Phosphorylation of $\alpha\alpha$- and $\beta\beta$-Tropomyosin and Synthetic Peptide Analogues†

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ABSTRACT: A tropomyosin kinase partially purified from chicken embryos was used to study the phosphorylation mechanism of $\alpha\alpha$- and $\beta\beta$-tropomyosin and synthetic peptides containing the site of phosphorylation at Ser-283 and corresponding to residues 264–284 of the tropomyosin isoforms. The apparent K_m is 47 μM for $\alpha\alpha$- and 265 μM for $\beta\beta$-tropomyosin, whereas the V_{max} values are similar. The α[264–284] and β[264–284] peptides have apparent K_m values of 500 μM and 650 μM, respectively, and V_{max} values similar to that of the intact tropomyosin. This indicates that the conformation of the phosphorylation site at the COOH-terminal end of tropomyosin contributes significantly to the phosphorylation of the substrate. Furthermore, the marginal difference in the K_m values of the α- and β-peptide cannot account for the 5-fold difference in the K_m of the native $\alpha\alpha$ and $\beta\beta$ isoforms, suggesting that the conformations of $\alpha\alpha$- and $\beta\beta$-tropomyosin at the phosphorylation sites are significantly different. Phosphorylation of β-peptide analogues, each with a single substitution corresponding to the α sequence, indicates that His-276 and Ile-284 have negative influences on the phosphorylation of the β-peptide, whereas Met-281 improves it. Direct analyses of the time courses of phosphorylation of $\alpha\alpha$-tropomyosin at 37 °C, where head-to-tail polymerization is minimized, show that a single exponential can fit the data satisfactorily. This indicates a random phosphorylation of two identical chains. At 25 °C, where tropomyosin exists as head-to-tail aggregates, two exponentials are needed to fit the time course data, indicating that phosphorylation of the two chains is ordered due to either nonidentical chain conformations or a negative cooperativity. In contrast, the time courses of phosphorylation for $\beta\beta$-tropomyosin are biphasic at either 25 or 37 °C, indicative of a stronger polymerizability for the $\beta\beta$ isoform.

Tropomyosin and the troponin complex regulate the Ca^{2+}-sensitive contraction of the vertebrate striated muscle (Ebashi et al., 1972; Adelstein & Eisenberg, 1980; Smillie, 1979; Sellers & Adelstein, 1987). Tropomyosin polymerizes in a head-to-tail fashion, involving an overlap of eight to nine amino acid residues at the NH_2- and COOH-terminal ends, to form long filaments lying along the two grooves of the F-actin strands. Each tropomyosin molecule, M_r 66 000, consists of two α-helices arranged in a nonstaggered and parallel manner to form a coiled-coil structure interacting with seven actin monomers and one troponin complex on each side of the F-actin filaments.

Phosphorylation of thin filament proteins in the skeletal muscle is well documented, but the effect of phosphorylation on the regulation of muscle contraction is not clear (Bârány & Bârány, 1980). Troponin I (Stull et al., 1972) and troponin T (Perry & Cole, 1973) from cardiac and skeletal muscle are phosphorylated in vivo (Gusev et al., 1980), and both are phosphorylated by cAMP-dependent protein kinase and phosphorylase kinase in vitro. Although the role of troponin T phosphorylation remains an enigma, several studies suggest that troponin I phosphorylation alters the Ca^{2+}-binding properties of cardiac troponin C (Robertson et al., 1982). Phosphorylation of tropomyosin has been demonstrated in skeletal muscle from human, rabbit, frog, and chicken (Ribolow & Barany, 1977; Montarras et al., 1981, 1982; O'Conner et al., 1978; Heeley et al., 1982a,b). Recently, a tropomyosin kinase was partially purified from chicken embryos (Montgomery & Mak, 1984; deBelle & Mak, 1987), and the phosphorylation sites in vitro and in vivo are identical, which locate at Ser-283 the penultimate residue in skeletal muscle α- and β-tropomyosin (Mak et al., 1978). Tropomyosin from smooth muscle, however, has not been shown to be phosphorylated (Montgomery & Mak, 1984), whereas a tropomyosin-like protein from human platelet appears to be phosphorylated in response to phorbol ester treatment (Bourguignon et al., 1984).

The physiological significance of tropomyosin phosphorylation is clearly demonstrated by the findings that the level of phosphorylation of tropomyosin in striated muscle declines sharply during development of the chicken embryo and rat fetus, from over 90% phosphorylation in the 10 day old chick embryo to less than 15% in the adult muscle (Montarras et al., 1981, 1982; O'Conner et al., 1978; Heeley et al., 1982a,b). At the molecular level, the strategic location of the phosphorylation site at Ser-283 (Mak et al., 1978; Montgomery & Mak 1984), which is involved in the head-to-tail polymerization of tropomyosin, strongly suggests a functional role for tropomyosin phosphorylation. This region is involved in several key functions of tropomyosin: interaction of tropomyosin with troponin T (Mak & Smillie, 1981; Pato et al., 1981; Brisson et al., 1986), cooperative binding of tropomyosin to F-actin (Mak & Smillie, 1981a,b), and perhaps cooperative binding of myosin subfragment 1 to regulated F-actin (Greene & Eisenberg, 1980). Although the detailed conformation of the head-to-tail overlap region is not known, X-ray crystallographic analyses (Phillips et al., 1986, 1980) and model buildings (McLachlan & Stewart, 1975) strongly suggest that the terminal ends are neither helical nor rigid and do not represent a simple stacking of two overlapping coiled coils. A more likely alternative is that the terminal ends interlock to form a flexible globular domain. The conformation of the

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COOH end is therefore expected to be quite different in the aggregated state and free state, and consequently, the phosphorylation of tropomyosin monomers and polymers may be different.

