Carbodiimide-Catalyzed Cross-Linking Sites in the Heads of Gizzard Heavy Meromyosin Attached to F-Actin†

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Received July 6, 1988; Revised Manuscript Received October 6, 1988

ABSTRACT: In the rigor complex between rabbit skeletal muscle F-actin and chicken gizzard heavy meromyosin (HMM), the direct contact between two HMM heads was demonstrated by using a zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]maleimide (EDC) [Onishi, H., Maita, T., Matsuda, G., & Fujiwara, K. (1989) *Biochemistry* (preceding paper in this issue)]. Here, the 60K peptide which was a product of the EDC cross-linking between two 24K heavy chain (tryptic) fragments of HMM was further fragmented with cyanogen bromide, and the location of the cross-linking sites on the amino acid sequence of the HMM heavy chain was investigated. The result showed that one site resided within the 77-residue peptide region (residues 1–77) on one head of HMM, whereas the other site belonged to the 40-residue peptide region (residues 164–203) on the other head. This finding suggests that the two HMM heads are in contact with each other at different sites. Ultracentrifugal fractionation revealed that the head-to-head cross-linked gizzard HMM could be reversibly released from F-actin in the presence of Mg-ATP. The yield of the head-to-head cross-linking was not significantly changed with the acto–HMM complex between actin/HMM head molar ratios of 1 and 4, and it was very slightly decreased even at a molar ratio of 8, where HMM molecules were attached sparsely to actin filaments. However, the 60K product of the head-to-head cross-linking was also generated by the EDC treatment of the rigor complex between skeletal muscle F-actin and gizzard myosin subfragment 1 (S-1), which was a single head of myosin. These findings suggest that although the majority of cross-linked heads originate in the same HMM molecule, the neck that connects the two heads is not absolutely required for the head-to-head cross-linking.

The sliding of myosin filaments along actin filaments, coupled with the hydrolysis of ATP, is the driving force of the muscular contraction (Huxley, 1969). The sliding force is developed by the cross-bridge between the actin and myosin filaments. Electron microscopic studies of the vertebrate striated muscle revealed that the cross-bridges consist of the globular heads of myosin molecules projected from each myosin filament. Although each myosin molecule has two heads (Slayter & Lowey, 1967), isolated single heads (subfragment 1) show Mg-ATPase activity and can bind to actin filaments (Lowey et al., 1969). Thus, each myosin head is capable of forming a cross-bridge, a force-generating contractile apparatus. Since no biochemical and morphological approaches had shown direct contact between two myosin heads, it was thought that the two heads of a myosin molecule did not interact with each other during the cyclic process of cross-bridge sliding along actin filaments. However, recently, we have found that the two heads of chicken gizzard HMM[1] can be cross-linked by a zero-length cross-linker, when gizzard HMM is bound to F-actin (Onishi et al., 1989). This finding suggests that the two myosin heads attached to two neighboring actin subunits within an actin filament are also in contact with each other. It suggests further that the two heads of a single myosin molecule may be functionally associated when cross-bridges are formed during their sliding along an actin filament.

In order to elucidate the physiological significance of the head-to-head contact during cross-bridge sliding, the two following questions are investigated: (1) whether or not the contact between two myosin heads occurs at specific affinity sites, and (2) whether or not the linkage between two heads via a neck is essential for the observed head-to-head contact. To answer the first question, we have attempted to identify the sites involved in the cross-linking by EDC. Our previous study revealed that cross-linking sites of HMM heads both reside within the NH_2-terminal 24K segment region of the HMM heavy chain (Onishi et al., 1989). A 60K homodimer of 24K tryptic HMM heavy chain fragments was isolated from the EDC cross-linked rigor complex formed between F-actin and tryptically digested gizzard HMM. In the present study, the 60K cross-linked product was fragmented with cyanogen bromide, and the location of the cross-linking sites in the sequence of the 24K polypeptide was investigated. We found that the cross-linking sites were located at different positions in the 24K fragment sequence.

Cross-linking studies using myosin subfragment 1 (S-1) are useful for answering the second question, because S-1 is a single head of myosin. Such a study has been reported by Marianne-Pêpin et al. (1985). However, in their study, F-actin

† This work was supported by Grant-in-Aids for Special Project Research, "Calcium Ions and Cell Function" and "Metabolic Researches of Blood Vessels", from the Ministry of Education, Science and Culture of Japan and by a Research Grant for Cardiovascular Diseases (60C-1) from the Ministry of Health and Welfare.

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[1] Abbreviations: HMM, heavy meromyosin; S-1, subfragment 1; ATPase, adenosine 5′-triphosphatase; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

0006-2960/89/0428-1905$01.50/0

was first activated with EDC, and then the cross-linking reaction was initiated by mixing activated F-actin with gizzard S-1. Their results showed no observable cross-linking between two S-1 molecules. In our study, the rigor complex was first formed by mixing gizzard S-1 with F-actin, and then the cross-linking reaction was initiated by adding EDC. In contrast to their result, we observed cross-linking between two S-1 molecules.

Materials and Methods

Protein Preparations. Chicken gizzard myosin was prepared as described in our previous report (Ikebe et al., 1978). Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (1971).

Isolation of AEDANS-Labeled HMM. Gizzard myosin (5 mg/mL) was labeled at 20 °C for 50 min with an 80 molar equiv of *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) in 1.5 mM ATP, 0.15 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5). The reaction was stopped by adding 30 mM 2-mercaptoethanol. Labeled gizzard myosin was digested with trypsin, and HMM was then isolated from the tryptic digest as described in the preceding paper in this issue (Onishi et al., 1989). The tryptically digested HMM consisted of five polypeptide components; three heavy chain fragments of approximate molecular weights 24K, 50K, and 68K, the 17K light chain, and a fragment of the 20K light chain (Onishi & Watanabe, 1985). Among these HMM components, only the 24K heavy chain fragment and the 17K light chain were heavily labeled with fluorescent AEDANS (Onishi, 1985; Onishi et al., 1989).

