The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin

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Abstract

We report here that in vitro exposure of monomeric actin to hydrogen peroxide leads to a conversion of 6 of the 16 methionine residues to methionine sulfoxide residues. Although the initial effect of H₂O₂ on actin is the oxidation of Cys374, we have found that Met44, Met47, Met176, Met190, Met269, and Met355 are the other sites of the oxidative modification. Met44 and Met47 are the methionyl sites first oxidized. The methionine residues that are oxidized are not simply related to their accessibility to the external medium and are found in all four subdomains of actin. The conformations of subdomain 1, a region critical for the functional binding of different actin-binding proteins, and subdomain 2, which plays important roles in the polymerization process and stabilization of the actin filament, are changed upon oxidation. The conformational changes are deduced from the increased exposure of hydrophobic residues, which correlates with methionine sulfoxide formation, from the perturbations in tryptophan fluorescence, and from the decreased susceptibility to limited proteolysis of oxidized actin.

Keywords: actin oxidation; conformational changes; cyanogen bromide; cysteine; methionine; surface hydrophobicity

It has been established in several different animal models, including man, that there is an exponential increase in the level of oxidized proteins during aging and exposure to oxidative stress, such as hyperoxia, forced exercise, ozone, ischemia-reperfusion, cigarette smoke, or chronic alcohol treatment. Accumulation of oxidized proteins is associated with a number of diseases, including rheumatoid arthritis, muscular dystrophy, amyotrophic lateral sclerosis, Alzheimer's disease, cataractogenesis, respiratory distress syndrome, progeria, and Werner's syndrome (for a review, see Stadtman & Berlett, 1998).

In vivo, oxygen-containing radicals are generated in a variety of cells (e.g., neutrophils), leading to the oxidation of several amino acids, including methionine, which, together with cysteine and tryptophan, belongs to the most easily oxidized amino acids. The oxidation of methionine results in the conversion of this thioether to its sulfoxide counterpart. This is a reversible reaction in which the Met residue can be regenerated either by reducing agents or

by the ubiquitous enzyme methionine sulfoxide reductase. The reversibility of methionine oxidation suggests that it could act as a cellular regulatory mechanism. It has also been proposed that methionine residues could constitute an important endogenous antioxidant defense mechanism for proteins (Levine et al., 1996). Surface exposed methionine residues create an extremely high concentration of reactant, available as an efficient oxidant scavenger. Thus, other residues within the critical regions of the protein would be protected. The existence of a repair mechanism by reduction of the methionine sulfoxide to methionine would allow the antioxidant system to function catalytically.

The oxidation of methionine to its sulfoxide derivative is often concomitant with a complete inactivation or a decrease in the biological activity of proteins. However, there are numerous examples of sulfoxide formation of specific Met residues in proteins having little or no effect in structure or activity or leading to an increase in biological activity (for a review, see Vogt, 1995).

Cytoskeletal alterations as well as damage in cytoskeletal protein thiols observed, for example, in rat hepatocytes, human red blood cells, and P388D₁ murine cell line exposed to oxidative stress, suggest that cytoskeletal elements represent one of the cellular targets of biological oxidants (Hinshaw et al., 1988; Mirabelli et al., 1988; Lii & Hung, 1997).

A dynamic network of actin filaments is one of the most prevalent features of the cytoskeleton. In cultured cells, many of the observed alterations have been associated with hydrogen peroxide-

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Abbreviations: G-actin, monomeric actin; F-actin, filamentous (polymerized) actin; MetSO, methionine sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl) ethylenediamine; ANSA, 8-anilino-1-naphthalene sulfonic acid; NTCB, 2-nitro-5-thiocyanobenzoate.

induced oxidative modifications of actin (Hinshaw et al., 1991; Omann et al., 1994; Mocali et al., 1995). In endothelial cells, hydrogen peroxide (H₂O₂) induces the rapid formation of focal adhesion complexes at the ventral face of the cells and a major reorganization of the actin cytoskeleton, including sustained recruitment of actin to the cytoskeleton and actin filament rearrangement, with disruption of the dense peripheral bands and formation of stress fibers (Huot et al., 1998; Zhao & Davis, 1998).

Previous studies demonstrated that oxidative conditions alter the kinetics of actin polymerization (DalleDonne et al., 1995, 1999). It has been exhaustively demonstrated by chemical or proteolytic modifications of purified actin that the correct conformation in the local or global structure of actin is necessary for the correct interaction between adjacent subunits in actin filaments as well as between actin and actin binding proteins (Hegyi et al., 1974; Malm, 1984; Schwyter et al., 1989; Crosbie et al., 1991; Khaitlina et al., 1993). The present study examines the oxidative modification of actin by hydrogen peroxide, with the purpose of establishing the susceptibility to oxidation of cysteine, methionine, and tryptophan residues, the three preferred targets of oxidizing compounds. Furthermore, it investigates whether oxidation of one or more of such oxidizable amino acids residues induces alterations of the three-dimensional (3D) structure of the protein.

