various size standards for characterization of the sizes of compounds resolved by homochromatography were prepared by exhaustive digestion of STNV [^{125}I]RNA with RNase A.

Radioiodination of mRNA. STNV RNA was labeled in vitro with ^{125}I by a procedure patterned after that of Commerford (1971), including a second heat step at pH 7.6 to remove all significant quantities of radiolabeled uridine hydrate. The resultant STNV [^{125}I]RNA contained less than 5% of its ^{125}I label as 5-[^{125}I]iodouridine and greater than 95% of its ^{125}I label as 5-[^{125}I]iodocytidine. Specifically, 200 μg

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[1] Abbreviations used: STNV, satellite tobacco necrosis virus; [^{125}I]-RNA, an RNA containing 5-[^{125}I]iodocytidine residues as a result of in vitro radioiodination by the procedure of Commerford (1971); N, any ribonucleotide; KMH buffer, 0.1 M KCl, 3 mM $Mg(OAc)_2$, 20 mM Hepes-K salt, pH 7.6.

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of STNV RNA (approximately 0.5 mg/mL) was precipitated as the lithium salt by mixing in an equal volume of 4 M LiCl and 0.1 M NaOAc, pH 4.5, and storing overnight at 4 °C. The precipitate was then isolated by centrifugation (20 min, 10000 rpm) and redissolved in 200 μL of H_2O. This solubilized lithium salt of STNV RNA was added to a reaction system previously prepared by mixing (in turn) 72 μL of H_2O, 18 μL of 2 M NaOAc, pH 4.5, 40 μL of 5×10^{-5} M KI, and 30 μL of carrier-free Na[^{125}I]I (100 mCi/mL, Amersham), and the radioiodination was then performed by subsequent addition of 40 μL of 1×10^{-3} M $TlCl_3$ in 0.1 M NaOAc, pH 4.5, and heating at 60 °C for 20 min in a covered tube. The resultant STNV [^{125}I]RNA was isolated by chilling at 0 °C, addition of 0.8 mL of absolute ethanol, storage overnight at −20 °C, and centrifugation (20 min, 10000 rpm). The STNV [^{125}I]RNA was redissolved in 200 μL of 20 mM Tris–OAc, pH 7.6, 2 mM Na_2EDTA, and 0.5% sodium laurylsarcosine and then heated at 60 °C for 20 min, cooled to 0 °C, diluted with 10 μL of 2 M NaOAc, pH 4.5, prior to reisolation by the addition of 420 μL of absolute ethanol, stored overnight at −20 °C, and centrifuged (20 min, 10000 rpm). This precipitate was washed twice by resuspension in 0.6 mL of cold (0 °C) 70% ethanol and 0.1 M NaOAc, pH 4.5, and centrifugation (5 min, 10000 rpm) and then further washed by resuspension in 0.6 mL of cold (0 °C) absolute ethanol and centrifugation (5 min, 10000 rpm). This pellet of STNV [^{125}I]RNA was then dried by lyophilization and finally redissolved in 100 μL of H_2O prior to use.

Formation and Isolation of ^{125}I-Labeled Protected Fragments of STNV [^{125}I]RNA. Wheat germ 80S translation–initiation complexes were formed by incubating 1.0-mL reactions containing 0.4 mL of wheat germ S-30 extract, 0.1 M KCl, 3 mM $Mg(OAc)_2$, 1.2 mM dithiothreitol, 20 mM Hepes–K salt, pH 7.6, 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate, 40 μg of creatine phosphokinase, 60 mM L-methionine, 10 μM sparsomycin, and 15–25 μg of STNV [^{125}I]RNA for 10 min at 25 °C. Indicated levels of RNase T_1 or RNase A were then added, and the 25 °C incubation was continued for 10 min prior to quick cooling (ice bath) and layering of individual reactions onto 7-mL aliquots of cold (1 °C) 15% sucrose in KMH buffer and centrifugation for 1.5 h at 160000*g*. The surface of each resultant ribosome pellet was gently washed (layering and decantation) with 0.5 mL of cold KMH buffer and then each pellet was resuspended in 0.5 mL of cold (1 °C) KMH buffer. Each separate ribosome suspension was diluted with a 1% volume of 10% sodium dodecyl sulfate ($NaDodSO_4$) and an equal volume of 0.2 M potassium acetate, pH 6.0. The ^{125}I-labeled 80S initiation fragments were isolated by extraction with an equal volume of water-saturated phenol and subsequent precipitation of the macromolecules from the aqueous phase by the addition of 2 volumes of ethanol and storage (−20 °C, 12 h).

Wheat germ 40S translation–initiation complexes were formed like the 80S initiation complexes except that the reactions were scaled down to 0.25-mL volumes, and the incubations contained 0.4 mM guanosine 5′-(β,γ-methylene)triphosphate instead of GTP. The incubated reactions were then treated with specific nucleases in a manner identical with that for the 80S complexes, layered onto cold 10-mL exponential gradients of 10–25% sucrose in KMH buffer, and centrifuged for 6 h at 36000 rpm in a Beckman SW-41 ultracentrifuge rotor. The resolved solutions were fractionated into 0.45-mL fractions, and the 4 or 5 fractions near the middle of the gradients containing the peak of ^{125}I-labeled 40S ribosomes were pooled, diluted with an equal volume of 0.1% $NaDodSO_4$, and extracted with an equal volume of water-saturated phenol. The resultant aqueous phase was mixed with a 1/30 volume of 3 M potassium acetate, pH 6.0, and the ^{125}I-labeled 40S initiation fragments and associated macromolecules in the aqueous phase were then precipitated by the addition of 2 volumes of ethanol and storage (−20 °C, 12 h).

