Incorporation and Distribution of Selenium into Thiolase from *Clostridium kluyveri*

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*Clostridium kluyveri* incorporates selenium as selenomethionine into its acetoacetyl-CoA thiolase when grown in media containing normal sulfur-to-selenium ratios. Antibodies raised against the purified enzyme permitted quantitative immunoprecipitation of thiolase from crude cell extracts and thus facilitated the systematic analysis of the effects of wide variation in sulfur-to-selenium ratios on selenium incorporation into the enzyme. The extent of incorporation of selenium into thiolase was found to be dependent on the form of selenium supplied. When \[^{75}\text{Se} \text{selenomethionine}\] was the source of selenium, the incorporation of selenium into thiolase was inversely proportional to the level of added methionine. However, similar levels of methionine failed to decrease the incorporation of selenium from selenite. To study the location of selenomethionine and methionine residues in the polypeptide chain of the enzyme, thiolase was prepared from cells cultured in the presence of \(\text{H}_2\text{S}^{35}\text{S} \text{O}_4\) or \(\text{Na}_2\text{S}^{75}\text{SeO}_3\). The \(^{35}\text{S}\) or \(^{75}\text{Se}\)-labeled protein was treated with trypsin and the resulting peptides were isolated by reverse phase high performance liquid chromatography. The peptide maps of the enzyme indicated that selenium was distributed throughout the primary structure in a manner that paralleled methionine. From these studies, it is concluded that selenium occurs in thiolase adventitiously and is not required for any biological function.

The occurrence of selenium as a constituent of several proteins and bacterial tRNAs has been well documented \(1, 2\). In two of these proteins, mammalian glutathione peroxidase \(3, 4\) and protein A of clostridial glycine reductase complex \(1, 5\), the selenium moiety, which is present as selenocysteine, has been shown to occur at a specific residue within the polypeptide chain. In addition, both of these proteins contain cysteine residues. Thus, in spite of the fact that selenium resembles sulfur in many of its chemical properties, mechanisms apparently exist in nature for discrimination between compounds containing either element. The interest of selenium in biochemistry stems from both its essentiality and toxicity. The daily requirement for selenium in humans is about 80 \(\mu\)g. Yet the lower limit of the toxic value is only about an order of magnitude higher \(6\). Organisms which require selenium must differentiate it from sulfur in an environment which usually contains sulfur at concentrations a thousand times greater than selenium. Mechanisms by which this differentiation is accomplished are not understood. Conversely, selenium toxicity can occur when selenium randomly substitutes for or competes with sulfur in biochemical processes. The concentration of selenium analogs an organism can tolerate appears to vary with respect to whether their analogs are intermediates in the sulfur metabolic pathways of the organism in question \(1\). Selenium compounds which are analogs of normal sulfur metabolites are considerably more toxic \(7\). This presumably is due to the fact that many enzymes, which metabolize sulfur-containing compounds exhibit little or no selectivity with respect to selenium analogs \(8\). *Clostridium kluyveri* has been shown to incorporate selenium at high \(S\) to \(Se\) ratios into one of its major enzymes, thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9) \(9\). The selenium moiety of this enzyme was identified as selenomethionine. In this report, factors which influence the incorporation of selenium into thiolase are presented. The distribution of selenium throughout the primary structure of the protein is shown to mimic methionine which is present in relatively high amounts in this enzyme.

**EXPERIMENTAL PROCEDURES**

**Enzyme Assays**—Thiolase activity was determined in the thiolytic direction by monitoring the decrease in absorbance at 303 nm \(10\). The assay mixture contained 100 \text{mM Tris-HCl, pH 8.0}, 1 \text{mM 1,4-dithiothreitol, 10 mM MgCl}_2, 50 \text{mM acetoacetyl coenzyme A, 200} \mu \text{M coenzyme A and enzyme. The extinction coefficient used was 14,000} \text{M}^{-1} \text{cm}^{-1} \(11\).

**Growth of Bacteria**—*C. kluyveri* was grown on ethanol and acetate in a mineral salts medium as described previously \(12\). Selenium was offered to the cultures as either selenomethionine \((0.5 \mu\text{M})\) or sodium selenite \((0.5-1 \mu\text{M})\). Cells labeled with \(^{75}\text{Se}\) were obtained by adding 0.1-1 \text{mCi of either} \[^{75}\text{Se} \text{selenomethionine (Amersham)}\] or \(^{78}\text{SeO}_3^-\) \(\text{New England Nuclear) per liter of culture. In a series of experiments 0.25-6.0 mM L-methionine was also added to the culture medium. Additional sulfur in the form of} 1 \text{mM Na}_2\text{S}\) or 0.18 \text{mM Na}_2\text{S}_2\text{O}_3, which was used as a reducing agent was also present.