The level of phosphorylation in $\alpha\alpha$-tropomyosin in vivo is always higher than that in the $\beta\beta$ isoform in skeletal muscle in rat, rabbit, and chicken (Montarras et al., 1981; Heeley et al., 1982b; Mak et al., 1978), and the $\alpha\alpha$-tropomyosin is phosphorylated in vitro at a faster rate than the $\beta\beta$-isoform (Montgomery & Mak, 1984). This is surprising since the amino acid sequences at the sites of phosphorylation in the $\alpha\alpha$ and $\beta\beta$ isoforms are very similar with only one substitution involving a charge change, i.e., His-276 in $\alpha\alpha$-tropomyosin replaced by an Asn in $\beta\beta$-tropomyosin (Mak et al., 1980).

Structural and functional differences between the $\alpha\alpha$- and $\beta\beta$-tropomyosin isoforms are of considerable physiological significance due to the variation in the expression and phosphorylation of the two isoforms during development of different striated muscle types (Smillie, 1979; Montarras et al., 1982). Each $\alpha\alpha$- and $\beta\beta$-tropomyosin consists of two potential sites of phosphorylation, one on each chain. The rates of phosphorylation of the two sites may not be identical depending on whether the sites preexist in identical environments or whether phosphorylation of one site would affect the phosphorylation of the neighboring site. In addition, the states of polymerization of tropomyosin may also affect the phosphorylation of the individual sites. Thus, valuable information about the conformation of the COOH end of tropomyosin can be obtained by analyzing the time courses of phosphorylation of the intact $\alpha\alpha$- and $\beta\beta$-tropomyosin. In this study, we have investigated whether the difference in the phosphorylation of the $\alpha\alpha$- and $\beta\beta$-tropomyosin isoforms is due to differences in their conformations at the terminal ends by comparing the K_m and V_{max} values for the phosphorylation of intact tropomyosin and synthetic peptides containing the phosphorylation sites of the α and β isoforms, but having no secondary structures. We have also obtained the phosphorylation rates of the individual chains in $\alpha\alpha$- and $\beta\beta$-tropomyosin at 25 °C where the protein is engaged in a head-to-tail interlocking state and at 37 °C where polymerization is minimized (Iida & Ooi, 1967).

Materials and Methods

[^{32}P]ATP was purchased from Amersham. Phosphocellulose paper (P-81) was from Whatman. Phosphoserine and phosphothreonine standards were from Sigma. All other materials were of reagent grade.

Protein Preparations. Tropomyosin kinase was partially purified from 14 day old fresh chicken embryos as described previously (Montgomery & Mak, 1984; deBelle & Mak, 1987), except that the 0.2 M KCl extract, ammonium sulfate precipitation, and the first DEAE[1] column were carried out within 24 h to minimize loss in yield due to proteolysis of the kinase. Enzyme preparations were used within 1 week for the kinetic studies on intact tropomyosin and synthetic peptides.

$\alpha\alpha$-Tropomyosin was prepared from rabbit cardiac muscle as described (Stone & Smillie, 1978). $\beta\beta$-Tropomyosin was purified from rabbit skeletal muscle according to the method described (Lohmeier, 1982) without carboxymethylation of the cysteine residues. Contaminating cyanate ions were removed from urea by AX 501 mixed-bed resins. Dephosphorylated tropomyosin was used for all time course analyses and was prepared by treatment of tropomyosin with alkaline phosphatase at 37 °C for 1 h at an enzyme:substrate weight ratio of 1:100 in 5 mM $MgCl_2$ (Lewis & Smillie, 1980). Alkaline phosphatase was removed from the tropomyosin by hydroxyapatite chromatography.

