is somewhat anomalous and are presently exploring this point.

In general terms these results agree with the fluorescence results of Klausner & Wolf (1981). As discussed above, the application of simple solution theories to the determination of phase partition in this system is limited. Partition is not sufficiently defined by the effect of solute upon transition temperature. Furthermore, use of fluorescence as a measure of the transition is complicated by the aggregation state and relative fluorescence quantium efficiency of the probe in the two phases as well as by the algorithm used to determine transition temperature. Calorimetric studies typically report T_m as defined here, while fluorescence studies define T_m as the temperature of half-effect (Klausner & Wolf, 1980). Figure 5 suggests that such a definition would considerably alter the dependence of of T_m upon N. Application of fluorescent amphiphiles as probes of domain organization requires a knowledge of both their absolute partition and the partition of fluorescence.

Registry No. DMPC, 18194-24-6; DPPC, 63-89-8; DSPC, 816-94-4; C_{10}DiI, 84109-06-8; C_{12}DiI, 84109-08-0; C_{14}DiI, 84109-10-4; C_{16}DiI, 84109-11-5; C_{18}DiI, 41085-99-8; C_{20}DiI, 84109-13-7; C_{22}DiI, 84109-15-9.

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Mapping of Hydrophobic Sites on the Surface of Myosin and Its Fragments†

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ABSTRACT: The interaction of the hydrophobic probe conjugated polyene fatty acid *cis*-parinaric acid (PA) with myosin and its fragments was studied by measuring the enhancement of PA fluorescence following binding. The measurements point to the presence of about 1.34 hydrophobic sites per mol of myosin or per mol of double-headed fragment heavy meromyosin and 0.65 site per mol of myosin subfragment 1 (S-1). The binding constants were all in the range of 10^7 M^{-1}. The S-1 isoenzyme containing alkali light chain 1 [S-1(A1)] bound strongly close to 1 mol of PA per mol of S-1(A1) while the isoenzyme containing alkali light chain 2 [S-1(A2)] bound no PA. Isolated alkali light chains A1 and A2 bound PA very weakly. Mg–nucleotide binding to the S-1 active site had little effect on the stoichiometry or affinity of PA binding; binding of actin to myosin carrying the probe either in solution or in an organized system of myofibrils had no effect on the magnitude of probe fluorescence but dramatically increased the rotational relaxation time of bound PA. These data indicated that parinaric acid bound to the hydrophobic pocket formed between the 41-residue region at the N-terminal end of A1 and the heavy chain of S-1 and that the hydrophobic pocket is distant from both actin and the nucleotide binding sites.

The question of the effective (surface) hydrophobicity of the contractile proteins has not received much attention in the past. Although many hydrophobic residues are buried in the interior of native proteins, some hydrophobic groups may remain exposed at the molecular surface or in crevices; in fact, the nucleotide binding site of myosin may be such a crevice. It is conceivable that some hydrophobic groups are involved in the hydrolysis of ATP and in the process of force development in muscle. For example, hydrophobic groups may have their accessibility to the solvent changed as a result of orientational alteration of the cross-bridge attitude believed to occur during muscle contraction (Huxley, 1969; Huxley & Simmons, 1971). Further, binding of substrate or change in the area of the

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actin–myosin interface may alter the accessibility to the hydrophobic groups at, or near, the nucleotide binding site or at the point of contact between the thin filament and the tip of the cross bridge. Therefore, the study of the surface hydrophobicity of contractile proteins can potentially furnish useful information about cross-bridge attitude and conformational changes, occupancy of the enzymatic site, and changes at the actin–myosin interface.

A series of original investigations demonstrated the usefulness of the hydrophobic probes in the study of myosin conformation and nucleotide binding: thus, in an investigation using a macromolecular probe [8-anilino-1-naphthalenesulfonate (ANS)][1] which was believed to bind to hydrobhobic regions of proteins [e.g., see McClure & Edelman (1966)], Duke et al. (1966) found a small enhancement of the ANS fluorescence upon binding to native myosin and a very large enhancement upon binding to the enzyme modified by *p*-(chloromercuri)benzoate (PCMB). Lim & Botts (1967) have examined the effects of temperature and aging on the fluorescence of the myosin–ANS complex, and Cheung & Morales (1969) have shown that the efficiency of transfer of the excitation energy in the UV region from myosin chromophores to bound ANS decreased with modification of the enzyme with PCMB. These ANS fluorescence changes were interpreted in terms of the presence of different conformational states of native and modified myosin. Later, also using energy transfer from myosin chromophores to bound ANS, Cheung (1969) for the first time found evidence that myosin suffers a reversible change in localized structure during ATP catalysis.