Results

Methionine residues are remarkable for their high susceptibility to oxidation by a variety of physiologic and nonphysiologic systems, with the product generally being methionine sulfoxide (MetSO). Hydrogen peroxide is a relatively nonspecific oxidizing agent that has been extensively used for the specific oxidation of Met residues in proteins under low pH conditions. However, H₂O₂ has been shown to be an effective methionine oxidant at neutral or slightly alkaline pH for a variety of proteins (e.g., Teh et al., 1987).

Since actin in the mature form contains 16 methionine residues (Table 1), its susceptibility to Met oxidation when treated with H₂O₂ was investigated. The formation of MetSO may induce conformational changes in protein structure leading to an exposure of hydrophobic residues (Vogt, 1995). This possibility is supported by results of recent studies showing that the oxidation of Met residues in *Escherichia coli* glutamine synthetase leads to extensive changes in protein conformation (Berlett et al., 1996). Among

the techniques aimed at following conformational changes of proteins, binding of the fluorescent probe ANSA has been used to detect accessibility of hydrophobic regions on actin. Binding can be easily monitored since it is accompanied by an increase in fluorescence and a blue shift of the emission maximum associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment. Therefore, to test the hypothesis that H₂O₂ could induce conformational changes in monomeric actin, we measured the hydrophobicity of G-actin upon treatment with 1–5 mM H₂O₂. As revealed by an increase in the ANSA fluorescence, H₂O₂-oxidized G-actin binds ANSA (Fig. 1). The increase in hydrophobicity was quite significant (about 30%) after treatment with H₂O₂ for 1 h. As the exposure time to the oxidant was increased, the increase in surface hydrophobicity become dramatic, particularly at the higher levels of oxidation upon modification by 5 mM H₂O₂. There is then increased exposure of hydrophobic residues when going from native actin to oxidized actin.

The increase in surface hydrophobicity correlates with an increase in MetSO formation (Chao et al., 1997). The H₂O₂ oxidation of actin Met residues was investigated by partial cyanogen bromide cleavage. CNBr treatment causes cleavage of proteins at the level of their methionines, but not if Met residues are oxidized to MetSO. The SDS-PAGE patterns of fragments generated by the CNBr fragmentation of actin after prolonged exposure to H₂O₂ revealed that H₂O₂ oxidizes some Met residues (Fig. 2A). However, given the large number of Met residues in the actin molecule, the partial CNBr treatment generates a lot of fragments. This determines a very complex SDS-PAGE pattern that does not permit the unequivocal identification of fragments.

Oxidized Met residues were then identified by CNBr treatment of G-actin labeled at Cys374 with 1,5-IAEDANS; consequently,

Table 1. Rabbit skeletal muscle actin primary structure^a

DEDETTALV	C10	D	N	G	S	G	L	V	K	A	G	F	A	G	D	D	A	P	R	A	V
F	P	S	I	V	G	R	P	R	H	Q	G	V	M44	V	G	M47	G	Q	K	D	S
S	K	R	G	I	L	T	L	K	Y	P	I	E	H	G	I	T	N	W79	D	D	M82
T	F	Y	N	E	L	R	V	A	P	E	E	H	P	T	L	L	T	E	A	P	L
T	Q	I	M123	F	E	T	F	N	V	P	A	M132	Y	V	A	I	Q	A	V	L	S
T	G	I	V	L	D	S	G	D	G	V	T	H	N	V	P	I	Y	E	G	Y	A
L	A	G	R	D	L	T	D	Y	L	M190	K	I	L	T	E	R	G	Y	S	F	V
D	I	K	E	K	L	C217	Y	V	A	L	D	F	E	N	E	M227	A	T	A	A	S
Y	E	L	P	D	G	Q	V	I	T	I	G	N	E	R	F	R	C257	P	E	T	L
E	S	A	G	I	H	E	T	T	Y	N	S	I	M283	K	C285	D	I	D	I	R	K
M299	S	G	G	T	T	M305	Y	P	G	I	A	D	R	M313	Q	K	E	I	T	A	L
K	I	K	I	A	P	P	E	R	K	Y	S	V	W340	I	G	G	S	I	L	A	S
W356	I	T	K	Q	E	Y	D	E	A	G	P	S	I	V	H	R	K	C374	F		

^aIndividual amino acid residues are indicated with the single-letter code. Positions of methionine, tryptophan, and cysteine residues are evidenced.

Fig. 1. Determination of G-actin surface hydrophobicity by ANSA binding. After treatment with 1 mM (open squares) and 5 mM (open circles) H₂O₂ for various times and removal of the excess of H₂O₂, the change in fluorescence intensity associated with the binding of ANSA to actin surface hydrophobic regions was measured at 480 nm with λ_{ex} set at 370 nm, as described under Materials and methods. The ANSA-dependent fluorescence intensity of the nonoxidized actin was taken as a point of reference: we have arbitrarily set this value as 1.0 (arbitrary unit, a.u.). Surface hydrophobicity of all other samples is expressed as values with respect to the control actin reference value. Error bars represent the standard deviations of three independent experiments.