The inhibition of the conversion of 40S initiation complexes to 80S initiation complexes by guanosine 5′-(β,γ-methylene)triphosphate is not complete and, therefore, the sucrose gradient resolution procedure used in the above 40S initiation fragment isolation also resolves 80S initiation complexes as a peak of ^{125}I-labeled material near the bottom of the sucrose gradients. The ^{125}I-labeled 80S initiation fragments obtained by phenol extraction of these initiation complexes are identical with those obtained by the ribosome pelleting procedure used to isolate 80S initiation fragments. Thus the above 40S initiation fragment isolation procedure also provides 80S initiation fragments if desired.

Gel Electrophoretic Resolution of Specific Initiation Fragments. The precipitated initiation fragments were dissolved in a minimum of cold water. Aliquots containing a desired quantity of ^{125}I cpm were mixed with a 1/2 volume of 50% sucrose, 30% urea, 0.2% xylene cyanol FF, and 0.2% bromophenol blue, heated at 100 °C for 1 min, and then quickly cooled to assure denaturation of the ^{125}I-labeled oligonucleotides. The heated and cooled samples were then resolved by electrophoresis (1000 V until the xylene cyanol FF migrates halfway into the gel) by the method of Maxam & Gilbert (1977) on a 28 cm long, 13 cm wide, 0.75 mm thick 20% polyacrylamide gel containing 7 M urea, 1 mM Na_2-EDTA, and 50 mM Tris–borate, pH 8.3. Size markers were resolved in tracks adjacent to the ^{125}I-labeled initiation fragments. The gel electrophoretically resolved materials were detected by radioautography or by staining (Stains-all, Miles Laboratories, Inc.).

Specific bands of gel containing ^{125}I-labeled initiation fragments were cut out, and the ^{125}I-labeled oligonucleotide was recovered from the gel in approximately 90% yield by a stepwise extraction procedure that employed autoclaved (i.e., nuclease-free) reagents and components. First, a glass rod was used to mash the gel into fine pieces in the bottom of 0.75-mL polypropylene conical tube (Sarstedt Co.) that had been prefitted with a 13 mm diameter fluted paper disk (740-E penecillin assay disk, Schleicher & Schuell Co.) jammed into the bottom of the tube. Second, 0.2 mL of cold (1 °C) 0.15 M NaCl, 1 mM Na_2EDTA, and 50 mM Tris–borate, pH 8.3, was added to each mashed gel slice, and the polypropylene tubes were then agitated on an Evapo-mix shaker (Buchler Instruments) without vacuum for 3–4 h at 4 °C. Third, each polypropylene tube was punctured at the bottom with a thumbtack. The punctured tube was placed inside a 1.5-mL polypropylene conical tube (Sarstedt Co.), and then the fluid containing the extracted ^{125}I-labeled oligonucleotide was forced from the punctured tube into the base of the surrounding 1.5-mL polypropylene tube by 5 min of 4 °C centrifugation in a microfuge (Brinkmann Instrument Co.). Fourth, the residual gel in the 0.75-mL polypropylene tube was washed by resuspension (gently shaking) in 0.05 mL of cold (1 °C) 0.15 M NaCl, 1 mM Na_2EDTA, and 50 mM Tris–borate, pH 8.3, and repetition of the two-tube microfuge centrifugation procedure above. Fifth, the ^{125}I-labeled oligonucleotide in the combined extract and wash solution was precipitated by stepwise addition of 10 μg of nuclease-free carrier RNA (STNV RNA in our case) and 2 volumes of ethanol and storage (−20 °C, 12 h). The precipitated materials were lastly

washed by resuspension in 0.05 mL of cold (0 °C) ethanol, centrifugation (5 min in microfuge at 4 °C), and aspiration of the supernatant ethanol prior to final lyophilization of the ^{125}I-labeled initiation fragment(s) and associated carrier RNA.

Two-Dimensional Electrophoresis–Homochromatographic Fingerprint Characterization of Individual ^{125}I-Labeled Initiation Fragments. The resolved ^{125}I-labeled initiation fragments and associated carrier RNA were taken up in 10 μL of cold water and then either digested with RNase A (RNA/RNase A = 20:1) for 45 min at 37 °C or digested with RNase T_1 (RNA/RNase T_1 = 75 μg:1 unit) for 60 min at 37 °C. The reaction mixtures were then spotted onto a 3 × 50 cm cellulose acetate strip prewetted with pyridine–acetate buffer, pH 3.5 (5% acetic acid, 0.5% pyridine), 2 mM Na_2-EDTA, and 7 M urea and subjected to 2000 V until an added xylene cyanol FF dye marker migrated 8 cm toward the + pole. The ^{125}I-labeled materials on the cellulose–acetate strip between the origin and a point 20 cm toward the + pole were then transferred by the method of Southern (1974) to a 20 × 20 cm thin-layer chromatographic plate coated with DEAE-cellulose (Polygram Cel 300 DEAE/HR-2/15, Brinkman Instruments Co.) and further resolved in the second dimension (often with an added set of ^{125}I-labeled size markers) by homochromatography (Silberklang et al., 1977) at 60 °C with a 25 mM KOH homochromatographic mixture. The resolved ^{125}I-labeled oligonucleotides on the two-dimensional fingerprint were detected by radioautography.

Specific ^{125}I-labeled oligonucleotides detected on the two-dimensional fingerprints were characterized by the method of Brownlee (1972). This involved scraping of zones of the thin-layer plates containing individual ^{125}I-labeled compounds, extraction of the compounds from the DEAE-cellulose powder with 30% triethylamine bicarbonate, pH 9.0, lyophilized removal of triethylamine bicarbonate, exhaustive digestion of aliquots of the extracted ^{125}I-labeled compounds with RNase T_1 and RNase A or partial digestion of aliquots of the extracted ^{125}I-labeled compound with RNase U_2 and RNase T_2, and final one-dimensional electrophoretic resolution of the digested samples of DEAE-cellulose paper in 7% formic acid in the presence of standards. The ^{125}I-labeled compounds detected by radioautographic analysis of the electrophoretically resolved reaction mixtures were identified by any or all of three methods, namely, (1) comparison with known standards, e.g., purchased dinucleotides and trinucleotides and previously characterized ^{125}I-labeled products from specific nuclease digestions of ^{125}I-labeled oligonucleotides, (2) extraction and further size characterization by homochromatography against known sizes of ^{125}I-labeled oligonucleotides, and (3) calculation of *M* values (Brownlee, 1972) of all the products detected from partial digestions of specific ^{125}I-labeled compounds. The structures of the original specific ^{125}I-labeled oligonucleotides were then defined on the basis of the ^{125}I-labeled compounds released by the partial and total digestions with the specific nucleases and by comparison with the known nucleotide sequence of the 5′ terminus of STNV RNA (Leung et al., 1979).