The present work is concerned with additional aspects of the interaction between contractile proteins and hydrophobic probes. The probe used here, a conjugated polyene fatty acid, *cis*-parinaric acid (PA), has been shown by Sklar et al. (1975, 1977) to be a useful molecule for studying the reversible binding to proteins; advantages of *cis*-parinaric acid as a fluorescence membrane probe have been reported by Sklar et al. (1976). Most importantly, Kato & Nakai (1980) have recently demonstrated that the "effective" hydrophobicity of a variety of proteins as determined by fluorescence enhancement following binding *cis*-parinaric acid correlates very well with the classical parameters of hydrophobicity such as the hydrophobic partition coefficient, interfacial tension, and the emulsifying activity index. Such a correlation may not hold for ANS because it has been shown (Penzer, 1972) that the fluorescence enhancement following binding of this substance to biological structures does not necessarily identify the binding site as hydrophobic.

It is shown here that myosin, at both high and low ionic strength, has 1.3 high-affinity binding sites for reversible binding of *cis*-parinaric acid. Heavy meromyosin (HMM) is also shown to contain a little more than one binding site while S-1 is found to bind on the average 0.65 mol of *cis*-parinaric acid/mol of S-1 (with the same affinity). S-1 isoenzymes containing A1 and A2 alkali light chains [S-1(A1) and S-1(A2), respectively] were found to differ greatly in their ability to bind PA: S-1(A1) bound PA with 1:1 stoichiometry and high affinity while S-1(A2) bound PA very weakly, if at all. Isolated alkali light chains also bound PA very weakly. On the basis of these observations, it is suggested that the hydrophobic binding site is formed between the 41-residue polypeptide at the N-terminal end of the alkali light chain 1 and the heavy chain of S-1. The fluorescence energy transfer between S-1 tryptophans and the hydrophobic probe and the influence of both nucleotide and actin binding on the environment of the PA binding site and on the polarization of fluorescence of the bound fluorophore have also been examined.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; A1, alkali light chain 1; A2, alkali light chain 2; PCMB, *p*-(chloromercuri)benzoate; bis(ANS), 5,5′-bis(8-anilinonaphthalene-1-sulfonate); S-1, myosin subfragment 1; HMM, heavy meromyosin; BHT, butylated hydroxytoluene; PA, *cis*-parinaric acid; BSA, bovine serum albumin; LMM, light meromyosin; Nbs_2 light chain, 19000 molecular weight subunit of myosin dissociated by treatment with 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

Materials and Methods

Proteins. Myosin was prepared from the back muscles of rabbit by the method of Tonomura et al. (1966). HMM and S-1 were routinely prepared from myosin by chymotryptic digestion according to Weeds & Pope (1977). The two S-1 isoenzymes were isolated by the procedure of Weeds & Taylor (1975). Alkali light chains A1 and A2 were prepared according to Holt & Lowey (1975), except that precipitation of the heavy chain was achieved by dialysis of the mixture of heavy chains and light chains against low ionic strength buffer containing 80 mM potassium phosphate (pH 6.0) and 0.1 mM DTT. The light chains were fractionated by chromatography on DEAE-cellulose columns (2.5 × 60 cm). They were applied to the above buffer and eluted with a linear gradient to 0.27 M potassium phosphate and 0.1 mM DTT. All proteins were assayed for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis. A1 always had a small contamination of the Nbs_2 light chain. Myofibrils were obtained from glycerinated rabbit psoas muscle as previously described (Borejdo, 1982).

