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Hydrogen Peroxide Triggers the Formation of a Disulfide Dimer of Muscle Acylphosphatase and Modifies Some Functional Properties of the Enzyme*

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Acylphosphatase is expressed in vertebrates as two molecular forms, the organ common and the muscle types. The former does not contain cysteine residues, whereas the latter contains a single conserved cysteine (Cys-21). We demonstrated that H_2O_2 at micromolar levels induces, *in vitro*, the formation of a disulfide dimer of muscle acylphosphatase, which displays properties differing from those of the reduced enzyme. In particular, we observed changes in the kinetic behavior of its intrinsic ATPase activity, whereas the kinetic behavior of its benzoyl phosphatase activity does not change. Moreover, the disulfide dimer is capable of interacting with some polynucleotides such as poly(G), poly(C), and poly(T) but not with poly(A), whereas the reduced enzyme does not bind polynucleotides. Experiments performed with H_2O_2 in the presence of increasing SDS concentrations demonstrated that disulfide dimer formation is prevented by SDS concentrations higher than 300 μM, suggesting that a non-covalently-linked dimer is present in non-denaturing solvents. Light-induced cross-linking experiments performed on the Cys-21 → Ser mutant in the pH range 3.8–9.0 have demonstrated that a non-covalently-linked dimer is in fact present in non-denaturing solutions and that an enzyme group with a pK_a of 6.4 influences the monomer-dimer equilibrium.

Acylphosphatase (ACP)[1] is an enzyme widespread in all organisms; two isoenzymes, the muscle type (MT) and the organ-common type (CT), codified by two distinct genes, are expressed in vertebrates. The MT isoform is prevalently expressed in skeletal muscle and heart, whereas the CT isoform is prevalently expressed in erythrocytes, in brain, and in testis (1, 2). Several reports on the possible physiological roles of the enzyme have been produced: Some indicate ACP as an enzyme involved in the control of membrane ion pumps (Refs. 3, 4, and citations therein), because it displays hydrolytic activity against the aspartyl-phosphate intermediates formed during the action of Na^+,K^+- and Ca^{2+}-ATPases. Other reports involve ACP in cell differentiation and apoptosis (5–7); this is because the enzyme is overexpressed when cells are induced to differentiate by certain agents, and it is able to migrate into the nucleus during both differentiation and apoptosis (8, 9). The primary structure of the two isoenzymes differs in about 50% of amino acid positions, but they display very similar folds: Both consist of a five-stranded antiparallel twisted β-sheet flanked on one side by two antiparallel α-helices running parallel to the β-sheet (10). This peculiar ACP fold is very similar to that found in small RNA-binding domains of several RNA-binding proteins (11, 12); in fact, many RNA-binding proteins have modular structures consisting of RNA-binding modules and auxiliary domains that perform additional functions (13). This prompted us to investigate the involvement of ACP in polynucleotide processing. Chiarugi *et al.* (14) found that, *in vitro*, ACP catalyzes the hydrolysis of both DNA and RNA.

This paper deals with the induction of MT ACP to form a disulfide dimer in the presence of micromolar concentrations of H_2O_2 and reports a study on the biochemical properties of the modified enzyme. Severe oxidative insults, such as the one occurring during phagocytosis in leukocytes (15), drastically modify the cellular redox status by producing reactive oxygen species (ROS), which then react with protein thiol groups thereby producing structural and functional modifications.

MATERIALS AND METHODS

Benzoyl phosphate was synthesized according to Camici *et al.* (16). ATP and 30-mer poly-deoxyribonucleotides (poly(G), poly(A), poly(T), and poly(C)) were purchased from Sigma Chemical Co. Recombinant MT-ACP was expressed and purified as previously described (17). The Cys-21 → Ser mutant of MT-ACP was obtained by oligonucleotide-directed mutagenesis using a USE mutagenesis kit based on the unique site elimination (USE) method developed by Deng and Nickoloff (18). The mutations were confirmed by DNA sequencing according to Sanger *et al.* (19) and by amino acid analysis of the purified proteins. All other reagents were the purest commercially available.

Preparation of the Fully Reduced Form of ACP—The enzyme (0.4 μmol) in 0.1 M Tris-HCl buffer, pH 8.5, was concentrated to 0.5 ml by ultrafiltration using Centricon YM10 and mixed with 4.5 ml of 9 M freshly deionized urea. Then, solid dithiothreitol (0.5 M final concentration) was added, and the mixture was incubated overnight at 4 °C. The reduced enzyme was purified from low M_r reagents by Superdex-75 chromatography (2.6 × 60 cm column); the gel was previously equilibrated with 0.1 M sodium formate buffer, pH 3.5, containing 0.15 M NaCl. The elution was performed at a flow rate of 2 ml/min with the same buffer, and fractions of 3 ml were collected. The fractions containing the reduced enzyme were pooled, concentrated by ultrafiltration with YM10 Centricon, and conserved for weeks at −20 °C.

Enzyme Assays—The benzoyl phosphatase activity of ACP was assayed at 25 °C by a continuous optical test, using 5 mM benzoyl phosphate as substrate (20) (the final test volume was 1.5 ml). The ATPase activity of ACP was assayed at 37 °C by adding the enzyme to test

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[1] The abbreviations used are: ACP, acylphosphatase; MT, muscle type; CT, organ-common type; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoate); ROS, reactive oxygen species; GSSG, oxidized glutathione; GSH, reduced glutathione.

