Tryptic Digestion of Rabbit Skeletal Myofibrils: An Enzymatic Probe of Myosin Cross-Bridges†

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ABSTRACT: Tryptic digestion of rabbit skeletal myofibrils under physiological ionic strength and pH conditions was used as a probe of cross-bridge interaction with actin in the presence of nucleotides and pyrophosphate. Under rigor conditions, digestion of myofibrils at 24 °C results in the formation of 25K, 110K [heavy meromyosin (HMM)], and light meromyosin (LMM) fragments as the main reaction products. Very little if any 50K peptide is generated in such digestions. In the presence of magnesium pyrophosphate, magnesium 5′-adenylyl imidodiphosphate (MgAMPPNP), and MgATP, the main cleavage proceeds at two positions, 25K and 75K from the N-terminal portion of myosin, yielding the 25K, 50K, and 150K species. The relative amounts of the 50K, 110K, and 150K peptides and the rates of myosin heavy-chain digestion in the presence of pyrophosphate and AMPPNP indicate partial dissociation of myosin from actin. Direct centrifugation measurements of the binding of HMM and subfragment 1 (S-1) to actin in myofibrils confirm that cross-bridges partition between attached and detached states in the presence of these ligands. In the presence of MgADP, HMM and S-1 remain attached to actin at 24 °C. However, tryptic digestion of myofibrils containing MgADP is consistent with the existence of a mixed population of attached and detached cross-bridges, suggesting that only one head on each myosin molecule is attached to actin. As shown by tryptic digestion of myofibrils and the measurements of HMM and S-1 binding to actin, nucleotide- and pyrophosphate-induced dissociation of cross-bridges is more pronounced at 4 than at 24 °C. We also confirm that temperature has a marked effect on the proteolytic susceptibility of the myosin hinge in myofibrils and find that this hinge is cleaved at a slower rate in actomyosin than in myofibrils.

It is generally assumed that the contractile process in a muscle cell involves some changes in the structure and the angle of attachment of myosin cross-bridges to actin (Huxley, 1969; Huxley & Simmons, 1971; Huxley et al., 1981, 1983). Taken together, the biochemical and structural studies have described two states of cross-bridges. In the rigor orientation, each cross-bridge assumes an angle of about 45° with the actin filament (Reedy et al., 1965; Reedy, 1967; Borejdo & Putnam, 1977), and in the relaxed state, if indeed there is a unique orientation of detached cross-bridges, this angle is about 90° (Reedy et al., 1965, 1983; Dos Remedios et al., 1972; Nihei et al., 1974; Goody & Holmes, 1983). The search for additional states of bound cross-bridges, states which would be pertinent to the swinging cross-bridge model for muscle contraction (Huxley, 1969; Huxley & Simmons, 1971), has yet to be successfully completed. Much attention has been focused on the state of cross-bridges in the presence of magnesium 5′-adenylyl imidodiphosphate (MgAMPPNP)[1] in the hope that this nucleotide analogue might shed light on states that exist only transiently in the presence of MgATP. Indeed, mechanical studies, X-ray diffraction, electron microscopy, and a variety of spectroscopic techniques provide convincing evidence for AMPPNP-induced changes in the orientation of cross-bridges. Interpretations differ as to the nature of the AMPPNP state, i.e., whether it represents a mixture of rigor and relaxed orientations (Thomas & Cooke, 1980; Dos Remedios et al., 1972), a new loosely attached state (Goody et al., 1976; Marston et al., 1979; Yanagida, 1981), or perhaps cross-bridges with only one head attached to actin (Reedy et al., 1983).

Equally intriguing is the state of myosin cross-bridges in the presence of MgADP. According to stiffness measurements, they remain attached to actin, forming a ternary actomyosin–ADP complex (Marston, 1973; Marston et al., 1979; Rodger & Tregear, 1973). Yet spectroscopic studies suggest the existence of two populations of cross-bridges with different orientations with respect to actin (Yanagida, 1981). An alternative view is that MgADP imposes a local deformation on the cross-bridge, thereby changing the spatial attitude of the dye-containing domain on the myosin head (Borejdo et al., 1982).

Most recently, a new experimental tool for probing the state of cross-bridges has been made available. Limited tryptic proteolysis of myosin subfragment 1 leads to the formation of three stable peptides (25K, 50K, and 20K), which remain associated under nondenaturing conditions (Yamamoto & Sekine, 1979; Balint et al., 1978; Mornet et al., 1979). Most importantly, the 50K/20K cut is virtually blocked upon binding of myosin to actin (Mornet et al., 1979; Yamamoto & Sekine, 1979). Lovell & Harrington (1981) used this finding in order to determine the fraction of myosin heads attached to actin under rigor conditions in rabbit skeletal and flight muscles (Lovell et al., 1981). They have demonstrated that quantitation of the amount of the 50K peptide formed by digestion of myofibrils provides a reliable measure of the fraction of attached (in rigor) and detached cross-bridges.

In this work, we extend the application of the proteolytic technique to the investigation of other states of cross-bridges, in particular in the presence of MgAMPPNP and MgADP. We show that although the proteolytic reactions do not yield any information on the spatial orientation of cross-bridges, they resolve between the bound (rigor and nonrigor type) and de-

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[1] Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2; BSA, bovine serum albumin; AMPPNP, 5′-adenylyl imidodiphosphate; AP_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; LC-2, Ca^{2+}-binding 19 000 molecular weight subunit of myosin; PMSF, phenylmethanesulfonyl fluoride.

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tached states of myosin and sense structural changes in their heads. In conjunction with direct measurements of HMM and S-1 binding to actin in myofibrils, the tryptic digestion reactions indicate partitioning of cross-bridges between attached and detached states in the presence of $MgPP_i$ and MgAMPPNP at 4 and 24 °C. In the presence of MgADP at 24 °C, the partitioning between the two states of cross-bridges may occur between the two heads on each myosin molecule. We conclude that the proteolytic techniques supplement the structural and spectroscopic studies on cross-bridges and facilitate their interpretation. A preliminary account of this work has been presented (Chen & Reisler, 1983).

Materials and Methods

Materials. Trypsin, soybean trypsin inhibitor, α-chymotrypsin, catalase, and dithiothreitol as well as the nucleotides ATP, ADP, and AMPPNP were products of Sigma Chemical Co. (St. Louis, MO). AP_5A was purchased from Boehringer Mannheim (Indianapolis, IN). All reagents used were analytical grade.

