Biochemical Consequences of the Cardiofunk (R177H) Mutation in Yeast Actin*

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The zebrafish cardiofunk actin mutation, R177H, causes abnormal heart development. We have introduced this mutation into yeast actin to assess its biochemical consequences. R177H G-actin exhibited reduced thermal stability and an accelerated nucleotide exchange rate. R177H actin has an increased critical concentration and polymerizes with a greatly extended nucleation phase but a faster elongation process, suggesting that significant fragmentation accompanies filamentation. P$_i$ release from R177H actin is tightly coupled to polymerization, as with wild type (WT) actin, suggesting that the R177H mutation does not affect ATPase activity and P$_i$ release. R177H actin shows no polymerization-dependent decrease in intrinsic Trp fluorescence, and the fluorescence yield of a pyrene at polymerization-dependent decrease in intrinsic Trp fluorescence in zebrafish cardiogenesis.

Arg$^{177}$, conserved in all actins, is located in subdomain II of actin near the base of the protein’s interdomain cleft, where the P$_i$ that is generated from bound ATP during actin polymerization is thought to exit the protein. Crystallographic and protein structure modeling studies suggest that the guanidinium group of Arg$^{177}$ is involved in a number of interresidue interactions that potentially can affect both actin structure and function (3, 4). It can form a hydrogen bond with the carbonyl oxygen of the peptide bond involving His$^{73}$, resulting in a potential cross-cleft stabilization of the protein. It can also bond potentially with the side chain carboxyl of Asp$^{179}$ in subdomain IV. In the filament model of Holmes (5), it may interact with Ser$^{199}$, Phe$^{200}$, and Val$^{201}$ of a monomer in the apposed strand of the filament across the interstrand space, potentially leading to cross-strand stabilization within the actin helix. Molecular dynamics modeling suggested that, because of its location near the exit channel for P$_i$, the interaction between the guanidinium and phosphate groups (6). This residue can be ADP-ribosylated by bacterial toxins such as the C3 toxin from C. botulinum, thereby rendering the actin nonpolymerizable (7, 8).

The role of Arg$^{177}$ in actin function had been previously addressed at a more mechanistic level in three studies. In an alanine-scanning mutagenesis study of yeast actin, Arg$^{177}$ and Asp$^{179}$ were simultaneously converted to Ala (9). When expressed as the sole actin in the cell, this double mutant resulted in sensitivity to elevated temperature and hyperosmolar medium, and actin cables were only slightly detectable using anti-actin antibodies as a detection tool. Buzan and Frieden (10) further characterized this double mutant at the biochemical level and reported that the double mutation resulted in poor polymerization. Schuler et al. (11) changed Arg$^{177}$ to the oppositely charged Asp in chick $\beta$-actin, with the result being a 10-fold increase in critical concentration, lowered thermostability, and more rapid exchange of bound nucleotide. All of these results point to the importance of Arg$^{177}$ in actin function. However, the nature of the mutations employed for these studies makes the significance of the results uncertain. In the first study, not only have two oppositely charged residues been completely neutralized, but the two mutations together make it impossible to assess the contribution of the R177A mutation alone. In the second, the reversal of charge at this position in a region containing a number of other acidic residues may have altered the structure of the protein significantly due to charge repulsion.

We wished to understand the biochemical basis for the lethality of the cardiofunk mutation in the homozygous state and its partial heterozygous dominance as well as to better define the role of Arg$^{177}$ in actin filament formation and control of P$_i$ release. Toward this end, we have carried out a series of in vitro...
experiments with yeast actin in which Arg177 was changed to His, the cardiac myosin mutation. This is a much milder mutation than those used above, since it maintains at least a partially positive charge at this residue and alters only one residue at a time. Besides characterizing the biochemical properties of the mutant actin alone, we have also assessed its behavior in the presence of WT yeast actin to approximate the heterozygous situation, and we have examined the stabilization of the actin by tropomyosin, a major protein involved in regulation of thin filament function in cardiac muscle.

