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^{19}F Nuclear Magnetic Resonance Studies of Selectively Fluorinated Derivatives of G- and F-Actin†

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ABSTRACT: G-Actin is a globular protein (M_r 42 300) known to have three cysteine residues that are at least partially exposed and chemically reactive (Cys-10, -284, and -374). When G-actin was reacted with 3-bromo-1,1,1-trifluoropropanone, three resolvable ^{19}F resonances were observed in the ^{19}F NMR spectrum. This fluorinated G-actin derivative remained fully polymerizable, and its ^{31}P NMR spectrum was not significantly different from that of unmodified G-actin, indicating that the chemical modification did not denature the actin and the modified residues do not interfere with the extent of polymerization or the binding of adenosine 5′-triphosphate. One of the three ^{19}F resonances was assigned to fluorinated Cys-374 on the basis of its selective reaction with *N*-ethylmaleimide. This resonance was dramatically broadened after polymerization of fluorinated G-actin, while the other two resonances were not markedly broadened or shifted. Thus, Cys-10 and -284 are not involved in or appreciably affected by the polymerization of G-actin, while the mobility of the ^{19}F label at Cys-374 is markedly reduced.

Fluorine-19 nuclear magnetic resonance (NMR) has proven to be an exceptionally useful probe for studying the conformation and mobility of proteins, membranes, and other biological macromolecular systems (Sykes & Weiner, 1980; Gerig, 1978). It offers the advantages of high sensitivity (close to that of ^1H NMR), 100% natural abundance, a wide chemical shift range (~2000 ppm), and a low background of naturally occurring resonances. ^{19}F must, in general, be introduced into the system by bioincorporation of fluorinated

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amino acids into labeled proteins, by addition of an appropriate fluorinated substrate analogue, or by specific chemical modification involving a fluorine-containing reagent. This latter approach, employing 3-bromo-1,1,1-trifluoropropanone (BTFP) as the modifying reagent, has been used to selectively alkylate the exposed cysteine residues of troponin C (Seamon et al., 1977), ribonuclease (Brown & Seamon, 1978), hemoglobin (Huestis & Raftery, 1972), papain (Bendall & Lowe, 1976), glyceraldehyde-3-phosphate dehydrogenase (Bode et al., 1975; Long & Dahlquist, 1977), and aspartate aminotransferase (Critz & Martinex-Carrion, 1977).

Actin is a ubiquitous protein that is essential for muscle contraction, cell structure and shape, exocytosis, cytoplasmic streaming, and various aspects of cellular and organelle movement (Korn, 1980). This protein can exist in a globular, monomeric form (G-actin) or polymeric, filamentous form (F-actin) (Oosawa & Kasai, 1970). The protein, in either form, generally has a bound nucleotide (ATP or ADP) and one or more bound divalent cations (usually Mg^{2+} or Ca^{2+}). NMR studies have been limited to the G-actin form (Brauer & Sykes, 1981a,b, 1982; Barden et al., 1980; Burns & Burtnick, 1981) because the resonances of the F-actin form are too broad to be observed (Highsmith & Jardetzky, 1980).

The reactivity of the cysteine residues of actin to various sulfhydryl reagents has been studied. Of the five cysteine residues of actin, three are known to react to some degree under native conditions. G-Actin was found to be alkylated at Cys-10, -284, and -374, with some additional alkylation of Cys-256 when the protein was denatured (Lusty & Fasold, 1969).[1] Cys-217 was not alkylated under any conditions. G-Actin with Cys-10, -284, and -374 alkylated could still be fully polymerized. Alkylation with *N*-ethylmaleimide (NEM) was found to be selective for Cys-374 (Elzinga & Collins, 1975). Reaction at Cys-374 was found not to inhibit polymerization of the G-actin, ATP binding, or other characteristics of native actin (Knight & Offer, 1978), and various ESR and fluorescent labels have been directed to this site (Tao, 1978; Ikkai et al., 1979; Porter & Webec, 1979). Other alkylating reagents can preferentially react with Cys-10 under certain conditions (Sleigh & Burley, 1973; Bridgen, 1972). The binding of nucleotide and divalent metal ion to G-actin is known to shield some of the cysteine residues from the alkylating reagents (Katz, 1963, 1965; Faulstich et al., 1984).