Results

Rationale for Ribosome Protection Experiments with STNV [^{125}I]RNA. Figure 1 shows the sequence of the first 54 nucleotides at the 5′ terminus of STNV RNA. Previous work (Leung et al., 1979) has established the sequence of the first 42 nucleotides of Figure 1. Several lines of evidence establish that the longer sequence of Figure 1 is correct. First, the indicated codons yield the known (Leung et al., 1979) N-terminal amino acids of STNV coat protein. Second, the sequence is identical with a characterization of the first 86

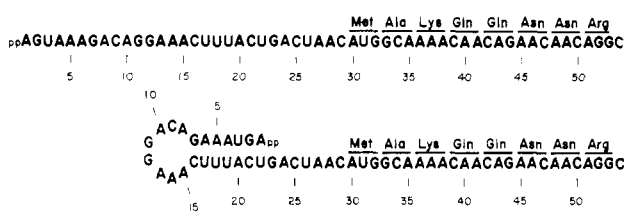

FIGURE 1: The 5′-terminal nucleotide sequence of STNV RNA.

nucleotides of STNV [5′-^{32}P]RNA (R. E. Lockard, personal communication). Third, the sequence agrees with analyses of large specific oligonucleotides generated from STNV RNA (Donis-Keller, 1979; H. Donis-Keller, personal communication). Lastly, analyses of a DNA clone of a reverse transcript of STNV RNA (Ysebaert et al., 1980) confirm the indicated sequence.

Figure 1 also shows that the 5′-terminal nucleotide sequence of STNV RNA can fold into an energetically favorable 5′-terminal stem and loop secondary structure (Leung et al., 1979). The close proximity of this stem and loop conformation to the initiator AUG codon of STNV RNA suggests that the stem and loop may function in the formation of translation–initiation complexes. This concept is particularly intriguing in cases of eucaryotic translation of STNV RNA because similar stem and loop conformations have been implicated in the formation of 40S initiation complexes between wheat germ ribosomes and reovirus mRNAs (Kozak & Shatkin, 1977a,b).

Ribosome protection experiments can characterize the region(s) of a radiolabeled mRNA that bind with, and presumably function with, ribosomes in translation–initiation complexes (Steitz, 1969). STNV [^{125}I]RNA will form specific initiation complexes with eucaryotic wheat germ ribosomes (Leung et al., 1976). Accordingly, we utilized ribosome protection of STNV [^{125}I]RNA by wheat germ 80S and 40S translation–initiation complexes to identify the portions of the 5′ terminus of STNV RNA that function in mRNA recognition during the initiation of STNV RNA translation.

Protection of STNV [^{125}I]RNA by Wheat Germ 80S Ribosomes. Incubation of a wheat germ S-30 extract with STNV [^{125}I]RNA under appropriate conditions in the presence of sparsomycin results in the formation of 80S translation–initiation complexes (Leung et al., 1976). Treatment of these 80S complexes with increasing concentrations of RNase T_1 or RNase A followed by isolation and gel electrophoretic resolution of the ribosome bound ^{125}I-labeled material(s) yields a series of ^{125}I-labeled 80S "initiation fragments" (Figure 2). As can be seen, characterization of the "limit 80S initiation fragments", i.e., the largest initiation fragments protected by 80S ribosomes from high concentrations of specific ribonucleases, is somewhat subjective. Yet 80S ribosomes clearly protect an approximately 32 nucleotide long initiation fragment from high concentrations of RNase T_1 and an approximately 28 nucleotide long fragment from high concentrations of RNase A. We therefore define these specific initiation fragments as the limit initiation fragments of 80S ribosome protection of STNV RNA from RNases T_1 and A, respectively.

The nucleotide sequence near the initiator AUG of STNV RNA (Figure 1), the sizes of the limit 80S initiation fragments (Figure 2), and the specificities of RNases T_1 and A all restrict the potential sources of these limit 80S initiation fragments of STNV RNA and suggest that wheat germ 80S ribosomes protect the limit initiation fragments of STNV RNA indicated on Figure 3. Significantly, exhaustive secondary digestions of each of these proposed limit 80S initiation fragments by

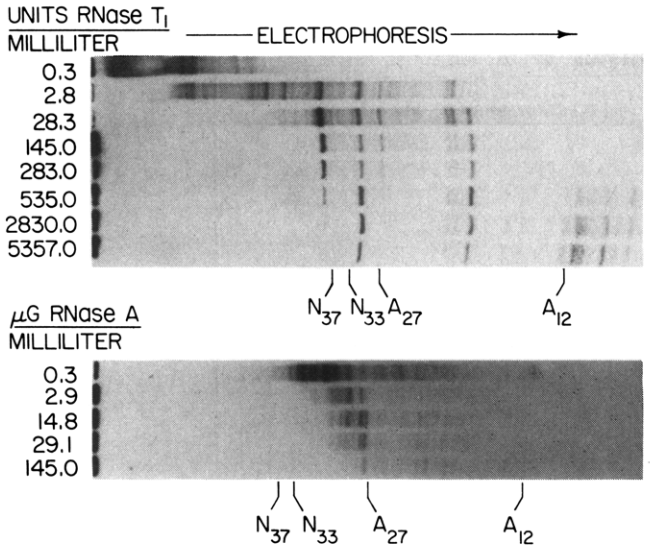

FIGURE 2: Radioautograms of the gel electrophoretic resolution of the ^{125}I-labeled initiation fragments of STNV [^{125}I]RNA protected from increasing concentrations of RNase T_1 and RNase A by wheat germ 80S translation–initiation complexes. N_{37}, N_{33}, A_{27}, and A_{12} indicate the location of specific size standards, all resolved in parallel tracks on the gels.

