Stability of mutant actins

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Mutants of the *Drosophila Act88F* actin gene were transcribed and translated *in vitro* and their relative stabilities were examined using urea gradient gel electrophoresis. Most of the mutant actins (*E334K*, *E364K*, *G366D*, *G368E* and *R372H*) were as stable as the wild-type. *V339I* had a slight decrease in stability, and *E316K* was the least stable. The causes of the differences are discussed and contrasted with the behaviour of the mutants *in vivo*, where *E316K* has normal stability and *V339I* is the least stable.

INTRODUCTION

We have examined the effects of mutations in the *Act88F* actin gene of *Drosophila* on the structure and function of the flight muscle (Drummond *et al.*, 1990, 1991). The mutant actins *E316K*, *E334K* and *G368E* were stable and accumulated to normal levels in the flight muscle. *E364K* and *G366D* actins were present at decreased levels, and very little *V339I* actin accumulated. [³⁵S]-Methionine labelling experiments *in vivo* suggested that *V339I* actin was very unstable. In this paper we examine possible causes of the instability of the mutant actins.

Several factors, including size, charge, flexibility of structure, thermal stability and assembly, have been suggested as being important in determining the stability of proteins *in vivo* (reviewed by Goldberg & Dice, 1974; Goldberg & St John, 1976; Rechsteiner *et al.*, 1987). However, no unambiguous correlation has been found.

Within the series of mutant actins examined, size remains unaltered and there is no obvious relationship between charge changes and stability. All of the actins with decreased stability failed to assemble into normal myofibrils. However, it is unclear if this is simply because the actin is intrinsically unstable, and therefore fails to accumulate and assemble, rather than being due to the actin's inability to assemble leading to its subsequent breakdown. Possible causes of intrinsic instability include changes in the conformation or thermal stability of the mutant actin. Therefore, to try and determine the cause of decreased stability *in vivo*, we have examined the conformation and thermodynamic stability of the mutant actins *in vitro*.

It is difficult to obtain sufficient actin from the flight muscle of *Drosophila* for biochemical measurements, especially from mutants with unstable actin. Even expression in *E. coli* gives very poor yields of functional actin (Frankel *et al.*, 1990). We have therefore used an *in vitro* transcription/translation system to produce small quantities of radioactively labelled actin (Drummond *et al.*, 1991). The stability of the different mutants was then examined using the urea gradient gel system of Creighton (1979). This gives good correlations with other measures of protein thermodynamic stability, such as guanidinium-HCl and temperature denaturation (Goldenberg & Creighton, 1984). Coupled with the *in vitro* transcription/translation system it provides a rapid means of screening mutant proteins for changes in stability or folding kinetics.

EXPERIMENTAL

Actins

Rabbit actin was prepared using the method of Pardee & Spudich (1982) and was stored as G-actin in liquid N₂.

Act88F actin was transcribed and translated *in vitro* as described in Drummond *et al.* (1991). After translation 100 µg of RNAase A/ml (EC 3.1.27.5) was added to the reticulocyte lysate and incubated at 37 °C for 10 min. The lysate was then dialysed against three changes of 2 mM-Tris/HCl (pH 8.0)/0.1 mM-CaCl₂/0.2 mM-ATP/0.5 mM-dithiothreitol (DTT) for 1 h each at 4 °C, and then stored at –80 °C.

Non-denaturing gels

The gel system of Safer (1989) was used. Gels were run in a Bio-Rad miniProtean II gel system (1 mm gel, length 5.0 cm) at 140 V for 1 h before loading the samples. Proteins were electrophoresed at 140 V for 45 min at room temperature (20 °C).

Act88F actin samples were prepared by adding 0.2 vol. of 10 mM-Tris/HCl (pH 8.0)/0.5 mM-CaCl₂/1.0 mM-ATP/0.5 mM-DTT/50 % (v/v) glycerol, centrifuging at 10000 g in a micro-centrifuge for 1 min, and then immediately loading on to the gel. A sample volume of 2 µl gave optimal resolution. After electrophoresis the gel was fixed, dried down and autoradiographed.

Urea gradient gels

Non-denaturing gels containing transverse gradients of 0–8 M-urea and counter gradients of 7.5–5.5 % (w/v) acrylamide were prepared as described by Goldenberg & Creighton (1984).