In this paper, we have reacted G-actin with BTFP alone and BTFP after reaction with NEM and used ^{19}F and ^{31}P NMR to characterize the modified protein. Three resolvable ^{19}F resonances corresponding to the three modified cysteines of G-actin have been observed, and the changes in these resonances upon polymerization of the protein to F-actin have been studied in relation to the structure of G- and F-actin.

Materials and Methods

Actin was prepared by the method of Spudich and Watt (1971). G-Actin was prepared by dialysis of F-actin with a depolymerization buffer of 2 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris), 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.2 mM adenosine 5′-triphosphate, pH 7.8, at 4 °C for 3 days. Residual F-actin was removed by ultracentrifugation at 80000*g* for 3 h at 4 °C. Fluorination of the G-actin with BTFP (PCR Inc., Gainesville, FL) was done in the following manner. G-Actin (7–8 mg/mL) in depolymerization buffer at 4 °C was warmed to 25 °C, and the pH was adjusted to pH 8.0. Urea was added as a solid to 1.5 M concentration, and BTFP (5 mM final concentration) was immediately added. The pH was maintained at 8.0 throughout the reaction, with a pH-stat. The reaction was allowed to proceed for 20 min, and the solution was then dialyzed again with depolymerization buffer at 4 °C for another 2–3 days. For NMR analysis, the fluorinated G-actin solution was concentrated to ~15 mg/mL (at 4 °C) in a Minicon B15 concentrator, diluted 1:1 with D_2O, and reconcentrated to 15 mg/mL again. pH values were checked before NMR spectra were run.

To selectively block fluorination of Cys-374, a solution of G-actin (7 mg/mL) was warmed to 25 °C, the pH was adjusted to pH 8.0, and NEM was added, with stirring, to a final concentration of 1.5 mM. After 30 min, the reaction was quenched by adding an excess of dithiothreitol (final concentration 3.0 mM), and the reaction mixture was dialyzed against depolymerization buffer at 4 °C for 2 days before undergoing the fluorination reaction.

^{19}F NMR spectra of fluorinated G-actin in 50% D_2O were taken on a Bruker HXS 270 NMR spectrometer (254-MHz ^{19}F frequency). An excitation pulse of 90° was used with a repetition time of 0.70 s, a spectral width of ±5000 Hz, and data blocks of 4096 points. The free induction decay was exponentially multiplied to decrease spectral noise, resulting in a 1-Hz line broadening. Chemical shifts are relative to trifluoroacetic acid with upfield shifts negative. T_1 values were determined by the progressive saturation method (Freeman & Hill, 1971). Spectra were run at 4 °C in 10-mm flat-bottom NMR tubes with delrin vortex plugs. Spectral simulation studies were done with the Nicolet curve analysis program (NTCCAP). ^{19}F NMR spectra of fluorinated G-actin in 15% and 85% D_2O were taken on a Nicolet 300WB NMR spectrometer (282-MHz ^{19}F frequency) with 12-mm flat-bottom NMR tubes. All other experimental conditions were kept constant. ^{31}P NMR spectra were obtained as previously described (Brauer & Sykes, 1981a).

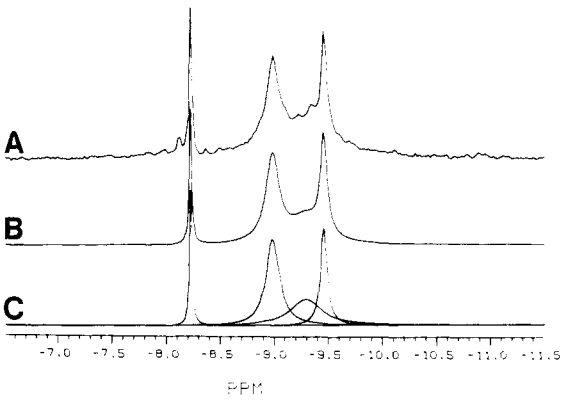

FIGURE 1: ^{19}F NMR spectra of fluorinated G-actin. (A) Experimentally obtained spectrum of fluorinated G-actin (13 mg/mL) in 50% D_2O/50% depolymerization buffer, pH 7.8, 4 °C, after acquisition of 10000 scans with 1-Hz line broadening. (B) Simulated spectrum based on the summation of four theoretical Lorentzian line shapes. (C) Simulated spectrum showing the four individual Lorentzian line shapes used to generate spectrum B.