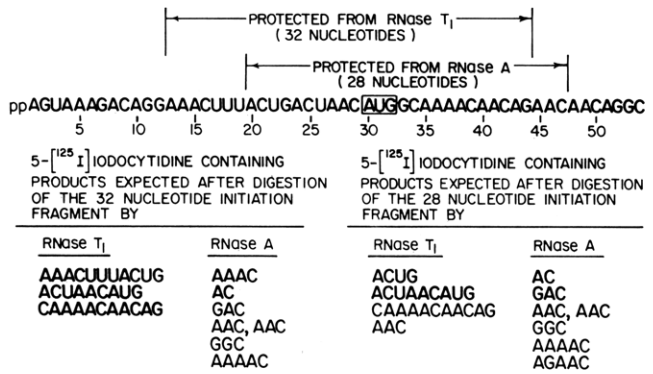

FIGURE 3: Proposed origins for two 80S initiation fragments of STNV [^{125}I]RNA and a listing of the 5-[^{125}I]iodocytidine-containing products expected after exhaustive digestion of these initiation fragments with RNase T_1 and RNase A.

RNases T_1 and A will yield specific 5-[^{125}I]iodocytidine-containing oligonucleotides that can establish the validity of these assignments. For example, exhaustive digestion of the proposed 32 nucleotide limit 80S initiation fragment with RNase A should yield ^{125}I-labeled AAAC (Figure 3), a product only obtainable if the 5′ end of the 32 nucleotide initiation fragment starts at position 13 from the 5′ terminus of STNV RNA. Similarly, exhaustive digestion of the 28 nucleotide limit initiation fragment with RNase A will yield ^{125}I-labeled AC and ^{125}I-labeled AGAAC only if this fragment spans positions 20–47 from the 5′ terminus of STNV RNA.

Fingerprint resolutions of the 5-[^{125}I]iodocytidine-containing products obtained from exhaustive digestions of the 32 and 28 nucleotide initiation fragments with RNase T_1 and RNase A (Figures 4 and 5) yield all, and only, the ^{125}I-labeled products predicted in Figure 3. Further, quantitative analyses of the ^{125}I label present in each spot and the radioautographic intensity of the observed spots generally agree with the stoichiometric yield predictions of Figure 3 in spite of all the potential losses and transfer errors inherent in oligonucleotide fingerprint procedures. The predictions of Figure 3 are therefore correct, and wheat germ 80S ribosomes protect nucleotides at positions 20–44 from the 5′ terminus of STNV RNA.

These characterizations of the 32 and 28 nucleotide long 80S initiation fragments of STNV RNA also establish the conformation of STNV RNA in wheat germ 80S initiation complexes. Specifically, the RNase A dependent 28 nucleotide limit initiation fragment requires a cleavage of STNV RNA between positions 19 and 20 from the 5′ terminus of STNV RNA. RNase A does not cleave double-stranded RNAs (Uchida & Egami, 1971). Positions 19 and 20 are in the middle of the double-stranded region of the stem and loop structure potentially available at the 5′ terminus of STNV RNA (Leung et al., 1979). The conformation of the 5′ terminus of STNV RNA in 80S wheat germ ribosomes must therefore be single stranded, lacking the potential 5′-terminal stem and loop structure.

Figure 2 also reveals a series of ^{125}I-labeled specific initiation fragments that are less protected than the 32 and 28 nucleotide long limit 80S initiation fragments characterized in Figures

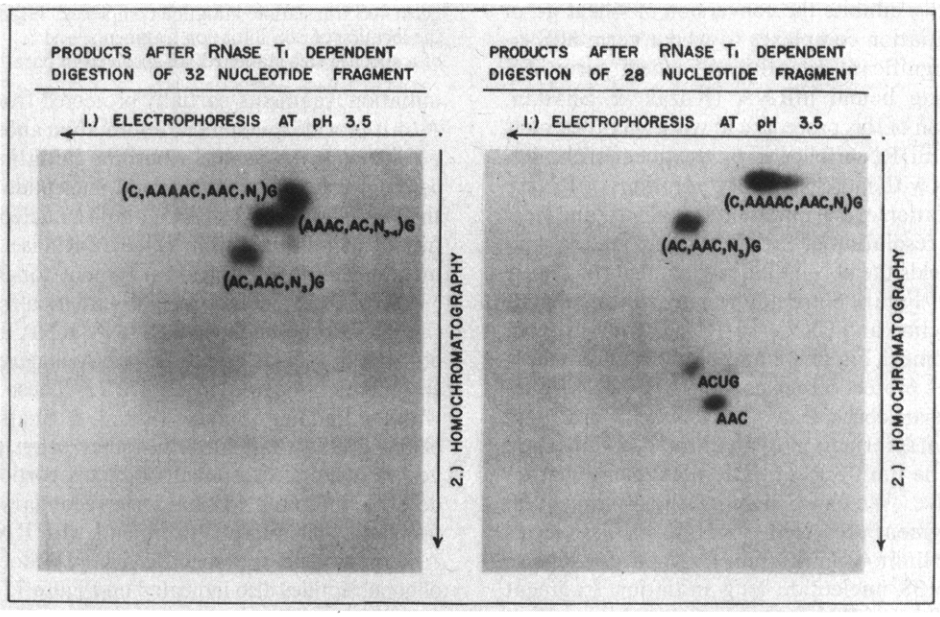

FIGURE 4: Radioautograms of the two-dimensional fingerprint resolution (Brownlee, 1972) of the ^{125}I-labeled products released from 80S initiation fragments by exhaustive digestion with RNase T_1.