Myofibrils were prepared from rabbit psoas muscle according to the procedure of Rome (1967). Approximately 20 g of muscle was shredded and suspended in 200 mL of buffer containing 20 mM KCl, 10 mM Bis-Tris, and 4 mM EDTA (pH 6.85). The suspension was homogenized in a Waring blender for 5 s at low speed and then at high speed for 2 × 30 s. The homogenate was centrifuged in a Sorvall RC-5 centrifuge at 5000 rpm for 15 min. The pellet was washed 3 times in 10× volume of the above buffer, by centrifugation. After the washing, the myofibrils were suspended in the same buffer, made 50% in glycerol, and stored at −20 °C.

Myosin was prepared from rabbit psoas muscle as previously described (Godfrey & Harrington, 1970). Subfragment 1 was prepared by chymotryptic digestion of myosin according to Weeds & Pope (1977). Actin was prepared by the method of Spudich & Watt (1971).

Digestion of Myofibrils with Trypsin. Myofibrils stored in 50% glycerol were washed 4 times in 5× volume of rigor buffer containing 0.1 M NaCl, 20 mM imidazole, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT at pH 7.0 by centrifuging at 3000 rpm for 5 min in a Sorvall RC-5 centrifuge. The protein concentration of a myofibrillar suspension was determined spectrophotometrically in 5% (w/v) SDS by assuming $E^{1\%}_{280nm} = 7.0$ (Sutoh & Harrington, 1977). The myofibrils were adjusted to a concentration of 4 mg/mL and digested with trypsin (0.1 mg/mL) in rigor buffer at 4 and 24 °C, in the presence or absence of nucleotides. Digestions carried out in the presence of MgADP also included up to 200 μM AP_5A, as well as glucose and hexokinase. At given time intervals, aliquots were removed from the digestion mixture, and proteolysis was terminated by addition of soybean trypsin inhibitor to 0.3 mg/mL. Catalase was added to each aliquot as an internal standard for quantitation of protein bands. The samples were then denatured and subjected to SDS–polyacrylamide gel electrophoresis.

Tryptic Digestion of Actomyosin and Acto-S-1. Myosin (3 mg/mL) and S-1 (1 mg/mL) were combined with actin (2.5 mg/mL) in rigor buffer. The mixtures were incubated at 24 °C for 30 min to form the appropriate complexes. Subsequent digestions were carried out at a trypsin concentration of 0.015 mg/mL, at 4 and 24 °C, in the presence and absence of nucleotides. At indicated time intervals, aliquots were withdrawn from the reaction, and the digestion was terminated by addition of soybean trypsin inhibitor to 0.045 mg/mL.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970). In most cases, a two-phase resolving gel of 10% (upper, w/v) and 15% (lower, w/v) acrylamide was employed.

Densitometric Measurement. The optical densities of protein bands and the appropriate mass distributions were determined with a Helena Quick Scan R&D gel scanner equipped with an integrator. To account for experimental variations, the intensities of all protein bands in a given lane were first normalized to the intensity of the catalase band in the same lane. The differences in dye adsorption were corrected by dividing the normalized intensities by the molecular weights of the respective protein bands. In order to examine the time course of production of all the fragments under different reaction conditions, we quantitated their formation relative to the extent of heavy-chain digestion. In other words, the normalized amount of each fragment formed at a given time was divided by the theoretical maximum amount of this fragment that could be produced from the cleaved fraction of the heavy chain.

Identification of the Digestion Products. The main products of tryptic digestion of myosin in myofibrils were identified on the basis of previous assignments of Lovell & Harrington (1981) according to comigration of protein bands with the products of tryptic digestion of S-1 (75K, 70K, 50K, 26K, and 20K bands) and by their solubility in the rigor solvent (150K, 130K, and 110K bands). The solubility experiments were carried out as described by Lovell & Harrington (1981). Following proteolysis, the myofibrils were centrifuged (30000 rpm for 2 h, at 4 °C) in the rigor solvent and in the presence of 1 mM $MgPP_i$ which dissociated actin from myosin. The soluble products (HMM) remained in the supernatant whereas myosin, rod, and LMM were concentrated in the pellet. The weights of the proteolytic fragments were measured against the following standards: myosin (225K); HMM (130K); myosin rod (120K); S-1 (95K); catalase (55K); actin (42K); LC-2 light chain (19K) (Weeds & Pope, 1977).

Binding Experiments. The binding of HMM and S-1 to actin was measured in myofibrils under conditions employed in the proteolytic digestion experiments. Prior to the binding measurements, the myofibrils were adjusted to pH 8.0 in 0.1 M NaCl and 20 mM Tris-HCl and were digested with α-chymotrypsin (0.10 mg/mL) for 30 min at room temperature. The reaction was terminated with PMSF. Between 80% and 90% of myosin was converted to HMM and S-1. Throughout this digestion, the generated fragments remained bound to actin.

To determine the dissociating effect of nucleotides and PP_i on HMM and S-1, the predigested myofibrils were equilibrated with the rigor solvent at either 4 or 24 °C. Immediately before their centrifugation (for 1 h at 40000 rpm at either 4 or 24 °C), the myofibrils were made up to one of the following: 1 mM $MgPP_i$, 5 mM MgATP, 1 mM MgAMPPNP, and 1 mM MgADP (+AP_5A). After the centrifugation, the pellet was resuspended in the original volume of the solvent, and both supernatant and pellet were examined on SDS gels. The bands corresponding to HMM and S-1 were quantitated, and the partitioning of these fragments between the pellet and supernatant, i.e., between the bound and dissociated states, was determined.