Synthesis and Purification of Peptides. The peptides were synthesized according to the general procedures for solid-phase peptide synthesis on a Beckman Model 990 as described by Parker and Hodges (1985). The amino acids were protected at the α-amino positions with the Boc group, and the following side-chain protecting groups were used: Lys(2-chlorobenzyloxycarbonyl), Ser(Bzl), Thr(Bzl), Asn(4,4′-dimethoxybenzhydryl), Asp(OBzl), Glu(OBzl), His(Tos), and Tyr(2,6-Cl_2-Bzl). The COOH-terminal amino acid of the peptide sequence was esterified as the cesium salt to chloromethylated poly(styrene-*co*-1% divinylbenzene) resin. Deprotections and neutralizations were carried out with 50% TFA/CH_2Cl_2 and 5% DIEA/CH_2Cl_2, respectively. The protected amino acids were stepwise attached to the support by the DCC-activated double-coupling program. On completion of the synthesis, the peptide was cleaved from the resin with 20 mL of hydrofluoric acid and 2 mL of anisole per gram of resin at 4 °C for 35 min. The resin was washed with ether and the peptide extracted with TFA (5 × 5 mL). The combined TFA washes were evaporated, and the residue was redissolved in water and lyophilized. The crude peptide was dissolved in 0.1% TFA/H_2O and applied to a reversed-phase HPLC column (SynChropak RP-P, 250 × 10 mm, SynChrom, Inc.) and eluted with a linear AB gradient (A = 0.1% TFA in H_2O and B = 0.05% TFA in acetonitrile) increasing at 1% B/min at a flow rate of 2 mL/min. The absorbance was recorded at 210 nm. Amino acid compositions of all the peptides were analyzed after hydrolysis at 110 °C for 24 h on a Beckman amino acid analyzer. All peptides contained the expected amino acid composition within 10% error.

Phosphorylation of Peptides and Tropomyosin. The tropomyosin kinase was incubated at 25 or 37 °C for 15–30 min in the assay buffer containing 0.5 mM [γ-^{32}P]ATP (250 μCi/μmol), 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM DTT. The reaction was started by the addition of the substrate in the same buffer and temperature. Covalently bound phosphate was measured as described in Kemp (1979) by spotting 10-μL aliquots of the reaction mixture onto phosphocellulose P-81 papers, and the [γ-^{32}P]-ATP was removed by washing in 75 mM H_3PO_4. Background phosphorylation was determined by omitting the substrate in the reaction mixture. For the analyses of time courses of phosphorylation of tropomyosin, the phosphorylated and nonphosphorylated proteins were also separated by alkaline–urea gel electrophoresis (Lewis & Smillie, 1980), and the percent of phosphorylation of the protein was determined by scanning the Coomassie blue stained bands with a densitometer in order to confirm the phosphocellulose paper results; 100% phosphorylation represents one phosphate per Ser residue.

For measuring kinetic parameters, the rates of phosphorylation were always constant with time for all substrate concentrations, and the levels of phosphorylation were less than 15%. The experiments were performed at 37 °C, and the K_m and V_{max} values were analyzed by double-reciprocal plots. The data were fitted to the Michaelis–Menten equation by the method of least squares. Since partially purified tropomyosin

[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; DFP, diisopropyl fluorophosphate; SAP, *Staphylococcus aureus* protease; DTT, DL-dithiothreitol; DEAE, diethylaminoethyl; Boc, *tert*-butoxycarbonyl; Tos, tosyl; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DIEA, diisopropylethylamine.

Table I: Amino Acid Sequences of the Synthetic Peptide Substrates[a]

peptide	sequence
	265 270 275 280
α[264-284]	NH_3 - K - L - K - Y - K - A - I - S - E - E - L - D - **H** - A - L - N - D - **M** - T - S(P) - **I** - COOH
β[264-284]	NH_3 - K - L - K - Y - K - A - I - S - E - E - L - D - **N** - A - L - N - D - **I** - T - S(P) - **L** - COOH
His-276-β[264-284]	NH_3 - K - L - K - Y - K - A - I - S - E - E - L - D - **H** - A - L - N - D - **I** - T - S(P) - **L** - COOH
Met-281-β[264-284]	NH_3 - K - L - K - Y - K - A - I - S - E - E - L - D - **N** - A - L - N - D - **M** - T - S(P) - **L** - COOH
Ile-284-β[264-284]	NH_3 - K - L - K - Y - K - A - I - S - E - E - L - D - **N** - A - L - N - D - **I** - T - S(P) - **I** - COOH

[a] Amino acid substitutions are enclosed in a box.

kinase was used, the V_{max} values were only relative, and they were determined with the same preparation of enzyme.

For the analyses of time courses of phosphorylation, intact tropomyosin concentrations between 10 and 50 μM were used. The plots of log of substrate remaining versus time were similar for the different substrate concentrations. A concentration of 100 μM was used for the peptides. The percent phosphorylation of the substrate was plotted against time, and the data were computer-fitted with one of three equations involving one or two exponentials (Sellers et al., 1983; Orsi & Tipton, 1979). The first equation, with a single exponential, deals with the random phosphorylation of a single population of sites. The second equation, with two exponentials, involves the phosphorylation of two even populations of sites at different rates, k_1 and k_2. The third equation is used for the negatively cooperative model of phosphorylation, as described in Sellers et al. (1983).

Location of Phosphorylation Sites. ^{32}P-Labeled peptides were hydrolyzed at 110 °C for 2 h, and the phosphorylated amino acid was identified on thin-layer electrophoresis at pH 1.8 with internal standards of phosphoserine and phosphothreonine as described before (Montgomery & Mak, 1984).

