Limited Trypsinolysis Changes the Structural Dynamics of Myosin Subfragment 1[†]

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ABSTRACT: The effects of limited trypsinolysis of myosin subfragment 1 (S1) on its structural dynamics were investigated by using the method of transient electric birefringence. Conversion of S1 by trypsin to produce S1(T) did not change the specific Kerr constant [$(8.1 \pm 0.3) \times 10^{-7}$ and $(8.0 \pm 0.3) \times 10^{-7}$ cm²/statvolt² for S1(T) and S1, respectively] or the degree of alignment in a weak electric field, suggesting that the size of S1 and its permanent electric dipole moment are not modified by trypsin. On the other hand, the relaxation time for the field-free rotation, after achieving a steady-state birefringence signal, was reduced from 316 ns for S1 to 269 ns for S1(T), at 3.7 °C, suggesting that trypsinolysis increases the flexibility of the connections between S1 segments or introduces additional segmental motions. For both S1 and S1(T), the rate of decay for a steady-state signal was independent of the field strength, between 3.34 and 20.3 statvolt/cm. Shortening the duration of the weak electric field pulses to 0.35 μs, so that steady-state signals were not achieved, decreased the relaxation times for S1 and S1(T) to 240 and 210 ns, respectively, which is consistent with the segmented flexible S1 structure proposed earlier [Highsmith, S., & Eden, D. (1986) *Biochemistry 25*, 2237]. When the strength of the electric field was increased to above 10 statvolt/cm, in order to make the interaction energy for the S1(T) electric dipole moment in the electric field greater than the thermal energy, the relaxation time after a 0.35-μs pulse decreased from 210 to 170 ns as the field was increased from 7 to 20 statvolt/cm. The percent decrease for S1 was about the same. Thus, the elastic distortion of S1 structure that is observed when short strong electric fields are used may not be significantly changed by the action of trypsin on S1.

Recent improvements in transient electrical birefringence instrumentation (Eden & Elias, 1983) have made it possible to collect precise data using dilute solutions of proteins of molecular mass in the 10^5 dalton range. When this technique was applied to the contractile protein heavy meromyosin (HMM),[1] the segmental flexibility between the subfragment 1 (S1) and subfragment 2 regions (Mendelson et al., 1973) was quantitatively confirmed by using a weak electric field and a variable pulse length procedure to align the HMM to various degrees and then monitoring its rate of rotation back to equilibrium (Highsmith & Eden, 1985). In that procedure, the interaction energy ($U = \hat{\mu}_p \cdot \hat{E}$) for the electric dipole moment ($\hat{\mu}_p$) of the molecule in the electric field ($\hat{E}$) is kept too small to distort the molecular structure; so the molecule is simply aligned by the electric field. A long pulse rotates the entire molecule. If a molecule is segmented and flexible, as HMM is, the smaller segments align more rapidly, and their alignment and field-free motion back to random orientation can be resolved by using a sufficiently short pulse. This weak field, variable pulse length procedure also indicated that S1 itself is segmented and flexible (Highsmith & Eden, 1986). In addition, when the electric field strength was increased to make the interaction energy greater than the thermal energy ($U > 3kT/2$), the rate of rotation of S1 after a short pulse was increased. This increased rate of rotation was interpreted in terms of electric field induced reversible distortion of the S1 structure against an elastic restoring force, and the in-

teraction energy required to distort the S1 structure was shown to be comparable to the energy available from one ATP hydrolysis (Highsmith & Eden, 1986). Results are presented here for the application of the above procedures in order to compare S1 to trypsinized S1, or S1(T).

