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Energetics of the Equilibrium between Two Nucleotide-Free Myosin Subfragment 1 States Using Fluorine-19 Nuclear Magnetic Resonance†

John W. Shriver and Brian D. Sykes*

ABSTRACT: A new fluorine-containing reagent has been synthesized and used to specifically label the reactive sulfhydryl [sulfhydryl-1 (SH_1)] of myosin subfragment 1 (S-1). The labeled S-1 (S-1-CF_3) demonstrates activated calcium and magnesium adenosinetriphosphatase (ATPase) activities relative to S-1 and a lower potassium ethylenediaminetetraacetate (EDTA) ATPase activity. Maximal effect is obtained with the modification of one thiol per S-1. The ^{19}F NMR spectrum of S-1-CF_3 contains only one resonance with a line width of 110 Hz, which implies a rotational correlation time of 2.3×10^{-7} s. The chemical shift of this resonance is sensitive to temperature, pH, ionic strength, and nucleotides bound in the active site. The temperature dependence of the chemical shift clearly indicates two limiting states for the S-1-CF_3 with a highly temperature-dependent equilibrium between 5 and 40 °C. The low-temperature state appears to be identical with the state resulting from the binding of Mg·ADP or Mg·AMPPNP at 25 °C. The energetics of the conformational change have been studied under various conditions. At pH 7 in 25 mM cacodylate, 0.1 M KCl, and 1 mM EDTA, $\Delta H°$ = 30 kcal/mol and $\Delta S°$ = 105 cal deg^{-1} mol^{-1}. A decrease in pH to 6.5 results in an increased population of the low-temperature state with $\Delta H°$ = 31 kcal/mol and $\Delta S°$ = 107 cal deg^{-1} mol^{-1}. Similarly, the low-temperature state is favored by low ionic strength. In 5.8 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) and 1 mM EDTA (pH 7), $\Delta H°$ = 8 kcal/mol and $\Delta S°$ = 27 cal deg^{-1} mol^{-1}. We have also obtained ^{19}F NMR spectra of S-1-CF_3 in D_2O solution with 30% ethylene glycol at pH 7.1. Increasing concentrations of ethylene glycol progressively stabilize the high-temperature state.

Recent work with ^{31}P nuclear magnetic resonance (NMR)[1] (Shriver & Sykes, 1980, 1981a,b) and ultraviolet and fluorescence spectroscopy (Morita, 1977; Bechet et al., 1979) has indicated that myosin subfragment 1 can exist in two discrete states or conformations in the presence of ADP or AMPPNP. Transient kinetic experiments have provided evidence for two myosin S-1 ADP and AMPPNP complexes in addition to a transitory recognition complex (Trybus & Taylor, 1979). In addition, transient and steady-state kinetic experiments have provided evidence for two M·ADP·P ternary complexes (Bagshaw & Trentham, 1974). This body of work has implied that there are two fundamental states of myosin subfragment 1 with the relative population of the two states being dependent on the nucleotide occupying the ATPase active site and the temperature. However, to this date, no evidence for two states of S-1 in the absence of nucleotide has been provided. We show here that a fluorine probe attached to the SH_1 of myosin S-1 may be used as a sensitive monitor of the conformational state of S-1 in the absence of nucleotide. The variation with temperature of the chemical shift of this probe clearly demonstrates that myosin S-1 in the absence of nucleotide may exist in two discrete states which differ significantly in structure as indicated by large $\Delta H°$ and $\Delta S°$ values for the transition. As seen with the two M·ADP and M·AMPPNP states, $\Delta G° \approx 0$ at physiological temperatures.

A wide variety of probes have been attached to the SH_1 sulfhydryl of myosin S-1 (Quinlivan et al., 1969; Seidel et al., 1970; Takashi et al., 1976; Nihei et al., 1974; Thomas, 1978), and the effects of the modification on the characteristics of the ATPase have been well documented. Aromatic derivatives of iodoacetamide have been shown to have a high specificity

† From the Medical Research Council Group on Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received October 20, 1981.* This work has been supported by the Medical Research Council of Canada (postdoctoral fellowship to J.W.S. and grant to the Group on Protein Structure and Function) and by the Muscular Dystrophy Association of Canada (postdoctoral fellowship to J.W.S.).

[1] Abbreviations: SH_1, sulhydryl-1; S-1, myosin subfragment 1; S-1-CF_3, myosin subfragment 1 labeled with *N*-[4-(trifluoromethyl)-phenyl]iodoacetamide; DTT, dithiothreitol; ADP, adenosine 5′-diphosphate; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance.

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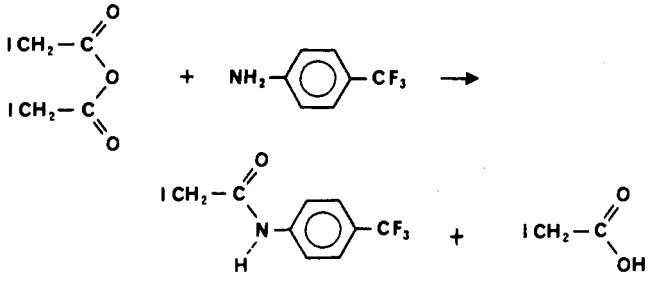
for the SH_1 position. We present here the synthesis of a new fluorine-containing reagent, *N*-[4-(trifluoromethyl)phenyl]-iodoacetamide, which reacts rapidly under very mild conditions to label myosin subfragment 1. The three fluorine nuclei per label, the 100% natural abundance of the ^{19}F isotope, and the high intrinsic NMR sensitivity of the ^{19}F nucleus make this reagent very attractive and allow ^{19}F NMR studies to be performed on samples of relatively low concentration. ^{19}F NMR has been used by a number of investigators in studies of protein structural changes and interactions with ligands (Huestis & Raftery, 1971; Bode et al., 1975; Brown & Seamon, 1978; Sakai & Dallas, 1978; Hull & Sykes, 1975; Hagen et al., 1979).

