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Biochemistry

Oxidation of the methionine residues of *Escherichia coli* ribosomal protein L12 decreases the protein's biological activity

(methionine sulfoxide/hydrogen peroxide/protein dimerization/ribosomal protein L7)

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ABSTRACT **Oxidation of ribosomal protein L12 with hydrogen peroxide converts the three methionine residues to methionine sulfoxide. The oxidized protein has a decreased ability to bind to ribosomes, interact with ribosomal protein L10, be precipitated by L12 antiserum, and serve as substrate for the acetylating enzyme that converts L12 to L7. Full activity of L12 is regained when the protein is reduced with 2-mercaptoethanol. Sedimentation equilibrium analysis shows that oxidation of the methionine residues in L12 causes the conversion of the protein from the dimer to the monomer form, and the results indicate that the dimer is the active form of the protein in the above reactions.**

The ease with which *Escherichia coli* ribosomal protein L12, and its acetylated derivative L7, can be specificially removed from the ribosome (1) has permitted detailed studies on their role in protein synthesis. The 50S ribosomal subunit lacking L7/L12 cannot interact properly with the soluble factors involved in the initiation (2–5), elongation (1, 6, 7), and termination (8) steps of protein synthesis, and, therefore, the hydrolysis of GTP that is associated with these partial reactions does not occur with L7/L12-depleted ribosomes (1–7). The activity of L7/L12-depleted 50S subunits can be restored by the addition of L7/L12 because these proteins rapidly bind to the large subunit (9). It is of interest that there are four equivalents of L7/L12 per ribosome, whereas the other *E. coli* ribosomal proteins are present in one copy per ribosome (10, 11). Although the ratio of L7 to L12 on the ribosome varies under different growth conditions (12), no function of L12 has been observed that cannot be performed by L7, and vice versa (3, 8, 9, 13).

There also is considerable information available on the chemical nature of the L7 and L12 proteins. They have molecular weights of 12,200, and the amino acid sequences are known (14, 15). Serine is the NH_2-terminal amino acid, and the difference between the two is that L7 has an acetylated NH_2-terminus. The enzyme that catalyzes the acetylation has been purified, and it has been shown that free L12 is acetylated 10 times faster than ribosomal-bound L12 (16). It is also of interest that these proteins lack cysteine, histidine, tryptophan, and tyrosine, are very acidic (pI = 4.7–4.9), and have a high degree of secondary structure with >45% helical content (17–19). The proteins in solution form dimers (14, 20, 21) that may be the functional form on the ribosome and also bind tightly to ribosomal protein L10 (22, 23). Other results suggest that L10 on the ribosome is also required for L7/L12 to bind to the ribosome (24, 25). In this regard an NH_2-terminal fragment of L12, containing 73 amino acids, can bind to L7/L12-depleted ribosomes but does not restore their biological activity (26).

Because little is known about the structure–function relationship of either L12 or L7, the present study was undertaken to determine whether oxidation of the methionine residues in L7/L12 would affect the biological activity of these proteins. During the course of these studies, Koteliansky *et al.* (27) reported that oxidation of the methionines in L7 renders the protein inactive in poly(U)-dependent polyphenylalanine synthesis and elongation factor G-dependent GTP hydrolysis.

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MATERIALS AND METHODS

Ribosomal proteins L7 and L12 were isolated and purified as previously described (1, 14), and L10 was a kind gift from H. G. Wittmann. The purified proteins were labeled by reductive methylation (28) using either ^{14}C- or ^{3}H-labeled formaldehyde (New England Nuclear Corp.). Antibodies to the purified proteins were raised in rabbits with Freund's complete adjuvant. Iodo[^{14}C]acetic acid (14 mCi/mol) (1 Ci = 3.7×10^{10} becquerels) was purchased from the New England Nuclear Corp. and L7/L12-depleted ribosomes were prepared by the method of Hamel *et al.* (1).

Oxidation of L12 with Hydrogen Peroxide. The procedure used was a modification of the method of Neumann (29). Reaction mixtures containing L12 at 5–15 mg/ml were adjusted to pH 3.2 with perchloric acid and incubated at 30°C in the presence or absence of 0.3 M H_2O_2. After one hour of incubation, the samples were chilled and dialyzed for 16 hr at 4°C against a buffer containing 10 mM each of Tris·HCl at pH 7.4, $MgCl_2$, and NH_4Cl (buffer A).

Reduction of Oxidized L12. The dialyzed oxidized and control proteins were incubated for 48 hr at 37°C in the presence of 0.8 M 2-mercaptoethanol and then dialyzed for 16 hr against buffer A.