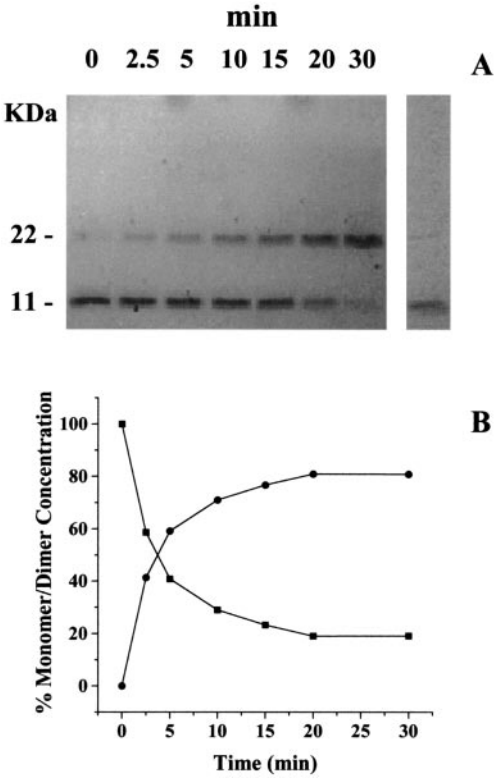


FIG. 1. **Hydrogen peroxide induces muscle ACP to form a disulfide dimer.** The reaction mixture, which contained 118 μM reduced enzyme in 0.1 M Tris-HCl buffer pH 8.0 and 160 μM H_2O_2, was incubated at 25 °C. At the indicated times, aliquots were taken and mixed with an equal volume of 2× non-reducing Laemmli sample buffer for SDS-PAGE analysis (*A*). Gel was stained by Coomassie Blue. *First lane* (0 min), control without H_2O_2; *other lanes*, time course formation of a covalently stabilized dimer. To test the nature of the band at 22 kDa, after 30-min incubation with H_2O_2, an aliquot of the mixture was mixed with an equal volume of 2× reducing Laemmli sample buffer, containing 1.4 M 2-mercaptoethanol (*last lane*). *B*, time courses of both dimer formation (22-kDa band) and disappearance of the monomeric reduced ACP (11-kDa band). The relative concentrations were measured by densitometry of the SDS-PAGE bands.

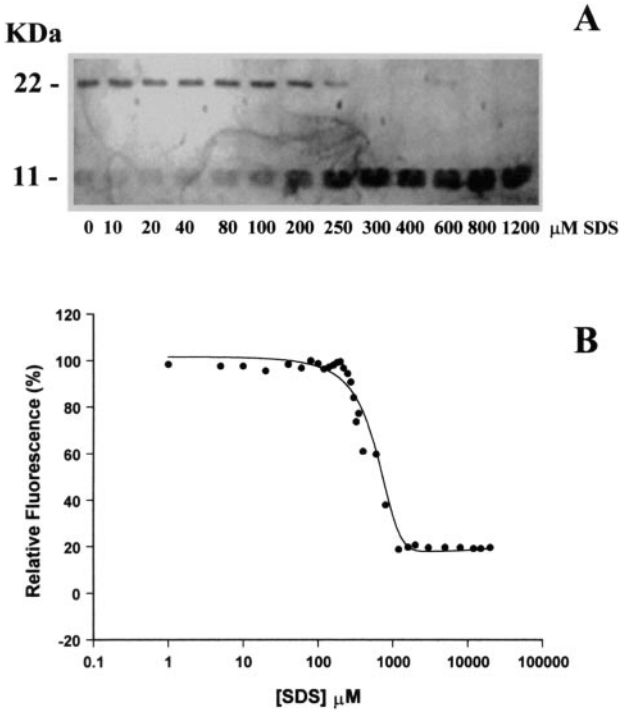


FIG. 2. **Increasing SDS concentration prevents ACP cross-linking by H_2O_2.** *A*, SDS-PAGE analysis of H_2O_2-induced cross-linking of ACP at increasing concentrations of SDS. The experimental conditions were the same as in Fig. 1, except that the gel was silver-stained. *B*, effect of SDS on intrinsic tryptophan fluorescence. The excitation and emission wavelengths were 280 and 334 nm, respectively. The midpoint of the transition is 545 ± 12 μM SDS.

mixtures containing 1 mM ATP (the final test volume was 0.81 ml). Aliquots were withdrawn at the appropriated times, and then the P_i released from ATP was determined by the method of Baginski *et al.* (21). The buffer used for both assays was 0.1 M sodium acetate, pH 5.3. All assays were done at least in duplicate, taking into account the non-enzymatic hydrolysis of the benzoyl phosphate and of the nucleotide. The main kinetic parameters (K_m and V_{max}) were determined by measuring the initial rates at differing substrate concentrations (ranging from 0.1 to 5 mM for benzoyl phosphate, and 0.01 to 2.5 mM for ATP). The experimental data were analyzed using the appropriate equations and linear or non-linear fitting programs (Fig.P, Biosoft, UK).

SDS-PAGE Analysis—SDS-PAGE was performed according to Laemmli (22). Samples were dissolved in a non-reducing 2× Laemmli sample buffer or, alternatively, in a reducing 2× Laemmli sample buffer (containing 4% 2-mercaptoethanol).

Titration of Thiol Groups with DTNB—The reactions were carried out at 25 °C in a 1-cm light path spectrophotometer cuvette. Enzyme (9 μM) was dissolved in 0.8 ml of 0.1 M buffer at the appropriate pH value, and the reaction was started by adding a 5-fold molar excess of DTNB. Measurements were taken with a Beckman model 7500 diode-array spectrophotometer; the absorbance values were acquired at the rate of 10 points per second. Reaction progress was fitted to a single exponential to calculate the first order kinetic constant. The second order kinetic constants at the tested pH values were then calculated by dividing the observed first order rate constants by the DTNB concentration.

RESULTS

Hydrogen Peroxide Induces ACP to Form a Disulfide Dimer—ACP (118 μM) in 0.1 M Tris acetate buffer, pH 8.0, was mixed with H_2O_2 (160 μM, final volume 0.3 ml). The mixture was incubated at 25 °C; aliquots were withdrawn at various time intervals to assay benzoyl phosphatase and ATPase activities. Other aliquots, taken during the incubation time, were mixed with a nonreducing 2× Laemmli sample buffer and then incubated for 3 min at 100 °C for SDS-PAGE analysis. Fig. 1*A* shows that H_2O_2 induces a time-dependent disappearance of the reduced form of ACP (11 kDa) as well as the appearance of a new protein band (22 kDa), a dimeric form of the enzyme, which prevails after 30 min. The weak band of dimer visible in *lane 1* is caused by the handling of the reduced enzyme in the presence of atmospheric oxygen. This band is absent in the freshly purified enzyme (data not shown). The time courses of H_2O_2-induced dimer formation and monomer loss are reported in Fig. 1*B* (the relative concentrations of monomer and dimer were measured by densitometry). Furthermore, another aliquot of the mixture containing ACP, previously incubated for 30 min at 25 °C with 160 μM H_2O_2, was mixed with a reducing 2× Laemmli sample buffer (*i.e.* containing 4% 2-mercaptoethanol) and then incubated at 100 °C for 3 min prior to performing the SDS-PAGE analysis. The *last lane* of Fig. 1*A* shows that reducing conditions cause the disappearance of the 22-kDa band, suggesting that it consists of a disulfide dimer of ACP. We also performed a time course experiment incubating the Cys-21 → Ser mutant of ACP with 160 μM H_2O_2 at the above experimental conditions. SDS-PAGE analysis, performed after 30-min incubation at 25 °C, shows the presence of the 11-kDa band only (data not shown), thus demonstrating the essential role of Cys-21 in the H_2O_2-induced dimer formation.

