Interaction of Tropomyosin and Troponin T: A Proton Nuclear Magnetic Resonance Study†

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Received December 10, 1985; Revised Manuscript Received February 24, 1986

ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) has been used to study the nature of the interaction between tropomyosin (TM) and troponin T (Tn-T). Resonances corresponding to the histidine residues in fragments of both TM and Tn-T can be resolved and assigned in the ^{1}H NMR spectrum. Changes in the pH titration profiles of these resonances when the various fragments are mixed provide probes of the interaction sites between the proteins. Fragment T1 (residues 1–158) of Tn-T appears to interact weakly but specifically with fragments of TM in which the NH_2-terminus (residues 1–10) is intact. While fragment CB2 (residues 71–151) of Tn-T interacts weakly (dissociation constant of 0.1–0.2 mM) with NH_2-terminal fragments of TM, this appears to be nonspecific since the absence of residues 1–10 and 128–189 of TM does not affect the observed perturbations of the titration profiles of His-79 of CB2. Although a strong interaction between T1 and the COOH-terminal Cy2 fragment (residues 190–284) of TM has been previously demonstrated, no perturbation of His-276 of Cy2 or of His-7, -23, -29, or -36 of T1 was observed in a mixture of T1/Cy2. The pK_a of His-276 was also not affected in a mixture of Cy1/Cy2 (where Cy1 is residues 1–189 of TM) but was significantly decreased in the ternary complex T1/Cy1/Cy2. The importance of residues 1–70 of Tn-T in its binding to TM is illustrated by the specificity it confers on the T1/Cy1 interaction and by the absence of His-276 perturbation in the mixture CB2/Cy1/Cy2.

Central to the structure of the thin filament of skeletal muscle, and the role of that structure in the mediation of the regulation of muscle contraction by calcium ions, is the head to tail polymerization of tropomyosin (TM)[1] molecules in the grooves of the F-actin helix and the effects on that polymerization of the binding of troponin (Tn) to TM. The head to tail aggregation of TM molecules, first described by Bailey (1946, 1948), is known to be markedly influenced by salt concentration (Bailey, 1948; Tsao et al., 1951; Kay & Bailey, 1960; McCubbin & Kay, 1969) and by pH (Iida & Ooi, 1967; Dabrowska et al., 1980), and to be significantly reduced by chemical modification of lysine-7 in the NH_2-terminal region of the molecule (Johnson & Smillie, 1977).

The preparation of nonpolymerizable TM, in which 11 residues are enzymically and quantitatively cleaved from its COOH-terminus (residues 274–284), completely abolishes this head to tail interaction and dramatically reduces binding of TM to F-actin (Mak & Smillie, 1981a,b), presumably by eliminating the cooperative interaction of contiguous TM molecules in that binding process (Yang et al., 1979; Wegner, 1979; Walsh & Wegner, 1980). The overlap of NH_2- and COOH-termini involved in this head to tail polymerization has been estimated to be 8–11 amino acid residues (residues 1–11 and 273–284) and may well be in a structure different from that of a simple overlap of regular coiled coils (Phillips et al., 1979, 1981; Stewart, 1981; Stewart & Roberts, 1983).

Tn binds to TM through its troponin T (Tn-T) component and significantly induces further head to tail polymerization as indicated by the large viscosity increases upon addition of Tn to TM at low ionic strength (Ebashi & Kodama, 1965; Yamamoto & Maruyama, 1973; Jackson et al., 1975). This effect of whole Tn is almost exactly mimicked by Tn-T and its cyanogen bromide fragment CB1 (residues 1–151), but not by subfragments CB2 (residues 71–151) or CB3 (1–70) nor by other fragments derived from the COOH-terminal region (residues 152–259) of the Tn-T polypeptide chain (Jackson et al., 1975). Recent studies of the binding of fragments of Tn-T to TM and its NH_2- and COOH-terminal fragments have shown that CB1 binds close to or at the COOH-terminal end of TM (Pato et al., 1981a; Mak & Smillie, 1981a; Pearlstone & Smillie, 1982) and induces the formation of a ternary complex with NH_2- and COOH-terminal fragments of TM under conditions in which no interaction between these fragments by themselves could be detected. The formation of this ternary complex was dependent on intact NH_2-terminal sequences of the TM fragments and did not occur with those fragments in which residues 1–11 were absent (Pato et al., 1981a). While no direct interaction between NH_2-terminal fragments of TM and CB1 [or T1 (residues 1–158 of Tn-T)] was detected in these studies, the observation that Tn and Tn-T induce cooperative binding of NPTM to F-actin (Mak et al., 1983) suggested the possibility that the CB1 (or T1) region of Tn-T may bridge the gap between the ends of NPTM molecules by interacting both with a residual binding site for T1 on NPTM (e.g., residues in the region of Tyr-261 and -267)

† This work was supported by the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

[1] Abbreviations: Tn, troponin; Tn-T, troponin T; TM, tropomyosin; NPTM, nonpolymerizable tropomyosin; Cy1, residues 1–189 of TM; T1-TM, residues 1–133 of TM; Cn1A, residues 11–127 of TM; Cy2, residues 190–284 of TM; CB1, residues 1–151 of Tn-T; T1, residues 1–158 of Tn-T; CB3, residues 1–70 of Tn-T; CB2, residues 71–151 of Tn-T; NMR, nuclear magnetic resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-4548$01.50/0

Table I: pK_a Values Determined from Histidine C_2–H Proton Resonances of Tropomyosin and Troponin T Fragments[a]

sample[b]	[KCl] (mM)	histidine pK_a						
		1 (His-153)	2a (His-276)	A	B	C	Da	E (His-79)
Cy1	50	7.30						
T1	50			7.33	7.42	7.61	7.82	*e*
T1/Cy1[c]	50	7.26		7.32	7.40	7.59	7.80	*e*
T1/Cy1	50	7.19		7.30	7.41	7.62	7.84	*e*
T1/T1-TM	50			7.29	7.39	7.59	7.81	*e*
T1/Cn1A	50			7.30	7.40	7.59	7.81	*e*
Cy1	100	7.26						
T1	100			7.31	7.42	7.60	7.79	*e*
T1/Cy1[c]	100	7.20		7.31	7.41	7.63	7.84	*e*
T1/Cy1	100	7.23		7.32	7.43	7.62	7.82	*e*
T1/Cn1A	100			7.37	7.45	7.65	7.85	*e*
CB3	50			7.33	7.42	7.62	7.88	
CB3/Cy1	50	7.20		7.34	7.43	7.63	7.88	
CB2	100							6.83
CB2/CB3	100			7.28	7.37	7.57	7.79	6.84
CB2/Cy1	100	7.18						7.04
CB2/T1-TM	100							7.08
CB2/Cn1A	100							7.12
Cy2	100		7.99					
Cy1/Cy2	100	7.16	7.97					
T1/Cy2	100		7.98	7.30	7.40	7.61	7.78	*e*
T1/Cy1/Cy2	100	7.18	7.27	7.27	7.36	7.57	7.77	*e*
CB2/Cy2	100		7.97					7.48
CB2/Cy1/Cy2[d]	100	7.14	7.83					*e*