Fig. 2. Cleavage of actin at methionine residues by cyanogen bromide. After treatment with H₂O₂ and removal of the excess of the oxidizing agent, both (A) unlabeled G-actin and (B) G-actin labeled at Cys374 with 1,5-IAEDANS was cleaved at Met residues with 1% (w/v) CNBr, in the presence of 0.1% SDS. The cleavage reaction was allowed to proceed for 15 min at 25 °C and then samples were lyophilized. SDS-PAGE analysis of undigested actin (lanes U), control (nonoxidized) actin (lanes C), actin fragmented after oxidation with 1 mM H₂O₂ for 1, 3, or 16 h; actin fragmented after oxidation with 5 mM H₂O₂ for 1, 3, or 16 h. Lanes F show the polypeptide bands of CaCl₂-polymerized Ca-F-actin generated after a 40 min digestion with subtilisin at an enzyme-to-protein mass ratio of 1:200. Lanes Cys show the actin polypeptide bands obtained after chemical cleavage at Cys residues with NTCB. On the left of both panels, segments derived from subtilisin digestion and NTCB cleavage are indicated. A: Coomassie Blue staining of actin polypeptide bands. B: Fluorescence profiles of polypeptide bands generated from AEDANS-actin.

the fluorescent CNBr fragments generated from the fluorescent actin monomer necessarily contain the C-terminus. However, to assign these fluorescent fragments in the actin sequence, we needed some “sequence markers,” obtained by cleavage of AEDANS-Ca-F-actin with subtilisin (Fig. 2B, lane F) or AEDANS-Ca-G-actin with NTCB (Fig. 2B, lane Cys). On the basis of the information from sequence analysis, the identification of fragments containing the C-terminus, and the apparent molecular masses of the fragments, the products of subtilisin digestion were clearly aligned within the primary structure of actin (Strzelecka-Golaszewska et al., 1996). In particular, the C-terminal fragment spanning residues 48–375, with an apparent molecular weight of 35K, derives from the splitting between Met47 and Gly48. NTCB selectively reacts with cysteine residues and cleaves peptide chains (Jacobson et al., 1973). When AEDANS-actin was treated with NTCB to cleave the polypeptide chain at Cys residues (Cys10, Cys217, Cys257, and Cys285), three fluorescent fragments were generated (Fig. 2B, lane Cys). From their apparent molecular weights, these fragments were identified as fragments spanning residues 217–374, residues 257–374, and residues 285–374. The product of cleavage at Cys10 would co-migrate with intact actin under the electrophoresis conditions used. Figure 2B shows that prolonged exposure of AEDANS-actin to 1 mM H₂O₂ as well as treatment with 5 mM H₂O₂ cause oxidation of some Met residues. The partial or total absence of the 35 kDa fragment, which is the highest molecular weight product that results after cleavage by CNBr at positions 44 and/or 47, indicates that oxidation of both Met44 and Met47 occurred. Met269 and Met355 were also found to be affected by exposure to the oxidant. At the highest level of treatment, Met176, Met190, and Met269 were completely oxidized to the sulfoxide form. Furthermore, the reduction of the diffusion of the low molecular weight broad band, generated by cleavages of AEDANS-actin at Met299, Met305, Met313, and Met325, would indicate that one or more of these Met residues has been oxidized to at least some degree.

Actin contains five cysteine residues (Cys10, Cys217, Cys257, Cys285, and Cys374; Table 1) existing in the reduced form (Elzinga et al., 1973). The native protein exposes only one thiol

group, that of quickly reacting Cys374, located at the C-terminus (Takashi, 1979). To examine the effect of H₂O₂ on actin thiol groups, the number of remaining SH groups in the oxidized protein was determined. When reacted with Ellman’s reagent, the number of exposed Cys residue(s) in folded actin diminished from about 1 in native G-actin to about 0.5 in G-actin oxidized for a longer time and/or with the higher (5 mM) H₂O₂ concentration (not shown). As expected, we found about five Cys residues per unfolded monomer by titration with DTNB in the presence of 6 M GdmCl. In oxidized G-actin solutions, the number of Cys residues per unfolded monomer decreased from five to about four (not shown). These observations indicate that oxidation of actin thiol groups produced by H₂O₂ involves only Cys374.

Tryptophan residues are among the preferred targets for the attack of oxidizing compounds, resulting in the oxidation of the indole ring (e.g., Ray et al., 1995). Therefore, experiments were done to test for tryptophan oxidation by H₂O₂. Monitoring the possible loss of tryptophans by the change in the absorbance spectrum at 280 nm (Matsushima et al., 1980; Ray et al., 1995), we did not observe the characteristic UV absorption spectrum associated with tryptophan oxidation (not shown).