Isolation of S-1 and Its AEDANS-Modified Derivative. Gizzard myosin (1 mg/mL) in 50 mM KCl, 10 mM EDTA, and 20 mM Tris-HCl (pH 7.5) was digested with papain (weight ratio: protease/myosin = 1/250) at 20 °C for 11 min. The digestion was terminated by adding 1 mM iodoacetic acid. The digest was dialyzed overnight against 5 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (Sigma Chemical Co.), 20 mM KCl, 10 mM $MgCl_2$, 0.3 mM DTT, and 10 mM Tris-HCl (pH 7.5). After centrifugation of the dialyzed solution at 100000*g* for 30 min, the supernatant was salted out by ammonium sulfate at pH 7.2. The protein precipitated at 62% ammonium sulfate saturation was collected by centrifugation and dialyzed against the buffer containing 0.3 M KCl, 2 mM $MgCl_2$, 0.3 mM DTT, and 20 mM Tris-HCl (pH 7.5). Papain S-1 thus obtained (1.25 mg/mL) was further digested with trypsin (weight ratio: protease/S-1 = 1/125) at 20 °C for 15 min. The digestion was terminated by adding trypsin inhibitor (weight ratio: inhibitor/protease = 2/1). S-1 was collected again by salting out with 62% saturated ammonium sulfate. The tryptically digested S-1 consisted of three major heavy chain fragments of approximate molecular weights 24K, 50K, and 21K. The AEDANS-modified S-1 derivative was prepared from AEDANS-modified gizzard myosin in the same procedure described for unmodified S-1.

DACM Labeling of Actin. Fluorescently labeled rabbit skeletal muscle actin was obtained by incubating F-actin with *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) under the conditions specified in the preceding paper (Onishi et al., 1989).

Cross-Linking of Acto–HMM or Acto–S-1. Tryptically digested gizzard HMM (0.5 mg/mL) or S-1 (0.35 mg/mL) in 0.1 M KCl, 10 mM $MgCl_2$, and 10 mM imidazole hydrochloride (pH 7.0) was mixed with skeletal muscle F-actin at a molar ratio of actin/HMM head or actin/S-1 = 1. The cross-linking reaction of acto–HMM or acto–S-1 was performed by incubating the protein with freshly prepared 5 mM EDC for 1 or 2 h at 24 °C. The reaction was terminated by adding 30 mM 2-mercaptoethanol. The details of the procedure were described in the preceding paper (Onishi et al., 1989).

Isolation of the 24K Peptide and Its 60K Cross-Linked Homodimer. This procedure was presented in the preceding paper (Onishi et al., 1989). Briefly, following the EDC-induced cross-linking, the rigor complex between skeletal muscle F-actin and tryptically digested gizzard HMM was dissolved in a denaturing solution containing 6 M guanidine hydrochloride, 5 mM EDTA, and 0.5 M Tris-HCl (pH 8.0), reduced with 2-mercaptoethanol, and S-carboxymethylated with iodoacetic acid (Crestfield et al., 1963). The S-carboxymethylated acto–HMM sample was then submitted to gel filtration on a Sephadex G-100 column, and the first and the second peaks were collected as the 60K- and 24K-rich fractions, respectively. Both fractions were further purified by chromatography on a CM-52 column.

Cyanogen Bromide (CNBr) Cleavage and Separation of CNBr Peptides. Purified 24K and 60K peptides (5 mg) were separately lyophilized and then resuspended with a 150-fold molar excess of CNBr in 70% formic acid to initiate the peptide cleavage. The amount of CNBr was 150-fold more than the calculated number of methionine residues in the 24K or 60K peptide. The reaction was allowed to proceed at 25 °C for 24 h. The cleavage was terminated by diluting the reaction mixture with 10 volumes of distilled water and then by drying in vacuo. CNBr peptides were dissolved in a small volume of 0.1% trifluoroacetic acid and subjected to high-performance liquid chromatography (HPLC), using a reverse-phase column (0.4 × 15 cm) of Cosmosil $_5C_{18}$ resin (Nakarai Chemicals Co.). Peptides were eluted with a 64-min linear gradient of acetonitrile concentration from 0 to 60% containing 0.1% trifluoroacetic acid. The eluate was monitored by the absorbance at 220 nm. Some fractions were further purified by gel filtration on a Sephadex G-75 superfine column which had been equilibrated with 5% acetic acid (for details, see Results). Peptides were eluted with the same buffer as the one used for equilibrating the column at a flow rate of 3.5 mL/h.

Amino Acid Composition. Peptide samples were hydrolyzed, derivatized with phenyl thiocyanate, and analyzed by reverse-phase HPLC as described in the preceding paper (Onishi et al., 1989).

Gel Electrophoresis. Gel electrophoresis in the presence of 0.1% $NaDodSO_4$ was carried out according to Laemmli (1970), using polyacrylamide slab gels containing 11% acrylamide and 0.3% bis(acrylamide). Aliquots from the cross-linking reaction mixtures were applied on the gels. Gels were observed under a long-wavelength UV lamp and then stained with Coomassie brilliant blue. Gel electrophoresis in the presence of 0.1% $NaDodSO_4$ and 8 M urea was performed by the method of Swank and Munkres (1971), using polyacrylamide slab gels containing 15% acrylamide and 0.5% bis(acrylamide). The CNBr fragments of the 24K or 60K peptide were dissolved in 40% sucrose, 0.1% $NaDodSO_4$, 0.14 M 2-mercaptoethanol, and 0.1 M phosphoric acid (pH was adjusted to 6.8 with Tris). Samples were heated at 60 °C for 15 min before application on the gels.

ATPase Activity. To obtain the time course of the changes in the Mg-ATPase activity of acto–HMM or HMM alone during the EDC cross-linking reaction, aliquots were withdrawn from the reaction mixture at various times and submitted for assay. The ATPase activity was determined as specified in the preceding paper (Onishi et al., 1989).

