Effect of glutathione deficiency on the pool of CoA–glutathione mixed disulfide in *Escherichia coli*[1]

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The formic acid extracts of several glutathione-deficient strains of *Escherichia coli* have been assayed for the presence of the mixed disulfide of CoA and glutathione, CoASSG. Strains deficient in γ-glutamyl-cysteine synthase (EC 6.3.2.2) produced only CoA dimer. Strains deficient in glutathione synthase (EC 6.3.2.3) produced the mixed disulfide of CoA and the γ-glutamyl-cysteine dipeptide. The pool size of total CoA in the cell did not change significantly even in the absence of glutathione.

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Nous avons vérifié la présence du disulfure mixte de CoA et de glutathion, CoASSG, dans les extraits à l'acide formique de plusieurs souches de *E. coli* déficientes en glutathion. Les couches déficientes en γ-glutamylcystéine synthase (EC 6.3.2.2) ne produisent que le dimère CoA. Les souches déficientes en glutathion synthase (EC 6.3.2.3) produisent le disulfure mixte de CoA et d'un dipeptide, le γ-glutamylcystéine. La grandeur du pool du CoA total dans les cellules ne change pas de façon significative même en absence de glutathion.

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Introduction

CoASSG has been shown to be a major component of the CoA pool in yeast (1), mammalian tissue (2, 3), and *Escherichia coli* (4, 5). During logarithmic growth on glucose, CoASSG constituted 40–50% of the CoA pool and this proportion increased to greater than 85% under certain growth conditions (6). Whether or not CoASSG is formed enzymatically is not known but the fact that *E. coli* produces much larger amounts of glutathione than most other bacteria (7–9) may facilitate the formation of the mixed disulfide. Its abundance and the presence of an enzyme activity capable of reducing it (5), suggest that the mixed disulfide has some biological significance regardless of its origin. For example, CoASSG has been shown to inhibit RNA polymerase (10, 11).

In 1975 several glutathione-deficient mutants were described which had defects either in gsh B (12, 13) or in the gsh A (13). Because little or no detectable glutathione was produced by these mutants making this aspect of their metabolism similar to many other bacteria (7) but not fungi (14), it was of interest to study the effect of these defects on the CoA pool. Specifically this work reports that CoASSG is not present in any of the glutathione mutants and that there is no change in the overall pool size of CoA in these mutants.

Materials and methods

Chemicals and enzymes

All biochemicals and enzymes were obtained from Sigma.

Bacterial strains

The strains were obtained directly from the laboratory which originally reported their isolation. They were: KMBL54 (F^-, thi, thy, pyr, and lac), 129 (F^-, thi, thy, pyr, lac, and gsh A), AB1157 (F^-, thr, leu, pro, thi, his, arg, lac, gal, ara, xyl, mtl, and $tsx^R str^R$), 821 (from AB1157 and gsh A), and 830 (from AB1157 and gsh B) from Ref. 13.

UV spectra

UV spectra were measured with 1-cm path length cuvettes in a Beckman Acta III spectrophotometer.

Ion-exchange chromatography

Cultures of 10 L of the various strains were grown overnight at 30°C to stationary phase in Tris–salts–glucose minimal medium (4) supplemented with any nutrients required by the individual strains as described in Ref. 13. The cells were collected by centrifugation and resuspended in 120 mL of 1 *M* formic acid for 30 min at 0°C. After centrifugation, the extraction was repeated one time. The combined formic acid extracts were neutralized to pH 6.5 with concentrated ammonia, diluted to 1 L with water, and

ABBREVIATIONS: CoASSG, CoA–glutathione mixed disulfide; gsh A, γ-glutamyl-cysteine synthase; gsh B, glutathione synthase; UV, ultraviolet; TEAB, triethylammonium bicarbonate.