Materials and Methods

Iodoacetic acid, α,α,α-trifluoro-*p*-toluidine, and dicyclohexylcarbodiimide were obtained from Sigma Chemical Co. Iodoacetic acid was recrystalllized from cyclohexane. *N*-[4-(Trifluoromethyl)phenyl]iodoacetamide (I) was synthesized



(I)

by using standard haloacetyl synthetic techniques (Wilchek & Givol, 1977). Iodoacetic acid (2.7 g) was dissolved in dry ethyl acetate (15 mL). Dicyclohexylcarbodiimide (1.53 g in ethyl acetate) was added via an addition funnel slowly and with stirring. After 1 h at room temperature, the dicyclohexylurea was removed by filtration, and the iodoacetic anhydride was used immediately. To the iodoacetic anhydride solution was added 1.17 g of α,α,α-trifluoro-*p*-toluidine in ethyl acetate. After 1 h, the solvent was removed on a rotary evaporator. The product was dissolved in benzene and washed 3 times with 0.1 N NaOH. It was dried over anhydrous Na_2SO_4, and the benzene was removed on a rotary evaporator. The resulting crystals were twice recrystallized from $CHCl_3$ and petroleum ether and finally from toluene. Purity was checked by thin-layer chromatography with benzene (Eastman chromagram sheets, no. 6060) which indicated a highly pure preparation of the fluorine label (I). Iodoacetic acid was monitored by spraying the thin-layer chromatography plates with a 0.5% ethanol solution of bromophenol blue. The identity and purity of the fluorine label (I) were confirmed by ^1H and ^{19}F NMR.

Myosin subfragment 1 (S-1) was prepared as previously described (Shriver & Sykes, 1981a). S-1 was labeled with I by procedures developed for labeling the SH_1 position with fluorescence and ESR iodoacetamide probes (Seidel et al., 1970; Takashi et al., 1976). A 1.5-fold excess of I was dissolved in acetonitrile and mixed with a myosin S-1 solution (approximately 5 mg/mL in 0.05 M Tris, pH 7.9, 0 °C). The reaction was allowed to proceed for 10 min in the dark at 0 °C and then stopped with a 200-fold excess of DTT. Excess unreacted label and DTT were removed by passing the reaction mixture through a Bio-Gel 200 column equilibrated with 5 mM Tris, pH 7.9, and 0.1 mM NaN_3. The fluorine-labeled protein was then concentrated by using a Minicon B15 concentrator.

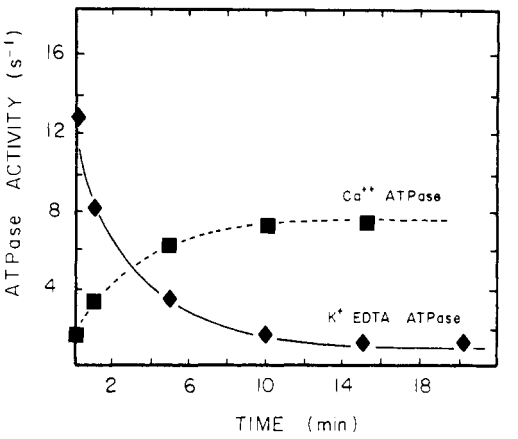


FIGURE 1: Time dependence of the labeling of myosin subfragment 1 with *N*-[4-(trifluoromethyl)phenyl]iodoacetamide using the change in ATPase activity of S-1 as an indicator of the reaction. Conditions for the labeling and ATPase assays are given under Materials and Methods.

The time course of the labeling reaction was followed by monitoring the ATPase activity of the S-1 and quantitation of the loss of the sulhydryl groups. After a reaction was started, 200-μL aliquots were removed at specific time intervals and quenched with 1.8 mL of 5 mM DTT in 50 mM Tris (pH 7.9). These samples were dialyzed extensively against 0.05 M Tris (pH 7.9) and the Ca^{2+} and K^+ EDTA ATPase activities determined by using a Radiometer pH stat. The Ca^{2+} ATPase activity was measured in 0.6 M KCl, 10 mM $CaCl_2$, and 5 mM ATP at 25 °C and pH 7.9. The K^+ EDTA ATPase activity was measured in 0.6 M KCl, 5 mM EDTA, and 5 mM ATP at 25 °C and pH 7.9. The loss of reactive S-1 groups was followed by using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (Habeeb, 1972). The Mg^{2+} ATPase activity was measured in 0.1 M KCl, 5 mM $MgCl_2$, and 2.5 mM ATP at pH 7.9 and 25 °C.

