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Reactive Lysyl of Myosin Subfragment 1: Location on the 27K Fragment and Labeling Properties†

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ABSTRACT: The limited tryptic digestion of the heavy chain of chymotryptic myosin subfragment 1 resulted in five peptides with approximate molecular weights of 75K, 50K, 29.5K, 27K, and 20K. Of the five peptides, two, 75K and 29.5K, were transient and disappeared during the digestion. Our data suggest that the 27K fragment is generated by two parallel routes: directly from the 75K fragment and through a 29.5K precursor. A method was developed to isolate the final products, 50K, 27K, and 20K fragments, of the tryptic hydrolysis of the heavy chain of myosin subfragment 1. Using this method, it was found that the reactive lysyl residue, labeled by a trinitrophenyl moiety, resides in the 27K fragment. The reactive lysyl residue was also present in the 29.5K fragment. The trinitrophenylation of the reactive lysyl residue was inhibited by magnesium pyrophosphate in the 27K but not in the 29.5K fragment. This may indicate that the two routes of generating the 27K peptide correspond to the proteolysis of two qualitatively different subfragment-1 heads as suggested by Tonomura [Tonomura, Y. (1972) *Muscle Proteins, Muscle Contraction and Cation Transport*, University of Tokyo Press, Tokyo, and University Park Press, Baltimore].

It is generally believed that myosin subfragment 1 (S-1)[1] is the segment of the myosin molecule that catalyzes the hydrolysis of adenosine 5′-triphosphate (ATP) and thereby impels actin. So the structure and "internal mechanics" of S-1 acquire great importance. Groups with various suggestive functionalities (reactive thiols, a reactive lysine, certain tryptophans, etc.) reside on S-1. Balint et al. (1978) showed that limited tryptic hydrolysis cuts S-1 into three major fragments (so-called "50", "27K", and "20K") without much additional proteolysis. Recently, Mornet et al. (1979) and Yamamoto & Sekine (1979) showed that the 50K/20K cut abolishes actin activation while leaving unaffected the various adenosine triphosphatases (ATPases) of S-1 alone. Szilagyi et al. (1979) showed that at least the purine moiety of the ATP substance binds to the 27K fragment. However, groups that strongly affect ATPase, such as the reactive thiols "SH_1" and "SH_2", reside on the 20K fragment (Balint et al., 1978). Kassab has suggested to us that perhaps the P–O–P–O–P moiety of ATP binds to the 20K fragment. The researches of Balint et al. (1978) and of Mornet et al. (1979) opened a new line of investigation. Locating known functionalities among the fragments is an early step along this line. Here, we show that the reactive lysyl residue (RLR) resides on the 27K fragment.[2] We also report observations indicating that among the final products the 27K RLR-bearing fragments are generated by two routes of proteolysis. This would be expected if the starting substrate (S-1) were of two qualitatively different kinds.

The RLR is defined by its reaction with 2,4,6-trinitrobenzenesulfonate (TNBS), and by the consequent effects on myosin ATPase (Kubo et al., 1960; Fabian & Muhlrad, 1968). Under the conditions employed here, binding and enzyme kinetic studies (Muhlrad & Takashi, 1980) indicate that about 70% of the trinitrophenyl (TNP) group that binds to S-1 binds to the RLR; presumably 30% binds to the remaining 82 lysyls of S-1 and has no enzymatic effects.

Materials and Methods

Chemicals. TNBS and *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylaminesulfonic acid (IAEDANS) were from Aldrich Chemical Co. [^{3}H]TNBS, an Amersham product, was a generous gift from Professor Y. Tonomura. TPCK-trypsin, soybean trypsin inhibitor, and chymotrypsin were from Worthington Biochemical Co. All other chemicals were of reagent grade.

Proteins. Myosin and actin from back and leg muscles of rabbits were prepared by well-established methods [Tonomura et al. (1966) and Spudich & Watt (1971), respectively]. S-1 was prepared by digestion of myosin filaments with chymotrypsin (Weeds & Taylor, 1975) and purified by filtration through Sephacryl S-200 in 20 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes), pH 7.0.

Protein Concentrations. Unlabeled myosin and S-1 concentrations were calculated from their absorbances, assuming

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[1] Abbreviations used: S-1, myosin subfragment 1; RLR, reactive lysyl residue; TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, trinitrophenyl; IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylaminesulfonic acid; TPCK-trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PP_i, inorganic pyrophosphate; $NaDodSO_4$, sodium dodecyl sulfate.

[2] Although we are unacquainted with the approach or details of their as yet unpublished work, we are aware that Tonomura and his associates reached this same conclusion well before us.

