Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 466-475

PROXIMITY OF ALKALI LIGHT CHAINS TO 27K DOMAIN OF THE HEAVY CHAIN IN MYOSIN SUBFRAGMENT 1

J.P. LABBE, D. MORNET, P. VANDEST and R. KASSAB

Centre de Recherches de Biochimie Macromoléculaire du C.N.R.S.
Route de Mende B.P. 5051 34033 Montpellier Cedex, France

Received June 24,1981

SUMMARY.- When myosin chymotryptic subfragment-1 was treated with dimethylsuberimidate or dithiobis (succinimidylpropionate) under nearly physiological ionic conditions, the alkali light chains A1 and A2 were selectively and intramolecularly cross-linked to the 95K heavy chain. Experimental conditions were developed with both reagents for optimal production of A1 and A2-containing dimers. After conversion of reversibly cross-linked S-1 (A1+A2) into (27K-50K-20K)-S-1 derivative by restricted tryptic proteolysis, the light chains were found to be attached to the NH_2-terminal 27K segment of the heavy chain.

(S-1) of skeletal muscle myosin contains the ATPase catalytic site and the actin binding site (1,2). It provides the simplest structural model for the study of the myosin head assembly. S-1 is an oligomeric protein made up of a heavy chain of Mr 95K and an alkali light chain of Mr 20,7K (A1) or 16,5K (A2) (3,4). The light component is an asymmetric polypeptide (5) the removal of which is accompanied by loss of ATPase activity; it does modulate the interaction of actin with myosin head both in the absence and in the presence of Mg^{2+}-ATP (4,6). However, the molecular basis of these important functions, in particular the positioning of the light subunit within the head and its critical binding interactions with the heavy chain are unknown. Distances at the molecular level between thiol groups in the two subunits were recently investigated by spectroscopic techniques (7,8). Intramolecular chemical cross-linking of the two subunits represents an alternative powerful approach towards a solution of this problem. The cross-linking reactions previously applied to analyse the radial disposition of cross-bridges within myosin filaments (9-11) and to probe the dynamic contacts between heads within myosin in solution (12) have failed to reveal internal cross-linking of the heavy chain to the light subunits. In the following study we provide evidence that, under appropriate and mild experimental conditions, intramolecular covalent cross-links, induced by two different bifunctional reagents, can be established between the alkali light chains and the 95K heavy chain of native chymotryptic S-1. This unprecedented light chain-heavy chain chemical cross-linking has

0006-291X/81/170466-10$01.00/0

allowed us to characterize for the first time the possible interaction of the light chains with the NH_2-terminal 27K segment of the heavy chain; earlier, this portion of the head was proposed as a potential component of myosin ATPase site (13,14).

MATERIALS AND METHODS

Enzyme preparations : Rabbit skeletal muscle myosin was prepared according to Offer et al. (15); S-1 was prepared by digestion of myosin filaments with chymotrypsin (2), purified as a mixture of S-1 isoenzymes (S-1 A1+A2) by gel filtration over Sephacryl S-200 eluting with 50 mM Tris HCl, pH 8,2 and resolved into pure S-1 A1 and S-1 A2 species by ion exchange chromatography (2). S-1 solutions were dialyzed overnight at 4°C against the appropriate buffer of cross-linking experiments and were used within 2 days of preparation. Enzyme concentration was determined by spectrophotometry assuming an $E^{1\%}_{280\ nm}$ = 7.5 cm^{-1} M^{-1} (4) and a molecular weight of 115.000 (2). Limited tryptic proteolysis of cross-linked S-1 was carried out, after dialysis against 0.1 M potassium bicarbonate pH 8, as described (16) using a protease to S-1 weight ratio = 1:10.

