Steinmetz, M. A., & Deal, W. C. (1966) *Biochemistry 5*, 1399-1405.
Tanford, Ch. (1966) *J. Biol. Chem. 241*, 1521-23.
Tanford, Ch. (1968) *Adv. Protein Chem. 23*, 121-182.
Tanford, Ch. (1970) *Adv. Protein Chem. 24*, 1-95.
Timasheff, S. N., & Townend, R. (1970) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. I., Ed.) Part B, pp 147-212, Academic Press, New York.

Internal Motions in Myosin. 2†

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ABSTRACT: The high-resolution ^1H NMR detected internal motions in myosin and myosin subfragment 1 (S1) [Highsmith, S., Akasaka, K., Konrad, M., Goody, R., Holmes, K., Wade-Jardetzky, N., & Jardetzky, O. (1979) *Biochemistry 18*, 4238-4244] were unperturbed by induced changes in the rate of protein tumbling, and the mobile regions proved inaccessible to added surface-directed paramagnetic probes. The rate of tumbling was changed by changing the solvent viscosity for S1 or by aggregation to thick filaments for myosin. Neither manipulation caused a measurable broadening of the narrow lines in the spectrum. Sulfhydryl-directed covalently attached nitroxide spin-labels, soluble nitroxide spin-labels, and $MnCl_2$ were used to probe the surface. Unique labeling at the fastest reacting thiol of S1 had no effect on the NMR spectrum. Multiple labeling of thiols caused a small but detectable broadening of the narrow peaks. Soluble spin-labels and $MnCl_2$ had a very small effect on the narrow bands even in great excess. The results substantiate the notion that myosin has internal motions that are independent of the overall rate of rotation and suggest that the mobile structure is mainly in the interior of the S1 moiety. This supports a model in which actin quenches the internal motions of myosin by changing the structure of myosin upon binding.

Force generation in muscle by the actomyosin-nucleotide complex very likely involves a substantial change in the overall dimensions of the complex. Most contemporary working hypotheses stem from the original cross-bridge models of Huxley (1969) and Huxley & Simmons (1971). In the simplest versions, actin and myosin reorient relative to one another without either individual protein changing its structure. Another possibility is that actin and/or myosin themselves undergo a structural change as part of the total conformational change of the complex. Thus far, no evidence has been obtained to demonstrate any structural changes. Recently, a change in the dynamics of myosin due to actin binding was reported which is large enough to suggest a possible structural change for myosin when it binds actin (Highsmith et al., 1979). It was shown by high-resolution ^1H NMR[1] measurements that more than 20% of the structure of subfragment 1 (S1) of rabbit skeletal myosin is undergoing rapid internal motion. Neither MgATP nor a selection of its analogues appeared to have any effect on the internal motions. However, when actin was bound to S1, it caused the narrow peaks associated with the mobile structure to disappear. Small perturbations in the rate of free S1 rotation in solution had no effect on the narrow peaks in its spectrum. Therefore, it was suggested that actin binding eliminated the narrow NMR peaks of S1 because it changed the structure of S1 in such a way as to quench its internal motions. However, the possibility that the loss of narrow peaks was due to the trivial mechanism of a reduced rate of overall molecular rotation was not eliminated. The location of the mobile structure in S1 was also not identified beyond the exclusion of the nucleotide binding site and the homologous parts of the alkali light chains. In particular, the possibility that the mobile 20% of S1 was composed of amino acid side chains on the surface was not to be eliminated.

Two experimental approaches to this problem are reported here. First, the rate of rotation of S1 was reduced without actin to determine how much of the actin binding effect is due to the slowing of the overall rotation of S1. Second, paramagnetic probes were used to estimate the fraction of the mobile structure accessible to the solvent and therefore presumed to be on the surface of the protein. The results of these experiments indicate that the rate of rotation of S1 has very little effect on the line widths of the narrow resonance peaks. In addition, it appears that almost all of the mobile structure is inaccessible to small solute molecules in the solvent. These results indicate that the "internal motions" occur in the interior of the protein and that actin quenches the internal motions when it binds by altering a substantial part of the structure of S1.