To locate the site of phosphorylation, the ^{32}P-labeled peptide was digested with SAP for 16 h at 37 °C with a substrate:enzyme molar ratio of 1:100 in 50 mM NH_4HCO_3, pH 8.0. The SAP digest was fractionated on a C18 HPLC column eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid over 60 min. The radioactive peptide was monitored by Cherenkov counting and absorbance at 214 nm, pooled, and freeze-dried. Half of the sample was analyzed by thin-layer electrophoresis, and the radioactive peptide was located by ninhydrin staining followed by autoradiography. Mobility of the peptide relative to Asp was measured and compared to the calculated value on the basis of the method of Offord (1966). The other half of the HPLC sample was hydrolyzed for 24 h at 110 °C, and the amino acid composition was determined on a Beckman 6300 amino acid analyzer.

Other Methods. The concentration of tropomyosin was determined by measuring the absorbance at 280 nm with $E_{280nm}^{1mg/mL} = 0.33$ (Côté & Smillie, 1981). Concentration of the peptides were determined by amino acid analyses. Thin-layer electrophoresis, SDS–polyacrylamide gel electrophoresis, and autoradiography were performed as described before (Montgomery & Mak, 1984).

Results

Identification of Phosphorylation Site on Synthetic Peptides. The sequences of the synthetic peptides used in this study are shown in Table I. The α[264–284] and β[264–284] peptides correspond to residue 264–284 of the αα- and ββ-tropomyosin which contain the in vitro and in vivo phosphorylation sites at Ser-283. These peptides were chosen because each has three Lys residues corresponding to residues 264, 266, and 268 in the native proteins which provide the necessary positive charges for attaching the peptides to the phosphocellulose paper used for the assay of phosphorylation as described in Kemp (1979).

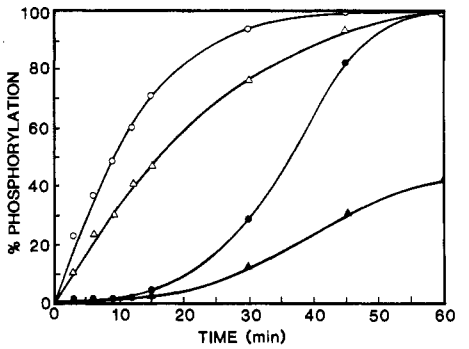

FIGURE 1: Activation of tropomyosin kinase by ATP. Time course of phosphorylation of rabbit cardiac αα-tropomyosin and α[264–284] peptide. The tropomyosin kinase was preincubation for 10 min at 25 °C with [γ-^{32}P]ATP before addition of αα-tropomyosin (○) or α[264–284] peptide (△). Alternatively, the substrates were preincubated with [γ-^{32}P]ATP for 10 min, (●) αα-tropomyosin or (▲) α[264–284], before addition of the tropomyosin kinase. [Kinase] = 20 μg/mL, [ATP] = 1 mM, [tropomyosin] = 152 μM, and [peptide] = 445 μM.

Only phosphoserine was identified in the partial acid hydrolysates of ^{32}P-labeled peptides, not shown. To locate the site(s) of the phosphoserine(s) at residues 271 and/or 283, ^{32}P-labeled α[264–284] was digested with SAP and separated on a C18 column by HPLC. The amino acid composition of the only radioactive fragment was 3.5 Asp, 0.5 Thr, 0.6 Ser, 1.2 Ala, 0.6 Met, 0.8 Ile, 2.0 Leu, and 1.1 His, which corresponds to residues 274–284 indicating that SAP cleaved predominately at Glu-273 and that only Ser-283 was phosphorylated. This was also confirmed by analysis of the radioactive SAP digest by thin-layer electrophoresis at pH 6.5 followed by autoradiography. The mobility of the radioactive spot was −0.6 with respect to Asp which agreed well with the calculated value based on the method of Offord (Offord, 1966). A maximum of 1 mol of phosphate/mol of peptide can be incorporated as shown in Figure 1.

It is interesting to note that Ser-271 appears in the sequence Ser-Glu-Glu, which resembles the recognition sites for casein kinase I (Hathaway & Traugh, 1982). The present result that

Table II: Kinetic Parameters of the Phosphorylation of the Synthetic Peptides and Intact Tropomyosin[a]

substrate	K_m (μM)	V_{max} (nmol of PO_4 min^{-1} mg^{-1})
αα-tropomyosin	47 ± 5	36
ββ-tropomyosin	264 ± 30	33
α[264–284]	500 ± 60	34
β[264–284]	660 ± 45	37
His-276-β[264–284]	1480 ± 100	72
Ile-284-β[264–284]	1360 ± 120	29
Met-281-β[264–284]	330 ± 10	55

[a] K_m values were the average of three determinations using three different preparations of kinases.

tropomyosin kinase does not recognize Ser-271 is consistent with earlier reports (Montgomery & Mak, 1984) that, although tropomyosin kinase has properties similar to casein kinases I and II, the three enzymes are not the same.