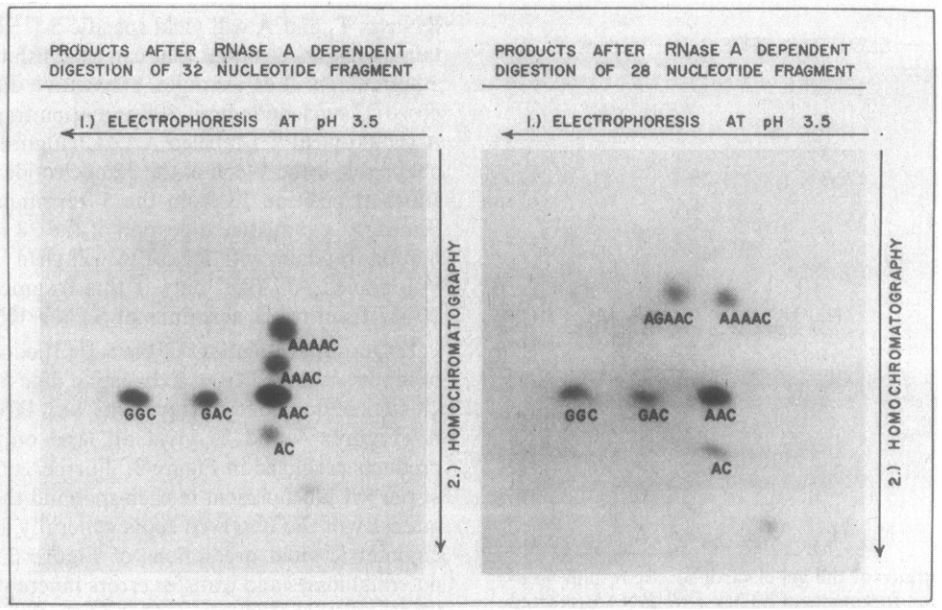

FIGURE 5: Radioautograms of the two-dimensional fingerprint resolution (Brownlee, 1972) of the ^{125}I-labeled products released from 80S initiation fragments by exhaustive digestion with RNase A. The unnamed spot in the lower right of each fingerprint is a low (less than stoichiometric) level of 5-[^{125}I]iodocytidylate that occasionally occurs due to low-level nonspecific binding of STNV [^{125}I]RNA by wheat germ ribosomes.

3–5. These other initiation fragments must also reflect, in part, the topography of STNV RNA in wheat germ 80S initiation complexes. Accordingly, we used the characterization procedures of Figures 3–5 to establish that the 40 and 29 nucleotide long initiation fragments of STNV [^{125}I]RNA protected from low levels of RNase T_1 by 80S initiation complexes involve protection of positions 13–52 and 24–52 from the 5′ terminus of STNV RNA. We did not attempt similar analyses of the many initiation fragments protected from low levels of RNase A by 80S initiation complexes. However, these initiation fragments protected from low levels of RNase A must reflect some degree of protection up through position 54 from the 5′ terminus of STNV RNA for preliminary ribosome protection studies with 80S ribosomes and low levels of RNase A (Leung et al., 1976; Smith & Clark, 1979) yield the ^{125}I-labeled AGGC expected from positions 51–54 of STNV RNA.

Protection of STNV [^{125}I]RNA by Wheat Germ 40S Ribosomes. The GTP analogue guanosine 5′-(β,γ-methylene)-triphosphate partially inhibits the conversion of wheat germ 40S translation–initiation complexes to wheat germ 80S ribosomes to yield significant quantities of wheat germ 40S ribosomes containing bound mRNA (Kozak & Shatkin, 1977a,b). Application of this procedure to wheat germ extracts containing STNV [^{125}I]RNA followed by treatment of the 40S initiation complexes with increasing concentrations of RNase T_1 or RNase A, isolation of the initiation fragments, and final gel electrophoretic resolution of the ribosome-bound ^{125}I-labeled material(s) yields the 40S initiation fragments of Figure 6. Two features of Figure 6 are noteworthy. First, a comparison of the protection of STNV [^{125}I]RNA by wheat germ 80S and 40S ribosomes (Figures 2 and 6) shows that wheat germ 40S ribosomes protect larger portions of STNV [^{125}I]-RNA from intermediate concentrations of RNase T_1 or RNase A. Second, the characterization of the limit 40S initiation fragments is, like the limit 80S initiation fragment characterizations, subjective. We chose to define the prominent 50 nucleotide long fragment protected from intermediate levels of RNase T_1 as the limit 40S initiation fragment for RNase T_1. Similarly, the 38 nucleotide long initiation fragment protected from 15.0 μg of RNase A/mL is an obvious limit 40S initiation fragment for RNase A. Yet the larger 40S

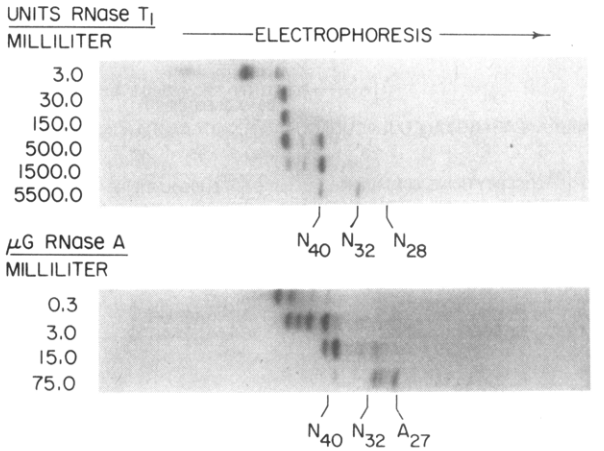

FIGURE 6: Radioautograms of the gel electrophoretic resolution of the ^{125}I-labeled initiation fragments of STNV [^{125}I]RNA protected from increasing concentrations of RNase T_1 and RNase A by wheat germ 40S translation–initiation complexes. N_{40}, N_{32}, and N_{28} indicate the location of 80S initiation fragments, and A_{27} indicates the location of a specific size standard, all resolved in parallel tracks on the gels.

initiation fragments partially protected from 3.0 μg of RNase A/mL provide much more information about the conformation of STNV RNA in 40S ribosome initiation complexes. Accordingly, we investigated the 51 nucleotide long fragment (i.e., the largest of the 51, 47,45, and 41 nucleotide long initiation fragments protected from 3.0 μg of RNase A/mL) as the most meaningful 40S initiation fragment for RNase A.

