The Tritium–Hydrogen Exchange of Myosin and Its Proteolytic Fragments*

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ABSTRACT: The tritium–hydrogen exchange of myosin, light meromyosin (LMM), and heavy meromyosin (HMM) has been studied by means of the gel filtration technique of Englander. Computer analysis of the exchange curves allowed a small and discrete number of exchanging classes of markedly differing rate constants to be identified. A detailed examination of the pH and temperature dependence of exchange for each cluster of hydrogen atoms revealed that the results are consistent with a Linderstrøm–Lang-type exchange mechanism in myosin and its proteolytic fragment LMM. Estimates of the half-times of exposure of the exchanging groups to solvent ranged between 20 and 2000 min while the rates of closure are about 0.1 sec. Calculation of the free energies for the opening reaction show that these vary from $\Delta F^\circ = 3$ to >9 kcal/mole and that the more stable clusters are present in the heavy meromyosin portion of the molecule. The light meromyosin segment, which is presumed to be a coiled coil of α helices, contains five classes of exchanging hydrogens. The rate of opening of the hydrogen clusters in this segment of myosin is less (by a factor of 10^9) than that reported for α-helical polyglutamic acid.

Over the past few years an increasingly well-defined model for the structure of myosin has emerged from a combination of solid-state and solution physical–chemical studies. Most of the solution techniques which have been employed measure time-independent properties which are averaged over all molecules, and thus give information relevant only to the most abundant conformations within the molecules. Additional insight into the structure can be obtained in principle by isotopic exchange techniques which provide a measure of the sizes and stabilites of various motile structural regions within the protein. Since the myosin molecule is now known to be composed of two morphologically distinct segments (fibrous and globular), it would be expected that these regions would show quite different and informative exchange properties. Indeed, Hartshorne and Stracher (1965) and Willumsen (1966) have reported that the peptide hydrogen atoms of the fibrous portion of the molecule (LMM)[1] and the globular portion (HMM) are markedly different in exchange behavior. The combined results of these two groups indicate that the deuterium–hydrogen-exchange rates of myosin, LMM, and HMM are all strongly pH dependent.

These findings have prompted us to investigate the exchange reaction of myosin and its proteolytic fragments over closely spaced pH and temperature intervals in an attempt to isolate and identify the kinetic classes of hydrogens originating from structural fragments within the molecule. Kinetic analysis of the time-dependent loss of bound tritium indicates that the over-all exchange process is a composite of several distinct and independent classes of exchanging hydrogens in both the LMM and HMM segments of the molecule. The variation of the kinetic parameters of these classes with pH and temperature conforms closely to that expected for a Linderstrøm-Lang-type hydrogen-exchange mechanism (Linderstrøm-Lang, 1955). This simple model has allowed us to make quantitative estimations of the stabilities of the exchanging fragments and their rates (opening and closing) of exposure to the solvent. The results would seem to be relevant to the mechanism of exchange in other protein systems as well.

Materials and Methods

The rate of loss of protein-bound tritium from myosin and its proteolytic fragments, HMM and LMM, was followed by the method of Englander (1963). In this technique the protein is equilibrated with tritiated water, and the exchange reaction was initiated by passing the equilibrated solution through a Sephadex G-25 column. A major difficulty arises from the necessity of tritiating the sample prior to experimentation, a procedure which often results in irreversible denaturation of the protein. We have

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[1] Abbreviations used: LMM and HMM, light and heavy meromyosin; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; ATPase, adenosine triphosphatase; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; PLGA, poly-L-glutamic acid.

found in the present study that the most effective method of tritiation is to equilibrate the experimental sample with tritiated water for 1 hr at pH 9.5 and 0°, followed by further equilibration at pH 7–8 at 4° overnight. The effectiveness of the high-pH treatment is undoubtedly a result of the well-known base catalysis of the exchange of proteins and polypeptides (Bryan and Nielsen, 1960; Klotz and Frank, 1965; Hvidt and Kanarek, 1963). Myosin treated in this way retained most (82%) of its ATPase activity, and no changes in sedimentation properties were observed. It is also known from the work of Lowey (1965) that there is no observable change in the polypeptide chain conformation of myosin, LMM, and HMM, as judged by the invariance of the Moffit parameter, b_0, in the pH range 6–10. Although the high-pH procedure appears to greatly enhance the degree of tritiation without significantly altering the native conformation of myosin and its proteolytic fragments, the possibility still exists that some of the potentially exchangeable hydrogens may not have reached equilibrium with the solvent. Such hydrogens are, therefore, not detected in the present experiments.

In the one-column experiments where only rapidly exchanging hydrogens were measured, equilibration with tritiated water was carried out overnight at the pH of the exchange reaction. F-actin was allowed to equilibrate with tritiated water for 2 days at pH 8.1 in 0.1 M Tris–0.5 M KCl at 4°.

The columns used in the present study are 28 × 150 mm, water-jacketed chromatography columns with Teflon stopcocks (Scientific Glass Apparatus Company, Bloomfield, N. J.). To eliminate dead space, the lower parts of the columns were filled with about a 1-cm layer of fine glass beads. Small circular pieces of filtering silk (Joymar Scientific, Inc., New York, N. Y.), placed beaneath this layer, were used to hold the beads in the columns. Coarse-grained Sephadex G-25 was deaerated under vacuum and poured over the glass beads to form a 9–12-cm column. The gel was then stirred and allowed to settle. A hollow Perspex cylinder similar to the commercial Sephadex sample applicator was placed on top of the gel with the result that buffer or sample could be applied rapidly to the column without disturbing the top. With these columns elution requires approximately 1 min and 1-ml samples can be purified 10^5–10^6-fold from tritium by a single elution. No change in the ATPase activity or sedimentation pattern of myosin was observed following elution from these columns. It was found, however, that the concentration of myosin solutions had to be kept below 0.4%, and F-actin below 0.1%, to obtain adequate chromatographic separation.

Ultracentrifugation studies on the various myosin preparations showed no detectable aggregation (Schlieren optics) at the lowest pH (5.6) used for exchange experiments. On the other hand, significant aggregation was observed in the LMM preparations below pH 6.0 and exchange experiments are, therefore, not reported in this range. It should be noted that both myosin and HMM retain only a fraction of their ATPase activity at pH 6.0 and 5.6.

Both the one-column and the two-column methods of Englander (1963) were employed. In one-column experiments, temperature was controlled to ±0.3° by circulating water from a constant-temperature bath through the jackets on the columns. In two-column experiments, exchange reactions were carried out in separate water-jacketed vessels, and temperature was regulated to ±0.05°. Hydrogen exchange was followed in 0.5 M NaCl, with either maleate, Tris, or borate buffers, adjusted to the desired pH with a Radiometer PHM4c pH meter, which had been standardized against National Bureau of Standards phthalate and phosphate buffers. Maleate, which has a low temperature coefficient, was brought to the desired pH at room temperature, but the pH of borate and Tris were adjusted at 0°. The observed pH of Tris was corrected to 3.3°, the temperature at which exchange experiments in this buffer were carried out, by subtracting 0.1 pH unit. The pH values reported are accurate to an estimated ±0.02 pH unit.

Protein concentrations were determined spectrophotometrically from ultraviolet absorptions using the following extinction coefficients: 543 cm^2/g (λ 279 mμ) for myosin (Gellert and Englander, 1963), 647 and 369 cm^2/g (λ 280 mμ) for HMM and LMM, respectively (Young *et al.*, 1964), and 507 cm^2/g (λ 276 mμ) for myosin in 5 M guanidinium chloride (Kielley and Harrington, 1960). Protein concentrations were also estimated by the microbiuret method (Zamenhof, 1956). Biuret extinction coefficients were determined from the biuret absorptions of samples of myosin, HMM, and LMM of known 280-mμ absorption. Tritium was assayed either with a Nuclear-Chicago Model 6804 or with a Packard Model 3365 liquid scintillation counter. The scintillation fluid was that described by Bray (1960), except that POPOP and ethylene glycol were omitted. Quench corrections were applied in the guanidinium chloride studies by the addition of internal standards. It was found that quench corrections were unnecessary in other studies. The number of unexchanged hydrogen atoms per molecule was calculated from the specific activity of effluent protein using the formula given by Englander (1963) and molecular weights of 5.95×10^5 for myosin (Woods *et al.*, 1963), 1.62×10^5 for LMM, and 3.62×10^5 for HMM (Young *et al.*, 1964). Results of experiments in which the exchange of actin was measured are reported as unexchanged hydrogens/10^5 g assuming a biuret extinction coefficient equal to that of HMM.

In experiments where the exchange of one protein was followed in the presence of another, the two-column procedure was employed. After the first gel filtration step, the biuret absorption of the tritiated (exchanging) protein was determined. A weighed amount of this protein solution was then added to a predetermined amount of the second, nonradioactive protein (in the same solvent). The fraction of the optical density due to the tritiated protein was calculated and used to normalize the specific activity of the

effluent protein from the second column fractionation to the number of unexchanged hydrogen atoms per tritiated protein molecule. Since the first chromatography step results in a 10^4–10^5-fold reduction in solvent tritium, the uptake of tritium by the added protein is negligible when compared to the tritium bound to the exchanging protein. That all proteins exhibited the same elution behavior on our Sephadex G-25 columns was indicated by constant specific activities in the protein peak following the second gel filtration step.

Rabbit myosin was prepared by the method of Kielley and Bradley (1956). LMM fraction 1 was prepared from "75-sec" tryptic digests of myosin (weight ratio of trypsin to myosin 1:250, 25° at pH 7; reaction was terminated by the addition of a twofold weight excess of soybean trypsin inhibitor) by the method of Szent-Györgyi (1953) and Szent-Györgyi *et al.* (1960). HMM was prepared from the same digests by centrifuging the low ionic strength supernatant (after removal of LMM and undigested myosin) overnight at 50,000 rpm in a Spinco Model L ultracentrifuge. The resulting HMM pellet was redissolved by gentle stirring and dialyzed against the appropriate buffer. At all stages in the preparation of HMM following digestion, the solvent was maintained 0.1 M in 2-mercaptoethanol to reduce the disulfide-caused aggregation which is frequently observed in HMM preparations. F-actin was prepared by the method of Mommaerts (1951), and oxidized ribonuclease by the method of Hirs (1956). Oxidized ribonuclease solutions were passed through Sephadex G-25 columns to select for fronting material before use.

All chemicals used in these experiments were reagent grade. Tritiated water was purchased from Volk Radiochemical Co. Trypsin, soybean trypsin inhibitor, and ribonuclease were obtained from Worthington Biochemical Corp., and ATP was purchased from the Sigma Chemical Co. Guanidine·HCl was obtained from the Eastman Chemical Co. and was recrystallized once from a methanol–ether solution. Hydrolysis of ATP was determined by assaying inorganic phosphate as the phosphomolybdate complex (Kielley and Bradley, 1956).

Kinetic Analysis of Exchange Data. Exchange data were analyzed as a sum of independent first-order reactions by the method of Prony (Hildebrand, 1956) with the aid of an IBM 7094 digital computer. In this procedure a set of $m + 1$ evenly spaced points, f(0), f(1), ... f(m) is approximated by the following equation.

$$f(x) = C_1 r_1^x + C_2 r_2^x + \ldots\ldots\ldots C_n r_n^x, \qquad x = 0, 1, 2 \ldots m$$

The particular program employed in the present study fit the data with any desired number of exponential terms, n, providing $n \leqslant 50$ and $m + 1 \geqslant 2n$. Our procedure has been to draw the best smooth curve through the data points, and to take directly from this curve evenly spaced values (usually one point was taken every 15 min) of the number of unexchanged hydrogens per molecule. These values were then used to make the Prony approximation (after subtracting infinite time values (see below)), and each r_i and C_i was normalized to a first-order rate constant and class size, respectively.