[a] The average error of the pK_a values is ±0.02. All measurements were done at 10 °C in 10 mM K_2DPO_4, 0.5 mM EDTA, 0.2 mM DSS, and D_2O with 50 mM KCl or 100 mM KCl. The pK_a values are uncorrected for D_2O. [b] All mixtures are at equimolar ratios except where indicated. [c] Molar ratio = 2:1. [d] Molar ratio = 2:1:1. [e] His E was too broad to be observed.

and with the NH_2-terminus of a neighboring NPTM molecule. However, other interpretations are possible, and the recent demonstration (Walsh et al., 1985) that the addition of Tn (±Ca^{2+}) to NPTM at a variety of ionic strengths has no effect on its viscosity suggests that if such interactions do occur, they are of a relatively weak nature.

In the present investigation, ^1H nuclear magnetic resonance (NMR) spectroscopy (Edwards & Sykes, 1978, 1980) has been used to further probe the nature of the interactions between the NH_2-terminal region of Tn-T (residues 1–158) and the molecular ends of the TM molecule. With the Tn-T and TM fragments shown in Figure 1, resonances corresponding to the histidine residues in each of the fragments could be resolved and assigned in the ^1H NMR spectra. Since histidine residues are located in the NH_2-terminal region of Tn-T and in the COOH-terminal region of TM (Figure 1), changes in the pH titration profiles of these resonances when the various fragments were mixed provided probes for the interaction sites between the proteins.

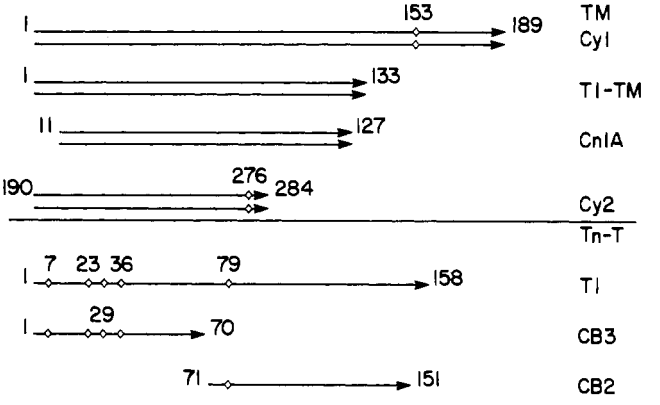

FIGURE 1: Fragments of TM and Tn-T used in the ^1H NMR monitored pH titrations. The positions of the histidine residues in the sequence are denoted by open diamonds.

Experimental Procedures

The preparation of α-TM and its fragments was done according to the methods of Pato et al. (1981b). Fragments of Tn-T were prepared as previously described (Pearlstone et al., 1981, 1977).

The NMR samples were prepared by (1) dialyzing the fragments against H_2O, (2) determining the protein concentration by amino acid analysis, (3) mixing the fragments in specific ratios,[2] (4) adding 0.5 mL of buffer (10 mM K_2HPO_4, 0.5 mM EDTA, and 50 or 100 mM KCl), (5) exchanging twice with D_2O, and (6) redissolving in 0.5 mL of D_2O.

The pH titrations were performed by adding aliquots of NaOD or DCl directly to the sample in the NMR tube. The pH was measured with an Ingold microelectrode. All measurements on the NMR sample and H_2O standard buffers (pH 4, 7, and 10) were taken at 10 °C with the aid of a circulating water bath. All pH measurements are uncorrected for D_2O.

Proton NMR spectra were recorded at 10 °C on a 300-MHz Nicolet NT-300 wide-bore spectrometer. Depending on the protein concentration (0.5–3 mM), 10000–1000 transients were acquired with 8192 data points in the double precision mode. Before the Fourier transformation, a line broadening of 1–2 Hz was used to increase the signal to noise ratio of the spectrum. The HDO resonance was suppressed with homonuclear decoupling. The internal reference for the chemical shifts was the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

The variation of the observed chemical shift of a histidine resonance, δ_{obsd}, with pH (Figure 2) was analyzed by using the equation:

$$\delta_{obsd} = \delta_A + (\delta_{AH} - \delta_A)\frac{[H]}{K_a + [H]}$$

where K_a is the dissociation constant, δ_A is the chemical shift of the resonance from unprotonated histidine, and δ_{AH} is the

[2] When molar ratios are mentioned relative to tropomyosin or its fragments, these indicate moles of dimer and not moles of the individual chains of the coiled coil.

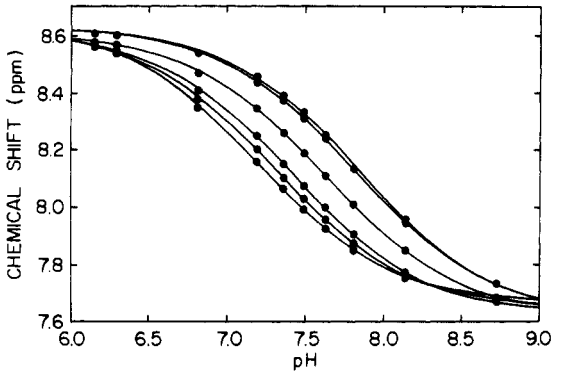

FIGURE 2: ^{1}H NMR monitored pH titration of the histidine residues of 2:1 T1/Cyl in 10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS at 10 °C and least-squares fit of the data (solid lines).

