

THE β AND γ CYTOPLASMIC ACTINS ARE DIFFERENTIALLY THERMOSTABILIZED
BY MgADP; γ ACTIN BINDS MgADP MORE STRONGLY

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SUMMARY: Using the technique of thermal denaturation in a temperature gradient followed by two-dimensional gel electrophoresis of the fractions, the thermostabilities of the cytoplasmic actins have been investigated. Addition of 5 mM MgADP (but not NADH or CaCl_2) to stripped, radiolabeled leukocyte lysate thermostabilizes the actins by about 16°C relative to stripped lysate alone, producing a slightly stronger stabilization in γ actin. This result will allow preparative fractionation of β and γ actins and additional biochemical characterization of their differences.

INTRODUCTION

Actin is a major protein of most, if not all, cells of higher organisms, generally (though not always) associated with structure and movement. It is interesting that although purified actins from many tissues display similar biochemical properties (ability to polymerize, bind myosin, tropomyosin, etc.), at least seven distinct actin sequences have been detected in the cow (1), with two cytoplasmic forms predominant in most mammalian non-muscle tissue (2). It is not presently understood why there should be two forms of actin in a single cell type, since no functional differences between them appear to have been reported. In the course of a study designed to detect binding of small molecules by soluble human leukocyte proteins through altered thermostability, I have discovered that MgADP preferentially stabilizes γ -actin. Although a difference in ADP binding may turn out to be quite important, the main present usefulness of this observation is in the possibility it provides of preparative fractionation of β and γ actins for further characterization by other methods.

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MATERIALS AND METHODS

Human leukocytes were prepared from 35 ml pooled blood as follows: to three volumes of blood was added 2 volumes 5% Ficol 400 in phosphate buffered saline (PBS). After 30 minutes, the red cells had settled and the supernatant containing leukocytes was collected. Leukocytes were pelleted by centrifugation and washed once in RPMI 1640 and once in Iscove's medium 479 (3) without methionine (and without five other amino acids). The cells were then suspended in 3 ml medium 479 (as before) supplemented with penicillin (1000 U/ml) streptomycin (100 μ g/ml), gentamycin (200 μ g/ml), and 150 μ l 35 S-methionine (~ 1 mCi), and were rocked in a 25 cm² culture flask for 24 hr at 37°C, 5% CO₂. After incubation cells were collected (viability $\sim 90\%$) and washed twice in PBS and once in RPMI 1640. The cell pellet was then suspended in 5 ml of a 1:5 PBS extract of rabbit psoas muscle (~ 19 mg/ml, as carrier protein) and sonicated. After centrifugation at 40,000 rpm for 20 minutes ($\omega^2 t = 3.1 \times 10^{10}$), the clear supernatant was applied to the top of a P-4 Biogel column equilibrated with PBS and the proteins (excluded peak) stripped of low molecular weight compounds, collected, and frozen at -80°C.

Thermal denaturation was carried out as previously described (4) using aliquots at twenty temperature points over the range 36.6 to 83.8°C. In addition to stripped proteins, rows of aliquots supplemented with 5 mM MgADP, 5 mM NADH, or 5 mM CaCl₂ were also run in the same experiment. All protein solutions were supplemented with 0.1% mercaptoethanol, and each contained 11,400 cpm 35 S protein/ μ l before denaturation. The 80 samples were denatured in the temperature gradient (aliquots 1 at 83.8° and aliquots 20 at 36.6°, others linearly between these) for 5 minutes, then cooled on ice for 2 minutes, spun in a Beckman microfuge B for 2 minutes and the supernatants (75 μ l of undenatured protein) collected. To each was added 25 μ l 9 M urea, 2% NP-40, 2% 3.5-10 LKB ampholines, 0.5% mercaptoethanol. All 80 samples (30 μ l each) were then analyzed by two-dimensional electrophoresis (5,6), first on iso-electric focusing gels (2/3 pH 5.7, 1/3 pH 3.5-10 Ampholines) and then on 10-20% gradient acrylamide SDS slab gels. After fixing and staining, the gels were soaked in 2% glycerol, dried and autoradiographed for 14 days on Kodak XR-5 X-ray film. Segments of gels shown have focusing dimension horizontal (acid end to left) and SDS dimension vertical.

RESULTS

Stripped cytoplasmic β and γ actins are both approximately 50% depleted by thermal denaturation into insoluble forms at well 15 ($\sim 49^\circ\text{C}$) under the experimental conditions used here (Fig. 1a). Based on two-dimensional gaussian fitting of autoradiograph spots (to be described elsewhere), 75% of the actin density is in β actin. Another leukocyte protein of similar molecular weight (marked τ in Fig. 1) is considerably more thermostable. Proteins of widely varying thermostabilities can be found in other parts of the gel (not shown).

Upon addition of 5 mM MgADP, the actins are stabilized by about 16°C, showing 50% depletion at wells 8 or 9 (66.4° or 64°C ; Fig. 1). The γ -form