Curves were reconstructed from kinetic parameters with the aid of another computer program. The number of unexchanged hydrogen atoms per molecule, $H(t)$, was calculated for various values of t, using the equation $H(t) = \Sigma H_i e^{-k_i t}$.

To determine the proper number of exponential terms (n) needed to describe a given curve, a set of data points was approximated with various numbers of terms. It was found that if n was increased stepwise (while using the same values of f(x)), after a certain value of n, all additional terms were of insignificant magnitude and usually imaginary. All of our curves were described by three or less parallel first-order reactions. Between 30 and 40 points were used in our analyses and six-term approximations were made. Small and oscillating terms were discarded, and the curves were reconstructed from the significant terms in order to check the approximation. To test our method of analysis, values of $H(t)$, calculated from the equation

$$H(t) = 1029e^{-0.00171t} + 1176e^{-0.0105t} + 1190e^{-0.0798t} + H_\infty,\ t = 0, 15, 30 \ldots\ldots 585 \text{ min}$$

TABLE I: Computed Rate Constants from the Curve[a] $H(t) = 1029e^{-0.00171t} + 1176e^{-0.0105t} + 1190e^{-0.0798t} + H_\infty$. Forty Evenly Spaced Points from $t = 0$ to 585 min Were Used in This Calculation.

H_∞	H_3	k_3	H_2	k_2	H_1	k_1
0	1031	1.72×10^{-3}	1175	1.06×10^{-2}	1189	8.01×10^{-2}
100	1068	1.38×10^{-3}	1232	1.02×10^{-2}	1192	7.96×10^{-2}
300	1211	9.98×10^{-4}	1290	9.86×10^{-3}	1192	7.96×10^{-2}
600	1476	7.13×10^{-4}	1325	9.70×10^{-3}	1190	7.95×10^{-2}
1000	1842	5.04×10^{-4}	1358	9.50×10^{-3}	1186	7.92×10^{-2}

[a] The constants used in this curve are similar to those which describe the exchange of myosin.

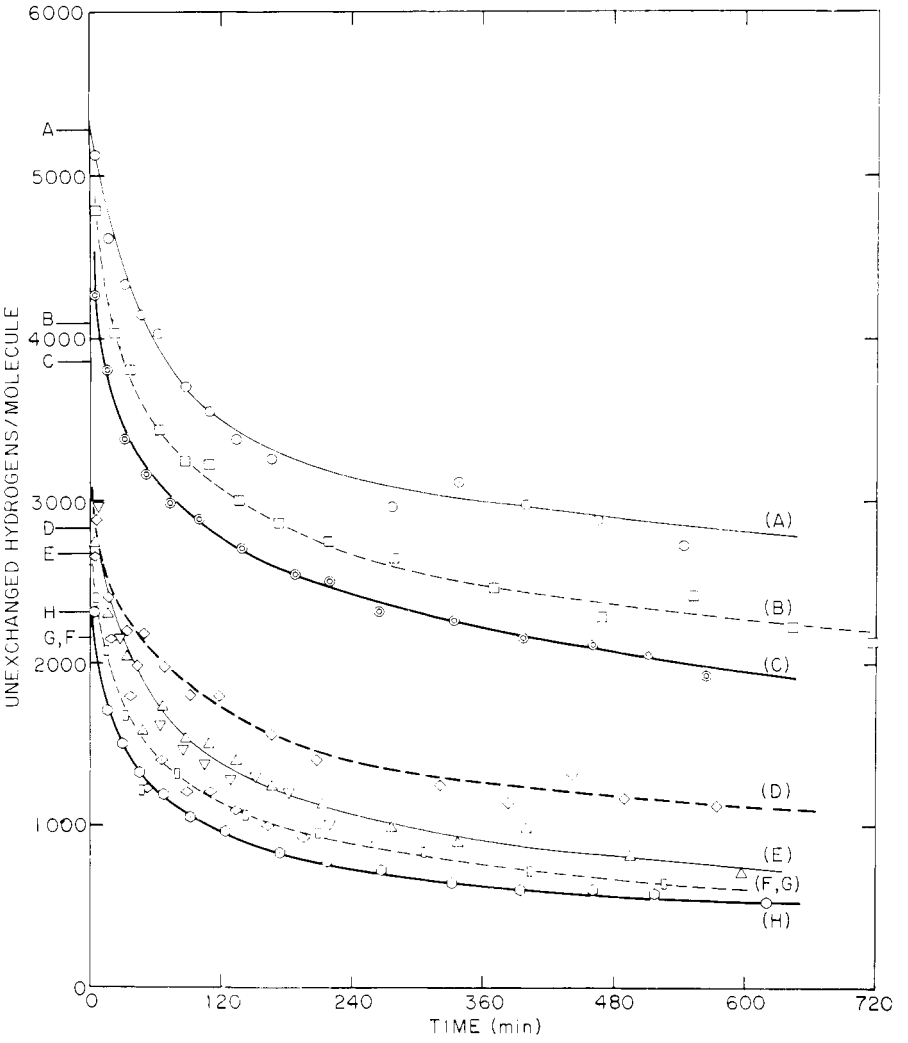

FIGURE 1: The time-dependent exchange of myosin in 0.5 M NaCl at 3.3° and various pH values. (A) pH 5.61 (—⊙—), 0.05 M maleate; (B) pH 6.00 (- -⊡- -), 0.05 M maleate; (C) pH 6.50 (—ⓞ—), 0.05 M maleate; (D) pH 6.99 (- -◈- -), 0.05 M maleate; (E) pH 7.52 (—▽—), 0.01 M Tris, and (—△—), 0.05 M Tris; (F) pH 7.98 (- -⊡- -), 0.05 M Tris; (G) pH 8.46 (- -◈- -), 0.02 M borate; and (H) pH 8.94 (—▯—), 0.05 M borate.

were used to make a six-term Prony approximation. The constants used in this equation are similar to those describing the exchange of myosin. Table I presents the significant rate constants and class sizes retrieved for various values of H_∞. H_∞ can be considered as a class of exchanging hydrogens with rate constant $k = 0$. Although we let $n = 6$, in each case only three large, nonoscillating terms were recovered. As can be seen, when $H_\infty = 0$ the approximation is excellent. However, when $H_\infty > 0$, the slowest reaction (reaction 3 of Table I) analyzes as a single first-order reaction of rate constant between $k_3 = 1.71 \times 10^{-3}$ and 0. The difficulty in resolving the slow reaction from the zero rate reaction (H_∞) is not surprising since the slow reaction is followed in the analysis for only about 1.5 half-times. In the present experiments, infinite time extrapolated values of unexchanged hydrogen atoms per molecule are not, in general, zero. Therefore, in order to increase the accuracy in the determination of slow-reaction parameters, plots of the *rate* of hydrogen exchange *vs.* the number of unexchanged hydrogens per molecule were extrapolated to zero rate. The intercepts gave the number of hydrogens present at "infinite time" (H_∞), and these were subtracted from values taken directly from the exchange curve, before analysis.

Often as a result of extended extrapolations and large scatter in rates, it was difficult to obtain an accurate size for the "zero-rate" reaction. It was observed, however, that rate plots from exchange reactions carried out in a narrow range of pH or temperature tended to extrapolate to similar values. Therefore, the somewhat arbitrary procedure of picking the best value (*i.e.*, shortest extrapolate) from a cluster of H_∞ values and using it for all curves of the cluster was adopted. In cases where only fast reaction rates

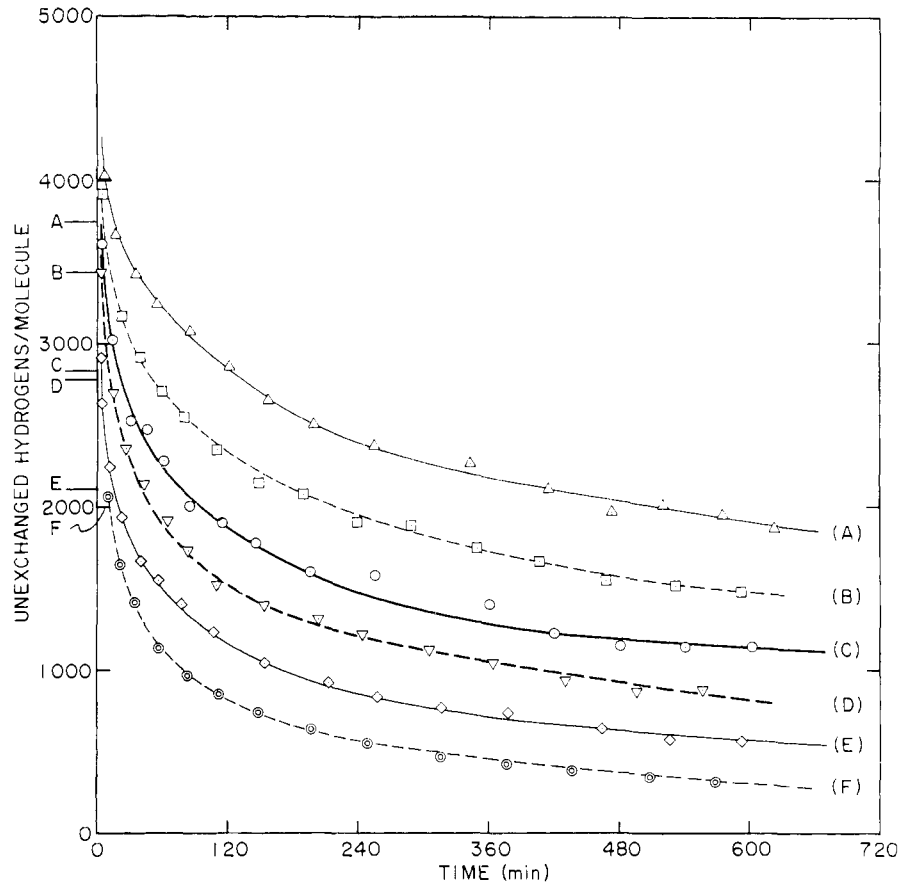

FIGURE 2: The time-dependent exchange of myosin in 0.5 M NaCl–0.05 M maleate, pH 6.50, at various temperatures. (A) (—△—) 3.3°; (B) (- -□- -) 8.1°; (C) (—○—) 13.1°; (D) (- -▽- -) 18.0°; (E) (—◇—) 23.1°; and (F) (- -⓪- -) 28.0°.

and class sizes were needed, infinite time values were not subtracted.

Results

Hydrogen Exchange of Myosin and Its Proteolytic Fragments

Hydrogen Exchange of Myosin. Figure 1 presents the results of a series of exchange studies of myosin at 3.3° as a function of pH. In each experiment myosin in 0.5 M KCl was allowed to equilibrate at pH 9.5, 0°, for 1 hr with tritiated water (*ca.* 1 mc/ml of solution). The solution was then titrated to neutrality and allowed to stand overnight. Exchange experiments were initiated by passing 2-ml aliquots through Sephadex columns preequilibrated with the solvent used in the experiment and the number of unexchanged hydrogens per molecule determined according to the procedure described in Materials and Methods.