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absorption coefficients, $A^{1\%,1cm}_{280nm}$, of 5.7 and 7.5, respectively; for actin, we assumed an $A^{1\%,1cm}_{290nm}$, of 6.3; in each case, light-scattering corrections were applied. Concentrations of labeled proteins were measured by the Folin phenol method, using the unlabeled protein as a standard. We assumed that S-1 and actin monomer had molecular weights of 110K and 42K, respectively.

Labeling of S-1. It has been shown elsewhere (Takashi et al., 1980) that although IAEDANS attached to SH_1 and TNP attached to the RLR interact the final situation is unaffected by the order in which the ligands attach; arbitrarily, we first reacted the RLR, then SH_1. To react the RLR, we mixed 40 μM S-1 with 80 μM TNBS in 0.1 M tris(hydroxymethyl)-aminomethane (Tris), and waited 5 min, at pH 7.7, 25 °C, in the absence or presence of 0.5 mM magnesium pyrophosphate ($MgPP_i$). The resulting mixture was then dialyzed against a relatively large volume (usually 1 L) of 10 mM potassium maleate, pH 6.2, at 4 °C. The number of attached TNP groups was calculated frm the 345-nm absorbance, assuming a $\Delta\epsilon^{M}_{1cm}$ of 1.45×10^4. To react S-1 (or TNP-S-1) with IAEDANS, we incubated overnight with equimolar IAEDANS in 0.15 M KCl and 10 mM Tes at pH 7.0, 0 °C.

Tryptic Digestion of S-1. S-1 was digested by $^1/_{100}$th its concentration (w/w) of TPCK-trypsin, in 0.1 M $KHCO_3$, for 30 min at pH 8.0, 25 °C (Mornet et al., 1979). Soybean inhibitor was added to twice the TPCK-trypsin concentration (w/w) to terminate digestion. Then sodium dodecyl sulfate ($NaDodSO_4$) and β-mercaptoethanol were added to a final concentration of 1% and 0.1%, respectively, and incubated in a boiling water bath for 3 min. Either the digest was analyzed directly by $NaDodSO_4$ gel electrophoresis or the fragments were separated by gel filtration.

Resolution of the S-1 Fragments. Digested S-1 was dialyzed against a solution of 50 mM Tris, 1% $NaDodSO_4$, and 0.1% β-mercaptoethanol at pH 6.8 and room temperature and then filtered through a 1.5 × 160 cm column of Bio-Gel P-100 preequilibrated with the same solution at room temperature. Filtration aliquots of 2.5 mL were collected. Electrophoretograms were obtained by using slab gels (Laemmli, 1970) of 15% acrylamide and 0.8% bis(acrylamide).

Analysis of Separated S-1 Fragments. When S-1 had been labeled only with TNBS, the TNP content of a fragment aliquot was calculated from its 345-nm absorbance; if the S-1 had also been IAEDANS labeled, the TNP content was calculated from the 420-nm absorbance of the aliquot.

A Hitachi Perkin-Elmer MPF-4 spectrophotofluorometer operating at room temperature in the ratio mode was used for fluorescence measurements. Bound IAEDANS was excited at 340 nm, and its 500-nm emission was recorded. Protein concentration was measured by the Folin phenol method. Glycerol and brom phenol blue was added (to 10% and 0.001%, respectively) to aliquots of the column eluate for application to slab gels.

After electrophoretic separation, a gel slice containing a Coomassie-stained band of the fragment of interest was extracted with 20% (v/v) pyridine–water for 5 h at 37 °C, and the fragment concentration was taken to be proportional to the 605-nm absorbance of the eluted dye. That muscle peptides in this size range give absorbances that obey Beer's Law has been previously demonstrated (Fenner et al., 1975). Alternatively, the relative band intensities were assessed by scanning the gels in a Helena Quick Scan densitometer. When [^{3}H]TNBS was used for trinitrophenylation, bands were cut after scanning and dissolved in 30% hydrogen peroxide after overnight incubation at 70 °C. Ascorbic acid was added to the dissolved gels, and radioactivity was measured in a Beckman LS-150 liquid scintillation counter. Molecular weights were estimated by using authentic marker proteins and from the known molecular weight of myosin light chains.