EXPERIMENTAL PROCEDURES

Materials—DNase I (grade D) was purchased from Worthington. Affi-Gel 10 activated resin and Bio-Spin® 30 Tris columns were purchased from Bio-Rad. The EnzChek™ phosphate assay kit and 1, N'-ethenoadenosine 5'-triphosphate (εATP) as a 5 mM stock solution in 50 mM Tris-Cl, pH 7.5, were purchased from Molecular Probes, Inc. (Eugene, OR) and stored at −20 °C. The QuickChange site-directed mutagenesis kit was purchased from Stratagene Corp. (La Jolla, CA), and the DNA primers used for site-directed mutagenesis were obtained from Integrated DNA Technologies (Coralville, IA). N-(1-pyrenyl)maleimide was purchased from Sigma. Yeast cakes for preparation of wild type actin and frozen beef heart for preparation of tropomyosin were obtained locally. All other chemicals used were reagent grade quality.

Oligodeoxynucleotide-directed Mutagenesis—Using site-directed mutagenesis, we replaced the codon for Arg177 in yeast actin with a His codon. The actin coding sequence was obtained from the centromeric plasmid pRS14 (12) adjacent to the ACTI promoter. The set of DNA primers (5'-CTATCCACGGCATTTCGATCATGGATTTGGCCGTTAG-GAG-3' and 5'-CTCTACGCCCAATATGATGCCAAATTTGCGGTA-GGTAG-3') was used in which the codon for Arg177 (AGA) was changed to His (CAT). A haploid yeast strain expressing the mutant actin as the only actin in the cell was generated as described previously using a plasmid shuffling procedure (13). The resultant plasmid was rescued from the cell and sequenced to ensure that the mutation was intact.

Protein Preparation—Yeast wild-type actin was purified in the calcium form by a DNase I affinity chromatography/DEAE ion exchange chromatography protocol as previously described with modifications (13). The major modification was the use of 75 mM of 0.6 M NaCl to wash the DNase I affinity column bound with yeast cellular proteins in order to eliminate the possible binding of coflin to G-actin. The quality of the G-actin preparation was analyzed with SDS-PAGE, and the bands were visualized by Coomassie Blue staining. G-actin was stored in G-buffer (5 mM Tris-Cl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl2, and 0.1 mM dithiothreitol) at 4 °C and used within 3 days. The actin concentration was determined by the UV absorbance at 290 nm using the extinction coefficient ε = 25.6 cm−1·M−1. Bovine cardiac tropomyosin (BVC TFM) was purified according to Landis (14), and the concentration (dimers) of TFM was determined by the UV absorbance at 280 nm using the extinction coefficient ε = 15.36 cm−1·M−1. εATP-bound actin was prepared as described in Wen et al. (15) and used within 1 day.

Pyrene-labeled Actin Preparation—Sufficient N-(1-pyrenyl)maleimide freshly dissolved in dimethyl sulfoxide at a final concentration of 3.3 mM was mixed with a G-actin solution (without dithiothreitol) to yield a final concentration of 45 μM, and the resulting solution was incubated at 20 °C for at least 30 min in the dark. This step was repeated until the molar ratio of total pyrene to G-actin was 1.1.1. This pyrene-labeled G-actin was polymerized by adding MgCl2 and KCl to 2 and 50 mM, respectively, at 20°C for 1 h in the dark. The pyrene-labeled F-actin solution was collected by centrifugation in a Beckman TL centrifuge in a TLA 100.2 rotor at 75,000 rpm, 25 °C for 1 h, and depolymerized by dialysis against G buffer. The extent of labeling was determined by UV absorbance at 344 nm using the extinction coefficient ε = 22 mm−1·cm−1.