Fatty Acid. cis-Parinaric acid was purchased from Molecular Probes, Junction City, OR. PA solutions were freshly prepared each time by dissolving in ethanol at a concentration of 3–4 mM; equimolar butylated hydroxytoluene (BHT) was added as an antioxidant. All solutions were purged with nitrogen; the excitation slit of the fluorometer was kept as small as possible (see below). These procedures ensured negligible decomposition of the fatty acid. The concentration of parinaric acid was determined by the absorbance at 318 nm (=74000 M^{-1} cm^{-1}).

Spectroscopic Measurements. Absorption spectra were obtained on a Cary 118 spectrophotometer. Fluorescence measurements were made at 20 °C on a Hitachi Perkin-Elmer MPF-4 spectrofluorometer in the ratio mode. The excitation and emission monochromator slits were set at 2 and 8 nm, respectively. Polarization of fluorescence was measured in a homemade apparatus by using the mercury lamp excitation, the excitation light monochromator set at 325 nm, and the emission barrier filter with a 400-nm cutoff.

The fluorescence enhancement following mixing of protein and *cis*-parinaric acid developed rapidly and then kept increasing very slowly with time. For consistency reasons, therefore, at each probe/protein molar ratio, the fluorescence intensity recording began immediately after mixing and continued for exactly 3 min afterward. The initial reading was taken as a measured value. The sample was slowly stirred throughout the experiment. Energy transfer efficiency was calculated from fluorescence quenching of the tryptophan residues by using the equation $T = 1 - F/F_0$ where F is the fluorescence of PA–protein conjugates at 330 nm when excited at 280 nm and F_0 is the fluorescence of the protein alone.

ATPase Measurements. ATPase assays were performed by a pH-stat method in a Radiometer titrigraph at pH 7.6 and at 25 °C. The Ca^{2+}-stimulated activity assay solution (3 mL) contained 50 mM KCl, 5 mM $CaCl_2$, and 2.5 mM ATP. The EDTA assay solution contained 0.6 M KCl, 1 mM EDTA,

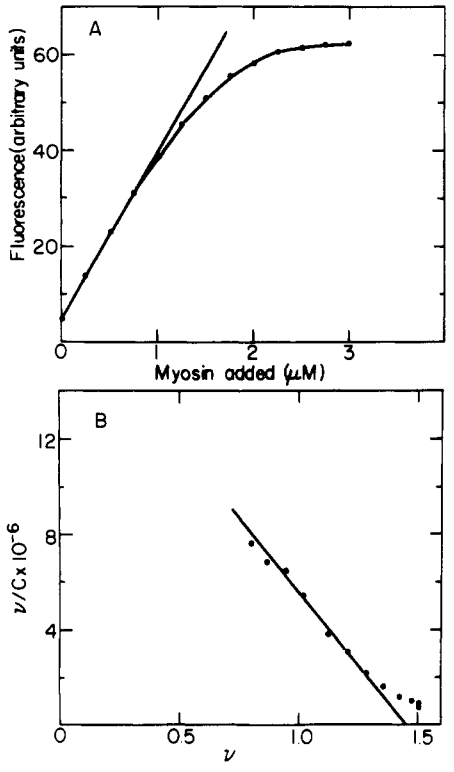

FIGURE 1: Binding of the hydrophobic probe to myosin measured by the enhancement of fluorescence. (A) Titration of PA (2.5 μM) with myosin. Here and in all other experiments, the excitation wavelength was 325 nm (slit 2 nm) and the emission wavelength 410 nm (slit 8 nm), unless otherwise indicated. No correction for tryptophan fluorescence was made. (B) Scatchard plot of the data shown in (A). ν is the number of moles of PA bound per mole of myosin. Myosin was in high ionic strength buffer containing 0.6 M KCl–5 mM phosphate buffer, pH 7.0.

and 2.5 mM ATP. Actin-activated ATPase was measured at pH 7.2 in 50 mM KCl, 5 mM $MgCl_2$, and 2.5 mM ATP. F-Actin was added to a final concentration of 0.05–1.5 mg/mL. All assay solutions contained 0.3 mg of S-1.

Results

PA Binding to Myosin. The quantum yield of PA in an aqueous solution is less than 0.001 (Sklar et al., 1977), and it increases 13-fold when the probe binds to myosin. The shape of the absorption and emission spectrum of PA was otherwise unchanged after binding to myosin or to its fragments.