As will be seen in Figure 1, the number of unexchanged hydrogen atoms present in the myosin molecule at any given time is strongly dependent on the pH of the experiment and decreases with increasing pH. The dependence of the number of slowly exchanging hydrogens on pH is greatest in the low pH range (pH 5.6–7.0). Above pH 7.0, a much smaller pH dependence is observed and, in fact, the pH 8.0 and 8.5 curves superimpose within experimental error. A striking feature of these curves is the large shift in the number of slowly exchanging hydrogens over the range pH 6.5–7.0. To anticipate the findings presented in a later section, this unusual feature is not observed in either of the two proteolytic fragments of myosin, LMM or HMM. Although profound changes are observed in the hydrogen-exchange behavior of myosin as a function of pH, the polypeptide chain conformation of myosin is not significantly altered over the pH range 5.6–9.0, as judged by optical rotatory studies (Lowey, 1965).

The effect of temperature on the exchange behavior of myosin at a fixed pH (6.5) is shown in Figure 2, demonstrating that the number of slowly exchanging hydrogens at any given time decreases in a regular manner with increasing temperature. As in the pH dependence of exchange, no comparable changes in the structure of myosin are observed over this temperature range.

Although no simple correlation exists between the hydrogen-exchange behavior of myosin as a function of pH and temperature and the polypeptide chain con-

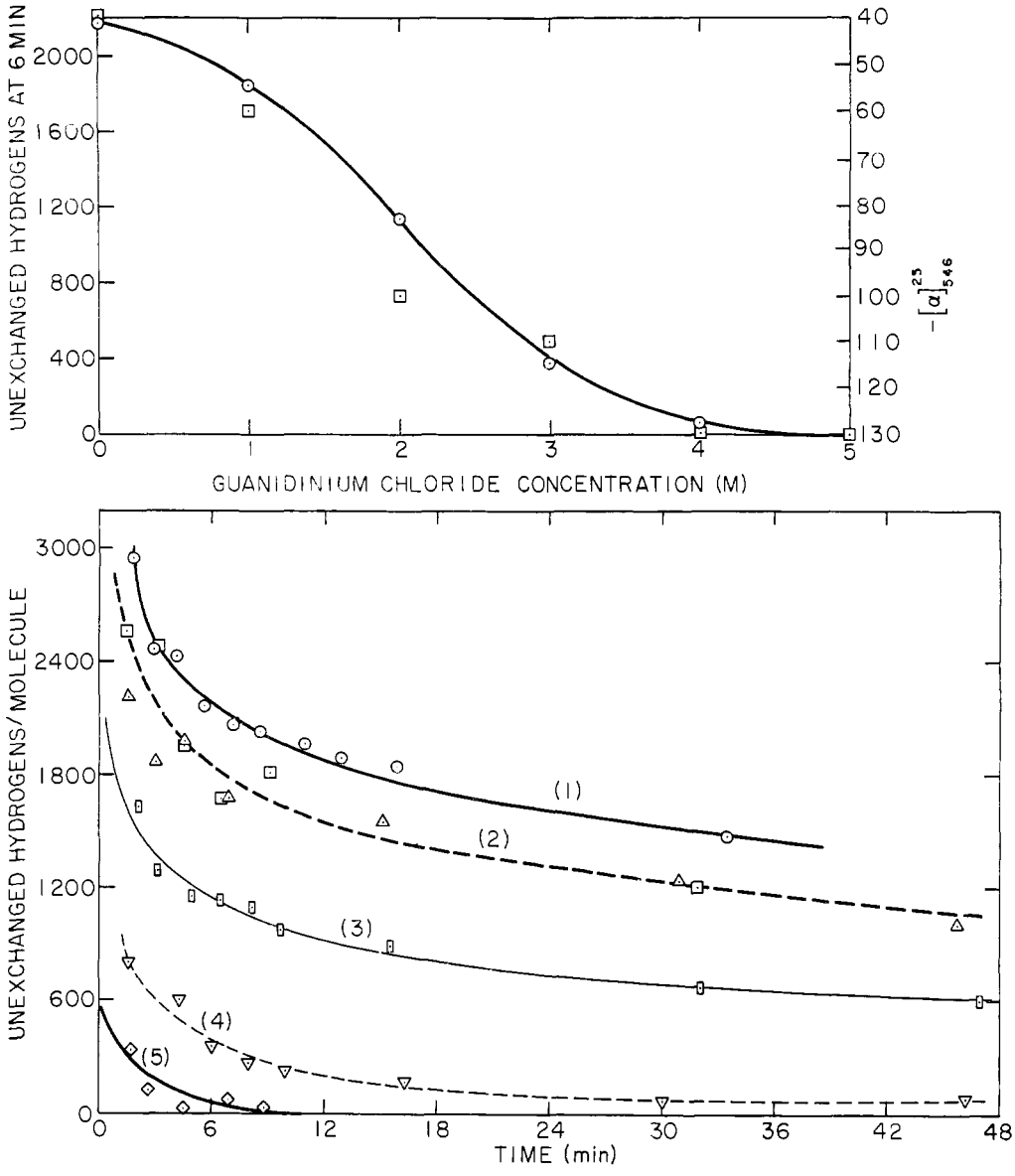

FIGURE 3: The guanidinium chloride induced denaturation of myosin (upper) and the hydrogen exchange of myosin as a function of time at various concentrations of guanidine·HCl (lower). Unexchanged hydrogens at 6 min; (⊡) — $[\alpha]^{25}_{546}$ (upper) (Young *et al.*, 1962). (1) (—⊙—) 0.0 M guanidinium·HCl; (2) (—△—⊡—) 1.0 M guanidinium·HCl; (3) (—⊡—) 2.0 M G·HCl; (4) (- -▽- -) 3.0 M G·HCl; (5) (—◇—) 4.0 M G·HCl. Exchange experiments were carried out using the "one-column" procedure. In addition to G·HCl, the solvent was 0.01 M Tris (pH 7.0)–0.5 M NaCl, temperature 3.6°.

formation, we do not mean to imply that hydrogen exchange is not intimately related to the three-dimensional structure of this protein. In fact, a close correspondence between the number of slowly exchanging hydrogen atoms of myosin and the degree of secondary–tertiary structure is clearly shown in Figure 3, which presents the number of unexchanged hydrogens per myosin molecule at the arbitrary time of 6 min (H_6) and $[\alpha]_{346}$ as a function of guanidine·HCl concentration. The transition from the folded to the unfolded state of myosin in this strong denaturing agent is closely paralleled by the change in H_6. The close correspondence in the H_6 and $[\alpha]_{346}$ *vs.* guanidine·HCl profiles strongly suggests that the denaturing agent is not playing any significant role in the general catalysis of the exchange rates (see, for example, Klotz and Frank, 1965).

Quantitative interpretation of the exchange curves shown in Figures 1 and 2 requires a comprehensive kinetic analysis. In the present study we have assumed that each exchange curve represents a sum of independent first-order processes. Analysis was carried out

TABLE II: First-Order Rate Constants (k, min^{-1}) and Class Sizes (H, hydrogens/5.95×10^5 g) for the Exchange of Myosin at a Temperature of 3.3° and Various pH Values.

pH	k_1	H_1	k_2	H_2	k_3	H_3	H_∞
5.61	2.43×10^{-1}	900	2.01×10^{-2}	1711	2.11×10^{-3}	1066	2500
6.00	5.42×10^{-1}	1738	9.01×10^{-3}	1393	5.24×10^{-4}	1587	1100
6.50	2.65×10^{-1}	1208	1.44×10^{-2}	1037	1.17×10^{-3}	1715	1100
6.99	3.43×10^{-1}	1720	4.09×10^{-2}	405	6.13×10^{-3}	1323	1100
7.52	1.89×10^{-1}	1170	1.96×10^{-2}	1188	2.14×10^{-3}	1059	420
7.98[a]	2.27×10^{-1}	1675	1.88×10^{-2}	929	2.46×10^{-3}	803	420
8.46[a]	2.60×10^{-1}	1658	1.88×10^{-2}	929	2.46×10^{-3}	803	420
9.00	2.10×10^{-1}	1397	1.25×10^{-2}[b]	649[b]	2.81×10^{-3}	555	420
			5.93×10^{-2}	682			

[a] The pH 7.98 and 8.46 curves superimpose within experimental error. [b] Analyzed as two classes.

TABLE III: First-Order Rate Constants (k, min^{-1}) and Class Sizes (H, hydrogens/3.62×10^5 g) for the Exchange of HMM at a Temperature of 3.3° and Various pH Values.

pH	k_1	H_1	k_2	H_2	k_3	H_3	H_∞
5.63	4.31×10^{-1}	785	1.73×10^{-2}	97	7.70×10^{-3}	773	1540
5.99	3.88×10^{-1}	981	1.53×10^{-2}	314	2.97×10^{-3}	728	990
6.53	*b*	*b*	8.64×10^{-3}	470	4.41×10^{-3}	341	1040
6.98	3.85×10^{-1}	925	1.51×10^{-2}	476	1.95×10^{-3}	747	500
7.46	*b*	*b*	1.37×10^{-2}	702	2.20×10^{-3}	489	500
7.95	*b*	*b*	1.96×10^{-2}	709	2.65×10^{-3}	471	180
8.49[a]	*b*	*b*	1.36×10^{-2}	414	2.43×10^{-3}	412	120
			5.89×10^{-2}	386			
8.95[a]	4.10×10^{-1}	1186	1.36×10^{-2}	414	2.43×10^{-3}	412	120
			5.89×10^{-2}	386			

[a] The pH 8.49 and pH 8.95 curves superimpose within experimental error. [b] Not determined.

using the method of Prony (Hildebrand, 1956) with the aid of an IBM 7094 digital computer (see Materials and Methods), and it was found that a maximum of three exponential terms was required to fit each curve although in most cases two terms sufficed. At each pH the first-order rate constants for the exchanging kinetic classes generally differed by a factor of about 10. The finding that the exchange curves can be fitted by so few exponential terms of widely differing rate constants lends confidence to the calculated results and greatly facilitates the analysis.

First-order rate constants and class sizes for the exchange of myosin at 3.3° at various pH values are presented in Table II. Classes 2, 3, and H_∞ of Table II are derived from the results presented in Figure 1 while class 1 parameters were obtained from separate one-column experiments. The points shown in Figure 1 are the experimentally determined values, while the curves were synthesized from the kinetic parameters of classes 2, 3, and H_∞ (Table II) at times greater than 20 min. Classes of rapidly exchanging hydrogens with half-lives of the order of 10 min are also present in all experiments with the exception of the study carried out at pH 5.6. This reaction could not be followed accurately with either the one- or two-column techniques and its associated kinetic parameters have, therefore, been omitted from Table II. The total number of unexchanged hydrogen atoms at zero time in classes 2, 3, and H_∞ are indicated on the ordinate of Figure 1 at each pH. Curves were fitted to the experimental points by eye for the initial 20-min period.

Before attempting a detailed interpretation of the kinetic results, some estimate of the reproducibility is required. Figure 4a demonstrates the good agreement obtained between two separate experiments carried out under identical conditions, while Figures 4b and c show that the exchange behavior is unaffected by the various buffer systems employed in the present study.[2] An estimate of the maximum error in the

[2] The experiments demonstrating reproducibility were carried out on samples which were tritiated separately and thus provide evidence of reproducibility of the level of tritiation.

