Separation and Recombination of the Ethylene Glycol Bis(β-
aminooethyl Ether)-N, N'-Tetraacetic Acid-sensitizing
Factor Obtained from a Low Ionic Strength
Extract of Natural Actomyosin*

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SUMMARY
The ethylene glycol bis(β-aminoethyl ether)-N, N'-tetra-
acetic acid-sensitizing factor extracted from natural acto-
myosin at low ionic strength was concentrated in the fraction
obtained between 40 and 60% ammonium sulfate saturation.
Fractionation within narrower salt limits and at low protein
concentrations yielded a troponin-rich fraction at 40 to 53%
saturation and a tropomyosin-rich fraction at 53 to 60% sat-
uration. These ammonium sulfate fractions could be further
purified by isoelectric precipitation at pH 4.6. The super-
natant of the 40 to 53% fraction was shown to contain prima-
arily troponin, while the precipitate of the 53 to 60% fraction
was pure tropomyosin. It was found that for maximal EGTA-
sensitizing factor activity a ratio of tropomyosin to troponin of
1:1.3, w/w, was required. This combination appears to form
a complex which sediments as a single peak in the ultracen-
trifuge with a sedimentation constant of 5.15 S.

The Mg++-activated adenosine triphosphatase activity
of actomyosin extracted directly from skeletal muscle (natural
actomyosin) is inhibited by the removal of small amounts of
Ca++. Ebashi (1) has shown this effect to be mediated by a
protein factor, the ethylene glycol bis(β-aminoethyl ether)-N,
N'-tetraacetic acid-sensitizing factor, and it has been sub-
sequently proposed (2) that two proteins, tropomyosin and
troponin, are essential for its activity. This hypothesis was not
universally accepted and several reports (3-6) indicated the
uncertain nature of this factor.

This study was undertaken in an attempt to clarify the situa-
tion. A low ionic strength extract of natural actomyosin
was subjected to ammonium sulfate and isoelectric fractionation.
Each fraction was assayed for EGTA-sensitizing factor activity
and troponin content. The finding of Schaub, Hartshorne,
and Perry (7) that tropomyosin depressed the Ca++-activated
ATPase activity of actomyosin was used to estimate the latter.
These results were then correlated with the schlieren boundaries
observed with the analytical ultracentrifuge. A more detailed
report of these and other studies on the EGTA-sensitizing factor
is in preparation.

Natural actomyosin prepared by the method of Schaub et al.
(7) was dialyzed against several changes of 2 mM Tris-HCl
(pH 8.9)-2 mM dithiothreitol for 48 hours. The partially
desensitized actomyosin was removed by centrifugation at
78,480 x g for 16 hours and re-extracted with its original volume
of solvent. The two supernatants were combined and dialyzed
gainst 0.2 M KCl-10 mM Tris-HCl (pH 7.6)-2 mM dithio-
threitol prior to ammonium sulfate fractionation. Precipitates obtained
at 40 to 60, 40 to 53, and 53 to 60% ammonium sulfate saturation
(designated as P40--60, P40--53, and P53--60, respectively) were dis-
solved in 1.0 M KCl-10 mM Tris-HCl (pH 7.6)-2 mM dithio-
threitol, and dialyzed against this solvent. These solutions
were used for ultracentrifugal analysis and isoelectric precipita-
tions at pH 4.6. The latter were performed at 0° over a period
of approximately 18 hours. The precipitates and the superna-
tants were separated by centrifugation.2

For the assay of EGTA-sensitizing factor activity and tropo-
myosin, the proteins in the 1.0 M KCl solvent were diluted to
approximately 0.5 mg per ml with 10 mM Tris-HCl (pH 7.6)-2
mM dithiothreitol. In the cases where the ionic strength of this
dilution was above 0.1, the protein was dialyzed against the
latter solvent.