Results

The ^{19}F NMR spectrum of G-actin in 50% D_2O after chemical modification with BTFP showed the presence of a sharp ^{19}F resonance at −8.2 ppm from trifluoroacetic acid, two somewhat more broad resonances at −9.0 and −9.4 ppm, and a very broad resonance at about −9.3 ppm (Figure 1A). (The latter resonance is more readily apparent in subsequent spectra, such as Figure 3B,C.) The line widths, chemical shifts, and

[1] Note the change in amino acid sequence numbers since some of these earlier studies were done (Vandekerckhove & Weber, 1978).

Table I: Spectral Parameters Determined for the ^{19}F Resonances Observed in Fluorinated G-Actin

	1	2	3	4
chemical shift (ppm from trifluoroacetic acid)				
in 15% D_2O[a]	−8.17	−8.95	−9.24	−9.37
in 50% D_2O[b]	−8.23	−8.97	−9.27	−9.43
in 85% D_2O[a]	−8.27	−9.01	−9.30	−9.47
SIIS[c]	0.14	0.09	0.09	0.14
line width (Hz)				
in 15% D_2O[a]	5	35	~100	20
in 50% D_2O[b]	6	35	~100	14
in 85% D_2O[a]	4	35	~100	22
T_1 (s)				
in 15% D_2O[a]	0.73	0.26	0.23	0.26
in 50% D_2O[b]	1.00	0.36	0.37	0.32
in 85% D_2O[a]	0.88	0.38	0.40	0.34
relative intensity				
in 50% D_2O[b]	0.3	1.0	0.7	0.6

[a] Spectra taken at 282-MHz ^{19}F frequency. [b] Spectra taken at 254-MHz ^{19}F frequency. [c] Solvent-induced isotope shift (SIIS) = (extrapolated chemical shift in 100% D_2O) − (extrapolated chemical shift in 0% D_2O).

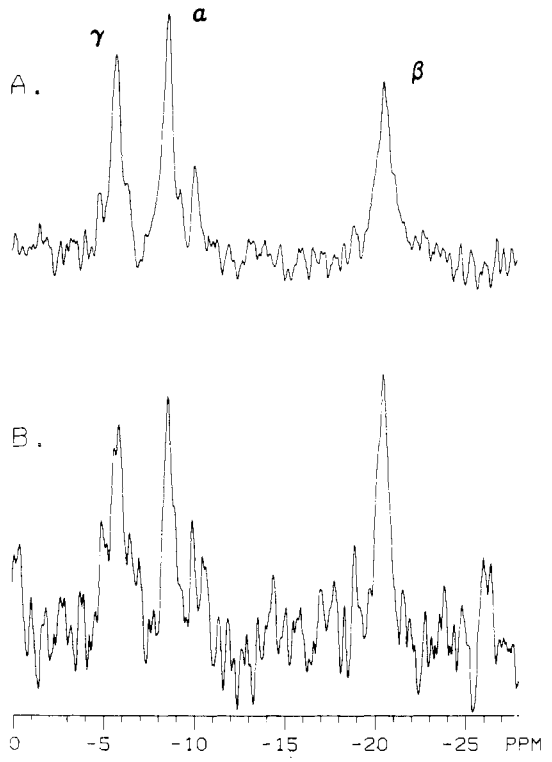

FIGURE 2: ^{31}P NMR spectrum of unmodified G-actin (18 mg/mL, 27000 scans) (A) and of the fluorinated G-actin derivative (13 mg/mL, 40000 scans) (B). The spectra were taken at 109.29-MHz ^{31}P frequency with a 78° excitation pulse, a spectral width of ±2500 Hz, a repetition time of 2.0 s, and a line broadening of 10 Hz. Chemical shifts are relative to 85% phosphoric acid as external standard. Sample conditions are the same as those in Figure 1A. The γ-, α-, and β-phosphate resonances of the G-actin-bound ATP are indicated.