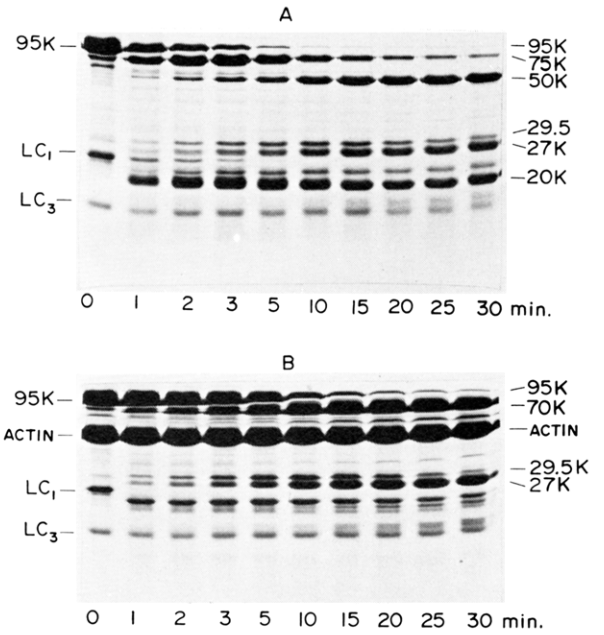


FIGURE 1: Electrophoretograms of S-1 digested by trypsin for different time intervals. Tryptic digestion was carried out as described under Materials and Methods. (A) Digestion in the absence of F-actin; (B) digestion in the presence of F-actin.

ATPase Assays. ATPase activities [μmol of P_i (mg of S-1)$^{-1}$ min^{-1}] were calculated from the inorganic phosphate (P_i) content (Fiske-Subbarow method) of timed 2-mL aliquots of reaction mixture containing 50 μg of S-1 and 2 mM ATP at 25 °C. Never more than 15% of the ATP was hydrolyzed. The remaining composition of the reaction mixture depended on the assay, as follows: (a) for Mg^{2+} activated, 400 mM KCl, 50 mM potassium maleate, and 2 mM $MgCl_2$, pH 6.4; (b) for K^+(EDTA) activated, 600 mM KCl, 50 mM Tris-HCl, and 5 mM EDTA, pH 8.0; (c) for Ca^{2+} activated, 600 mM KCl, 50 mM Tris-HCl, and 10 mM $CaCl_2$, pH 8.0; (d) for actin-Mg^{2+} activated, 3 mM KCl, 20 mM Tes, 1 mM $MgCl_2$, and 400 μg of F-actin, pH 7.0.

Results and Discussion

Tryptic Digestion of S-1. Chymotryptic S-1 in the presence and absence of F-actin was digested with trypsin. At prescribed times, the reaction was stopped by adding soybean trypsin inhibitor, and the digest was analyzed by gel electrophoresis (Figure 1). The resulting electrophoretograms were very similar to those of Mornet et al. (1979); i.e., in the absence of actin, the 95K heavy chain was cleaved first to 70K and 20K fragments. Then the 75K fragment was cut further, resulting finally in 50K and 27K fragments. In the presence of actin, the 50K/20K junction was protected, so 70K and 27K fragments were the final products. However, we noticed a new feature: our electrophoretograms always showed a doublet at the 27K fragment location. The molecular weight of the faster band of the doublet was found to be 27K and that of the slower 29.5K, based on comparison with the mobility of markers of known molecular weight run under the same conditions. The appearance of the doublet was unaffected by the presence of actin during the digestion. The qualitative distribution of the heavy-chain fragments obtained from the digestion in the absence of actin was assessed from the absorbance of the Coomassie blue dye attached to the peptides.

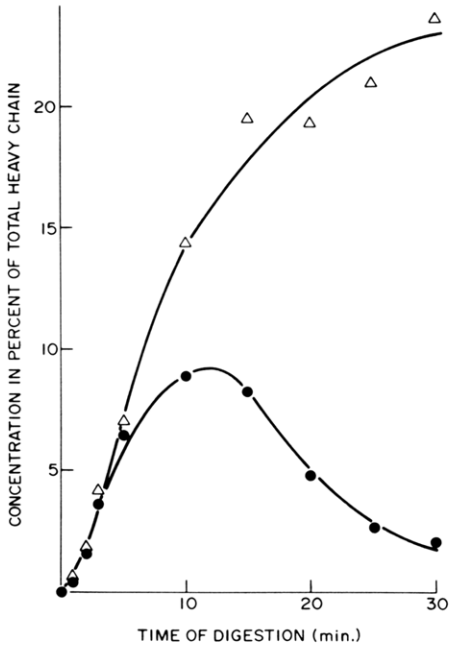
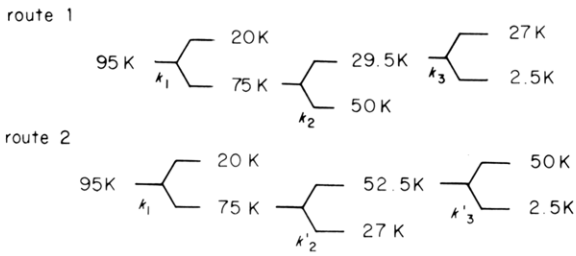


FIGURE 2: Distribution of the 29.5K and 27K fragments during the course of tryptic digestion of S-1. Values presented are intensities of fragments, which were obtained by densitometric scanning of the electrophoretograms of Figure 1A, expressed as the percent of total intensities of all fragments of the heavy chain: (△) 27K; (●) 29.5K.