*C. kluyveri* cells were labeled with \(^{35}\text{S}\) of known specific activity, by growth in media supplemented with 1-10 \text{mCi of carrier-free} \(\text{H}_2\text{S}^{35}\text{S} \text{O}_4\) \(\text{ICN}) and 0.6 \text{mM titanium (III) citrate} \(13\). The use of titanium citrate as a reducing agent allowed for accurate determination of available sulfur and eliminated the inclusion of sulfur contributed by sodium sulfide or dithionite. Cells were harvested by centrifugation and extracts were made by sonication with a Bronson LS-75 sonifier.

**Purification of Thiolase**—In early experiments, thiolase was purified as described \(9\) with several modifications. Because a loss of activity was experienced on the final purification step, chromatography on Matrex gel green A, an alternative procedure was sought. Chromatography of partially purified thiolase on CM-Sephadex 50 as described elsewhere \(14\) yielded nearly homogeneous enzyme with a specific activity comparable to that obtained earlier. By optimization of this technique, crude extracts of *C. kluyveri* were chromatographed directly on CM-Sephadex \(14\), and highly purified enzyme was obtained in a single step.

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Immunization and Immunoprecipitation—The peak thiolase fractions from CM-Sephadex chromatographic profiles were used for the production of sheep antibodies. For the initial injection, emulsions were made with an equal volume of Freund’s complete adjuvant and 1 mg of purified thiolase. Multiple site, subcutaneous injections were administered on day 0. On day 15, similar injections were given except that Freund’s incomplete adjuvant was used. A second booster was injected on day 27 and the animal was subjected to plasmapheresis on day 35 to obtain 1 liter of immune plasma. Serum was obtained by adjusting the plasma to 30 mM CaCl₂ and warming to 30 °C for 1 h to allow for complete clot formation. Immunoglobulin G was purified by precipitation with 40% saturated ammonium sulfate (15). Quantitative immunoprecipitation was performed by incubating 1.6 units of thiolase with 0.48 pfu of anti-thiolase IgG (20 mg/ml) in phosphate-buffered saline. Reactions were allowed to proceed overnight at room temperature and the immunoprecipitates were collected by centrifugation. The supernatants were assayed for thiolase activity.

Pelage were washed with 170 μl of H₂O, resuspended in 100 μl of H₂O, and assayed for radioactivity.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed in a vertical slab apparatus by the method of Laemmli (16) using either 10 or 15% acrylamide. Gradient SDS gels (10-20% polyacrylamide) were obtained from Separation Sciences. Standards for the calibration of gels were obtained from Bio-Rad and consisted of: phosphorylase b (92,500), bovine serum albumin (66,200), carbonyl anhydrase (31,000), soybean trypsin inhibitor (21,500), and β-galactosidase (14,400).

Isolation of Thiolase Subunits by Reverse Phase HPLC—Purified thiolase was chromatographed on a Waters C₁₈ μBondapak column to isolate individual subunits. Solvent A is 0.05% trifluoroacetic acid; Solvent B is acetonitrile containing 0.05% trifluoroacetic acid. Thiolase in 50 mM potassium phosphate buffer, pH 7.1, and 1 mM L-histidine hydrochloride was applied directly to the columns which were eluted with 35% solvent B. The solvent program was 10 min 65% solvent A, 30 min linear gradient from 35 to 50% solvent B. Chromatography was performed using an IBM 9533 Liquid Chromatograph with an IBM 9000 computer. The detector used was an HP1040A photodiode array detector, which was capable of monitoring eight wavelengths simultaneously, as well as taking and storing spectra (17).

Reduction and Labeling of Sulphydryl Groups in Thiolase—To obtain complete enzymatic digestion of thiolase with protease, it was necessary to reductively denature the protein and then block its sulphydryl groups. Since selenoethers are susceptible to alkylating agents, a very mild reagent, DTNB, was chosen. The formation of the DTNB mixed disulfide afforded a unique chromophore which absorbed at 328 nm and, when coupled with ³⁵S label, helped to differentiate methionyl from cysteinyl residues. Thiolase labeled with ³⁵S was mixed with ³²Se-labeled thiolase (~0.5 mg each) and dialyzed overnight against 8 M urea and 200 mM β-mercaptoethanol. The redispersed denatured protein was dialyzed against 8 M urea with two changes in less than 1 h. To each milliliter of protein solution, 0.2 ml of 10 mM DTNB (in pH 7.0, 50 mM potassium phosphate buffer) was added and allowed to react for 10 min. The DTNB-labeled enzyme was then dialyzed against 1 liter of 1 M urea containing 0.05% trifluoroacetic acid.