Cross-linking reactions with dimethyl suberimidate and dithiobis (succinimidyl-proprionate) : S-1 (1 mg/ml) was amidinated in 100mM triethanolamine-HCl, pH 8,5 at 20°C with varying concentrations of dimethyl suberimidate (0-4 mg/ml) and for different reaction times (0-60 min.). Stock solutions (0.07M) of DMS in 100 mM triethanolamine pH 8,5 were prepared immediately before use. The cross-linking reaction could be quenched at any time by adjusting the solution to 100 mM glycine (pH 8.0). 50 µl samples were subsequently withdrawn and were immediately prepared for electrophoresis by the addition of an equal volume of 10% $NaDodSO_4$, 10% mercaptoethanol solution and incubation for 2 min. at 100°C.

S-1 (1 mg/ml) was acylated in 100 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) buffer, pH 7,5 at 20°C with varying concentrations of dithiobis (succinimidylproprionate) (0-0,5 mg/ml) and for various reaction times (0-15 min.). Stock solutions (0,05 M) of DSP were made in dry acetone immediately prior to use; the amount of acetone introduced into the sample solution never exceeded 5% (v/v) and had no apparent effect on the enzyme as judged by measurements of ATPase activities. The reactions were terminated by the addition of 0,1 volume of 1 M glycine and 50 µl aliquots were taken, mixed with an equal volume of 10% $NaDodSO_4$ solution (without mercaptoethanol) and denatured by heating at 37°C for 90 min. (17).

$NaDodSO_4$ polyacrylamide gel electrophoresis : one dimensional $NaDodSO_4$ electrophoresis was carried out in the slab gel mode (18) (15x15x0,3 cm) according to Laemmli (19). The acrylamide and bisacrylamide concentrations were 10% and 0.28% respectively; reducing agents were omitted with proteins cross-linked by DSP. Gels were run for 18 h. at 4 mA. They were stained with Coomassie Blue R-250 and destained in 10% acetic acid. A Joyce-Loebel densitometer was used for scanning gels. Quantification of protein bands on gels of cross-linked samples was performed and expressed according to Sutoh and Harrington (9) with actin as internal standard protein.
The molecular weight of the cross-linked heavy chain-light chains species was estimated on 10% polyacrylamide gels calibrated with the following marker proteins : phosphorylase b (20); α and β subunits of phosphorylase b kinase (21); skeletal muscle myosin light chain kinase and chymotryptic S-1 heavy chain (22). Two-dimensional diagonal gel electrophoresis was accomplished as follows : an 8 mm wide strip was cut from an unstained first-dimension gel (5-18% gradient acrylamide) and placed horizontally above a 1 cm high top gel layer which was 1% (w/v) agarose in the $NaDodSO_4$ buffer containing 10% (v/v) mercaptoethanol.