^{19}F NMR spectra at 254 MHz were collected on a Bruker HXS-270 instrument interfaced to a Nicolet 1180 computer and a 293 B pulse programmer. Spectra were obtained in the Fourier-transform mode with quadrature phase detection. Sweep widths of ±5000 Hz were used with an acquistion time of 0.2 s and a pulse delay of 0.8 s. Generally, 1000 transients were accumulated for each spectrum (16.75-min accumulation time) with 4096 data points. Chemical shifts were measured relative to the shift of the label I (~3 mM) reacted with DTT and dissolved in 50% ethanol/50% D_2O at the same temperature.

Preparations of ethylene glycol solutions of S-1-CF_3 were performed following the precautions and procedures given in the literature (Douzou et al., 1976; Bechet et al., 1979). Addition of ethylene glycol to a pH 6.9 cacodylate-buffered solution to make a 30% solution results in an increase in pH to 7.1. The temperature dependence of the pH is negligible for cacodylate-buffered solutions.

The temperature dependence of the chemical shift was fit by using a nonlinear least-squares fit [see Hull (1975)] of the data to the van't Hoff equation assuming a two-state equilibrium. Spectra were simulated by using an exchange program developed by Lewis Kay or a simulation program developed by Nicolet Magnetics Co.

Results

Figure 1 shows the alteration of the Ca^{2+} and K^+ EDTA ATPase activities as a function of the reaction time of myosin S-1 with *N*-[4-(trifluoromethyl)phenyl]iodoacetamide. The modification reaction is essentially complete after 10 min under

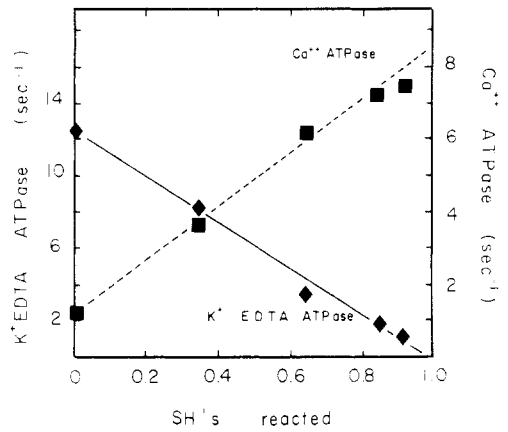


FIGURE 2: Change in Ca^{2+} and K^+ EDTA ATPase activities of myosin subfragment 1 as a function of the number of thiols reacted with *N*-[4-(trifluoromethyl)phenyl]iodoacetamide. Freely reacting thiols were quantitated by using Elman's reagent.

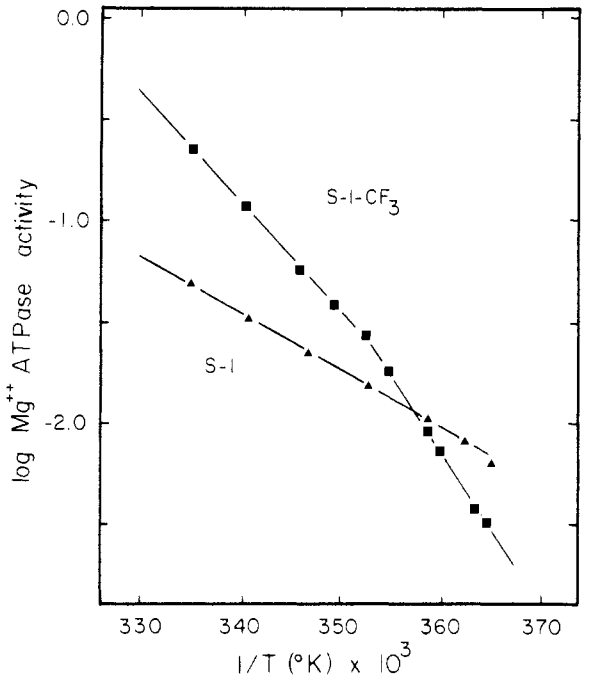


FIGURE 3: Arrhenius plots of the Mg^{2+} ATPase activities of myosin S-1 and S-1-CF_3.

these very mild conditions with a 1.5-fold excess of labeling reagent over S-1. Modification of the S-1 results in an activation of the Ca^{2+} ATPase and a loss of K^+ EDTA ATPase. This is characteristic of modification of the fast-reacting thiol, SH_1. Quantitation of the free sulhydryls of S-1 shows that the observed changes in ATPase activities parallel modification of a single thiol. Figure 2 demonstrates that the modification of one thiol results in complete loss of the K^+ EDTA ATPase and an 8-fold activation of the Ca^{2+} ATPase.

In Figure 3, we show the Mg^{2+} ATPase activity for S-1 and S-1-CF_3 as a function of temperature. After correction for the fact that the preparation of S-1-CF_3 actually contained 30% unlabeled S-1, the activation enthalpy of S-1-CF_3 ATPase was determined to be 24 kcal/mol above the break point and 44 kcal/mol below. The activation enthalpy for S-1 is 13 kcal/mol. The Mg^{2+} ATPase of S-1 is activated by modification of SH_1 only at temperatures greater than approximately 7 °C. Below this temperature, the Mg^{2+} ATPase is actually decreased.