The time course of the appearance and disappearance of the parent 95K peptide and the daughter 75K, 50K, and 20K fragments was followed and found to be very similar to that observed by Mornet et al. (1979). The time course of the appearance and disappearance of the 29.5K and 27K fragments is shown in Figure 2. After a short lag, both fragments appear simultaneously. The concentrations of these two fragments are roughly the same in the first 3 min of digestion, and then the amount of the 27K fragment steadily increases to a plateau at 30 min, while the amount of the 29.5K fragment reaches a maximum at 12 min from the onset of the reaction and declines thereafter; by 30 min, its concentration is rather negligible. As the 27K fragment was shown to be the N-terminal peptide of S-1 (Lu et al., 1978) and the 27K and 29.5K fragments appear simultaneously, we infer that there are *two* equally vulnerable peptide bonds, distant 27K and 29.5K from the N terminus of the heavy chain. On this assumption, our observations can be rationalized by assuming that two routes of fragmentation proceed in parallel:[3]



Assuming all rate constants to be first order, we can calculate k_1 from the half-life of 95K; it is 0.012 s^{-1}. For simplicity, we set $k_2 = k'_2 = k_3$. Because of 52.5K was not seen, we assumed $k'_3 >> k_3$. Satisfactory fits to the experimentally observed time course of the appearance and disappearance of the various fragments could be obtained if $k_2 = k'_2 = k_3 = 0.0011$ s^{-1}. We will return to these two pathways below.

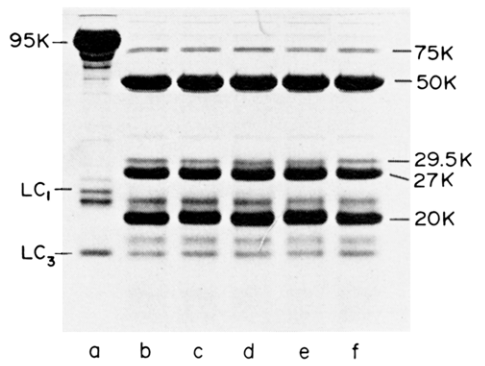


FIGURE 3: Electrophoretograms of the tryptic digest of S-1, IAEDANS–S-1, TNP-S-1, and IAEDANS–TNP-S-1. Tryptic digestion was for 30 min as described under Materials and Methods. (a) Nondigested S-1; (b and f) digested S-1; (c) digested IAEDANS–S-1; (d) digested TNP-S-1; (e) digested IAEDANS–TNP-S-1.

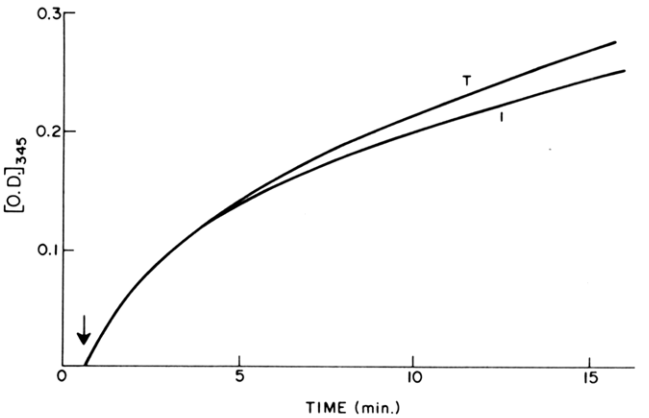


FIGURE 4: Trinitrophenylation of intact and trypsinized S-1. TNBS (100 μM) was added to 11 μM S-1 in 100 mM Tris-HCl, pH 7.7, at the time indicated by the arrow. Reaction was followed by recording the change in optical density at 345 nm in a Cary 118C spectrophotometer by using thermostated cells at 25 °C. (T) Trypsin digested; (I) intact S-1. Tryptic digestion of S-1 was carried out for 30 min as described under Materials and Methods, stopped by the addition of soybean trypsin inhibitor, and dialyzed against 100 mM Tris-HCl, pH 7.7, overnight.

Location of the RLR on S-1. In native S-1, there are strong interactions between a TNP ligand at the RLR and an IAEDANS ligand at SH_1 (Takashi et al., 1980); in fact, these two ligands are within energy-transferring distance of one another. Thiol SH_1 resides on the 20K fragment (Balint et al., 1978), not too far from the 20K/50K junction (Gallagher & Elzinga, 1980). Since our objective was to assign the RLR to one of the three fragments usually obtained from unlabeled S-1, we had first to establish certain premises. Electrophoretic analysis shows (Figure 3) that the pattern of tryptic cutting is the same, whether or not S-1 has been previously (doubly) labeled. Reciprocally, the states at certain sites are the same, whether or not S-1 has been previously cut, e.g., the state at the RLR as revealed by its rate of modification (Figure 4)[4] or by its MgADP-induced difference spectrum (Figure 5) (Muhlrad, 1977), and the state at the ATPase site as revealed by the ATPase activities (Table I, no actin cases). These observations reassure that the three final heavy-chain fragments to be discussed below are the same as those studied by Balint et al.