Determination of the Methionine Residues of L12 by Alkylation with Iodo[^{14}C]acetic Acid. The amount of methionine residues was determined by exhaustive alkylation of the protein with iodoacetic acid (29). Each incubation mixture (100 μl) contained 8 M urea, oxidized or control L12 (0.5–1 mg), and iodo[^{14}C]acetic acid in a 400-fold molar excess to the protein. The pH was adjusted to 3.0 with HCl and the reaction was carried out at 40°C for 24 hr in the dark. An aliquot of the incubation mixture was diluted into 2 ml of 10% Cl_3CCOOH and then filtered through a nitrocellulose filter. The filter was washed with 10 ml of 5% Cl_3CCOOH and the radioactivity retained on the filter was assayed in a Beckman liquid scintillation spectrometer. The moles of alkylated methionine residues per mole of L12 were then calculated.

Polyphenylalanine Synthesis. Each incubation mixture contained in a final volume of 50 μl: 0.05 M Tris·HCl at pH 7.4, 0.02 M $MgCl_2$, 0.08 M NH_4Cl, 0.04 M 2-mercaptoethanol, 3 mM phospho*enol*pyruvate, 0.2 mM GTP; 2 μg of pyruvate kinase, 27 pmol of 30S subunits, 7 pmol of 50S subunits (treated with ethanol/NH_4Cl at 0°C to remove L7/L12), 5 μg of poly(U), 9 units of elongation factor (EF)-Tu, 75 units of EF-Ts,

2 μg of EF-G, and control or treated L12 as indicated. The mixtures were preincubated for 60 sec at 37°C and then the reaction was started by the addition of 8 pmol of purified [^{14}C]Phe-tRNA and incubated for an additional 2 min. The incubation was terminated by the addition of 2 ml of 10% Cl_3CCOOH, and, after heating for 10 min at 90°C, the mixture was filtered through a nitrocellulose filter. The filter was washed with 15 ml of 5% Cl_3CCOOH and assayed for radioactivity.

Binding of [^{14}C]L12 to Ethanol/NH_4Cl-Extracted Ribosomes. [^{14}C]L12, treated as described in the text, was assayed for its ability to bind to ethanol/NH_4Cl-extracted ribosomes (L12 depleted) as previously described (9). The ribosomes were recovered by centrifugation at 200,000 × *g* for 1 hr and the amount of [^{14}C]L12 per A_{260} was determined.

Acetylation of L12. The assay for the acetylation of L12 (which converts it to L7) was carried out as previously described (16). Each incubation mixture contained in a total volume of 50 μl: 50 mM Tris·HCl at pH 7.4, 10 mM $MgCl_2$, 10 mM NH_4Cl, 25 mM KCl, 10 μM ^{3}[H]acetyl coenzyme A, 3.2 μg of partially purified transacetylase, and 250 pmol of treated or control L12. After 5 min at 37°C, the reaction mixture was diluted with 3 ml of cold buffer A and passed through a nitrocellulose filter (HAWP 0.45 μm, Millipore Corp.). The filter was washed three times with 3-ml aliquots of the buffer and then assayed for radioactivity. Under these conditions, L7 and L12 are quantitatively retained by the filter.

Immunoprecipitation of L12. The complete reaction mixture (250 μl) contained 0.05 M Tris·HCl at pH 7.4, 0.5 M NaCl, 1% Triton X-100, 60 μl of antiserum to L12, and 440 pmol of treated or control [^{14}C]L12. After incubation at 37°C for 60 min the reaction mixture was diluted with 0.5 ml of the above detergent solution and passed through a nitrocellulose filter that had been previously washed with this detergent solution (22). Under these conditions, only the immunoprecipitated protein is retained by the filter. The filter was then assayed for radioactivity and the amount of immunoprecipitated L12 was determined.

Binding of L10 to L12. The conditions for the formation of an L10–L12 complex have been described (22). The basis of the assay is the ability of antiserum to protein L10 to precipitate L12 when the latter is bound to L10. Each incubation contained 200 pmol of [^{3}H]L10 and 440 pmol of treated or control [^{14}C]L12. The two proteins were preincubated for 5 min at 37°C and then 120 μl of L10 antiserum was added and the incubation was continued for an additional 60 min. The amount of L12 bound to L10 was then determined by passing the immunoprecipitate through detergent-washed filters and determining the retained radioactivity (22).