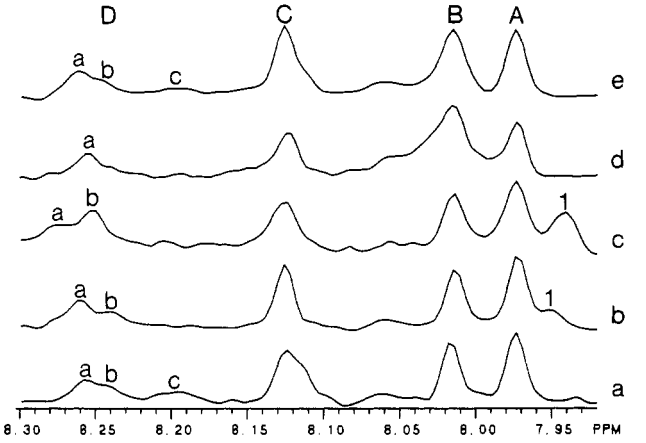

FIGURE 3: Spectra of the histidine C_2–H region of T1 and tropomyosin fragments at 10 °C, pH 7.61 (10 mM K_2DPO_4, 50 mM KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) T1, 1 mM. (b) T1:Cyl ratio = 2:1 = 0.5 mM:0.25 mM. (c) T1:Cyl ratio = 1:1 = 0.5 mM. (d) T1:T1-TM ratio = 1:1 = 0.2 mM:0.2 mM. (e) T1:Cn1A ratio = 0.7 mM:0.7 mM. The histidine resonances of T1 are labeled A, B, C, and D. Resonance D is split into different signals (a, b, and c) corresponding to different conformers of T1. The population of these conformers varies with the composition of the samples. Resonance 1 is from His-153 of Cyl.

chemical shift from protonated histidine (Edwards & Sykes, 1978). A minimum of 10 points were analyzed for each pH titration shown in Table I. All pH titrations were reversible.

RESULTS

Individual Fragments. The various fragments used in the ^{1}H NMR monitored pH titrations and the position of the histidine residues in these fragments are indicated in Figure 1. The ^{1}H NMR resonances from the C_2–H protons of the various histidine residues are well separated downfield (7.6–8.7 ppm) from the other ^{1}H NMR resonances of the protein and resolved from one another at pH values near the pK_a's of the histidines (Edwards & Sykes, 1978, 1980). While only spectra at a few pH values are shown for the most part, complete pH titrations were performed for all samples and yielded data of the quality indicated in Figure 2.

For pH titrations involving Cyl, a single resonance was observed for the C_2 protons of the two His-153 residues of the coiled-coil dimer. This resonance is denoted by the number 1 in Figures 3–9. Its pK_a values could be determined for all the pH titrations performed (Table I). Throughout all the experiments, similar δ_A and δ_{AH} values were calculated for the His-153 C_2 protons of Cyl (Table II).

For Cy2, at certain pH values, three separate signals (denoted 2a, 2b, and 2c in Figure 7b) were observed for the C_2–H

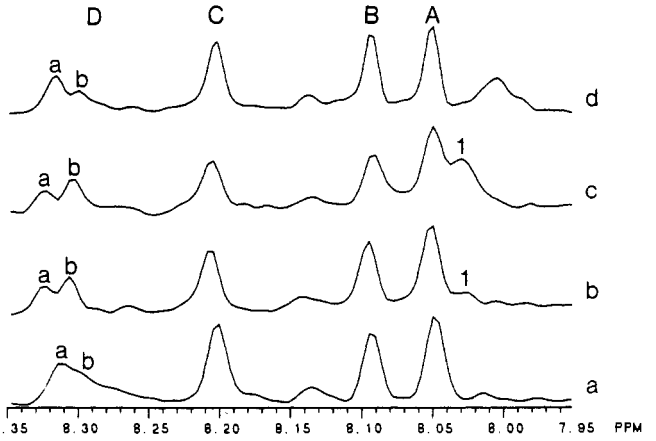

FIGURE 4: Spectra of the histidine C_2–H region of T1 and tropomyosin fragments at 10 °C, pH 7.47 (10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) T1, 1 mM. (b) T1:Cyl ratio = 2:1 = 0.5 mM:0.25 mM. (c) T1:Cyl ratio = 1:1 = 0.5 mM:0.5 mM. (d) T1:Cn1A ratio = 1:1 = 0.2 mM:0.2 mM. The labeling of the resonances is the same as in Figure 3.

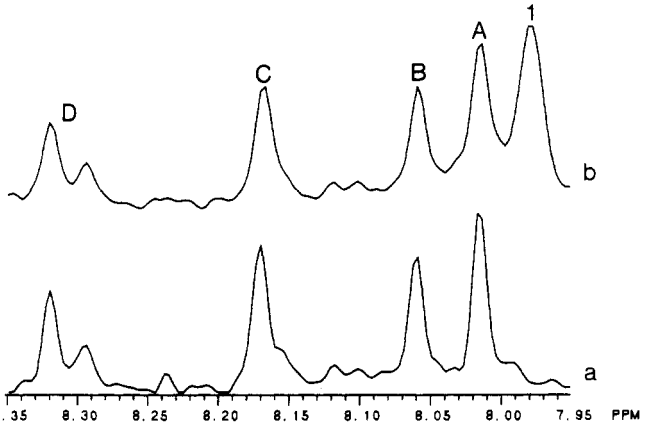

FIGURE 5: Spectra of the histidine C_2–H region of CB3 and tropomyosin fragments at 10 °C, pH 7.56 (10 mM K_2DPO_4, 50 mM KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) CB3, 1 mM. (b) CB3:Cyl ratio = 1:1 = 0.25 mM:0.25 mM. Resonances A–D are from CB3, and resonance 1 is from Cyl.