Results

The rate of tryptic digestion of the myosin heavy chain (225K) in myofibrils and the type of products generated depend on the particular reaction conditions, the presence of

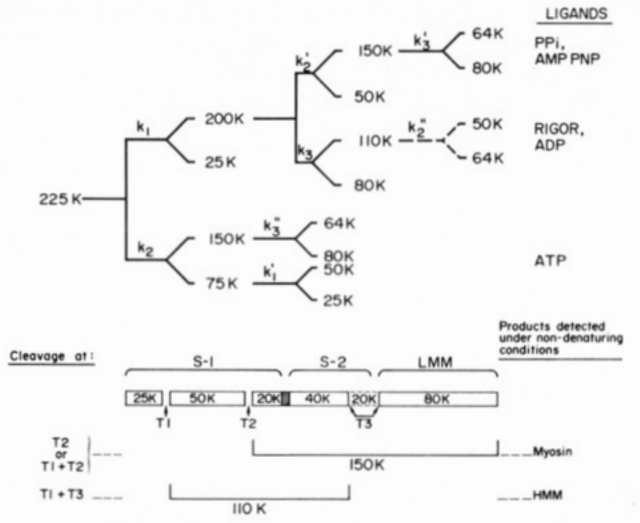

FIGURE 1: Scheme for tryptic digestion of the myosin heavy chain in myofibrils at 24 °C. The degradation of the 110K peptide into 50K and 64K products (at rate k_2'', dashed route) is slow under rigor conditions. At 24 °C, except in the presence of MgATP, $k_1 > k_2$, and the flow proceeds along the upper branches of the scheme. The lower pathway is utilized to a greater extent in digestions carried out at 4 °C. At this temperature, the formation of the 110K peptide is greatly suppressed. The different digestion products are aligned along the myosin molecule in the lower part of this figure.

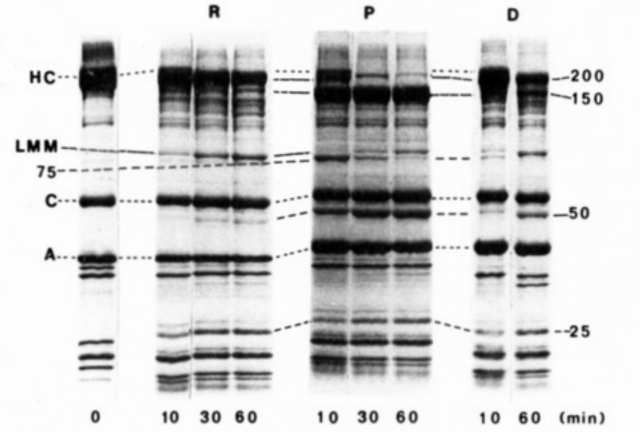

FIGURE 2: Representative SDS–polyacrylamide gels showing tryptic digestion (1:40 w/w) of myofibrils (4 mg/mL) at 4 °C. The digestions were carried out under rigor conditions (R) and in 0.1 M NaCl, 20 mM imidazole, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT (pH 7.0) in the presence of 1 mM $MgPP_i$ (P) and 1 mM MgADP (D). Digestion times (in minutes) are indicated under each lane. The molecular size of tryptic fragments is given in kilodaltons on both sides of the gel. HC refers to the myosin heavy chain (225K), C corresponds to catalase, and A is actin.

nucleotides, the ionic strength, and the temperature. The cleavage scheme shown in Figure 1 refers to tryptic digestions at 24 °C but can be consulted for clarification and identification of digestion products in reactions carried out at 4 °C.

Tryptic Digestion of Myofibrils at 4 °C. (A) Rigor. As shown by Lovell and Harrington (1981), the fragmentation of myosin in rigor myofibrils is rather slow and leads mainly to the formation of the 25K and 200K fragments (Figure 2). Subsequent slow degradation of the 200K heavy chain results in the formation of small amounts of LMM (~80K) and HMM (130K and 140K bands). Most importantly, the production of the 50K peptide and the transient 75K species (25K + 50K) is practically blocked under rigor conditions [Figure 2; see also Lovell & Harrington (1981)]. Any traces of the 50K peptide obtained in such digestion reactions can be attributed to myosin heads extending beyond the overlap region.

Table I: Fraction of the Myosin Heavy Chain Cleaved into 150K, 110K, and 50K Fragments during Tryptic Digestion of Myofibrils at 4 °C[a]

ligand present	concn (mM)	myosin degradation, $t_{1/2}$ (min)[b]	150K	110K	50K
none (rigor)		40	[c]	0.10–0.20	
MgADP	1	10	0.30	0.10	0.20–0.30
$MgPP_i$	1	2.5	1.00		0.90–1.00
MgAMPPNP	1	2.5	0.80		0.80
MgATP	5	0.5	1.00		1.00

[a] The fraction of each product generated in myosin digestions was calculated relative to the amount of degraded myosin. For each time point of tryptic digestion of myofibrils, we determined the amount of heavy chain cleaved at that time and calculated the maximum theoretical amount of each fragment that could be obtained from the digested portion of myosin. The fractions reported here represent the ratios between the actual amounts of generated fragments and such theoretical values. They showed little variation with the time of digestion (between 5 and 60 min) in spite of increasing cleavage of myosin and were reproducible within 10% (±0.1) in three separate experiments. For details of digestion reactions, see Materials and Methods. [b] $t_{1/2}$ for myosin degradation corresponds to an interpolated digestion time at which 50% of the myosin heavy chain (225K + 200K species) is cleaved into smaller fragments. The digestion of the heavy chain was monitored as shown in Figure 6B. [c] Under rigor conditions, the HMM fragments (130K and 140K) amount to up to 20% of cleaved myosin.

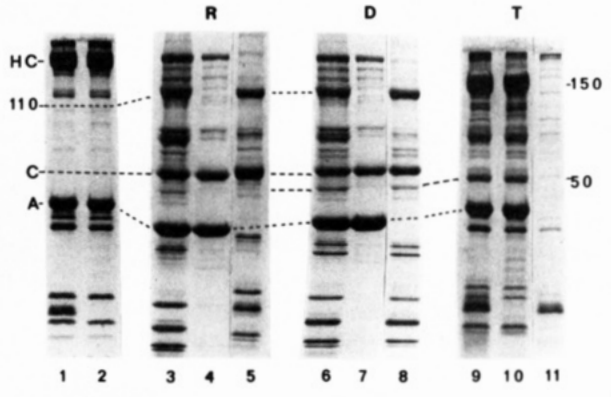

FIGURE 3: Identification of the 110K and 150K products of tryptic digestion of myofibrils. Representative SDS–polyacrylamide gels show the tryptic fragments of myofibrils after their centrifugation in the presence of MgATP (for details, see Materials and Methods). (1) Undigested myofibrils; (2) pellet obtained from centrifugation of (1); (3) myofibrils digested under rigor conditions for 30 min at 24 °C; (4) pellet and (5) supernatant obtained after centrifugation of (3); (6) myofibrils digested for 30 min at 24 °C in the presence of 1 mM MgADP; (7) pellet and (8) supernatant of (6); (9) myofibrils digested for 5 min at 24 °C in the presence of 5 mM MgATP; (10) pellet and (11) supernatant of (9).