As intrinsic fluorescence emission of proteins is highly sensitive to changes in the environment of the chromophore, fluorescence is an excellent spectroscopic probe to investigate conformational changes of proteins. Changes in protein conformation, such as unfolding, very often lead to large changes in fluorescence emission. In proteins that contain tryptophan, both shifts in wavelength and changes in intensity are generally observed upon unfolding. In the present study, we have used tryptophan intrinsic fluorescence as a probe for alterations in G-actin conformation induced by H₂O₂, as suggested by the increased surface hydrophobicity. Tryptophan emission spectra (Fig. 3) reveal a reduction of the fluorescence intensity of oxidized G-actin compared to that of native protein, without the spectral red shift that is usually connected with the accessibility of the Trp residue to the solvent molecules. In particular, a loss of 15–25% in tryptophan fluorescence was ob-

Fig. 3. Intrinsic fluorescence emission spectra of G-actin. Tryptophan fluorescence emission spectra of both native and actin oxidized with (A) 1 mM and (B) 5 mM H_2O_2 were taken with λ_{ex} set at 297 nm after removal of the oxidant. In both A and B, G-actin exposed to the oxidant for 1 h (thin solid trace), 3 h (long dashed trace), or 16 h (short dashed trace); the thick solid trace is the fluorescence intensity of nonoxidized G-actin.

served upon exposure to 1 mM H_2O_2 for 1–16 h, respectively, while treatment with 5 mM H_2O_2 led to a 30% decrease in the tryptophan fluorescence.

Limited proteolysis is a valuable tool for probing the tertiary structure of actin. We used proteolytic susceptibility as a probe for identifying regions of the actin polypeptide chain where structural rearrangements took place as a consequence of the oxidation produced by H_2O_2 . Proteases with different specificity, subtilisin, α -chymotrypsin, and trypsin, were used, which allows for potential cleavage in different regions of the protein. Time courses of cleavage by subtilisin of the peptide bond between Met47 and Gly48 to produce a 35 kDa C-terminal fragment (Schwyter et al., 1989) are illustrated in Figures 4A and 4B. Oxidation of G-actin results in a decrease in the rate of protein digestion. A similar decrease in the digestion rate was observed during proteolysis by α -chymotrypsin at Met44 and Val45 (not shown). The decrease in the rate of proteolysis by subtilisin and α -chymotrypsin could be due to different rates of proteolysis by the enzymes because the oxidized Met residues are immediately adjacent to the proteolytic sites. Therefore, proteolytic digestion of oxidized vs. nonoxidized G-actin was performed using denatured actin as the substrate, i.e., in the presence of low concentrations of SDS, where both enzymes keep their activity (Allen, 1989; Carrey, 1990) and accessibility is not an issue. The activity of subtilisin and α -chymotrypsin was the same whether digestion was carried out on oxidized or nonoxidized G-actin (not shown). Another alternative explanation for the reduction in proteolysis is that increased surface hydrophobicity may cause oxidized G-actin to aggregate, limiting access to proteases. We checked for aggregation by both sedimentation and nondenaturing electrophoresis, finding no aggregates in the oxidized samples (not shown). Therefore, H_2O_2 -induced oxidation renders the subtilisin, as well as the α -chymotrypsin-cleavage site less accessible, resulting in a decreased protease susceptibility of the surface loop 38–52 in subdomain 2 of the actin polypeptide chain. In contrast, oxidized samples displayed no significant change in their susceptibility to proteolysis by trypsin, which is known to cleave G-actin at Arg62 and at Lys68 (data not shown). Normal-mode

Fig. 4. Time courses of decay of nonoxidized and H_2O_2 -oxidized G-actin during enzymatic digestion with subtilisin. Subtilisin digestions of G-actin (A, B) and actin blocked at Cys374 with 1,5-IAEDANS (C, D) were carried out at an enzyme-to-protein mass ratio of 1:1,500. The concentration of undigested actin is expressed as percentage of initial actin concentration. Nonoxidized actin (filled circles), actin exposed to 1 mM (A, C) or 5 mM (B, D) H_2O_2 for 1 h (open triangles), 3 h (open squares), or 16 h (open circles). Error bars represent the standard deviations of three replicate measurements.

analysis of the actin structure predicts considerable movements of subdomains and individual loops in the molecule (Tirion et al., 1995). Subdomain 2 and its DNase-I-binding loop are the most mobile regions of the molecule (reviewed in Egelman & Orlova, 1995). Our observation that oxidation produced by H_2O_2 renders the loop 38–52 less susceptible to proteases suggests that in H_2O_2 -treated actin this region assumes a more compact (less mobile) structure compared to native actin. However, the structural perturbation of subdomain 2 has also been observed in actin oxidized only at Cys374 (DalleDonne et al., 1999). Therefore, to examine whether methionine oxidation may contribute, at least partly, to the H_2O_2 -induced structural alterations, we probed the conformation of the segment 38–52 by limited proteolysis of actin oxidized only at Met residues. For this purpose, actin was first labeled with 1,5-IAEDANS to block Cys374, and then exposed to the oxidant. When Met-oxidized actin was subjected to limited proteolysis by subtilisin, inhibition of proteolytic cleavage was still observed (Fig. 4C,D), suggesting that Met oxidation may have a protective effect on the DNase-I-binding loop against protein digestion. The pattern of fragmentation of oxidized actin by α -chymotrypsin also confirms the inhibitory effect of Met conversion to MetSO on the accessibility of the DNase-I-binding loop (not shown).