Characterization of G-actin—The apparent melting temperature of G-actin was determined by circular dichroism according to Yao et al. (16). Briefly, 1 μM G-actin in G buffer was heated at a constant rate of 1 °C/min from 25 to 80 °C with constant stirring. Changes in the actin ellipticity were monitored at 222 nm using an AVIV 62 DS spectropolarimeter. Data were normalized as the fraction of native protein based on the net change in ellipticity and then fitted to a two-state model (17) with a single transition between a native and a denatured form of the protein by using Microsoft Excel (Seattle, WA). The apparent Tm value was defined as the temperature at which 50% of the G-actin was denatured.

The rate of εATP release was determined by the change in the fluorescence of an εATP-bound G-actin solution as described by Wen (15). Briefly, 2.5 μM calcium εATP-bound actin in the absence of free nucleotide was added to a 300 μM ATP solution at 25 °C. The fluorescence change due to the release of actin-bound εATP was recorded as described below, and the rate constant for the release was obtained by fitting the data with a first-order reaction equation with Microsoft Excel.

Actin Polymerization—The procedures for initiating actin polymerization and observing the critical concentration are essentially the same as described elsewhere (18). Briefly, each G-actin solution was mixed with MgCl2 and KCl to give final concentrations of 2 and 50 mM, respectively, to trigger the polymerization reaction. To measure the critical concentration (C.c.) of actin polymerization in the absence or the presence of BVC TFM, the net change in either light scattering or pyrene fluorescence of an actin polymerization reaction was measured as a function of increasing actin concentration. The critical concentration of actin was obtained by drawing a line through the points and determining its intersection on the x axis. To assess the effect of TFM on actin polymerization, a 6.9 μM G-actin solution was mixed with 3.5 μM TFM, and polymerization was induced as described above.

To monitor actin polymerization in the pH range from 6.5 to 8, a G-actin solution (pH 7.5) was processed through a Bio-Spin® 30 Tris column (Bio-Rad) preequilibrated with pH 8 G-buffer (5 mM Tris-HCl, pH 8, 0.2 mM CaCl2, and 0.2 mM ATP). To prepare the 5 mM G-actin solution at pH 7 and 6.5, a 5 mM G-actin solution (pH 7.5) was mixed with an equal volume of MES G buffer (5 mM MES at pH 6.8 and 6.1, 0.2 mM CaCl2, and 0.2 mM ATP). The final pH of each G-actin solution was checked by a pH meter.

Fluorescence Assays—Fluorescence measurements were obtained using a FluoroMax (Jobin Yvon Inc., Edison, NJ) fluorescence spectrometer with a thermostatted water bath attached to the cuvette chamber. Actin polymerization (described below) was monitored by a change in light scattering with excitation and emission wavelengths set at 360 nm. It was also monitored by the change in pyrene fluorescence with excitation and emission wavelengths set at 365 and 386 nm, respectively. The pyrene fluorescence emission spectra were obtained by exciting the sample at 365 nm and recording the fluorescence emission from 375 to 450 nm. The actin intrinsic Trp fluorescence emission spectrum was obtained by exciting at 295 nm and recording the fluorescence emission from 305 to 70 nm. The εATP fluorescence intensity change was monitored with excitation and emission wavelengths of 340 and 410 nm, respectively.

Simulation of Actin Polymerization—KinTekSim version 2.03 (available on the World Wide Web at www.kintek-corp.com) was utilized to simulate and fit actin polymerization kinetics data with the actin polymerization scheme adopted and modified from Buxton and Frieden (10) and Du and Frieden (19) as described below, where A is an actin monomer and A2p is an actin polymer.

\[
\begin{align*}
A & \rightarrow A + \Delta A \\
A_2p & \rightarrow A_2p + \Delta A \\
A & \rightarrow A + \Delta A \\
A_2 & \rightarrow A + A + \Delta A \\
A_3 & \rightarrow A + A + A + \Delta A \\
A_4 & \rightarrow A + A + A + A + \Delta A \\
A_5 & \rightarrow A + A + A + A + A + \Delta A \\
A_6 & \rightarrow A + A + A + A + A + A + \Delta A \\
A_7 & \rightarrow A + A + A + A + A + A + A + \Delta A \\
7A & \rightarrow 2A + \Delta A \\
8A & \rightarrow 2A + \Delta A
\end{align*}
\]

Scheme 1

The approximate fit of the data to Scheme 1 was obtained using the Simulation-Setup function within KinTekSim, and these approximations were further used to globally fit the data using the FitSim function within KinTekSim.