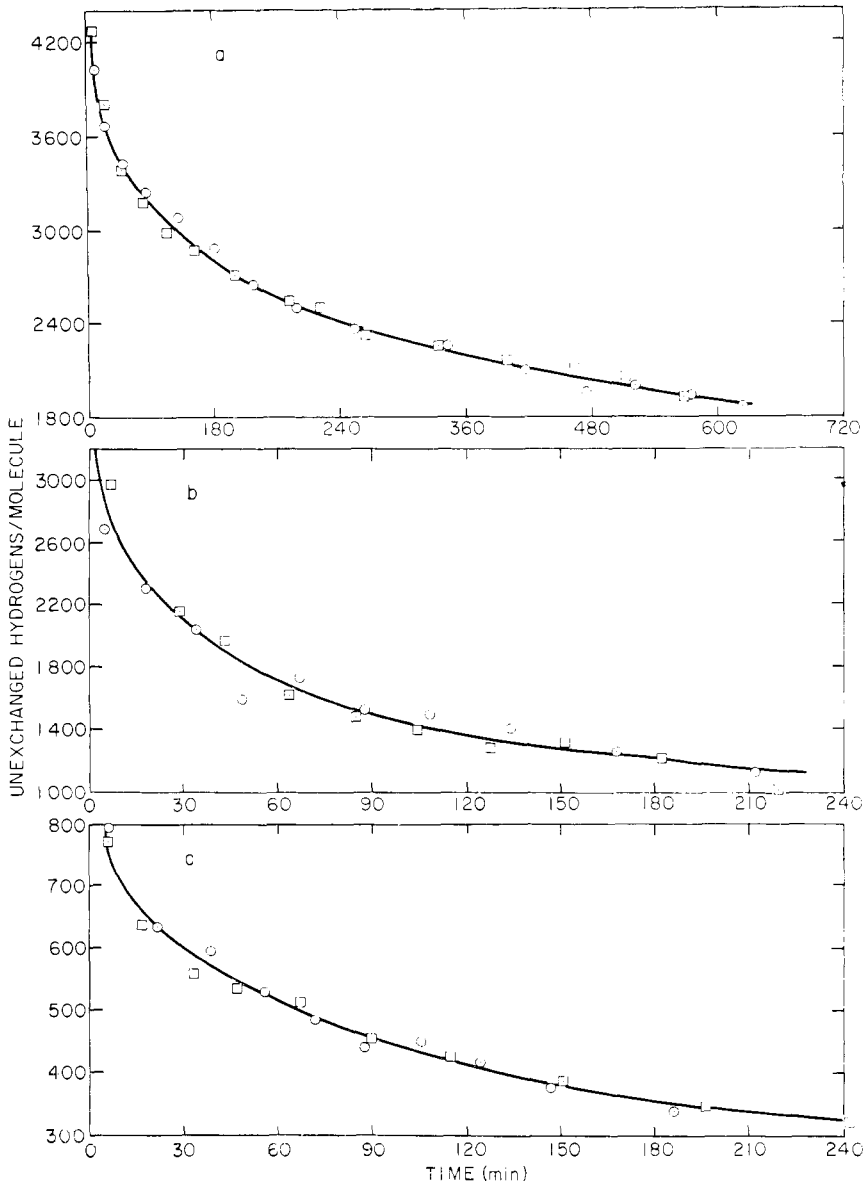

FIGURE 4: (a) Two identical exchange experiments (⊙ and ⊡) of myosin in 0.5 M NaCl–0.05 M maleate, pH 6.50, at 3.3°. (b) The exchange of myosin in 0.05 M NaCl, pH 7.50, at 3.3°: (⊡) in 0.01 M Tris; (⊙) in 0.05 M Tris. (c) The exchange of LMM in 0.5 M NaCl, pH 7.00, at 3.3°: (⊙) in 0.01 M Tris; (⊡) in 0.05 M maleate.

kinetic parameters was obtained from an analysis of curves drawn through the lower and upper extremes in the data points presented in Figure 1. Even under these exaggerated cases the kinetic parameters differed by at most a factor of 2.

Hydrogen Exchange of HMM and LMM. For purposes of localizing the various exchanging classes in the myosin molecule we have also investigated the exchange behavior of the two proteolytic fragments of myosin, HMM and LMM. Figures 5 and 6 show the time-dependent loss of bound tritium from HMM and LMM, respectively, at differing pH values. As in the case of myosin, the number of unexchanged hydrogen atoms at any given time decreases with increasing pH for both fragments, with the greatest effect being observed in the low pH range. The large shift in the number of slowly exchanging hydrogens between pH 6.5 and 7.0 noted in the myosin experiments is not observed in either HMM or LMM.

Kinetic parameters derived from Figures 5 and 6 are presented in Tables III and IV. The number of bound tritium atoms remaining in HMM at any given time was in general appreciably greater than that observed for LMM under identical experimental conditions.

Identification of Exchanging Clusters. Casual inspection of Tables II–IV reveals that there is apparently no orderly progression either in the size of a given

TABLE IV: First-Order Rate Constants (k, min^{-1}) and Class Sizes (H, hydrogens/1.6 × 10^5 g) for the Exchange of LMM at a Temperature of 3.3° and Various pH Values.

pH	k_1[a]	H_1[a]	k_2	H_2	k_3	H_3	H_∞
5.98	3.21×10^{-1}	299	1.85×10^{-2}	239	1.19×10^{-3}	407	295
6.49	*b*	*b*	3.31×10^{-2}	117	2.91×10^{-3}	427	290
6.92	3.42×10^{-1}	421	1.01×10^{-2}	346	8.69×10^{-4}	184	140
7.45	*b*	*b*	*c*	—	7.67×10^{-3}	277	140
8.03	3.25×10^{-1}	429	1.55×10^{-2}	203	1.83×10^{-3}	146	0
8.49	*b*	*b*	2.55×10^{-2}	188	2.80×10^{-3}	133	0

[a] Determined in separate one-column exchange experiments. [b] Not determined. [c] Only one term needed to fit curve.

TABLE V: Tentative Class Assignments for the Exchanging Hydrogens of Myosin at Various pH Values.

	pH 5.61		pH 6.00		pH 6.50		pH 6.99	
Class	k	H	k	H	k	H	k	H
A	2.11×10^{-3}	1066	9.01×10^{-3}	1393	1.44×10^{-2}	1037	4.09×10^{-2}	405
B	0	1400	5.21×10^{-4}	1587	1.17×10^{-3}	1715	6.13×10^{-3}	1323
C	0	700	0	700	0	700	0	700
D	0	400	0	400	0	400	0	400
E	2.01×10^{-2}	1711		—	—	—	—	—
Total H[a]		5277		4080		3852		2828
Total H + fast H		6177		5738		5060		4548

	pH 7.52		pH 7.98		pH 8.46		pH 9.00	
A	—	—	—	—	—	—	—	—
B	1.96×10^{-2}	1188	1.88×10^{-2}	929	1.88×10^{-2}	929	1.25×10^{-2}	649
							5.93×10^{-2}	682
C	2.14×10^{-3}	1059	2.46×10^{-3}	803	2.46×10^{-3}	803	2.81×10^{-2}	555
D	0	420	0	420	0	420	0	420
E	—	—	—	—	—	—	—	—
Total H[a]		2677		2152		2152		2306
Total H + fast H		3837		3827		3810		3703

[a] Myosin has 5200 peptide hydrogens.

class or the velocity constant of exchange with increasing pH or temperature. In fact, however, this apparent complexity is largely a reflection of the manner in which the kinetic analyses have been presented. This is so because the class numbers presented in Tables II–IV do not necessarily refer to the same exchanging group of the protein at each pH or temperature.

Assume for purposes of clarity that a given structural region in the myosin molecule contains a group of hydrogen atoms which exchange with a rate approaching zero at pH 6.5. In the kinetic analysis this group will be included in the H_∞ class. If its rate of exchange is raised into the measurable range at pH 7.0, this same group will now be designated class 3, and the class which was designated "3" at pH 6.5 will now be included in the class 2 category at pH 7.0. This is the reason that both the rate constants and the class sizes show such large and apparently random fluctuations within a given class category.

In order to identify a given group of exchangeable hydrogens throughout the pH ranges of Tables II–IV, it is necessary to make two assumptions. (1) All hydrogen atoms which form a discrete kinetic class (i) under a given set of experimental conditions will respond in a similar manner to environmental variations. The result will be that the observed rate constant (k_i) of that class will vary, while the class size (H_i) remains constant. (2) The rate of exchange of a given group

TABLE VI: Tentative Class Assignments for the Exchanging Hydrogens of LMM at Various pH Values.

	pH 5.98		pH 6.49		pH 6.92	
Class	k	H	k	H	k	H
A	1.85×10^{-2}	239	3.31×10^{-2}	177	–	–
B	1.19×10^{-3}	407	2.91×10^{-3}	427	1.01×10^{-2}	346
C	0	150	0	150	8.69×10^{-4}	184
D	0	140	0	140	0	140
Total H		936		894		670
Total H[a] + fast H		1235		–		1091
	pH 7.45		pH 8.03		pH 8.49	
A	–	–	–	–	–	–
B	–	–	–	–	–	–
C	7.67×10^{-3}	277	1.55×10^{-2}	203	2.55×10^{-2}	188
D	0	140	1.83×10^{-3}	146	2.80×10^{-3}	133
Total H		417		349		321
Total H[a] + fast H		–		778		–

[a] LMM has approximately 1400 peptide hydrogens.

TABLE VII: First-Order Rate Constants (k, min^{-1}) and Class Sizes (H, hydrogens/5.95×10^5 g) for the Exchange of Myosin at pH 6.50 and at Various Temperatures.

Temp (°C)	k_1	H_1	k_2	H_2	H_∞
3.3	8.13×10^{-3}	1226	1.00×10^{-3}	1397	1130
8.1	1.61×10^{-2}	889	2.42×10^{-3}	1426	1130
13.1	a	–	6.62×10^{-3}	1692	1130
18.0	1.85×10^{-2}	1164	1.76×10^{-3}	1244	390
23.1	1.31×10^{-2}	916	2.61×10^{-3}	777	400
28.0	1.71×10^{-2}	1095	1.61×10^{-3}	807	0

[a] Only one term needed to fit curve.

increases with increasing pH and temperature. This assumption is supported by the results of a number of workers (see Hvidt and Nielsen, 1966) which demonstrate an increase in the rate of hydrogen exchange in model compounds and proteins with increasing pH and temperature.

As an illustration of our method for following the pH and temperature-dependent shifts in rate of exchange of a specific group of labile hydrogens, consider the alteration in the H_∞ class of myosin between pH 5.61 and 6.00 (Table II). A decrease of about 1400 exchangeable hydrogens occurs in this range. Paralleling this change, the velocity constant of class 3 drops by approximately a factor of 4 (from $k = 2.11 \times 10^{-3}$ to 5.24×10^{-4} min^{-1}), while the size of this class increases from 1070 to 1590. We interpet this behavior as resulting from a transfer of about 1500 exchangeable hydrogens as a group from the H_∞ class to class 3. As the pH increases from 6.00 to 7.00, the H_∞ class remains constant and the velocity constant of exchange of class 3 increases monotonically over this pH range. In the pH range between 7.0 and 7.5, another group of exchanging hydrogens moves into class 3 and it will be observed that the velocity constant again exhibits a drop (of about threefold). A similar transfer of clusters of exchanging hydrogens will be seen to occur between the class 3 and class 2 columns of Table II in this pH range.