Use of these sizes for the 40S initiation fragments along with the nucleotide sequence of STNV RNA and the known specificities of RNase T_1 and RNase A suggests the 40S initiation fragments indicated on Figure 7. These assignments agree with the findings of others (Kozak & Shatkin, 1977a,b; Legon et al., 1977) that 40S ribosomes often protect larger and generally more 5′ terminally directed portions of mRNAs than do 80S ribosomes. Exhaustive secondary digestion of these proposed 40S initiation fragments with RNase T_1 and RNase A should yield the specific 5-[^{125}I]iodocytidine-containing oligonucleotides also indicated on Figure 7. Gel electrophoresis at different pHs provides better resolutions of larger ^{125}I-labeled oligonucleotides than do traditional two-dimensional

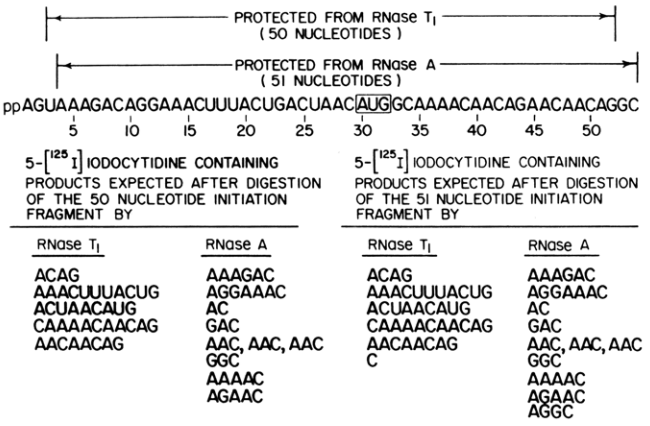

FIGURE 7: Proposed origins for two 40S initiation fragments of STNV [^{125}I]RNA and a listing of the 5-[^{125}I]iodocytidine-containing products expected after exhaustive digestion of these initiation fragments with RNase T_1 and RNase A.

fingerprint procedures. Thus we used gel electrophoresis at different pHs to resolve and characterize the various ^{125}I-labeled oligonucleotides produced by RNase T_1 dependent exhaustive digestions of the 50 and 51 nucleotide 40S initiation fragments (Figure 8). As can be seen, gel electrophoretic resolution at pH 3.5 (a process largely dependent upon nucleotide composition) resolves the RNase T_1 dependent digestion products from the 50 and 51 nucleotide long 40S initiation fragments into a diffuse band of ^{125}I-labeled ACAG and three other diffuse bands of ^{125}I-labeled material classified here as bands 1, 2, and 3. Band 2 must be the sequence AACAACAG from positions 45–52 from the 5′ terminus of STNV RNA for it is present in the 40 nucleotide long 80S initiation fragment but is lacking in the 32 nucleotide long 80S initiation fragment (Figure 3). Extraction of bands 1, 2, and 3 followed by additional gel electrophoretic resolution at pH 8.3 (a process largely dependent upon oligonucleotide size) resolves band 3 into two ^{125}I-labeled compounds and also demonstrates that the ^{125}I-labeled compounds of band 1 and both ^{125}I-labeled compounds of band 3 are larger than the band 2 octanucleotide AACAACAG. Exhaustive further digestion of each of these pH 8.3 resolved components with RNase A followed by traditional two-dimensional fingerprint analysis yields the 5-[^{125}I]iodocytidine-containing products shown on Figure 8. Combination of these data with the relative sizes of the ^{125}I-labeled oligonucleotides obtained from the 40S initiation fragments after digestion with RNase T_1 (Figure 8) and the known sequence of STNV RNA (Figure 1) establishes the sequences indicated for the ^{125}I-labeled oligonucleotide released from the 50 and 51 nucleotide long 40S initiation fragments by RNase T_1. These characterizations, plus traditional two-dimensional fingerprint characterization of the ^{125}I-labeled products produced by RNase A dependent exhaustive digestions of the 50 and 51 nucleotide long 40S initiation fragments (Figure 9), account for all, and only, the ^{125}I-labeled products predicted in Figure 7. Thus the data of Figures 8 and 9 establish that Figure 7 correctly defines the origins of these 40S initiation complexes.

Similar characterizations of the 47, 45, 41, and the limit 38 nucleotide long 40S initiation fragments partially protected from 3.0 and 15.0 μg of RNase A/mL reveal these 40S initiation fragments arise from positions 4–50, 10–54, 10–50, and 10–47, from the 5′ terminus of STNV RNA, respectively. Thus wheat germ 40S ribosomes convey partial protection over the region 3–54 nucleotides from the 5′ terminus of STNV RNA and provide limit protection (i.e., protection against high levels of RNases) over the region 10–47 nucleotides from the 5′ terminus of STNV RNA. Significantly, these data also establish that RNase T_1 and RNase A cleave 40S ribosome-bound STNV RNA at positions 2 and 3 nucleotides from its 5′ terminus. RNase T_1 and RNase A are single strand specific RNases (Uchida & Egami, 1971), and positions 2 and 3 from the 5′ terminus of STNV RNA are in the stem of the proposed 5′-terminal stem and loop conformation (Figure 1). Therefore, wheat germ 40S ribosomes must feature a linear form of STNV RNA lacking the potential 5′-terminal stem and loop structure.