Phosphate Release Assay—The inorganic phosphate released from F-actin during polymerization was measured in a coupled assay using the EnzChek assay developed by Webb (20) with modifications. Briefly, each reaction contained actin at the desired concentration, 0.5 units/ml

The abbreviations used are: εATP, 1,N'-ethenoadenosine 5'-triphosphate; MES, 2-(N-morpholino)ethanesulfonic acid; BVC, bovine cardiac; TFM, tropomyosin; C.c., critical concentration; WT, wild type.
purine nucleoside phosphorylase, and 200 μM 2-amino-6-mercaptopurine riboside. P_i released from actin in P buffer (5 mM Tris-HCl, pH 7.5, 2 mM MgCl_2, 50 mM KCl, 0.2 mM CaCl_2, 0.2 mM dithiothreitol, and 0.2 mM nucleotide triphosphate) upon actin polymerization was monitored continuously by the change in absorbance at 360 nm. A standard curve allowed conversion of the absorbance change to P_i concentration.

Electron Microscopy—To prepare the samples for electron microscopy, 6.9 μM actin was induced to polymerize as described above. A 3-μl aliquot sample from the actin solution was removed at time 0, 5, and 10 min and applied to carbon-coated Formvar grids and visualized following negative staining with 1.5% (w/v) uranyl acetate using a Hitachi 7000 electron microscope (University of Iowa Electron Microscope Facility).

RESULTS

Effect of the R177H Mutation on G-actin Structure—To assess the changes in G-actin structure caused by the mutation, we examined its effect on thermal stability, rate of nucleotide exchange, and stability to limited proteolysis of the protein. εATP exhibits a much higher fluorescence when bound to actin than when it is free in solution, and its release from actin can be followed in the presence of a large excess of ATP to prevent rebinding of the fluorescent analogue (21). Fig. 1 shows a faster decay curve for the mutant than the WT actin. Fitting the data to a first order equation (see "Experimental Procedures"), and the simulations (solid lines) along with the experimental points are shown. ΔF is the decrease in εATP fluorescence. The experiments presented in this figure have been performed twice with essentially identical results.

gets cleavage sites at the top of subdomain II, however, did not show differences between the WT and mutant proteins (data not shown), suggesting that changes in conformation caused by the mutation may have been restricted to near the base of the interdomain cleft where the residue lies.

Polymerization of R177H Actin—We had previously shown that, in comparison with WT actin, the mutation caused a premature level of termination of polymerization, an apparently elongated nucleation time, and a faster than expected rate of filament formation based on the amount of polymerizable actin present in the solution. To better understand the nature of the defect, we simulated the data previously obtained with a model based on that of Buzan and Frieden (10), which incorporated a fragmentation/annealing step not required for the WT protein. We then used the model to fit the original data points. The results of the fit are shown in Fig. 3 and Table I, and the kinetic constants for the individual steps in the model are listed in Table I. For the association rate constant k_a of WT actin, we used a value of 2 μM⁻¹ s⁻¹, the same as the one used by Buzan and Frieden (10). For the WT actin, nucleation is assumed to be trimer or tetramer formation. For the mutant, a best fit was obtained with the nucleus assumed to be a pentamer. In general, in comparison with WT actin, the mutant is characterized by slower monomer on-rates, faster monomer off-rates, and a significant propensity for fragmentation, leading to increased numbers of filament nucleation sites following the onset of polymerization. Using the plateau levels for each of the concentrations of actin polymerized, we also calculated a C.c. for the R177H actin of 2 ± 0.4 μM (n = 4), -7-fold higher than that of WT actin (0.3 ± 0.2 μM, n = 3).