The enhancement of PA fluorescence following complex formation was used to estimate the affinity and stoichiometry of PA binding to myosin and its fragments. Two methods of titration were used. In the first method (method I), the PA concentration was fixed (usually at 2.5 μM), and aliquots of myosin or fragments were added to obtain the binding curve. In the second method (method II), the concentrations of myosin or fragments were held fixed, and aliquots of PA were added.

Figure 1A shows fluorescence enhancement of a solution of PA when it is titrated with the myosin at high ionic strength (method I). The initial linear increase in fluorescence extrapolates to a value close to 0.68 mol of myosin/mol of PA, and there is little further fluorescence increase when the myosin/PA ratio exceeds 1.0. The Scatchard plot of the data of Figure 1A is shown in Figure 1B. It has been assumed that maximum fluorescence corresponds to 100% binding, i.e., that the concentration (c) of free PA is given by $[PA]_{free} = (1 - I/I_{max})[PA]_{tot}$ where I_{max} is the maximum fluorescence intensity and $[PA]_{tot}$ is the added (total) concentration of PA.

Table I: Affinity and Stoichiometry of PA Binding to Myosin and Its Fragments

moiety	K ($\times 10^7$ M^{-1})	ν_{max}	N^a
myosin	1.29 ± 0.37[b]	1.34 ± 0.12	14
HMM	1.01 ± 0.42	1.10 ± 0.03	5
S-1	1.65 ± 0.88	0.65 ± 0.09	15

[a] Number of experiments. [b] Mean ± standard deviation.

ν in Figure 1B is the number of moles of PA bound per mole of myosin. The slope of the Scatchard plot is the binding constant. The straight-line plot of Figure 1B indicates a simple binding site. The horizontal axis intercept indicates that there are, in this case, on the average $\nu_{max} = 1.45$ binding sites per mol of myosin. There may be other binding sites of low affinity as suggested by curvature of the plot at high ν values. In 14 experiments of this kind, the average value of the association constant for binding of PA to the high-affinity site in myosin was $(1.29 \pm 0.37) \times 10^7$ M^{-1}. The mean number of binding sites was 1.34 ± 0.12 (Table I).

A similar picture was obtained when myosin was titrated with PA (method II, not shown). Here, the free PA concentration was computed as $[PA]_{tot} = IV_{max}/I_{max}$ where I_{max} was the maximum fluorescence intensity and V_{max} was the maximum concentration of bound PA. In this method, V_{max} had to be assumed known a priori. In most cases, all values where $V_{max} > 1.5[M]_{max}$ (where $[M]_{max}$ was the added myosin concentration, usually fixed at 2.5 μM) could be rejected because then all, or most, of the ν/c values were negative. In five titrations by method II, the average value of the binding constant and the average number of binding sites were $(1.16 \pm 0.48) \times 10^7$ M^{-1} and 1.2 ± 0.05, respectively.

In an attempt to see whether the PA binding site overlaps, at least partially, with the nucleotide binding site of myosin, PA was titrated with myosin at low and high ionic strength in the presence of MgADP and MgATP. Neither K_a nor ν_{max} was influenced by either MgATP or MgADP binding, indicating that the nucleotide and the PA binding sites were distinct. We next probed the question of whether the hydrophobic binding is affected by the state of aggregation of myosin. To this end, PA was titrated with myosin at low ionic strength. The mean values of K_a and ν_{max} determined from six such experiments were $(1.60 \pm 0.28) \times 10^7$ M^{-1} and 1.35 ± 0.11, respectively, not significantly different from the values obtained at high ionic strength.

PA Binding to Myosin Fragments. The typical Scatchard plots for binding of PA to the double-headed HMM and to S-1 are shown in Figure 2A. The K_a values for binding to either fragment were similar; the affinity of binding was as strong as in the case of myosin. The stoichiometry was on the average 0.65 ± 0.09 for S-1 and 1.1 ± 0.03 for HMM. These data are summarized in Table I. MgATP and MgADP had no effect on PA binding to S-1 or HMM.