[3] In genealogical schemes based on electrophoretic mobility measurements, true *mass* balances cannot always be achieved. The relationship between mobility and mass is inexact, and also it cannot always be assumed that Coomassie blue stains all peptides uniformly.

[4] This is true up to the equivalence point. Beyond that extent of reaction, lysyl groups other than the RLR react slightly faster in cut than in uncut S-1.

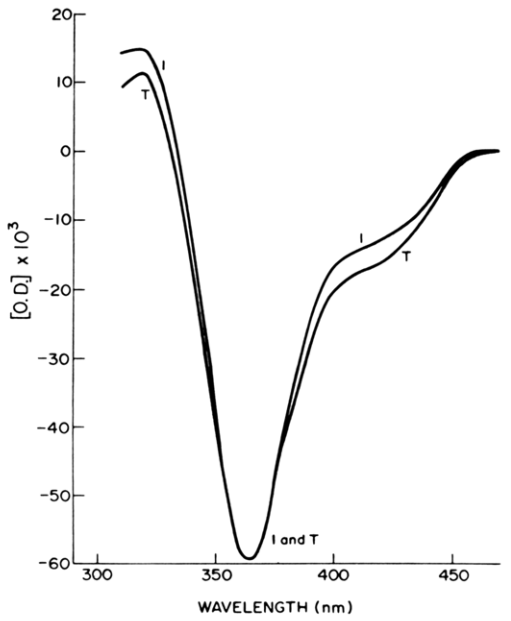


FIGURE 5: Difference spectra of trinitrophenylated intact and trypsin-digested S-1 induced by MgADP. Intact and trypsin-digested S-1 contained 1.31 and 1.16 TNP/S-1, respectively. Trinitrophenylation and tryptic digestion (30 min) were carried out as described under Materials and Methods. Trypsinized S-1 was trinitrophenylated after tryptic digestion. Conditions of the difference spectra measurements are as follows: 1 mM MgADP was added to 40 μM S-1 in 30 mM KCl and 10 mM Tes, pH 7.1, in thermostated (0 °C) 10-mm matched silica cells of a Cary 118C recording spectrophotometer, with MgADP in the sample and without MgADP in the reference position. (T) Trypsin digested; (I) intact S-1.

Table I: ATPase Activities of Intact and Trypsinized S-1[a]

ATPases	activity [mol of P_i (mg of S-1)$^{-1}$ min^{-1}] intact S-1	trypsinized S-1
Ca^{2+} activated	0.60	0.58
K^+(EDTA) activated	2.14	2.25
Mg^{2+} activated	0.016	0.019
actin activated[b]	1.13	0.17
K^+(EDTA) activated after TNBS treatment	0.28	0.31[c]
Mg^{2+} activated after TNBS treatment	0.12	0.13[c]
Ca^{2+} activated after IAEDANS treatment	1.38	1.42[c]

[a] For the conditions for measurement of ATPase activities, see Materials and Methods. [b] Actin/S-1 = 8:1 (molar ratio). [c] Chemical modifications were carried out after limit proteolysis by trypsin as described under Materials and Methods.

(1978) and by Mornet et al. (1979) and that the state of the RLR on a fragment is the same as the state previously studied in myosin or intact S-1.

A central result of this work is seen in Figure 6A: ca. 68% of all the TNP residues that bind to S-1 bind to the 27K fragment after 30 min of tryptic digestion; 19% binds to the 50K fragment and 10% to the 20K fragment. Since elsewhere we have shown (Muhlrad & Takashi, 1980) that TNBS is ca. 70% specific for the RLR, this result shows that the RLR resides on the 27K fragment.