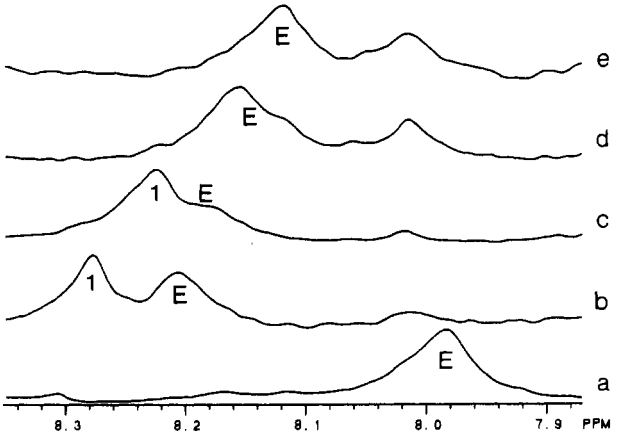

FIGURE 6: Spectra of the histidine region of CB2 and tropomyosin fragments at 10 °C (10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) CB2, 1 mM, pH 7.11. (b) CB2:Cyl ratio = 2:1 = 0.26 mM:0.13 mM, pH 6.92. (c) CB2:Cyl ratio = 1:1 = 0.25 mM:0.25 mM, pH 7.07. (d) CB2:T1-TM ratio = 1:1 = 0.12 mM, pH 7.09. (e) CB2:Cn1A ratio = 1:1 = 0.15 mM, pH 7.21. Resonance E is from CB2, and resonance 1 is from Cyl. Additional resonances in (d) and (e) near 8.0 ppm do not shift with pH.

resonances of His-276. These multiple resonances arise from intermediates in the thermal unfolding of the tropomyosin

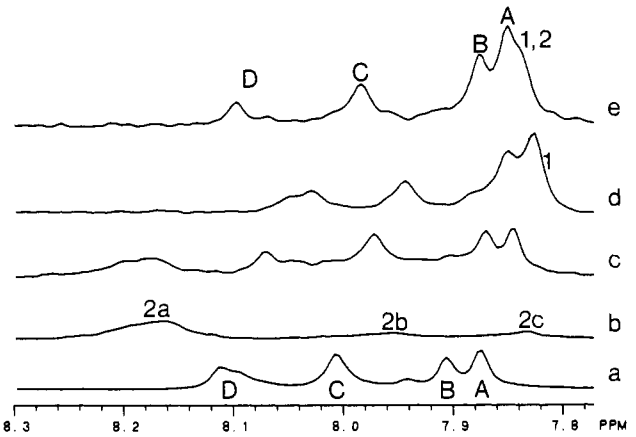

FIGURE 7: Spectra of the histidine region of T1 and tropomyosin fragments at 10 °C (10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) T1, 1 mM, pH 7.84. (b) Cy2, 2 mM, pH 7.84. (c) T1:Cy2 ratio = 1:1 = 0.4 mM:0.4 mM, pH 7.82. (d) T1:Cy1 ratio = 1:1 = 0.5 mM:0.5 mM, pH 7.94. (e) T1:Cy1:Cy2 ratio = 1:1:1 = 0.12 mM:0.12 mM:0.12 mM, pH 7.86. Resonances A, B, C, and D are from T1, resonance 1 is from His-153 of Cy1, and resonances 2a, 2b, and 2c are from His-276 of Cy2.

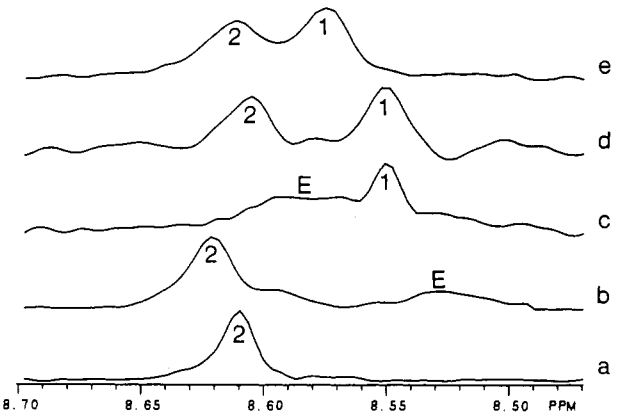

FIGURE 8: Spectra of the histidine region of CB2 and tropomyosin fragments at 10 °C (10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) Cy2, 0.6 mM, pH 6.17. (b) CB2:Cy2 ratio = 0.5 mM:0.5 mM, pH 6.01. (c) CB2:Cyl ratio = 1:1 = 0.25 mM:0.25 mM, pH 6.15. (d) CB2:Cy1:Cy2 ratio = 2:1:1 = 0.14 mM:0.07 mM:0.07 mM, pH 6.18. (e) Cy1:Cy2 ratio = 1:1 = 0.4 mM:0.4 mM, pH 5.83. Resonances 1, 2, and E are from Cy1, Cy2, and CB2, respectively.

Table II: Chemical Shift Values δ_A and δ_{AH} for Histidine C_2 Proton Resonances of Tropomyosin and Troponin T Fragments[a]

histidine	δ_A (ppm)	δ_{AH} (ppm)
1	7.66	8.65
2a	7.65	8.62
A	7.64	8.62
B	7.63	8.61
C	7.64	8.61
Da	7.64	8.63

[a] The average error for δ_A and δ_{AH} is ±0.01 ppm.

coiled coil (Edwards & Sykes, 1980). At low temperatures, conformer 2a is dominant (Figure 7b), while at high temperatures, conformer 2b becomes the predominant conformational state for Cy2 (unpublished results). To simplify the spectra when various fragments were mixed together, all pH titrations were performed at 10 °C where Cy2 should exist in its most stable conformation. The pK_a values for His-2a are given in Table I, and the δ_A and δ_{AH} values are given in Table II. The δ_A and δ_{AH} values for Cy2 did not vary for different titrations.

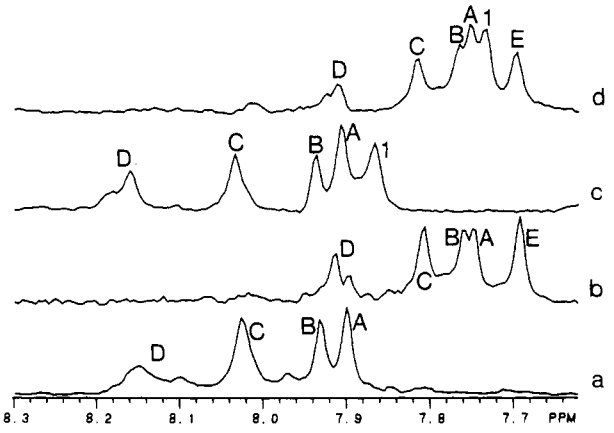

FIGURE 9: Temperature variation of T1 and tropomyosin fragments, pH 7.6 (10 mM K_2DPO_4, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mM DSS). (a) T1, 1 mM, 10 °C. (b) T1, 1 mM, 60 °C. (c) T1:Cy1 ratio = 1:1, 10 °C. (d) T1:Cy1 ratio = 1:1, 60 °C.