Because the simulation suggested that filament fragmentation of the mutant F-actin might be significant, we examined the actin by electron microscopy as a function of time following initiation of the polymerization reaction. Fig. 4E shows that with the mutant after 5 min only short actin oligomers or amorphous aggregates are observed. After 10 min (Fig. 4F), longer filaments begin to appear in a mass of otherwise short choppy looking filaments. In contrast, after 5 min, a comparable amount of WT actin formed a mass of long, well structured filaments like those seen in Fig. 4B. These data are consistent with the suggestion of massive fragmentation early during
polymerization due to filament instability followed by a rapid phase of fragment growth due to more extensive nucleation and annealing of preformed filament fragments. However, the early appearance of fragments and kinked actin in the case of the mutant may simply reflect a normal process that is more readily apparent because of the polymerization delay observed with this protein.

Effect of Tropomyosin on R177H Actin Polymerization—To try to resolve the two possible explanations for the EM observations, we examined the effect of tropomyosin on the polymerization of the mutant actin because tropomyosin has been shown to prevent fragmentation (22). Moreover, within the context of the sarcomere, the actin thin filament is saturated with tropomyosin, which has been shown to be able to stabilize actin filaments. We thus tested whether inclusion of cardiac tropomyosin with pyrene-labeled R177H actin at a level sufficient to saturate the actin would decrease the hyperfragmentation we hypothesized occurred with the mutant actin. Fig. 5 shows that tropomyosin had two effects on the pure pyrene-actin. First, its increase in pyrene fluorescence that the addition of salt to induce polymerization of the R177H actin leads to significant postnucleation abortive oligomer formation during which F-actin monomer conformation—The location of Arg177 near the bottom of the nucleotide cleft possibly in association with His73 of subdomain I suggested that introduction of His for the Arg might weaken interdomain connections enough to control the conformation of the actin monomer within the confines of the filament structure. To begin to test this possibility, we employed intrinsic

tation prior to the attainment of the steady state. This result is consistent with the fragmentation behavior proposed for the mutant actin.

Effect of the R177H Mutation on Polymerization-induced P_i Release—Because Arg177 had originally been implicated as being important in determining P_i release rates from F-actin following polymerization-induced hydrolysis of ATP, we assessed the effects of the cardiofunk mutation on this process. Fig. 6 shows that the rate of P_i release tracks with the elongation phase of actin polymerization, just as it does for WT actin. However, for the mutant, we repeatedly detected a significant rate of P_i release during the lag phase preceding elongation, where there is no net filament formation. This result suggests that introduction of His for the Arg might weaken interdomain connections enough to control the conformation of the actin monomer within the confines of the filament structure.

To begin to test this possibility, we employed intrinsic

TABLE I

| Step | k_+ | k_- | k_+ | k_- |
|------|-----|-----|-----|-----|
| 1    | 2   | 83,467 | 0.63 | 10^6 |
| 2    | 2   | 366   | 0.63 | 9000 |
| 3    | 2   | 1     | 0.63 | 100  |
| 4    | 2   | 1     | 0.63 | 10   |
| 5    | 2   | 1     | 0.63 | 1    |
| 6    | 2   | 0     | 0.63 | 0    |
| 7    | 2   | 1     | 0.63 | 1    |
| k_+  | k_- | k_+  | k_-  |
| 8 (fragmentation/annealing) | — | — | 0.045 | 0.763 |

