on actin responsible for the energy transduction process. In Fig. 4*c*, we illustrate these effects by plotting force increase or inhibition by these peptides (at 50 μM) as a percentage of control force at 2.5 μM Ca^{2+} in the absence of peptide. In this way, an activity profile is obtained over the stretch of SI peptide comprising residues 680–745 (shown on the abscissa), which also includes the inactive flanking regions.

It should be emphasized that at maximally activating concentrations of Ca^{2+}, all the peptides inhibit force development to some degree (see Fig. 4*b* for example); this is to be expected should they be interfering with the actomyosin interaction, as we have noted in the actin–SI Mg^{2+}-ATPase solution assays (Fig. 3*a*, *b*). In skinned fibres, however, peptides from the N-terminal region (Y608, Y629, Y668, Y669 and Y762) are only slightly inhibitory (~15%; Fig. 4*b*, upper panel), but this effect is accompanied by an increase in force development at submaximal activating concentrations of calcium which is significant compared with controls (32 ± 10% in the case of peptide Y629 for example; Fig. 4). It is tempting to explain the enhancement of force at submaximal calcium concentrations in terms of a reduction of the apparent crossbridge-detachment rate constant (compare ref. 6). If this were the case, however, the force developed at maximal calcium concentration would have to increase rather than decrease. A dual effect of the peptides would be more feasible if they were competing with both troponin-I and myosin for their respective binding sites on actin.

We confirmed that the synthetic peptides affect both ATPase and contractile activity by their direct interaction with actin, using ^{1}H-NMR to monitor binding to actin. These experiments show that the peptides with biological activity bind to actin, whereas those from the flanking sequences do not. They also indicate that only selected amino-acid side chains or groups of amino acids along this sequence are perturbed by actin-binding. This will be commented on more fully elsewhere in the light of NMR data on the interaction of actin and troponin-I (ref. 7). Morita and colleagues[8] also reported, while these experiments were in progress, that a small peptide from this region (residues 702–708) can interfere with SI binding to actin. We cannot be sure that this is the only site on the 20K domain that is involved in actin binding, and indeed crosslinking studies may indicate otherwise[9].

Our results, however, indicate that the region 690–725 on the myosin head is likely to span two functionally distinct (and possibly spatially distinct) sites on actin in the actin–myosin complex. The crossbridges attaching to actin are known to increase the calcium sensitivity of the contractile system[10]. It is therefore plausible that the calcium-sensitizing effects of the N-terminal region could result from this segment weakening the actin–troponin-I interaction, either by direct competition with troponin-I for the same site on actin (both troponin-I (ref. 7) and SI (refs 1, 2) bind to the N-terminal region of actin) or as a result of some allosterically transmitted event. In either case, the peptides would mimic the 'potentiating effect' of SI on actin[11] and switch on the thin filament. The C-terminal half, whose inhibitory effects on force overshadow these calcium-sensitizing properties, should then bind at another site on actin, giving rise to the idea of a multi-site docking of the interacting partners in the force-generating cycle, an idea implicit in many of the crosslinking studies[1,2,9]. This could arise from the blocking of the actin–SI interaction directly, or from switching off the thin filament by some other means. We are using these peptides as specific inhibitors of interacting actin–myosin sites to investigate the kinetic stages of the contractile event and to relate these more precisely with molecular events. □

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Relative helix-forming tendencies of nonpolar amino acids

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AN important issue in understanding the relationship between protein sequence and structure is the degree to which different amino acids favour the formation of particular types of secondary structure. Estimates of the 'helix-forming tendency' of amino acids have been made based on 'host–guest' experiments, in which copolymers are made of the amino acid of interest (the 'guest') and a host residue (typically hydroxypropyl- or hydroxybutyl-L-glutamine)[1]. Recently, however, short alanine-based peptides were found to form stable monomeric helices in water[2], contrary to the result predicted from host–guest experiments[2]. We have now measured the helix-forming tendency of five different nonpolar amino acids (Ala, Ile, Leu, Phe, Val) by substituting each in turn for alanine in a 17-residue alanine-based peptide and determining the extent of α-helix formation. Our results differ from those of host–guest experiments both in the degree of variation in helix-forming tendency of different amino acids, and in the rank order of the helix-forming tendency. We conclude that the helix-forming tendency of a particular amino acid depends on the sequence context in which it occurs; and the restriction of side-chain rotamer conformations is important in determining the helix-forming tendency.