The limited action of trypsin on S1 that was prepared from myosin by using α-chymotrypsin is to convert the 97-kDa heavy chain into 25-, 50-, and 20-kDa fragments, which can be resolved by gel electrophoresis in the presence of sodium dodecyl sulfate (Mornet et al., 1979; Yamamoto & Sekine, 1979a; Balint et al., 1978). In the absence of denaturants, the fragments stay associated, even if the light chains are removed (Burke & Kamalakannan, 1986). S1(T) has unmodified Ca- and Mg-ATPase activities in the absence of actin and unmodified actin binding properties in the absence of nucleotides. However, the actin-activated ATPase activity is greatly reduced (Mornet et al., 1979; Yamamoto & Sekine, 1979c; Botts et al., 1982; Furukawa & Arata, 1984), suggesting that although the nucleotide and actin binding sites are not modified, the interaction between the sites is modified by limited trypsinolysis (Botts et al., 1982). Nucleotide binding affects the proteolytic susceptibility of S1 (Hozumi, 1982; Applegate & Reisler, 1984), which is consistent with the idea that the trypsin binding sites on S1 are involved in the transduction mechanism.

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[1] Abbreviations: S1, myosin subfragment 1; S1(T), trypsinized S1; τ, rotational relaxation time; Φ, orientation function; μ_p, permanent electric dipole moment; Δn_{av}, steady-state birefringence; Δn_s, saturation birefringence; E, electric field; K_{sp}, specific birefringence; Tris, tris(hydroxymethyl)aminomethane; U, interaction energy; HMM, heavy meromyosin; kDa, kilodalton(s); SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

Understanding the nature of this trypsin-induced modification of the mechanochemical energy-transducing properties of myosin is important.

Several results suggest that the denatured resolved primary sequences (25, 50, and 20 kDa) have tertiary structural counterparts in the form of domains of S1 and that the domains have different functions. Only the 50- and 20-kDa fragments are cross-linked to actin (Yamamoto & Sekine, 1979b; Mornet et al., 1981; Sutoh, 1982), whereas only the 25-kDa fragment is photoaffinity labeled, using ATP analogues (Okamoto & Yount, 1985). The 50-kDa portion of skeletal S1 is selectively made proteolytically susceptible by heat (Setton & Muhlrad, 1984). The fragments may even have independent structural integrity when isolated. The 20-kDa fragment attached to the myosin rod, with apparent tertiary structure, can be isolated and seen in negatively stained electron micrographs (Winkelmann et al., 1984). The 20-kDa fragment also can be isolated from S1(T) and when freed from denaturant will bind to actin (Muhlrad & Morales, 1984), as will a 10-kDa fragment derived from the 20-kDa fragment (Katoh et al., 1985). Skeletal muscle S1 is flexible, suggesting it is segmented (Highsmith & Eden, 1986). A segmented flexible scallop S1 structure also has been proposed, on the basis of electron micrographs (Vibert et al., 1986). On the basis of these suggestions of the apparently independent structural and functional integrity of the fragments, there has been widespread speculation that S1 function depends upon interactions between structural domains that consist of the 25-, 50- and 20-kDa primary sequence fragments.

Thus far, there is little evidence to suggest that limited trypsinolysis causes a global conformational change in S1. The rate of rotational Brownian motion, determined by time-resolved fluorescence anisotropy decay measurements on S1 and S1(T) that were modified with an extrinsic fluorescent probe, was not changed within experimental error by trypsinolysis (Botts et al., 1982). Conformational changes have been detected by fluorescence energy transfer measurements on S1 and S1(T) modified at two locations with energy transfer donor–acceptor pairs, which suggest limited trypsinolysis causes certain intramolecular distances to change by 1–5%, assuming that the local orientations of the donor and acceptors remain unchanged (Cheung et al., 1985). Reported here are data that suggest limited trypsinolysis causes a substantial change in S1 structural dynamics.

EXPERIMENTAL PROCEDURES

S1 was prepared from rabbit dorsal muscle myosin by the action of α-chymotrypsin in the absence of Mg^{2+} (Weeds & Pope, 1976) and purified by size-exclusion chromatography by using Sephacryl-S400. Light chains 1 and 3 were present, light chain 2 was absent, and the 95 000-dalton heavy-chain fragment was homogeneous when analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Weeds & Pope, 1976; Highsmith & Eden, 1986). Limited trypsinolysis of S1 was done according to Mornet et al. (1979), and the size-exclusion column purified S1(T) had its heavy chain more than 95% converted to 50-, 25-, and 20-kDa fragments, when analyzed by gel electrophoresis in the presence of SDS. ATPase activities at 25 °C in 100 mM KCl, 5 mM $CaCl_2$, 2 mM ATP, and 10 mM MOPS (pH 7.5) were typically 3.1 s^{-1}. Samples of 5–20 μM S1 and S1(T) were dialyzed together against 10 mM Tris–acetate (pH 7.0) and 0.1 mM $MgCl_2$ at 4 °C and centrifuged at 80000g for 1 h before birefringence measurements were made. The stock protein solutions were diluted with the final dialysate to obtain the desired protein concentration.