The ^{19}F NMR spectrum of S-1-CF_3 shows a single resonance with a line width of 110 Hz (Figure 4) which has a

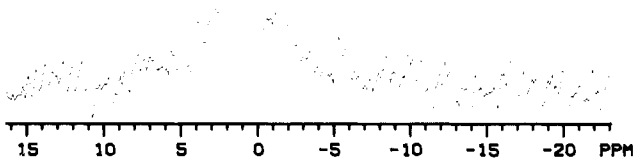


FIGURE 4: ^{19}F NMR (254 MHz) spectrum of S-1-CF_3 (0.4 mM), in 10 mM Tris (pH 7.9) and 0.2 mM EDTA, at 10 °C. NMR conditions: 5000 transients, 0.205-s acquisition time, 0.8-s pulse delay, ±5000-Hz sweep width, 20-μs pulse width (approximately 90° pulse angle). The chemical shift is measured relative to the free label reacted with DTT and dissolved in 50% ethanol/50% D_2O.

Table I: ^{19}F NMR Chemical Shifts of S-1-CF_3

metal/ligand	pH[a]	temp (°C)	shift (ppm)[b]
EDTA[c]	5.5	3	1.44
	6.5	5	1.45
	6.9	33	1.17
	7	30	1.18
	8	25	1.18
	8	30	1.18
Mg^{2+}	7.9	33	1.18
Mg·ADP	8	25	1.45
Mg·AMPPNP	7.9	25	1.46

[a] pH was adjusted at 25 °C and buffered with 50 mM buffer throughout except for the experiment at pH 6.9 where 25 mM cacodylate was used. The other buffers were as follows: pH 5.5, Mes; pH 6.5, Pipes; pH 7, Pipes; pH 7.9 and 8, Tris. [b] Chemical shifts are referenced relative to *N*-[4-(trifluoromethyl)phenyl]iodoacetamide reacted with DTT in 50% ethanol/50% D_2O. [c] KCl (0.1 M) was used throughout. Other concentrations were the following: EDTA, 1 mM; $MgCl_2$, 5 mM; ADP or AMPPNP, 1 mM.

chemical shift of 16.35 ppm relative to an external capillary of neat trifluoroacetic acid. For removal of any temperature effects inherent in the chemical shift of the (trifluoromethyl)phenyl group, or of the D_2O resonance to which the spectrometer is field/frequency locked, the chemical shift reference throughout this work is taken as the chemical shift of the label reacted with a large excess of dithiothreitol and dissolved in 50% ethanol/50% D_2O. The chemical shift of the fluorine probe is a linear function of temperature between 0 and 40 °C. It is assumed that the inherent temperature dependence of the probe is not affected by the protein environment and therefore can be used to correct the S-1-CF_3 chemical shifts for the intrinsic temperature dependence of the chemical shift of the label. The results presented herein and numerous other unpublished observations consistently support this assumption.

In Table I, we give the chemical shift of the ^{19}F probe on S-1-CF_3 relative to the label reacted with DTT under various conditions. It is interesting to note that the chemical shift of the probe attached to SH_1 is clearly sensitive to the binding of Mg·ADP or Mg·AMPPNP in the active site at pH 8.0 and 25 °C. It is expected, therefore, that the probe should serve as a sensitive monitor of structural changes in S-1 at various

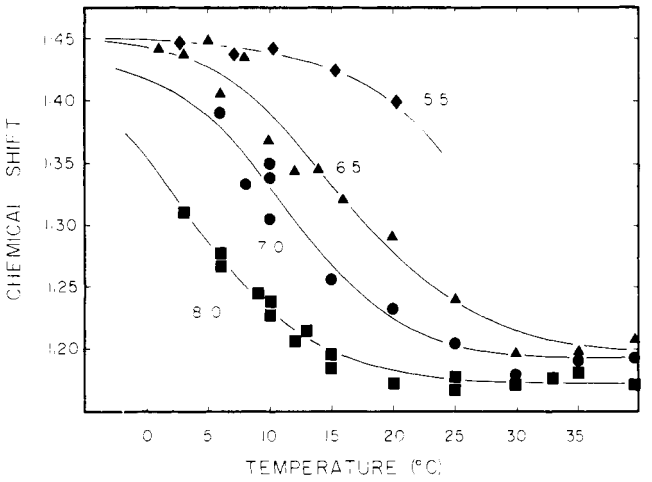


FIGURE 5: Variation of the ^{19}F NMR chemical shift of S-1-CF_3 as a function of temperature. pH was adjusted to the values shown at 25 °C. Buffers were as follows: (♦) pH 5.5, Mes; (▲) pH 6.5, Pipes; (●) pH 7.0, Pipes; (■) pH 8.0, Tris. Conditions: 0.4 mM S-1-CF_3, 50 mM buffer, 0.1 M KCl, 1 mM EDTA, and 0.1 mM NaN_3. Approximately 1000 transients were collected for each data point. Chemical shift is relative to free label reacted with DTT.

stages of ATP hydrolysis and that the energetically large conformational changes detected at the active site by using ^{31}P NMR (Shriver & Sykes, 1981a,b) should also be expressed at the SH_1 position.