In intact tropomyosin, the C_2–H resonances of His-153 appeared as multiple signals. However, in Cy1 the C_2–H histidine resonances are not split into multiple peaks. The difference in behavior of these resonances in intact tropomyosin and Cy1 can be attributed to differences in the thermal unfolding of the coiled coil of TM and Cy1 (Edwards & Sykes, 1980). By cleaving TM at Cys-190, the rate of exchange between various conformational states of the coiled coil is altered in residues 1–190, as judged by differences in helical content and melting temperatures between TM and Cy1 (Pato et al., 1981b).

At certain pH values and low temperatures, four out of five C_2–H histidine resonances of T1 were clearly resolved (A, B, C, and D in Figure 3). The histidine D resonances were split into several signals. The fifth histidine resonance (E) was not observed due to the effects of intermediate exchange. However, at temperatures above 40 °C, the His E resonance could be observed (Figure 9), indicating either that the exchange rate between intermediates increased or that one of the conformers became predominant.

Histidines A–D were located in the NH_2-terminal end of T1 (His-7, -23, -29, or -36 in Figure 1) since the C_2–H resonances of CB3 (residues 1–70) had similar pK_a values and similar δ_A and δ_{AH} values to those of T1 (Tables I and II). The resonances of His D in CB3 were also split into two signals of unequal intensities (Figure 5a), indicating two conformational states of CB3 which have unequal populations. Since the conformational equilibrium of His D was different in CB3 compared to T1 (at least three states for the His D resonances), residues 71–151 in T1 must have an influence on the conformational states of the NH_2-terminal end of T1. Conversely, residues 1–70 of T1 have an influence on the conformational state of residues 71–151 of T1 since the behavior of His E in T1 vs. CB2 (residues 71–151) was different. In CB2, the C_2–H resonance of His E (His-79) is clearly observed at low temperatures (Figure 6) while in T1, under similar conditions, His E is not observed due to the effects of intermediate exchange between conformers. Hence, the presence or absence of residues 1–70 affects the rate of exchange between conformers observed for His E (His-79) in T1 compared to CB2.

Mixtures of T1 with Cy1, T1-TM, and Cn1A. To determine if the NH_2-terminal region of Tn-T was binding with the NH_2-terminal end of tropomyosin, fragments Cy1, T1-TM, and Cn1A of TM were mixed with fragment T1 of Tn-T. In Figure 3a, four histidine resonances (labeled A, B, C, and D) of T1 are observed. Resonance D is split into a number of signals due to local conformational differences which affect

its pK_a. At least three different conformers were observed (peaks a, b, and c in Figure 3a). [Note the labeling with lower case letters (a, b, c, etc.) of the resonances corresponding to different conformers in Figure 3 does not imply a consistent assignment under different experimental conditions. The resonances are labeled if and only if they appear consistently throughout the titration and then are labeled in order of increasing pK_a.] Upon addition of Cyl to Tl (1:2 ratio), the broad resonance from His-153 of Cyl is observed (peak 1 in Figure 3b). Also, a change in the population distribution for resonance D can be detected. When Cyl is added to Tl, peak c of resonance D is no longer observable, and only the two major conformers, represented by peaks a and b in Figure 3b, are now observed. This shift in the population distribution of conformers for resonance indicates an interaction between Tl and Cyl. Upon addition of more Cyl to the mixture, to a final ratio of 1:1 for Tl/Cyl, it can be observed in Figure 3c that the intensity of the C_2–H resonance of Cyl (peak 1) has increased and that the population distribution for resonance D has changed. This indicates that the interaction between Cyl and Tl depends on the concentration of the two proteins with respect to each other. The spectra in Figure 3 represent only one spectrum out of each full titration, so that the line-shape changes were observed at many pH values (cf. Figure 2). Further, these experiments were repeated several times with reproducible changes in the NMR line shapes.

In Figure 2, the variation of the chemical shift of the C_2–H resonances of the histidine residues for a 2:1 Tl:Cyl ratio is shown. As can be observed, all the resonances were resolved, and the pK_a, δ_A, and δ_{AH} values of each resonance could be determined. Apart from the change in population distribution of the His D conformers, the pK_a of His-153 of Cyl did decrease slightly upon addition of Tl. The pK_a of His-153 in TM is very sensitive to the stability of the coiled coil of TM (Edwards & Sykes, 1980). Since the binding of Tl to Cyl will affect the stability of the coiled coil, this in turn could affect the pK_a of His-153.

For the mixture Tl/Tl-TM (Figure 3d), the population distribution of His D of Tl is still perturbed by the presence of Tl-TM. Since Tl-TM lacks residues 134–189 of Cyl, this result indicates that Tl does not bind near His-153 of Cyl and that Tl probably binds to a region near the NH_2-terminal end of Cyl. For such a case, one would expect the population distribution of His D conformers to be similar for Tl/Cyl and Tl/Tl-TM. However, the line shape of His D is not exactly the same in Figure 3c and Figure 3d, indicating that Tl binds in a different manner to Cyl than Tl-TM. A probable cause for this effect is that the thermal stability of the coiled coil of tropomyosin is different for Cyl and Tl-TM (Pato et al., 1981b). Hence, removing residues near His-153 of Cyl would affect the stability of the coiled coil which in turn would affect the binding of the tropomyosin fragments with Tl and thus alter the population distribution of His D conformers.

The importance of an intact NH_2-terminal fragment of TM for binding to Tl can be demonstrated when Tl and CnlA (residues 11–127) of TM are mixed together (Figure 3e). In such a case, the signals for the C_2–H resonance of His D are quite similar to the ones for Tl by itself in Figure 3a. However, some interaction between the two fragments can still be postulated because the intensity of peak c of His D in Tl/CnlA is decreased as compared to the intensities of peak c for Tl. (Among the experiments presented in Figure 3, only for Tl and Tl/CnlA could resonance c be followed throughout the titration.) This effect is more clearly observable at a different salt concentration.