Steady-state electric birefringence measurements were made by using the high-power pulse generator and the instrument described previously (Eden & Elias, 1983; Highsmith & Eden, 1985, 1986). In each series of measurements, for the first two voltages used, data consisting of one-tenth the number of electric field pulses needed for good statistics were collected, stored, and used to obtain a steady-state birefringence that corresponded to a fresh S1 sample. These values for the birefringence were always equal, within experimental error, to the values obtained subsequently with 10 times as many pulses. The field strengths were varied randomly, and at least one early measurement was repeated at the end of a series to ensure further that the electric field was not altering the samples. The electric field pulses were 5 and 7 μs long and varied between 2.47 and 24.7 statvolts/cm (1 statvolt = 299.75 V).

The time courses of the decay of the birefringence signals were determined by analyzing the decay after a steady-state signal was obtained by using a 7-μs pulse, or by using fresh samples of S1 or S1(T) and 0.35-μs pulses produced by a cable discharge pulser. For the short pulses, many more applications of the electric field were required to obtain a signal that provided adequate statistics. The CaATPase activity was determined after measurements and was within 90% of the original value for the data shown here.

The decay of the birefringence signal, after correcting for the signal obtained with the laser beam blocked (Highsmith & Eden, 1986), was fit by using a multiexponential decay analysis program titled "DISCRETE" (Provencher, 1976a,b). The base line (typically $\sim$1% of the maximum signal) was a floating parameter, and the program determined the minimum number of exponential decays that were required to fit the data, given its accuracy. In over 85% of the cases, the best fit was obtained with a single-exponential decay. In the remaining cases, the single exponential was the second best fit. There was no correlation between the cases where the single-exponential decay was second best and the strength of duration of the pulse, or the signal to noise ratio. All the decay constants (τ) reported here are for single-exponential functions using the equation $\Delta n/\Delta n_{max} = A \exp(-t/\tau) + B$, where Δn is the birefringence, t is the time, and A and B are constants.

RESULTS AND DISCUSSION

Solutions were prepared as described under Experimental Procedures to obtain [S1] and [S1(T)] near 4 μM. With the use of the apparatus described earlier (Highsmith & Eden, 1985, 1986), a steady-state birefringence signal was achieved for either protein when the duration of the applied electric field was longer than 5 μs. A typical trace of the increase in signal due to the applied field and its decay after the field is removed is shown in Figure 1 for S1(T). The slowness of the signal increase (downward in Figure 1) in comparison to the return of the signal to the base line is consistent with an orientation mechanism for S1(T) that is dominated by the permanent electric dipole moment of the molecule, with negligible rapidly induced dipole moment contributions (Tinoco & Yamaoka, 1959), as was observed for S1 (Highsmith & Eden, 1986).