In Figure 5, the chemical shift of the fluorine probe is plotted as a function of temperature at pH 5.5, 6.5, 7.0, and 7.9. It is apparent from these curves that there are two limiting values for the chemical shift, approximately 1.19 and 1.45 ppm, indicative of the two states. The four curves indicate a temperature-dependent titration from one form to the other. The chemical shift of the low-temperature state, the L state, is determined to be 1.45 ppm from the pH 5.5 data, and also data collected in the presence of Mg·ADP or Mg·AMPPNP where a temperature-independent shift is observed over the 5–35 °C range (Table I and unpublished results). The shift of the high-temperature state, the H state, varies slightly with pH. The three curves for pH 6.5, 7.0, and 7.9 can be fit by a nonlinear least-squares program by assuming a two-state temperature-dependent equilibrium using the van't Hoff equation:

$$\left(\frac{\partial R \ln K}{\partial 1/T}\right)_P = -\Delta H°$$

where K is equal to $(\delta_L - \delta_{obsd})/(\delta_{obsd} - \delta_H)$ where δ_L and δ_H are the chemical shifts of the L and H states, respectively, and δ_{obsd} is the observed chemical shift. The data were used to fit δ_L, δ_H, and $\Delta H°$. $\Delta H°$ derived from these fittings is approximately 32 kcal/mol for all three curves, and $\Delta S°$ is approximately 115 cal deg^{-1} mol^{-1} (see Table II). Simulation of NMR spectra representing two-site exchange using line widths and chemical shifts such as those observed here indicated that a lower limit cannot be placed on the exchange rate between the two forms. In addition, simulations demonstrated that the variation of the chemical shift with temperature accurately reflects the $\Delta H°$ and $\Delta S°$ values for a two-state equilibrium even in the limit of very slow exchange. This results from the width of the lines relative to the chemical shift difference.

The pHs of the solutions used for the experiments in Figure 5 were set at 25 °C as indicated. The pH changed slightly over the temperature range indicated due to the heat of ionization of the buffer and protein. No attempt was made to adjust the pH for each experiment since problems with dilution and ionic strength would result. A slight upward curvature in the chemical shift points is seen at high temperature possibly due to a decrease in pH with increasing temperature. The Pipes, Mes, and Tris solutions can be expected to decrease in pH by approximately 0.17, 0.22, and 0.62 pH unit with a 20 °C increase in temperature. There is no obvious upward curvature of the high-temperature portion of the pH 8 curve even though Tris is temperature sensitive. This is probably due to the fact that at pH 8 and 25 °C the equilibrium between the two forms is already very far toward the H form and a slight decrease in pH is outweighed by the increase in temperature. The chemical shift of the H form is therefore taken to be approximately 1.175 ppm from the fitting of the pH 8 curve.

Table II: Thermodynamic Parameters Associated with the S-1 Change in State Observed by ^{19}F NMR

pH[a]	buffer[b]	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal deg^{-1} mol^{-1})	temp where $K = 1$ (K)
7	cacodylate	30 (±3)[c]	105 (±10)	286 (±0.4)
7	Pipes	34 (±7)	120 (±20)	283 (±0.7)
6.5	Pipes	31 (±4)	107 (±14)	289 (±1)
8	Tris	32 (±3)	116 (±10)	276 (±0.3)
7	Pipes (0.0 M KCl)	8.0 (±0.5)	26 (±2)	302 (±0.9)
7	Pipes (0.5 M KCl)	33 (±8)	120 (±30)	275 (±0.7)

[a] pH was adjusted at 25 °C and varied slightly according to the heat of ionization of the buffer and protein. [b] The cacodylate concentration was 0.025 M. All other buffer concentrations were 0.05 M except in the 0.0 M KCl experiment where the Pipes concentration was 5.8 mM. Except where indicated, the KCl concentration was 0.1 M and EDTA concentration was 1 mM. [c] Standard deviation.

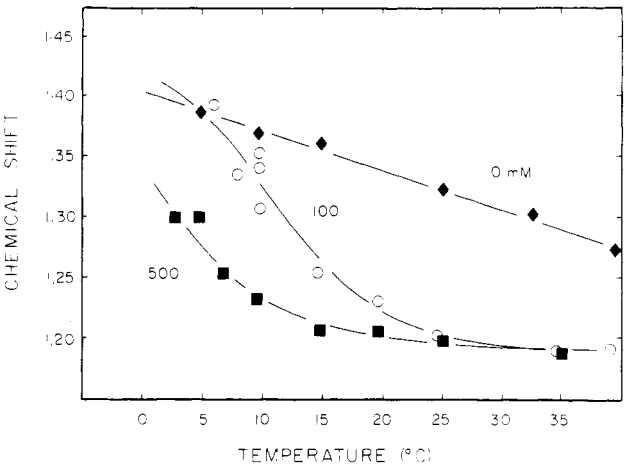


FIGURE 6: Variation of the ^{19}F NMR chemical shift of S-1-CF_3 as a function of temperature at three different ionic strengths using KCl at pH 7 in the absence of nucleotide. (○) Data from Figure 5 shown for comparison. (■) Same as (○) but with 0.5 M KCl. (♦) No KCl and with 5.8 mM Pipes.

The temperature dependence of the ^{19}F chemical shift of S-1-CF_3 in cacodylate buffer is shown in Figure 7. Within experimental error, the results are the same as those seen with Pipes at pH 7: $\Delta H°$ = 30 kcal/mol and $\Delta S°$ = 105 cal deg^{-1} mol^{-1}. The temperature dependence of the cacodylate pK is very small (Douzoue et al., 1976; Bechet et al., 1979), and no upward curvature of the high-temperature points is seen in Figure 7. The chemical shift of the H form is essentially the same as that seen with the pH 8 curve, 1.162 ppm.