(B) $MgPP_i$ and MgAMPPNP. Addition of 1 mM $MgPP_i$ to the rigor system results in striking changes in the pattern and rate of digestion of myofibrils (Lovell & Harrington, 1981). The rate of myosin cleavage is much greater than in rigor (Figure 2, Table I), with respective half-times ($t_{1/2}$) for heavy-chain degradation of $t_{1/2}(PP_i)$ = 4 min and $t_{1/2}$(rigor) = 40 min (Table I). The major proteolytic product, the 150K band [Figure 2; corresponds to the 160K band in Lovell & Harrington (1981)], is generated by a tryptic cut at the 50K/20K junction on the myosin head. The assignment of the 150K band to the rod portion of myosin is based on the presence of this peptide (as well as the 50K and 25K fragments) in the pelleted myofibrils after their centrifugation in

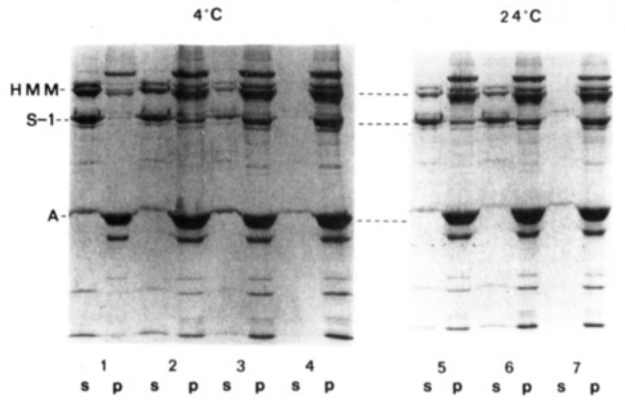

FIGURE 4: Binding of HMM and S-1 to actin in myofibrils at 4 and 24 °C. Myofibrils were digested with α-chymotrypsin to convert their myosin content into HMM and S-1. Subsequently, they were centrifuged at both temperatures under rigor conditions (lanes 4s and 4p) and in the presence of 1 mM $MgPP_i$ (lanes 1s and 1p and 5s and 5p), 1 mM MgAMPPNP (lanes 2s and 2p and 6s and 6p), and 1 mM MgADP (lanes 3s and 3p and 7s and 7p). The supernatants (s) and pellets (p) of the centrifuged myofibrils were run on SDS–polyacrylamide gels (lanes 1–7), and the respective amounts of HMM and S-1 in each phase (s and p) were quantitated. Fragments found in the pellet (p) correspond to bound HMM and S-1; those found in the supernatant (s) represent the dissociated fraction of myosin fragments.

the presence of 1 mM $MgPP_i$ (Figure 3; Lovell & Harrington, 1981). The second product, the 75K peptide, exists only transiently and is converted into the stable 50K and 25K peptides. The amount of 50K generated (or 50K + 75K) corresponds to the total amount of digested heavy chain (Table I), indicating that all the myosin heads are cut at the 50K/20K junction in the presence of 1 mM $MgPP_i$. Thus, according to the proteolytic probe, 1 mM $MgPP_i$ dissociates the cross-bridges from actin at 4 °C.

By the criteria of proteolytic digestion, the state of cross-bridges in the presence of MgAMPPNP appears to be similar to that induced by $MgPP_i$. The rates of heavy-chain digestion in the presence of AMPPNP and PP_i are very close (Table I), and the accumulating products (150K, 50K, and 25K bands) are identical, albeit the recovery of the 50K material is less complete (80%) with the nucleotide analogue than in the presence of ATP or PP_i.

(*C*) *MgATP.* Essentially the same digestion pattern and products are observed during tryptic attack on myofibrils in the presence of 5 mM MgATP (+EGTA) at 4 °C. However, in line with the fact that MgATP dissociates the actomyosin complex better than $MgPP_i$ and MgAMPPNP, the rate of heavy-chain cleavage into 150K and 75K peptides is faster in the presence of ATP (Table I). The accumulation of the 50K peptide accounts for the complete recovery of this fragment from the original heavy chain (Table I).

(*D*) *MgADP.* In reactions carried out in the presence of 1 mM MgADP (and the kinase inhibitor AP_5A), only a fraction (20–30%) of the digested heavy chain yields the 50K peptide. This and the relatively slow degradation of the myosin heavy chain are characteristic traits of the rigor cleavage system. Yet, the very presence of the 50K peptide along with the 150K fragment is suggestive of partial dissociation of cross-bridges from actin in the presence of MgADP.

Dissociation of Cross-Bridges from Actin at 4 °C. In view of the observed effects of nucleotides on tryptic digestion of myosin heads in myofibrils, we have searched for an independent estimate of actomyosin binding in our system. The adopted procedure involves chymotryptic digestion of myofibrils at pH 8.0 (in rigor) to cleave a large fraction of myosin into HMM and S-1 (Figure 4). When the digested myofibrils are then centrifuged at 4 °C, under rigor conditions, all the HMM and S-1 is concentrated in the pellet. Thus, as expected, in the absence of nucleotides and PP_i all the myosin heads are bound to actin.

When the same centrifugation of myofibrils is carried out in the presence of 5 mM MgATP, almost all the S-1 and HMM remain in the supernatant, indicating nearly complete dissociation of myosin heads from actin. Centrifugations done in the presence of 1 mM $MgPP_i$ also lead to substantial recovery of S-1 and HMM in the supernatant (Figure 4 and Table II). In the presence of MgAMPPNP, the dissociation of cross-bridges from actin is less effective than that by PP_i or ATP; S-1 and HMM can now be found in both the supernatant and the pelleted myofibrils (Table II). MgADP has the least effect on actomyosin at 4 °C. Only a small fraction of HMM and S-1 is dissociated from actin by MgADP (Figure 4). In all cases, the ratio of the dissociated to the bound protein is between 3- and 4-fold greater for S-1 than for HMM (Table II).