Table III: Chemical Shift Values of δ_A and δ_{AH} for Histidine C_2 Proton Resonances of the Troponin T Fragment CB2 (Residues 71–151)

sample	δ_A (ppm)	δ_{AH} (ppm)
CB2	7.67 ± 0.01	8.62 ± 0.02
CB2/CB3	7.66 ± 0.01	8.60 ± 0.01
CB2/Cyl	7.66 ± 0.01	8.69 ± 0.01
CB2/Tl-TM	7.66 ± 0.01	8.63 ± 0.01
CB2/CnlA	7.65 ± 0.01	8.64 ± 0.02
CB2/Cy2	7.64 ± 0.01	8.56 ± 0.01

Table IV: Change in Chemical Shift of the C_2–H Resonance of His E of CB2 as a Function of Total Cyl Concentration[a]

[Cyl] (mM)	Δδ(CB2) (ppm)	Δδ(calcd) (ppm)
0.030	0.016	0.016
0.052	0.028	0.026
0.069	0.034	0.034
0.081	0.041	0.041
0.100	0.049	0.048
0.138	0.066	0.063
0.157	0.069	0.071
0.169	0.075	0.075
0.186	0.077	0.081
0.208	0.088	0.088
0.238	0.097	0.096
0.250	0.100	0.100

[a] [CB2] = 0.25 mM. Measurements were done at 10 °C in 10 mM K_2DPO_4, 100 mM KCl, 0.5 mM EDTA, and 0.2 mM DSS, pH 6.92.

At 0.1 M KCl, the interaction between Tl and Cyl is still present as judged by the differences in the population distribution of His D conformers for Tl and Tl/Cyl (Figure 4a–c). For the mixture Tl/CnlA, the population distribution of the His D conformers is quite different from the one for Tl/Cyl, indicating the importance of an intact NH_2-terminal of TM for binding to Tl. However, the line shape of His D is slightly different than the one for Tl, indicating that CnlA still binds to Tl. These line-shape and chemical shift differences were consistent and observed throughout the titration.

Mixture of CB3 and Cyl. By comparing the line shape of His D in CB3 (Figure 5a) and in CB3/Cyl (Figure 5b), it can be observed that Cyl has no effect on the population distribution of His D conformers. However, the pK_a of His-153 of Cyl (Table I) was still affected by the presence of CB3, indicating an interaction between the two protein fragments.

Interaction of CB2 with the NH_2-Terminal Region of Tropomyosin and with CB3. In Figure 6, representative spectra from the pH titrations for CB2, CB2/Cyl, CB2/Tl-TM, and CB2/CnlA are shown. As can be observed, the C_2–H resonance of His-79 (His E) of CB2 was clearly observed under all conditions, thus allowing the determination of its pK_a, δ_A, and δ_{AH} parameters (Tables I and III). For each of these mixtures of CB2 and TM fragment, the variation in the pK_a values for CB2 with TM fragments was greater than the experimental error in the measurements. Hence, CB2 bound to all the TM fragments studied. In the case of a mixture of CB2 and CB3, on the other hand, there was no indication of an interaction between them (Tables I and III).

An estimate of the binding constant between Cyl and CB2 was obtained from the change in the chemical shift (or pK_a) of the His-79 resonance as a function of total Cyl concentration (Table IV). Assuming one to one binding, the dissociation constant was determined to be between 0.1 and 0.2 mM. Since at a 1:1 ratio the pK_a values of His-79 for CB2/Tl-TM and CB2/CnlA were similar to the one for CB2/Cyl, the binding of CB2 to Tl-TM and CnlA should have the same order of magnitude as the one for Cyl. Thus, the weak binding of CB2 on these TM fragments appeared

to be nonspecific as compared to that of T1 since it was not significantly affected by the absence of residues 1–10 or 128–189.

Interaction of T1 and Cy2. For the pH titration of T1/Cy2, the C_2–H signal corresponding to the major conformer of His-276 (resonance 2a in Figure 7) could easily be distinguished from the C_2–H resonances of T1 (resonances A–D in Figure 7). By comparing the spectra for T1, Cy2, and T1/Cy2 in Figure 7a–c, respectively, it can be noticed that the only effect due to mixing of T1 and Cy2 is a slight change in the population distribution of resonance D. The variation in chemical shift is due to slight differences in the pH of the samples for the spectra presented. Since T1 and Cy2 are known to bind tightly together (Pato et al., 1981a), it can be concluded that histidines A–D of T1 and His-276 of Cy2 are not directly involved in the binding of these two proteins.

Interaction of T1 with Cy1 and Cy2. T1 (or CB1) has previously been shown to promote head to tail polymerization of TM molecules (Jackson et al., 1975) and to form a ternary complex with fragments Cy1 and Cy2 under conditions in which no detectable interaction between these latter fragments could be detected (Pato et al., 1981a). Evidence for the involvement of His-276 in the formation of this ternary complex is presented in Figure 7. By comparing the spectra for T1/Cy2, T1/Cy1, and T1/Cy1/Cy2 at similar pH values, it can be observed that the C_2–H resonance of His-276 which resonated near 8.17 ppm in T1/Cy2 (Figure 7c) now appears at 7.84 ppm in T1/Cy1/Cy2 (Figure 7e) overlapping with the C_2–H resonances A of T1 and His-153 of Cy1. For Cy2, Cy1/Cy2, and T1/Cy2, the pK_a of His-276 of Cy2 is near 7.97, while for T1/Cy1/Cy2 the pK_a value is decreased to 7.27 (Table I). Thus, it is only in the formation of the ternary complex of T1 with Cy1 and Cy2 that one observes a perturbation of His-276.

Interaction of CB2 with Cy1 and Cy2. Previous studies (Jackson et al., 1975) have shown that CB2 is significantly less effective than CB1 (and therefore T1) or whole Tn in promoting head to tail polymerization of TM molecules as indicated by its reduced ability to increase the viscosity of TM solutions. This suggests that the CB3 region of CB1 (and therefore T1) plays an important role in this induction of polymerization. This role was more clearly defined by considering the pH titrations shown in Figure 8. At low pH values, the C_2–H resonances of the histidine in each fragment can be clearly resolved in Cy2 (Figure 8a), CB2/Cy2 (Figure 8b), and CB2/Cy1 (Figure 8c). The interaction of CB2 and Cy2 greatly perturbs the pK_a of His-79 (His E) of CB2. Upon mixing of CB2, Cy1, and Cy2, the C_2–H resonances of Cy1 and Cy2 are clearly observed while the resonance arising from CB2 is now too broad to be detected (Figure 8d). For T1/Cy1/Cy2, resonances 1 and 2 had similar pK_a, δ_A, and δ_{AH} values and overlapped at all pH values. However, for CB2/Cy1/Cy2, resonances 1 and 2 are clearly distinct in Figure 8d and have pK_a values which are similar to those for Cy1/Cy2 (Table I). These results indicate that His-276 of Cy2 is not as much perturbned upon mixing of CB2 with Cy1 and Cy2. Hence, CB2 must not induce the same degree of polymerization of Cy1 and Cy2. Thus, the NH_2-terminal end of T1 (i.e., that portion of T1 represented by fragment CB3) is necessary for the full induction of polymerization of TM molecules.