— not required.
tryptophan fluorescence measurements. Actin polymerization is usually characterized by a decrease in intrinsic tryptophan fluorescence (23) caused by an alteration in the environment of three tryptophans in subdomain 1 (24). Fig. 7 shows that at 4.6 μM the G-actin intrinsic tryptophan fluorescence of both WT and R177H actins are essentially the same. However, whereas the usual postpolymerization decrease is observed with WT actin, no such decrease at all is seen with the mutant, suggesting that these tryptophans in the mutant remain in a much more hydrophobic environment than they do in the WT protein. Another way of examining conformation changes in subdomain I is to assess the fluorescence behavior of a pyrene attached either as a pyrene-acetamide or pyrene succinimide group to the sulfhydryl residue of Cys374 adjacent to the protein's C terminus. Polymerization normally produces an increase in pyrene fluorescence (25). We thus assessed the change in pyrene fluorescence of labeled 4.6 μM WT and R177H actins. Because of critical concentration differences, this amount of mutant G-actin should have yielded about 2.6 μM F-actin, producing about 50% of the fluorescence of that amount of WT actin (Fig. 8, line 5). Fig. 8 shows, however, that the percentage change in fluorescence of the mutant was only 25% of that of a comparable amount of the WT protein. This result, a decrease in fluorescence yield, again demonstrates a profound change in the conformation of subdomain I caused by the introduction of this mutation.

It is possible that the tropomyosin-dependent increase in pyrene fluorescence shown in Fig. 5 resulted in part from an increase in fluorescence yield of the pyrene itself, due to a tropomyosin-induced conformation change in the actin. To distinguish between this possibility and a tropomyosin-induced lowering of the actin critical concentration, we assessed the dependence of critical concentration of both WT and R177H pyrene-labeled actin on the presence of cardiac tropomyosin. Fig. 9 demonstrates that there was little effect of tropomyosin on the C.c. of WT actin. However, tropomyosin reduced the critical concentration of the R177H actin from 2.1 μM, similar to that determined by light scattering, to 1 μM, a decrease consistent with the increase in fluorescence we observed upon polymerization of the actin. It should be noted that this C.c. value for the mutant actin in the presence of tropomyosin is still higher than that of WT actin. Additionally, the similarity in slopes of the critical concentration lines of the R177H actin in the presence and absence of tropomyosin indicates that the fluorescence yield of the pyrene in each case is the same.

Effect of Lowered pH on R177H Actin Polymerization— Whereas the pKₐ of an arginine guanido group is about 12–14, that of a histidine imidazole is typically about 6–7, resulting in a decrease in positive charge at this position as a result of the substitution at neutral pH. If part of the defect resulting from the R177H mutation derives from the decrease in positive charge, then decreasing the pH might be expected to rescue the
polymerization defect. We thus studied the effect of decreasing the pH from 7.5 to 6.5 on R177H actin polymerization using light scattering as an assay. We did not go to lower pH values, because below pH 6.5 there was a rise in the light scattering of the G-actin unrelated to filament formation that caused a decrease in sensitivity of the assay. Fig. 10A shows that for WT yeast actin, the decrease in pH causes an acceleration of polymerization with no significant change in the apparent critical concentration as judged by the steady state polymerization level attained. For the R177H (Fig. 10B), the effect is even more dramatic. Acidification results in a shortening of the nucleation phase, a more drastic increase in the elongation phase, and a gradual increase in the steady state level of polymerization, although at pH 6.5 it is still below that attained by the WT actin. For the mutant, a significant portion of the increased rates of nucleation and elongation may have resulted from the decreased critical concentration caused by acidification, since these other two processes are dependent on the concentration of polymerization-competent actin. To determine to what extent acidification restores the conformation of subdomain I to a WT state, we determined the effect of polymerization on the intrinsic tryptophan fluorescence spectra of the WT and mutant actin at pH 6.5. As was the case at pH 7.5, the fluorescence of the WT actin decreased, but that of the mutant did not change (data not shown). Together, the results suggest that the charge on this residue is important for allowing formation of the intermonomer contacts needed for proper actin polymerization.