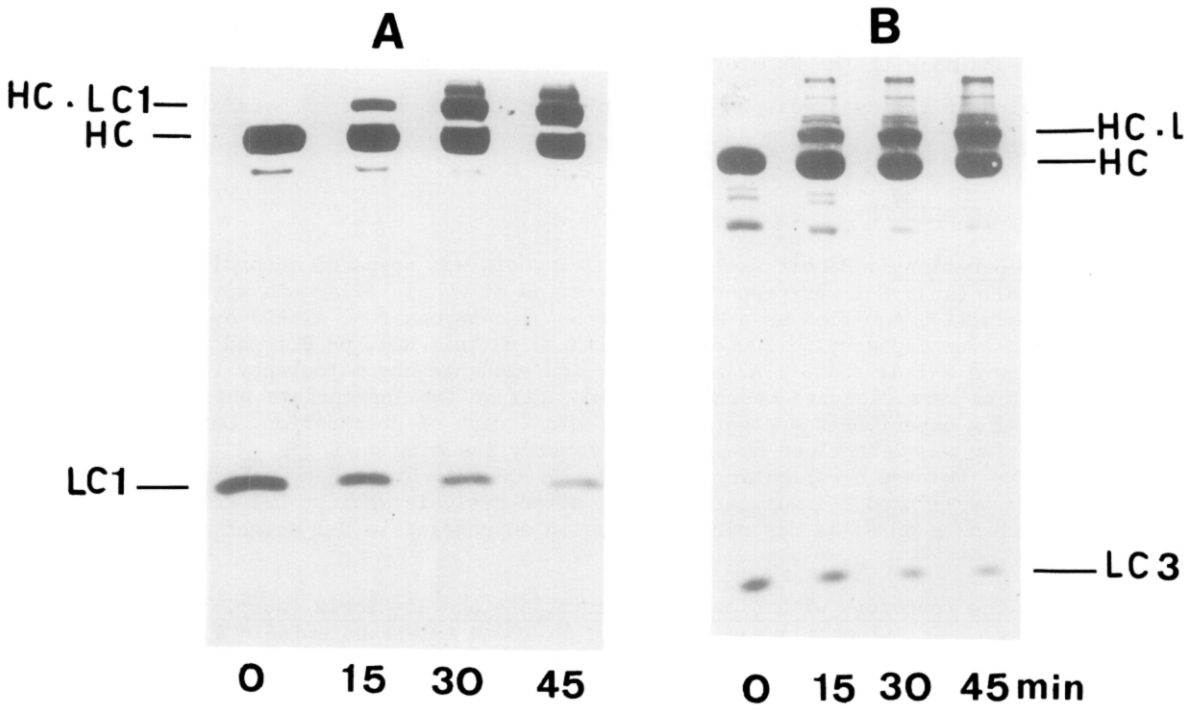

Figure 1.- Dimethyl suberimidate-induced cross-linking of the heavy and light chains in myosin S-1. A/ S-1 (A1) B/ S-1 (A2) (1 mg/ml) were treated with DMS (0.200 mg/ml) in 100 mM triethanolamine pH 8,4, 20°C. Samples were withdrawn at the times indicated and analyzed on $NaDodSO_4$- 10% acrylamide slab gels. All lanes contained 25 μg of protein; HC = 95K heavy chain; HC-LC = presumed heavy chain-light chain cross-linked species.

A lower layer of 5-18% polyacrylamide slab gel was then cast underneath the reducing agarose gel and used as a second dimension gel. The slabs were run 2 h. at 2 mA to ensure cleavage of disulfide-containing cross-links and then 18 h. at 4 mA.
Ammonolysis of DMS-treated samples was attempted by soaking the first dimension gel strip in a solution containing 35% ammonium hydroxyde, 10% $NaDodSO_4$, glacial acetic acid (12:1:1, v/v) for 24 h. at 25°C (23).

RESULTS

Cross-linking of S-1 isoenzymes with dimethyl suberimidate : When a mixture of S-1 isoenzymes (A1+A2) or when pure S-1 (A1) and S-1 (A2) (Fig.1A and 1B) were treated with DMS under approximately physiological ionic conditions excluding intermolecular interactions between S-1 molecules and using an optimal weight ratio of reactants (100 mM triethanolamine, pH 8, 20°C, 0.200 mg reagent for 1 mg protein/ml) a major new protein band, with a mobility lower than the initial 95K heavy chain, appeared on $NaDodSO_4$- 10% polyacrylamide gels; its apparent mass was approximately 120K. Examination of the time course of changes in the electrophoretic pattern of the enzyme (Fig.1A) showed clearly that conco-

mitantly with the formation of this component there was a significant progressive decrease of the intensity of the bands corresponding to the original heavy and light chains. No significant alteration in this pattern was noticed when the salt concentration was increased to 200 mM nor when the protein concentration was lowered to 0.500 mg/ml. DMS was without effect on the electrophoretic profile of native S-1 when it was preincubated for 30 min. at 20°C, pH 8,5 before being added to the enzyme.