Discussion

We have previously used high-resolution ^{1}H NMR spectroscopy to study the titration behavior of the histidine residues (His-153 and -276) of tropomyosin (Edwards & Sykes, 1978, 1980). The resonances corresponding to the C_2 proton of the histidines could be resolved and assigned in the spectrum, and the pK_a's of the histidines, as well as the chemical shifts of the protonated and unprotonated forms, could be determined by following the observed chemical shifts as a function of pH. In these studies, the titration profiles of His-153 and -276 were found to be mutually interacting, sensitive to local conformational states of the COOH-terminal region of tropomyosin, and sensitive to the head to tail polymerization of tropomyosin. These interactions were not reflected in the limiting chemical shift values of the protonated or unprotonated forms of the histidine residues, but in the pK_a's of the histidines. Consequently, when several conformational states of tropomyosin were present, for example, resulting in different pK_a values for a given histidine in the different states, this was only reflected in the C_2 proton resonance of the histidine at pH values near its pK_a where the observed chemical shifts for the various forms were different.

In this paper, we have applied the same ^{1}H NMR methods to the study of fragments of tropomyosin and troponin T and their interactions. The overall form of the results of the ^{1}H NMR monitored pH titration profiles of the histidine residues is the same; interactions are reflected in the perturbation of the pK_a's of histidine residues involved or in a change of the population distribution between several conformational states of the fragment which is seen as a change in the intensity distribution of several forms of a histidine resonance that result from different pK_a's for the histidine in the different conformational states. The major results can be summarized in two categories, the differences between fragments and the intact proteins indicating intramolecular interactions and the perturbations of the fragments when mixed with other fragments indicating intermolecular interactions. The sensitivity of NMR is such that high protein concentrations ($\simeq$0.2–1.0 mM) are employed for the measurements so that very few situations result in no perturbations. Thus, in the present work, a number of interactions have been observed between various fragments for which there had been no evidence in previous studies (Pato et al., 1981a; Mak & Smillie, 1981a; Pearlstone & Smillie, 1982, 1983) and in which much lower protein concentratons had been used. In some cases (e.g., T1 and Cy1), these weak interactions appear to be of a specific nature. In others (e.g., CB2 with Cy1, T1-TM, and Cn1A), they are probably nonspecific.

In Cy1, His-153 is not split into several forms as it was in TM, supporting the original conclusion that the conformational states observed reflected local thermal unfolding in the COOH-terminal region of TM. In Cy2, His-276 shows several conformational states, with one predominating at low temperatures. In T1, four out of five histidine residues (A–D) are observed at low temperatures; the fifth histidine (E) is only seen at higher temperatures where it is sharper. His E is assigned to His-79 by comparison of T1 with CB2 and CB3. His-79 is observed at all temperatures in CB2, indicating the importance of the integrity of T1 for the normal conformational properties of its two major components, CB3 and CB2. This is further supported by His D which shows multiple forms with different populations in CB3 and T1. His A–C are very similar between CB3 and T1.

As described previously, fragment CB1 (residues 1–151 and therefore considered equivalent to T1 in the present work) mimics whole Tn and Tn-T in its ability to induce head to tail polymerization of TM. Fragment CB2 (residues 71–151) is less effective in this respect, suggesting that the CB3 region (residues 1–70) has an important structural role in this phe-

nomenon. Previous studies have also indicated that CB1 binds close to or at the COOH-terminal end of the TM molecule, perhaps involving the heat to tail overlap region. However, previous work had provided no evidence for a direct interaction of CB1, CB2, or CB3 with TM fragments containing an intact NH_2-terminal sequence. In the present work and using much higher protein concentrations, interactions are indicated by changes in the intensity distribution of the several conformational states of His D and small but significant changes in the pK_a of His-153. The populations of the multiple His D resonances are perturbed when T1 is mixed with Cyl and T1-TM, and largely unperturbed when T1 is mixed with Cn1A. These results indicate the importance of NH_2-terminal residues 1–10 of TM in this interaction, and the lesser influence of residues 134–189. The pK_a of His-153 is also lowered significantly in mixtures of T1 and Cyl. The interaction is weakened by going from 50 mM KCl to 100 mM KCl. That these interactions are relatively weak, and therefore not detected in earlier studies, is indicated by the effects of changing the molar ratios of the components on the population distribution of His D resonances.

In contrast to the weak but apparently specific interactions of T1 with the NH_2-terminal fragments of TM, we have concluded that the weak interactions of CB2 with Cyl, T1, and Cn1A are nonspecific since the perturbations of the pK_a of His-79 of CB2 were equally great with Cyl, T1-TM, and Cn1A. The titration of CB2 and Cyl yielded a dissociation constant for the complex of 0.1–0.2 mM. This observation plus the fact that Cyl had no influence on the population distribution of His D resonances of fragment CB3 emphasizes the importance of intact T1 for these interactions. It would appear that the CB3 region of intact T1 has an important function in conferring specificity on the latter's interaction with the NH_2-terminus of TM.

Considering now the interaction of T1 with the COOH-terminus of TM and the effects of T1 on head to tail polymerization, we observe that while the pK_a of His-276 is not perturbed in mixtures of T1 and Cy2, nor in mixtures of Cyl and Cy2, there is a very significant drop in its pK_a when all three components are combined to form a ternary complex (see Table I). The lack of perturbation of His-276 in the T1/Cy2 mixture is in spite of the fact that there is good experimental evidence for the interaction of these fragments at much lower protein concentrations than those used in the present experiments (Pato et al., 1981a). That this interaction is specific for the COOH-terminus of TM is indicated by the much reduced affinity between NPTM (residues 1–273) and CB1 (Mak & Smillie, 1981; Pearlstone & Smillie, 1982). In spite of this, it would appear that the environment of His-276 is not affected by the formation of the T1/Cy2 binary complex. The absence of a pK_a change for His-276 where only Cyl and Cy2 are mixed may suggest that these two fragments do not interact even at these high protein concentrations. Alternatively, it is possible that the NH_2-terminus of Cyl and the COOH-terminus of Cy2 do interact in a head to tail manner but that this interaction does not lead to a change in the environment of His-276. The change in the pK_a of His-153 of Cyl from 7.26 to 7.16 in the Cyl/Cy2 mixture indicates that this is occurring but this pK_a change could arise from a nonspecific interaction. In any case, the important point is that the perturbation of His-276 is only observed in a mixture of all three components and is thus a useful parameter for the detection of head to tail polymerization induced by T1.