R177H Actin Exerts a Partially Dominant Negative Effect on WT Actin in Vitro—As mentioned previously, the R177H mutation exerts a partially dominant negative effect in zebrafish. It is thus possible that this behavior arises from a negative effect of the mutant actin on the polymerization behavior of the WT actin in the same cell in the heterozygous state. To explore this possibility, we assessed the effect of increasing concentrations of R177H actin on WT actin polymerization. Fig. 11 shows that a 1:1 mixture of the two acts polymerizes to the same extent as an equivalent amount of WT actin alone. Thus, based on the extent of polymerization, at this ratio, the WT actin has lowered the apparent critical concentration of the R177H protein. However, there is still a perceptible nucleation lag, and the rate of elongation is about half of that of WT actin. As the percentage of mutant actin increases, there is a sharp increase in critical concentration and a prolongation of the nucleation phase, although the degree of fragmentation seems to be curtailed by the presence of the WT protein. Clearly, at a 1:1 level, based on these results, the mutation can adversely impact the rate of actin polymerization.
We next repeated the tropomyosin experiment with a 1:1 mixture of WT and mutant actins (Fig. 12), using light scattering as a measure of polymerization. We observed basically the same polymerization curve for the actin mixture in the presence of tropomyosin (•) that we did with just WT actin plus tropomyosin (○), and in both cases the tropomyosin caused a slight retardation in the rate of polymerization of the actin (data not shown), much less than that observed with the mutant actin alone.

DISCUSSION

It had previously been shown that mutation of actin Arg177 to an anionic residue led to a disruption of normal actin function in vitro (10, 11). Furthermore, it has recently been demonstrated that the defect in heart development caused by the cardiofunk mutation results from an R177H substitution. Modeling studies had indicated that a major factor in the proposed role of Arg177 in controlling the rate of Pi release was the positive charge on its guanido side chain (6, 11). Contrary to the other mutant alleles at this position studied previously, the His still maintained at least a partial positive charge. Because of this and the biological consequences of the R177H change, we examined the properties of this mutant to better define the role of Arg177 in normal actin function and to gain insight into the molecular basis for the phenotype caused by the R177H mutation in vivo.

The more rapid nucleotide exchange we detected with the mutant actin and the slightly depressed melting temperature were consistent with an interruption in proposed hydrogen bonding and ionic interactions between the two domains of the protein near the base of the interdomain cleft involving the backbone carbonyl oxygen of His73 and the side-chain carboxyl group of Asp179. However, other parameters were consistent with at best a minimal change in actin monomer structure.
Susceptibility to proteases and the intrinsic tryptophan fluorescence of WT and mutant G-actin were the same. We detected a very different picture, however, when we examined the polymerization properties of the mutant protein. The increased critical concentration, prolonged apparent nucleation phase, and faster than expected elongation rate based on the amount of polymerizable actin present suggested that even a small decrease in the positive charge of the residue at 177 produced significant effects on the ability of monomers to associate with one another to form a stable filament. This proposed weakening of intermonomer contacts was further substantiated by modeling of the polymerization kinetics, which required an enhanced monomer off-rate and a significant degree of filament fragmentation to suitably fit the data. Based on these parameters, one can assemble the following polymerization picture: decreased ability to form a filament nucleus, abortive oligomer formation before the incipient filament is long enough to acquire the stability necessary for detectable net polymerization, facile fragmentation of these growing filaments leading to the establishment of enhanced numbers of barbed ends for faster extension, and decreased filament mass due to energetically less favorable monomer-monomer contacts. Fragmentation was required to model the polymerization of WT yeast actin in the presence of cofilin, a well established actin filament-severing protein (19), and the fragmentation rate constant they calculated for a 1:1 actin/cofilin mixture was approximately the same as what we observed for the mutant actin alone. In contrast, no such fragmentation was required by Buzan and Frieden (10) to describe the behavior of the R177A/D179A double mutant. In comparison with the double Ala mutation, the more disruptive polymerization effect observed with the R177H mutation may arise from the combination of the reduced positive charge in the continued presence of the anionic Asp179 negative charge coupled with the larger mass of the histidine imidazole. The double Ala mutation would have decreased crowding and maintenance of a charge balance compared with the R177H protein.