We measured the rank order of helix-forming tendency of five nonpolar amino acids by substitution experiments using a 17-residue alanine-based peptide as a reference. Our three underlying objectives were: (1) to determine whether other nonpolar amino acids share with Ala the ability to form an α-helix in short peptides; (2) to determine whether the results of amino-acid substitution experiments are consistent with predictions from host–guest results; and (3) to find the factors that determine the helix-forming tendency of a nonpolar amino acid. We have deferred the study of other amino acids, because polar amino acids can disrupt the helix by hydrogen-bonding to main-chain NH or CO groups, and charged amino acids have additional complications imposed by electrostatic interactions.

The reference peptide that we used for the substitution experiments (Table 1) contained four Lys^+ residues inserted for peptide solubility in water, and one Tyr residue (N-terminal) to allow us to determine peptide concentration accurately by

TABLE 1 Peptide sequences and helix-forming properties

Peptide	Sequence*¶	$-[\theta]_{222}$†	Residue	% Helix‡	s§	$P\alpha$‖
3Ala	CH_3CO-YKAAAAKAAAAKAAAAK-NH_2	25,800	Ala	78	1.08	1.60
3Leu	CH_3CO-YKAA**L**AKAA**L**AKAA**L**AK-NH_2	26,300	Leu	80	1.10	1.50
3Phe	CH_3CO-YKAA**F**AKAA**F**AKAA**F**AK-NH_2	7,600	Ile	41	1.25	1.31
3Ile	CH_3CO-YKAA**I**AKAA**I**AKAA**I**AK-NH_2	13,400	Phe	23	1.06	1.45
1Val	CH_3CO-YKAAAAKAA**V**AKAAAAK-NH_2	16,200	Val	17	0.88	1.09
2Val	CH_3CO-YKAA**V**AKAA**V**AKAAAAK-NH_2	11,200				
3Val	CH_3CO-YKAA**V**AKAA**V**AKAA**V**AK-NH_2	5,600				

* The α-NH_2 is blocked by an acetyl group, and the α-COOH is blocked by an amide group.

† Mean residue ellipticity at 222 nm. Conditions: 0 °C, pH 7.0, 1.0 M NaCl, 1 mM each sodium citrate, sodium phosphate and sodium borate. Units: degrees cm^2 $dmol^{-1}$.

‡ The % helix is taken to be proportional to $-[\theta]_{222}$. The values for 100 and 0% helix are 33,000 and 0 degrees cm^2 $dmol^{-1}$ (see text). Values for peptides with three residue substitutions (except for Ala) are shown.

§ Host-guest values for s (taken from refs cited in ref. 5) at 0 °C, based on experiments with random sequence copolymers in which the host residue is hydroxypropyl-L-glutamine (HPLG) or hydroxybutyl-L-glutamine; s is the helix propagation parameter in the helix-flexible chain transition theory.

‖ $P\alpha$ is the helical preference[6] given by the frequency of an amino acid in α-helices (the three residues at each end are excluded), relative to its frequency in the entire protein.

¶ Peptide synthesis: Peptides were synthesized by solid-phase peptide synthesis on an automated Milligen 9050 synthesizer, using standard 9-fluorenylmethoxycarbonyl methodology[13]. The N terminus was acetylated with acetic anhydride and the peptides were cleaved from the benzhydrylamine resin by incubation with 95:5 trifluoroacetic acid:phenol mixture for at least 2 h yielding an amide at the C terminus. Peptide Purification: Peptides were purified by gel filtration and reverse phase chromatography as previously described[2]. Peptide identity was verified by mass spectrometry.

measuring tyrosine absorbance[2]. We substituted the chosen nonpolar amino acid for Ala at one to three positions (Table 1) spaced at least five residues apart to minimize intrahelical interactions between the substituted nonpolar residues. The 17-residue peptides were in the same size range as those of helical segments found in globular proteins, and the results show how differences in helix-forming tendency of an amino acid can affect the stability of a short helix.