The ionic strength dependence of the temperature variation of the S-1-CF_3 chemical shift is shown in Figure 6. The ionic

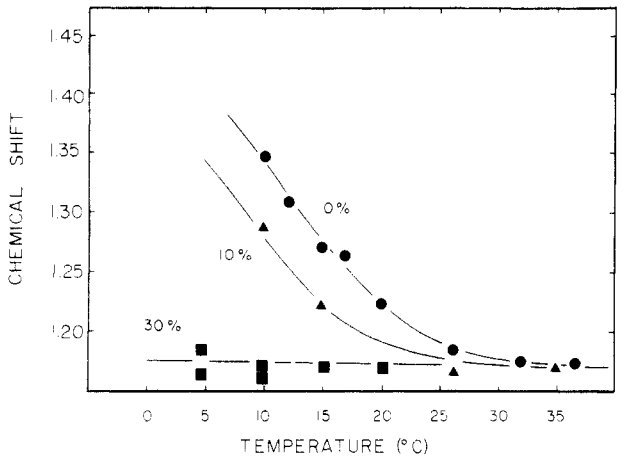


FIGURE 7: Temperature variation of the ^{19}F NMR chemical shift of S-1-CF_3 as a function of temperature in the presence of varying concentrations of ethylene glycol: (●) 0%; (▲) with 10% ethylene glycol; (■) with 30% ethylene glycol. Spectra run in 0.025 M cacodylate buffer at pH 6.9 with 0.1 M KCl and 1 mM EDTA.

strength was varied by using KCl. Within experimental error, the $\Delta H°$ does not change with an increase in ionic strength from 100 to 500 mM KCl (Figure 6 and Table II). Clearly, the equilibrium constant between the two forms is very ionic strength dependent. The accuracy of the data is not sufficient to attribute the difference between the 0.1 and 0.5 M curve specifically to either $\Delta H°$ or $\Delta S°$ differences and is probably due to small differences in both. At very low concentrations of KCl, there is a dramatic decrease in $\Delta H°$ and $\Delta S°$ relative to what is seen at 0.1 M KCl: $\Delta H°$ = 8.0 kcal/mol and $\Delta S°$ = 26 cal deg^{-1} mol^{-1}.

The effect of ethylene glycol on the energetics of the L ⇌ H equilibrium is shown in Figure 7. With increasing concentrations of ethylene glycol, the equilibrium between the two forms is shifted toward the H form. The effect of ethylene glycol on the pH of a solution buffered with cacodylate at pH 7.0 is minimal. With 30% ethylene glycol, only the H state was observed down to 5 °C. Experiments at lower temperatures were not feasible due to the high viscosity of 30% ethylene glycol solutions. Only a small increase in $\Delta S°$ would be required to shift the equilibrium to the left so that most of the titration occurs below 0 °C, and the data obtained at 30% ethylene glycol can be explained with $\Delta H°$ = 30 kcal/mol (i.e., unchanged relative to 0%) and $\Delta S°$ = 113 cal deg^{-1} mol^{-1}.

Discussion

Myosin contains a specific thiol, sulfhydryl-1 (SH_1), which is highly reactive toward sulfhydryl-modifying reagents. SH_1 has been used by numerous workers for specifically attaching fluorescence and nitroxide spin probes. These probes have normally been derivatives of iodoacetamide or maleimide, and the nature of the specific modification reaction has been characterized [see, for example, Rainford et al. (1964), Sekine & Kielley (1964), Takashi et al. (1976), and Botts et al. (1979)]. In particular, using peptide mapping, it has been shown that modification of a single fast-reacting thiol per myosin head results in a 7–8-fold activation of the Ca^{2+} ATPase and complete loss of the K^+ EDTA ATPase activity at 25 °C. In addition, the velocity and specificity of the reaction are increased by using an aromatic derivative. At lower temperatures, thiol modification of myosin results in loss in Ca^{2+} ATPase activity rather than an activation, and the Arrhenius plot of the activity demonstrates a break at approximately 15 °C (Levy et al., 1962).

We have demonstrated that the fluorine-containing iodoacetamide derivative introduced here reacts rapidly with myosin subfragment 1 under mild conditions with a high degree of specificity for a particular thiol. The velocity of the reaction and the characteristics of the modified S-1 indicate that the thiol which has been labeled is the SH_1 group. At pH 7.9 and 0 °C with a 1.5-fold excess of reagent over S-1, the labeling reaction was nearly complete within 10 min. The modification of one sulfhydryl per S-1 resulted in complete loss of the K^+ EDTA activity and activation of the Ca^{2+} ATPase by 8-fold at 25 °C. These data indicate that the observed effects are the result of modification of a single sulfhydryl per S-1. For minimization of the chances of secondary labeling, the reaction was routinely performed with only a 1.5–2-fold excess of reagent over S-1, and the time of the reaction was limited to 10 min; i.e., the reaction was never pushed to completion, and the S-1 was underlabeled routinely.

The Arrhenius plot of the Mg^{2+} ATPase of S-1 and modified S-1 (S-1-CF_3) demonstrates that modification of SH_1 with the fluorine probe (I) results in a higher activation enthalpy for k_{cat}. In addition, for S-1-CF_3, a break in the Arrhenius plot appears at approximately 12 °C. Bechet et al. (1979) have recently shown that the Arrhenius plot of heavy meromyosin contains a break at approximately 0 °C with an activation enthalpy of 15 kcal/mol above 0 °C and 29 kcal/mol below 0 °C. The break in the Arrhenius plot observed for S-1-CF_3 is not a result of modification, but labeling results in the shifting of the break point by approximately 10–15 °C. The break point can similarly be shifted by substituting Ca^{2+} or Mn^{2+} for Mg^{2+}, or by using ITP in place of ATP (Levy et al., 1962; Sekine & Kielly, 1964; Ishigami & Morita, 1977). Modification also increases the activation energy of the rate-limiting step at high temperatures, i.e., decay of the M**·ADP·P intermediate, and also at low temperature, presumably the release of ADP. It should be noted that muscle fibers with SH_1-modified myosin will contract (Borejdo & Putnam, 1977; Thomas & Cooke, 1980), although the differences caused by modification need to be further characterized.