Discussion

This report defines the regions of STNV RNA that are partially and more fully ("limit") protected by wheat germ ribosomes during the formation of 40S and 80S translation-initiation complexes. Most importantly, the observed cleavage of 80S ribosome-bound STNV RNA at position 19 from the 5′ terminus during limit protection from RNase A (Figure 3)

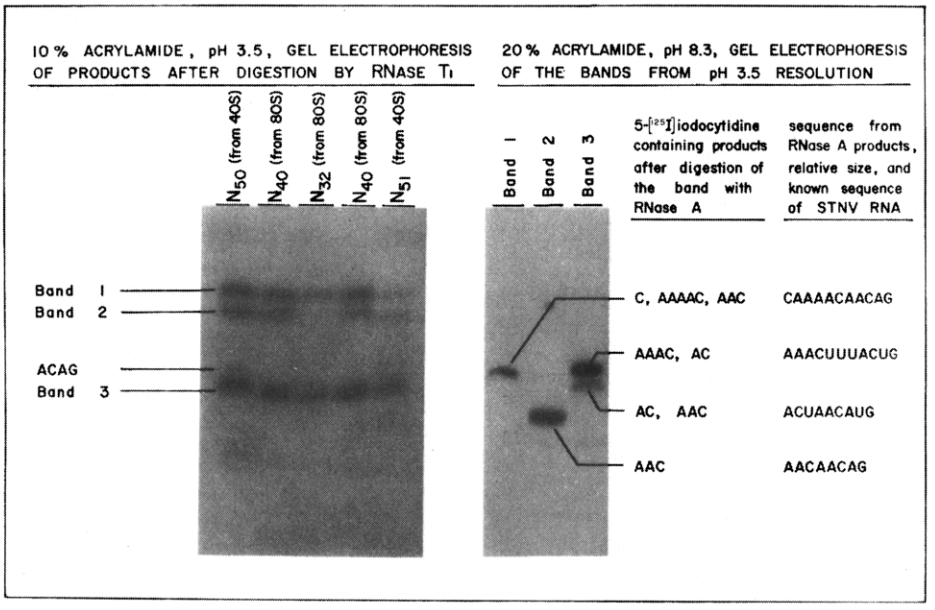

FIGURE 8: Radioautograms of two gel electrophoretic resolutions of the ^{125}I-labeled products released from 40S initiation fragments by exhaustive digestion with RNase T_1. The gel resolution of ACAG and bands 1–3 was run in 10% acrylamide containing 7 M urea and 0.025 M citrate, pH 3.5. The subsequent resolution of bands 1–3 was as in Figures 2 and 6.

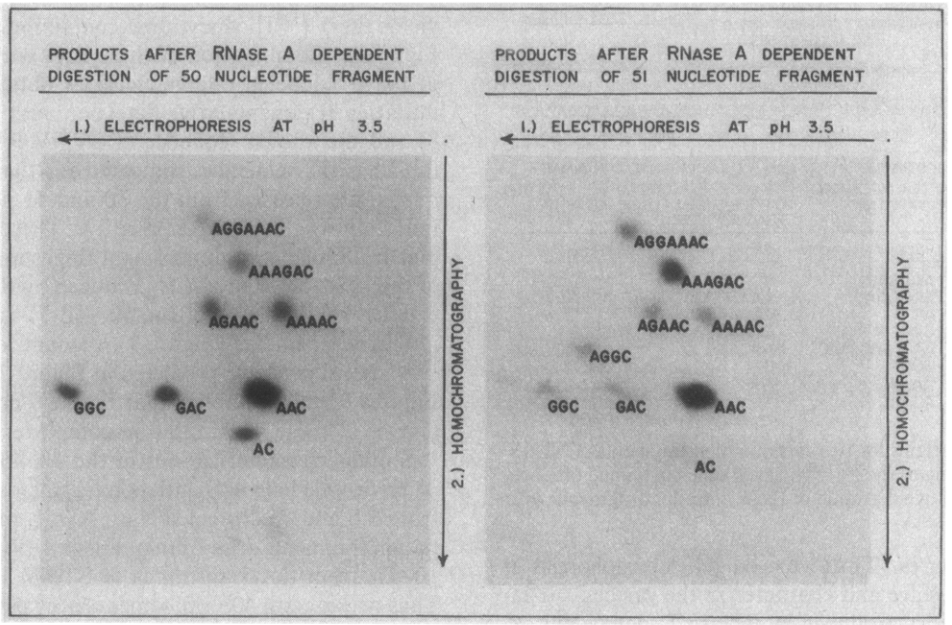

FIGURE 9: Radioautograms of the two-dimensional fingerprint resolution (Brownlee, 1972) of the ^{125}I-labeled products released from 40S initiation fragments by exhaustive digestion with RNase A.

and the observed cleavages of 40S ribosome-bound STNV RNA at positions 2 and 3 from the 5′ terminus during limit and partial protection from RNase T_1 and RNase A, respectively (Figure 7), dictate that both 40S and 80S wheat germ ribosomes bind a linear conformation of STNV RNA lacking the proposed (Leung et al., 1979) 5′-terminal stem and loop secondary structure. The 5′-terminal stem and loop conformation of STNV RNA is more energetically stable than the 5′-terminal linear conformation of STNV RNA (Leung et al., 1979). This 5′-terminal secondary structure of STNV RNA should therefore predominate in our ribosome binding assays performed in a high-salt environment [0.1 M KCl, 3 mM $Mg(OAc)_2$, etc.] that favors secondary structure in RNAs. It follows that one must consider two alternate mechanisms for the binding of STNV RNA by wheat germ ribosomes. First, one can envision that wheat germ ribosomes bind to members of the small population of 5′ terminally linear STNV RNA molecules present during the initiation complex formation. Alternately, one can envision that some mechanism of the wheat germ system opens the favored 5′-terminal stem and loop structure of STNV RNA during initiation complex formation. Our data, and STNV RNA translation studies by others (Kemper & Stolarsky, 1977; Herson et al., 1979), do not dictate a preferred choice between these two mechanisms.