The reaction with fresh DMS produced also a small amount of polymers appearing at the top of the gels; the level of this material was substantially increased by the use of reaction times exceeding 30 minutes or reagent concentrations higher than 0.200 mg for 1 mg protein. The two latter non optimal conditions resulted also in some diffusion and spreading of the major protein band in the gel. Quantitative scanning of the gels of DMS-treated S-1 (A1) showed that the 95K heavy chain and A1 were lost at about the same rate which was also quite similar to the rate of appearance of the new protein species in the cross-linking reaction. Multiple estimations based on band intensities corresponding to the free and covalently combined subunits of S-1 (A1) present in the gel indicated that the maximal extent of DMS-induced cross-linking was 40% $\pm$ 5%. This yield could not be improved by supplementing the reaction medium with fresh DMS after an initial 30 min. cross-linking period. In contrast, it was greatly diminished when aged S-1 preparations were employed. Our best results were obtained with one day-old S-1 material. As expected from the pH-dependence of the reaction of imidoesters with amino groups, the extent of cross-linking decreased significantly below pH 8 (10% and 20% at pH 7,0 and 7,5 respectively for S-1 (A1). Lowering the temperature to 2°C did not change the extent of modification observed at 20°C.

Because the electrophoretic mobility of cross-linked proteins may be changed (24), another cross-linking approach was developped to identify unequivocally the protein content of the 120K species.

Reversible cross-linking of S-1 (A1+A2) with the cleavable dithiobis (succinimidylproprionate) : This disulfide cross-linking agent offers the advantages of a different reactive group and a longer lifetime in aqueous solution. The time course of the reaction of DSP with S-1 (A1+A2) under optimized experimental conditions (100 mM Hepes, pH 7,5, 20°C, weight ratio of cross-linker to S-1, 0,025 : 1) was studied using gradient gel electrophoresis (Fig.2). The band pattern showed the formation of two components in the molecular weight range of 120K; these were clearly distinguishable at an early stage of the reaction (1 min.)

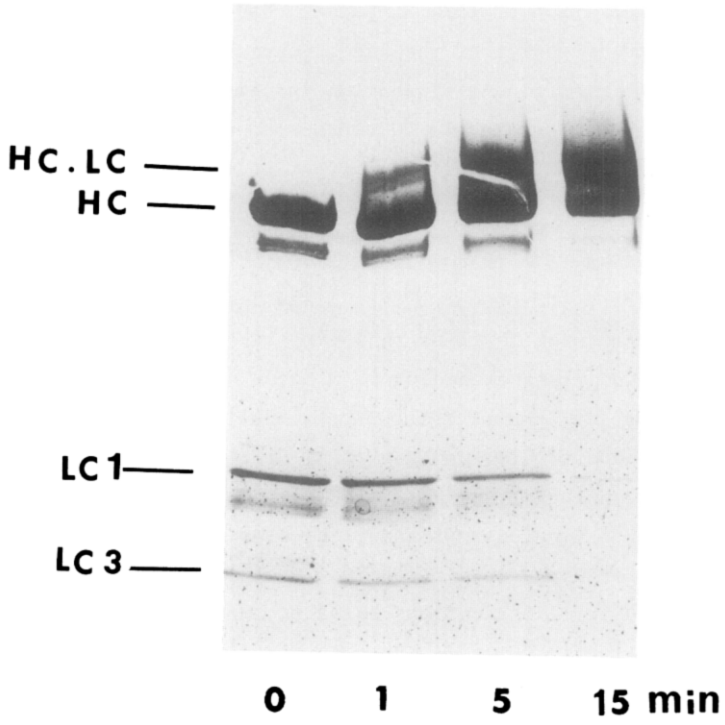

Figure 2.- Acylation of S-1 (A1+A2) with dithiobis (succinimidylpropionate). S-1 (A1+A2) (1 mg/ml) was treated with DSP (0.025 mg/ml) in 100 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid buffer, pH 7,5, 20°C. At the times indicated samples were taken for analysis on 5-18% acrylamide gels. All lanes contained 50 µg protein.

The DSP-induced reaction was of much greater efficiency than observed with DMS as the maximal intensity of the 120K band was reached within a 15 min. period using 8-fold less reagent.