Activation of the Tropomyosin Kinase by Preincubation with ATP. Figure 1 shows the time course of phosphorylation of intact αα-tropomyosin and the peptide α[264–284] with and without preincubation of the enzyme with ATP. In both cases, a time lag was observed if the substrate was incubated with the enzyme and ATP simultaneously as reported previously (Montgomery & Mak, 1984). This time lag was removed when the enzyme was preincubated with ATP for 10 min before the addition of the substrate. The mechanism of the activation of the tropomyosin kinase is not clear at present. Tropomyosin kinase preactivated with ATP was used for all subsequent kinetic studies.

Kinetic Parameters for the αα- and ββ-Tropomyosin and the Synthetic Peptides. The apparent K_m and V_{max}, calculated from the Lineweaver–Burk plots, are summarized in Table II. The apparent K_m for the ββ-tropomyosin was 5–6 times that for the αα-tropomyosin, and V_{max} is about the same for both substrates. This result agrees with previous reports that at low concentrations of substrates, about 15 μM, phosphorylation of αα-tropomyosin is about 5–10 times faster than that of ββ-tropomyosin.

The apparent K_m values for the peptides, α[264–284] and β[264–284], are 500 μM and 660 μM, respectively; the V_{max} is similar to that observed for the native proteins. The 10-fold increase in K_m for the α-peptide over that of αα-tropomyosin indicates that the native conformation of the αα-tropomyosin isoform contributes significantly to the recognition of the phosphorylation site by the kinase. A smaller increase in K_m, about 2-fold, is observed for the β-peptide.

In contrast to the 5-fold difference in the K_m values of the intact αα- and ββ-tropomyosin isoforms, the α- and β-peptides have very similar K_m values. Since the peptides are too short to have the same secondary structures as the tropomyosin molecules, the relatively large difference in the K_m values between the intact tropomyosin isoforms must be due to their conformational differences at the COOH ends. There are three differences in the amino acid sequences near the phosphorylation sites of the αα- and ββ-tropomyosin isoforms. Two are conservative substitutions at residues 281 and 284 involving Met/Ile and Ile/Leu, respectively. The other involves a charge change at residue 276, His/Asn. Replacing Asn-276 in the β-peptide with a His increases the K_m by 2-fold to 1500 μM and the V_{max} to 70 nmol min^{-1} mg^{-1}. This slight increase in K_m may be due to the introduction of a weak positive charge by His, since tropomyosin kinase is known to have properties similar to those of casein kinases which recognize acidic residues near the phosphorylation sites (Hathaway & Traugh, 1982). Substituting Leu-284 in the β-peptide by Ile also increases K_m to 1360 μM, whereas replacing Ile-281 with a Met lowers the K_m to 330 μM. It is apparent that His-276 and Ile-284 have negative effects on the phosphorylation of the β-peptide analogue whereas Met-281 improves the phosphorylation kinetics.

Analyses of the Time Course of Phosphorylation of αα- and ββ-Tropomyosin and Peptide Analogues. The conformation of the COOH end of tropomyosin is expected to be quite different in the aggregated and free state. This difference can be investigated by analyzing the time courses of phosphorylation of tropomyosin at 37 and 25 °C in buffer of low ionic strength and ~1 mg/mL protein. Under these conditions at 37 °C, head-to-tail aggregation is minimized as measured by viscosity experiments (Iida & Ooi, 1967).

Provided that the decreasing rate of the reaction with increasing time is only due to substrate depletion and if K_m exceeds S_0, the Michaelis–Menten rate equation becomes

$$\frac{dp}{dt} = \frac{V}{K_m}(S_0 - p)$$

where p = product concentration, S_0 = initial concentration of substrate, V = maximum velocity, and K_m = Michaelis constant (Orsi & Tipton, 1979).

This first-order equation can be integrated to give

$$\ln \frac{S}{S_0} = -\frac{V}{K_m}t = -kt$$

or

$$\frac{p}{S_0} = 1 - e^{-kt}$$

which describes the entire time course of a single-substrate reaction, where S = concentration of substrate remaining at t = time, $S = S_0 - p$, and $k = V/K_m$.

The time-course at 37 °C for αα-tropomyosin is shown in Figure 2A. The single-exponential equation, $p/S_0 = 1 - e^{-kt}$ is sufficient to fit over 95% of the entire curve at this temperature, and a straight line is obtained for the plot of log (S/S_0) versus time (inset in Figure 2A). The rate constant, k, is 0.053 min^{-1}. This is indicative of a random phosphorylation of the two identical chains of αα-tropomyosin which is not distinguishable by the kinase.