Experimental Section

Materials. Myosin was prepared from dorsal muscle of New Zealand rabbit by the method of Nauss et al. (1969) and purified by $(NH_4)_2SO_4$ fractionation. Its concentration in solution was determined by using the constants $M_r = 450\,000$ and $\epsilon^{1\%}_{280nm} = 5.7$. S1 was prepared from myosin by the method of Weeds & Taylor (1975) and purified as a mixture of isozymes by $(NH_4)_2SO_4$ fractionation. S1 prepared this way has

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[1] Abbreviations used: NMR, nuclear magnetic resonance; S1, myosin subfragment 1; ATP, adenosine 5'-triphosphate; Tempo-IAA, 4-[(2-iodoacetyl)amino]-2,2,6,6-tetramethylpiperidinyl-1-oxy; Tempop, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy dihydrogen phosphate (ester); HSA, human serum albumin.

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light chains 1 and 3 but no light chain 2. Its concentration in solution was determined by using the constants M_r = 100 000 and $\epsilon^{1\%}_{280nm}$ = 7.7. Purified human serum albumin (HSA) was a gift from Hans-Helmut Paul. Its concentration in solution was determined with the constants M_r = 66 000 and $\epsilon^{1\%}_{280nm}$ = 5.8. Myosin thick filaments were prepared from fresh myosin in 0.6 M KCl by dialysis to various conditions that have been characterized by Kaminer & Bell (1966). In the NMR experiments, thick filaments were converted to clear solutions of solubilized myosin by adding solid KCl directly to the NMR tube to obtain 0.6 M KCl. All protein samples were kept below 5 °C except during measurements at higher temperatures.

All inorganic chemicals were at least reagent grade in purity. Spin-labels were purchased from Syva Chemical Co. ^2H_2O (99.98% ^2H) was purchased from Bio-Rad. α-Chymotrypsin was from Sigma. Deuterated glycerol was obtained from Merck and Co.

Methods. S1, which was spin-labeled only at the SH_1 position, was prepared by incubating S1 and 4-[(2-iodoacetyl)amino]-2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo-IAA) (1:1 M:M) for 18 h at 4 °C in the dark in 0.15 M KCl and 0.01 M phosphate, p^{2}H 7.0. Ca–ATPase activity of the spin-labeled S1 was 8-fold greater than that of unlabeled S1, indicating the SH_1 was labeled (Kielley & Bradley, 1956). Electron spin resonance measurements, kindly performed by Dr. Roger Cooke on the same S1 sample used for the NMR studies, yielded a spectrum (not shown) characteristic of an "immobilized" spin-label. More extensively labeled S1 was prepared by incubating S1 and a 10-fold excess of spin-label (Tempo-IAA) for 18 h under the same conditions as above. The noncovalently attached spin-label 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy dihydrogen phosphate (ester) (Tempop) and $MnCl_2$ were added to a solution from 1 h to immediately before making a measurement.

Solutions of proteins in ^2H_2O were prepared by dialysis. The p^{2}H was determined without further correction by adding 0.4 to the value measured by use of a pH meter with a glass electrode. All samples except the thick filaments were centrifuged at 100000*g* for 1 h before a measurement to remove any aggregated material that may have accumulated during dialysis.

^{1}H NMR measurements were made with a modified Bruker HXS-360 spectrometer equipped with quadrature detection. The computer and the software package (NTCFT-1180) were from Nicolet Technology Corp. Typical measurements were on protein solutions that were less than 50 μM, and good signal-to-noise ratios were obtained within 1 h. The sweep width was ±3000 Hz in the S1 experiments and ±4000 Hz in the myosin experiments. The ^{2}HHO resonance was not saturated with a homonuclear decoupling pulse because it has been shown that, at least for myosin, this can distort the spectrum (Akasaka et al., 1979). The temperature was maintained with a temperature controller that was calibrated by using the chemical shift differences of the methyl hydrogen and hydroxyl hydrogen peaks of methanol. Chemical shifts are given in parts per million from external tetramethylsilane at 4 °C.