Table I: Amino Acid Compositions of Fractions Obtained by Reverse-Phase HPLC of the 24K CNBr Fragments[a]

	column peaks						
	1	2	3	4	5	6	7
Asp		2.00 (2)	1.06 (1)	3.32 (3)	10.97 (11)	3.89 (3.5)	4.54 (4)
Glu	1.04 (1)	1.04 (1)		6.16 (6)	12.41 (12)	5.70 (5.5)	5.69 (5)
cmCys[b]				0.70 (1)		0.31 (1.5)	0.68 (2)
Ser		0.93 (1)	0.90 (1)	3.55 (4)	5.68 (6)	3.62 (4.0)	3.72 (4)
Gly	1.06 (1)			3.93 (4)	3.30 (3)	3.04 (3.0)	2.71 (2)
HSE[c]	0.77 (1)	0.84 (1)	0.80 (1)		0.88 (1)	0.56 (0.5)	1.13 (1)
His	0.94 (1)		0.98 (1)	1.06 (1)	1.13 (1)	1.14 (1.0)	1.15 (1)
Arg	1.15 (1)		1.22 (1)	1.25 (1)		2.15 (1.5)	2.81 (2)
Thr			1.05 (1)	2.98 (3)	2.19 (2)	2.44 (2.5)	2.53 (2)
Ala			3.06 (3)	2.96 (3)	5.04 (5)	2.68 (2.5)	2.45 (2)
Pro		2.00 (2)	1.94 (2)		4.12 (3)	1.52 (1.0)	2.00 (2)
Tyr	1.00 (1)		1.92 (2)	0.98 (1)		2.81 (3.0)	4.09 (5)
Val		1.06 (1)		2.14 (3)	6.70 (7)	2.13 (3.0)	2.16 (3)
Ile			1.95 (2)	1.69 (2)	2.07 (2)	2.72 (3.5)	3.78 (5)
Leu				3.18 (3)	6.16 (6)	4.83 (5.0)	6.40 (7)
Phe		1.02 (1)			3.97 (4)	1.21 (1.0)	2.20 (2)
Trp					*d* (2)		
Lys	2.93 (3)	1.99 (2)		5.81 (5)	11.99 (12)	3.55 (3.5)	1.63 (2)
total no. of amino acids	9	11	15	40	77	45.5	51
identified polypeptides	CN4	CN2	CN5	CN6	CN1	0.5 × CN3 + 0.5 × CN6	CN3
amount recovered (nmol)	7.4	6.2	7.1	2.1	9.5	9.1	1.4

[a] The numbers of amino acid residues in parentheses are values from the sequence data (Maita et al., 1987). [b] *S*-(Carboxymethyl)cysteine. [c] Homoserine. [d] Present but not quantitated.

Protein Concentration. The protein concentrations were determined by using the biuret method (Gornall et al., 1949).

Results

Identification of the Cross-Linking Sites between Two Globular Heads of Gizzard HMM Attached to F-Actin. As shown previously (Onishi et al., 1989), the EDC cross-linked rigor complex between F-actin and trypsin-cleaved gizzard HMM contained a cross-linked complex (M_r approximately 60K) between two NH_2-terminal 24K tryptic fragments of the HMM heavy chain. Here, to identify the cross-linking sites within the amino acid sequence of the 24K fragment, both the 24K and 60K peptides were fragmented with cyanogen bromide (CNBr) under the same conditions, and peptide fragments were analyzed by reverse-phase HPLC.

The reverse-phase HPLC profile of CNBr fragments of the 24K peptide showed seven major peaks (Figure 1A). Since the complete sequence of the 24K peptide had already been reported by Maita et al. (1987) and Yanagisawa et al. (1987) and since the cleavage sites by CNBr were known, the amino acid composition for each CNBr fragment could be determined. Six fragments are expected to be produced by CNBr cleavage. From the NH_2-terminal end, these fragments are sequentially named CN1, CN2, CN3, CN4, CN5, and CN6 (Maita et al., 1987). As shown in Table I, the amino acid compositions of peaks 1, 2, 3, 4, 5, and 7 in the HPLC profile (Figure 1A) were very similar to the amino acid compositions determined for CN4, CN2, CN5, CN6, CN1, and CN3, respectively. The amino acid composition of peak 6 could be obtained from the equimolar mixture of CN3 and CN6. Since CN3 and CN6 were separated by $NaDodSO_4$ gel electrophoresis of the peak 6 (Figure 2, lane d), the covalent link appeared to be absent between these CNBr fragments.

CNBr fragments obtained from the 60K peptide revealed marked similarities in the reverse-phase HPLC profile to those obtained from the 24K peptide (Figure 1B). For example, the 60K peptide profile also showed seven major peaks. The amino acid compositions of peaks 1, 2, 3, 4, and 7 shown in Table II were found to agree with the values calculated for CN4, CN2, CN5, CN6, and CN3, respectively. However, the

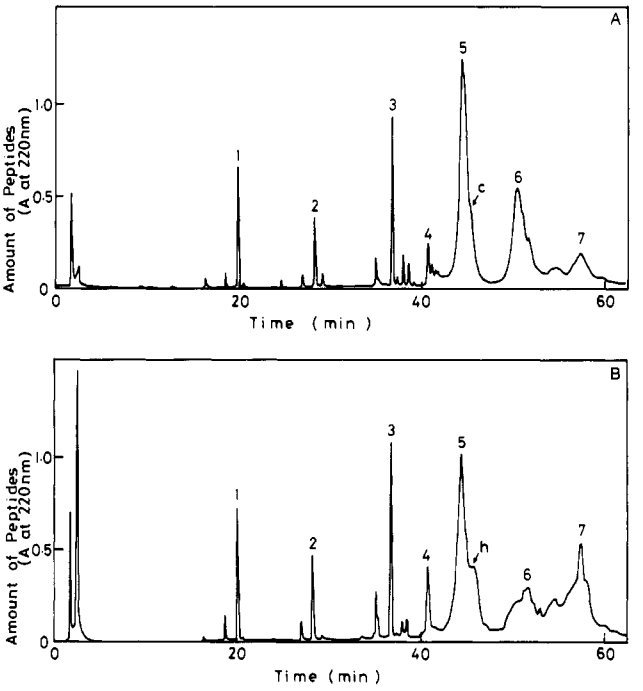

FIGURE 1: Reverse-phase HPLC elution profiles of CNBr fragments of 24K (A) and 60K (B) peptides. Samples (approximately 0.4 mg) were applied on a reverse-phase HPLC column (0.4 × 15 cm) of Cosmosil $_5C_{18}$ and eluted as described under Materials and Methods. The elution profiles are almost identical and exhibit seven major peaks which are identified by the numbers 1–7 in each panel. The amino acid composition of polypeptides contained in each of these peak fractions is presented in Tables I and II. The positions from which gel samples are taken for $NaDodSO_4$ gel analysis presented in Figure 2 are identified in the figure (c and h).

composition of peak 5 did not agree with the value calculated for pure CN1. The best-fitting analysis between the observed and calculated values indicated that this peak is composed of CN1 and CN6 with a molar ratio of 2:1. The $NaDodSO_4$ gel electrophoretic profile revealed that a major peptide in addition to CN1 was present in peak 5 (Figure 2, lane g). The M_r of the major peptide was estimated to be approximately 17K from