Protein content of the DSP-cross-linked 120K species : A direct evidence that the 120K product was a cross-linked dimer of 95K heavy chain and alkali light chain was provided by a diagonal electrophoretic analysis of the enzyme treated for 5 min. with DSP. This reaction time was selected in order to minimise the formation of polymers; it provides also sufficient amount of cross-linked light chains for clear identification on the gels. Upon reduction of the cross-links, the 120K band was quantitatively converted into 3 off-diagonal proteins migrating identically to the original 95K heavy chain and A1 + A2 subunits (Fig. 3). When the dimethyl suberimidate-cross-linked enzyme was subjected to diagonal analysis (substituting ammonolyse for disulfide reduction) a fractional but distinguishable release of the same components from the 120K species was also obtained.

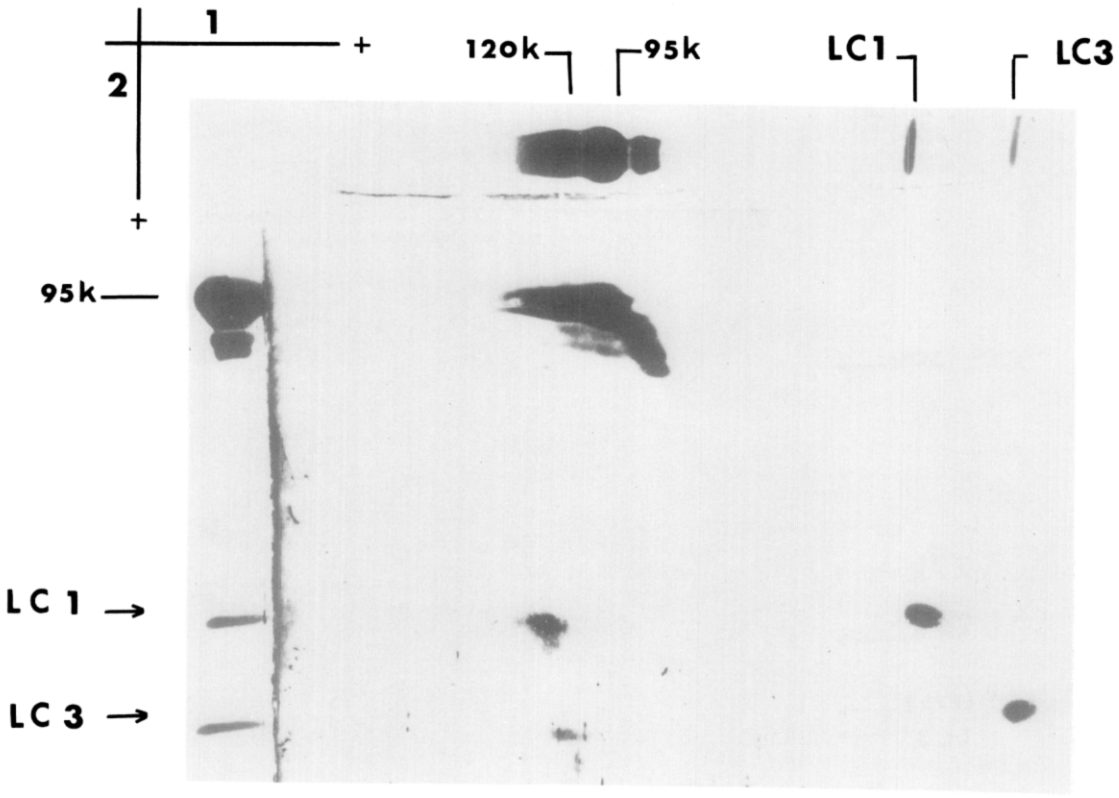

Figure 3.- Diagonal analysis of DSP-cross-linked S-1 (A1+A2). An unstained sample lane was cut out from a first-dimension gel, embedded above a two-layer gel slab and reelectrophoresed at 90° to the original direction to display proteins released by thiolysis of DSP cross-links in the upper agarose gel layer. A stained duplicate strip of the first-dimension gel was placed over the cross-linked products. A stained lane of native S-1 (A1+A2) is shown at left for comparison.