Tryptic Digestion of Myofibrils and Synthetic Actomyosin at 24 °C. (*A*) *Rigor.* Tryptic digestion of the myosin heavy chain in myofibrils at 24 °C is significantly different from that observed at 4 °C. The major products of proteolysis at 24 °C are the 110K band, which accounts for 70–75% of the digested myosin (Figures 3 and 5, Table III), the 25K peptide, and the LMM fragments. In contrast to this, very little 110K peptide is generated during the digestion of rigor myofibrils at 4 °C (Figure 2). When the digested (at 24 °C) myofibrils are

Table II: Effect of Ligands on the Binding of HMM and S-1 to Actin in Myofibrils at 4 and 24 °C[a]

ligand present	concn (mM)	fragment	4 °C F_{dis}	4 °C F_{assoc}	24 °C F_{dis}	24 °C F_{assoc}
none (rigor)		HMM	0	1.00	0	1.00
		S-1	0	1.00	0	1.00
MgADP	1	HMM	0.15	0.85	0	1.00
		S-1	0.30	0.70	0	1.00
MgAMPPNP	1	HMM	0.30	0.70	0.20	0.80
		S-1	0.65	0.35	0.40	0.60
$MgPP_i$	1	HMM	0.70	0.30	0.30	0.70
		S-1	0.90	0.10	0.60	0.40

[a] The binding of HMM and S-1 to actin was measured in chymotryptically predigested myofibrils, in which most of their myosin content was cleaved into HMM and S-1. The binding of HMM and S-1 to actin was determined by centrifuging myofibrils in the presence of the appropriate ligand at either 4 or 24 °C. The dissociated fragments remained in the supernatant whereas the bound fragments were collected in the pellet. Their respective amounts were quantitated by SDS gel electrophoresis of the pellet and supernatant proteins. F_{dis} and F_{assoc} are the fractions of dissociated and associated fragments, respectively.

Table III: Fraction of the Myosin Heavy Chain Cleaved into 150K, 110K, and 50K Fragments during Tryptic Digestion of Myofibrils at 24 °C[a]

ligand present	concn (mM)	150K	110K	50K
none (rigor)			0.70–0.75	0–0.05
MgADP	1	0.20–0.30	0.40–0.50	0.40–0.50
$MgPP_i$	1	0.60–0.70	0.20–0.25	0.70–0.80
MgAMPPNP	1	0.70–0.80	0.20–0.30	0.70–0.80
MgATP	5	0.90–1.00		0.90–1.00

[a] The fractions of the different fragments were calculated as described in Table I. The tabulated range of results corresponds to their reproducibility in three separate experiments. For details of digestion reactions, see Materials and Methods.

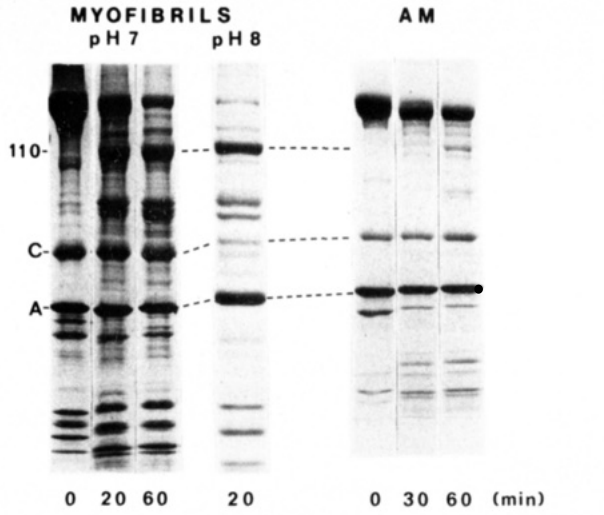

FIGURE 5: Representative SDS–polyacrylamide gels showing tryptic digestion (1:40 w/w) of myofibrils (4 mg/mL) under rigor conditions (as in Figure 2) at pH 7.0 and 8.0 at 24 °C. The comparable digestions of actomyosin (AM) were carried out in the same rigor solvent, at pH 7.0 and 24 °C. Digestion times in minutes are indicated under each lane. C refers to catalase and A to actin.

centrifuged in order to separate the soluble products, all the 110K and 25K material remains in the supernatant (Figure 3). Since the supernatant protein displays normal Ca^{2+} and K^+(EDTA) stimulated ATPase activities, we conclude that the 110K product represents HMM cleaved at the 25K/50K junction of the head (Figure 1). Thus, under rigor conditions and at 24 °C, trypsin efficiently cleaves the HMM/LMM junction (Figure 1). In agreement with previous studies utilizing α-chymotrypsin (Ueno & Harrington, 1981; Reisler & Liu, 1982; Reisler et al., 1983), the cleavage of the HMM/LMM hinge is faster at pH 8.0 than at pH 7.0 (Figure 5). The proteolytic susceptibility of this junction is much smaller in the synthetic actomyosin system, in which at most 20% myosin yields the 110K product (Figure 5). However, the myofibril and actomyosin systems are similar in that both produce only trace amounts of the 50K peptide (0–5%) under rigor digestion conditions.

(*B*) *$MgPP_i$, MgAMPPNP, and MgATP.* Examination of the gel patterns shown in Figure 6 suggests that $MgPP_i$ (between 1 and 5 mM) does not induce complete dissociation of cross-bridges from actin at 24 °C. Only 60–70% of digested myosin is converted into the 150K peptide (Table III), which is normally obtained under relaxing conditions. The formation of the 110K band (20–25% of digested myosin, Figure 6 and Table III) points to the existence of rigor-type bonds in the presence of $MgPP_i$. In line with this argument, only 70–80% of the myosin heavy chains yields the 50K peptide (Table III), as opposed to practically total recovery of this fragment at 4 °C digestions in the presence of $MgPP_i$ (Table I). The rates of myosin digestion in rigor and in the presence of $MgPP_i$, MgADP, and MgAMPPNP are closer to each other at 24 °C than at 4 °C.