RESULTS

Peroxidation of L12 Methionine Residues. Treatment of proteins with H_2O_2 is known to oxidize several amino acids, including cysteine, tryptophan, tyrosine, histidine, and methionine. Of these, only methionine is present in L12 and therefore mild oxidation by H_2O_2 should specifically oxidize the three methionine residues in L12* to the corresponding sulfoxide derivative. Methionine sulfoxide can be reduced back to methionine and, in fact, the standard procedure (HCl hydrolysis) used to hydrolyze proteins for amino acid analysis chemically reduces any sulfoxide present back to methionine. Thus, this method could not be used to determine the extent of the oxidation of methionine. A simple quantitative assay was therefore used to measure the extent of methionine oxidation. The assay takes advantage of the fact that exhaustive alkylation of a protein with iodo[^{14}C]acetate will carboxymethylate the methionine residues, but not the methionine sulfoxide residues. Under these conditions it was possible to demonstrate that after treatment of L12 with H_2O_2 there was a large decrease in the amount of methionine in the protein and the appearance of methionine sulfoxide. Table 1 shows the results of a typical assay. By this procedure there are 2.5 methionine residues in native L12, a figure close to the value of 3 determined by the amino acid sequence (15). After treatment with H_2O_2 the amount of methionine drops to 0.2. A control in which the protein was carried through the procedure in the absence of H_2O_2 showed no loss of methionine. As also shown in Table 1, reduction of the oxidized protein with 2-mercaptoethanol restored the methionine value almost to the control level. It appeared from these studies that it was possible to oxidize all three methionine residues in L12 and subsequently reduce them almost quantitatively. It was therefore possible to examine the effect of oxidation of L12 on a variety of reactions that involve this protein.†

Table 1. Peroxidation of L12 decreases its methionine content

L12 treatment	Methionine, mol/mol L12
None	2.5
+ H_2O_2	0.2
− H_2O_2*	2.5
+ H_2O_2, + 2-mercaptoethanol	2.1
− H_2O_2, + 2-mercaptoethanol	2.4

* These L12 samples were subjected to the oxidation conditions but H_2O_2 was not added.

Comparison of L12 and Oxidized L12. Table 2 compares the activities of native L12, L12 after oxidation with H_2O_2 (oxidized protein), and the oxidized protein after reduction with mercaptoethanol (oxidized–reduced protein). The first column shows that the oxidized protein cannot bind to L12-depleted ribosomes. The native protein, and the oxidized and subsequently reduced protein both bind well, suggesting that the methionine residues play an important role in the binding of L12 to the 50S subunit. It would be expected that oxidized L12 would not restore the activity of depleted ribosomes to function in a polyphenylalanine synthesis system, because oxidized L12 does not bind to the 50S subunit. This in fact is the case, as shown in the second column of Table 2. Once again the oxidized–reduced protein functions about as well as the native and control L12. It is known that ribosomal protein L12 forms a tight complex with ribosomal protein L10 (24, 25). The interaction of L12 with L10 can be conveniently assayed by using antiserum to either L10 or L12 (23). The third column of Table 2 summarizes the results of such experiments. Once again oxidized L12 reacts much more poorly than native L12 or the oxidized–reduced protein. In addition, the fourth column of Table 2 shows that oxidized L12 does not react well with antiserum prepared against native L12. Reduction of the oxidized

* Although the data presented in this study were obtained with L12, the same results were obtained when L7 was used.

† Other experiments have shown directly that the loss of methionine residues was accompanied by an increase in the methionine sulfoxide content. Purified L12 that had been labeled *in vivo* with [^{35}S]methionine was oxidized with peroxide and then hydrolyzed with KOH (29) in the presence of carrier methionine and methionine sulfoxide. The neutralized hydrolysate was subjected to paper chromatography (butanol/acetic acid/H_2O 65:15:20 by volume), and the areas corresponding to methionine and methionine sulfoxide were cut out and assayed for radioactivity. After oxidation with H_2O_2 there was almost complete disappearance of methionine in the hydrolysate with a corresponding increase in the formation of methionine sulfoxide.

Table 2. Peroxidation of L12 blocks various biological reactions

L12 treatment	Ribosome binding, pmol/pmol ribosome	Polyphenylalanine synthesis, pmol	L10 binding, pmol	Antibody precipitation, pmol	Acetylation, pmol
None	2.3	2.4	24.4	250	14.5
+ H_2O_2	0.3	0.2	6.5	38	3.0
− H_2O_2	2.2	2.6	23.1	310	14.7
+ H_2O_2, + 2-mercaptoethanol	2.0	2.1	21.5	300	11.2
− H_2O_2, + 2-mercaptoethanol	2.3	2.8	24.4	250	15.8

protein reverses this effect. Other experiments, however, showed that when higher levels of antiserum were used, the oxidized L12 could be precipitated just as well as untreated L12, indicating that the L12 antibodies can recognize oxidized L12, but much less efficiently than the native protein. Finally, it was possible to show (last column of Table 2) that oxidized L12 is not a substrate for the enzymatic acetylation reaction yielding L7.