The 254-MHz ^{19}F NMR spectrum of S-1-CF_3 shows a single line consistent with the labeling of one site on S-1 (Figure 4). It is certainly possible that secondary sites could have been labeled which have the same chemical shift as the site of interest. However, as shown under Results (and in other work to be published), under a wide variety of conditions which cause the observed ^{19}F peak to shift to various values, only one peak is observed. That is, only one population of probes appears to exist. Thus, these results in addition to the chemical evidence provided above imply that under the labeling conditions used here, i.e., near-stoichiometric quantities of label and a very brief labeling reaction period, essentially only one specific thiol in S-1 is being labeled.

The line width of the single observed ^{19}F peak is on the order of 110 Hz and may be used to calculate a rotational correlation time for the probe, assuming that relaxation is dominated by the chemical shift anisotropy interaction [see, for example, Hull & Sykes (1975)]. The principal elements of the chemical shift tensor are taken from a single-crystal study of silver trifluoroacetate (Griffin et al., 1972), and rapid rotation of the fluoromethyl group has been assumed; i.e., for rotation about the C–CF_3 bond, $\tau_c < 10^{-11}$ s. The rotational correlation time may be straightforwardly calculated following Hull & Sykes (1975) and is approximately $(2.3 \pm 0.2) \times 10^{-7}$ s. This is very close to the values obtained from fluorescence probes and nitroxide spin-labels attached to S-1 at the fast-reacting thiol, i.e., in the range of 2×10^{-7} s at 25 °C (Thomas, 1978; Mendelson et al., 1973). It is noteworthy that the rotational

correlation time of the β-phosphate of bound Mg·ADP is approximately 0.8×10^{-7} s, at 25 °C (Shriver & Sykes, 1981), which is consistent with a protein of molecular weight 100000. The longer correlation times observed for probes attached to SH_1 are not consistent with a protein the size of S-1. This discrepancy has been attributed to the nonspherical nature of myosin subfragment 1 and the resulting anisotropic character of its rotation in solution (Thomas, 1978). Probes attached to SH_1 appear to be sensitive to the motion of the long axis of S-1.

The results presented in Table I clearly show that the fluorine probe attached to SH_1 is sensitive to the nucleotide in the active site. The distance between the active site and SH_1 has been shown to be approximately 20 Å by fluorescence energy transfer measurements (Perkins et al., 1980). Under the conditions used here, both ADP and AMPPNP induce a similar conformational state which is different from that seen with labeled S-1 in the absence of ligand. Mg^{2+} on its own does not induce a conformational state different from that seen with EDTA.

As shown in Figure 5, the chemical shift of the fluorine probe in the absence of nucleotide changes with temperature in a manner which implies a two-state equilibrium. Decreasing the temperature tends to drive the labeled S-1 into a state which would appear to be essentially identical with that induced by the binding of nucleotide at 25 °C. The data at pH 7 in cacodylate buffer (Figure 7) can be fit by assuming a two-state temperature-dependent equilibrium with $\Delta H° = 30$ (± 3) kcal/mol and $\Delta S° = 105$ (± 10) cal deg^{-1} mol^{-1}. Clearly, these two states represent significantly different structures. It should be pointed out that cacodylate buffer has nearly a zero heat of protonation so that the pH remains essentially constant over the observed temperature range. As shown in Figure 5, the equilibrium between the two states is dependent on pH, with increasing pH favoring the high-temperature state. In the temperature range studied here, $\Delta H°$ and $T\Delta S°$ are compensatory so that small differences in $\Delta H°$ or $\Delta S°$ due to pH result in a significant shift of the temperature profile. Our experimental error is not, however, low enough to allow us to discern trends in the measured $\Delta H°$ and $\Delta S°$ values.

The energetics of the equilibrium are also dependent on ionic strength (or more explicitly, the concentration of KCl) with high salt favoring the high-temperature state as shown in Figure 6. $\Delta H°$ does not change within experimental error between 0.1 and 0.5 M KCl. However, at very low ionic strength, $\Delta H°$ decreases significantly as well as $\Delta S°$.

We have attempted to perform similar experiments in cryosolvents to allow titration curves to be obtained below 0 °C. Bechet et al. (1979) and Travers & Hillaire (1979) have demonstrated that myosin will hydrolyze ATP in ethylene glycol presumably in a manner similar to that observed in aqueous solution. We have been able to dissolve S-1-CF_3 in ethylene glycol at concentrations suitable for performing NMR experiments. These experiments show that ethylene glycol significantly changes the relative energies of the two states observed here (Figure 7). Increasing concentrations of ethylene glycol favor the high-temperature state, and at 30% ethylene glycol at pH 7.1 (in cacodylate buffer), the S-1-CF_3 stays in one state over the observed temperature range, indicating that $\Delta G°$ has been shifted by as much as 2.5 kcal/mol.