Digestion with trypsin can also be used to probe conformational transitions in the C-terminal region of actin, since the enzyme removes the three C-terminal residues in Ca-G-actin as a result of cleavage at Lys373 and Arg372. Proteolytic removal of the tripep-

tide Lys373–Cys374–Phe375 from the actin polypeptide chain can be conveniently monitored by using actin labeled with 1,5-IAEDANS at Cys374 and following the decrease in its fluorescence intensity, as the presence of the AEDANS probe does not affect the cleavage rate (Mossakowska et al., 1993). The results presented in Table 2 indicate a decrease in the proteolysis rate by trypsin of the C-terminal segment of oxidized actin.

Discussion

We report here that mild oxidation of monomeric actin with hydrogen peroxide leads to a substantial increase in surface hydrophobicity, conversion of some, among the 16 methionine residues, to MetSO residues, and oxidation of the SH group of Cys374, next to the actin C-terminus. In addition, the conformations of subdomains 1 and 2 are changed upon oxidation.

X-ray diffraction analysis of 3D crystals of skeletal muscle G-actin complexed with DNase I (Kabsch et al., 1990) revealed that the monomer consists of two domains, for historical reasons called large and small, although they turn out to be of almost the same size, with the nucleotide (i.e., ATP or ADP) and its associated divalent cation (Ca^{2+} , following standard *in vitro* preparation, or Mg^{2+} , under physiological conditions) bound in the cleft between the two domains. Each domain can be further subdivided into two subdomains (Fig. 5A). Subdomain 1 (residues 1–32, 70–144, 338–372) and subdomain 2 (residues 33–69) constitute the “small” domain, whereas subdomain 3 (residues 145–180, 270–337), and subdomain 4 (residues 181–269) constitute the “large” domain (Kabsch et al., 1990). The exact structure of the three C-terminal residues (Lys373, Cys374, Phe375) is still uncertain: in fact, their location in the actin-DNase I complex was modeled, being they removed by tryptic digestion before crystallization (Kabsch et al., 1990). In the case of G-actin, the C-terminal segment is exposed on the surface of the molecule, accessible to solvent and highly mobile. Upon polymerization, it adopts a different conformation that leaves Cys374 solvent-accessible (Milligan et al., 1990; Orlova & Egelman, 1995).

The actin molecule contains 16 Met residues located at positions 44, 47, 82, 119, 123, 132, 176, 190, 227, 269, 283, 299, 305, 313, 325, and 355, some of which are partially exposed (with different degrees of exposure), whereas others are completely buried within the polypeptide chain (Fig. 5B). Comparison of the CNBr cleavage patterns for nonoxidized and oxidized AEDANS-actin has permitted the identification of six methionyl residues converted to sulfoxide. Although the initial effect of H_2O_2 on actin is the oxidation of Cys374, we have found that Met residues at positions 44, 47, 176, 190, 269, and 355 are the other sites of the oxidative modification following actin exposure to hydrogen per-

oxide. The oxidized Met residues are found in all four subdomains of actin. Met44 and Met47, in subdomain 2, are the methionyl sites first oxidized. Analysis of the methionine relative accessibility in the actin monomer, done on the basis of atom coordinates of the actin-DNase I complex (Kabsch et al., 1990), revealed that six Met residues (i.e., Met82, Met119, Met123, Met132, Met299, and Met313) are completely buried within the actin molecule. The relative solvent accessibility of the other partially exposed Met residues resulted in the following: Met47 > Met355 > Met44 > Met227 > Met283 > Met269 > Met325 > Met176 > Met305 > Met190 (Fig. 5B). Our results indicate that the H_2O_2 -induced oxidative modification of individual methionines is not directly related to their solvent exposure, suggesting alterations in the tertiary structure of actin provoked by Met oxidation. Moreover, the slow time course, i.e., 16 h, of the oxidation reactions seems to suggest that such structural rearrangements must be necessary to oxidize some of the partially exposed methionines.

The demonstration that actin surface hydrophobicity, which correlates with MetSO formation, increases with exposure to H_2O_2 indicates changes in protein conformation (Vogt, 1995). As a measure of hydrophobicity, the increase in fluorescence associated with the binding of ANSA to hydrophobic regions of actin was used. Binding of ANSA could be related to an increase in the flexibility of the protein exposing hydrophobic clusters on the molecule surface allowing ANSA binding.

