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TROPOMYOSIN'S END-TO-END POLYMERIZATION IS IRREVERSIBLY LOST ON EXPOSURE TO UREA OR CYANATE[+]

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Tropomyosin's low-salt viscosity which is due to end-to-end polymerization, is irreversibly lost upon incubation in 8M urea at room temperature. This effect is due to the chemical modification of lysine residues by cyanate in the urea. In the absence of urea, cyanate alone has the same effect. A loss in tropomyosin binding to actin accompanies the loss in viscosity, consistent with the view that tropomyosin's end-to-end interaction is necessary for strong binding to actin. During column chromatography in 8M urea, used to separate the α and β chains of tropomyosin, the loss of viscosity can be minimized by using freshly-prepared urea and by reducing the time during which the protein and urea are in contact.

Tropomyosin binds end-to-end along the actin thin filament of skeletal muscle and thereby takes part in the regulation of contraction (1). Tropomyosin's end-to-end interaction is responsible for its polymerization at low salt, as measured by viscosity (2,3), and is thought to be involved in its cooperative binding to actin (4-6), in the cooperative binding of myosin to the thin filament (7,8), and in the cooperative regulation of actomyosin ATPase activity by Ca^{2+} (9,10) and myosin (11-13).

Rabbit skeletal muscle tropomyosin is a dimer which consists of two types of polypeptide chains, α and β, present in an α / β ratio of approximately 4/1 (14). The two chains are generally separated by chromatography on CM-cellulose at room temperature in the presence of 8M urea

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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which dissociates the two chains (14). We observed that the α chain prepared in this way and renatured to form α-α tropomyosin had a low-salt viscosity which was reduced considerably compared to that of the unchromatographed tropomyosin. Since tropomyosin's end-to-end interaction is an important parameter in the regulation of muscle contraction and since the chromatographic separation of tropomyosin chains for a variety of muscle and non-muscle tropomyosins is performed in the presence of urea we decided to try to understand this loss of viscosity. Further investigation indicated that the loss of viscosity is accompanied by a reduction in actin binding and is due to a chemical modification of lysine residues by cyanate present in the urea.

METHODS, RESULTS AND DISCUSSION

Tropomyosin, prepared from rabbit back and leg muscle (15,16), was separated into α and β chains by room temperature chromatography on CM-cellulose in the presence of 8M urea (Schwartz-Mann Ultra Pure) (14). The α tropomyosin fraction was renatured to form α-α tropomyosin by dialyzing vs 1M NaCl, 5mM Mops , 1mM EDTA, pH 7.5 and then exhaustively vs 2mM Mops, 0.1mM EDTA, pH 7.5. The specific viscosity of this α - α tropomyosin, in the low-salt buffer, was measured in an Ostwald-type viscometer (Cannon 150/A554) at 24^{o} at 0.83 mg/ml and found to have a value of 0.2. This was considerably less than the specific viscosity of the unchromatograhped tropomyosin which had a value of 1.6.

In order to understand this drop in viscosity, tropomyosin was dissolved in either 1M NaCl, 8M urea (freshly prepared), or 8M guanidinium chloride, which also dissociates tropomyosin chains, with all solutions containing 10mM Mops, 1mM EDTA, pH 7.5. After incubation at room temperature for variable times each sample was dialyzed vs 1M NaCl, 5mM Mops, 1mM EDTA, pH 7.5 and then exhaustively vs 2mM Mops, 0.1mM EDTA, pH 7.5. There was no loss in tropomyosin low-salt viscosity after 7 days in 1M NaCl and only a 13% loss of viscosity for tropomyosin in 8M guanidinium chloride for 7 days. However