The effects of AMPPNP and PP_i on the proteolysis of myofibrils at 24 °C are strikingly similar. These two systems resemble each other in the overall rate and pattern of myosin digestion and in the accumulation of the 50K, 110K, and 150K products (Figure 6 and Table III). A clearly different situation arises in the presence of 5 mM MgATP. The rate of cleavage of the myosin heavy chain is faster in the presence of ATP than with AMPPNP (Figure 6), and the 110K peptide which is formed when the analogue is bound to actin (Table III and Figure 6) cannot be detected in reactions containing ATP. The

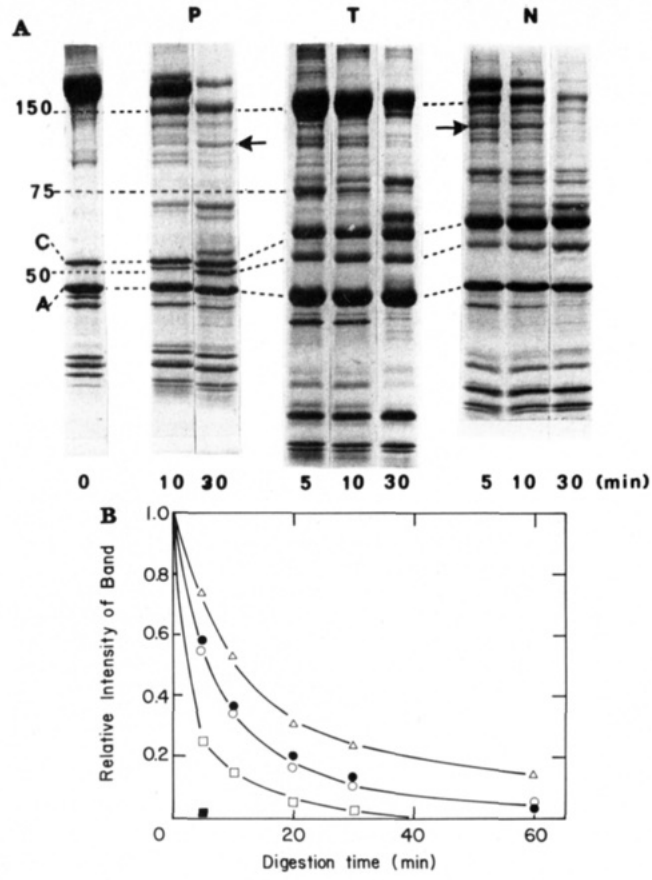

FIGURE 6: (A) Representative SDS–polyacrylamide gels showing tryptic digestion of myofibrils at 24 °C in the presence of 1 mM $MgPP_i$ (P), 5 mM MgATP (T), and 1 mM MgAMPPNP (N). Other conditions of digestion are the same as those in Figure 2. Digestion times in minutes are indicated under each lane. The 110K band is indicated by an arrow, and the size of other tryptic products is given in kilodaltons on the left side of the gel. C refers to catalase and A to actin. (B) Time course of tryptic digestion of the myosin heavy chain (the combined area under the 225K and 200K bands) in myofibrils at 24 °C. (△) Reaction under rigor conditions; (○) in the presence of 1 mM MgADP; (●) 1 mM $MgPP_i$ added; (□) in the presence of 1 mM MgAMPPNP; (■) with 5 mM MgATP. Other reaction conditions are the same as those in Figure 2.

total recovery of the 50K peptide (90–100% of degraded myosin) and the appearance of the 75K intermediate are representative of a relaxed system. The formation of this last intermediate indicates efficient dissociation of myosin from actin (Mornet et al., 1979; Yamamoto & Sekine, 1979; Muhlrad & Hozumi, 1982). The 75K band can be easily detected in digestions of relaxed myofibrils, i.e., in the presence of MgATP at 4 and 24 °C and with $MgPP_i$ at 4 °C. However, very little if any 75K peptide is formed upon cleavage at 24 °C of myofibrils containing either MgAMPPNP or $MgPP_i$ (Figure 6).

(*C*) *MgADP.* The most prominent products of tryptic digestion of myofibrils in the presence of between 1 and 3 mM MgADP are the 110K and LMM peptides. About 50% of the digested myosin yields the 110K fragment (Figure 7 and Table III) which we identify as cleaved HMM particles. However, in contrast to rigor conditions where we do not find any 50K product, the reaction in the presence of MgADP (and up to 200 μM AP_5A, glucose, and hexokinase) generates substantial amounts of the 50K peptide, up to 50% of degraded heavy chain (Figure 7 and Table III). The formation of the 50K peptide does not proceed via the 75K intermediate, whose presence is indicative of relaxing conditions. In synthetic acto-S-1 and actomyosin systems, the binding of MgADP to

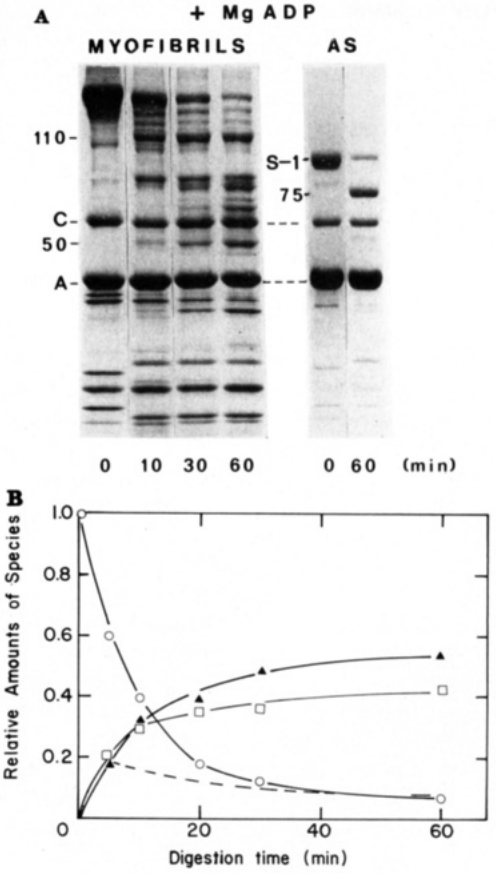

FIGURE 7: (A) Tryptic digestion of myofibrils and acto-S-1 (AS) in the presence of 1 mM MgADP at 24 °C. Digestion times in minutes are indicated under each lane. Other reaction conditions are the same as those in Figure 2. (b) Relative amounts of products formed during tryptic digestion of myofibrils in the presence of 1 mM MgADP at 24 °C (Figure 7A). (○) Heavy chain of myosin (225K + 200K bands); (▲) 50K peptide; (□) 110K peptide; the broken curve corresponds to the 150K product. The amounts of each fragment were calculated as shown in Table I.

the rigor complexes does not have any significant effect on the limited proteolysis of the myosin head and does not lead to the production of the 50K peptide [Figure 4; also see Muhlrad & Hozumi (1982)].