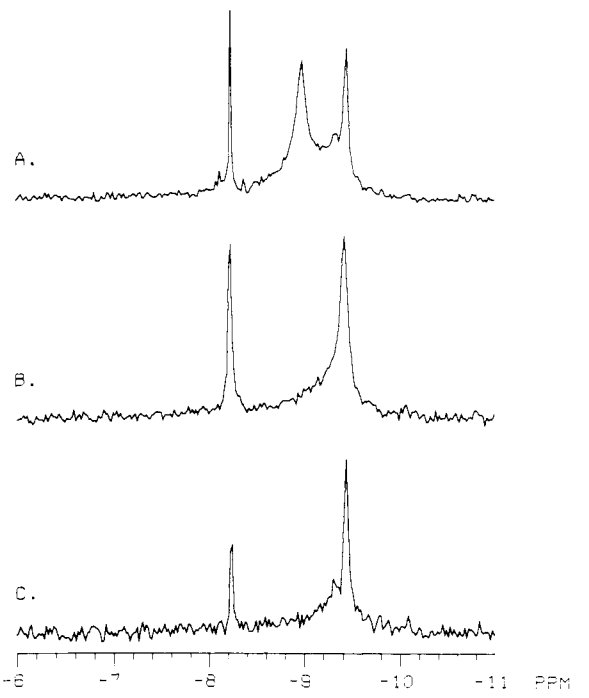

FIGURE 3: ^{19}F NMR spectra of fluorinated G-actin (A), fluorinated F-actin (B), and fluorinated G-actin with Cys-374 blocked with NEM (C). All three spectra were taken under identical experimental conditions as were used in Figure 1A.

relative areas of the partially overlapping resonances were quantitated by a spectral simulation procedure (Figure 1B,C; Table I). The sharp resonance at −8.2 ppm could be readily assigned to the base-catalyzed hydrolysis product of BTFP, 3-hydroxy-1,1,1-trifluoropropan-2-one (Long & Dahlquist, 1977), since this resonance disappears with more extensive dialysis. The other three resonances represent protein-bound fluorine labels since their intensities did not diminish with prolonged dialysis. In keeping with the well-characterized reactivities of Cys-10, -284, and -374 (Lusty & Fasold, 1969), these three ^{19}F resonances were assigned as a group as (trifluoroacetonyl)cysteine (TFA-Cys) residues 10, 284, and 374. The chemical shifts of these resonances were constant from pH 7.8 to pH 9.5. (Below pH 7.5, G-actin was found to polymerize readily.) The T_1 values for the three resonances were virtually identical and were about half the repetition time between scans (0.70 s) so that the relative areas of the resonances are proportional to relative concentrations. The spectral parameters of the four observed ^{19}F resonances are summarized in Table I.

^{31}P NMR studies of G-actin have been used to investigate the microenvironments of the three phosphates of ATP bound to G-actin (Brauer & Sykes, 1981a,b, 1982). This bound nucleotide is essential to the structural stability of G-actin. When the ^{31}P NMR spectrum of fluorinated G-actin was obtained (Figure 2B) and compared to that of unmodified G-actin (Figure 2A), no appreciable change in the spectra was noted. This indicates that alkylation of Cys-10, -284, and -374 does not significantly affect the microenvironments of the bound nucleoside phosphates and that these cysteine residues are not directly involved in the binding of ATP nor does their modification influence the ATP binding site.

When fluorinated G-actin was polymerized in the presence of 50 mM KCl, the resonance at −9.0 ppm disappeared because

of severe line broadening, while the other resonances were not appreciably affected (Figure 3A,B).[2] When subjected to the standard ultracentrifugation procedure (80000*g* for 3 h), <4% of the protein remained in the supernatant, indicating a negligible amount of denatured actin present in the sample. This also indicates that the three cysteine residues are not essential for the polymerization of G-actin.

Cys-374 selectively reacts with NEM (Elzinga & Collins, 1975). When G-actin was pretreated with NEM prior to the fluorination reaction with BTFP, Cys-374 was blocked from reacting with BTFP. The ^{19}F NMR spectrum of this fluorinated G-actin was similar to that of the original fluorinated G-actin, except for the loss of the resonance at −9.0 ppm (Figure 3C). The resonance at −9.0 ppm is therefore assigned to TFA-Cys-374. Thus, during the polymerization of G- to F-actin, the resonance for TFA-Cys-374 is broadened to the degree that it can no longer be observed, while the line widths for the resonances of TFA-Cys-10 and TFA-Cys-284 are not significantly broadened (Figure 3B).