At 25 °C, light scattering experiments demonstrated that tropomyosin forms head-to-tail polymers of apparent M_r 570 000 (Ooi et al., 1962). At this temperature, the time course of phosphorylation of αα-tropomyosin appears as a biphasic curve and no longer can be fitted by a single exponential. This can be shown more clearly by the log (S/S_0) verses time plot which is nonlinear (inset in Figure 2A). This can be explained by either one of two simplest modes of reaction (Sellers et al., 1983; Persechini & Hartshorne, 1983). The first depicts a model where the two chains preexist in nonidentical environments and phosphorylation of the two chains proceeds with different rates. To test this model, the data were fitted by computer with the method of least squares with a rate equation, $P/S_0 = 0.5(1 - e^{-k_1 t}) + 0.5(1 - e^{-k_2 t})$, consisting of the sum of two exponentials each representing 50% of the population. The data can be fitted satisfactorily by this equation as shown in Figure 2A, indicating that the time course data can be explained by the preexisting asymmetry model. The rate constant, k_1, for the first chain is about 12 times that of k_2 for the second chain, 0.187 and 0.016 min^{-1}, respectively. The second model describes a negatively cooperative phosphorylation of the two chains such that phosphorylation of one chain adversely affects the phosphorylation of the second chain. In this case, the time course was fitted

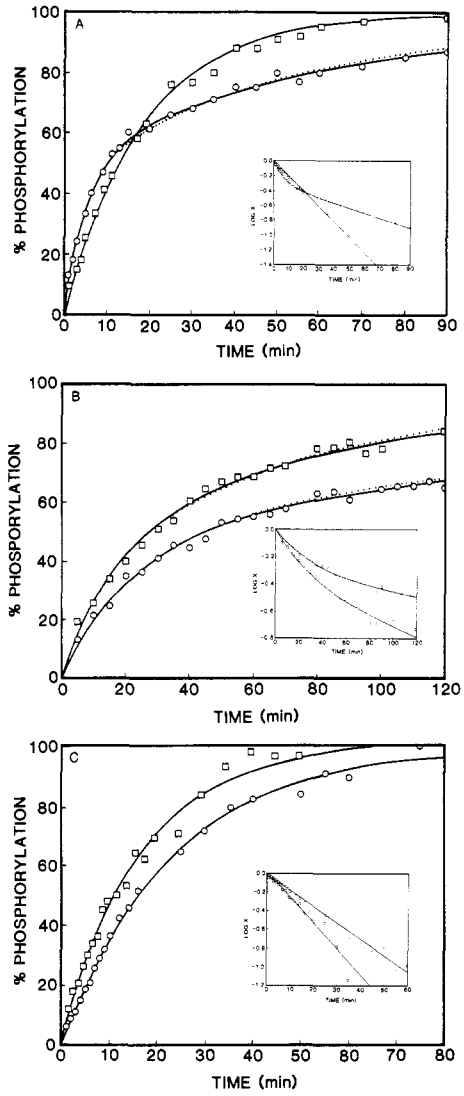

FIGURE 2: Analysis of the time courses of phosphorylation of $\alpha\alpha$- and $\beta\beta$-tropomyosin and α[264–284] peptide. Phosphorylation was carried out as described under Materials and Methods. [Kinase] = 50 μg/mL; [ATP] = 0.5 mM. A 100% phosphorylation was equivalent to 1 mol of phosphate per chain of tropomyosin or peptide. Inset is a plot of log X versus time, where X = fraction of substrate remaining. The dotted lines in the figures are the computer-drawn best fit to the data with the equation for a negatively cooperative model (see Results). (A) Rabbit cardiac $\alpha\alpha$-tropomyosin, 10 μM: (○) 25 °C computer-drawn best fit to the data with the equation with two rate constants, $k_1 = 0.187 \pm 0.007$ min^{-1} and $k_2 = 0.016 \pm 0.001$ min^{-1}; (□) 37 °C computer-drawn best fit to the data with the equation with a single rate constant, $k = 0.053 \pm 0.001$ min^{-1}. (B) Rabbit skeletal $\beta\beta$-tropomyosin, 20 μM: (○) 25 °C where $k_1 = 0.044 \pm 0.002$ min^{-1} and $k_2 = 0.004 \pm 0.000$ min^{-1}; (□) 37 °C where $k_1 = 0.054 \pm 0.003$ min^{-1} and $k_2 = 0.009 \pm 0.001$ min^{-1}. At both temperatures, the computer-drawn best fit to the data was with the equation with two rate constants. (C) α[264–284] peptide, 25 μM: (○) 25 °C where $k = 0.041 \pm 0.002$ min^{-1}; (□) where $k = 0.060 \pm 0.004$ min^{-1}. At both temperatures, the computer-drawn best fit to the data was based on the equation with a single rate constant.

with a more complex equation, $P/S_0 = 2 + [1/(k_1 - k_2)][e^{-k_1 t}(2k_2 - k_1) - k_1 e^{-k_2 t}]$, describing a sequential phosphorylation process as described in Sellers et al. (1983), yielding k_1 and k_2 values of 0.215 and 0.017 min^{-1}, respectively. As shown in Figure 2A, the observed time course at 25 °C can be fitted equally well by the two equations based on the two models, and kinetically it is not possible to distinguish between the two modes of reaction.