Table II: Amino Acid Compositions of Fractions Obtained by Reverse-Phase HPLC of the 60K CNBr Fragments

	column peaks						
	1	2	3	4	5	6	7
Asp		2.16 (2)	1.21 (1)	3.21 (3)	12.28 (12.5)	7.01 (7.0)	4.35 (4)
Glu	0.93 (1)	1.04 (1)	0.47	6.41 (6)	15.38 (15.0)	11.76 (8.9)	5.42 (5)
cmCys[b]				0.47 (1)	0.30 (0.5)	0.31 (1.7)	0.74 (2)
Ser		0.93 (1)	1.00 (1)	3.70 (4)	7.20 (8.0)	4.74 (5.8)	3.87 (4)
Gly	1.04 (1)		0.10	3.89 (4)	4.81 (5.0)	2.93 (3.5)	2.14 (2)
HSE[c]	0.82 (1)	0.82 (1)	0.87 (1)		1.02 (1.0)	0.66 (1.0)	0.80 (1)
His	1.04 (1)		1.07 (1)	1.05 (1)	1.71 (1.5)	1.39 (1.3)	1.10 (1)
Arg	1.04 (1)		1.18 (1)	1.25 (1)	1.19 (0.5)	3.20 (1.7)	2.70 (2)
Thr			1.11 (1)	2.74 (3)	3.60 (3.5)	3.64 (2.9)	2.63 (2)
Ala			2.95 (3)	3.02 (3)	6.50 (6.5)	4.56 (3.8)	2.24 (2)
Pro		1.89 (2)	1.81 (2)	0.46	4.15 (3.0)	2.03 (2.3)	2.28 (2)
Tyr	1.02 (1)		1.88 (2)	0.98 (1)	0.79 (0.5)	2.77 (3.8)	4.79 (5)
Val		1.00 (1)	0.14	2.20 (3)	7.35 (8.5)	3.36 (5.1)	2.11 (3)
Ile			1.88 (2)	1.84 (2)	2.77 (3.0)	3.42 (4.7)	3.89 (5)
Leu			0.18	3.10 (3)	8.02 (7.5)	8.39 (7.6)	7.01 (7)
Phe		1.03 (1)			3.81 (4.0)	1.91 (2.6)	2.00 (2)
Trp					*d* (2.0)	*d* (0.6)	
Lys	3.03 (3)	2.02 (2)	0.20	5.63 (5)	13.91 (14.5)	6.74 (6.5)	1.49 (2)
total no. of amino acids	9	11	15	40	97	70.8	51
identified polypeptides	CN4	CN2	CN5	CN6	0.5 × CN1·CN6[e] + 0.5 × CN1	0.3 × CN1·CN6 + 0.7 × CN3 + ?[f]	CN3
amount recovered (nmol)	7.7	8.8	11.4	3.7	12.6	5.0	8.4

[a] The numbers of amino acid residues in parentheses are values from the sequence data (Maita et al., 1987). [b] *S*-(Carboxymethyl)cysteine. [c] Homoserine. [d] Present but not quantitated. [e] CN1·CN6 = cross-linked product between CN1 and CN6. [f] ? = unidentified fragment.

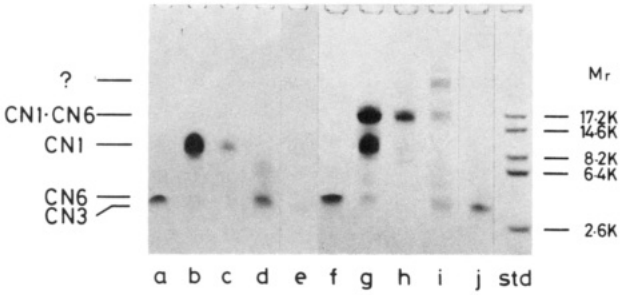

FIGURE 2: $NaDodSO_4$ (urea) gel electrophoretic patterns of selected peaks shown in Figure 1. Gel samples shown in lanes a–e are peaks from Figure 1A: (a) peak 4, (b) peak 5, (c) a shoulder of peak 5 ("c" in Figure 1A), (d) peak 6, and (e) peak 7. Lanes f–j contain peaks from Figure 1B: (f) peak 4, (g) peak 5, (h) a shoulder of peak 5 ("h" in Figure 1B), (i) peak 6, and (j) peak 7. One-twenty-fifth aliquot (by volume) of the reverse-phase HPLC peak fractions was applied per lane. The lane marked "std" = CNBr cleavage product of myoglobin as a molecular weight marker. Gels are stained by Coomassie blue. The positions of CNBr fragments are identified on the left margin. The molecular weights of CN1 and CN1·CN6 were estimated to be 9.5K and 17.2K, respectively.

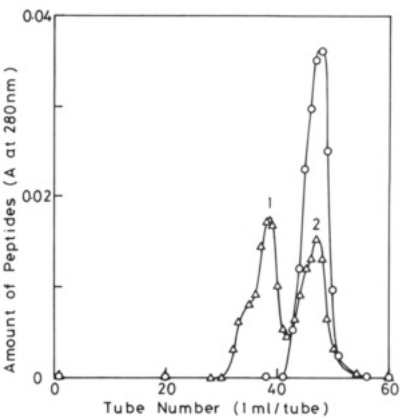

FIGURE 3: Gel filtration elution profiles showing separation of CN1 and CN1·CN6 present in peak 5 in Figure 1B by a Sephadex G-75 superfine column (1.2 × 95 cm). The elution profile of peak 5 in Figure 1A (○) shows a single peak of CN1. The elution profile of peak 5 in Figure 1B (△) shows two separate peaks (1 and 2). Peak 1 is the CN1·CN6 complex while peak 2 is CN1. The amino acid composition of these two peaks is shown in Table III.

the semilogarithmic plot of the migration distance against the M_r. These results were interpreted to mean that peak 5 is a mixture of CN1 and a cross-linked complex between CN1 and CN6. The determination of components in peak 6 was much more difficult. The $NaDodSO_4$ gel electrophoretic pattern indicated that CN3, CN1·CN6, and an unknown high molecular weight peptide were present in peak 6 (Figure 2, lane i).