Peptide Mapping—Each milliliter of TNB-thiolase was adjusted to pH 8.0 by the addition of 230 μl of 1 M Tris-HCl, pH 8.0. TPCK-treated trypsin, dissolved in 1 mM HCl, was then added at a proteinto-trypsin ratio of 20:1 and the digestion was allowed to proceed overnight at 37 °C. An additional aliquot of trypsin was added the next morning and the incubation was continued for an additional 2 h. The pH of the solution was adjusted to 2 by the addition of HCl.

Peptide maps of digested thiolase were generated by HPLC on a C₁₈ μBondapak column using the same instrumentation described above. The column was equilibrated with 0.05% trifluoroacetic acid. A linear gradient from 0 to 50% acetonirole, 0.05% trifluoroacetic acid was developed in 50 min at a flow rate of 1 ml/min. Fractions (0.5 min) were collected and assayed for ³²Se radioactivity using a Beckman γ Counter 5000. Half of each fraction was then added to 10 ml of Aquasol (New England Nuclear) and assayed for ³⁵S and ³²Se radioactivity using an LKB Rack γ scintillation counter. The remaining fractions were then recounted for ³²Se radioactivity. Radioactivity due to ³²Se was calculated by difference between scintillation counts and γ counts after relative efficiency corrections were made.

RESULTS

Isolation of Thiolase—A method for the purification of thiolase from C. kluyveri involving a series of chromatographic and salt fractionation steps was developed previously (9). More recently a single-step simultaneous procedure for the isolation of both thiolase and 3-hydroxybutyryl-CoA dehydrogenase had been developed using CM-Sephadex (14). Isolation of thiolase by either procedure yielded a homogenous protein as judged by native gradient gel electrophoresis and isoelectric focusing under native conditions, as well as in the presence of 8 M urea. SDS-gel electrophoresis (see Fig. 1, lane 2) of the purified enzyme under optimal conditions revealed two bands differing in molecular weight by less than 1000. The possibility that one of the bands was the result of partial proteolysis was considered. Extracts were made in the presence of the following protease inhibitors: 1 mM PMSF, 0.1 mg/ml of α-macroglobulin, 0.05 mg/ml of leupeptin, 20 mM ε-aminocaproic acid, and 50 mM EGTA. Thiolase was isolated as described above or isolated by precipitating with antibody raised against the purified enzyme. The same band pattern was observed in the presence or absence of the protease inhibitors. Thus, the occurrence of two types of thiolase subunits does not appear to be a manifestation of an artificial proteolytic event occurring during the course of isolation.

Quantitative Immunoprecipitation of Thiolase—To monitor

Fig. 1. SDS-polyacrylamide gel electrophoresis. Lane 1, crude thiolase preparation; lane 2, purified thiolase; lanes 6-8, immunoprecipitates of thiolase with 10, 15, 20, 25, and 30 μl of purified IgG (20 mg/ml). All incubations contained 1.6 units of thiolase, obtained from C. kluyveri cultures grown on selenite. Lane 8 is an IgG control. Immunoprecipitates were collected by centrifugation and redissolved in 4% SDS, 10% β-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. Samples were then heated at 100 °C for 5 min and then electrophoresed as described under “Experimental Procedures.”
the incorporation of selenium into thiolase an immunological method was developed. Sheep antibodies raised against the purified enzyme resulted in total immunoprecipitation of thiolase activity (O—O). The supernatants were assayed for thiolase activity (x—x). Cultures used in these experiments were grown on selenite, as described under "Experimental Procedures."

SDS-gel electrophoretic analysis of an immunoprecipitation experiment is shown in Fig. 1. In lane 1, a crude preparation of thiolase has been applied. Lane 2 shows the multiple subunits that occur with the purified enzyme. Lanes 3–8 are immunoprecipitates with increasing amounts of antibody added to the incubation mixtures. These data, along with those presented in Fig. 2, allow for the quantitative evaluation of selenium incorporation into thiolase directly from crude extracts.

**Incorporation of Selenium into Thiolase under Various Growth Conditions**—Since the selenium moiety in thiolase had been identified as selenomethionine, it was reasoned that if nonspecific incorporation of selenomethionine for methionine was occurring then selenium incorporation should be markedly decreased when methionine was added to the culture medium. In contrast, a mechanism of selenium incorporation invoking a post-translational modification of thiolase (as is envisioned for selenoenzymes containing selenocysteine) would not be expected to be affected by the concentration of methionine added to the culture medium. The form in which selenium was offered to the cells might also affect the incorporation of selenium into thiolase and the resulting effect of methionine.