Dissociation of Cross-Bridges from Actin at 24 °C. The binding of cross-bridges to actin in the presence of ligands was estimated as described for myofibrils maintained at 4 °C. When predigested myofibrils containing HMM and S-1 were centrifuged under rigor conditions at 24 °C, all the fragments were concentrated in the pellet (Figure 4). Addition of 1 mM MgADP had no effect on this result, suggesting that the cross-bridges remain attached to actin in the presence of this nucleotide (Figure 4). Similar centrifugations of digested myofibrils with either $MgPP_i$ or MgAMPPNP resulted in the partitioning of HMM and S-1 between the pellet and supernatant phases (Figure 4). Judging by the relative amounts of HMM and S-1 in these two phases (Table II), PP_i and AMPPNP induce only partial dissociation of cross-bridges from actin. MgATP has a more dramatic effect on actomyosin and leads to its dissociation at 24 °C. As in previous separations at 4 °C, the amount of dissociated S-1 is greater than that of HMM (Table II).

Discussion

The extension of proteolytic digestion methods to the study of myosin cross-bridges in the presence of nucleotides required additional and independent information on the binding of myosin to actin under the employed digestion conditions. The chymotryptically digested myofibrils, which contain the effective actin concentration of myofilament lattice and S-1 and HMM in full correspondence to the original myosin content, are most appropriate for such binding measurements. The bound S-1 and HMM are rapidly separated from the dissociated fragments by the fast sedimentation of myofibrils. The presence of both myosin fragments provides for competitive binding conditions and sheds light on their relative affinities for actin. In general, the results of our binding experiments agree well with the studies of Greene (1981) and Greene & Eisenberg (1980). In all cases, the dissociation of S-1 from actin by nucleotides and PP_i is greater than that of HMM, and the dissociation of both fragments is more efficient at 4 °C than at 24 °C. The results of these experiments verify the presence of a mixed population of attached and detached cross-bridges in intact myofibrils in the presence of nucleotides, their exact distribution cannot be determined. The quantitative extrapolation from the HMM system is limited by at least two factors. First, the HMM binding method is not sensitive to dissociation of only one of the two heads, and second, it does not account for the different steric constraints of myosin.

Myofibrils at 4 °C. The "pseudorelaxed" state of myofibrils induced by 1 mM $MgPP_i$ at 4 °C and the quantitative formation of the 50K peptide during their tryptic digestion [Table I; also see Lovell & Harrington (1981)] are consistent with the binding information. Since under such conditions the majority of HMM (about 70%) is dissociated from actin, and some of it must be bound through one head only, the efficient cleavage of the 50K/20K link in myosin is not surprising. The rate of this cleavage is slower than in the presence of MgATP, presumably due to the different rates of actomyosin dissociation by ATP and PP_i (Goody & Holmes, 1983). In this work, we have made no attempt to optimize the digestion reactions in order to better monitor the different rates of actomyosin dissociation.

The digestion of myofibrils in the presence of MgADP at 4 °C bears greatest resemblance to reactions carried out under rigor conditions. The slow cleavage of the myosin heavy chain is consistent with the slow dissociation rate of myosin heads from actin (Greene et al., 1982; Goody & Holmes, 1983). The amount of 50K peptide, which we detect in such digestion reactions, is compatible with the amount of dissociated HMM (about 15%), in particular since no correction is made for myosin attached to actin through one head.

The cleavage of the myosin heavy chain, and the formation of the 50K peptide in the presence of MgAMPPNP, can be correlated with the observed dissociation of HMM (about 30%) and the likely presence in myofibrils of cross-bridges attached to actin via one head (Greene, 1981). Thus, although our data do not exclude the possibility that the weak binding states of myosin–AMPPNP have a unique orientation of cross-bridges, the digestion experiments do not call for such a hypothesis. In fact, the presented results are consistent with the views of Thomas & Cooke (1980) and Borejdo et al. (1982), according to whom the cross-bridges partition in the presence of PP_i and AMPPNP between the rigor and relaxed conformations. It is not clear whether the evidence against cross-bridge dissociation by MgAMPPNP obtained from stiffness measurements on flight muscle (Marston et al., 1979; Marston et al., 1976) is directly applicable to rabbit muscle.

Myofibrils at 24 °C. The dissociating effects of MgAMPPNP and $MgPP_i$ on the actomyosin complex are about equal at 24 °C [Table II; see also Greene & Eisenberg (1980)]. In these two cases, we find between 20 and 30%

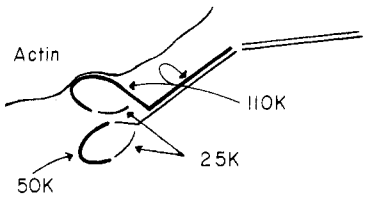

FIGURE 8: Expected tryptic cleavage of myosin if only one of its two heads is bound to actin. Such hypothetical formation of the 110K, 50K, and 25K peptides corresponds to the observed digestion pattern of myofibrils in the presence of MgADP at 24 °C.

dissociated HMM (and 40–60% dissociated S-1) under the ionic conditions (0.1 M NaCl) employed in this work. The main difference, when compared to the actomyosin complex at 4 °C, is in the properties of the myosin–PP_i complex, which has a significantly lower affinity for actin at cold temperatures.