Using this general procedure the specific groups of exchanging hydrogens have been tentatively identified and tabulated for each environmental condition in Tables V and VI for myosin and LMM. The complexity of the exchange pattern of HMM has precluded a similar analysis for this structure. All of the groups which are

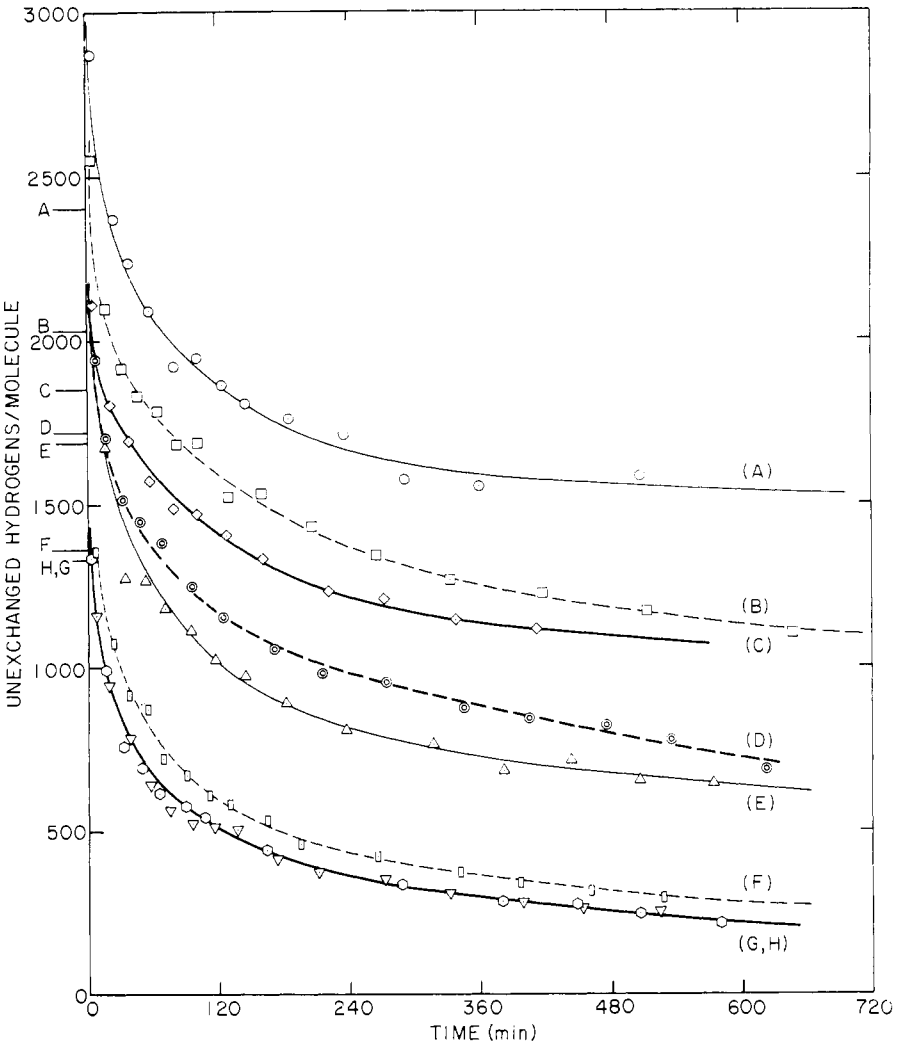

FIGURE 5: The time-dependent exchange of HMM (75 sec) in 0.5 M NaCl, at 3.3° at various pH values. (A) pH 5.63 (—⊙—), 0.05 M maleate; (B) pH 5.99 (- -⊡- -), 0.05 M maleate; (C) pH 6.53 (—◇—), 0.05 M maleate; (D) pH 6.98 (- -⓪- -), 0.05 M maleate; (E) pH 7.46 (—△—), 0.05 M Tris; (F) pH 7.95 (- -⊡- -), 0.01 M Tris; (G) pH 8.49 (—⌶—), 0.05 M Tris; and (H) pH 8.95 (—▽—), 0.02 M borate.

TABLE VIII: Tentative Class Assignments for the Exchanging Hydrogens of Myosin at Various Temperatures.

	3.3°		8.1°		13.1°	
Class	*k*	*H*	*k*	*H*	*k*	*H*
A	8.13×10^{-3}	1226	1.61×10^{-2}	889	–	–
B	1.00×10^{-3}	1397	2.42×10^{-3}	1426	6.62×10^{-3}	1692
C	0	700	0	700	0	700
D	0	430	0	430	0	430
Total H		3753		3445		3822
	18.0°		23.1°		28.0°	
A	–	–	–	–	–	–
B	1.85×10^{-2}	1164	1.31×10^{-2}	916	1.71×10^{-2}	1095
C	1.76×10^{-3}	1244	2.61×10^{-3}	777	1.61×10^{-3}	807
D	0	390	0	400	–	–
Total H		2798		2093		1902

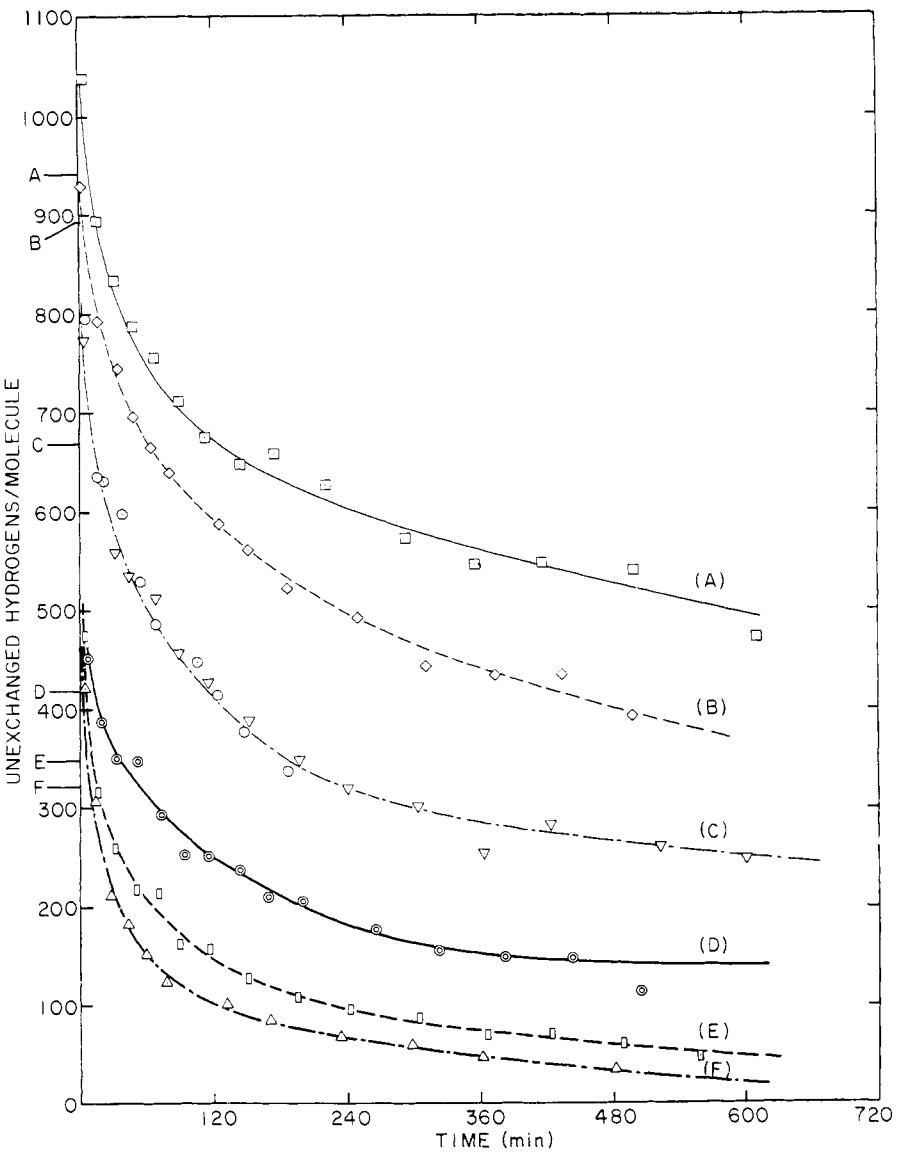

FIGURE 6: The time-dependent exchange of LMM (75 sec) in 0.5 M NaCl at 3.3° at various pH. (A) pH 5.98 (—□—), 0.05 M maleate; (B) pH 6.49 (- -◇- -), 0.05 M maleate; (C) pH 6.92 (—○—), 0.01 M Tris–0.05 M maleate; (D) pH 7.45 (—⓪—), 0.01 M Tris; (E) pH 8.03 (- -□- -), 0.05 M Tris; and (F) pH 8.49 (- -△- -), 0.05 M Tris.

measurable show a strong monotonic increase in rate with pH over the range of 5.6–7.0, but show little pH dependence above pH 7.0, in accord with the convergence of the exchange curves in Figures 1 and 2. Qualitatively similar behavior is observed in the temperature studies (Tables VII and VIII). The rate of exchange of each cluster increases with temperature to about 18°, but shows little temperature dependence at higher temperature. For the most part each cluster size remains constant within experimental error with variations in the environmental conditions (pH and temperatures), but in a few cases a significant fall in cluster size occurs. A generalized interpretation of the pH and temperature dependence of exchange kinetics is given in the following section.

The Mechanism of Hydrogen Exchange in Myosin and Its Proteolytic Fragments

In spite of the complexity of the observed exchange behavior, a general pattern emerges from the data which conforms very well to a model for the hydrogen exchange of proteins first developed by Linderstrøm-Lang (1955) and Berger and Linderstrøm-Lang (1957) and later extended and refined by Hvidt (1964) and Hvidt and Nielsen (1966). The model is based on the fundamental postulates of statistical mechanics. A solution of chemically homogeneous protein molecules must be considered to exist in a large number of interconverting conformational states, the relative abundance of each particular state being a function of its conformational energy. The essential feature

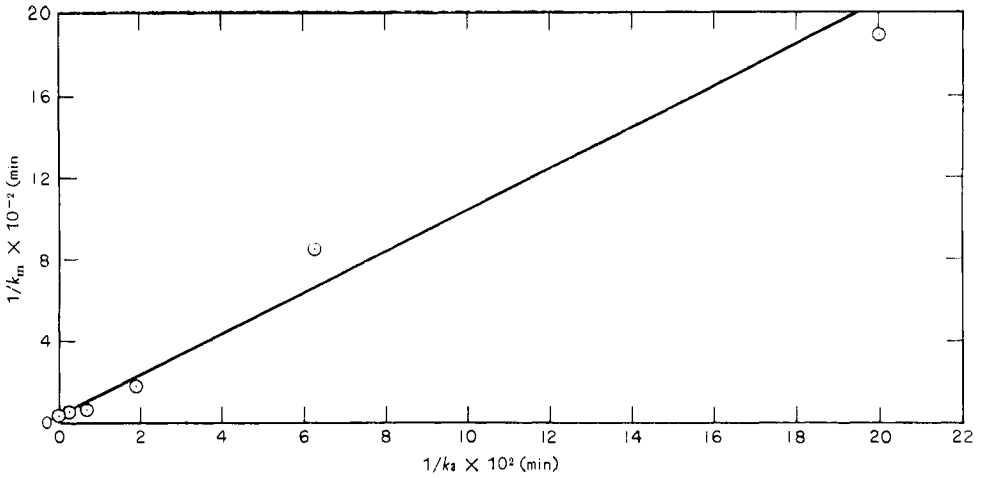

FIGURE 7: Plot of $1/k_m$ *vs.* $1/k_3$ for myosin class B at 3.3°. Values of k_3 were calculated from eq 3.

of the Linderstrøm-Lang model is that the instantaneous conformational states of all groups of protein molecules which contain labile hydrogen atoms can be divided into two classes: those which exchange at rates characteristic of small peptides (designated "I") and those which exchange at zero rate (presumably as a result of solvent exclusion) (designated "N"). Exchange is possible because of the interconversion of N and I forms.