Identification of the heavy chain segment cross-linked to the alkali light chains : Restricted tryptic proteolysis of native chymotryptic S-1 (A1+A2) converts the enzyme into (27K-50K-20K)S-1 derivative the heavy chain of which is a complex of three fragments (14,16); while the A2 light chain remains intact, the A1 subunit is degraded at its lysine-rich NH_2-terminal portion and largely converted into a 17K polypeptide (16). Mindful of these important proteolytic properties, we attempted to identify the heavy chain segment (s) cross-linked to the alkali light chains using diagonal gradient gel electrophoresis of reversibly cross-linked and tryptically fragmented S-1 (Fig.4). When the digest of DSP-cross-linked S-1 was analyzed in the first dimension, its protein band pattern showed two major differences as compared to that of the unmodified enzyme. 1- the amount of 27K fragment was significantly lower. 2- a new protein component was present with Mr 45K comigrating with actin used as marker. Upon

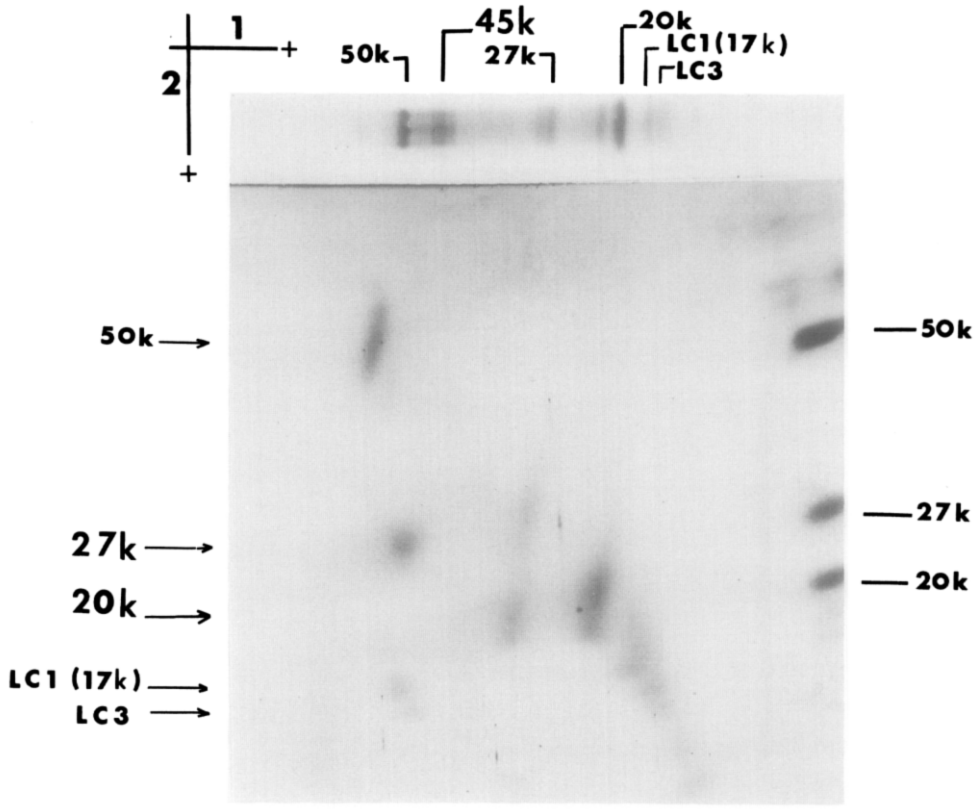

Figure 4.- Diagonal analysis of DSP-cross-linked S-1 (A1+A2) after limited tryptic proteolysis. S-1 was reacted with DSP for 15 minutes. Other experimental conditions were as in Fig.3. A stained lane of (27K-50K-20K)-S-1 (A1+A2), prepared as described (25), is shown at right for comparison.