Our results also reveal two other features of STNV RNA that relate to the mechanism of initiation of mRNA translation. First, our data show that the 5′ terminus of STNV RNA is not protected by, and therefore not associated with, wheat germ ribosomes. An added 5′-terminal $m^7G(5')ppp(5')$... capping group on STNV RNA should also project beyond wheat germ ribosomes and not interact in ribosomal reactions during initiation of translation. This concept is supported by the observation (Smith & Clark, 1979) that an added 5′-terminal capping group does not enhance the translation initiation properties of intact STNV RNA. Second, our studies established that translation begins at the first AUG present near the 5′ terminus of the linear conformation of STNV RNA present in initiation complexes with wheat germ ribosomes. This linear conformation is intriguing in light of the model (Kozak, 1978) that selection of the initiator AUG in eucaryotes involves reading in from the 5′ terminus of the mRNA until the system reaches the first AUG.

The data reported here establish the [^{125}I]mRNA prepared by the cytidine-specific radioiodination procedure of Commerford (1971) can serve a useful role in mRNA structure and function studies. Our results show that [^{125}I]mRNA will function specifically and correctly in ribosome protection experiments to yield unique initiation fragments. We also establish that if the nucleotide sequence of the translation region of a mRNA is known, identification of the 5-[^{125}I]iodocytidine-containing compounds released from unique initiation fragments by specific nucleases and comparison of these identifications with the nucleotide sequence of the mRNA allow one to characterize the ribosome binding sites of the [^{125}I]mRNA in question. This capability will prove useful with readily available viral mRNAs such as plant viral mRNAs. For example, any of the recently developed RNA sequence methods can yield the nucleotide sequence of the potential translation–initiation sites in the mRNA. Subsequent ribosome protection studies with the [^{125}I]mRNA can then identify and characterize the translation–initiation sites in question.

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Conformational Change of Human Prothrombin Induced by Calcium Ions: An X-ray Scattering Study†

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ABSTRACT: The scattered X-ray intensities from dilute solutions of prothrombin and Ca^{2+}–prothrombin at 21 °C in 0.1 M Tris-HCl buffer of pH 7.4 indicate that the prothrombin molecule attains a more extended conformation when Ca^{2+} ions are bound. This is indicated from the distance distribution function: the largest distance within the prothrombin molecule increases from 130 to 150 Å when Ca^{2+} ions are bound; the radius of gyration increases from 35.5 to 39.5 Å. A comparison of the experimental scattering curves with theoretical scattering curves calculated for various triaxial bodies shows that the shape of the prothrombin molecule can be represented by two ellipsoids and that the effect of Ca^{2+} binding can be represented by a change in the angle between their major axes.

The last zymogen activation step in the blood coagulation "cascade" is the conversion of prothrombin into thrombin via the cleavage of two peptide bonds in prothrombin by factor X_a (Davie & Fujikawa, 1975; Mann, 1976; Suttie & Jackson, 1977). Thrombin then catalyzes the limited proteolysis of soluble fibrinogen which yields insoluble fibrin. Prothrombin as well as other vitamin K dependent plasma proteins binds Ca^{2+} ions; in the presence of Ca^{2+} ions each of these proteins forms complexes with phospholipid surfaces (Suttie & Jackson, 1977; Nelsestuen et al., 1978; Nelsestuen, 1978). Prothrombin seems to bind Ca^{2+} ions via its γ-carboxyglutamic acid residues; they are formed by posttranslational vitamin K dependent carboxylation of the ten glutamic acid residues situated in the amino-terminal end of the molecule (Stenflo & Suttie, 1977; Olson & Suttie, 1977). When prothrombin is cleaved by thrombin, an amino-terminal fragment of 22 000 is formed which is denoted fragment 1 (Mann, 1976; Suttie & Jackson, 1977). This fragment, which contains the Ca^{2+} binding residues, has recently been crystallized (Aschaffenburg et al., 1977). There are several pieces of indirect evidence which indicate that both fragment 1 and prothrombin undergo a conformational change when Ca^{2+} ions are bound (Suttie & Jackson, 1977; Bloom & Mann, 1978; Björck & Stenflo, 1973).

The aim of this work has been to study the interaction of Ca^{2+} ions with prothrombin by analyzing the size and shape of both prothrombin and Ca^{2+}–prothrombin by using the low-angle X-ray scattering method. The results indicate that the prothrombin molecule can be described by one large and one small ellipsoid. When Ca^{2+} ions are bound to prothrombin, the change in the X-ray scattering curve can be explained if it is assumed that the smaller ellipsoid moves so that the molecule attains a more extended conformation.

Materials and Methods

Prothrombin was prepared and checked for dimerization as described in a previous communication (Stenflo, 1976). The concentration of prothrombin was calculated from the absorbance at 280 nm by using the extinction coefficient $E_{1cm}^{1\%} = 14.5$ (Stenflo, 1972).

The X-ray small-angle scattering data were recorded with a camera developed by Kratky & Skala (1958). The entrance slit width of the camera was 0.10 mm and the counter slit width 0.25 mm. The scattering angle was set by an on-line Hewlett-Packard computer, 2100S, which also received and recorded the intensity data (B. G. Wingren, B. Sjöberg, and R. Österberg, unpublished experiments). Monochromatization was achieved with a nickel β filter and a pulse height discriminator in conjunction with a proportional counter. As indicated by both gel electrophoresis and low-angle X-ray scattering of some prothrombin samples, prothrombin behaved as a native protein after the low-angle X-ray scattering curve was recorded. Also, recording of a second X-ray scattering curve with the same sample was in good agreement with the first one, indicating that no major changes occurred during X-ray exposure.

All measurements were made at 21 °C. The sample container was a thin-walled Mark capillary with a diameter of 0.85 mm, and the distance between the sample and the plane of registration was 20.5 cm. The absolute scattered intensities

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