Circular dichroism (CD) spectra of the peptides measured at 0 °C in 0.1 M KF, pH 7, are shown in Fig. 1. With the exception of the peptide in which three Ala residues had been substituted by Phe (peptide 3Phe), all the peptides have CD spectra expected for mixtures of α-helix and random chain, with a first minimum at 222 nm, a second minimum between 197 and 207 nm, and a maximum at about 190 nm. The helix content (% helix) is taken as directly proportional to the mean residue ellipticity at 222 nm, $-[\theta]_{222}$. The existence of an isodichroic point (203 nm) is consistent with the presence of just two conformations for each residue, helix and random chain. The spectrum of 3Phe is anomalous; it does not contain the isodichroic point shown by the other peptides, and its value for % helix derived from $-[\theta]_{222}$ is tentative. We determined the value of $-[\theta]_{222}$ corresponding to 100% helix from the maximum value given by trifluoroethanol (TFE) titration[4] at 0 °C (data not shown). The same maximum value, within the limits of experimental error, was found for each peptide: $33{,}000 \pm 1{,}000$ degrees cm^2 $dmol^{-1}$.

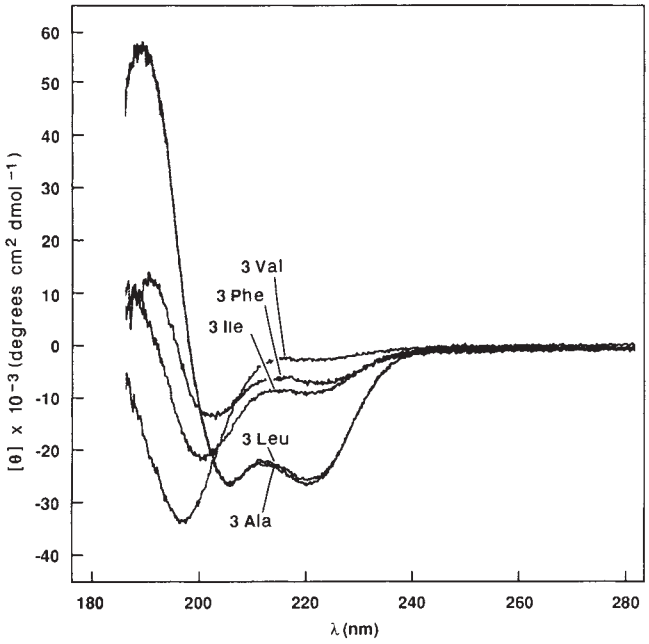

FIG. 1 CD spectra of the peptides taken at 0 °C, 0.1 M KF, 1 mM KPO_4, pH 7.0. The mean residue ellipticity (degrees cm^2 $dmol^{-1}$) is plotted. In order of decreasing values of $-[\theta]_{222}$, the spectra are for peptides 3Leu, 3Ala, 3Ile, 3Phe and 3Val (see Table 1).

METHODS. CD spectra were taken on an Aviv 60DS spectro-polarimeter. Samples were prepared as described[2]. The spectra shown above were taken at a peptide concentration of 50 μM in a 1-mm path length cuvette. Data were taken with a 0.2-nm step size, 1-s average time, and the results averaged over four scans. Identical spectra were obtained in a 1-cm path length cuvette with a 15 μM peptide concentration.

The extent of helix formation in similar alanine-based peptides is independent of peptide concentration throughout the helix/random chain transition[2]. Figure 2 shows that the reference peptide, 3Ala, is within the limits of experimental error, monomeric in the concentration range tested (up to 6 mg ml^{-1}), as judged by measurements of its apparent relative molecular mass (M_r) in sedimentation equilibrium experiments, using both Rayleigh interference and light absorption optical systems.