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charged on a 2.5 cm × 20 cm column of DEAE Sephadex A25 equilibrated with 0.2 *M* TEAB pH 7.6. The column was washed with 500 mL of 0.2 *M* TEAB pH 7.6, and a linear gradient of 500 mL 0.2 *M* TEAB and 500 mL 1.5 *M* TEAB was applied collecting 5.5-mL fractions. The absorbance at 260 nm of the fractions was determined using a Unicam SP500 spectrophotometer. Relevant fractions were pooled, concentrated, and purified further by gel filtration on a 1.2 cm × 100 cm Sephadex G50 column eluted with 0.1 *M* TEAB pH 7.6 as previously described (5). Relevant fractions were pooled, concentrated, and characterized as described in the text.

Amino acid analysis

Portions of various pooled fractions from Sephadex G50, were treated with performic acid for 3 h at 0°C (15), evaporated, taken up in 6 *M* HCl, sealed, and incubated for 16 h at 105°C. After concentration, the samples were analyzed both on a Technicon NC2P amino acid analyzer operated by the Department of Chemistry, University of Manitoba and by two-dimensional thin-layer chromatography as previously described (5).

Glutathione and CoA assays

Portions of 5–20 μL of the formic acid extracts were assayed for glutathione using the cycling assay of Tietz (16) as modified by Fahey *et al.* (14). All determinations were performed in duplicate at two different extract concentrations. The formic acid extracts were also assayed for CoA using the cycling assay of Allred and Guy (17). The assays were performed in the presence of dithiothreitol to assay for the total amount of CoA in the extract. CoASSG was shown to be a substrate for both the glutathione assay and the CoA assay. Results are expressed in terms of nanomoles per milligram dry weight where the dry weight was the total weight of the cells prior to extraction.

Results

A procedure similar to that initially used to isolate CoASSG from *E. coli* extracts was adapted to assay the extracts of various glutathione-deficient strains for the presence or absence of CoASSG. Formic acid extracts of stationary phase cultures of the various glutathione-deficient mutants and their respective parental strains were prepared and subjected to ion-exchange chromatography on DEAE Sephadex A25. Elution of this resin with a TEAB gradient clearly separated CoASSG and CoA dimer from most other cellular components. As shown in Figs. 1 and 2, the elution profiles of the various parental and mutant strains are organized such that the profiles for the respective parent–mutant groups can be compared.

Centered at fraction 84 in the elution profiles of all of the parent strains, there was one predominant peak which, after Sephadex G50 gel filtration, was identified as CoASSG. The criteria used for this identification have been described previously (5) and include the UV absorbance spectrum, R_f values after paper chromatography alongside authentic samples, characteristic elution volumes from DEAE Sephadex A25 and Sephadex G50, amino acid analysis, and more recently enzymatic assays for CoA (17) and glutathione (14). Centered at fraction 96

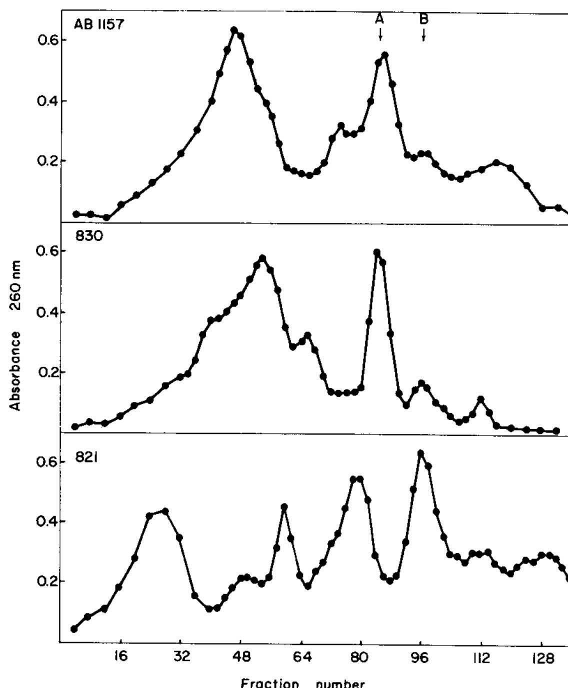

FIG. 1. Elution profile from DEAE Sephadex A25 of the formic acid extracts of strains AB1157 (parent), 821 (gsh A) and 830 (gsh B). The experimental conditions are described in Materials and methods. Peak A corresponds to the location of CoASSG and peak B corresponds to the location of oxidized CoA.