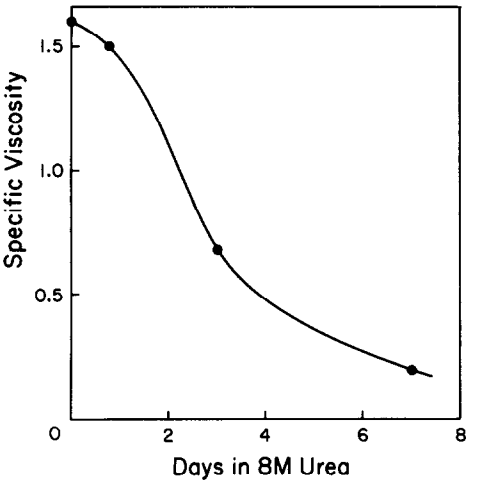

Figure 1. Specific viscosity of tropomyosin at 0.83 mg/ml at 24° in 2mM Mops, 0.1mM EDTA, pH 7.5 as a function of incubation time in 8M urea, 10mM Mops, 1mM EDTA, pH 7.5 at room temperature.

tropomyosin in 8M urea lost 87% of the initial viscosity over the same time period (figure 1). This large loss in viscosity is not due to improper renaturation since there was very little loss when guanidinium chloride was used as the denaturant or when urea was used for short incubation times (figure 1). Tropomyosin similarly incubated in 8M urea, 50mM formate, pH 4.0, conditions used for column chromatography to separate tropomyosin's chains (14), lost 56% of the initial viscosity. In order to test the effect of temperature on the urea-induced loss of viscosity, tropomyosin was incubated at 0° and room temperature in 6M urea, 10mM Mops, 1mM EDTA, pH 7.5 for 7 days and then dialyzed as above. The tropomyosin incubated at room temperature lost 82% of its viscosity while the sample incubated at 0° showed no loss.

These observations indicate that exposure of tropomyosin to urea results in a decrease of its low-salt viscosity and that this decrease is greater with increasing exposure time and temperature. Taking advantage of this knowledge we prepared α - α tropomyosin by chromatography as above, except that the tropomyosin was processed as fast as possible to minimize its contact time with urea and when it was in urea before and after chromatography it was kept on ice. The urea used was either freshly prepared or kept at 4°. The resulting α - α tropomyosin had a low-salt viscosity which was 82% that of the unchromatographed protein. This further indicates that the large loss

of viscosity of our previous α - α preparations was due to contact with urea.

These results suggest that some chemical modification of tropomyosin in the presence of urea weakens its end-to-end interaction. Indeed, urea in solution is in equilibrium with a small but significant amount of cyanate (17) which can chemically modify lysine residues over a wide pH range (18,19). Cysteine residues may also become modified (18) but the reaction should be reversed at pH > 7 (19,20). Many proteins have been found to be chemically modified in the presence of urea or by cyanate directly (21,22 and references therein). Urea reaches equilibrium with cyanate very slowly (several days) at room temperature and at 4^{o} very little, if any, cyanate is formed even after several days (17). These facts are consistent with our observations that in the presence of urea at room temperature tropomyosin's viscosity is lost slowly (figure 1) and not at all at 0^{o}. We also found that viscosity was lost more slowly when incubating in urea at pH 4.0 than when at 7.5. This is also consistent with cyanate modification of lysine residues since, although the reaction of cyanate with lysine is relatively insensitive to changes in pH (19,20), acidic conditions can result in a decomposition of cyanate (17,18).

In order to test whether cyanate alone can decrease tropomyosin's low-salt viscosity, tropomyosin was incubated overnight at room temperature in 0.1M KCNO (Curtin Matheson), 1M NaCl, 5mM Mops, 1mM EDTA, pH 7.5 and its viscosity was measured after exhaustive dialysis vs 2mM Mops, 0.1mM EDTA, pH 7.5. The viscosity of this cyanate-treated tropomyosin was 5% that of tropomyosin similarly incubated but in the absence of cyanate. This result provides further support for the direct involvement of cyanate in the loss of tropomyosin's viscosity during urea treatment. It also indicates that unfolding of tropomyosin (by urea) is not necessary for the modification by cyanate to lead to loss of viscosity.

Since it has been reported that cyanate can be removed by reaction with glycylglycine (19), tropomyosin was incubated in 8M urea as above for 5 days with the additional presence of 0.5M glycylglycine and then its low-salt

viscosity was measured. This tropomyosin lost 33% of its viscosity while the control tropomyosin, incubated in the absence of glycylglycine, lost 67% of its viscosity indicating that glycylglycine affords some protection from cyanate modification. Lysine provided less protection and Tris buffer or dithiothreitol gave no protection.