Table 1 shows the helix content at 0 °C, pH 7, for all the peptides studied. The peptide 3Leu forms a stable α-helix, comparable to 3Ala. Only leucine shares the ability of alanine to support α-helix formation in this short-peptide system. Ile, Val and Phe are helix destabilizing relative to Ala. Results for the series of peptides 1Val, 2Val and 3Val indicate that peptides with $\geq$4 Ala → Val substitutions would abolish measurable helix formation.

Table 1 shows that the rank order of helix-forming ability is clearly different from that predicted by host-guest values of s (ref. 5), the helix propagation parameter. The host-guest values of s for Ala, Leu and Phe are the same, within the limits of experimental error, and s for Ile is higher than it is for Ala (Table 1), whereas in our alanine-based peptide system both Phe and Ile are definitely helix-destabilizing compared with Ala and Leu. To compare the changes in helicity with predictions based on host-guest data, we used an approximate two-state model (see legend to Fig. 3), which gives an upper limit to the change expected in a substitution experiment. The changes that we observed for Ile, Val and Phe were large compared with this predicted upper limit. Consequently, the differences in helix-forming tendency that we measured are much larger than those predicted from host-guest values of s. Whether the differences in helix-forming tendency are large (the results presented here) or small (host-guest results) is important in determining whether local sequence effects or tertiary interactions are dominant in determining α-helix locations in proteins. Our results indicate that the helix-forming tendencies of the amino acids contribute

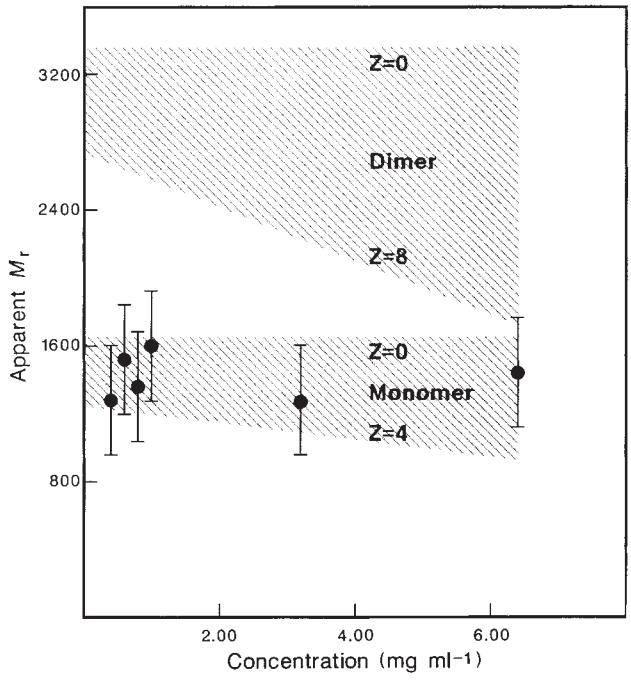

FIG. 2 Concentration dependence of the apparent M_r of peptide 3Ala (Table 1), measured by sedimentation equilibrium[14] using both Rayleigh interference and absorbance (275 nm) optical systems, at 20.0 °C, in 0.1 M NaCl, and 1 mM sodium citrate, phosphate and borate, pH 7.0. The partial specific volume was calculated[15] from peptide composition to be 0.76 ml g^{-1}; the measured buffer density was 1.0021 g ml^{-1}, and NONLIN[16] was used to fit the sedimentation results. Rotor speeds of 40,000–48,000 r.p.m. were used. The experiments at 0.91 and 0.45 mg ml^{-1} were made using absorbance optics; the other experiments used interference optics. Both short (0.7 mm) and medium (3 mm) solution columns were used. The net charge on the peptide (Z) introduces some uncertainty into the results: the magnitude of the charge effect has been estimated from equations 62 and 63 of ref. 17, and is shown by the shaded areas on the graph. The known monomer M_r (including four Na^+) is 1,676.