Discussion

Chemical modification studies of G-actin with a variety of alkylating agents have indicated one readily reactive cysteine residue, Cys-374 (Faulstich et al., 1984; Elzinga & Collins, 1975; Detmers et al., 1981), and two less reactive cysteine residues, Cys-10 and -284, which can still be alkylated under relatively native conditions (Lusty & Fasold, 1969; Bridgen, 1972). Thus, the presence of three protein-bound ^{19}F resonances most likely reflects the reaction of BTFP with Cys-10, -284, and -374.

The modification of the three cysteine residues in G-actin by BTFP did not result in an inhibition of the polymerizability of the G-actin derivative, as determined by ultracentrifugation. Further, ^{31}P NMR results indicate that Cys-10, -284, and -374 are likely not directly involved in ATP binding. Similar results have been reported for G-actin derivatives in which the three cysteine residues were alkylated with 3,2′-dicarboxy-4′-(iodoacetamido)azobenzene (Lusty & Fasold, 1969) and with iodoacetamide (Bridgen, 1972). Alkylation of Cys-374 has been reported to actually increase the rate of actin polymerization (Tait & Frieden, 1982).

The three modified cysteine residues have unique microenvironments within the G-actin protein, since each ^{19}F resonance has a different chemical shift and line width. The chemical shifts of the TFA-Cys resonances are affected by ring current effects, van der Waals interactions, static electrical fields, and specific bonding interactions, including hydrogen bonding and ionic interactions between the ^{19}F nucleus and the water or appropriate groups on the protein (Emsley & Phillips, 1971). These interactions are obviously different for each of the three TFA-Cys residues. While interpretation of the chemical shifts in terms of specific interactions is not possible, downfield shifts correlate in general with a more buried ^{19}F nucleus in closer contact with more of the protons in the protein (Sykes & Weiner, 1980). (For example, the chemical shift of the TFA-Cys residues of denatured G-actin was found to be −10.1 ppm.) Thus, TFA-Cys-374 is likely in a more hydrophobic environment than the other two residues, since its ^{19}F resonance is the furthest downfield.

^{19}F chemical shifts are affected by the solvent used. Solvent-exposed fluorine-containing moieties exhibit a maximum change in chemical shift when the solvent is changed from H_2O to D_2O (typically 0.1–0.3 ppm upfield), while protein-buried moieties exhibit little or no change in chemical shift (Sykes & Weiner, 1980; Gerig, 1978). This solvent-induced isotope shift (SIIS) was used to assess the degree to which each TFA-Cys residue was exposed or buried within the G-actin protein. Table I shows that the ^{19}F resonances at −8.2 and −9.4 ppm exhibited a SIIS of 0.14 ppm while those at −9.0 and −9.3 ppm exhibited a SIIS of 0.09 ppm. The resonance at −8.2 ppm represents free 3-hydroxy-1,1,1-trifluoropropan-2-one, which must be fully exposed to the solvent. Since the TFA-Cys residue at −9.4 ppm exhibited the same SIIS, it is likely also fully exposed to the solvent. The two TFA-Cys residues at −9.0 (TFA-Cys-374) and −9.3 ppm exhibited diminished SIIS's and are likely more buried within the G-actin protein structure.