electrophoresis at right angle in the presence of reductant, this protein entity was entirely split into 3 off-diagonal proteins; one migrated as 27K fragment and the two others as 16K and 17K peptides. The latter two could only represent the A2 light chain and the breakdown product of the labile A1 subunit respectively. A small amount of a signle component migrating as 20K peptide was also observed to lie off-diagonal; in the first dimension it was moving with Mr = 27K. This finding suggest that, before disulfide reduction, a fraction of the 20K fragment was associated with a peptide segment which, owing to its small mass, could not be identified in the free state on the same gel.

DISCUSSION

The present cross-linking study shows the formation, under highly controlled experimental conditions, of a covalent stable complex between the heavy and

alkali light chains A1 and A2 within chymotryptic S-1. The ability of the light subunits to be readily joined to the heavy chain is illustrated by the comparable results obtained with the two different cross-linkers employed, dimethyl suberimidate which bears a straight chain and dithiobis (succinimidyl-proprionate) a reagent with an angled structure (26). Although the yield of cross-linking obtained with both reagents is not quantitative, a substantial amount of covalent heavy chain-light chain dimer can be derived under conditions where formation of high molecular weight polymers is kept minimal. Manipulation of reagent concentration or incubation time resulted in accumulation of heavily cross-linked products in particular when DMS was used. DSP proved to be the most useful cross-linking agent; it induced a large extent of cross-linking between the heavy and light chains with, under the conditions employed very little intramolecular cross-links within the heavy chain as illustrated by the diagonal peptide map of the cross-linked fragmented protein. Cross-linking experiments with bifunctional reagents of different chain length and structure should be useful in probing further the molecular interactions existing between the heavy and light chains.

The heavy chain of skeletal myosin subfragment-1 appears to be composed of three structural domains of Mr 27K, 50K and 20K (27) which offer the possibility for precise characterization of the sites of covalent attachment of the light chains. The use of (27K-50K-20K)-S-1 has revealed unequivocally that the NH_2-terminal 27K segment of the heavy chain was involved in the DSP-induced cross-linking process. Identification of the 27K peptide was made possible because cross-linking occurred between it and the proteolytically stable homologous C-terminal portions of the light chains. The linkage of the basic amino-terminal region of A1 to any heavy chain peptide will be more difficult to assess because it is readily split by trypsin. However, the observed behaviour of the 20K fragment on the gels offers the possibility that cross-linking between it and the NH_2-terminal segment of A1 might have occurred. Alkali light chains are known to affect both the affinity of S-1 to actin and the kinetic parameters of the acto-S-1 ATPase (4). These properties are, most probably, elicited by specific heavy chain-light chain interactions. The NH_2-terminal 27K peptide of S-1 heavy chain contains the site of attachment of an inhibitory arylazido analogue of ATP (13) as well as the reactive lysine residue whose trinitrophenylation specifically alters the ATPase activities (14); in a forthcoming paper we will describe the specific structural changes induced in the 27K domain by metal-nucleotide binding to S-1. The data presented in this work suggest the possible interaction of the alkali light chains with this potent constituent of the myosin ATPase site and provide, for the first time, a molecular basis for their mode of action. The molecular relationship between the

ATPase site and alkali light chains is obvious from a number of previously reported observations; in particular, nucleotide binding to myosin was shown to impair dissociation of the light chains by chaeotropic salts (28) and interaction of Mg-ATP with gizzard myosin is accompanied by structural changes in the 17K subunit (29); also, occupation of myosin ATPase site by nucleotides affects the thiol reactivities in the alkali light chains (30) and enhances the rate of their affinity labeling with a purine disulfide analogue of ATP (31). The proximity of the alkali light chain to the 27K domain rationalizes all these data and may be essential for the function of the actomyosin complex. Studies are underway to assess the impact of the cross-linking events on the functional properties of the heads.