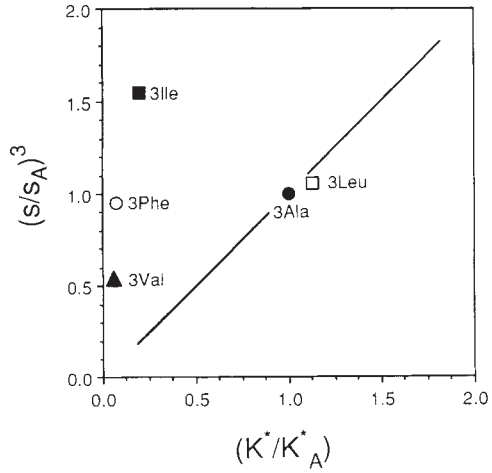

FIG. 3 Comparison of the effect of amino-acid substitution with the maximum effect predicted from the host–guest values of s (given in Table 1). Points on the line agree with the prediction from host–guest experiments, and points off the line show larger than expected changes in helicity as a result of making three substitutions (for example, Ala→Val) in the reference peptide. The peptide labelled 3Ala is the reference peptide and falls on the line by definition. The apparent two-state equilibrium constant K^* is $f/(1-f)$, where f is the fraction helix (Table 1) and K^*_A refers to the reference peptide 3Ala. For a two-state reaction, K^*/K^*_A is given by $(s/s_A)^3$ (ref. 18), where 3 is the number of substitutions of the chosen amino acid for Ala. Computations based on the Lifson–Roig[19] theory of helix formation, in collaboration with work of J. A. Schellman, indicate that the two-state approximation gives an upper limit (data not shown) to the effect expected for an amino-acid substitution.

strongly to determining whether a given sequence will form an α-helix in a protein. Experiments with short dimeric coiled-coil helices show that helix-forming tendency varies widely among the 20 amino acids, and substitution of a single residue can have a marked effect on helix stability (W. F. DeGrado, personal communication), in agreement with the results reported here.

It is interesting to compare these measurements of helix-forming tendency with the helical preference $P\alpha$, given by the frequency of an amino acid in protein α-helices relative to its frequency in the whole protein. Data for $P\alpha$, taken from a study[6] of protein X-ray structures, are given in Table 1. We were surprised to find that Phe (which is helix-destabilizing in our experiments) has the third highest $P\alpha$ value of any amino acid. This indicates that $P\alpha$ depends on other factors in addition to helix-forming tendency. It is likely that $P\alpha$ depends also on the frequency that an amino acid occurs in a helix–helix or helix–sheet pairing site.

What determines the order of helix-forming tendency in nonpolar amino acids? Hydrophobicity of the side chain cannot be the determining factor, because Ala and Leu have nearly equal helix-forming tendencies but very different hydrophobicities, and their helix-forming tendencies differ markedly from those of Val, Ile and Phe. Our finding that Val and Ile are helix-destabilizing is consistent with the view[8], based on results for α-helix versus β-sheet formation by synthetic polypeptides in organic solvents, that β-branching causes an amino acid to be helix-destabilizing. Host–guest results[5], which indicate that Ile is a better helix-former than Ala or Leu, have indicated that β-branching is only a minor factor in determining helix-forming tendency. Our results show further that Phe is helix-destabilizing. The side-chain rotamer conformations permitted in an α-helix are severely restricted by β-branching[8], and the bulky aromatic ring of Phe also restricts the side-chain conformations that can occur in a helix[8]. Recent work has emphasized the energetic importance of the occurrence of particular side-chain rotamer conformations in the hydrophobic core of a protein[9–12]. Our results indicate that restriction of side-chain rotamer conformations in the α-helix is similarly an important factor in determining the helix-forming tendency of a nonpolar amino acid.

Note added in proof: The distribution of side-chain chi 1 rotamer angles is known to be different in α-helices than in β-sheet or unordered segments of proteins (McGregor, M. J., Islam, S. A. and Sternberg, M. J. E., *J. molec. Biol.* **198,** 295–310 (1987)). For α-helices, the altered distribution of rotamer angles is, we suppose, correlated with a reduction in side-chain conformational entropy in the helix compared with the flexible chain.

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