The tryptic digestions of myofibrils in the presence of AMPPNP and PP_i proceed in a similar manner and yield the same products in similar amounts, albeit at somewhat different rates. In both cases, we note the formation of the 110K peptide which detects the presence of rigor bonds at room temperature. However, neither the amount of the 110K peptide nor the amounts of the 150K and 50K products can be taken as accurate measurements of the respective fractions of rigor and relaxed states. Bearing in mind that the lifetimes of attached states (in the presence of nucleotides) are short compared to the digestion times, the proteolysis cumulatively samples the dissociated heads. In other words, whereas dissociation of cross-bridges from actin is probably underestimated in the HMM binding experiments, it is most likely overestimated when determined by the relative amounts of tryptic products (50K, 110K, and 150K) in digested myofibrils. In spite of this quantitative limitation of the two methods, they establish that nucleotides partition the cross-bridges between attached and detached states. In analogy to digestions of myofibrils carried out at 4 °C, no unique states of cross-bridges need to be evoked to explain the proteolysis of the myosin heavy chain in the presence of AMPPNP and PP_i. In this context, it is appropriate to mention the recent spectroscopic work of Trayer & Trayer (1983), who conclude that there is no large change in structure of the acto-S-1 complex when it binds ADP or AMPPNP.

The state of cross-bridges in the presence of MgADP merits special attention. Our binding experiments do not detect any dissociation of HMM (or S-1) from actin by ADP at 24 °C. Yet the digestion of myofibrils in the presence of MgADP has common traits with reactions carried out under both rigor and relaxed conditions. The formation of the 110K peptide resembles the rigor system, whereas production of the 50K material (about 50%) is characteristic of dissociated heads. This result is consistent with the previous studies of Yanagida (1981) and Schaub et al. (1975), who found that about 50% of the myosin heads show altered spectroscopic or chemical properties in the presence of ADP. Borejdo et al. (1982) have further substantiated the unique effect of MgADP on the bound cross-bridges, although their results do not reveal what fraction of the heads is affected by the nucleotide. It is quite clear that the production of the 50K peptide cannot be explained by dissociation of myosin from actin. Thus, the simplest interpretation of the observed digestion pattern is to assume that in the presence of MgADP the two myosin heads have different affinities for actin. Such a possibility is consistent with the findings of Greene (1980) and might be related to the different affinities of these heads for 1,N^6-ethenoadenosine diphosphate (ϵ-ADP) (Yanagida, 1981) or ADP (Schaub & Watterson, 1981). Tight binding of one myosin head to actin would account for the rigor-type stiffness of muscle fibers in the presence of ADP (Marston, 1973; Yanagida, 1981; Marston et al., 1979) and the formation of the 110K product in our tryptic digestions of myofibrils. The tightly bound head would be protected from tryptic cleavage at the 50K/20K link. The weakly bound head would then account for the accumulation of the 50K peptide (up to 50%), as shown in the digestion scheme presented in Figure 8.

The formation of the 110K peptide during tryptic digestion of myofibrils is characteristic of the rigor state of cross-bridges at 24 °C. This product, identified as HMM, is hardly detectable in digestions carried out at 4 °C [see also Lovell & Harrington (1981)] but stands out as the main fragment made at room temperature. The strong temperature dependence of 110K (HMM) formation is in line with the documented temperature sensitivity of the HMM/LMM hinge in myosin (Ueno & Harrington, 1984). The digestion of the hinge is facilitated by releasing the S-2 portion of myosin from the filament backbone. This can be achieved in a dramatic manner by changing the charge balance on the myosin filament (by raising the pH to 8.0; Ueno & Harrington, 1981; Liu & Reisler, 1982; Reisler et al., 1983) and to some extent by binding the myosin heads to actin. It appears that the organizational confinement of myofibrils is necessary for the latter event to affect the hinge, since in synthetic systems actin has at best a minor influence on hinge digestion. At present, the extent and the fraction of cross-bridges released from the filament backbone in myofibrils under rigor conditions and at pH 7.0 are not clear. A more detailed analysis of the state of the myosin hinge requires the determination of the rates of its cleavage in myofibrils as a function of temperature in both relaxed and rigor conditions.

In summary, the results of tryptic digestions of myofibrils and measurements of HMM binding to myofibrillar actin show that AMPPNP and PP_i partition myosin cross-bridges between attached and detached states. In these experiments, we do not find any evidence for additional conformational states of cross-bridges. In the presence of MgADP at 24 °C, each myosin molecule appears to have one attached head and one detached or weakly bound head. We also find that in myofibrils the binding of myosin to actin affects the conformation of the myosin hinge.

Registry No. MgATP, 1476-84-2; MgADP, 7384-99-8; $MgPP_i$, 19262-94-3; MgAMPPNP, 69977-25-9.

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Inhibition of Protein Synthesis in Vesicular Stomatitis Virus Infected Chinese Hamster Ovary Cells: Role of Virus mRNA-Ribonucleoprotein Particle†

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ABSTRACT: Although host protein synthesis is preferentially inhibited, there is a steady decline in the ability of Chinese hamster ovary (CHO) cells infected with vesicular stomatitis virus (VSV) to synthesize both host and viral proteins. We previously reported finding an mRNA-ribonucleoprotein particle (mRNP) that contained all five VSV mRNAs and viral N protein exclusively. This particle apparently regulates translation by sequestering a majority of the VSV mRNA made late in infection and thus rendering it unavailable for protein synthesis. In the present investigation the mRNP was also shown to inhibit in vitro protein synthesis in rabbit reticulocyte and wheat germ lysates programmed with mRNA isolated from VSV-infected cells. The synthesis of the eIF-2·GTP·Met-tRNA (ternary) complex, the first step in initiation of protein synthesis, was markedly inhibited by the mRNP. The inhibition was partially reversed by addition of purified eIF-2 to the inhibited lysate or ternary complex formation reaction. These results indicate a dual role of the mRNP in regulating protein synthesis during infection. Nucleocapsid also inhibited in vitro protein synthesis, although this inhibition was not reversed by eIF-2. Nucleocapsid did not inhibit ternary complex formation in vitro. Consequently, nucleocapsid may also regulate in vivo protein synthesis, but by a mechanism different from the mRNP.

Early during infection of cells by vesicular stomatitis virus (VSV) host protein synthesis is selectively inhibited, and viral proteins are made (Mudd & Summers, 1970; McAllister & Wagner, 1976; Wertz & Youngner, 1972; Wagner, 1975). Later in infection, both host and viral protein syntheses are markedly inhibited. At all times both host and VSV mRNAs are competent for translation in in vitro protein synthesis systems (Lodish & Porter, 1980; Ehrenfeld & Lund, 1977).

The mechanism by which VSV selectively inhibits host protein synthesis has not been fully elucidated. Nuss et al. (1975) argue that VSV mRNAs initiate protein synthesis

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