Consider a small region, A, of the protein molecule. By assumption, all of the labile hydrogen atoms are either in the nonexchangeable state, N, or the exchangeable state, I. When this region is equilibrated with tritium and exchange is measured against an unlabeled solvent, the rate of loss of bound tritium from the region will be a first-order process with rate constant, k_m. According to the Linderstrøm-Lang (1955) hypothesis the exchange process for the region A can be written schematically as

$$N_A^* \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I_A^* \xrightarrow{k_3} E_A \qquad (1)$$

For the region A, N_A^* is the concentration of the unexchanged hydrogen atoms in the N forms, I_A^* the concentration of unexchanged hydrogen atoms in the I forms, and E_A the concentration of exchanged hydrogen atoms. The complete solution of the rate equations defining this process has been given by Hvidt (1964), and is closely approximated by $k_m = k_1k_3/(k_1 + k_2 + k_3)$. Experimentally, k_m will be the measured rate constant of a given class of exchanging hydrogen atoms. Each class will be comprised of all labile hydrogens from exchanging units, such as A, which have similar rate constants. It has been observed in the present and previous studies that the over-all exchange of protein molecules can be described by a small number of kinetic classes, with widely differing rate constants.

Since the polypeptide chains of myosin exchange much more rapidly in the I form (*i.e.*, in 4 M guanidine·HCl) than in the native state, it is clear that the N forms must predominate in the native molecule. Thus, $k_2 \gg k_1$ and the observed rate constant can be written as

$$k_m = \frac{k_1k_3}{k_2 + k_3} \qquad (2)$$

Bryan and Nielsen (1960) have estimated the dependence of k_3 on pH and temperature for poly-DL-alanine which appears to exchange at rates similar to those reported for random polypeptide chains. The rate of exchange of the deuterated polypeptide with water is given by the approximate equation

$$k_3 = 50(10^{-pH} + 10^{pH-6})10^{0.05(t-20)} \text{ min}^{-1} \qquad (3)$$

where t is the temperature in degrees centigrade. Although the absolute value of k_3 will depend somewhat on the buffer and the isotope used, the pH and temperature dependence should be closely similar in all random-chain systems. In the present studies k_m has been determined for each class as a function of k_3 by varying the temperature and pH. At constant temperature, k_3 increases approximately tenfold per pH unit above about pH 4. Thus subsitution of values of k_3 (eq 3) at constant temperature into eq 2 shows that k_m should increase hyperbolically with increasing pH, providing that k_1 and k_2 are independent of pH. A plot of $1/k_m$ *vs.* $1/k_3$ should consequently be linear with slope = k_2/k_1 and intercept = $1/k_1$. It will be seen from Figure 7 that such behavior is followed by the exchange kinetics of class B of myosin. From Tables V and VI it can be seen that the rate constants, k_m, of those exchanging groups which can be measured over an appreciable pH span (clusters B and C in myosin and C and D in LMM) do indeed show hyperbolic behavior with increasing pH. Moreover, k_m

shows a tenfold increase per pH unit in the low pH range, but is essentially independent of pH in the high pH range. For those classes which cannot be measured over a wide pH interval, the pH dependence is still consistent with that expected from eq 2.

It will be seen that under conditions where k_3 is small compared to k_2 (low pH and low temperature) eq 2 reduces to

$$k_m = \frac{k_1}{k_2}k_3 \qquad (k_3 \ll k_2) \tag{4}$$

In this case k_m should increase tenfold per pH unit and the ratio $k_m:k_3$ should give the equilibrium constant for the transconformational reaction

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I$$

That this is in agreement with our results is shown, for example, in Table IX where it will be observed that

TABLE IX: Equilibrium Constant for the $N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I$ Reaction for Class B of LMM at Three pH Values.

pH	k_3 (min^{-1})[a]	k_{obsd} (min^{-1})	$K \times 10^4$
6.0	5.0	1.19×10^{-3}	2.38
6.5	1.6×10^1	2.91×10^{-3}	1.82
7.0	5.0×10^1	1.01×10^{-2}	2.02
Av = 2.07×10^{-4}, ΔF = 4.7 kcal/mole			

[a] Estimated from eq 4.

an equilibrium constant ($K = 2.1 \times 10^{-4}$) is obtained for cluster B of LMM, which is independent of pH over the range pH 6.0–7.0.

On the other hand, when k_3 is much greater than k_2

$$k_m = k_1 \qquad (k_3 \gg k_2) \tag{5}$$

and k_m should be sensibly independent of pH as is observed at higher pH values in Tables V and VI. Thus the measured values of k_m at the two asymptotes of the k_m *vs.* pH hyperbola allow us to estimate the rate constants of interconversion of the N and I forms for a given kinetic class as well as the standard free energy of the transconformational reaction.

The rate constants and free energies estimated for the various exchanging groups of myosin and LMM are summarized in Table X. Perhaps the most striking feature of this table is the surprisingly low estimated rate constants of opening of the exchanging units. Half-lives vary for this reaction from about 20 to

TABLE X: Characterization of Kinetic Classes of Exchanging Hydrogens in Myosin and LMM. ΔF, k_1, and k_2 Refer to Reaction $N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I$.

Class	Approx Size	ΔF (kcal/mole)	k_1 (min^{-1})	k_2 (min^{-1})
		I. Myosin		
A	1000	3.0	–	–
B	1000–1500	5.1	1.9×10^{-2}	1.8×10^2
C	800	6.7	2.8×10^{-4}	5.0×10^1
D	400	>9	–	–
E	1700	2	–	–
Fast	1500			
		II. LMM		
A	200	3.2	–	–
B	400	4.7	–	–
C	180	5.3	3.3×10^{-2}	5.3×10^2
D	140	6.6	3.7×10^{-3}	5.2×10^2
Fast	300–400	Low		

2000 min. In contrast, the half-lives of closing of the exchanging units are of the order of 0.1 sec. The free energies estimated from the equilibrium constants (k_1/k_2) are a measure of the stability of the fundamental exchanging unit in each cluster. Since we have no knowledge of the number of these units within each kinetic class, it is not possible to estimate the free energy of opening of an entire class.

Further support for the Linderstrøm-Lang mechanism of hydrogen exchange is provided by the studies of the temperature dependence of exchange. As in the pH studies, the exchange process is intimately dependent on the magnitude of k_3, and the Linderstrøm-Lang theory predicts a transition in the form of the exchange rate constant with temperature. At constant pH, k_3 will vary with temperature according to eq 3. Following the discussion of the pH dependence of exchange, we see that at low temperature $k_m = (k_1/k_2)k_3$, while at high temperature $k_m = k_1$, providing that k_1 and k_2 are relatively independent of temperature. The exchange results of class B of myosin (Tables VII and VIII) are relevant in that the temperature dependence of k_m changes markedly over the interval 13–18°. Below 13° the temperature coefficient of k_m of this class is large, whereas at temperatures above 18° k_m is nearly independent of temperature (Figure 8). According to the present interpretation this means that in the range 13–18° k_3 becomes greater than k_2, and thus k_m changes in form. Indeed, a transition in the mechanism of exchange is to be expected in this temperature interval from the pH dependence of exchange of class B of myosin (Table V). As we have seen, k_3 becomes greater than k_2 in the pH range 7.0–7.5 (3.3°). In this pH range k_3 increases from 5.0

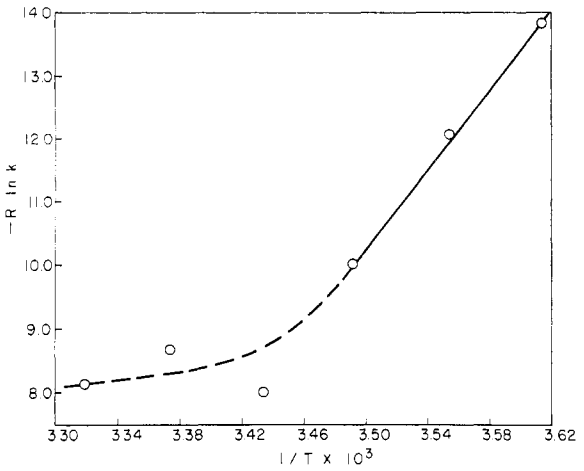

FIGURE 8: Arrhenius plot for myosin class B at pH 6.50. An activation energy of E^*_{app} = 32 kcal/mole was derived from the slope in the range 3.3–13.1°.

$\times 10^1$ to 1.6×10^2 min^{-1}, while at pH 6.5 (the pH of the temperature studies) k_3 passes through the same range as the temperature is increased from 10 to 20°.

The Linderstrøm-Lang mechanism can be utilized in conjunction with exchange data at various temperatures to obtain information on the stability of the various exchanging classes. Plots of log k_m *vs.* $1/T$ give an apparent activation energy, E_{app}*, for the exchange process. Under conditions where $k_m = k_1k_3/k_2$ for $k_3 \ll k_2$, $E_{app}^* = \Delta H + E^*$ where ΔH is the enthalpy of the N → I transconformational reaction and E^* is the activation energy associated with the exchange of simple peptides. A value of E^* = 23 kcal/mole has been reported by both Klotz and Frank (1964, 1965) and Bryan and Nielsen (1960).

From the slope of Figure 8 we estimate a value of E_{app}^* = 32 kcal/mole for the class B peptide hydrogen atoms of myosin. This unusually large value is the result of the high activation energy for the exchange of exposed peptide hydrogens and lends support to our interpretation of the exchange mechanism. Accepting a value of 23 kcal/mole for E^*, the enthalpy, ΔH, = 9 kcal/mole.[3]

[3] We note that the present experiments were performed at constant pH, not p(OH), and since k_3 is base catalyzed in the range above pH 3 (Bryan and Nielsen, 1960) a correction must be applied for the temperature dependence of K_w. The exchange studies of model compounds of Bryan and Nielsen (1960) and of Klotz and Frank (1964, 1965) were also carried out at constant pH (pD), and, therefore, the enthalpy of dissociation of water (or D_2O) is included in the 23-kcal/mole activation energy. Since this term amounts to about 14 kcal/mole, the true activation energy of peptide exchange will be the order of 9 kcal/mole. It should be recognized that this correction does not affect our value of the enthalpy of the N → I transconformational reaction as long as k_1 and k_2 are independent of the OH concentration. We thank Dr. S. W. Englander and P. Von Hippel for calling this to our attention.