Cross-linking by the H_2O_2 Requires a Pre-existing Dimeric Form of ACP—To clarify whether disulfide bridge formation is caused by collision during diffusion of two separate ACP molecules or by H_2O_2-induced cross-linking of two subunits of a pre-existing non-covalently stabilized dimer, we investigated

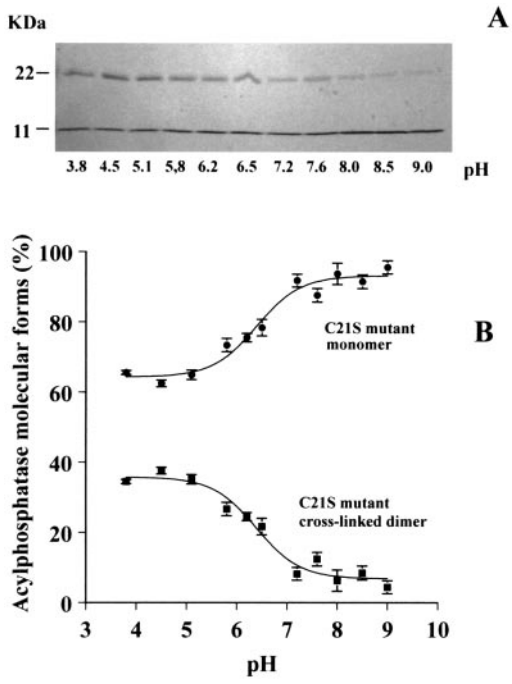


FIG. 3. **Effect of pH on light-induced cross-linking of the Cys-21 → Ser mutant of ACP in the presence of dipyridylruthenium(II) and ammonium persulfate.** The reaction mixtures contained 18 μM mutant in 110 μl of buffer adjusted to the indicated pH, 60 μM dipyridylruthenium(II), and 90 μM ammonium persulfate. Irradiation was performed for 15 s in 1.7-ml Eppendorf tubes positioned parallel to the beam of light at the distance of 20 cm from a 150-watt incandescent lamp. Immediately after irradiation, samples were quenched with 110 μl of 2× Laemmli sample buffer, heated to 100 °C for 5 min, after which SDS-PAGE analysis was performed. *A*, SDS-PAGE analyses; *B*, effect of pH on the concentrations (measured by densitometry of the SDS-PAGE separated bands) of monomer (11 kDa) and cross-linked dimer (22 kDa). The buffers used were: pH range 3.8–7.6, 0.1 M sodium 3,3-dimethylglutarate; pH range 8–8.5, 0.1 M Tris-HCl; pH 9.0, 0.1 M glycine-NaOH. The standard errors of the mean of four independent measurements are indicated.

the formation of the ACP disulfide dimer in the presence of both H_2O_2 and SDS, a denaturing agent. Fig. 2*A* shows that SDS prevents ACP cross-linking by H_2O_2 in a concentration-dependent manner. In particular, we observed a sharp transition in the cross-linking capability of ACP by H_2O_2, with a midpoint at 200 μM SDS. At SDS concentrations higher than 300 μM, no dimer formation was observed upon H_2O_2 treatment (Fig. 2*A*). To clarify the mechanism that produces this effect, we measured fluorescence spectra of ACP in solutions at increasing SDS concentrations. Fig. 2*B* shows the changes in tryptophan fluorescence intensity (excitation, 280 nm; emission, 334 nm) at varying SDS concentrations. It can be seen that ACP shows a sharp two-state transition profile. Using a non-linear least-squares analysis, experimental data points were fitted to a modified version of the Boltzman sigmoidal equation (23),

$$X_{[SDS]} = \frac{(X_N + m_N[SDS]) - (X_D + m_D[SDS])}{1 + \exp[([SDS] - SDS_{1/2}/dx] + (X_D + m_D[SDS])} \quad \text{(Eq. 1)}$$

where $X_{[SDS]}$ is the fluorescence intensity value at a particular concentration of SDS, and the remaining six terms are fitting parameters. The term X_N and X_D are the values of the measured fluorescence extrapolated to zero concentration of SDS, and m_N and m_D are the slopes for the dependence of X_N and X_D on SDS concentration. The transition midpoint, an SDS concentration, is termed $SDS_{1/2}$; dx is the width of the fitting subinterval. The calculated midpoint is 545 ± 12 μM SDS concentration. Taken together, our findings demonstrate that

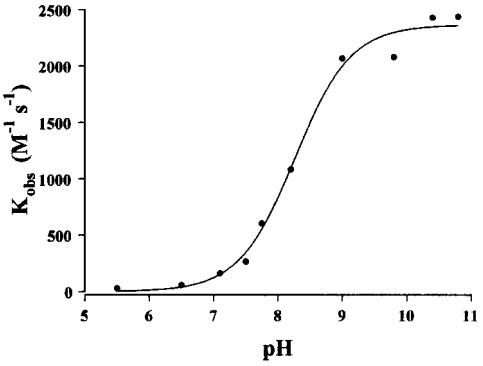


FIG. 4. **Determination of the pK_a of the exposed thiol group of muscle ACP.** Titration was performed with a 5-fold molar excess of DTNB. The following buffers, all containing 2 mM EDTA, were used: pH range 5.5–6.5, 0.1 M sodium cacodylate; pH range 7.1–8.6, 0.1 M Tris-HCl; pH range 9.0–10.8, 0.1 M glycine-NaOH.

the capacity of ACP to form a disulfide dimer upon H_2O_2 treatment is lost at SDS concentrations higher than 300 μM, a concentration substantially lower than the one necessary to cause a structural transition of the enzyme (midpoint = 545 μM SDS). This suggests the presence of a non-covalent dimeric form of ACP in non-denaturing solutions.