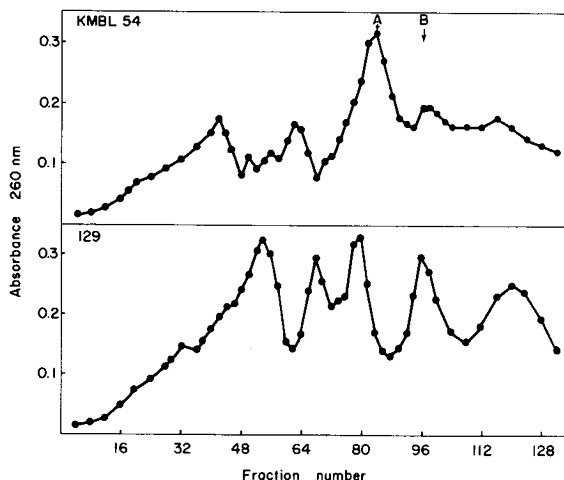

FIG. 2. Elution profile from DEAE Sephadex A25 of the formic acid extracts of strains KMBL54 (parent) and 129 (gsh A). The experimental conditions are described in Materials and methods. Peak B corresponds to the location of oxidized CoA.

TABLE 1. Total CoA and glutathione content in formic acid extracts of logarithmic and stationary growth phase culture

Strain	Total CoA, nmol·mg dry weight⁻¹		Glutathione, nmol·mg dry weight⁻¹	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
AB1157	8.67	8.55	11.53	16.40
821	9.00	10.06	ND*	ND
830	12.98	15.81	ND	0.29
KMBL54	5.45	4.26	7.20	16.80
129	3.03	2.54	ND	0.11

*ND, not detectable.

TABLE 2. Amino acid content of various CoA containing fractions isolated from formic acid extracts

Strain	Fraction	Adenosine*	Taurine	Cysteic acid	Glutamic acid	Glycine	β-Alanine
AB1157	A	2.11	2.12	2.05	2.20	2.10	2.18
	B	0.52	0.48	<0.02	0.38	<0.02	0.51
830	A	3.11	3.12	3.27	3.24	0.21	3.07
	B	0.43	0.40	<0.02	0.61	<0.02	0.43
821	B	2.28	2.31	<0.10	2.46	<0.10	2.14
KMBL54	A	1.46	1.43	1.41	1.49	1.51	1.52
	B	0.51	0.49	<0.02	0.41	<0.02	0.48
129	B	1.17	1.14	<0.10	1.25	<0.10	1.13

*Data is expressed as millimolar in the pooled fractions from Sephadex G50 after concentration to 1 mL.

there was a similar peak which, after Sephadex G50 gel filtration, was identified as CoA dimer but with a contaminating glutamate component. The same criteria used to identify CoASSG were also used to identify the CoA dimer and more recent work has shown the glutamate to result from a contaminant of *p*-aminobenzoic acid pentaglutamate (unpublished data).

The three mutants can be grouped on the basis of the DEAE Sephadex elution profiles. Strain 830 (Fig. 1) produced a profile differing only slightly in appearance from its parental strain, AB1157, with a single peak coincident with CoASSG and a smaller peak of CoA dimer. The second pair, 821 and 129 (Figs. 1 and 2, respectively), differed significantly from their respective parent strains with the peak corresponding to CoASSG being absent and replaced by a predominant peak of CoA dimer.