The fractions in peak 5 of Figure 1B were pooled and subjected to gel filtration on a Sephadex G-75 column. The elution profile shown in Figure 3 indicates that a high molecular weight peptide (peak 1) could be separated from CN1 (peak 2). The amino acid composition of the high molecular weight peptide was identical with the value calculated for the equimolar mixture of CN1 and CN6 (Table III). This supports our earlier interpretation that the 17K peptide is a cross-linked complex between CN1 and CN6. It should also be noted that in Table III the amounts of CN1 and CN1·CN6 recovered were 9.7 and 9.9 nmol, respectively. As already mentioned, the 60K peptide was the cross-linked homodimer of 24K fragments. Therefore, this peptide contains two identical CN1 regions. Since the amounts of CN1 and CN1·CN6 were equal, we concluded that only one of the two CN1 regions in the 60K peptide cross-linked with the CN6 region.

EDC Treatment of Acto–HMM Complexes Mixed at Different Molar Ratios of Actin/HMM Head. To determine whether the reaction of EDC predominantly occurs between the two heads of the same HMM molecule or between two heads of different HMM molecules, we examined the effect of the molar ratio of actin/HMM head (1–8) on the effectiveness of myosin head cross-linking. $NaDodSO_4$ gel electrophoretic analysis of the reaction products (Figure 4) showed that the yield of the cross-linked peptides of both 60K and 115K was not significantly changed between molar ratios of 1 and 4 and that the depression of the yield was very slight even at a molar ratio of 8, where HMM molecules were sparsely attached to actin filaments. High yield of the

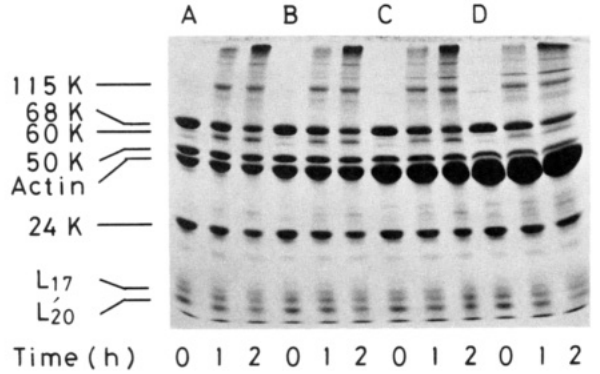

FIGURE 4: $NaDodSO_4$ gel electrophoretic patterns showing the effect of the molar ratio of actin/HMM head on the cross-linking reaction of the acto–tryptic gizzard HMM complex with EDC. The AEDANS-modified derivative of tryptic gizzard HMM (0.5 mg/mL) was mixed with skeletal F-actin at the molar ratio of actin/HMM head = 1 (A), 2 (B), 4 (C), or 8 (D). The cross-linking reaction was carried out as specified under Materials and Methods for 0 (not cross-linked), 1, or 2 h as indicated at the bottom of each lane. Each gel sample contained 10 μg of HMM. Gels were stained with Coomassie blue. The amount of the 60K cross-linked product remained high even in (D), indicating that the two heads involved in the cross-linking reaction are on the same HMM molecule.

Table III: Amino Acid Compositions of CN1·CN6 and CN1 Obtained by Sephadex G-75 Gel Filtration of Peak 5 in Figure 1B[a]

	CN1·CN6	CN1
Asp	14.37 (14)	10.94 (11)
Glu	18.48 (18)	12.68 (12)
cmCys[b]	0.48 (1)	
Ser	9.62 (10)	5.69 (6)
Gly	7.09 (7)	3.02 (3)
HSE[c]	0.68 (1)	0.65 (1)
His	2.01 (2)	1.01 (1)
Arg	1.43 (1)	0.39
Thr	5.12 (5)	2.27 (2)
Ala	8.84 (8)	5.21 (5)
Pro	3.86 (3)	3.19 (3)
Tyr	1.09 (1)	
Val	8.98 (10)	6.35 (7)
Ile	3.82 (4)	2.02 (2)
Leu	8.93 (9)	6.15 (6)
Phe	4.20 (4)	3.57 (4)
Trp	*d* (2)	*d* (2)
Lys	15.47 (17)	11.74 (12)
total no. of amino acids	117	77
amount recovered (nmol)	9.7	9.9

[a] The numbers of amino acid residues in parentheses are values from the sequence data (Maita et al., 1987). [b] *S*-(Carboxymethyl)cysteine. [c] Homoserine. [d] Present but not quantitated.

cross-linked products at such a high molar ratio of actin/HMM head suggests that the majority of the cross-linked heads belong to the same HMM molecule, not to two different HMM molecules.

Separation of HMM with Cross-Linked Heads from the Reaction Mixture. After ultracentrifugation of the EDC reaction mixture in the presence of Mg-ATP, both the pellet and the supernatant were analyzed by $NaDodSO_4$ gel electrophoresis (Figure 5). Before the treatment with EDC, most of the HMM components (L_{20}, L_{17}, 24K, 50K, and 68K) were found in the supernatant (lanes A and a). After 1-h treatment, about a half of the total HMM was cross-linked with F-actin. Thus, equal amounts of HMM components were present in both the pellet and the supernatant (lanes B and b). No 115K peptide was observed in the supernatant (lanes B and b). Nevertheless, HMM components in the supernatant contained a significant amount of fluorescent 60K peptide (lanes B and b). These results suggest that the fraction of HMM that was covalently bound to F-actin is completely sedimented by centrifugation, whereas the fraction of HMM with cross-linked heads remains suspended in the supernatant. After 2-h treatment, the 60K peptide could be still observed in the supernatant (lanes C and c).