Protein samples were prepared as for non-denaturing gels, except that the sample volume was increased to 50 µl and the sample was loaded across the whole width of the gel. Rabbit actin was denatured by resuspending in 8 M-urea/2 mM-Tris/HCl (pH 8.0)/0.1 mM-CaCl₂/0.2 mM-ATP/0.5 mM-DTT and incubating at room temperature for 30 min before loading. After electrophoresis the gels were stained with Coomassie Blue, photographed and then dried down and autoradiographed. Registration marks made using radioactive ink allowed precise alignment of the autoradiographs with the photographs of the Coomassie Blue-stained gels.

RESULTS AND DISCUSSION

Mutant actins were transcribed and translated *in vitro* and their mobilities on non-denaturing gels in the absence of urea were examined (Fig. 1). In non-denaturing gels protein mobility is determined by charge and shape. Mutants *E316K* and *E334K* (with a more positive charge) have decreased mobility compared with wild-type, *V339I* (no charge change) has the same mobility as wild-type, and *G368E* and *R372H* (more negative charge) have increased mobility. These results are consistent with these

Abbreviation used: DTT, dithiothreitol.

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Fig. 1. Autoradiograph of wild-type and mutant *Act88F* actins transcribed and translated *in vitro* and separated by non-denaturing gel electrophoresis

wt, wild-type; lane 1, *E316K*; 2, *E334K*; 3, *V339I*; 4, *E364K*; 5, *G366D*; 6, *G368E*; 7, *R372H*.

Fig. 2. Coomassie Blue-stained urea gradient gel of (a) native rabbit actin and (b) urea-denatured rabbit actin

mutants having largely normal structures, suggesting that there is no change in the structure of *V339I* to account for its decreased stability *in vivo*.

Only two mutants, *E364K* and *G366D*, had unusual mobilities. In both cases, although similar amounts of actin were loaded as for the other mutants (confirmed by SDS/PAGE; results not shown), less actin was visible as discrete bands. *E364K* forms two distinct well-separated bands, whereas *G366D* appears to have two less-well-resolved bands. This is consistent with these mutant actins being present as two distinct isomers which have a $t_{1/2}$ equivalent to at least the electrophoresis time of 45 min (Goldenberg & Creighton, 1984). Possibly one of the isomers is more susceptible to breakdown, leading to the decrease in *E364K* and *G366D* levels *in vivo*. The unusual behaviour of these two mutants in the continuous non-denaturing gel system of Safer (1989) was also found in the discontinuous system used previously (Drummond *et al.*, 1991). It may be significant that of the mutants we have examined only *E364K* and *G366D* caused a strong constitutive induction of heat-shock protein synthesis in flies (Drummond *et al.*, 1991).

To examine the relative thermodynamic stabilities of the mutant actins we used urea gradient gels with a transverse gradient of 0–8 M-urea (Goldenberg & Creighton, 1984). We first examined the behaviour of rabbit actin in this system (Fig. 2a). The native actin (with fast mobility) has a rapid transition to an unfolded form (with slower mobility) at higher urea concentrations. In the transition zone the protein band is discontinuous and smeared. This contrasts with the continuous band observed for many proteins which have a rapid equilibrium between the native and unfolded forms (Creighton, 1979, 1980). This suggests

Fig. 3. Photographs (a, c, e, g, i, k, m, o) and corresponding autoradiographs (b, d, f, h, j, l, n, p) of Coomassie Blue-stained urea gradient gels containing a mixture of native rabbit bulk actin and radioactive *Act88F* actin translated *in vitro*

(a, b) Wild-type; (c, d) *E316K*; (e, f) *E334K*; (g, h) *V339I*; (i, j) *E364K*; (k, l) *G366D*; (m, n) *G368E*; (o, p); *R372H*.

that the interconversion between the different forms of actin is slow. To investigate this further, rabbit actin was denatured using 8 M-urea before electrophoresis (Fig. 2b). No refolding was observed, but at low urea concentrations a series of bands was formed whose mobility was slower than that of the unfolded actin. These probably result from the formation of aggregates. Thus at high urea concentrations the rate of actin unfolding is rapid, whereas the rate of refolding is slow and remains slow even at lower urea concentrations. These results may help to explain the difficulties experienced by ourselves and others in obtaining native actin from inclusion bodies following expression in *Escherichia coli* (D. R. Drummond & A. J. G. Moir, unpublished work; Hitchcock-DeGregori, 1989).