Formic acid extracts of exponential growth phase cells were also prepared for all of the parent mutant groups, and after ion-exchange chromatography, the peaks corresponding to CoASSG and CoA dimer were isolated, purified further by gel filtration, and characterized using the same criteria as already described. For strains AB1157, KMBL54, and 830, there were slight differences in the elution profiles from exponential and stationary phase cultures with the ratio of CoA dimer to CoASSG (or CoAS-S dipeptide from 830) being slightly larger in exponential cells, a result of lower glutathione levels (8, 9). For strains 129 and 821, the elution profiles from exponential and stationary phase cultures were the same.

The sizes of the CoA dimer and CoASSG-like peaks from mutants relative to the same peaks from the parent strains suggested that the levels of CoA were unchanged despite the glutathione deficiency. This was confirmed by the direct assay of the total CoA pools in the various strains compiled in Table 1 and by quantitation of the pooled and concentrated fractions in Table 2. While there was some variation in the total CoA pools among the various parent–mutant groups, there was relatively little difference in the pools of the related parent and mutant strains. Only the pair of KMBL54 and 129 showed a significant difference and the lower level of CoA in 129 showed that increased CoA was not required to compensate for reduced glutathione. Furthermore, there was little or no difference in CoA levels in exponential and stationary phase cells. The glutathione levels in all of the strains were determined both in exponential and stationary phase (Table 1). Consistent with the literature data, the various glutathione-deficient strains produced much reduced levels of glutathione.

A second new peak also appeared in the extracts

of 821 and 129 (Figs. 1 and 2) centered at fraction 78. The absorbance spectrum and mobility during paper chromatography (4) suggested that the compound was guanosine tetraphosphate but this was not pursued further. The amino acid auxotrophic nature of the strains and the possibility of depletion of one component of the minimal growth medium would explain its appearance.

The presence of CoA in the peaks corresponding to CoASSG and CoA dimer was confirmed by several criteria including the enzymatic assay but glutathione was not present in all cases. The enzymatic glutathione assay simply confirmed the presence or absence of glutathione while amino acid analysis clearly defined what components were present or absent. The amino acid analyses of some of the CoASSG-like and CoA dimer peaks are presented in Table 2. The CoASSG peaks from all of the parent strains contained the five amino acids characteristic of CoASSG: taurine, β-alanine, glutamate, cysteine, and glycine. From strain 830, the CoASSG-like compound, which was identical in all respects to CoASSG except that it did not contain assayable glutathione, contained only four amino acids, taurine, β-alanine, glutamate, and cysteine while glycine was present at very low levels. This material was therefore concluded to be the mixed disulfide of CoA and the γ-glutamylcysteine dipeptide was formed in place of CoASSG in strain 830.

Discussion

The disulfide species of CoA found in the formic acid extracts of different glutathione-deficient mutants of *E. coli* can be correlated with the genotype of the mutants. There are two enzymes involved in combining the three amino acids to form glutathione. The γ-glutamyl-cysteine synthase catalyzes the first step to form the dipeptide and strains 129 and 821, which were deficient in this enzyme, were labelled gsh A (13). With no dipeptide being formed and cysteine existing at very low levels (8), CoA was the predominant sulfhydryl species in the cell and as expected, CoA dimer was found to be the principal CoA species in 821 and 129. The second enzyme, glutathione synthase, catalyzes the condensation of glycine with γ-glutamylcysteine and strain 830, which was characterized as being deficient in this enzyme, was labelled gsh B (13). The dipeptide was shown to accumulate in this strain in place of glutathione (13) and as expected this dipeptide was found joined to CoA by a disulfide link replacing CoASSG.

The levels of coenzyme A were not significantly altered in any of the glutathione-deficient strains relative to the parental strains. Even in strains 830 and 129 where no dipeptide was formed and CoA was the principal low molecular weight sulfhydryl form in the cell, the CoA level did not increase to compensate for the absence of glutathione. Perhaps significantly the CoA pool in *E. coli* is from three- to five-fold larger depending on the strain, than the CoA pool in *Bacillus megaterium* (18, 19) and this may more than satisfy the need for thiol in the cell even in the absence of glutathione.

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