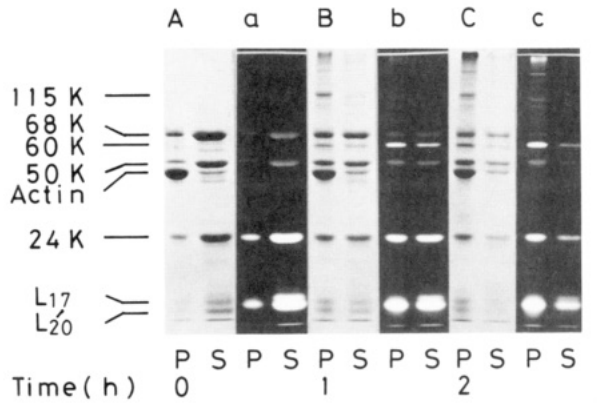

FIGURE 5: $NaDodSO_4$ gel electrophoretic patterns of the EDC cross-linked reaction products between the AEDANS-modified derivative of tryptic gizzard HMM (1.0 mg/mL) and skeletal F-actin at a molar ratio of actin/HMM head = 1. After the cross-linking (see Materials and Methods) for 0 (not cross-linked), 1, and 2 h as indicated at the bottom of the figure, the reaction mixture in 0.1 M KCl, 2 mM $MgCl_2$, 0.3 mM DTT, and 10 mM Tris-HCl (pH 7.5) was mixed with 1.25 mM ATP and then centrifuged at 150000*g* for 90 min. The pellet (P) and the superanatant (S) were submitted to $NaDodSO_4$ gel electrophoresis. Gels were stained with Coomassie brilliant blue (lanes A–C). The fluorographs were also taken by illuminating the gels with a UV lamp, before Coomassie blue staining, to detect AEDANS fluorescence (lanes a–c).

Cross-Linking of the Acto–Gizzard S-1 Complex with EDC. Although the EDC-catalyzed cross-linking between gizzard myosin single heads (S-1) and F-actin was demonstrated (Marianne-Pépin et al., 1985), no zero-length cross-linking between two S-1s that are bound to F-actin has so far been reported with either skeletal or gizzard S-1. The absence of cross-linking between two S-1 molecules may be due to the experimental condition used by the French investigators. Thus, we modified conditions of cross-linking experiments and reinvestigated whether or not two S-1s could be cross-linked by EDC (Figure 6). In our EDC cross-linking experiments, trypsin-fragmented gizzard S-1 was used. Since we focused on the interaction between two S-1s, the trypsin-fragmented S-1 was first mixed with skeletal F-actin and then treated with EDC.

Experiments were performed by using the combination of either actin and fluorescent S-1 (lanes B and b) or fluorescent actin and S-1 (lanes C and c). The protein band profiles of $NaDodSO_4$ gel electrophoresis (lanes B and C) indicated that, in both combinations, the reaction led to the formation of two new major products of M_r 60K and 68K. The cross-linking between actin and fluorescent S-1 produced the 60K fluorescent species (lanes b). This product had the same electrophoretic mobility as the 60K fluorescent species obtained by the EDC treatment of the acto–tryptic gizzard HMM complex (lanes A and a). On the other hand, the combination of fluorescent actin and S-1 resulted in the formation of the 68K fluorescent species (lanes c). This latter experiment indicates that the 60K product does not contain actin. Although data were not shown, we have also examined the EDC-catalyzed cross-linking of the rigor complex between F-actin and papain-digested gizzard S-1, of which the COOH-terminal 22K heavy chain fragment was labeled with fluorescent AEDANS, but the NH_2-terminal 72K heavy chain fragment was not. The

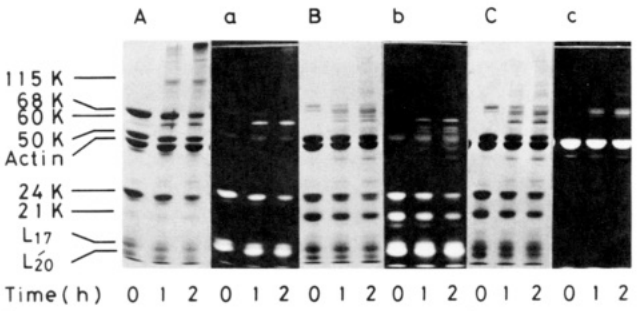

FIGURE 6: $NaDodSO_4$ gel electrophoretic patterns of carbodiimide cross-linked products between skeletal muscle F-actin and tryptic gizzard S-1. The cross-linking reaction was carried out for 0 (not cross-linked), 1, or 2 h as indicated in the figure. Coomassie blue stained gels (lanes A–C) and their fluorographs (lanes a–c) are shown as pairs. Lanes A and a: The AEDANS-modified tryptic HMM (0.5 mg/mL) was mixed with F-actin at a molar ratio of actin/HMM head = 1. Lanes B and b: The AEDANS-modified tryptic S-1 (0.35 mg/mL) was mixed with F-actin at a molar ratio of actin/S-1 = 1. Lanes C and c: Tryptic S-1 was mixed with F-actin as specified in lanes B and b, except that the S-1 was unmodified and the actin was made fluorescent with DACM. The cross-linking with EDC was carried out as described under Materials and Methods. Each gel sample contained 10 μg of HMM or S-1. The 21K band contains the COOH-terminal 21K segment of the S-1 heavy chain as a major component. However, the 21K band in lane b appears to be contaminated with a fluorescent species converted from the NH_2-terminal 24K segment of the S-1 heavy chain.

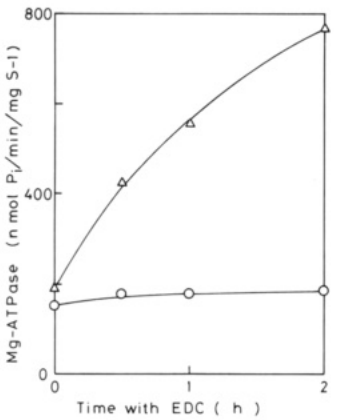

FIGURE 7: Enhanced Mg-ATPase activity of acto–gizzard S-1 complex induced by the chemical cross-linking. Tryptic gizzard S-1 (0.35 mg/mL) mixed with skeletal F-actin at a molar ratio of actin/S-1 = 1 (△) or without actin (○) was reacted at 24 °C with 5 mM EDC as specified under Materials and Methods. At time 0 h (not cross-linked), and at 0.5, 1, and 2 h following cross-linking, aliquots were withdrawn and mixed with excess 2-mercaptoethanol. Proteins (0.07 mg of S-1) were submitted to Mg-ATPase assay.

69K cross-linked product thus obtained was found to be fluorescent. On the basis of these observations, we concluded that the 60K species was the same cross-linked complex between two NH_2-terminal 24K fragments as already shown in the cross-linking experiments of the acto–HMM complex with EDC, whereas the 68K species (or the 69K species when papain-digested S-1 was used) was a cross-linked complex of actin and the COOH-terminal 21K tryptic fragment (or 22K papain fragment) of the S-1 heavy chain.