PA Binding to S-1(A1) and S-1(A2). The fact that one molecule of PA bound to on the average 0.75 molecule of myosin and 1.5 molecules of S-1 suggested that the hydrophobic site was in fact located not on the heavy chain but on one of the light chains of the S-1 molecule. This is because A1 and A2 light chain isoenzymes are known to be present in rabbit skeletal muscle in roughly 3:2 ratio (Sarkar, 1972; Weeds et al., 1972). Binding of PA to both S-1 isoenzymes was examined to test this possibility. The typical Scatchard plots for binding of PA to S-1(A1) and S-1(A2) are shown in Figure 2B. The affinity of binding to S-1(A1) was essentially the same as to unfractionated S-1 while the amount bound was now closer to 1 mol of PA/mol of S-1(A1). In

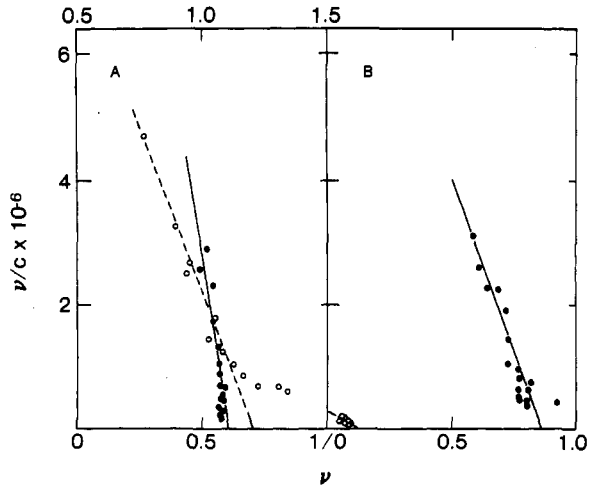

FIGURE 2: (A) Scatchard plots for binding of PA to S-1 (filled circles, solid line, bottom scale) and to HMM (open circles, dashed line, top scale). Fragments were in 80 mM KCl, 5 mM sodium phosphate buffer, pH 7.0, and 0.1 mM $MgCl_2$. PA concentration was fixed at 2.5 μM. (B) Scatchard plots for binding of PA to S-1(A1) (filled circles, solid line) and to S-1(A2) (open circles, dashed line). Fragments were in 80 mM KCl, 5 mM sodium phosphate buffer, pH 7.0, and 0.1 mM $MgCl_2$. PA concentration was fixed at 2.5 μM. otherwise, conditions as were described in Figure 1.

contrast, PA bound very weakly to S-1(A2) (Figure 2B).

PA Binding to Isolated Light Chains. The difference in PA binding to the S-1 isoenzymes could be due to binding either to the 41-residue difference piece at the N-terminal end of A1 or to the hydrophobic site involving the heavy chain which is present in S-1(A1) but not in S-1(A2). To distinguish between these possibilities, I have measured PA binding to the isolated light chains. To 2.5 μM PA in 80 mM KCl–5 mM phosphate buffer, pH 7.0, were added aliquots of 0.25 μM A1 (which had a small contamination of Nbs_2 light chain) or A2. PA bound at least 100-fold weaker to either light chain than to unfractionated S-1. The significance of this finding is dealt with under Discussion.

Energy Transfer from Tryptophan to PA. The spectral overlap between the absorption spectrum of PA and the emission spectrum of S-1 tryptophans should lead to considerable energy transfer between the chromophores, providing the distance between them is sufficiently small. We have chosen to follow the energy transfer by exciting the tryptophans at 240 nm, below the absorption limit of PA, and to follow the changes in the emission spectra of S-1 tryptophans. The change in the tryptophan fluorescence of S-1 associated with the binding of PA is shown in Figure 3A. The weighted energy transfer efficiency for these data calculated according to the equation defined under Materials and Methods is shown in Figure 3B. The low transfer efficiency for S-1 (25% even at 3.5 mol of PA/mol of S-1, cf. Figure 3B) is in line with the proposal of Kato & Nakai (1980) that the transfer efficiency correlates well with the effective hydrophobicity of a protein. By their standards, S-1 is weakly hydrophobic, and so the efficiency of transfer was expected to be low.