Conceivably, TNBS modification could affect the cutting pattern in the presence of actin, but here too, as in the case of Mornet et al. (1979) and Yamamoto & Sekine (1979), actin protects the 50K/20K junction, so that in the presence of actin 70K and 27K are the predominant fragments produced (Figure

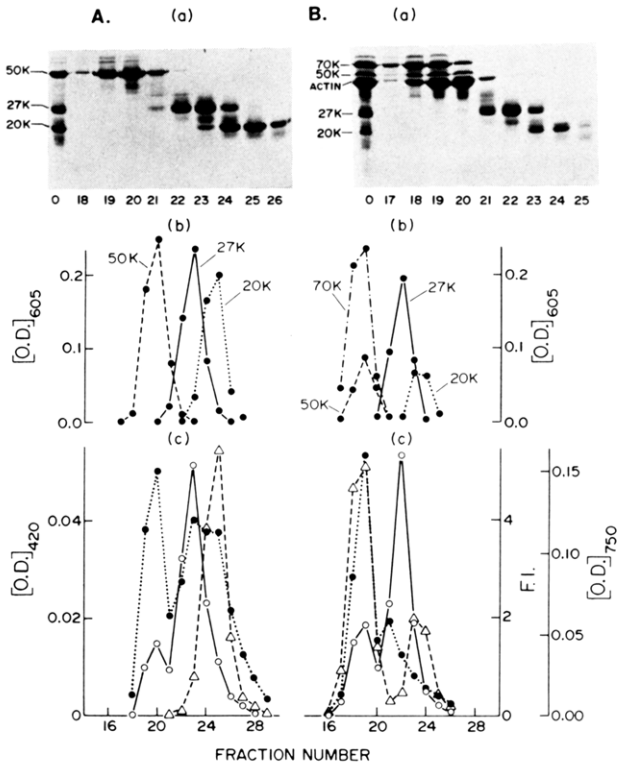


FIGURE 6: Separation and analysis of the tryptic digest of IAEDANS–TNP-S-1. IAEDANS–TNP-S-1 was digested by trypsin for 30 min in the absence (A) and presence (B) of F-actin, and the fragments were separated by gel filtration. Fractions obtained from gel filtration were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (a); the Coomassie blue stained bands of the fragments were cut from the gel and extracted by 20% pyridine, and the OD was read at 605 nm (b). The distribution of TNP-lysine (○) and IAEDANS-thiol (△) was evaluated by reading the $[OD]_{420}$ and fluorescence intensity (FI) of fractions obtained from gel filtration (c). The protein content (●) ($[OD]_{750}$) of the fractions was also determined (c). For detailed descriptions of the procedures, see Materials and Methods. Degree of trinitrophenylation, 1.0 TNP/S-1.

6B). As expected from assigning the RLR to the 27K fragment, most TNP absorbance remains associated with that fragment, the rest being in the unresolved sum of the 70K and 50K fragments. Also seen in Figure 6B is the IAEDANS fluorescence is now with the actin-protected 70K fragment. A study of various ATPase activities (Table I) further confirms the central finding of Mornet et al. (1979), viz., myosin ATPases are unaffected by the tryptic cuts, but actin-activated ATPase is 80% inhibited.

Effect of PP_i on Trinitrophenylation. Miyanishi et al. (1979) concluded that one of the two RLR's of duplex myosin is protected from TNBS modification by PP_i binding. If the two S-1 moieties of a myosin molecule are different (or if our preparation is a mixture of two isozymic myosins), then an S-1 preparation such as we used is a mixture of the said moieties. Figure 7 (in contrast to Figure 6) shows that if S-1 is similarly reacted with TNBS but in the presence of $MgPP_i$ TNP-lysine no longer correlates very well with 27K; i.e., the binding of $MgPP_i$ in some way reduces the specificity of the RLR for TNBS. quantitatively, this effect can be achieved in more than one way. For example, we can assume [as do Miyanishi et al. (1979)] that PP_i perfectly protects one-half of the RLR's (those in the "burst head"). Using the measured rate constants [ca. 2500 and 4.5 min^{-1} (A. Muhlrad and R. Takashi, unpublished experiments) for reaction with the RLR and with all other lysyls, respectively (pH 7.7, 25 °C)] in simulations, we find that a reduction in specificity from about 0.8 to about 0.6 (as indicated by our data) can be achieved

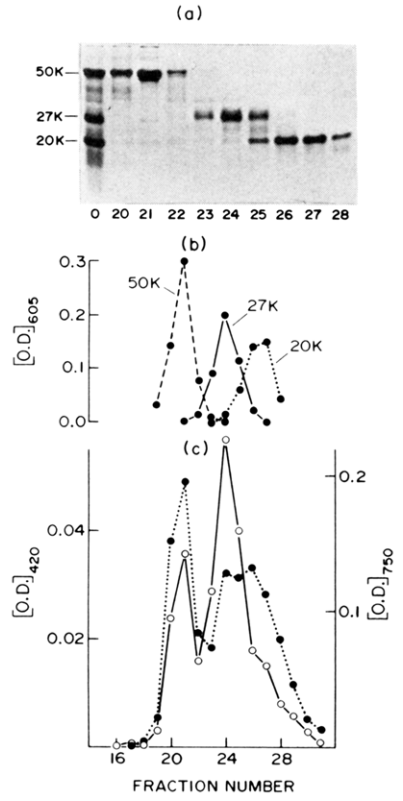


FIGURE 7: Separation and analysis of the tryptic digest of S-1 trinitrophenylated in the presence of $MgPP_i$. For symbols and procedures, see Figure 6 and Materials and Methods. Tryptic digestion was for 30 min in the absence of F-actin. Degree of trinitrophenylation, 1.1 TNP/S-1. S-1 was incubated with TNBS in the presence of $MgPP_i$ for 10 min.