The time courses of phosphorylation of the $\beta\beta$-tropomyosin at 25 and 37 °C are shown in Figure 2B. Clearly biphasic curves are obtained at both 25 and 37 °C. On the basis of the preexisting asymmetry model, $k_1 = 0.044$ min^{-1} and $k_2 = 0.004$ min^{-1} at 25 °C, whereas at 37 °C $k_1 = 0.054$ min^{-1} and $k_2 = 0.009$ min^{-1}. Again, both curves can be fitted equally well with the negative cooperative equation. The 10-fold difference in the k_1 and k_2 values at 25 °C suggests that, like its $\alpha\alpha$ counterpart, either the two chains of the $\beta\beta$-tropomyosin are asymmetric or the phosphorylation is negatively cooperative. Contrary to $\alpha\alpha$-tropomyosin, even at 37 °C, the progress curve for the $\beta\beta$-tropomyosin phosphorylation remains biphasic, although k_1 is only about 5 times that of k_2. It appears that end-to-end aggregation of $\beta\beta$-tropomyosin is not totally abolished at 37 °C.

A single-exponential equation can fit the data of the progress curves of the phosphorylation of the peptide α[264–284] at 25 and 37 °C as shown in Figure 2C. This is expected since the peptide lacks secondary structures at both temperatures and therefore only a single population of the peptide exists. Since the progress curves for the peptide are almost identical at the two temperatures, the temperature effect on the phosphorylation of the $\alpha\alpha$- and $\beta\beta$-tropomyosin is not due to changes in activity of the kinase.

Discussion

We have reported before that a time lag was observed in the phosphorylation of tropomyosin when the substrate was incubated simultaneously with ATP and tropomyosin kinase. This time lag can be removed if the tropomyosin kinase is preincubated with ATP for 10 min before the addition of tropomyosin. The mechanism for this enzyme activation is not clear at present, although preliminary work (Watson and Mak, unpublished results) suggests that it may involve the phosphorylation of the tropomyosin kinase itself.

Like many other studies on kinase specificity using synthetic peptide substrates, the intact tropomyosin serves as a better substrate than the peptides. This indicates that tropomyosin kinase recognizes some well-defined structures of higher orders in the intact protein more than simply the primary sequence of the phosphorylation site. Phosphorylation of the peptides probably involves an additional step in the kinetics which induces the formation of the correct conformation for phosphorylation after the initial recognition of the primary structure. Our observation that intact $\alpha\alpha$-tropomyosin is a much better substrate than $\beta\beta$-tropomyosin and yet the kinetic constants for the α- and β-peptide are very similar suggests strongly that the conformation of the COOH end of the intact $\alpha\alpha$-tropomyosin must be significantly different from that of the $\beta\beta$ isoform and the former is more recognizable by the tropomyosin kinase. Such conformational differences induced by the three substitutions at the COOH-terminal ends of the two isoforms would also affect their polymerizability, which has indeed been reported recently (Lohmeier-Vogel et al., 1987).

Although the α[264–284] and β[264–284] peptides have rather similar kinetic parameters, it is not clear whether it is due to a summation of opposing effects imposed by each of the three individual amino acid substitutions in the two peptides. For this reason, three β-peptide analogues, His-276-β-[264–284], Met-281-β[264–284], and Ile-284-β[264–284], each with a single substitution, were studied. His-276 and Ile-284 appear to have a negative influence on the phosphorylation of the β[264–284] peptide whereas Met-281 improves it. To what extent this would contribute to the relatively large difference in the phosphorylation of the intact $\alpha\alpha$- and $\beta\beta$-tropomyosin is not clear at present.

The biphasic time course for $\alpha\alpha$-tropomyosin at 25 °C can

be explained by two simple models. (1) The two chains of the same molecule are not identical in the aggregated state. Phosphorylation of the two chains is random, but each proceeds at a different rate. (2) The two chains are identical, but phosphorylation of one chain inhibits the phosphorylation of the second chain, i.e., negatively cooperative phosphorylation. Kinetically, it is impossible to distinguish between the two models. However, nuclear magnetic resonance studies (Edwards & Sykes, 1978) indicated that the two chains of $\alpha\alpha$-tropomyosin near the COOH-terminal end are in identical environments as reflected by unique chemical shifts of the protons of His-276. These findings seem to favor the latter model of negative cooperativity. The important point is that only a monophasic progress curve is observed at 37 °C when the COOH ends exist predominantly as free ends. This is consistent with our observation that at 25 and 37 °C the phosphorylation of the α[264–284] peptide follows a monophasic time course.

It should be pointed out that the observed biphasic time course can also be explained if two equal populations of the COOH-terminal ends exist; one is engaged in end-to-end overlaps and the other as free ends. This model is considered unlikely since light scattering and sedimentation experiments indicated that the apparent molecular weight of tropomyosin under conditions similar to ours at 25 °C is 570 000, which has nine monomeric units; i.e., only 10% of the molecules have COOH-terminal ends not engaged in end-to-end overlaps (Ooi et al., 1962).