Adenosine triphosphatase assays were performed at 25° in
25 mM Tris-HCl (pH 7.6)-2.5 mM ATP and either 2.5 mM MgCl2
or CaCl2. For the estimation of EGTA-sensitizing factor activ-
ity, 1 mM EGTA was included in the MgCl2 assay medium.
Synthetic actomyosin, prepared as previously described (3), was
washed four to six times with 2 mM Tris-HCl (pH 7.6) to remove
any residual EGTA-sensitizing factor. The determination of
inorganic phosphorus and protein concentration was as previously
described (3).

Sedimentation velocity diagrams of the fractions are shown in
Fig. 1. The schlieren pattern of the P40--53 fraction revealed

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1 The abbreviation used is: EGTA, ethylene glycol bis(β-amino-
ethy l ether)-N, N'-tetraacetic acid.

2 The precipitates from the isoelectric fractionation of these
fractions are designated IEP and the supernatants IES.
FIG. 1. Sedimentation velocity patterns of ammonium sulfate fractions (top row) from low ionic strength extract of natural actomyosin. Patterns of the isoelectric precipitates of the (NH₄)₂SO₄ fractions are shown in the middle, and of the isoelectric supernatants in the lower row of diagrams. The solvent was 1.0 M KCl-10 mM Tris-2 mM dithiothreitol, pH 7.6; temperature, 20°C; bar angle, 65°; speed, 59,780 rpm; the time of centrifugation (minutes at full speed is indicated above and the sample concentrations (milligrams per ml) below the boundaries.

FIG. 2. EGTA-sensitizing factor activity of fractions P₄₀₋₅₃ (O), P₅₃₋₆₀ (●), and P₆₀₋₆₅ (□). Actomyosin 0.38 mg/2 ml of assay. Amount of fraction protein as indicated in 2-ml assay volume.

FIG. 3. Effect of ammonium sulfate fractions on Ca²⁺-activated ATPase of actomyosin. P₄₀₋₅₃ (O), P₅₃₋₆₀ (●), and P₆₀₋₆₅ (□). Actomyosin, 0.38 mg/2 ml of assay. Amount of fraction protein as indicated in 2-ml assay volume.

essentially a single boundary with a sedimentation constant, sₐᵥ, of 5.15 S. A single boundary was also observed for the P₅₃₋₆₀ fraction. Its sedimentation constant, however, was 2.55 S, and was consistent with monomeric tropomyosin. The P₄₀₋₅₃ fraction showed a complex pattern, consisting of a sharp, fast sedimenting component similar to that of the P₅₃₋₆₀ fraction and a slower asymmetric boundary, bearing no resemblance to that of tropomyosin. This pattern was markedly influenced by the protein concentration at which the fractionation was performed. This aspect will be discussed further in a subsequent report.

Isoelectric precipitation of all these fractions resulted in supernatants free of tropomyosin (bottom row of Fig. 1). In the case of the P₄₀₋₅₃ fraction, the amount of protein in the P₄₀₋₅₃ IES was small (7 to 10%), and its removal caused no appreciable change in the sedimentation diagram of the P₄₀₋₅₃ IEP as compared with the source fraction. The isoelectric precipitation apparently
effected a slight purification mainly by the removal of nucleotide-rich contaminants. In the P_{40-53} and P_{40-60} fractions approximately 70% and 40%, respectively, appeared in the supernatants. These figures depend on the protein concentration at which both the ammonium sulfate and isoelectric fractionations were performed and for the experiments described here this was between 0.7 and 0.8 mg per ml. The schlieren diagrams of both supernatants showed a broad asymmetric boundary of higher sedimentation velocity than monomeric tropomyosin. Their removal resulted in a corresponding tropomyosin enrichment in the P_{40-53} IEP and the P_{40-60} IEP. Comparison of these schlieren diagrams with those of their source fractions indicated a decrease of the 5.15 S component and the appearance of a slower boundary consistent with monomeric tropomyosin.