Table II: Distribution of [^{3}H]TNP among Heavy-Chain Fragments during the Course of Tryptic Hydrolysis of S-1

		trinitrophenylation without $MgPP_i$ at time of tryptic digestion (min)			trinitrophenylation with $MgPP_i$ at time of tryptic digestion (min)		
frg	units	2	10	30	2	10	30
95K	frg %[a]	19.1			18.5		
	cpm/frg %[b]	29.4			17.4		
	cpm %[c]	22.3			21.1		
75K	frg %	40.6	21.4	0.5	41.2	19.4	0.9
	cpm/frg %	33.3	26.1	30.0	19.2	13.5	26.4
	cpm %	53.2	23.7	0.6	51.0	16.3	1.5
50K	frg %	6.8	30.3	47.8	7.1	31.2	46.7
	cpm/frg %	6.6	5.1	5.1	7.5	5.4	5.7
	cpm %	1.8	6.3	10.1	3.5	10.3	16.9
29.5K	frg %	1.9	6.2	0.4	1.7	5.5	0.2
	cpm/frg %	125.0	102.0	90.0	115.0	92.0	150.0
	cpm %	9.3	26.7	1.5	12.5	31.4	1.9
27K	frg %	2.1	11.9	21.3	1.9	11.5	20.7
	cpm/frg %	104.0	79.0	98.0	52.5	51.2	57.6
	cpm %	8.6	39.5	84.1	6.4	36.9	73.7
20K	frg %	29.5	30.2	30.0	29.6	32.4	31.5
	cpm/frg %	3.3	3.0	3.0	3.0	2.3	3.0
	cpm %	3.8	3.8	3.7	6.0	5.1	6.1

[a] Intensity of the fragment as a percent of the total intensities of the heavy chain. [b] Total radioactivity of the fragment divided by fragment % (see *a*). [c] The cpm in the fragment as a percent of the cpm in all fragments of the heavy chain. Degree of trinitrophenylation without $MgPP_i$, 0.48 TNP/S-1; with $MgPP_i$, 0.31 TNP/S-1. The recovery of radioactivity from the stained $NaDodSO_4$ gels was 85%.

in this way. But the same reduction is also obtained if we assume that PP_i reduces the TNBS–RLR rate constant of *all* RLR's by a factor of about 4.

In order to clarify further our data bearing on the question of the identity of myosin "heads", S-1 was trinitrophenylated by [^{3}H]TNBS in the presence and absence of magnesium pyrophosphate. After removal of all nonreacted reagents by dialysis, it was digested by trypsin for various periods, and the resulting fragments were separated by $NaDodSO_4$ gel electrophoresis. The quantitative distribution of radioactivity and peptides of the heavy chain was determined (Table II). A total of 84% of the radioactivity was found in the 27K fragment of the heavy chain in the S-1 preparation labeled by [^{3}H]TNBS in the absence of $MgPP_i$ and digested by trypsin for 30 min (Table II). This strongly supports the finding that the RLR is located in the 27K peptide. (The higher specificity of labeling observed in the radioactive experiment is due to the fact that there the number of attached TNP's per S-1 was 0.48 while in the nonradioactive experiment it was 1.0, and the higher value favors nonspecific labeling.) It is important to note that the 29.5K fragment was also labeled, in fact with a higher specific activity than the 27K fragment. This observation is consistent with assuming the 29.5K fragment to be a precursor of the 27K fragment as we do in route 1 (see above).

Total trinitrophenylation was less if $MgPP_i$ was present; specifically, less label appeared in the 27K fragment. This suggests that $MgPP_i$ reduces the reactivity of the RLR on the 27K fragment. On the other hand, the specific activity of the 29.5K fragment now was significantly higher than that of the 27K fragment, suggesting that trinitrophenylation of the 29.5K fragment is unaffected by $MgPP_i$. These observations can be rationalized by the hypothesis of Miyanishi et al. (1979), if we identify route 2 with their "head A", and rout 1 with their "head B". We would say that the favored route of tryptic hydrolysis of "head B" (where $MgPP_i$ does not influence the reactivity of the RLR) is route 1, while "head A" is digested mainly through route 2. At this stage, however, this interpretation of our data is not unique.