It is interesting that biphasic time courses were obtained for $\beta\beta$-tropomyosin at both 25 and 37 °C. If a biphasic progress curve is a true reflection on the aggregated state of the COOH ends of tropomyosin, this observation suggests that $\beta\beta$-tropomyosin polymerizes better than the $\alpha\alpha$ isoform and a certain degree of polymerization remains in $\beta\beta$-tropomyosin even at 37 °C. This is consistent with a recent report that the strength of head-to-tail interactions of $\beta\beta$-tropomyosin molecules is considerably greater than those of $\alpha\alpha$-tropomyosin (Lohmeier-Vogel et al., 1987). However, lacking a precise knowledge of the degree of polymerization of the $\beta\beta$-tropomyosin at 37 °C, one must consider this interpretation as tentative. It is also possible that the biphasic curve of $\beta\beta$-tropomyosin at 37 °C could be due to different rates of phosphorylation of the two chains even if the $\beta\beta$ molecule remains monomeric.

Although detailed structure of the head-to-tail overlap region of tropomyosin is not known, recent X-ray studies showed that the overlap region involves paired chains to form a compact and globular domain; each pair is made up of either two chains from the same tropomyosin molecule or one chain from each of the adjacent molecule (Phillips et al., 1986). It is conceivable that incorporation of a highly negatively charged phosphate group at Ser-283 of one chain can introduce a salt bridge between the phosphate and Lys-5, -6, or -7 of the adjacent tropomyosin molecule (Mak et al., 1978) and cause a conformational change at the joint such that Ser-283 of the second chain becomes less accessible to the tropomyosin kinase. However, detailed structure of this important region of tropomyosin, which is involved in key functions of the thin filament assembly, must await further studies.

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Registry No. α[264-284], 114394-75-1; β[264-284], 114394-76-2; His-276-β[264-284], 114394-77-3; Ile-284-β[264-284], 114394-78-4; Met-281-β[264-284], 114394-79-5; tropomyosin kinase, 90804-56-1.

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Orientation of Actin Monomer in the F-Actin Filament: Radial Coordinate of Glutamine-41 and Effect of Myosin Subfragment 1 Binding on the Monomer Orientation†

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ABSTRACT: We have employed the method of radial distance measurements in order to orient the actin monomer in the F-actin filament. This method utilizes fluorescence resonance energy transfer measurements of the distance between two equivalent chemical points located on two different monomers. The interprobe distance obtained this way is used to compute the radial coordinate of the labeled amino acid [Taylor, D. L., Reidler, J., Spudich, J. A., & Stryer, L. (1981) *J. Cell Biol. 89*, 362-367]. Theoretical analysis has indicated that if radial coordinates of four points are determined and six intramolecular distances are known, one can, within symmetry limits, position the monomer about the filament axis. The radial distance of Gln-41 that had been enzymatically modified with dansyl, rhodamine, and fluorescein derivatives of cadaverine was found to be approximately 40-42 Å. The determination of the radial distance of Cys-374 was accomplished by using monobromobimane and *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate as donors and *N*-[4-[[4-(dimethylamino)phenyl]azo]phenyl]maleimide as acceptor; the results were consistent with a radial coordinate for this residue of 20-25 Å. The effect of myosin subfragment 1 (S1) binding on the radial coordinates of (1) Gln-41, (2) Cys-374, and (3) the nucleotide binding site was also examined. S1 had a small effect on the radial coordinate of Gln-41, increasing it to 44-47 Å. In the two remaining cases the change in the radial coordinate due to the S1 binding was negligible. This finding excludes certain models of the interaction between actin and S1 in which actin monomer rotates by a large angle when subfragment 1 binds to it.

From low-resolution X-ray crystallography and electron microscopy it has been found that the structure of G-actin is bilobar (Suck et al., 1981; Kabsch et al., 1985; Taylor & Amos, 1983; Amos, 1985; Tajima et al., 1983; Egelman & DeRosier, 1983). The N-terminal portion of the molecule is in the smaller lobe whereas the C-terminus is in the larger. The phosphate moiety of the actin-bound nucleotide is located presumably in the interdomain space (Kabsch et al., 1985). While a high-resolution crystallographic structure of G-actin complexes with profilin (Carlsson et al., 1976; Schutt et al., 1985) and DNase[1] (Suck et al., 1984; Kabsch et al., 1985) is slowly emerging, the orientation of the monomer in the thin filament is still an open question.

Numerous attempts have been made to fit the available, low-resolution structure of monomeric actin into the filament [for a review see Egelman (1985)]. This is, of course, only the first step in the filament reconstruction since the difference in conformation between G-actin monomer and F-actin protomer is unknown. To date, however, in spite of efforts by several laboratories, no definite answer to the question of orientation has been obtained.

Besides crystallography and electron microscopy other techniques are helpful in orienting the monomer. Cross-linking

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[1] Abbreviations: S1, myosin subfragment 1; 1,5-IAEDNS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate; DABM, *N*-[4-[[4-(dimethylamino)phenyl]azo]phenyl]maleimide; DAB, [[4-(dimethylamino)phenyl]azo]benzene; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; ε-ADP, 1,N^6-ethenoadenosine 5′-diphosphate; DNC, dansylcadaverine; RHC, rhodamine cadaverine; FLC, fluorescein cadaverine; MBB, monobromobimane; FRET, fluorescence resonance energy transfer; rms, root mean square; DNase, deoxyribonuclease; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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