We have not attempted here to resolve the changes in transfer efficiency following the nucleotide binding because of the complicating effects of ATP on the emission spectrum of S-1 tryptophans. Such changes were previously detected by Cheung (1969) for the case of ANS and by Takashi et al. (1977) for bis(ANS) bound to myosin. Also, we have not attempted to decompose the observed total transfer efficiency into the contributions due to each donor separately because the measurement of the total transfer efficiency with just one type of acceptor does not yield enough information for the

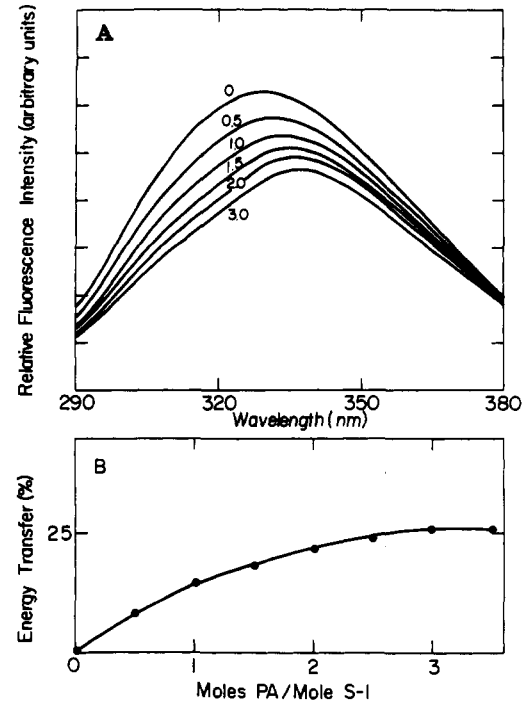

FIGURE 3: (A) Fluorescence emission spectra of S-1 in the presence of increasing concentrations of PA. The S-1 concentration was fixed at 2.5 μM. The numbers refer to ratios of moles of PA per mole of S-1. The excitation was at 240 nm (slit 4 nm); the emission slit was 20 nm. (B) Energy transfer efficiency as a function of the molar ratio of PA to S-1.

determination of the individual transfer efficiencies.

PA Binding to Actin and Acto-S-1. Polarization of Fluorescence of Bound PA. Under conditions where actin existed in the F form, we saw very weak binding of PA. The mean K_a was $<10^5$ M^{-1}. Because of this weak binding, it was possible to study the influence of actin on the fluorescence of S-1-bound PA, because actin does not effectively compete with S-1 for PA. S-1 (2.5 μM) was mixed with PA (2.5 μM); upon the addition of 10 μM F-actin, the PA fluorescence changed very little, suggesting that the fluorophore environment was not disturbed by actin. Similarly, addition of 0.1 mM MgATP to acto-S-1 (2.5 μM) in the presence of 2.5 μM PA gave no change in fluorescence. The excited state lifetime of bound PA was also completely unaffected by either actin or nucleotide binding. The fluorometric titrations with myofibrils confirmed the above observations. The Scatchard plot for PA binding to myofibrils resembled closely the PA–myosin binding and gave similar K_a and ν_{max} values. Dissolution of actomyosin complexes with 2 mM $MgPP_i$ had no effect on the PA fluorescence.

In an attempt to see whether the rotational motion of the myosin-bound fluorophore was affected by actin binding, we have measured rotational correlation time and the polarization of fluorescence of PA in the absence and in the presence of actin. PA (2.5 μM) was added to 5 μM S-1 in a solution containing 80 mM KCl–5 mM phosphate buffer, pH 7.0. The rotational correlation time of bound PA was 184 ns, suggesting that the fluorophore was rigidly attached to the protein. Upon addition of 10 μM actin the correlation time increased to about 2550 ns (the exact value of this correlation time could not be determined because of the short excited state lifetime of PA). The polarization of fluorescence of bound PA was $p = 0.391$. Addition of 10 μM actin increased the p value to $p = 0.411$.