Conformational rearrangements triggered by H_2O_2 were also deduced from the changes in Trp fluorescence and proteolytic susceptibility. Increasing actin oxidation is accompanied by a decrease in the tryptophan fluorescence intensity, without the spectral red shift characterizing denatured actin. The fluorescence spectrum of native actin is a relatively blue one ($\lambda_{\text{max}} = 322 \text{ nm}$). All of four actin Trp residues (Trp79, Trp86, Trp340, and Trp356) are confined to subdomain 1. Trp79 and Trp86 are in α -helix 74–92, Trp340 in α -helix 338–348, and Trp356 in the loop 355–359, the last being in a flexible region of the structure (Kabsch et al., 1990). As actin tryptophans are not oxidized by H_2O_2 , the lowering of intrinsic fluorescence observed after H_2O_2 treatment reflects structural alterations in the conformation of subdomain 1 of the actin molecule. The structural changes induced by H_2O_2 could be better localized if we define the contribution of each of four Trp residues to intrinsic fluorescence. The analysis of the 3D structure of the actin molecule shows that the density of the microenvironment of separate Trp residues varies greatly. Trp340 and Trp356 have a very high density of microenvironment. Although they are located close to the periphery of the actin molecule, they are apparently inaccessible to the solvent; therefore, their fluorescence spectra must be blue. The packing density of the microenvironment of Trp86 is lower than that of Trp340 and Trp356. At the same time,

Table 2. Removal from G-actin of AEDANS-labeled Cys374 during digestion with trypsin^a

	Control	1 mM H_2O_2			5 mM H_2O_2		
Duration of treatment	—	1 h	3 h	16 h	1 h	3 h	16 h
Digestion rate ($\mu\text{M s}^{-1}$)	0.265	0.261	0.266	0.148	0.263	0.165	0.111
	(± 0.03)	(± 0.04)	(± 0.03)	(± 0.05)	(± 0.03)	(± 0.04)	(± 0.03)

^aControl and oxidized AEDANS-actins were digested in the fluorescence cuvettes at a trypsin:actin mass ratio of 1:50. Errors represent the standard deviations of three replicate measurements.

Fig. 5. Molecular model of monomeric actin. **A:** A view of the X-ray crystal structure of actin (PDB Id: 1ATN), in which the protein backbone is depicted as a ribbon. **A:** Different actin subdomains are represented in yellow (subdomain 1), turquoise (subdomain 2), magenta (subdomain 3), and green (subdomain 4). Methionine residues are drawn in a space-filling representation and are labeled with the single-letter code and their sequence position number in rabbit skeletal muscle actin. **B:** The ribbon diagram of actin and methionine residues, drawn in a space-filling representation, are colored in accordance with their relative accessibility to the solvent, defined as described in Materials and methods. Dark blue color is attributed to completely buried methionines, whereas dark orange color is attributed to residues with at least 70% of their relative surface accessibility. H_2O_2 -oxidized methionines are labeled with the single-letter code and their sequence position number in rabbit skeletal muscle actin. In both **A** and **B**, ATP and Ca^{2+} are drawn in a space-filling representation and are colored white and red, respectively. The N- and C-terminus of the actin polypeptide chain are indicated.

this Trp residue is located far from the protein periphery, and it is also inaccessible to the solvent. Thus, the only Trp residue that can be regarded as exposed to the solvent is Trp79. In addition, there are no quenching groups in the vicinity of Trp340 and Trp356, while microenvironments of both Trp79 and Trp86 contain a number of sulfur atoms, which are known to be effective fluorescence quenchers (Kabsch et al., 1990; McLaughlin et al., 1993). Hence, the fluorescence spectrum of G-actin must mainly be formed by the buried blue spectrum residues Trp340 and Trp356 (Kuznetsova et al., 1996; Turoverov et al., 1999). If Trp340 and Trp356 determine actin fluorescence, we could conclude that the H_2O_2 treatment induces structural alterations in helix 338–348 and/or in the loop 355–359 within subdomain 1 of the actin molecule. The decrease in the susceptibility to proteolysis by trypsin in the carboxyl-terminus seems also to reflect a conformational rearrangement within subdomain 1. Nevertheless, we cannot rule out the possibility that the oxidation of Met355, the second most solvent accessible Met, could affect, at least partly, the fluorescence of Trp356 and/or proteolysis at Lys373.