Using a value of 5.1 kcal/mole for ΔF (calculated from the pH studies) and 9 kcal/mole for the enthalpy, the estimated entropy change for the opening of class B is ΔS = 14 eu. These thermodynamic parameters indicate a very stable exchanging unit with a calculated transition temperature (N → I) at about 640°K. It will be recognized that this calculation makes the rather unrealistic assumption that the exchanging units of class B open and close independently and are independent of the conformational stability of the remaining portion of the protein.

It is clear from Figure 8 that the apparent activation energy of the class B exchange above 18° is much lower than that observed in the low temperature range, but experimental errors in this region prevent a meaningful quantitative analysis. Since the temperature dependence of k_1 (the slope of the ln k_m *vs.* $1/T$ plot above 18° (Figure 8)) is small and since the temperature dependence of the ratio $k_1:k_2$ is also small (ΔH = 0 kcal/mole) it follows that k_2 is also relatively temperature independent. This observation is in accord with our finding that the mechanism of exchange undergoes a transition in the predicted temperature range (based on the assumption that k_2 is independent of temperature).

Exchange Studies on Myosin in the Presence of ATP and Actin. The possibility of detecting a substrate-induced conformational change in myosin has also been investigated in the present work, using the technique of tritium–hydrogen exchange. Indeed Hvidt and Kagi (1963) have reported a study on the conformational change induced in yeast alcohol dehydrogenase by DPN^+ and DPNH in which deuterium–hydrogen exchange was monitored by following changes in the amide II infrared band. These workers found that DPN^+ caused an approximately 4% increase in the number of slowly exchanging peptide hydrogens. In a subsequent paper, Di Sabato and Ottesen (1965) observed a much larger conformational change in chicken heart lactic dehydrogenase induced by interaction with DPN^+ and DPNH. In this study interaction with the low molecular weight substrates caused a lowering in the rate of exchange of as many as 30% of the peptide hydrogens. The only analogous exchange study on myosin is that of Hartshorne and Stracher (1965) who measured deuterium–hydrogen exchange using the infrared technique. Their studies indicate that the exchange of HMM is unaffected by ATP in the presence of Ca^{2+} or Mg^{2+}.

In the present study we have repeated and extended these experiments to include the effect of F-actin binding on the exchange properties of HMM. We have also measured the exchange of tritiated F-actin on combination with HMM. Results are presented in Figures 9 and 10 where it will be seen that no significant alteration in the exchange curves of HMM or actin occurs under our experimental conditions. Unlike the time-dependent exchange of HMM, most of the exchangeable hydrogens of actin are released before the first measurement can be completed (<5 min) at 4° and pH 6.5. Thus it is possible that a gross con-

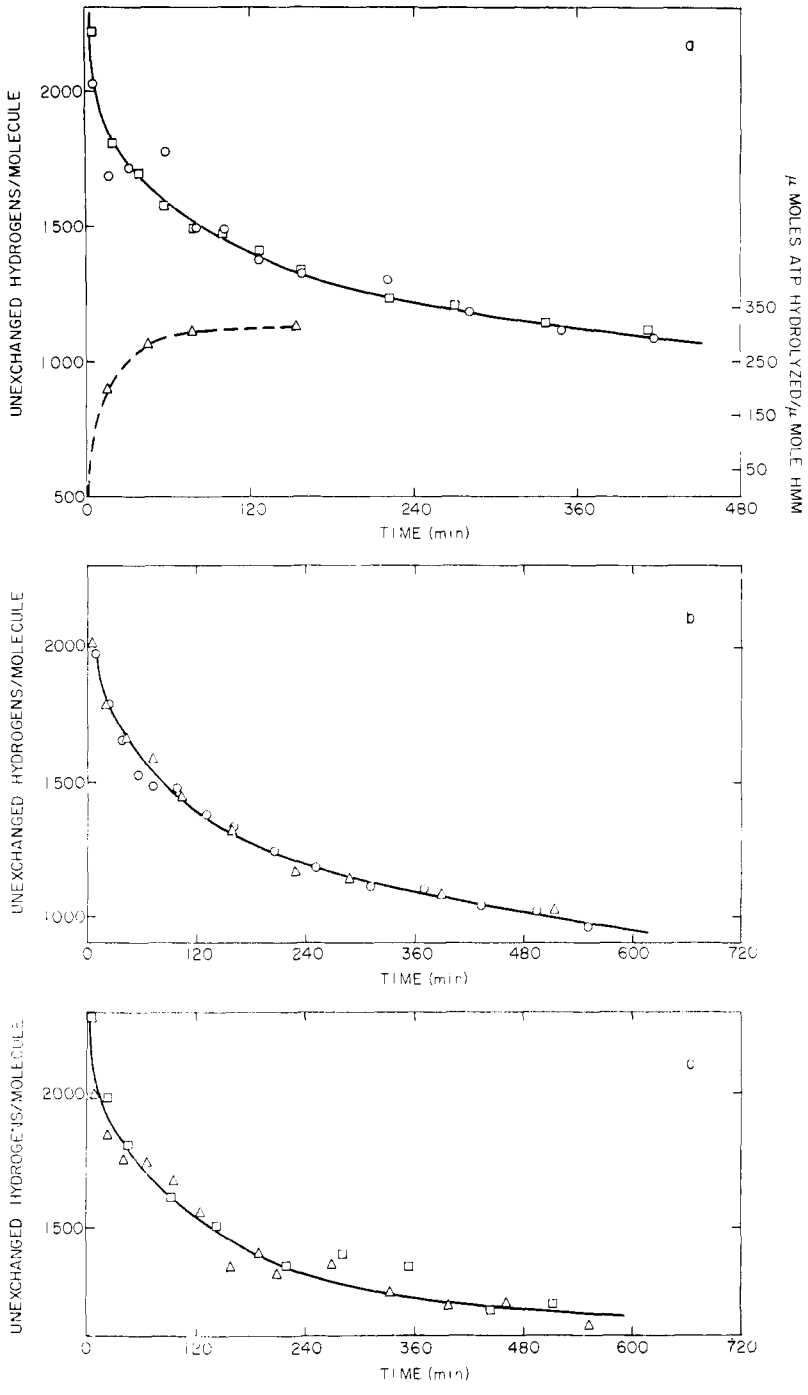

FIGURE 9: The time-dependent hydrogen exchange of HMM in 0.5 M NaCl. (a) 0.05 M maleate–0.005 M $CaCl_2$, pH 6.53 at 3.3°: (○) with 0.002 M ATP and 0.005 M Ca^{2+}; (□) no ATP; (Δ) micromoles of inorganic phosphate released per micromole of HMM. (b) 0.05 M maleate–0.001 M $MgCl_2$, pH 6.50 at 3.1°: (○) in the presence of F-actin; (Δ) no actin. (c) 0.05 M maleate–0.001 M $MgCl_2$, 0.01 M ATP, pH 6.50 at 3.3°: (Δ) in the presence of F-actin; (□) no actin.

formational change could have occurred within the actin molecule which would have escaped detection by this technique. Such a change is suggested by the small effect of HMM plus ATP upon the exchange of actin (Figure 10). However, the effect is small and its significance is therefore open to question.

Discussion

From a variety of studies with model compounds (see Hvidt and Neilsen, 1966) it has become apparent that exposed peptide hydrogens exchange at rates many orders of magnitude (~10^4) lower than labile hydrogen

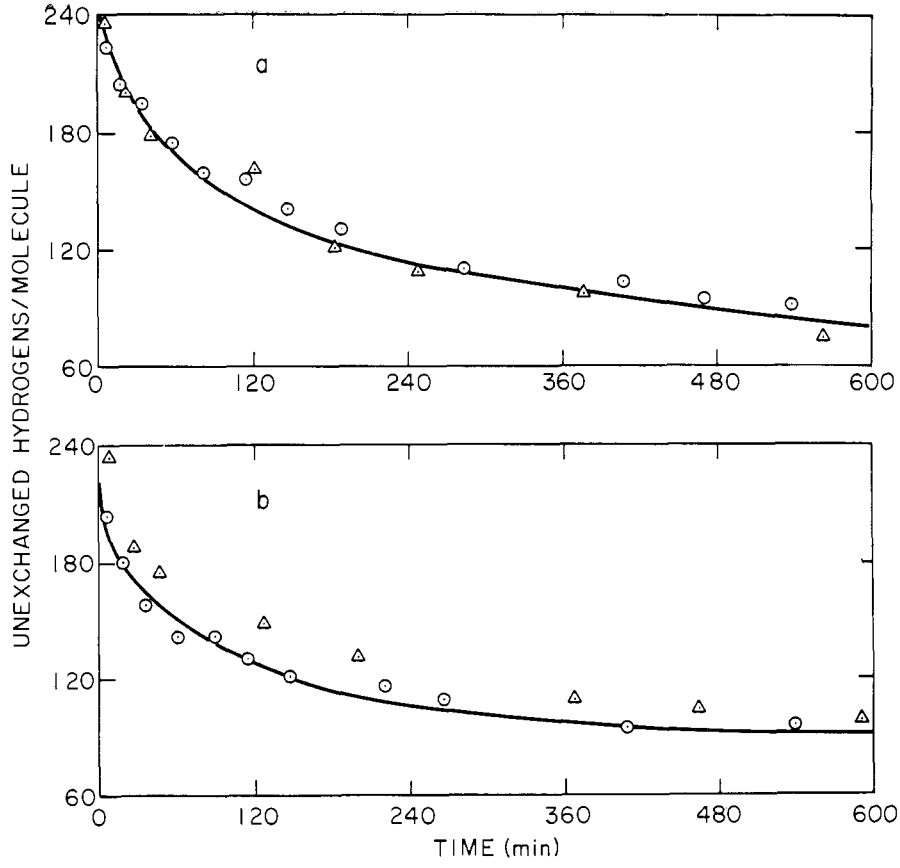

FIGURE 10: The time-dependent hydrogen exchange of actin. (a) In 0.5 M NaCl–0.05 M maleate–0.001 M $MgCl_2$, pH 6.50, at 3.3°: (⊙) in the presence of HMM; (△) in the presence of oxidized ribonuclease. (b) 0.05 M maleate–0.001 M $MgCl_2$, at 3.3°: (⊙) in the presence of HMM and 0.01 M ATP; (△) in the presence of HMM only.

atoms of exposed side-chain groups (with the possible exceptions of those of glutamine and asparagine). Therefore, in the Linderstrøm-Lang (1955) model, the functional group most likely to show the characteristic pH dependence of exchange (*i.e.*, when $k_2 \gg k_3$) is the peptide group. In fact, by comparing the exchange of lysozyme as followed by the gradient method (which measures *all* exchanging hydrogens) with that measured by the infrared method (which detects only peptide hydrogens), Hvidt (1963) has shown that most of the slowly exchanging hydrogens of lysozyme are peptide hydrogens. Similarly, the number of slowly exchanging hydrogens ($t_{1/2} > 20$ min) of myosin determined in the present study (pH 6.5, 23°) approximates the number reported in the infrared studies of Hartshorne and Stracher (1965) (pH 6.3, 25°).