Results

The rate of rotational Brownian motion of S1 was perturbed in three ways: (1) by changing the temperature of S1 solutions in ^2H_2O; (2) by adding 50% glycerol-d_8 and varying the temperature; (3) by polymerizing myosin to thick filaments.

Temperature. No effect on the line widths of narrow peaks in the spectrum of S1 could be observed in the accessible temperature region 277–303 K. The two lower traces in Figure 1 show spectra for S1 at 277 and 298 K in 0.15 M KCl and 0.010 M phosphate, p^{2}H 7.0. This temperature difference changes the viscosity of H_2O by about a factor of 2. If S1 is approximated as a sphere, then the rate of Brownian rotational motion at 277 K will be about one-half of what it is at 298 °C (Tanford, 1961).

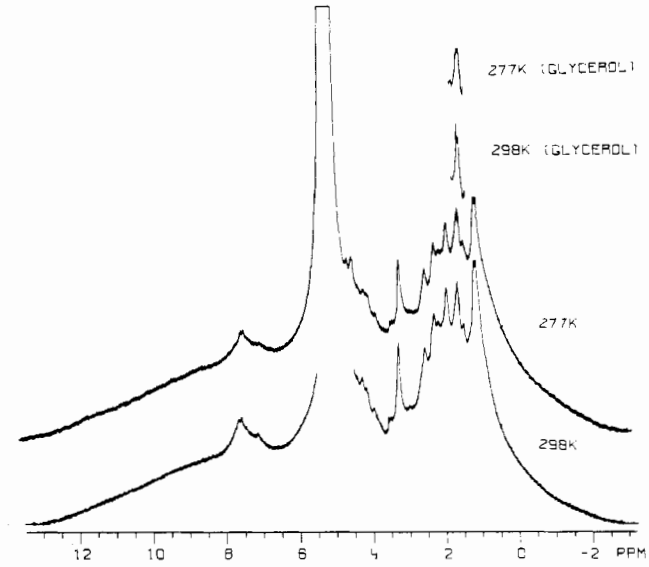

FIGURE 1: ^{1}H NMR spectra at 360 MHz of myosin subfragment 1 in 10 mM phosphate and 0.15 M KCl, p^{2}H 7.0, at 277 and 298 K (two lower traces) and a portion of the spectra of the same protein in 5 mM phosphate, 75 mM KCl, and 50% glycerol-d_8, p^{2}H 7.0, at 277 and 298 K (two upper traces). Intensity of the resonance absorption is plotted against the chemical shift in ppm.

Glycerol. Even with 99.8% deuterated glycerol-d_8, at 50% concentration there are several peaks the size of that for ^{1}HO^{2}H due to the ~20 mM hydrogenated glycerol. These peaks obscured all of the narrow peaks of S1 except the one at 1.5 ppm. This peak is shown in the upper two traces of Figure 1 for S1 in 50% glycerol, 0.15 M KCl, and 0.01 M phosphate, p^{2}H 7.0, at 277 and 298 K. At the lower temperature, the rotational correlation time of S1 is estimated from tables in the Handbook of Physics and Chemistry (1965) to be increased ~100-fold. Nonetheless, as can be seen in Figure 1, there is little broadening or loss of intensity for the peak at 1.5 ppm.

Thick Filaments. The spectrum of myosin thick filaments is similar to that of myosin in solution. This can be seen in Figure 2 for p^{2}H 6.8 at 298 K; similar results were obtained at p^{2}H 6.3 and 8.0 (not shown).