To further assess the existence of a non-covalent dimer form in solutions containing reduced ACP, we performed chemical cross-linking experiments triggered by light in the presence of tris-bipyridylruthenium(II), using the Cys-21 → Ser mutant of the enzyme. The method is based on a process that involves brief photolysis of tris-bipyridylruthenium(II) dication with visible light in the presence of the electron-acceptor ammonium persulfate and the protein of interest (24). The Cys-21 → Ser mutant of the enzyme (18 μM, final volume 110 μl) in the appropriate buffer solutions (3.8–9.0 pH range; the buffers used were: 0.1 M sodium 3,3-dimethylglutarate, pH 3.8–7.6; 0.1 M Tris-HCl, pH 8–8.5; 0.1 M glycine-NaOH, pH 9.0) was mixed with 60 μM dipyridylruthenium(II) and 90 μM ammonium persulfate (final concentrations). The solutions were placed in 1.7-ml Eppendorf tubes positioned parallel to the beam of light at a distance of 20 cm from a 150-watt incandescent lamp (Osram). The samples were irradiated for 15 s, and immediately after irradiation they were quenched with 110 μl of 2× Laemmli sample buffer, heated to 100 °C for 5 min; SDS-PAGE analysis was then performed. Fig. 3*A* shows the results obtained in experiments performed in the pH range 3.8–9.0. It can be seen that dipyridylruthenium(II) photolysis resulted in the production of a covalently linked dimer of the Cys-21 → Ser ACP mutant at all tested pH. We measured the relative concentration (%) of monomer and dimer at the tested pH by densitometry, and data were fitted to the Henderson-Hasselbalch equation,

$$k_{obs} = k_{min} + \frac{k_{max} - k_{min}}{1 + 10^{pKa\text{-}pH}} \quad \text{(Eq. 2)}$$

where k_{max} is the highest and k_{min} the lowest measured k_{obs} value, respectively. A pK_a value of 6.4 ± 0.1 (see Fig. 3*B*) for the cross-linking reaction was calculated. This finding indicates the presence of an enzyme group with pK_a 6.4 that influences the equilibrium between a non-covalently-linked dimer and the monomer in non-denaturing solvents. Experiments similar to those reported in Fig. 3*A* were performed with lysozyme at the same protein concentration and conditions, but they do not produce any dimeric form of lysozyme. Taken together, all findings clearly demonstrate that a non-covalent dimeric form of ACP exists in solutions of reduced enzyme prior to H_2O_2 treatment.

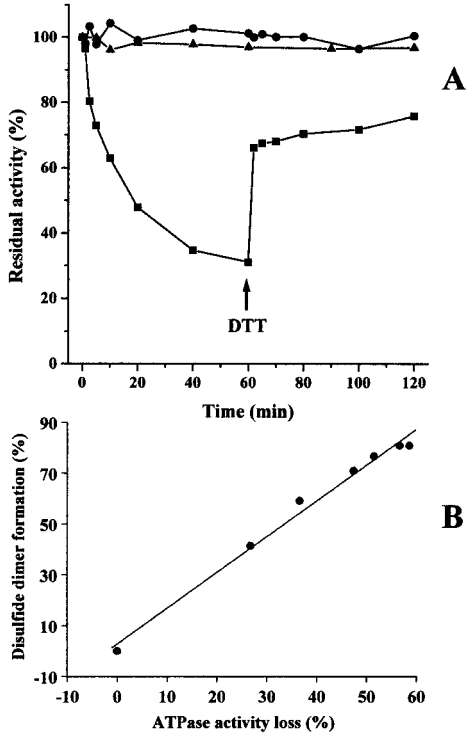


FIG. 5. **Hydrogen peroxide causes the loss of the ATPase but not of the benzoyl phosphatase activities displayed by ACP.** *A*, effect of H_2O_2 on the benzoyl phosphatase and the ATPase activities of muscle ACP. The following symbols indicate: ●, control of ATPase activity without H_2O_2 added; ■, time course of the ATPase activity in the presence of 160 μM H_2O_2; ▲, time-course of the benzoyl phosphatase activity in the presence of 160 μM H_2O_2. At the time point indicated by the arrow, dithiothreitol (*DTT*, 3.8 mM final concentration) was added. *B*, the residual ATPase activity, calculated on the basis of the time course analysis displayed in *A*, is plotted against the concentration of the disulfide dimer formed during reaction between reduced ACP and H_2O_2. The experimental conditions are the same as described for Fig. 1.

The Cys-21 Thiol of ACP Is Partially Buried in Non-denaturing Solvents—Reduced ACP (9 μM) dissolved in 0.1 M Tris-HCl buffer, pH 7.5, was titrated with excess DTNB to measure solvent-exposed thiol groups in the enzyme. We found that only 0.38 thiol residue per molecule of enzyme reacted with DTNB to produce the yellow DTNB thiolate anion. On the contrary, 0.83 thiol residue per molecule of enzyme reacted with DTNB when titration was performed in the presence of 35 mM SDS. The observed molar ratio of 0.83 in 35 mM SDS reflects the difficulty in obtaining the enzyme in full thiol form, because atmospheric oxygen also induces appreciable thiol cross-linking during laboratory handling of the enzyme (see Fig. 1*A*, *first lane*). Taking into account the results reported in Fig. 2, we realize that 35 mM SDS certainly produces structural modifications and thus the dissociation of the non-covalent dimeric form of the reduced enzyme. Because the ACP molecule contains a single thiol group (Cys-21), these findings also suggest that at least two molecular forms of the reduced enzyme exist in non-denaturing solvents: The former has a thiol group exposed to the solvent, whereas the latter (probably a non-covalent dimer) contains at least one thiol group non-exposed to the solvent.