A nonlinear least-squares fit of the UV difference spectroscopy data of Morita (1977, Figure 3) and the fluorescence data of Bechet et al. (1979, Figure 7) for AMPPNP indicates that $\Delta H°$ is approximately 33 kcal/mol and $\Delta S°$ is roughly 120 cal deg^{-1} mol^{-1}. It should be noted that the fluorescence data of Bechet et al. (1979) were collected in 30% ethylene glycol, and the midpoint of the titration is shifted to lower temperature by approximately 5 °C relative to that observed by Morita. Ethylene glycol (30%) appears to stabilize the high-temperature form in the presence of AMPPNP similar to the results reported here for the two S-1 forms with no bound nucleotide.

The data presented here clearly indicate that myosin subfragment 1, labeled at SH_1 with a fluorine-containing derivative of iodoacetamide, exists in two discrete states under a wide variety of conditions. The fact that we have also observed two states for M*·ADP and M*·AMPPNP with unlabeled S-1 by using ^{31}P NMR implies that labeling SH_1 has not created the two observed S-1-CF_3 states. This is the first clear evidence that myosin subfragment 1 can exist in two states in the absence of nucleotide. In addition, one of these states is identical, as far as the ^{19}F NMR probe can distinguish, with the state induced by the binding of Mg·ADP or Mg·AMPPNP. We believe this to be additional evidence supporting our hypothesis that myosin S-1 exists in two fundamental states, M_R and M_T, the relative populations of which are determined by the nucleotide in the active site and the temperature (Shriver & Sykes, 1981a). The two forms are freely interconvertible during ATP hydrolysis.

During ATP hydrolysis, two distinct conformations of myosin heads (namely, the M**·ADP·P_i and M*·ADP species) have been observed by UV spectroscopy (Morita, 1967), fluorescence spectroscopy (Werber et al., 1972), ESR (Siedel & Gergely, 1971), and chemical reactivity studies (Onishi & Morales, 1976; Watterson et al., 1975; Burke, 1980). We have presented arguments for these two species being representative of the two myosin states M_R and M_T (Shriver & Sykes, 1981a). Morales and co-workers (Rainford et al., 1964) and others (Levy et al., 1962) have proposed that myosin can exist in two forms [α and β in the Rainford et al. (1964) terminology] one of which is active, or "modified", and the other "inhibited". This has been offered as an explanation for the observed dependence of the ATPase pH profile on temperature, divalent cations, modification of sulfhydryls, and other parameters, and also for break points in Arrhenius plots. At present, the exact relationship of M_R and M_T to α and β is unclear. The pH profile of the myosin ATPase is determined by the rate-limiting step in the hydrolysis scheme. A change in the character of the profile between two distinct forms, i.e., from the "aberrant" to the "sigmoidal" form (Rainford et al., 1964), probably represents a change in the rate-limiting step in the ATPase reaction from the decay of M**·ADP·P_i to M*·ADP (Ishigami & Morita, 1977). Thus, α and β most likely do not represent two different forms of myosin with different activities but rather two different intermediates in the ATPase reaction, the decay of which is determined by the release of P_i or ADP. If this is correct, then α and β are intermediates representing the two possible states of myosin, α being an example of the M_R state and β being an example of the M_T state.

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Effect of Tyrosyl Modifications on Nucleosome Reconstitution: A Spin-Labeling Study†

Daniel C. F. Chan and Lawrence H. Piette*

ABSTRACT: An imidazole spin-label was used to study the role of tyrosyl residues in the reassociation process for the nucleosome core particle. The nucleosome core particle, containing 145 base pairs of DNA and a histone core (two each of the four histones H2A, H2B, H3, and H4), was isolated from chicken erythrocytes. Native particles were first dissociated in 2 M NaCl and labeled with varying concentrations of imidazole spin-label. The labeled histone core and endogenous DNA were then reassociated back by salt step dialysis. Reconstituted spin-labeled complexes, purified by an isokinetic sucrose gradient, were found to have physical properties identical with those of unlabeled native particles. Spin-labeling the surface tyrosines of the histone core did not interfere with proper reassociation of the nucleosome core complex. ESR spectra of the reconstituted nucleosome core complex are not of the strongly anisotropic type, suggesting that labeled surface tyrosines in the histone core are not involved in specific DNA–histone interaction nor does wrapping of DNA on the histone core involve very close contact with the label. When labeling was carried out under denaturing conditions following exposure of the histone core to urea, additional histone tyrosine residues were spin-labeled. The resulting histone–DNA complexes that formed after reassociation had physical properties different from those of the native nucleosome core. This result suggested that some of the "buried" tyrosines are essential for specific histone–histone interactions that lead to stable histone core structures. Spin-labeling the buried tyrosines prevented the compact supercoiling of DNA into the nucleosome core particle.

The nucleosome core particle, an elementary subunit of chromatin structure, consists of 146 base pairs of core DNA and an octomeric histone core comprised of two each of the core histones (H2A, H2B, H3, and H4) [for a review, see McGhee & Felsenfeld (1980a)]. The amino acid sequences for these four histones are highly conserved throughout evolution (Isenberg, 1979). Data from studies of histone complexing (Van Holde & Isenberg, 1975; Spiker & Isenberg, 1977) and chromatin cross-linking (Kornberg, 1977; Trifonov,

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