ATPase Measurements. The Ca^{2+}-activated activity of S-1 was 2.36 ± 0.14 ($N = 4$) μmol of P_i mg^{-1} min^{-1}. The addition of up to 15-fold molar excess of PA over S-1 (39 nmol of PA

per 2.6 nmol of S-1 in 3 mL of assay medium) had no effect whatever on the activity regardless of whether the ATPase was measured immediately or after up to 45 min of incubation of S-1 with PA. The EDTA-activated ATPase activity of S-1 was 2.65 ± 0.35 ($N = 4$) μmol of P_i mg^{-1} min^{-1}, and it was unchanged after the addition of up to 15-fold molar excess of PA over S-1. A 45-min incubation of S-1 with PA also had no effect on EDTA ATPase of S-1.

Discussion

The enhancement of fluorescence of the polyene fatty acid *cis*-parinaric acid upon binding to proteins has been used here to elucidate the question of the effective (i.e., surface) hydrophobic regions of the contractile proteins. This probe has been exploited here with the following significant advantages: (1) PA, in contrast to alternative environment-sensitive dyes, such as ANS, is specific for hydrophobic regions of proteins. For example, the binding constant of ANS to biological macromolecules is typically quite strong (Radda, 1971), yet the partition coefficient of this chromophore between water and hydrophobic solvents is near zero (Flanagan & Ainsworth, 1968). Binding of PA to a variety of proteins, on the other hand, has been experimentally correlated with the surface hydrophobicity parameters. (2) It does not affect the enzymatic activity of myosin, even at 15-fold molar excess.

The fluorometric titrations by the two methods showed that myosin has, on the average, 1.3 sites at which it can bind PA with high affinity. The fact that PA binds to HMM with similar affinity and stoichiometry clearly indicates that the hydrophobic site is not located in the LMM region. Our data indicate, further, that the hydrophobic binding site is located in the head region of the myosin molecule. This is because S-1 bound PA with affinity similar to that of HMM and with the stoichiometry of, on the average, 0.65 mol of PA/mol of S-1. The important implication of this observation was the suggestion that the alkali light chain 1 was specifically involved in the binding of PA; this possibility was directly verified by studying the binding of PA to fractionated S-1 isoenzymes. The fact that S-1(A1) strongly bound PA with approximately 1:1 stoichiometry while S-1(A2) hardly bound PA at all (Figure 2B) suggested that indeed alkali light chain 1 is involved in PA binding. The fact that isolated alkali light chains did not bind PA would seem to suggest that the hydrophobic binding site is not located exclusively on the 41-residue difference piece: if it were, it would have to be postulated that the light chains assume different conformations in solution and when bound to the heavy chain, specifically, that the difference piece is not accessible to PA when A1 is free in solution. It is difficult to imagine that the lysine-rich difference piece is completely shielded from the solvent when A1 is free in solution. Moreover, the direct binding of PA to the 41-residue difference piece is not likely in view of the fact that the difference piece does not contain an unusual abundance of hydrophobic side chains (Frank & Weeds, 1974). It therefore seems either that PA binds exclusively to the heavy-chain regions of S-1 which is obscured by A2, but not A1, or that the 41-residue difference piece together with the adjacent portion of the heavy chain forms a hydrophobic pocket accessible to PA. The former possibility seems unlikely because it would imply that the homologous regions of alkali light chains on the C-terminal side of A1 and A2 bound to the S-1 heavy chain in a conformation that is different for both light chains. Recently, Burke & Wang (1982) have shown that in spite of the extensive sequence homology between the two light chains at the C-terminal side, fluorophores attached at the thiol groups of the free A1 and A2 find themselves in quite a different environment. However, the environmental differences largely disappeared in the bound state, suggesting that indeed the homologous regions of A1 and A2 bound similarly to the heavy chain. We therefore conclude that PA binds to the hydrophobic pocket formed by the 41-residue difference peptide at the N-terminal end of A1 and by the underlying heavy chain of S-1.