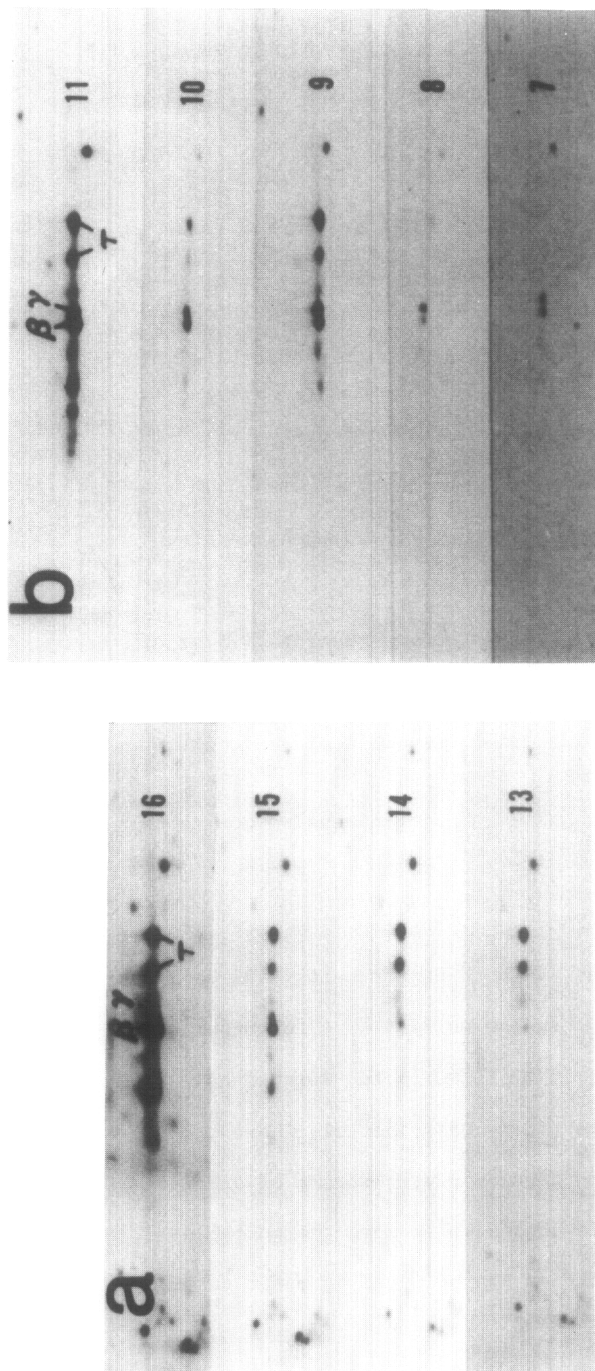


Fig. 1. The figure shows a small section (centered on actin) taken from a number of two-dimensional gel autoradiographs. Each panel shows the series of temperature points over which the actins (β, γ) are denatured, and hence disappear from the undenatured supernatant protein which was applied to the gels. In panel a (stripped proteins; i.e., low-molecular weight compounds removed), both actins disappear together, and are approximately 50% depleted at well 15. Panel b shows the result when 5 mM MgADP is added to the stripped protein solution before denaturation; both actins are thermostabilized (shifted to lower numbered, hotter wells) but γ actin is stabilized one or two wells more and hence is preferentially enriched in several fractions (8-10). In comparing the panels, it is the relative abundances of spots which is important, not the absolute abundances. The "reappearance" of more β actin in panel b7 is probably an artifact due to contamination of the supernatant with some of the β actin contained in the pellet; this well (of those shown) should contain the purest γ actin at the lowest total recovery. Spots to the right of actin (an unknown protein labeled τ) disappear at 8-9 with or without MgADP. Spots immediately to the left of actin are deamidated actins which behave the same as the native form during denaturation. Approximate temperatures in wells shown were: 16 (46.5°C), 15 (49°), 14 (51.5°), 13 (54°), 11 (59°), 10 (61.5°), 9 (64°), 8 (66.4°), 7 (68.9°).

is preferentially stabilized, as shown by its greater relative abundance in wells 8 and 9. Under the conditions pertaining in these aliquots, γ actin is enriched from 25% to 60-70% of the total actin autoradiographic spot density. The relative enrichment ought to increase at higher temperatures, while the total recovery should decrease. The unknown protein marked τ denatures in this range with or without added MgADP, indicating that it probably does not bind the complex. Addition of NADH did not stabilize either actin, and CaCl_2 significantly destabilized actin and most other cytoplasmic proteins (data not shown). Similar results have been obtained upon repetition of the experiment.

DISCUSSION

The thermal stability of a given protein is very sensitive to small structural changes often undetectable by other methods (short of sequencing). Electrophoretically "silent" enzyme variants are often detected this way. I have attempted to use denaturation temperature shifts caused by low-molecular weight compound (cofactor, substrate, etc.) binding as a means of detecting the binding protein spots on two-dimensional gels without knowledge of spot identities (4). The present results concerning the cytoplasmic actins are the first step in the analysis of human cellular proteins by this technique. The fact that β and γ actins are found to behave differently upon binding MgADP is both useful (for preparative separation) and indicative of the potential power of the technique for detecting molecular associations and resolving similar species. By screening other similar compounds, it may be possible to find one which would give even better differential stabilization of γ actin. It has been assumed here that since the presence of MgADP thermostabilizes the actins, the binding of this complex must energetically stabilize the normal, non-denatured structure. The preferential stabilization of γ actin indicates different and probably tighter binding to this form, following from the notion that stronger binding provides greater stabilization energy.

The interest in fractionating β and γ actins is due primarily to the suggestion that they have slightly different functions. Sequence data reported by Vandekerckhove and Weber (1) indicate that β and γ (as well as the muscle types) differ from each other in ways that are strongly conserved across species barriers. The apparent ubiquity of both forms in non-muscle tissue of higher animals (ref. 2 and my unpublished observations) also indicates that neither form can satisfactorily substitute for the other. It is, therefore, of some importance to characterize each biochemically, and, if possible, to prepare discriminating antisera in order to detect possible differences in cytoplasmic localization. Unfortunately, no mammalian cell type so far examined appears to have more γ than β . Thermal denaturation enrichment of γ actin in the presence of MgADP, therefore, appears to be a useful approach to the desired fractionation, since it is easy to accomplish on a preparative scale.

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