The EGTA-sensitizing factor activity of the original extract was concentrated in the P_{40-53} fraction. Both the P_{40-50} and P_{40-60} fractions exhibited lower activity (Fig. 2). It is of particular interest that the P_{40-60} fraction with negligible EGTA-sensitizing factor activity had the most marked influence on the Ca^{++}-activated ATPase activity of actomyosin (Fig. 3), whereas the most active EGTA-sensitizing factor fraction, the P_{40-50}, had an intermediate effect, and the P_{40-60} fraction, the least effect on the Ca^{++} curve. These findings confirmed that the P_{40-50} fraction contained the highest proportion of tropomyosin and indicated that this component was considerably reduced in the P_{40-53} fraction. It is clear that tropomyosin alone is not sufficient for EGTA-sensitizing factor activity. Also, on consideration of the above results, it would appear that the EGTA-sensitizing factor is a complex of tropomyosin and another component or components (troponin).

In order to clarify the nature of this complex, the EGTA-sensi-

![Image of schlieren diagrams](http://www.jbc.org/)

**Fig. 5**. a, sedimentation velocity diagrams of mixtures with varying ratios of P_{60-50} IEP (troponin) and P_{60-40} IEP (tropomyosin). The total sample concentration of all mixtures is 11.8 ± 0.2 mg per ml of protein. The boundary pattern of each mixture is illustrated by one schlieren photograph obtained after 176 min of centrifugation at 59,780 rpm at a temperature of 20° and a bar angle of 65°. The solvent for all experiments was 1.0 M KCl-10 mM Tris-2 mM dithiothreitol, pH 7.6. The ratio of P_{60-50} IES (troponin) to P_{60-40} IEP (tropomyosin) in the samples is indicated in each frame. b, boundary patterns of the two components at the concentrations prevailing in the 2:1, 1:1, and 1:2 mixtures of the corresponding samples in a. P_{60-50} IES (troponin) was centrifuged in a wedged-window double-sector cell and appears on top, P_{60-40} IEP (tropomyosin) was centrifuged in a standard window double-sector cell and appears on bottom of the schlieren diagrams. The concentration (milligrams per ml) of each component is indicated below the baselines. Photographs were obtained after 128 min of centrifugation at 59,780 rpm, 20°, and a bar angle of 65°. The solvent was 1.0 M KCl-10 mM Tris-2 mM dithiothreitol, pH 7.6.
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EGTA-sensitizing factor activities of mixtures of troponin and tropomyosin were examined. From the troponin-rich P$_{40-53}$-IES and the tropomyosin fraction (P$_{53-60}$-IEP), a titration curve was composed. The mixtures were analyzed for EGTA-sensitizing factor activity and sedimentation characteristics. The results based on EGTA-sensitizing factor activity are shown in Fig. 4. Maximum activity was obtained at a ratio of troponin to tropomyosin of 1.3:1 (w/w). This ratio must be regarded as tentative until the stability and purity of the troponin preparation is established. Fig. 5a shows the boundaries observed on ultracentrifugation of representative mixtures of troponin and tropomyosin. For comparison, the boundaries of the isolated components at the concentrations prevailing in the 2:1, 1:1, and 1:2 troponin to tropomyosin ratios are also shown (Fig. 5b). The troponin fraction revealed a single broad asymmetric boundary of higher sedimentation velocity than monomeric tropomyosin. Preliminary experiments suggest that this represented an aggregated form of troponin. There was no indication that troponin was a modified form of tropomyosin, and amino acid analyses of the two fractions showed significant differences, notably in the proline residue.

At approximately the maximal EGTA-sensitizing factor activity ratio of troponin to tropomyosin, 1.3:1.0, only one schlieren boundary was observed. This corresponded to the 5.15 S component of the P$_{40-53}$ fraction. With increasing excess of tropomyosin its slower sedimenting boundary became more prominent, while the faster sedimenting boundary of the tropomyosin-troponin complex decreased. Excess troponin, however, did not lead to the appearance of a second boundary. A definitive interpretation of this behavior must be deferred until further physicochemical characterization of the complex formation becomes available.

The data presented here support the hypothesis of Ebashi and Kodama (2) that the EGTA-sensitizing factor requires both tropomyosin and troponin, and it is suggested that for maximum activity, the EGTA-sensitizing factor complex contains troponin slightly in excess of tropomyosin, on a weight basis. Although the necessity of these proteins has been established, the possibility remains that a third factor may be involved in the complex formation.

REFERENCES

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