Molecular Weight of L12 after Oxidation. Previous studies have shown that ribosomal protein L12 exists in solution as a dimer (20, 21), probably in a head-to-tail configuration (30). Because, as shown above, the oxidation of L12 caused the loss of activity in a variety of unrelated reactions, it was thought that perhaps the common feature of all the reactions was the requirement for the dimer form of the protein. Therefore the molecular weights of the various species were analyzed by ultracentrifugation. Ribosomal protein L12 has a molecular weight of about 12,200 by amino acid analysis, and thus the dimer form would have a molecular weight of about 25,000. Table 3 shows that the values obtained by ultracentrifugation analysis of native L12, or the $-H_2O_2$ control, are in reasonable agreement with this value and confirms previous conclusions that the protein exists in solution as a dimer (20, 21, 30). However, when L12 is oxidized with H_2O_2, this value decreases to about 12,000, the molecular weight of the monomer. After the oxidized protein is treated with mercaptoethanol the protein again is present in the dimer form.

DISCUSSION

Ribosomal proteins L7 and L12 have three methionine residues, at positions 14, 17, and 26 (15), and the present study shows that when these methionines are oxidized to methionine sulfoxide the protein becomes inactive in several reactions. As shown in Table 2, oxidized L12 cannot bind to L12-depeleted ribosomes or function in polypeptide synthesis. In addition, oxidized L12 cannot interact with L12 or by acetylated to L7. Even the ability to react with antiserum to native L12 is altered if the methionines in L12 are oxidized. It is conceivable that one or more of the methionines in L12 is directly involved in all of these reactions, although a more probable explanation is that an L12 dimer is required for all of the above reactions and oxidation of the methionines in L12 prevents dimer formation. The inability of oxidized L12 to form dimers is shown in Table 3 and confirms the recently published results of Gudkov *et al.* (30). These authors also postulated that the biologically active form of L7/L12 is the dimer.

It is also clear from the present results that the effects of the oxidization of the L12 methionine residues can be reversed by reduction of the oxidized protein with 2-mercaptoethanol. Not only the biological activity but also the ability of the L12 to form dimers is restored, supporting the view that dimer formation is correlated with biological activity. However, it is possible that the two events are unrelated and oxidation of the methionines in L12 results in conformational changes that are responsible for the observed biological results. It should be noted that another study has suggested that oxidation of the methionine residues in an erythrocyte glycoprotein affects the ability of the protein to dimerize (31).

In yeast the reduction of free methionine sulfoxide to methionine is catalyzed by a pyridine nucleotide-dependent enzyme system (32). It is not known whether this enzyme system is equally effective when the sulfoxide is present in proteins, but this may represent one way that cells protect themselves against protein oxidation by peroxides and oxygen radicals. In fact, there is evidence that the oxidation of methionines in protein takes place *in vivo*. A recent report (33) has shown that cataractous human lens protein contains significant amounts of methionine sulfoxide as compared to normal lenses, and suggests that the etiology of cataracts may be due to this chemical change. It is of interest to speculate that, other pathological conditions may involve increased levels of methionine sulfoxide in proteins.

Table 3. Peroxidation of L12 halves its molecular weight

L12 treatment	Molecular weight
None	22,200
+ H_2O_2	11,450
− H_2O_2	22,400
+ H_2O_2, + 2-mercaptoethanol	21,600
− H_2O_2, + 2-mercaptoethanol	23,500

The protein solutions were dialyzed for 16 hr at 4°C against a buffer containing 0.02 M Tris·HCl at pH 7.4 and 0.15 M KCl. Molecular weights were determined by sedimentation equilibrium at 20,000 rpm (20°C) in a Beckman model E analytical ultracentrifuge equipped with UV optics set at 240 nm, photoelectric scanner, and a rotor temperature indicator control unit. The A_{240} was 0.4. An ANH-Ti rotor and cells composed of sapphire windows and double-sector, 12-mm, Kel F center pieces were utilized. A value of 0.76 ml/g was used for partial specific volume $\bar{v}$ (30) and ln A vs. r^2 plots were linear.

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