We determined the pK_a of the exposed thiol group by measuring the rate of thiol disulfide exchange reaction between ACP and DTNB in non-denaturing solvent conditions at varying pH in the range 5.5–10.8. The measured second order rate constants were plotted against pH (Fig. 4), and data were fitted to the Henderson-Hasselbalch equation (Equation 2), enabling us to calculate a pK_a of 8.3 ± 0.1 for the solvent-exposed thiol

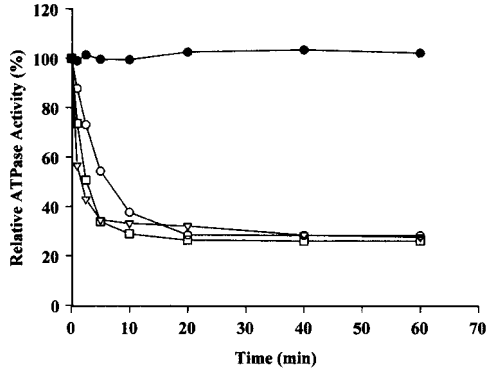


FIG. 6. **Hydrogen peroxide does not produce the complete loss of the ATPase activity of ACP.** The symbols indicate: ●, control without H_2O_2; ○, 0.12 mM H_2O_2; □, 0.24 mM H_2O_2; ▽, 20 mM H_2O_2. Other experimental conditions were the same as in Fig. 1.

group of ACP. That pK_a is in fact related to the exposed thiol group dissociation, because dimer-monomer equilibrium is scarcely affected by pH above 7.0, as demonstrated by Fig. 3*B*, which shows the effect of pH on the relative dimer and monomer concentrations of the Cys-21 → Ser mutant of ACP.

The Disulfide Dimer of ACP Shows Biological Properties Differing from Those of the Reduced Enzyme—Previously, we demonstrated that ACP displays nucleoside di- and tri-phosphatase activities in addition to its canonical activity (25). Thus, we performed experiments to study the effect of H_2O_2 on two known catalytic activities of ACP, using benzoyl phosphate and ATP as substrates. The reduced enzyme (118 μM) in 0.1 M Tris acetate buffer, pH 8.0, was treated with H_2O_2 (160 μM, final volume 0.3 ml), and the mixture was incubated at 25 °C. Aliquots were withdrawn at various time intervals to assay both the benzoyl phosphatase and the ATPase activities of the enzyme. Fig. 5*A* shows the time courses of both activities during the exposure of the enzyme to H_2O_2. We can observe that the benzoyl phosphatase activity remains unchanged, whereas the ATPase activity of ACP decreases in a time-dependent manner. Furthermore, we observed that the treatment of the reduced ACP with relatively high concentrations of H_2O_2 (up to 20 mM) does not result in a complete loss of ATPase activity, indicating that the S–S dimeric form maintains a fraction of the original ATPase activity (residual activity about 28%, data not shown). The different behaviors of the benzoyl phosphatase and the ATPase activities of ACP were unexpected, because previous studies demonstrated that this enzyme uses a unique active site to carry on both activities (25). The linear correlation between the formation of disulfide dimeric form of ACP and the loss of its ATPase activity is shown in Fig. 5*B*, demonstrating that H_2O_2 can regulate the ATPase activity of the enzyme. The partial loss of ATPase activity is reversible, as demonstrated by the following experiment: The mixture containing 118 μM ACP and 160 μM H_2O_2 in 0.1 M Tris acetate buffer, pH 8.0 (final volume 0.3 ml), was incubated at 25 °C for 60 min; then 4.4 mM dithiothreitol (final concentration) was added, and the time course of the recovery of ATPase activity was monitored for an additional 60 min. Fig. 5*A* shows that treatment of ATPase-inactivated enzyme with dithiothreitol causes a time-dependent recovery of this activity. All findings demonstrate that H_2O_2 not only induces the formation of a disulfide cross-linking between two ACP molecules, but also modifies at least one known functional property of the enzyme, *i.e.* its ATPase activity.

H_2O_2 Does Not Produce the Complete Loss of ATPase Activity of ACP—We observed that the treatment of ACP with relatively high concentrations of H_2O_2 (up to 20 mM) does not apparently produce the complete loss of ATPase activity, indi-

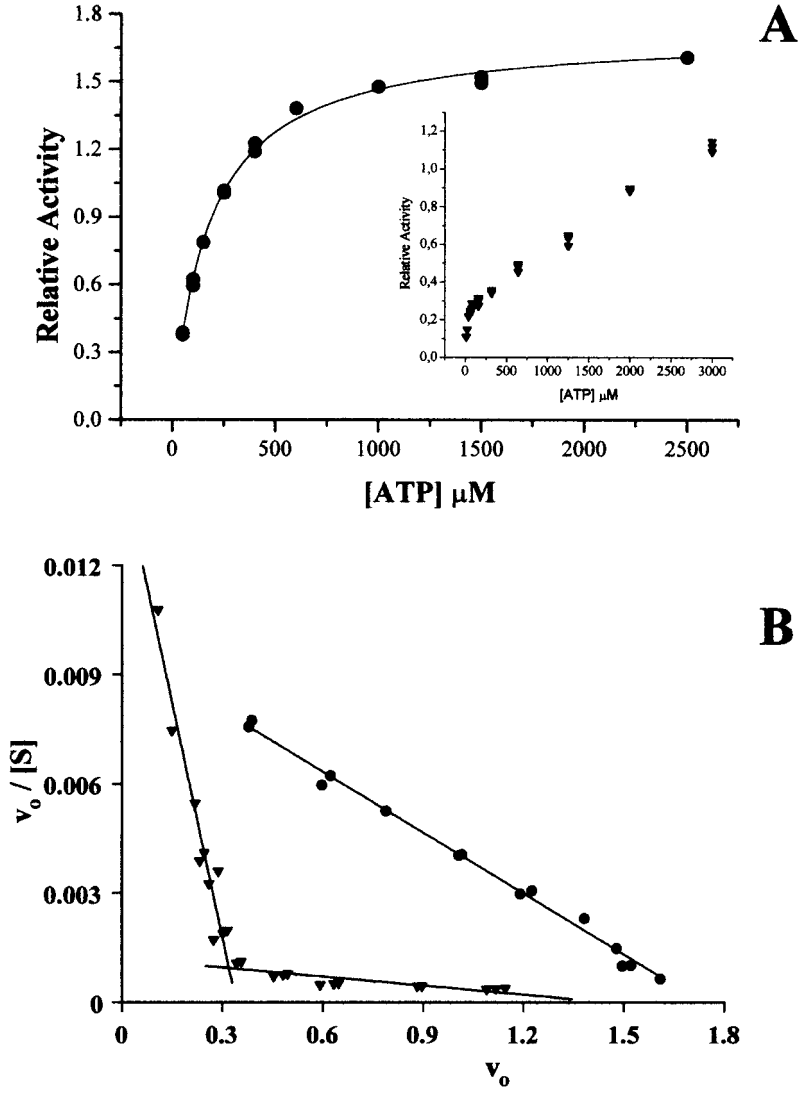


FIG. 7. **The kinetics of the ATPase activity of the disulfide dimer of ACP differs from that of the reduced enzyme.** *A*, plot of ATPase activity of the reduced enzyme against the ATP concentration. The *inset* reports a similar plot for the disulfide dimer. *B*, Eadie-Scatchard analysis of the data displayed in *A*. The clear biphasic behavior indicates that the disulfide dimer contains two active sites differing for binding affinity. The following symbols indicate: ●, reduced ACP; ▼, disulfide dimer of ACP.