Conclusions. In summary, we have developed a method for separating and isolating the products of tryptic hydrolysis of S-1. Using this method, we have found (as had Tonomura in earlier unpublished work) that the RLR resides in the 27K fragment. Our data further suggest that the 27K fragment is generated by the two routes, distinguishable by the effect of $MgPP_i$ on the trinitrophenylation of the starting peptides. conceivably, our "two routes" could correspond to the preteolysis of two qualitatively different S-1 heads as postulated by Tonomura (1972).

Acknowledgments

We are indebted to Professor Y. Tonomura for informing us about unpublished results of his group concerning the location of the RLR and for his generous gift of [^{3}H]TNBS. We also thank Professor M. F. Morales for several helpful discussions during the work and preparation of the manuscript and Dr. Julian Borejdo for developing the computer program simulating route 1 and route 2. T.H. is greatly indebted to Professor J. Botts for support and encouragement during his fellowship.

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Solid-Phase Synthesis of Crystalline Glucagon†

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ABSTRACT: Mammalian glucagon was synthesized by a stepwise solid-phase method. The support was an alkoxybenzyl alcohol resin, and the biphenylylisopropyloxycarbonyl group was used for temporary α-amino protection. After purification by gel filtration and ion-exchange chromatography, the 29-residue hormone was readily crystallized from water at alkaline pH. The product was homogeneous and indistinguishable from natural bovine glucagon by gel electrophoresis, ion-exchange chromatography, reverse-phase high-pressure liquid chromatography, fluorescence spectroscopy, and amino acid analysis. The synthetic hormone was fully active in the rabbit hyperglycemia assay.

The bihormonal hypothesis of Unger (Unger & Orci, 1975) has created renewed interest in glucagon physiology and its role in diabetes mellitus. It is generally agreed that some of the remaining questions about the mechanism of action of glucagon can best be answered by total synthesis of the hormone and of appropriate analogues. The early synthetic efforts on this 29-residue peptide proved to be quite difficult because of its unusual chemical structure (Schröder, 1967; Wünsch, 1966). The first successful synthesis of mammalian glucagon was accomplished in 1968 (Wünsch & Wendelburger, 1968) and made use of the classical fragment condensation method. The crystalline product was indistinguishable from the native hormone and was shown to be of high purity and full activity in several systems. The second synthesis, several years later, was by the Protein Synthesis Group in Shanghai (1975) who used a solid-phase fragment approach. The protected peptide-resin was cleaved and fully deprotected with HF to give an active product. Recently, Yajima reported solution syntheses of mammalian (Fujino et al., 1978) and avian (Ogawa et al., 1978) glucagons by a scheme which differed from the first two in the size of the fragments, the protecting groups, and the coupling reagent.

The first stepwise solid-phase synthesis of glucagon, briefly reported by us in 1977 (Merrifield et al., 1977), is described here in detail. Because of the presence of an Asp-Ser sequence, in addition to Trp and Met residues, we decided to rely on a mild acidolytic system to avoid α–β rearrangement and excessive alkylation side reactions. Therefore, the C-terminal residue was anchored to *p*-alkoxybenzyl alcohol resin through an ester bond (Wang, 1973). This bond, which was cleavable by 50% trifluoroacetic acid in dichloromethane, required the use of a temporary N^{α}-protecting group such as the biphenylylisopropyloxycarbonyl group (Sieber & Iselin, 1968) that could be removed selectively at each cycle of the synthesis with very mild acid. Side-chain protection was based on the *tert*-butyl group, *tert*-butyl esters for the three aspartic acid residues, *tert*-butyl ethers for the nine hydroxyl-containing residues, and a *tert*-butyloxycarbonyl group for the lysine. In addition, the guanidino groups of the two arginine residues were protected with nitro groups, thus providing the option of their removal by catalytic hydrogenation or by HF or of their retention in the final product. Histidine was protected against racemization during coupling by a dinitrophenyl group on the imidazole nitrogen. Since this residue was N terminal, it was incorporated with an N^{α}-Boc group. Special precautions were taken against nitrile formation during coupling of asparagine and glutamine by using DCC/HOBt.[1] All other couplings were with DCC alone. The protection scheme is summarized in Figure 1. This new synthetic approach to glucagon has provided a rapid and efficient synthesis in moderate yield of homogeneous, fully active hormone that

† From the Rockefeller University, New York, New York 10021. *Received October 2, 1980.* This work was supported in part by Grant AM 24039 from the U.S. Public Health Service.

[1] Abbreviations used: DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DCHA, dicyclohexylamine; CHA, cyclohexylamine; Bpoc, biphenylylisopropyloxycarbonyl; DMF, dimethylformamide; Dnp, dinitrophenyl; high-pressure LC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-2950$01.25/0