Mg-ATPase Activity of the Cross-Linked Acto–Gizzard S-1 Complex. The gizzard S-1 covalently cross-linked to F-actin showed an enhanced Mg-ATPase activity (Marianne-Pépin et al., 1985). Figure 7 illustrates the similar enhancement of the activity induced by the cross-linking of the acto–tryptic gizzard S-1 complex with EDC.

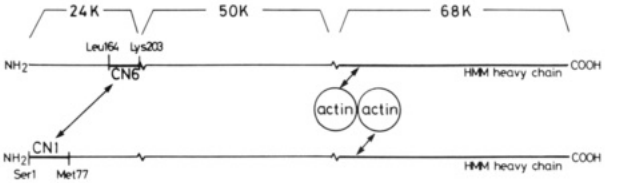

FIGURE 8: Schematic representation of the two covalent cross-linking sites by carbodiimide between two heavy chains (long double-headed arrow) and between actin and a myosin head (short double-headed arrows). CN1 and CN6 are fragments containing Ser 1–Met 77 and Leu 164–Lys 203, respectively, produced from the NH_2-terminal 24K segment of the HMM heavy chain by CNBr cleavage. Wavy lines indicate tryptic cleavage sites in the HMM heavy chain, so that three heavy chain fragments of 24K, 50K, and 68K are generated by tryptic digestion of HMM.

Discussion

In the preceding paper (Onishi et al., 1989) and the present paper, the protein–protein contact regions in the rigor complex between skeletal muscle F-actin and gizzard HMM were investigated by using a zero-length cross-linker EDC. To identify the location of the contact regions in HMM heads, the HMM heavy chains were cleaved with trypsin into three fragments of 24K, 50K, and 68K. These fragments were noncovalently bound with each other and retained the original HMM structure (Marianne-Pépin et al., 1983). This HMM could interact with actin to form the rigor complex. Two new polypeptide species were generated by the cross-linking reaction of the rigor complex with EDC. The 115K species was the covalent complex between the 68K fragment and actin (short double-headed arrows in Figure 8). We therefore concluded that two HMM heads were in contact with actin at their 68K heavy chain segments.

The 60K species was the covalently linked homodimer of 24K fragments. By reverse-phase HPLC (see Figure 1A) and subsequent gel filtration (see Figure 3) of the CNBr-cleaved fragments of the 60K product, the CN1·CN6 complex was isolated. Since each 24K fragment consists of CN1 and CN6 regions, it may be expected that 2 mol of the CN1·CN6 complex are formed from 1 mol of the 60K product. However, we found that only one of the two CN1s in the 60K species cross-linked with CN6 to form the CN1·CN6 complex and the other CN1 was isolated by itself (see Table III). Therefore, there is only one cross-link within in the 60K species (long double-headed arrow in Figure 8). Since CN1 and CN6 were localized at different positions in the primary sequence of the 24K segment as shown in Figure 8 (Maita et al., 1987), the two contact regions are different parts in the HMM head. The CN1·CN6 complex was the only detectable product of cross-linking between the two heads. Therefore, it seems likely that the two HMM heads are in contact with each other at specific sites. Previous cross-linking studies of scallop myosin indicated that interhead regulatory–regulatory light chain cross-linking occurred in the same myosin molecule (Hardwicke & Szent-Györgyi, 1985). Our finding is different from theirs, in that a zero-length cross-linker was used, the sites of cross-linking were located on the myosin heavy chain, and the attachment of myosin heads to F-actin was required for the cross-linking.

As Maita et al. (1987) have shown, CN6 is a 40-residue peptide from Leu 164 to Lys 203 (Figure 8). This peptide contains the consensus glycine-rich sequence (GXXGXGKT) present in many nucleotide-binding enzymes, which was first proposed by Walker et al. (1982). The crystal structure analysis of adenylate kinase (Pai et al., 1977) revealed that the adenine nucleotide binding site consisted of a β-sheet, a

glycine-rich loop, and an α-helix. The amino acid sequence of CN6 from gizzard myosin indicates that the polypeptide contains amino acid sequence capable of forming a β-sheet (residue numbers 171–176), a loop (residue numbers 177–184), and an α-helix (residue numbers 185–198) (Maita et al., 1987). A glutamic acid residue in the *Acanthamoeba* myosin was covalently labeled with UTP when irradiated with a UV lamp (Atkinson et al., 1986). On the basis of the sequence homology between *Acanthamoeba* and gizzard myosins, this residue corresponds to the glutamic acid at the position 186 in the sequence of gizzard myosin (Maita et al., 1987). Because the consensus glycine-rich sequence and UTP-photolabeled glutamic acid were localized in the CN6 region, it is suggested that this region contributes to an adenine nucleotide binding. The present finding that CN6 was cross-linked with CN1 indicates that, within an HMM molecule, one contact region is located near the nucleotide binding site.

The other cross-linking site was present in the NH_2-terminal end region of the HMM heavy chain (Figure 8), because CN1 was identified as a peptide containing Ser 1–Met 77 by Maita et al. (1987). There are several pieces of evidence suggesting that the NH_2-terminal end region of the HMM heavy chain influences the nucleotide binding of myosin and the interaction of myosin with actin. (1) Gizzard myosin heavy chains can be cleaved by α-chymotrypsin or papain within their CN1 regions (Okamoto & Sekine, 1981a; Bonet et al., 1987; Onishi & Watanabe, 1984), and the rate of cleavage is significantly accelerated if the nucleotide binding site of gizzard myosin is occupied by the substrate (Okamoto & Sekine, 1981a; Onishi & Watanabe, 1984). (2) The actin-activated Mg-ATPase of the gizzard HMM which was cleaved within CN1 regions showed the maximum turnover rate different from that of native HMM (Okamoto & Sekine, 1981b). At present, we are unable to determine if the contact between two heads as we have found in this study produces an effect on the interaction of HMM heads with actin. However, since one contact region appears to be close to the nucleotide binding site, the contact between two heads may affect the enzymatic properties of acto–gizzard HMM. Gizzard HMM, of which the two heads are covalently cross-linked, but not with actin (see Figure 5), may provide a valuable tool for investigating this problem.