The further observation that in the mixture CB2/Cyl/Cy2 the pK_a of His-276 is only slightly perturbed compared to that in the T1/Cyl/Cy2 complex is further evidence for the importance of residues 1–70 of Tn-T in the induction of head to tail polymerization. It is in agreement with the earlier demonstration that CB1 (and thus T1) is significantly more effective in this respect than is CB2 (Jackson et al., 1975).

In previous studies on the induction of cooperative binding of NPTM to F-actin by whole Tn ($-Ca^{2+}$) and Tn-T, we had suggested that the T1 region of Tn-T may bridge the gap between the ends of the NPTM molecules. Alternatively, or in addition, the cooperativity could have arisen from conformational changes transmitted through actin from one NPTM-Tn binding site to others. In light of the present results and the observation of Walsh et al. (1985), the former hypothesis now seems unlikely. The interaction between T1 and the NH_2-terminal end of TM is clearly weak in nature, at least in the absence of the structure comprising the head to tail overlap as is the case with NPTM. Further, the interacton between the COOH-terminal end of TM and the T1 fragment has also been largely eliminated with NPTM (Mak & Smillie, 1981a; Pearlstone & Smillie, 1982). These considerations are therefore consistent with the demonstration by Walsh et al. (1985) that the addition of Tn ($\pm Ca^{2+}$) to NPTM at a variety of ionic strengths has no effect on its viscosity. Thus, the cooperativity observed in the Tn-induced binding of NPTM to actin must arise from conformational effects transmitted through the actin.

This does not mean, of course, that the head to tail polymerization of TM molecules and the induction of that polymerization by Tn-T are not of significance in the intact system. While the replacement of TM by NPTM in a reconstituted actin/myosin subfragment 1/TM/Tn ATPase system appears to have little effect on the cooperativity of the Ca^{2+} activation curve (Walsh et al., 1985), there are other cooperative aspects of this complex system that must be investigated.

ACKNOWLEDGMENTS

We thank M. Nattriss for the amino acid analyses. We are also grateful for the helpful discussions with Dr. J. R. Pearlstone and Dr. C. Sanders.

Registry No. His, 71-00-1.

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Identification of Peptide Sequences at the tRNA Binding Site of *Escherichia coli* Methionyl-tRNA Synthetase†

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Received January 27, 1986; Revised Manuscript Received April 7, 1986

ABSTRACT: Four different structural regions of *Escherichia coli* $tRNA^{fMet}$ have been covalently coupled to *E. coli* methionyl-tRNA synthetase (MetRS) by using a tRNA derivative carrying a lysine-reactive cross-linker. We have previously shown that this cross-linking occurs at the tRNA binding site of the enzyme and involves reaction of only a small number of the potentially available lysine residues in the protein [Schulman, L. H., Valenzuela, D., & Pelka, H. (1981) *Biochemistry 20*, 6018–6023; Valenzuela, D., Leon, O., & Schulman, L. H. (1984) *Biochem. Biophys. Res. Commun. 119*, 677–684]. In this work, four of the cross-linked peptides have been identified. The tRNA–protein cross-linked complex was digested with trypsin, and the peptides attached to the tRNA were separated from the bulk of the tryptic peptides by anion-exchange chromatography. The tRNA-bound peptides were released by cleavage of the disulfide bond of the cross-linker and separated by reverse-phase high-pressure liquid chromatography, yielding five major peaks. Amino acid analysis indicated that four of these peaks contained single peptides. Sequence analysis showed that the peptides were cross-linked to $tRNA^{fMet}$ through lysine residues 402, 439, 465, and 640 in the primary sequence of MetRS. Binding of the tRNA therefore involves interactions with the carboxyl-terminal half of MetRS, while X-ray crystallographic data have shown the ATP binding site to be located in the N-terminal domain of the protein [Zelwer, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81]. The methods developed for the present studies should be applicable to determination of peptide sequences at the tRNA binding sites of other proteins.

The highly specific interaction of transfer RNAs with aminoacyl-tRNA synthetases represents an attractive model system for study of the molecular basis of RNA–protein recognition. The interaction of *Escherichia coli* initiator methionine tRNA ($tRNA^{fMet}$)[1] with *E. coli* methionyl-tRNA synthetase (MetRS) has been actively investigated in this laboratory for a number of years [for reviews, see Schulman (1979) and Schulman & Pelka (1977a, 1984)]. These studies have provided considerable information on the role of specific nucleotide bases in $tRNA^{fMet}$ in recognition by MetRS. In contrast, little is known about the peptide sequences which comprise the tRNA binding site of the enzyme. Covalent cross-linking of $tRNA^{fMet}$ to MetRS provides an approach to determining the relative orientation of the two macromolecules in solution. High-resolution X-ray crystallographic data for the tRNA (Woo et al., 1980) and for a biologically active

† This work was supported by Research Grant GM16995 from the National Institutes of Health. Partial support for L.H.S. was provided by National Cancer Institute Grant P30CA13330-10.
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[1] Abbreviations: $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; DTSP, dithiobis(succinimidyl propionate); PDA, propane-1,3-diamine; DTSP/PDA-$tRNA^{fMet}$, $tRNA^{fMet}$ modified with PDA followed by coupling to DTSP; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; DMF, dimethylformamide; TFA, trifluoroacetic acid; GdmCl, guanidinium chloride; TPCK, tosylphenylalanine chloromethyl ketone; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; DABITC, 4-(dimethylamino)azobenzene-4′-isothiocyanate; DABS or dabsyl, 4-(dimethylamino)azobenzene-4′-sulfonyl; ODS, octadecylsilane; DEAE, *N*,*N*-diethylaminoethyl.

0006-2960/86/0425-4555$01.50/0