The magnitude of the steady-state birefringence was measured for applied electric fields in the 2.47–19.8 statvolt/cm range for the S1 and S1(T) solutions at 3.7 °C, and the results are shown as a Kerr plot in Figure 2A. The Kerr law is obeyed up to about 150 $statvolt^2/cm^2$, and S1(T) has a specific birefringence, $(8.0 \pm 0.3) \times 10^{-7}$ $cm^2/statvolt^2$, equal to that of S1, $(8.1 \pm 0.3) \times 10^{-7}$ $cm^2/statvolt^2$, within experimental error. This result does not have an unambiguous interpretation. The observed birefringence signal is an average value
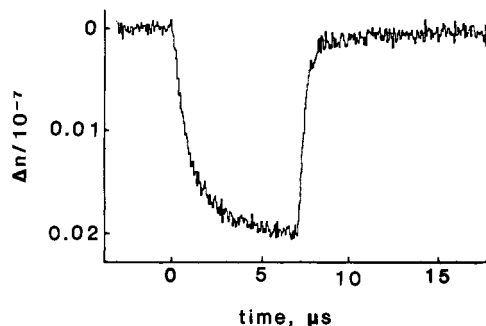
FIGURE 1: At time = 0, a solution of 4.0 μM S1(T), 10 mM Tris-acetate, and 0.1 mM $MgCl_2$, pH 7.0 at 3.7 °C, was subjected to a 7-μs 2.47 statvolt/cm electric field pulse. The values for Δn_{av} used to calculate K_{sp} and Φ (see Figure 2) were obtained from the data in the 6.3–6.8-μs region, and the decay time, τ, was obtained by analyzing the region beyond t = 7 μs. The positive birefringence is displayed downward for technical reasons. The trace shown was obtained by averaging data from 500 pulses.

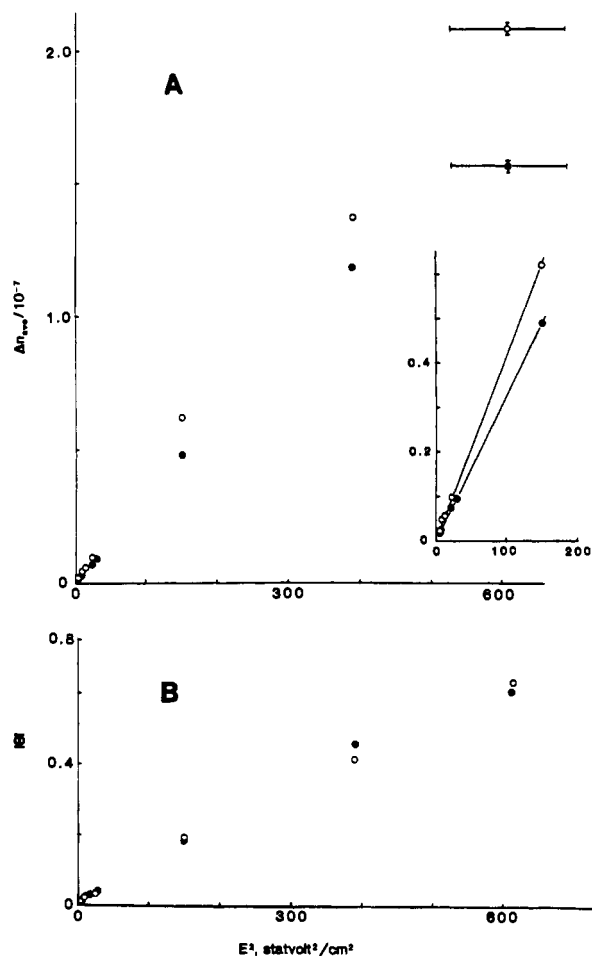FIGURE 2: (A) Steady-state birefringence signal (see Figure 1) is shown for 4.5 μM S1 (O) and 4.0 μM S1(T) (●) as a function of E^2. The inset shows the Kerr law region in greater detail. When the magnitudes of the birefringence signals were corrected for the differences in protein concentration, specific Kerr constants of 8.1×10^{-7} and 8.0×10^{-7} $cm^2/statvolt^2$ were obtained for S1 and S1(T), respectively. (B) Orientation function Φ (the relative degree of alignment) is shown over the same E^2 region.