In the present study, we also examined if the link between two myosin heads via the neck is essential for the EDC-catalyzed head-to-head cross-linking. The 60K species was produced by the treatment with EDC between S-1 fragments (see Figure 6). We thus concluded that the link via a neck is not absolutely necessary for cross-linking two HMM heads. In contrast, the attachment of HMM heads to actin was essential for the cross-linking between two HMM heads as shown by Onishi et al. (1989). However, from these experiments, it is not clear whether the cross-linked HMM heads belong to the same HMM molecule or different molecules. In order to determine which case occurs more efficiently, the cross-linking reactions were carried out at different molar ratios of actin/HMM head (see Figure 4). A detectable amount of 60K species was observed even at high actin/head ratios where different HMM molecules are rarely bound to adjacent actin subunits. This suggests that cross-linking occurs more efficiently between two heads of the same HMM molecule than between two heads of different HMM molecules.

Intermolecular cross-links between S-1 molecules and between F-actin and S-1 have previously been reported (Labbé et al., 1982). These authors used bifunctional reagents with 11-Å arm length, bis imidoesters, and skeletal acto–S-1. However, no zero-length cross-linking between two S-1s has so far been reported with skeletal acto–S-1.

From the CNBr fragments of the 24K peptide, an equimolar mixture of CN3 and CN6 could be separated from pure CN3 and CN6 by reverse-phase HPLC (see Figure 1A and Table I). Although these CNBr fragments were already denatured in 0.1% trifluoroacetic acid, this result suggests possible interaction between CN3 and CN6. CN3, a peptide consisting of residues Ala 89–Met 139, is extremely hydrophobic. The 34-residue sequence from Tyr 107 to Tyr 140 was devoid of charged side chains except for N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine 127 (Maita et al., 1987). This region could comprise a hydrophobic pocket for the binding of the myosin head, as proposed first by Tong and Elzinga (1983). Since CN6 also contributes to the adenine nucleotide binding site, the CN3–CN6 interaction may be involved in building the ATPase site in the native myosin head.

We have not yet obtained evidence that direct contact between two myosin heads occurs in cross-bridges of the contracting muscle. Nevertheless, we propose a hypothesis that the two heads of a myosin molecule interact with each other in the cross-bridge. This hypothesis provides a long-sought explanation for the fact that each myosin molecule has two heads.

ACKNOWLEDGMENTS

We thank Drs. Hironobu Sunada and Michitaka Masuda for their helpful discussions and Dr. Tadashi Tanabe for his valuable advice in analyzing amino acid compositions.

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Intermediates on the Reassociation Pathway of Phosphofructokinase I from *Escherichia coli*†

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Received July 5, 1988; Revised Manuscript Received October 5, 1988

ABSTRACT: The folding and association pathway of the allosteric phosphofructokinase from *Escherichia coli* has been investigated after complete denaturation of the protein in guanidine hydrochloride by spectroscopical methods, fluorescence and circular dichroism. Three successive processes can be observed during the renaturation of this protein. First, a fast reaction, detected by fluorescence, results in the formation of a (partially) structured monomer. Second, two monomers associate into a dimeric species. This step involves the shielding of the unique tryptophan residue, Trp 311, from the aqueous solvent, and it corresponds to the formation of the interface containing the effector binding site. The presence of ATP during renaturation increases the rate of formation of this dimeric species. The other ligands of the enzyme have no effect on this reaction as well as on the whole reactivation. Finally, the enzymatic activity is regained during the third slowest step. This last reaction is due to the association of two dimers into the native tetrameric structure. The presence of fructose 6-phosphate does not increase the rate of reactivation, even though this ligand strongly stabilizes the native enzyme against denaturation by bridging the interface corresponding to the active site. The self-assembly of phosphofructokinase from *E. coli* from its unfolded and separated chains follows a specific order in the formation of the interactions between subunits and involves a dimeric intermediate with a defined geometry.

Phosphofructokinase (PFK)[1] catalyzes the phosphorylation of fructose 6-phosphate (F6P) by adenosine triphosphate (ATP) to form fructose 1,6-bisphosphate (F-1,6-DP) in a reaction which controls the glycolysis in the cell:

$$\text{F6P} + \text{ATP} \xrightarrow{Mg^{2+}} \text{F-1,6-DP} + \text{ADP} \qquad (1)$$

In *Escherichia coli* the activity of PFK is regulated by different allosteric effectors; it is activated by the purine nucleotide diphosphates, ADP and GDP, and inhibited by phosphoenolpyruvate. Most of the enzymatic properties of *E. coli* PFK are consistent with a concerted model (Monod et al., 1965): the enzyme exists in two different conformational states in equilibrium, with the same catalytic efficiency and affinity for ATP and with largely different affinities for F6P and/or the effectors (Blangy et al., 1968).

The enzyme from *E. coli* is a tetramer of identical polypeptide chains of molecular weight 34 817. The complete amino acid sequence of 320 residues, deduced from the nucleotide sequence of the *pfK* A gene, is known (Hellinga & Evans, 1985). X-ray diffraction studies (Shirakihara & Evans, 1988) show that each subunit consists of two domains, with a cleft between them which forms the active site. Each subunit is in close contact with only two of the other subunits in the tetramer. The F6P binding site belongs to one of these contact areas, whereas the effector binding site belongs to the other. In the case of an oligomeric enzyme such as PFK, the reconstitution of the native quaternary structure after complete unfolding in guanidine hydrochloride must be the consecutive folding and association of the polypeptide chains (Jaenicke, 1982). The simplest scheme which describes the self-assembly of PFK involves the folding of the monomers followed by two successive association steps:

$$4\text{U} \rightarrow 4\text{M} \rightarrow 2\text{D} \rightarrow \text{T} \qquad (2)$$

In this model U and T stand for the enzyme in its unfolded and native state, M stands for the monomeric protein, and D stands for the dimeric protein. It was recently shown that PFK from *E. coli* can be reassembled into a fully functional species after separating and unfolding its constituent polypeptide chains. The enzyme activity was quantitatively recovered as well as the allosteric behavior (Martel & Garel, 1984). The kinetics of reappearance of the native PFK can be described by a biphasic mechanism composed of a first-order reaction, which is generally attributed to the monomolecular fold-

† This work was supported by the Centre National de la Recherche Scientifique and the Université Paris 6 (UER 58). W.T. is a recipient of an EMBO long-term fellowship.

[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); ANS, 8-anilino-1-naphthalenesulfonic acid; F6P, D-fructose 6-phosphate; DTT, DL-dithiothreitol; Gdn-HCl, guanidine hydrochloride; CD, circular dichroism.

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