TABLE I
Kinetic properties of muscle-type acylphosphatases

Substrate or inhibitor	Unit	Reduced form	Disulfide dimer
Benzoyl phosphate	K_m	394 ± 56 μM	308 ± 23 μM
	k_{cat}	539 ± 3 s^{-1}	503 ± 23 s^{-1}
ATP	K_m	171 ± 19 μM	
	k_{cat}	0.228 ± 0.021 s^{-1}	
	K_{m1}		23 ± 1 μM
	K_{m2}		1210 ± 180 μM
	k_{cat1}		0.047 ± 0.001 s^{-1}
	k_{cat2}		0.204 ± 0.018 s^{-1}
Poly(G)	K_i	No inhibition	169 ± 5 nM[a]
Poly(A)	K_i	No inhibition	No inhibition
Poly(T)	K_i	No inhibition	808 ± 17 nM[a]
Poly(C)	K_i	No inhibition	247 ± 1 nM[a]

[a] Competitive inhibition.

cating that the disulfide dimeric form maintains a fraction of the original ATPase activity (Fig. 6). We purified the S–S dimer by Superdex-75 chromatography; the SDS-PAGE analysis of this preparation shows a single band at 22 kDa (not shown). Then we studied some of its properties. Fig. 7*A* shows the plot of the residual ATPase activity of the reduced ACP *versus* the ATP concentration; the *inset* reports a similar plot for the disulfide dimer. It can be seen that, although the data for the reduced enzyme fit the Michaelis-Menten equation well and then display a hyperbolic curve, the data relative to the disulfide dimer do not agree with the equation of Michaelis, showing no evident saturation in the range 0–3000 μM ATP. Replotting the experimental points according to the Eadie-Scatchard equation,

$$\frac{v_o}{[S]} = \frac{-1}{K_m} \times v_o + \frac{V_{max}}{K_m} \qquad \text{(Eq. 3)}$$

where v_o is the relative activity, we can see that the curve relative to the disulfide dimer is clearly biphasic, whereas the reduced enzyme gives the expected *straight line* (Fig. 7*B*). Such kinetic behavior has been described for a mixture of two enzymes catalyzing the same reaction or for a multisite enzyme that has substrate-binding sites of different affinities or in the case of enzymes that display negative cooperativity (26). Taking into account that these experiments were carried out with a pure protein, the presence in the disulfide dimer of two binding sites with different affinities cannot be excluded: The linear fitting of the data displayed by the disulfide dimer in the low ATP concentration range (10–80 μM) enabled us to calculate a K_{m1} of 23 μM and a $kcat_1$ of 0.047 s^{-1}, whereas the fitting of the experimental points in the 0.16–3.00 mM ATP concentration range gives a K_{m2} of 1210 μM and a k_{cat2} of 0.204 s^{-1} (K_{m1} and k_{cat1} are kinetic parameters relative to active site 1, whereas K_{m2} and k_{cat2} are kinetic parameters relative to active site 2 of the dimer) (Table I).

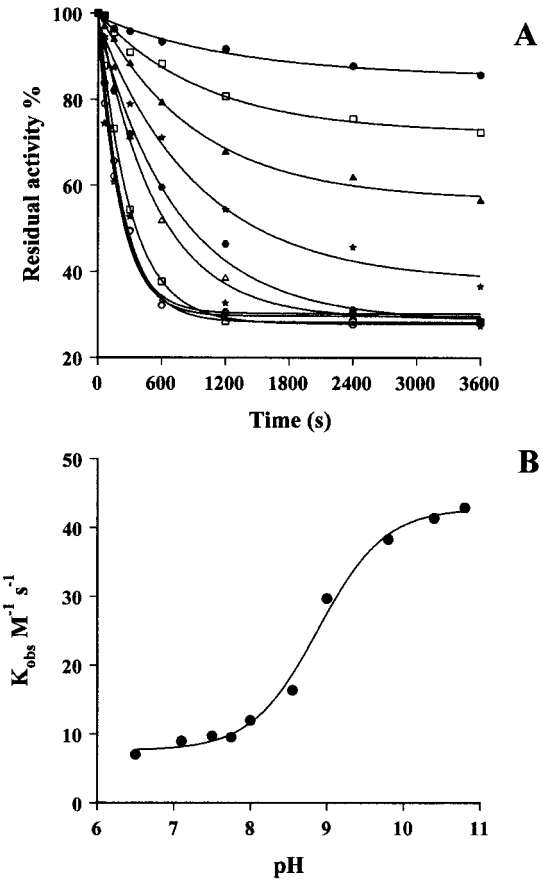
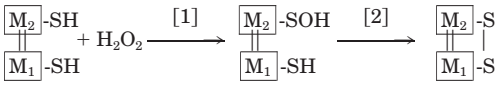


FIG. 8. **The disulfide dimer of ACP maintains part of the ATPase activity of the reduced ACP.** *A*, time course of ATPase loss at varying pH values. The experimental data were fitted to the single-exponential equation (*continuous lines*). The buffers used, all containing 2 mM EDTA, were as follows: pH 6.50, 0.1 M sodium cacodylate; pH 7.10–8.55, 0.1 M Tris-HCl; pH 9.00–10.80, 0.1 M glycine-NaOH. *B*, effect of pH on the loss of the intrinsic ATPase activity of reduced ACP; experimental data were fitted to the Henderson-Hasselbalch equation.

Taken together, the above kinetic data suggest that the observed apparent loss of ATPase activity of the disulfide dimer of ACP with respect to that of the reduced enzyme is mainly caused by the modification of its catalytic center due to the formation of two active sites with different affinity for ATP. Site 1 is completely saturated by the 1.0 mM ATP concentration used in the standard ATPase assay, because its K_m value is 23 μM, whereas site 2 is far from saturation, because its K_m is 1210 μM.