The three ^{19}F resonances of the TFA-Cys residues in fluorinated G-actin had significantly different line widths, again indicating different microenvironments for each residue. Two major T_2 relaxation mechanisms contribute to the observed ^{19}F line width within a protein, chemical anisotropy (CSA) and dipole–dipole (DD) interactions (Hull & Sykes, 1975). DD interactions may occur between the ^{19}F nucleus and the other two ^{19}F nuclei in the TFA moiety, nonexchangeable protons on the protein, and exchangeable protons in the solvent or on the protein. Since the observed line widths in 15% D_2O were not measurably broader than those in 85% D_2O (Table I), DD interactions involving exchangeable protons contribute negligibly to the final $1/T_2$ relaxation rate. However, Table I shows that the T_1's in 15% D_2O were shorter than in 85% D_2O, indicating that DD interactions between the ^{19}F nuclei and exchangeable protons contribute about 30% to the final $1/T_1$ relaxation rate. A detailed analysis of the contributions of the remaining interactions to the overall observed line width requires a priori knowledge of the chemical shift tensor elements for the TFA-Cys residue, distances between interacting nuclei, and the orientation and effective correlation times for each interaction (Hull & Sykes, 1975). Since there are six bonds between the ^{19}F nuclei of the TFA-Cys residue and the α-carbon of the polypeptide backbone, multiple links of internal rotation are possible, resulting in a shorter effective correlation time and narrower observed line widths (London, 1980).

While an unambiguous analysis of the ^{19}F resonance line widths in G-actin is not possible, the change in the line widths following polymerization to F-actin does yield information about residues 10, 284, and 374. The ^{19}F resonances at −9.3 and −9.4 ppm, corresponding to TFA-Cys-10 and -284, were not appreciably broadened following polymerization, indicating that the effective correlation times for residues 10 and 284 were not significantly altered. These two cysteine residues are thus not involved in or affected by the polymerization process. On the other hand, the ^{19}F resonances at −9.0 ppm, corresponding to TFA-Cys-374, was dramatically broadened from a line width of 35 Hz in G-actin to an undetectably broad resonance in F-actin (line width >300 Hz). The only parameter that could cause this large increase in observed line width is an increase in the effective correlation time of the TFA moiety due to decreased mobility in F-actin. Cys-374 may be immobilized as a result of the interaction between actin subunits in the F-actin assembly. If Cys-374 were directly involved in this interaction, one would expect that the modification of this residue would result in some inhibition of polymerization. In fact, fluorinated G-actin remains 100% polymerizable. Cys-374 may be close enough to the contact point between F-actin subunits to be immobilized, but it is not actually directly

[2] Milder reaction conditions for the fluorination of G-actin resulted in the appearance of only one ^{19}F resonance at −9.0 ppm. Polymerization of this derivative in either 50 mM KCl or 2 mM $MgCl_2$ resulted in the total loss of any observable resonance (data not shown).

involved in the binding between F-actin subunits. The immobilization of TFA-Cys-374 may alternatively involve some conformational change within the protein rather than simply the steric blocking action between F-actin monomers. Such a conformational change independent of actual F-actin formation has been reported, i.e., the "F-actin monomer" (Rich & Estes, 1976) and G* monomer (Rouayrenc & Travers, 1981), although others have disputed the existence of this conformational change (Curmi et al., 1982).

In electron spin resonance studies, a variety of spin-labels attached to Cys-374 has been found to become immobilized when G-actin is polymerized (Sleigh & Burley, 1973; Tao, 1978). Fluorescence labels covalently bound to Cys-374 (Porter & Webec, 1979) or to the adjacent Lys-373 (Detmer et al., 1981) exhibit increased fluorescence intensity after polymerization of the actin. Similar results were obtained upon addition of K^+ or Mg^{2+} to dilute fluorescence-labeled G-actin solutions below their critical actin concentration, indicating that the Cys-374 site may be involved in a conformational change that occurs independent of the polymerization process (Frieden et al., 1980). Peptide maps of chemically cross-linked actin dimers showed Cys-374 in one subunit to be linked to and within 8 Å of Lys-191, -213, or -215 in the other subunit (Sutoh, 1984). Thus, it seems clear that the C-terminal region of actin is involved in subunit interactions to form the F-actin polymers and/or conformational changes occurring before polymerization.

The TFA-Cys-10 and -284 resonances were not appreciably broadened as a result of actin polymerization. These two residues are likely far from the contact points between actin subunits in F-actin and likely remain mobile within the polymer. Since these two resonances remain observable in F-actin, this fluorinated derivative should prove useful for NMR studies of F-actin and its interactions with metal ions, other F-actin filaments, tropomyosin, myosin, and other proteins that interact with F-actin.

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Registry No. BTFP, 431-35-6; NEM, 128-53-0; Cys, 52-90-4.

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