Thick filament formation is known to reduce the rate of rotational motion of the S1 moiety about 5-fold (Mendelson et al., 1973; Thomas et al., 1975). Nonetheless, the narrow peaks in the aliphatic region of the myosin spectrum persist in thick filaments. The aromatic peak at 7 ppm is broadened beyond detection. The small changes occurring in the aliphatic region appear to be small losses of intensity rather than broadening and are probably due to immobilization of the small fraction of mobile side chains in the subfragment-2 region of myosin. Control measurements on S1 in 0.15 and 0.65 M KCl to determine the effects of ionic strength gave spectra (not shown) that were virtually identical. To ensure that the thick filament solutions did not contain significant free myosin, an aliquot of the thick filament solution was solubilized and its absorbance at 280 nm measured. The remaining suspension was then centrifuged at 80000*g* for 1 h and the absorbance at 280 nm measured for an equal aliquot of the supernate. The ratio of the absorbances indicated less

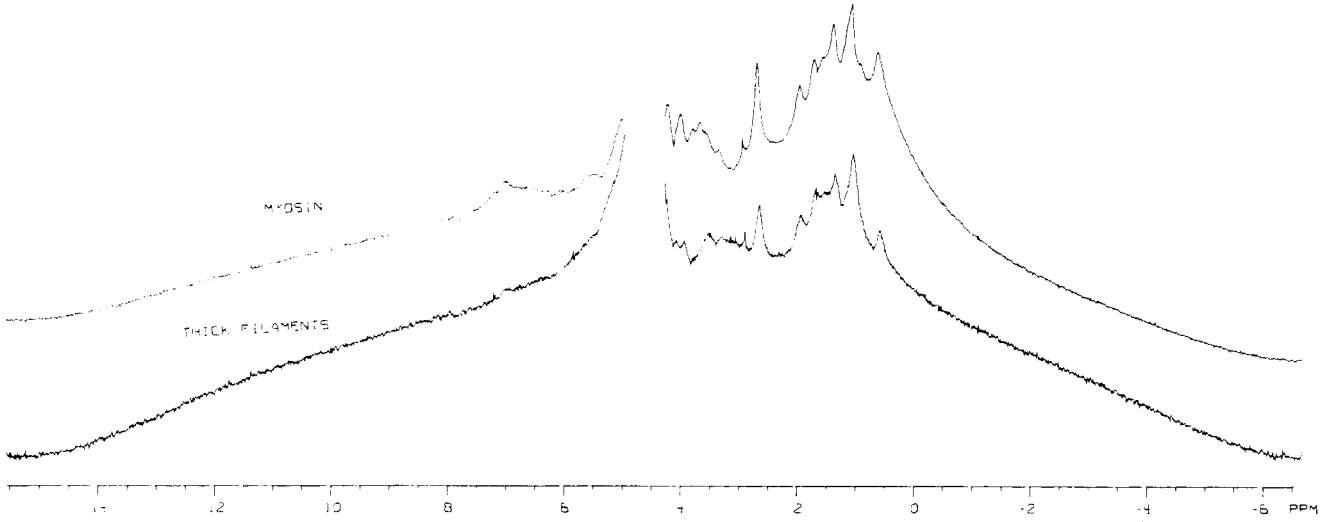

FIGURE 2: ^1H NMR spectra at 360 MHz of myosin thick filaments in 0.1 M KCl and 10 mM phosphate, p^{2}H 6.8, at 298 K (lower trace) and the same sample after solid KCl had been added to convert the thick filaments into myosin in solution (upper trace). Intensity of the resonance absorption is plotted against the chemical shift in ppm.