A decrease in the rate of proteolysis by subtilisin and α -chymotrypsin in the loop 38–52 on the top of subdomain 2 of actin was observed for the H_2O_2 -treated actin compared with the nonoxidized protein, probing structural differences between the various forms of actin. Proteolytic susceptibility changes significantly with the extent of methionine oxidation, confirming that the oxidative modification of actin Met residues has structural consequences within the monomer. Refinements of the atomic models of F-actin

(Lorenz et al., 1993; Tirion et al., 1995) have pointed out that subdomain 2, whose surface (i.e., residues 38–52) is implicated in the formation of subunit–subunit contacts, rotates and shifts toward subdomain 4 during polymerization of Ca-ATP-G-actin, inducing a reorientation of loop 38–52 relative to the G-actin:DNase I crystal structure. Therefore, the alterations in the tertiary structure of the oxidized monomer might have repercussions in the surface loops participating in the intermonomer interactions, and/or might influence the relative orientation of the domains or subdomains of actin. In fact, we have previously showed that oxidized G-actin has a lower capability to polymerize (DalleDonne et al., 1995, 1999).

The structural coupling between subdomain 2 and the C-terminus in G-actin has been shown by a number of studies (reviewed in Moraczewska et al., 1999). In particular, there is both intra- and intermonomer coupling between the DNase-I-binding loop and the C-terminal region. The intramonomer coupling is allosteric, because of the large distance between the two regions, and works in both directions (Khaitlina et al., 1993; Crosbie et al., 1994). The high mobility of the DNase-I-binding loop and the C-terminus and the intra- and intermolecular relationship between them are assumed to be essential for the proper functioning of the actin filament.

Furthermore, the bottom edge of the actin molecule and especially subdomain 1, where the C-terminal segment and Trp residues are located, is a region critical for the functional binding of different actin-binding proteins in both the G and F forms of actin (Kabsch & Vandekerckhove, 1992). Structural changes in sub-

domain 1 and in the surface loop 38–52 are expected to destroy or attenuate the interactions between actin and actin binding proteins, leading eventually to disorganization of the microfilaments, since it is through their association with actin that actin binding proteins modulate the actin polymerization dynamics, thus allowing remodeling of actin cytoskeleton in response to intracellular or extracellular stimuli, such as oxidative stress.

The question is whether actin methionine oxidation would occur in vivo. With the exception of $O_2^{\bullet-}$, all the biologically synthesized free radical species that play crucial roles in oxidative stress and tissue injury oxidize methionine residues to methionine sulfoxide. Considering the observation that exposure of cells to oxidative stress leads to alterations in the structure of the actin cytoskeleton and the here firstly described alterations in the molecular structure of actin following oxidative modification of Met residues, we cannot rule out the possibility that such oxidation can occur physiologically.

Materials and methods

Reagents

N-Iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS), 2-nitro-5-thiocyanobenzoate (NTCB), 8-anilino-1-naphthalene sulfonic acid (ANSA), subtilisin Carlsberg (EC 3.4.21.62, from *Bacillus licheniformis*), trypsin (EC 3.4.21.4, tosylphenylalanine-chloromethane-treated, type VII: from bovine pancreas), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and ATP (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Cyanogen bromide (CNBr) was purchased from Aldrich Chemical (Milan, Italy). All other reagents were of analytical grade.

Actin preparation

Monomeric actin (molecular mass 42.3 kDa) was prepared from rabbit skeletal muscle as described previously (DalleDonne et al., 1999). Prior to use, G-actin solutions were clarified by a 60 min ultracentrifugation at $110,000 \times g$; protein solutions and buffers were then filtered with $0.20 \mu\text{m}$ disposable filters. Actin concentration was determined by measuring the absorbance at 290 nm, using an extinction coefficient of $0.617 \text{ mg}^{-1} \text{ mL cm}^{-1}$ (Gordon et al., 1976). The experiments were executed in buffer A (2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl_2 , 1.5 mM NaN_3), at 25 °C, unless stated otherwise.

Modification of actin Cys374 with

N-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine

Actin fluorescently labeled at Cys374 with 1,5-IAEDANS was prepared according to Frieden et al. (1980). The concentration of label was determined from the absorbance at 337 nm using an extinction coefficient of $6,000 \text{ M}^{-1} \text{ cm}^{-1}$. Actin concentration was measured by the absorbance at 290 nm using an extinction coefficient of $0.63 \text{ mg}^{-1} \text{ mL cm}^{-1}$ and corrected for the absorbance due to the 1,5-IAEDANS by subtracting $0.21 A_{337}$ from A_{290} . The molar ratio of dye to actin in all preparations was at least 0.9.

Hydrogen peroxide treatment on actin monomers

Monomeric actin (5.9 μM) was treated for 1, 3, and 16 h with 1 and 5 mM H_2O_2 . The removal of H_2O_2 was accomplished

by exhaustive dialysis against buffer A. All the experiments were executed in the absence of H_2O_2 .