that is composed of contributions from all of the S1 structure. The individual contributions may be positive or negative; Thus, the unchanged Kerr constant could reflect an unchanged S1 structure with respect to the arrangement of the individual structural components that contribute to the average, or it could result from compensating changes that coincidentally give the same average value. The former interpretation is
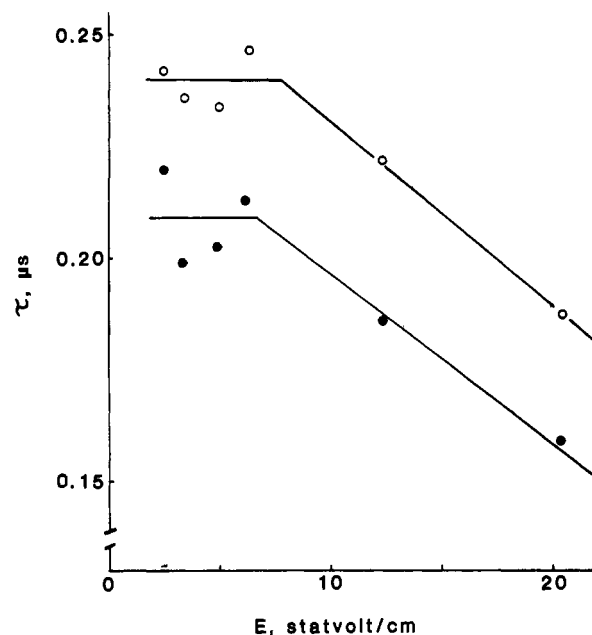

FIGURE 3: Rotational relaxation times, τ, for S1 (O) and S1(T) (●) are shown for increasing electric field strength pulses of 0.35-μs duration.

simpler, and there is no reason to doubt it, but it is not unique.

Values for the saturation birefringence, Δn_{sat}, were obtained by extrapolation to $1/E$ = 0 (Fredericq & Houssier, 1973), and the orientation function ($\Delta n/\Delta n_{sat}$ = Φ) is plotted against E^2 in Figure 2B. These data indicate that the relative degree of alignment at steady state is the same, within experimental error, for S1 and S1(T) when they are exposed to electric fields in the 2.47–19.8 statvolt/cm region. Thus, the two proteins have equal electric dipole moments under these conditions.

The field-free rate of rotation after a 7-μs square pulse was adequately fit by a single-exponential decay function (Provencher, 1976a,b). For decay from a steady-state signal, the S1 and S1(T) rotational relaxation times changed by less than 7% when the applied electric field was increased from 3.34 to 20.3 statvolt/cm. The average rotational relaxation times in that field strength range were 316 ± 7 and 269 ± 9 ns for S1 and S1(T), respectively. These data indicate that S1(T) is effectively more compact than S1,[2] with regard to rotational Brownian motion. S1(T) could be either more flexible or shorter than S1. Modeled as a prolate ellipsoid, S1(T) would have to be about 1 nm shorter. Given the equivalent Kerr constants and permanent dipole moments for the two proteins (Figure 2A,B), it is possible, but seems less likely, that S1 has been made shorter by trypsinolysis. The alternative that S1(T) is more flexible than S1 is supported by the results below.

When the field strength was kept at 3.34 statvolt/cm and the duration of the weak pulse was reduced to 0.35 μs, the field-free rotational relaxation times were reduced by about 20% to 250 ± 10 and 210 ± 12 ns for S1 and S1(T), respectively. These decreases in the observed rotational relaxation time when a weak-field pulse is shortened indicate segmental flexibility (Highsmith & Eden, 1985, 1986). The smaller value for S1(T) suggests that the segments being preferentially aligned during the brief pulse are smaller or more

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[2] In an earlier publication (Highsmith & Eden, 1986), the length of S1, when modeled as a prolate ellipsoid, was calculated to be 19–20 nm from the field-free rate of rotational Brownian motion. In that calculation, the value for the axial ratio was erroneously given as 3.5 instead of 4.3. The calculated length was correct and is consistent with the data given here.

flexible than their counterpart in S1. The rotational relaxation times observed for S1 and S1(T) after a 0.35-μs pulse (Figure 3) were independent of the electric field strength in the 2.4–6.2 statvolt/cm range (where $U < 3kT/2$), suggesting segmented flexible motion, rather than bending of a uniformly flexible material.