The fact that PA binds very weakly to F-actin makes it possible to meaningfully interpret PA fluorescence in the presence of actomyosin complexes. It was at first expected that if the hydrophobic binding site lay indeed near the 41-residue difference piece then fluorescence of myosin-bound PA will be affected by complexing with actin since there is evidence for the interaction of this piece with actin: proton NMR spectroscopy first applied to the problem of segmental motions in S-1 by Highsmith et al. (1979) shows immobilization of the light chain motion by actin (Prince et al., 1981), and affinity chromatography experiments of the thrombic digests of the A1 light chain (Henry et al., 1980) and cross-linking of A1 and actin (Sutoh, 1982) suggest directly the interaction of the 41-residue piece with actin. Fluorescence of bound PA would then be a convenient probe of actomyosin contact, and this contact could be assessed also in the presence of nucleotides because we have shown above that the binding of MgATP or MgADP by myosin or its fragments has little effect on bound PA: unlike bis(ANS) (Takashi et al., 1977), it is certainly not partially displaced, and it is apparently not very sensitive to the local conformational changes induced by nucleotide binding (Werber et al., 1972). In the event, no effect of actin binding on PA fluorescence was found either in acto-S-1 or in myofibrils, suggesting that the hydrophobic site is insensitive (as far as the probe environment is concerned) to changes occurring at the actin–myosin interface. However, we have observed a significant increase in the rotational relaxation time of PA upon binding of S-1 to actin. This effect is undoubtedly due to the partial immobilization of the rotational motion of PA upon binding of the fluorophore bearing S-1 to actin. This relaxation time can be used to estimate the mole fractions of free and bound S-1 much in the same way anisotropy decay curves have been used to measure association constants for S-1 binding to actin (Highsmith et al., 1976). There are several important advantages of PA as a probe: (a) it is insensitive to nucleotide binding; (b) it binds specifically near the A1 light chain and therefore has no detectable effect on the enzymatic properties of myosin and actomyosin; (c) PA has excellent polarization properties over the entire excitation band (Jameson, 1978); (d) it binds weakly to actin and therefore can be used in myofibrils; (e) PA is easily and rapidly applied.

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Isolation and Some Structural and Functional Properties of Macrophage Tropomyosin†

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ABSTRACT: Tropomyosin purified from rabbit lung macrophages is very similar in structure to other nonmuscle cell tropomyosins. Reduced and denatured, the protein has two polypeptides which migrate during electrophoresis in sodium dodecyl sulfate on polyacrylamide gels with slightly different mobilities corresponding to apparent M_r's of about 30 000. Following cross-linking by air oxidation in the presence of $CuCl_2$, electrophoresis under nonreducing conditions reveals a single polypeptide of M_r 60 000. Macrophage tropomyosin has an isoelectric point of 4.6 and an amino acid composition similar to other tropomyosins. It contains one cysteine residue per chain. In the electron microscope, macrophage tropomyosin molecules rotary shadowed with platinum and carbon are slender, straight rods, 33 nm in length. Macrophage tropomyosin paracrystals grown in high magnesium concentrations have an axial periodicity of 34 nm. On the basis of yields from purification and from two-dimensional electrophoretic analyses of macrophage extracts, tropomyosin comprises less than 0.2% of the total macrophage protein, a molar ratio of approximately 1 tropomyosin molecule to 75 actin monomers in the cell. Macrophage tropomyosin binds to actin filaments. Macrophage, skeletal muscle, and other nonmuscle cell tropomyosins inhibit the fragmentation of actin filaments by the Ca^{2+}–gelsolin complex. The finding implies that tropomyosin may have a role in stabilizing actin filaments in vivo.

The cytoplasm of most nonmuscle cells contains actin filaments and associated proteins (Weeds, 1982). One of these associated proteins is tropomyosin. It has been identified by immunofluorescence in epithelial cells (Lazarides, 1975) and has been purified from human platelets (Cohen & Cohen, 1972), brain (Fine et al., 1973), fibroblasts (Masaki, 1975), cultured mammalian cells (Schloss & Goldman, 1980), and porcine and equine platelets (der Terrossian et al., 1981; Côté & Smillie, 1981a,b; Côté et al., 1978a,b). Although tropomyosin is a component of the system that regulates the interactions between actin and myosin in striated muscles (Taylor, 1979; Mannherz & Goody, 1976), its role in nonmuscle cells is not clear.

As part of our endeavor to understand the structure and movement of cytoplasm in mammalian phagocytes, we have asked whether tropomyosin is present in rabbit lung macrophages and, if so, how it interacts with actin and actin-associated proteins. This paper documents the identification of tropomyosin in the macrophage and describes an inhibitory effect of this protein on the activity of gelsolin, a calcium-

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