Effect of pH on the Apparent Loss of ATPase Activity—ACP (10 μM), dissolved in the appropriate 0.1 M buffer (pH 6.5, sodium cacodylate; pH range 7.1–8.55, Tris-HCl; pH range 9–10.8, glycine-NaOH, all containing 2 mM EDTA), was incubated at 25 °C in the presence of 130 μM H_2O_2. At appropriate times, aliquots were withdrawn to measure the residual ATPase activity of the enzyme. Residual activity was plotted against reaction time, and progress data were fitted to a single exponential to calculate the first order rate constants for the apparent loss of ATPase activity (Fig. 8*A*). The observed first order rate constants divided by the concentration of H_2O_2 yielded the second order rate constants. The pK_a for the ATPase inactivation (8.9 ± 0.1) was estimated by fitting the data of the second order rate constants *versus* pH to the Henderson-Hasselbalch equation (Equation 1) (Fig. 8*B*). This pK_a value is 0.6 unit higher than that measured by DTNB titration of the exposed free thiol group of ACP, suggesting that the apparent loss of ATPase activity is not directly caused by the initial chemical modification of the Cys-21 thiol by H_2O_2, as shown in Scheme 1, Reaction 1,



SCHEME 1

where H_2O_2 first reacts with thiols forming sulfenic acids (or other reactive compounds), which successively can react with vicinal thiols forming intra- or intermolecular disulfide bridges (27). $\boxed{M_1}$ and $\boxed{M_2}$ are the two monomers of ACP; the symbol "‖" indicates non-covalent interactions.

This hypothesis agrees with the non-essentiality of Cys-21 for the catalytic hydrolysis of ATP. In fact, the kinetic parameters for the ATPase activity of the ACP Cys-21 → Ser mutant are the following: K_m = 0.23 ± 0.01 mM, and k_{cat} = 0.31 ± 0.01 s^{-1}, indicating that the mutant is fully active. Therefore, we suggest that the pK_a values of 8.9 for the H_2O_2-induced apparent loss of the ATPase activity of ACP is to be related to the dissociation of the thiol group involved in Reaction 2 of Scheme 1.

H_2O_2 Induces ACP to Interact with Some Poly-deoxyribonucleotides—Taking into account that ACP possesses a fold very similar to that contained in some polynucleotide-binding proteins (11, 12), we tested the ability of different 30-mer polynucleotides (poly(A), poly(G), poly(T), poly(C)) to inhibit the benzoyl phosphatase activity of both untreated ACP- and H_2O_2-produced disulfide dimer.

We measured the initial rates of benzoyl phosphate hydrolysis (concentration range 0.2–5 mM) in the presence of different polynucleotide concentrations. The data were fitted to the reciprocal Lineweaver-Burk equation,

$$\frac{1}{v_o} = \left(\frac{K_m}{V_{max}} \times \frac{1}{v_o}\right) + \frac{1}{V_{max}} \qquad \text{(Eq. 4)}$$

using a linear regression program. Fig. 9 shows that the disulfide dimer of ACP is inhibited by low micromolar concentrations in three out of four tested polynucleotides (poly(G), poly(T), and poly(C)), although it is not inhibited by poly(A). The reduced enzyme is not inhibited by the above polynucleotides. The graphs relative to the inhibition of the disulfide dimer by poly(G), poly(T), and poly(C) show V_{max} values very close to that measured in the absence of polynucleotides, indicating that the inhibition is competitive. The K_m values calculated from the graphs were re-plotted against the polynucleotide concentration, obtaining a *straight line* that intersects the abscissa at a point corresponding to $-K_i$ (Fig. 10). Furthermore the data summarized in Table I together with other kinetic parameters of both reduced and disulfide dimer ACP forms, show K_i values in the 10^{-6} to 10^{-7} molar range, demonstrating that poly(G), poly(T), and poly(C) possess high affinities for the disulfide dimer of ACP. These findings demonstrate that H_2O_2, by inducing the formation of disulfide dimer of ACP, modifies certain biological properties of the enzyme, such as its capacity to bind some kinds of polynucleotides, and its intrinsic ATPase activity.

Taken together, our results indicate that muscle ACP is extremely sensitive to oxidation; in fact, the exposition of the enzyme to atmospheric molecular oxygen during enzyme purification hinders the possibility of obtaining a completely purified thiol form (see above). Furthermore, we have demonstrated that H_2O_2 at micromolar concentrations induce the formation of a cross-linked disulfide dimer of the enzyme and modify some properties, suggesting that ACP also mediates some biological effects of ROS *in vivo*.