When selenium was offered to C. kluyveri as [\(^{75}\)Se]selenomethionine, its incorporation into the protein was totally competitive with methionine added to the culture (Table I). These results are in agreement with those of others (18) who have shown that selenomethionine can be used to label proteins by replacing methionine. Obviously different results were obtained when selenium was added as selenite in the presence of various levels of methionine (Table II). Selenium incorporation into thiolase was not decreased by the addition of increasing amounts of methionine to the culture. In fact, methionine appears to have enhanced selenium incorporation into the enzyme.

**Isolation of Thiolase Subunits by Reverse Phase HPLC**—Thiolase subunits were isolated by reverse phase HPLC to ascertain whether selenium was preferentially incorporated into either of the subunits. Chromatography of thiolase on a Waters C\(_{18}\)Bondapak resulted in excellent resolution of the thiolase subunits (Fig. 3). The peak eluting second from this
column corresponded to the lower molecular weight band separated by SDS-polyacrylamide gel electrophoresis (Fig. 1). Enzyme samples isolated from cultures labeled with $^{35}$S or $^{75}$Se were mixed and applied to the column. As shown in Fig. 3, both peaks contained selenium. However, the radioactivity profiles due to $^{35}$S and $^{75}$Se did not completely coincide. Enzyme obtained from a culture in which both isotopes were added simultaneously also showed this discrepancy, thus eliminating the possibility of differences in the two enzyme preparations (Fig. 3). This suggests that a sulfur-containing species of thiolase can be partially resolved from its selenium analog by this procedure.

**Peptide Mapping of Thiolase Labeled with $^{35}$S and $^{75}$Se—**To further elucidate the distribution of selenium into thiolase, enzyme labeled with $^{35}$S or $^{75}$Se (by growing cells in the presence of Na$_2$Se$_2$O$_3$) was subjected to proteolysis with TPCK-treated trypsin. Peptide maps were generated by reverse phase HPLC. Fractions were collected throughout the digestion of total thiolase, the selenium and sulfur profiles due to $^{35}$S and $^{75}$Se did not completely coincide. Enzyme obtained from a culture in which both isotopes were added simultaneously also showed this discrepancy, thus eliminating the possibility of differences in the two enzyme preparations (Fig. 3). This suggests that a sulfur-containing species of thiolase can be partially resolved from its selenium analog by this procedure.

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**DISCUSSION**

Cultures of *C. kluwyeri* grown on ethanol and acetate produce butyrate and caproate throughout their fermentation. We have found that thiolase and NADP$^+$-dependent 3-hydroxybutyril-CoA dehydrogenase can constitute nearly 20% of the total cellular protein from this organism (14). Because of the major role these enzymes have in cellular metabolism, levels of selenium in the media appear to be reflected in the composition of these proteins. It was observed initially that the incorporation of selenium into thiolase occurred at relatively high ratios of sulfur-to-selenium (9). Data presented here have shown that when selenium is offered as selenite to cultures of *C. kluwyeri* the incorporation of selenium is not reduced by the addition of methionine. A possible explanation for this phenomenon may be linked to the fact that *C. kluwyeri* has an extremely active sulfur metabolism responsible for the synthesis of large quantities of CoA. Cysteine, a precursor of CoA, may be continuously synthesized from available sulfur, other than methionine, even in the presence of high concentrations of methionine. In addition, because of this cysteine requirement, there may in fact be no feedback inhibition with respect to methionine synthesis. Similar results have also been obtained with 3-hydroxybutyril-CoA dehydrogenase labeled with $^{35}$S and $^{75}$Se (data not shown). These data, together with the apparent lack of a specific selenium requirement for enzymatic activity, indicate that the element is not an essential part of the protein structure.

Studies with *Escherichia coli* have shown that the efficient dispersal of selenium into methionine (22, 23) and subsequently into proteins, for example $\beta$-galactosidase, has little effect on catalytic activity or physical properties of the protein (22). However, in contrast to the studies described here with *C. kluwyeri*, the *E. coli* cultures were grown with 10 mM selenate or selenomethionine with little or no sulfur added to
the media. Prokaryotes and also yeasts and some higher plants appear to have the capacity of converting excess selenium into selenomethionine which can then be readily translated into proteins in place of methionine. The possibility that this mechanism of selenium storage occurs in mammals has yet to be established.

Examples of specific incorporation of selenium into proteins (24–27) and tRNAs also exist (28–30). The mechanisms by which this occurs is one of the central questions in selenium biochemistry. It is likely, in these instances that selenium incorporation in proteins occurs post-translationally (31). In several of the proteins that have been characterized the selenium moiety exists as selenocysteine. However, the precise location of selenium within the polypeptide chains of many of these proteins remains unknown. The data described here indicate that significant amounts of selenium can also exist in proteins as selenomethionine, but the distribution of selenium in these enzymes occurs throughout the primary structure of the protein rather than at an active, catalytic center.

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REFERENCES