When the strength of the electric field for the 0.35-μs pulse was increased above 10 statvolt/cm into the range where $U > 3kT/2$, the observed rotational relaxation times for S1 and S1(T) decreased (Figure 3). This behavior has been reported for S1 and interpreted by assuming a structural model for S1 that has at least two domains connected by a linkage that allows the domains to freely move in a restricted volume and to be further moved against a restoring force when the interaction energy is sufficiently large (Highsmith & Eden, 1986). Presumably, the torque of the electric field on the dipole moments that are more nearly perpendicular to the field transiently bends those S1's to a more compact form. If this is the case, the dependency of τ on E provides an estimate of the relative stiffness of the structural component of S1(T) which can be distorted by an electric field, compared to its counterpart in S1. The data in Figure 3 suggest that S1 and S1(T) do not have significantly different elastic properties under these conditions. Regardless of the mechanism by which a briefly applied strong electric field increases the rate of field-free rotation, the data in Figure 3 suggest that S1 and S1(T) are equally susceptible to it, within experimental error.

In conclusion, the data presented here are consistent with a segmented and flexible structure for the S1 portion of myosin (Highsmith & Eden, 1986; Vibert et al., 1986). Proteolytic action appears to increase the segmental flexibility. To the best of our knowledge, this is the first demonstration of a substantial structural difference between intact S1 and S1(T).

REFERENCES

Applegate, D., & Reisler, E. (1984) *Biochemistry 23*, 4779–4784.

Balint, M., Wolf, I., Tarcsafalvi, A., Gergely, J., & Streter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793–799.

Botts, J., Muhlard, A., Takashi, R., & Morales, M. F. (1982) *Biochemistry 21*, 6903–6905.

Burke, M., & Kamalakannan, V. (1985) *Biochemistry 24*, 846–852.

Cheung, H. C., Gonsoulin, F., & Garland, F. (1985) *Biochim. Biophys. Acta 832*, 52–62.

Eden, D., & Elias, J. G. (1983) in *Measurement of Suspended Particles by Quasi-elastic Light Scattering* (Dahneke, B., Ed.) p 401–438, Wiley, New York.

Fredericq, E., & Houssier, C. (1973) in *Electric Dichroism and Electric Birefringence* (Harrington, W., & Peacocke, A. R., Eds.) pp 1–60, Clarendon Press, Oxford.

Furukawa, K. I., & Arata, T. (1984) *J. Biochem. (Tokyo) 95*, 1343–1348.

Highsmith, S., & Eden, D. (1985) *Biochemistry 24*, 4917–4924.

Highsmith, S., & Eden, D. (1986) *Biochemistry 25*, 2237–2244.

Hozumi, T. (1983) *Biochemistry 22*, 799–804.

Katoh, T., Katoh, H., & Morita, F. (1985) *J. Biol. Chem. 260*, 6723–6727.

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

Mornet, D., Pantel, P., Audemand, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925–932.

Mornet, D., Pantel, P., Bertrand, R., Audemand, E., & Kassab, R. (1981) *FEBS Lett. 123*, 54–58.

Muhlrad, A., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1003–1007.

Okamoto, Y., & Yount, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1575–1579.

Provencher, S. W. (1976a) *Biophys. J. 16*, 27–41.

Provencher, S. W. (1976b) *J. Chem. Phys. 64*, 272–277.

Setton, A., & Muhlrad, A. (1984) *Arch. Biochem. Biophys. 235*, 411–417.

Sutoh, K. (1982) *Biochemistry 21*, 4800–4804.

Tinoco, I., & Yamaoka, K. (1959) *J. Phys. Chem. 63*, 423–427.

Vibert, P., Szentkiralyi, E., Hardwicke, P., Szent-Gyorgyi, A. G., & Cohen, C. (1986) *Biophys. J. 49*, 131–133.

Weeds, A., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.

Winkelmann, D. A., Almeda, S., Vibert, P., & Cohen, C. (1984) *Nature (London) 307*, 758–760.

Yamamoto, K., & Sekine, T. (1979a) *J. Biochem. (Tokyo) 86*, 1855–1862.

Yamamoto, K., & Sekine, T. (1979b) *J. Biochem. (Tokyo) 86*, 1863–1868.

Yamamoto, K., & Sekine, T. (1979c) *J. Biochem. (Tokyo) 86*, 1869–1881.