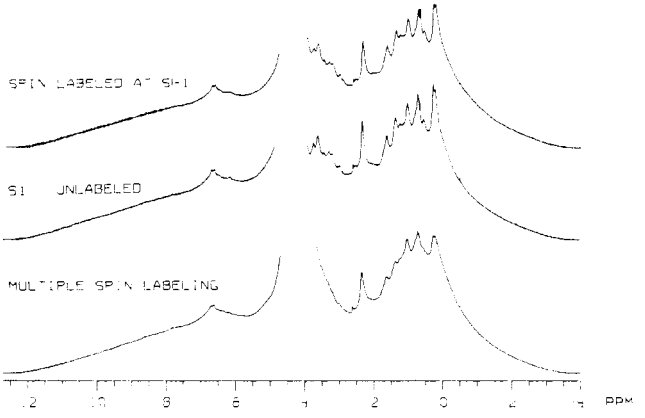

FIGURE 3: Effects of covalently linked spin-labels on the 360-MHz ^1H NMR spectrum of myosin subfragment 1 in 0.15 M KCl and 0.010 M phosphate, p^{2}H 7.0, at 273 K. The uppermost trace is for S1 labeled with one Tempo-IAA specifically at the sulfhydryl SH_1. The middle trace is S1 with no label. The lowest trace is for S1, which had as many as 10 spin-labels covalently attached. Intensity of resonance absorption is plotted against chemical shift in ppm.

than 4% of the myosin in the thick filament preparation was free.

Two types of paramagnetic probes were used to investigate the surface of S1: (1) covalent spin-labels attached to sulfhydryl groups and (2) paramagnetic ions free in solution.

Covalently Attached Spin-Labels. When the "fast thiol", or "SH_1", on S1 was uniquely labeled (see Experimental Section) with the iodoacetamide derivative of Tempo, there was no observable change in the spectrum of S1 as shown in Figure 3. Also shown in Figure 3 in the lower trace is the spectrum of S1 with spin-labels covalently attached to several sulfhydryl groups. (The labeling conditions are described above.) The spectrum of this more extensively labeled S1 shows slight broadening of the narrow resonance peaks at 0.3, 1.7, and 1.9 ppm. The peak at 0.3 ppm is the most broadened, but even there, the effect is small. In no case was the aromatic region altered, indicating that none of the mobile aromatic amino acid side chains are near the labeled sulfhydryl groups.

Nonattached Paramagnetic Probes. A nonreactive anionic Tempo derivative, Tempop, and the paramagnetic cation,