ACKNOWLEDGMENTS. - This research was supported by grants from CNRS and Délégation à la Recherche Scientifique et Technique (Action Concertée n°80.7.0291)/

REFERENCES

1. Jones, J.M. and Perry, S.V. (1966) Biochem.J. 100, 120-130.
2. Weeds, A.G. and Taylor, R.S. (1975) Nature, 257, 54-56.
3. Frank, G. and Weeds, A.G. (1974) Eur.J.Biochem., 44, 317-334.
4. Wagner, P.D. and Weeds, A.G. (1977) J.Mol.Biol., 109, 455-473.
5. Alexis, M.N. and Gratzer, W.B. (1978) Biochemistry, 17, 2319-2325.
6. Winstanley, M.A., Small, D.A. and Trayer, I.P. (1979) Eur.J.Biochem., 98, 441-446.
7. Moss, D.J. and Trentham, D.J. (1979) Int.Congr.Biochem.11th, 203.
8. Marsh, D. and Lowey, S. (1980) Biochemistry, 19, 774-784.
9. Sutoh, K. and Harrington, W.F. (1977) Biochemistry, 16, 2441-2449.
10. Sutoh, K., Yu-Chih Chen Chiao and Harrington, W.F. (1978) Biochemistry, 17, 1234-1239.
11. Yu-Chih Chen Chiao and Harrington, W.F. (1979) Biochemistry, 18,959-963.
12. d'Albis, A. and Gratzer, W. (1976) J.Biol.Chem., 251, 2825-2830.
13. Szilagyi, L., Balint, M., Streter, F.A. and Gergely, J. (1979) Biochem. Biophys.Res.Commun., 87, 936-945.
14. Mornet,D., Pantel, P., Bertrand, R., Audemard, E. and Kassab, R. (1980) Febs Lett., 117, 183-188.
15. Offer, G., Moss, C. and Starr, R. (1973) J.Mol.Biol., 74, 653-676.
16. Mornet, D., Pantel, P., Audemard, E. and Kassab, R. (1979) Biochem. Biophys.Res.Comm. 89, 925-932.
17. Wang, K. and Richard, F.M. (1974) J.Biol.Chem., 249, 8005-8018.
18. Studier, F.W. (1973) J.Mol.Biol., 79, 237-248.
19. Laemmli, U.K. (1970) Nature, 227, 680-685.
20. Titani, K., Koide, A., Hermann, J., Ericsson, C.H., Kumar, S., Wade, R.D. Walsh, K.A., Neurath, H. and Fischer, E.H. (1977) Proc.Natl.Acad.Sci., USA, 74, 4762-4766.
21. Cohen, PH. (1973) Eur.J.Biochem., 34, 1-14.
22. Weeds, A.G. and Pope, B. (1977) J.Mol.Biol. 111, 129-157.
23. Ludwig, M.L. and Byrne, R.(1962) J.Am.Chem.Soc., 84, 4160-4162.
24. Davies, G.E. and Stark, G.R. (1970) Proc.Natl.Acad.Sci.USA, 66, 651-656.
25. Mornet, D., Pantel,P., Bertrand,R., Audemard,E. and Kassab,R. (1981) Febs Lett., 123, 54-58.

26. Jocelyn, P.C. (1972) Biochemistry of the SH-group, Academic Press, New York.
27. Mornet, D., Bertrand, R., Pantel, P., Audemard,E. and Kassab, R. (1981) Biochemistry, 20, 2110-2120.
28. Dreizen, P. and Richards, D.H. (1973) Cold Spring Harbor Symp.Quant.Biol. 37, 29-45.
29. Okamoto, Y. and Sekine, T. (1980) J.Biochem. (Tokyo), 87, 167-178.
30. Yamamoto,K. and Sekine, T. (1980) J.Biochem. (Tokyo), 87, 593-599.
31. Wagner, P.D. and Yount, R.G. (1976) J.Biol.Chem., 251, 5424-5429.