Determination of cysteine residues

Determination of both exposed and total Cys residues of actin monomers was performed by the Ellman (1959) assay. Actin monomers (1 mL) were added with 50 μL of 3 mM DTNB, which reacts with the free sulfhydryl side chain of cysteine to form an S–S bond between the protein and a thionitrobenzoate (TNB) residue. After 2 h incubation, the number of cysteines was determined by measuring the absorbance of the released TNB anion. To unfold G-actin and expose all its cysteine residues, actin monomers (1 mL) were lyophilized under vacuum and then dissolved in 6 M guanidine hydrochloride (GdmCl). After addition of DTNB and incubation for 15 min, the number of cysteines was determined by measuring the absorbance of the released TNB anion. Solutions of DTNB and GdmCl were prepared in 0.1 M phosphate buffer, pH 7.3. The molar absorbance of the TNB anion is $E_{412} = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$ in 6 M GdmCl and $E_{412} = 14.15 \text{ mM}^{-1} \text{ cm}^{-1}$ in its absence (Riddles et al., 1983).

Determination of actin surface hydrophobicity

Actin surface hydrophobicity was determined according to Chao et al. (1997). The fluorescence emission spectra (excitation 370 nm) were monitored from 400 to 600 nm. The increase in fluorescence intensity at 480 nm resulting from the binding of ANSA (200 μM in buffer A) to G-actin (4.7 μM) was evidenced by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the different forms (native and oxidized) of G-actin.

Enzymatic cleavage with subtilisin and trypsin

Time courses of subtilisin digestions of control and oxidized G-actin, both labeled and unlabeled, were carried out at an enzyme:protein mass ratio of 1:1,500; fragmentation of CaCl_2 -polymerized AEDANS-Ca-F-actin was obtained at a protease:protein mass ratio of 1:200 for 40 min. Digestions with subtilisin were stopped with 2 mM PMSF. Protein samples were then denatured by incubation for 5 min at $\sim 95^\circ\text{C}$ with 6x SDS-PAGE sample buffer and analyzed by SDS-PAGE (Laemmli, 1970) on 15% (w/v) polyacrylamide slab gels. The extent of digestion was determined by densitometry of the actin bands on SDS-PAGE.

Hydrolysis by trypsin of the peptide bond between Lys373 and Cys374 was monitored by using actin labeled at Cys374 with 1,5-IAEDANS and following the decrease in the AEDANS fluorescence intensity. Digestions of control and oxidized AEDANS-G-actin (2.38 μM) were carried out in the fluorescence cuvettes, at a trypsin:protein mass ratio of 1:50. Rates of removal from G-actin of AEDANS-labeled Cys374 were calculated within the first 3 min of reaction.

Enzymatic digestions under actin denaturing conditions tolerated by the enzymes were performed in the presence of SDS at 0.5% (w/v) in the case of subtilisin (Allen, 1989) and at 0.1% (w/v) in the case of α -chymotrypsin (Carrey, 1990).

Spectroscopic measurements

The tryptophan intrinsic fluorescence of G-actin was obtained by excitation at 297 nm. Intensity changes in the emission fluores-

cence were measured at all wavelengths ranging between 300 and 400 nm. The fluorescence excitation and emission wavelengths for AEDANS-actin were 340 and 460 nm, respectively.

Chemical cleavage at methionine residues

Actin samples were treated with 0.1% (w/v) SDS and, soon afterwards, with 0.1 volume of 10% (w/v) CNBr, dissolved in 70% formic acid. After 15 min at 25 °C, formic acid and excess reagent were removed by rotary evaporation. Lyophilized samples were dissolved in 0.1 M NaOH and added with 6× SDS-PAGE sample buffer for the electrophoretic run on 15% (w/v) polyacrylamide slab gels. We have identified which methionine residues are susceptible to oxidation on the basis of the reasonable assumption that the fragments produced by limited cleavage with CNBr will migrate on SDS-PAGE in order of molecular weight.

Chemical cleavage at cysteine residues

Lyophilized AEDANS-labeled actin was dissolved in the modification buffer (0.2 M Tris acetate, pH 8.0, 6 M GdmCl) and incubated for 1 h at 37 °C. It was mixed with NTCB (0.5 M in dimethylformamide) to 40-fold molar excess over sulfhydryl groups and incubated at 37 °C for 20 min. Cleavage of the modified protein was achieved by adjusting to pH 9.0 with NaOH solution and incubation at 37 °C for 2 h. The cleavage reaction was quenched by addition of excess -mercaptoethanol and the resulting solution was exhaustively dialyzed against water.

Molecular modeling and methionine relative accessibility

Molecular modeling and analysis of the relative accessibility of Met residues in the actin molecule were performed by using the molecular visualization program Swiss-PdbViewer, version 3.5 (Glaxo Wellcome Experimental Research). Figure was created on the basis of atom coordinates of the actin-DNase I complex (file 1ATN in the Protein Data Bank (PDB); Kabsch et al., 1990). It was assumed that the structures of free actin and actin in complex with DNase I are the same. Maximum methionine accessibility is defined as being the accessible surface of a methionine in a pentapeptide GGMGG in extended conformation. This is only an approximate scale, but perfectly sufficient to differentiate core methionines from surface ones.

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