Tropomyosin's lysine content was measured by the methods of Habeeb (23) and Bohlen et al. (24) and the cysteine content by the method of Ellman (25) before and after a 7-day incubation in 8M urea at room temperature. Both lysine (78/tropomyosin) and cysteine (2.3/tropomyosin) content remained unchanged within experimental error. Cysteines are not expected to be modified by cyanate (see above) and the lack of change of lysine residues suggests that only a small number may have become modified by cyanate. A previous report has shown that the modification (by acetic anhydride) of only one lysine (lys-7) per chain can result in an almost complete loss of tropomyosin's low-salt viscosity (26).

The actual number of lysines modified was determined on a Beckman 118/119CL amino acid analyzer after protein hydrolysis in vacuo in 6N HCl at 110^{o} for 22 hours (18). The analysis was performed on a control tropomyosin, on tropomyosin treated with 8M urea for 7 days at room temperature, and on tropomyosin treated with 0.1M KCNO for overnight at room temperature. The chromatograms obtained were compared to that of a standard solution of lysine modified with cyanate (i.e. L-homocitrulline, obtained from U.S. Biochemical Corp.). A correction was made for the fact that approximately 24% of the homocitrulline formed from the protein hydrolysis can revert back to lysine during the hydrolysis (27). The results of this analysis showed that an average of approximately 1 lysine per chain was modified by cyanate for urea-treated tropomyosin and roughly 7 lysines per chain were modified for cyanate-treated tropomyosin. Therefore a greater loss in viscosity corresponds to a greater number of modified lysines providing further support for lysine modification being responsible for loss in end-to-end interaction.

Table I : ACTIN BINDING OF TROPOMYOSIN TREATED WITH UREA OR CYANATE

TROPOMYOSIN	% BOUND to ACTIN	
	OPTIMUM CONDITIONS	SUBOPTIMUM CONDITIONS
Control	92	82
Urea-treated	85	50
KCNO-treated	14	8

Tropomyosin was treated with 8M urea, 10mM Mops, 1mM EDTA, pH 7.5 for 7 days or 0.1M KCNO, 1M NaCl, 5mM Mops, 1mM EDTA, pH 7.5 for overnight and dialyzed vs 1M NaCl, 5mM Mops, 1mM EDTA, pH 7.5 and then 2mM Mops, 0.1mM EDTA, pH 7.5. Tropomyosin and F-actin were mixed to final concentrations of 0.18 mg/ml and 1 mg/ml, respectively, in solutions which were optimum (30mM NaCl, 5mM $MgCl_2$, 10mM Hepes, pH 7.5) and suboptimum (60mM NaCl, 10mM Hepes, pH 7.5) for binding (28). In all cases a control sample of tropomyosin alone was also prepared. The solutions were incubated for 15 min at room temperature and spun in a Beckman airfuge at 25 psi for 30 min. The supernatants were removed, incubated in 0.5% sodium dodecyl sulfate, 20mM dithiothreitol, electrophoresed on polyacrylamide gels in the presence of sodium dodecyl sulfate, and the bands were analyzed by densitometry (Biomed laser instrument). The fraction of tropomyosin bound to F-actin was calculated by comparing the absorbance of the tropomyosin band in the mixtures with tropomyosin alone.

The effect of this lysine modification on the binding of tropomyosin to actin was studied under optimum and suboptimum binding conditions (28). For tropomyosin treated with 8M urea the degree of binding was reduced slightly under optimum conditions and considerably under suboptimum conditions (Table I). Tropomyosin treated with 0.1M KCNO lost most of its actin-binding capacity under both conditions. Thus the modification of lysine residues by cyanate has a profound depressive effect on tropomyosin's end-to-end interaction and on its interaction with actin, consistent with the view that tropomyosin's end-to-end interaction is necessary for strong binding to actin (4-6).

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