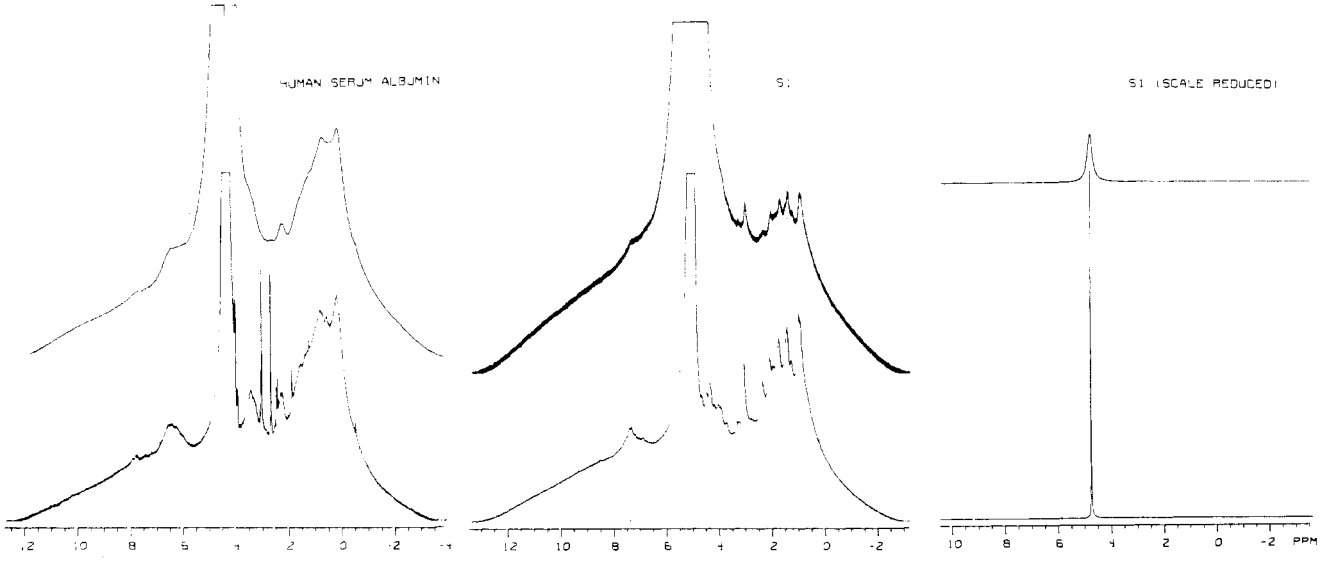

FIGURE 4: Effects of 1 mM Mn^{2+} on the 360-MHz ^1H NMR spectra of 50 M human serum albumin (on the left) in 0.1 M KCl, 10 mM phosphate, p^{2}H 7.0 and 0.1 mM EDTA at 293 K and 30 mM S1 in 0.15 M KCl and 10 mM phosphate, p^{2}H 7.0 (middle and right show same spectra with different application). The presence of EDTA (not shown) had no effect on the results. Intensity of resonance absorption is plotted against chemical shift in ppm.

Mn^{2+} were used to investigate the solvent accessibility of S1. In the case of Mn^{2+} parallel experiments on HSA were done as a control. S1 was titrated with Mn^{2+} up to a ratio of 1:50 (M:M), and there was very little broadening of the aliphatic narrow resonance peaks. The aromatic peaks, on the other hand, were substantially broadened by Mn^{2+} at a ratio near 1:1 (not shown). The spectra of S1 and S1 plus a 50-fold molar excess of Mn^{2+} are shown in Figure 4 along with the human serum albumin control experiment. The trace on the right shows the S1 and S1 plus Mn^{2+} spectra greatly reduced in scale, so that the HO^2H peak broadening due to Mn^{2+} is obvious. The resonance peaks of HSA and HO^2H are broadened substantially, as one would expect for solutions containing Mn^{2+}. However, the spectrum of S1 is changed very little beyond that introduced by the severely broadened water peak.

The addition of Tempop to solutions of S1 had even less effect on the narrow peaks of the S1 spectrum (not shown) than did Mn^{2+}. This experiment showed that the mobile region is relatively inaccessible to negatively as well as positively charged paramagnetic ions in solution.

Discussion

Rotational Brownian Motion and NMR Line Width. The temperature, glycerol, and thick filament experiments provide evidence for two conclusions on the actomyosin system. One is that the internal motions found in myosin (Highsmith et al., 1979) really are internal and depend very little, if at all, on the rate of rotation of the molecules as a whole. The other is that the quenching of these internal motions by actin binding is not due to a simple decrease in myosin rotation but to a more specific structural effect of the actin–myosin interaction.

Although the decrease in the rate of rotation for the S1 moiety of myosin in thick filaments is not as great (~5-fold) as for solutions of S1 in 50% glycerol (~100-fold), it has been inferred by other experiments that the motion of the S1 moiety in thick filaments is reduced by a degree comparable to that caused by binding S1 to actin. Mendelson & Cheung (1978) measured the rotational correlation time by using fluorescence depolarization and found S1 bound to actin about equal or slightly greater in mobility as compared to the S1 moiety in myosin thick filaments. Thomas et al. (1975) using saturation transfer ESR found S1 bound to actin to be about 30 times less mobile than it is in myosin thick filaments. However, the narrow NMR peaks of S1, which reflect internal rather than overall motion, persist in the thick filaments whereas they are eliminated by actin binding (Highsmith et al., 1979). These results and those of the glycerol addition experiments indicate that actin binding quenches the *internal* motions of myosin and does so by a mechanism other than by reducing S1 rotational mobility.