On the other hand, if the fast reaction class ($t_{1/2} \leqslant 2$ min) is included in the analysis, it is apparent from Table VIII that at pH 5.6 and 6.0 the total number of hydrogens exchanging with measurable rates in myosin exceeds (by about 20%) the total number of peptide hydrogens, implying that some of these stem from side chains. At pH 5.6 it is reasonable to assign the fast class to side-chain hydrogens and the remaining, more slowly exchanging, protons to the peptide groups, since at this pH the number of hydrogens in the slower classes closely approximates the total number of peptide hydrogens in myosin. At higher pH it is expected that some of the slowly exchanging hydrogens move into the fast class as a result of base catalysis. In either case the majority of the hydrogens of the slow classes would derive from the peptide groups. Based upon these considerations as well as upon the limited comparison with the infrared results of Hartshorne and Stracher (1965) and analogy with the lysozyme system we have assumed in the present study that the majority of the slowly exchanging hydrogens are from the backbone amide groups.

The Location of Exchanging Clusters within the Myosin Molecule. It is clear from the results of a variety of physical and chemical studies that the myosin molecule exhibits features characteristics of both a fibrous and a globular protein. The fibrous part of the molecule appears to be a multistranded supercoil of α helices. At the end of this rodlike segment each polypeptide chain folds into a more globular structure giving an over-all "tadpole" appearance to the native molecule. As is well known, the fibrous (LMM) and more globular (HMM) regions can be separated from each other by brief tryptic digestion

of a highly susceptible region within the rod segment. This feature allows us to locate the exchanging classes within these structurally distinct regions of the native molecule and obtain information pertaining to their relative stabilities and accessibility to the solvent.

To begin with let us compare the total number of slowly exchanging hydrogens of myosin with those of LMM and HMM under various experimental conditions. In Table XI the sums of the hydrogens in the various kinetic classes determined in the two column experiments ($t_{1/2} > 20$ min) are listed at each pH for myosin, and for LMM plus HMM. In the column marked Δ, the number of slowly exchanging hydrogens present in myosin but missing from LMM and HMM is indicated, and to show that the over-all picture is not dependent upon the kinetic analysis, this parameter, determined directly from the exchange curves at 30 min, is also listed. At pH 6.0 and 6.5, it is observed that myosin contains about 1100 more slowly exchanging hydrogens than LMM plus HMM, while above pH 6.5 this value decreases to about 450–500. This decrease, which is qualitatively visible as a large gap between the pH 6.5 and 7.0 curves of myosin (Figure 1), is not present in the HMM or LMM curves (Figures 5 and 6) and is apparently due to movement of about 650 hydrogens of class A of myosin out of the measurable range.

TABLE XI: A Comparison of the Total Number of Slowly Exchanging Hydrogens (from two-column experiments) in Myosin with the Sum of Those from LMM and HMM.

pH	H (myosin)	H (LMM + HMM)	Δ[a]	Δ 30 min[b]
6.0	4080	2968	1112	1070
6.5	3852	2745	1107	975
7.0	2828	2393	435	205
7.5	2667	2108	555	314
8.0	2152	1709	443	470
8.5	2152	1653	499	425

[a] The difference between the number of slowly exchanging hydrogens in myosin and in LMM plus HMM. [b] Values for H taken directly from curves at 30 min.

In a recent study (Segal *et al.*, 1967) it was shown that about 75–95 × 10^3 g/mole of myosin is released as low molecular weight peptides during the formation of HMM and LMM, and arguments were advanced to support the contention (Mihalyi and Harrington, 1959; Woods *et al.*, 1963) that most of this mass is derived from a single belt in the myosin rod lying between the HMM and LMM segments. Assuming a mean residue weight for the amino acids of this region of 115, there should be 650–850 less peptide hydrogens in LMM plus HMM than in myosin. Thus it seems likely that the 650 slowly exchanging hydrogens which are lost from the myosin molecule on increasing the pH from 6.5 to 7.0 reside in the proteolytic sensitive belt. From a comparison of Tables II–IV it will be seen that the 400–500 slowly exchanging hydrogens which are detected in myosin at high pH, but which are missing from LMM and HMM, derive primarily from the slowest classes of HMM. It will also be observed from Table VIII that at 25° the hydrogens of class A, as well as many of the very slowly exchanging hydrogens of myosin (and HMM), are not detectable. This is consistent with the finding of Hartshorne and Stracher (1965), who found that the total number of slowly exchanging hydrogens in the proteolytic fragments, HMM and LMM, is equal to that of the parent myosin molecule at this elevated temperature.

Assuming that class A hydrogens do, in fact, reside within the proteolytic enzyme-sensitive belt of myosin, then an average standard free energy of 3.0 kcal/mole (Table X) is required to open the exchanging units of this region. In contrast, we find that in other portions of the myosin rod (*e.g.*, LMM) the exchanging units tend to be considerably more stable, the values for ΔF ranging from 3.2 to 6.6 kcal/mole. The comparatively high availability of the peptide hydrogen atoms of the class A region for exchange with solvent molecules would be consistent with the observed high accessibility of these peptide bonds to proteolytic attack. This does not imply, however, that the region containing class A hydrogens in myosin is completely structureless. On the contrary, only 0.1% ($K_{equil} = 10^{-3}$) of the peptide hydrogens of this class are available for exchange at any given time.

It can be seen that above pH 7 a strong correlation exists between the exchange of myosin and that of HMM. For example, above pH 7 classes B and C of myosin (Table V) can be equated with classes 2 and 3 of HMM (Table III). From Table VI it is apparent that above pH 7 the LMM segment should not be expected to contribute significantly to the over-all exchange of myosin. Moreover, because LMM constitutes only about 25% of the mass of myosin, it should not (within experimental error) be expected to add discrete classes to the myosin exchange analyses, even at lower pH values, but rather should contribute to larger classes originating in the head portion (about $^2/_3$ of the myosin mass is found in HMM). Thus the loss of 400 hydrogens from the myosin class B (Table V) on elevating the pH from 7.0 to 7.5 is probably owing to the fact that class B of myosin is a composite of class B of LMM (Table VI) (which disappears in this range) and another class present in HMM.

One thousand hydrogens from class B of myosin have, therefore, been located in the HMM portion of the molecule. It has already been mentioned that classes C and D of myosin are also probably located in the head portion, and these contribute 800 and 400

hydrogens (the number in native myosin) to HMM, respectively. The sizes of these classes are somewhat smaller in HMM than in myosin, but it is reasonable to assume that the regions of HMM responsible for these classes have been somewhat altered during preparation as a result of proteolysis. The regions are probably not necessary for the actin-combining and ATPase functions, since those activities are unimpaired in HMM. In contrast to LMM, HMM is characterized by very slowly exchanging hydrogens, especially of classes C and D. This might be expected of a globular molecule with large hydrophobic regions, since the exchanging unit in this case would be large, and would require the rupture of many stabilizing, noncovalent bonds before exchange could occur. Classes B–D combined account for 2200 slowly exchanging hydrogens in HMM or about 70% of the total peptide hydrogens. Since about 50% of the amino acid residues of HMM are helical, as judged by optical rotatory dispersion, some of these slowly exchanging hydrogens must come from helical regions. These helices may be expected to exchange more slowly than those of LMM because they are located in interior regions, and are, therefore, less accessible to the solvent than the helices of LMM.

Originally it was thought that hydrogen exchange would be of use in the detection of various conformations in proteins and, in particular, would be of use as a check on optical rotatory dispersion measurements of α-helix content. In the past few years, however, it has become apparent (Hvidt and Nielsen, 1966) that there is no general relationship between the helix content and the hydrogen-exchange behavior of proteins. This is well demonstrated by the fact that five kinetic classes are required to describe the exchange behavior of only one kind of conformation, the α helix in LMM. In the present discussion the point of view has been adopted that hydrogen-exchange rates are measures of either the stability of exchanging units or of the rates at which exchanging units become exposed to the solvent. Assuming this point of view, it is not difficult to see how the helical residues of the multistranded, α-helical rope, LMM, can fall into more than one class with respect to exchange behavior. It should be expected, for instance, that residues lying on the surface of the LMM molecule would exchange more rapidly than those situated in the interior. In the absence of an exact model for LMM, however, the significance of five kinetic classes is not clear, and furthermore one or more of the classes may be artifacts, introduced by proteolysis during preparation.[4]

From the work of Lumry *et al.* (1964) and Schwarz (1965) the maximum half-time for opening (*i.e.*, for N → I) of helical residues in PLGA has been estimated to lie in the microsecond range, and for stable helices the rate of closing must be even greater. From the results of Table X it can be seen that if our interpretation of the pH dependence of the exchange of LMM is correct, the half-times of the N → I transconformational reactions for two classes of exchanging hydrogens of LMM vary from about 20 to 200 min. This is one billion to ten billion times greater than for α-helical PLGA, and, similarly, the rate of closing is at least 10^5 times slower in LMM than in PLGA. This means that ΔF^* for opening reactions of LMM is about 12 kcal/mole greater than for PLGA, and for the closing reaction the difference is at least of the order of 6 kcal/mole. It is apparent that the difference in ΔF^* for both reactions can easily be accounted for by assuming a difference in size of exchanging units, since the free energy of activation for breaking hydrogen bonds is most likely quite high (of the order of 5–6 kcal/mole residue). The tentative conclusion to be made from these comparisons is that a multistranded α-helical rope is much less motile (*i.e.*, k_1 and k_2 are much smaller) than a single-stranded helix, and that this is probably related to the fact that the multistranded helix is a much more highly cooperative structure than the single helix, resulting in an increase in the size of the exchanging unit.

Addendum

An alternative interpretation of the exchange data has been suggested by Dr. S. W. Englander. In contrast to the present interpretation, Englander proposes that each labile hydrogen atom responds in an identical manner to variations in environmental conditions over the entire pH and temperature range. In the ideal case in which kinetic class sizes are independent of pH and temperature, both proposals predict (Berger and Linderstrøm-Lang, 1957) that horizontal lines (constant H) constructed on Figures 1 and 2 should intersect any two adjacent exchange curves at times whose ratios remain constant over the whole curve. The interpretations differ, however, in that according to the present proposal the time ratios should vary from about 2 or 3 at low pH and temperature to 1 at higher values. Englander suggests the ratio should be constant over the entire pH and temperature range. It would appear that the pH studies support the former scheme, while the temperature data are more consistent with that suggested by Englander.

In either case, the assumption that the class sizes remain unaffected by environmental conditions renders the time ratio analysis highly questionable. Such changes in the class sizes should be expected to occur as a result of small conformational changes (*i.e.*, dependence of k_1 and k_2 on pH or temperature) and variations in the initial degree of tritiation. In fact it will be recalled that the curves of Figure 2 were constructed from the kinetic parameters of Table VII, and that these parameters indicate nonuniform changes in k's and H's with pH and temperature. Nevertheless these curves give relatively constant time ratios. Apparently the constancy of time ratios is consistent with

[4] Paralleling the preferential proteolysis at the trypsin-sensitive region of the myosin rod, other bonds within the rod segment are also cleaved (Mihalyi and Harrington, 1959; Young *et al.*, 1964). We estimate five peptide bonds to be cleaved in LMM under conditions used in the present study for preparation of HMM and LMM (75-sec digestion).

both a regular and a nonregular increase in rate constant with temperature, and will support either exchange mechanism. We therefore feel that meaningful information can be obtained only by direct kinetic analysis of the exchange curves, and that the time ratio analysis should be employed as a check of analyses in which class sizes have been shown to remain invariant.

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