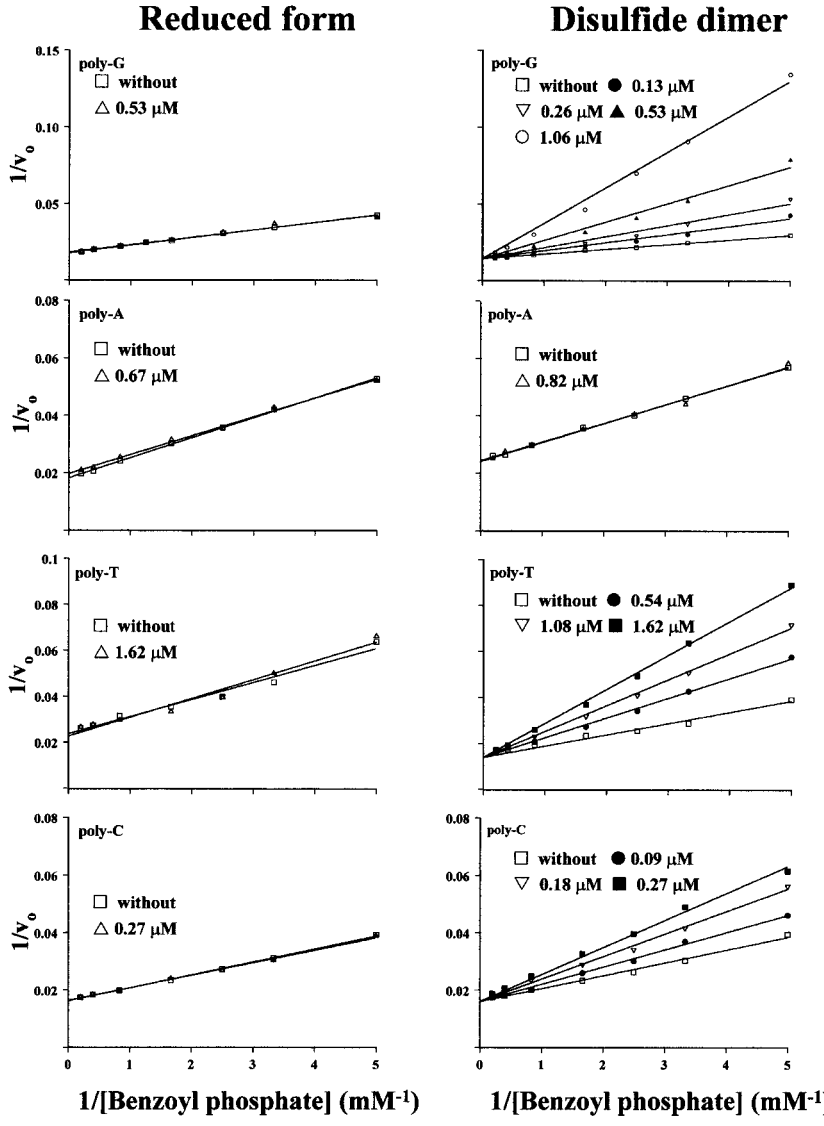


FIG. 9. **H_2O_2-induced S–S-cross-linking modifies the binding affinities of muscle ACP for poly-nucleotides.** The effect of poly(G), poly(A), poly(T), and poly(C) on the kinetic parameters of the reduced form and of the disulfide dimer of ACP. The enzyme activity was assayed using benzoyl phosphate as substrate; the initial rates (v_o) are expressed as arbitrary units. *poly-G*, *poly-A*, *poly-T*, and *poly-C* indicate 30-mer *d*-polynucleotides, whose concentrations are indicated. Experimental data were fitted to the double-reciprocal Lineweaver-Burk equation. Three polynucleotides (*i.e.* poly(G), poly(T), and poly(C)) display strong inhibition on disulfide dimer activity but not on that of the reduced enzyme.

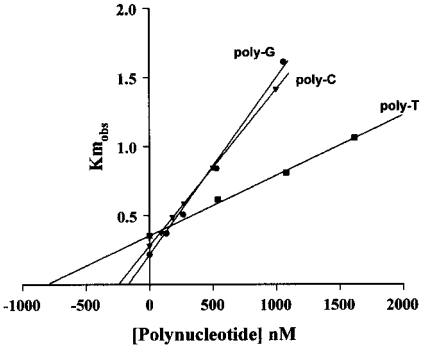


FIG. 10. **Replots of K_m(obs) against the concentration of polynucleotides.** The K_m(obs) values were calculated from the experiments reported in Fig. 9.

DISCUSSION

Changes of cell environment are always accompanied by an adaptation response that generally involves modifications of protein structure-function properties. Well-established mechanisms for such regulation are protein-protein interactions, allosteric changes induced by ligand binding, proteolytic processing, and chemical modifications such as acylation, alkylation, and phosphorylation. However, the molecular mechanisms of the response of cells to oxidative stress conditions, which generate ROS, are still poorly understood. ROS produced during oxidative stress may react with protein side chains such as thiol groups, with consequent modification of protein structure and/or function.

MT-ACP, purified by immunoaffinity chromatography, contained three ACP molecular forms that were separated by cation exchange chromatography (28): the first (68%) was the mixed disulfide with glutathione, the second (3%) remained uncharacterized, and the third (29% of total enzymatic protein) was an S–S dimeric form of the enzyme. On the other hand, we demonstrated that the main natural form of the enzyme contained in the freshly excised muscle is the reduced one (29). Taken together, the above findings indicate the strong tendency of MT-ACP first to be oxidized by the atmospheric molecular oxygen and then induced to form a mixed disulfide with cellular glutathione or a disulfide dimer during enzyme extraction and purification.

Here we report new results on the *in vitro* induction of MT ACP to form a disulfide dimer in the presence of hydrogen peroxide, which is also produced *in vivo* during oxidative stress conditions. The S–S cross-linking of the ACP is accompanied by modification of some biological properties; particularly, we observed an apparent decrease in its intrinsic ATPase activity (due to changes in the kinetic behavior), and a strong enhancement in its ability to bind with certain monotone polynucleo-

tides (poly(G), poly(T), and poly(C)). Neither the reduced nor the disulfide dimer form of ACP is able to bind poly(A).

Previous studies have reported that the MT-ACP isoenzyme migrates into the nucleus upon induction of differentiation (8) or apoptosis (9). On the contrary, the CT-ACP isoenzyme, which does not contain cysteine, remains in the cytoplasm, suggesting that the two isoenzymes perform different cell functions. Nevertheless, the mechanism that induces nuclear migration of this particular isoenzyme and the physiological significance of this event remained unknown.

In a recent review (30) Schafer and Buettner report data that suggest a correlation between the half-cell reduction potential (E_{hc}) of the GSSG/2GSH redox couple with the biological status of the cell. In particular, they indicate that an E_{hc} of about −240 mV correlates with the proliferating status, an E_{hc} of about −200 mV with the differentiation status, and an E_{hc} of about −170 mV with the apoptotic status and hypothesize that reduction potential-driven nano-switches move cells through different biological stages. The exact nature of these nano-switches remains to be determined, but clearly protein thiols are the principal candidates to sustain this function. The formation of ROS in particular cell conditions is generally the cause of cellular E_{hc} modification of the GSSG/2GSH redox couple.

The present findings indicate that the MT isoenzyme of ACP is one of the targets of the cellular action of ROS. We suggest that the observed migration into the nucleus of this particular isoenzyme (8, 9), which contains a single conserved cysteine, is triggered during differentiation and apoptosis by the formation of a disulfide cross-linked dimer caused by the more positive redox potential associated with both physiological processes (30). This modification of the enzyme is accompanied by changes in the capacity to bind polynucleotides and in its intrinsic ATPase activity. Our research does not clarify whether these changes have an active role in the initiation or progression of differentiation or apoptosis, but it certainly gives us new ideas for further studies of the still unknown physiological function of ACP, an enzyme widely expressed in metazoan tissues as two molecular forms.

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