Although actin contains at least two major domains (Kabsch *et al.*, 1990), only a single transition from folded to unfolded was observed. This contrasts with some other multidomain proteins which give several transitions thought to correspond to unfolding of the individual domains (Goldenberg & Creighton, 1984). It would be interesting to examine whether other proteins such as hexokinase (Fletterick *et al.*, 1975) and heat-shock cognate protein 70 (Flaherty *et al.*, 1990), which have similar structures to actin (Kabsch *et al.*, 1990), have the same folding properties.

Unfortunately, the slow interconversion of the actin forms

prevents a quantitative analysis of the thermodynamic stability of the protein (Goldenberg & Creighton, 1984). However, by including rabbit actin in the gels as an internal standard it was still possible to make a qualitative comparison of the relative stabilities of the mutant actins (Fig. 3). Rabbit bulk actin mixed with wild-type *Act88F* actin translated *in vitro* was electrophoresed and the gel was stained with Coomassie Blue (Fig. 3*b*). As the quantity of *Act88F* actin present was small (a few picograms), only the rabbit actin was visible in the Coomassie-stained gel. In addition, the endogenous proteins of the reticulocyte lysate are also visible, particularly globin, which forms an almost continuous band across the top of the gel. In the corresponding autoradiograph of the same gel only the radioactive *Act88F* actin was visible (Fig. 3*b*). Both *Act88F* and rabbit actin have similar stabilities in urea, with the rabbit actin appearing to be slightly more stable. This may result from some of the sequence differences between the two actins, or from a failure of the reticulocyte lysate to post-translationally modify *Act88F* actin (Hennessey *et al.*, 1991).

Mutants *E334K* (Figs. 3*e* and 3*f*), *G368E* (Figs. 3*m* and 3*n*) and *R372H* (Figs. 3*o* and 3*p*) had the same stability as wild-type actin. In addition, with the mutants *E364K* (Figs. 3*i* and 3*j*) and *G366D* (Figs. 3*k* and 3*l*), where more than one isomer is visible, both isomers have similar stabilities. *V339I* (Figs. 3*g* and 3*h*) had a slight decrease in stability compared with wild-type. Although valine to isoleucine is a conservative change, it does increase the size of the side chain by one methyl group. Such subtle differences have been shown to have a significant effect on protein stability, particularly if they affect the internal packing of the molecule (Kellis *et al.*, 1988; Matsumura *et al.*, 1988). The greatest decrease in stability was observed with *E316K* (Figs. 3*c* and 3*d*), which was less stable than *V339I*. From the structure of actin (Kabsch *et al.*, 1990), Glu-316 forms hydrogen bonds with amino acids 312 and 275 (K. Holmes, personal communication). Disruption of these bonds may decrease the stability of the native actin conformation. A second feature of the mutant *E316K* is that at lower urea concentrations there appears to be a form of actin whose mobility is intermediate between that of the native and unfolded forms. This form also appears to be present, although to a lesser extent, in *V339I*. This may represent a partially folded form of actin, possibly corresponding to the unfolding of one of the domains.

Despite its large decrease in stability in urea, *E316K* actin accumulates normally *in vivo* (Drummond *et al.*, 1991) and assembles into at least partially functional muscle (Drummond *et al.*, 1990). This contrasts with *V339I*, which has only a slight decrease in stability in urea, yet fails to accumulate *in vivo*. We conclude that there is no correlation between the stability of mutant actins in urea and their stability *in vivo*. This contrasts with the results of McLendon & Radnay (1978), who reported a good correlation between the half-life of proteins *in vivo* and their thermal denaturation, but agrees with the findings of

Rogers & Rechsteiner (1985) and Reichsteiner *et al.* (1987), who could find no correlation using a series of proteins injected into cells.

What is the specific feature that determines the stability of the mutant actins *in vivo*? Assembly into multisubunit structures is important for several proteins such as globin (Schaeffer, 1983), ribosomal proteins (Abovich *et al.*, 1985) and cytoskeletal proteins (Chiu & Goldman, 1984). However, for the mutant actins examined there was no simple correlation between assembly and stability. Although *E364K*, *G366D* and *V339I* all fail to assemble into normal myofibrils, *V339I* is more unstable than the others. Possibly the heat-shock proteins induced by *E364K* and *G366D* enhance their stability compared with that of *V339I* actin.

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