Our model is further substantiated by the detection of a salt-induced increase in P_i release during the apparent lag phase and the substantial elongation of the apparent nucleation phase and retardation of the elongation phase brought about by tropomyosin. The P_i data are consistent with a rapid and reversible formation of oligomers early in the process. The tropomyosin data are consistent with tropomyosin-dependent hyperstabilization of contacts between neighboring monomers along the strand of the actin helix, leading to decreased creation of free ends from both oligomer and filament fragmentation. These results are similar to what we previously observed for another actin mutant, V265G/L266G actin, with weakened monomer/monomer contacts leading to unstable oligomer formation and a vastly increased critical concentration (18).

The substantial alteration in polymerization behavior caused by the R177H mutation must reflect a mutation-dependent alteration in the conformation of the F-actin monomer leading to altered intermonomer contacts along the filament axis. We have two pieces of spectroscopic evidence that support this assertion: the lack of change in intrinsic tryptophan fluorescence following polymerization and the significant decrease in the polymerization-dependent fluorescence enhancement of a pyrene attached to Cys374 of the actin. Doyle et al. (24) showed that of the four tryptophans in subdomain 1 of actin, three, at positions 79, 86, and 340 are responsible for the polymerization-dependent change in intrinsic tryptophan fluorescence. However, we observed no such change with our mutant. Trp379 and Trp340 are located in the a-helix just after His73 (Fig. 13). If a tight interaction between His73 and Arg177 is needed for proper actin behavior, its disruption could easily perturb the surrounding area in the protein such that the normal polymerization-dependent alteration of the subdomain 1 conformation, and the subsequent change in tryptophan environment did not occur. The increase in fluorescence of a Cys374 pyrene is thought to arise from the probe being buried in the interface between two neighboring monomers in the same filament strand following polymerization. The significantly decreased fluorescence enhancement we observed with the labeled R177H protein is consistent with a weakened or more open interface resulting from a subdomain 1 alteration induced by the mutation.

To mimic the protein state in animals that are heterozygous for the cardiofunk mutation, we studied the polymerization behavior of mutant and WT actins in the presence and absence of tropomyosin. For the actins alone, a 1:1 mixture polymerized to the same extent as an equivalent amount of pure WT actin, showing that the presence of the WT protein can lower the critical concentration of the mutant actin. Although the lag phase observed with the mutant alone was greatly reduced, the elongation rate was only about half of that observed with the WT actin alone, and a similar behavior was observed at a 2:1 mutant/WT ratio. Thus, in these proportions, rescue of the mutant phenotype by the WT protein was incomplete. A dominant negative phenotype was obviously present. As the fraction of WT actin continued to increase up to 50%, the retardation in elongation rate also lessened. Interestingly, as the molar fraction of WT actin decreased below 33%, a distinct lag in nucleation rapidly reappeared, as did an elevation of the critical concentration, and these trends worsened as the molar fraction of the mutant actin continued to increase. In the 1:1 mixture, the presence of tropomyosin did not obviously alter the rate of polymerization of the protein, cause an extension in the lag phase, or alter the critical concentration of the mixture in comparison to WT actin alone with tropomyosin. Thus, an equivalent amount of the WT actin stabilized the mutant protein conformation sufficiently, so the fragmentation-based tropomyosin effect was virtually eliminated.

The actin isoform that carries the cardiofunk mutation is expressed during development at about the time of cardiac valve formation, and correct development must depend stringently on the correct temporal deposition of contractile proteins in the myocytes and proper sarcomere formation. Our in vitro
data with the pure cardiofunk mutant actin revealed severe deficiencies both in the timing and extent of actin polymerization, and the timing was even further disturbed by the binding of tropomyosin that occurs along the length of the thin filament in vivo. Apart from possible effects of the mutation on the development of contractile force once the sarcomeres are assembled, it is not difficult to imagine how homozygous expression of this actin could lead to lethality. With respect to polymerization timing, we also saw mild deleterious effects of the mutant actin in the presence of WT actin, and the effects worsened as the percentage of mutant actin increased. Again, the presence of these defects in the heterozygous state and the possibility of having different relative amounts of the two actins because of expression or stability differences could explain the low incidence of the mutant phenotype in heterozygous animals and the dependence of the appearance of this phenotype on the expression of certain alleles of other genes.

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