Effects of Paramagnetic Probes. The fact that a single spin-label attached to SH_1 does not cause any measurable broadening in the spectrum of S1 (see Figure 3) is reasonable. SH_1 may be near or in the nucleotide binding site which is thought to be rigid (Shriver et al., 1979). The case where several labels are attached to S1 (see Figure 3) does show broadening. This suggests that at least some, albeit a small percentage, of the mobile structure is near surface sulfhydryl groups, although the additive fields of several paramagnetic centers could also broaden groups located in the interior.

The results obtained when S1 was titrated with Mn^{2+} are surprising. As seen in Figure 4, even when Mn^{2+} is in a 50-fold molar excess over protein and sufficient to cause gross broadening of the spectra of HSA and water, the narrow aliphatic peaks of S1 are only slightly broadened. The narrow aromatic peak appears to be broadened, and the effect is seen at ratios of Mn^{2+} to S1 near 1:1. This is difficult to ascertain because of its small amplitude, but it seems to be true and suggests some specific binding interaction of Mn^{2+} with or near the aromatic groups. Since the relative intensity of the aromatic peak increases when S1 is produced proteolytically from myosin (Highsmith et al., 1979), perhaps some aromatic amino acid side chains are located in the swivel region between S1 and the remaining rod of myosin, which are exposed by proteolysis. Divalent cations are known to effect the swivel in heavy meromyosin and myosin (Highsmith, 1977). Electron spin resonance experiments on Mn^{2+} and S1 would argue against Mn^{2+} binding at any high-affinity binding sites in the absence of the light chain 2 (Bagshaw & Kendrick-Jones, 1979); however, residual fragments of the light chain 2 could still be present. In spite of any broadening of the small aromatic peaks, the dominant result is the general lack of broadening by Mn^{2+}. When the negatively charged Tempop was added to S1 in as much as 80-fold excess, it also failed to broaden the narrow peaks. These unexpected results suggest that for all practical purposes the entire mobile region of S1 is buried in the interior of the molecule and that the surface is less mobile by comparison.

References

Akasaka, K., Konrad, M., & Goody, R. (1979) *FEBS Lett. 96*, 287–290.

Bagshaw, C., & Kendrick-Jones, J. (1979) *J. Mol. Biol. 130*, 317–336.

Handbook of Physics and Chemistry 1965–1966 (1965) (Weast, R. C., Ed.) Section F, Chemical Rubber Publishing Co., Cleveland, OH.

Highsmith, S., Akasaka, K., Konrad, M., Goody, R., Holmes, K., Wade-Jardetzky, N., & Jardetzky, O. (1979) *Biochemistry 18*, 4238–4244.

Huxley, A. F., & Simmons, R. (1971) *Nature (London) 233*, 533–538.

Huxley, H. E. (1969) *Science (Washington, D.C.) 164*, 1356–1366.

Kaminer, B., & Bell, A. L. (1966) *J. Mol. Biol. 20*, 391–401.

Kielley, W. W., & Bradley, L. B. (1956) *J. Biol. Chem. 218*, 653–659.

Mendelson, R. A., & Cheung, P. H. C. (1978) *Biochemistry 17*, 2139–2148.

Mendelson, R. A., Morales, M. F., & Botts, J. (1973) *Biochemistry 12*, 2250–2255.

Nauss, K. M., Kitagawa, S., & Gergely, J. (1969) *J. Biol. Chem. 244*, 755–765.

Shriver, J. W., Baldo, J. H., & Sykes, B. D. (1979) *Biophys. J. 25*, 244a.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 443–451, Wiley, New York.

Thomas, D. D., Seidel, J. C., Hyde, J. S., & Gergely, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1729–1733.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–57.