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BIOCHEMICAL CHARACTERIZATION OF GLUTATHIONE-DEFICIENT MUTANTS OF *ESCHERICHIA COLI* K12 AND *SALMONELLA* STRAINS TA1535 AND TA100

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Abstract—Glutathione-deficient mutants of *Escherichia coli* K12/343/408 and *Salmonella typhimurium* TA1535 and TA100 were characterized biochemically by measuring the rate of formation of (^{14}C)γ-glutamylcysteine and (^{14}C)glutathione in cell-free extracts of the strains. γ-Glutamylcysteine synthetase activity was found to be absent in the NGR-2 mutant of *E. coli* and in the *Salmonella* mutants TA1535/NG-19, TA100/NG-57 and TA100/NG-11, while only low activities were found in the NGR-9 and NG-54 mutant of *E. coli* and *Salmonella* respectively. These results correspond with the decreased levels of glutathione found in these strains. Extracts of the parent strains have normal glutathione levels and show high γ-glutamylcysteine synthetase activities. It is concluded that the present GSH-deficient strains of *E. coli* and *Salmonella* are *gshA* mutants, analogous to those previously described in *E. coli*. In addition, the present results show that the fluorometric method used for the determination of glutathione, employing *o*-phthalaldehyde as a reagent, is not specific for glutathione (at pH 8.0), but also sensitively reacts with γ-glutamylcysteine.

In previous papers [1–3] we reported on the isolation of glutathione ‖(GSH)-deficient mutants of *E. coli* and *Salmonella typhimurium*, and on their usefulness in assessing the role of GSH in the activation or deactivation of mutagenic chemicals inside cells. The presence of GSH has been demonstrated in a wide variety of cells, including bacteria [4–6], and GSH-deficient mutants have already been described and characterized before [7–9]. The purpose of the present studies was to determine whether (and which) enzyme deficiencies of the GSH-biosynthesis pathway is (are) responsible for the decreased GSH levels observed in our previously isolated mutants of *E. coli* and *Salmonella*.

In *E. coli* GSH represents about 95% of the acid-soluble thiol fraction. The biosynthesis of GSH was shown to occur in two ATP-dependent steps [10]: glutamic acid + cysteine + ATP → γ-glutamylcysteine + ADP + P_i (I) γ-glutamylcysteine + glycine + ATP → GSH + ADP + P_i (II). The first is catalyzed by γ-glutamylcysteine synthetase and the second by GSH synthetase. The synthesis of GSH from glutamic acid, cysteine, and glycine in cell-free extracts of *E. coli* has been demonstrated by Samuels [11]. Two types of GSH-deficient mutants have been characterized in *E. coli* [7–9]. Those deficient in γ-glutamylcysteine synthetase (*gshA*) and others deficient in GSH synthetase (*gshB*). In the present paper, we employed the same enzymatic procedures as those described by Apontoweil and Berends [7], using their *gshA* and *gshB* mutants as reference.

MATERIALS AND METHODS

Chemicals. GSH and L-amino acids were obtained from Sigma Chemical Co. (St. Louis, MO) ATP, phosphoenolpyruvate, pyruvate kinase 500 (U/mg) and carboxypeptidase A (35 U/mg) from Boehringer Mannheim (F.R.G.); L-(U-^{14}C)glutamic acid and L-(U-^{14}C)glycine from Amersham (Buckinghamshire U.K.). γ-Glutamylcysteine was prepared from GSSG, obtained after oxidation of GSH, as described by Strumeyer and Bloch [12]; yield: 37%. The product formed was subjected to electrophoresis in a Whatman 3 MM paper strip for 16 hr at 6.5 V/cm and 4° in a buffer consisting of acetic acid/pyridine/water (75:15:2000), pH 3.9. Thereafter, the paper strip was sprayed with ninhydrin reagent and heated for color development indicative of amino acids, amines, or amino sugars. The product so formed, and examined by this procedure, showed one single spot at 17.5 cm in the direction of the positive electrode. All other chemicals were of analytical grade.

Bacterial strains. A list of the strains employed in this study is given in Table 1. The GSH-deficient mutants NGR-2 and NGR-9 were derived from *E. coli* K12/343/408 [9] and the mutants TA1535/NG-19, TA100/NG-57, TA100/NG-54 and TA100/NG-11 were isolated from *Salmonella* strains TA1535 and TA100 were kindly provided by Prof. B. N. Ames, Berkeley, U.S.A. The *E. coli* K12 strain AB1157

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‖ Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; OPT, *o*-phthalaldehyde.

Table 1. GSH$^{+/-}$ Derivatives of *E. coli* and *Salmonella* strains used

Strain	Relevant genotype	Origin or reference
E. coli K-12		
AB1157	*thi-1, thr-1, leu-6, proA2, hisG6, argE3, strA31, supE44, lacY1, galK2, ara-14, mtl-1 tsx-33*	Apontoweil and Berends [7]
821	as AB1157, *gshA2*	Apontoweil and Berends [7]
830	as AB1157, *gshB3*	Apontoweil and Berends [7])
343/408	*galR^s 18, arg-87, his-233, nad-62*	Mohn *et al.* [1]
343/408/NGR-2	as 343/408, *gshA50*	Mohn *et al.* [1] and this paper
343/408/NGR-9	as 343/408, *gshA51*	Mohn *et al.* [1] and this paper
Salmonella LT2		
TA1535	*hisG46, rfa, Δ (uvrb.bio)*	Ames *et al.* [13]
TA1535/NGR-19	as TA1535, *gshA4*	Kerklaan *et al.* [2]
TA100	as TA1535, (pKM 101)	Ames *et al.* [13]
TA100/NGR-57	as TA100, *gshA1*	Kerklaan *et al.* [3] and this paper
TA100/NGR-54	as TA100, *gshA2*	Kerklaan *et al.* [3] and this paper
TA100/NGR-11	as TA100, *gshA3*	Kerklaan *et al.* [3] and this paper

and its GSH-deficient derivatives 821 (*gshA*) and 830 (*gshB*) were obtained from Dr P. Apontoweil, Delft, The Netherlands.

Media. The composition of nutrient broth has been described elsewhere [13]. The minimal medium used was that described by Vogel and Bonner [14], supplemented with 0.2% glucose.

Preparation of extracts. Overnight cultures of the strains were prepared by growing in nutrient broth until the stationary phase was reached (≥16 hr at 37°). The cultures were then washed and re-suspended to reach a concentration of 1 g of wet cells per 5 ml of minimal medium. The bacterial suspensions were then sonicated for 5 × 1 min at 0° followed by centrifugation for 10 min at 12,000 *g*. The resulting supernatant fractions containing the cell-free extracts were frozen directly at −90° and kept at this temperature for up to 1 month. The protein concentration was determined by the method of Lowry *et al.* [15].

Assay of γ-glutamylcysteine synthetase. The method described by Apontoweil and Berends [5] was used to determine the γ-glutamylcysteine synthetase activity in bacterial extracts. The final assay mixture (0.5 ml, pH 8.5) contained: 2.5 μmol ATP, 5 μmol phosphoenolpyruvate, 0.004 mg pyruvate kinase, 5 μmol MgSO4, 50 μmol KC1, 7.5 μmol cysteine, 7.5 μmol (U-^{14}C)glutamic acid (sp. act. 120 μCi/mmol), 0.05 ml 1 M diethanolamine/HCl buffer pH 9.15, 0.5 mg bovine serum albumin and 0.1 ml cell-free extract of *E. coli* or 0.2 ml extract of *Salmonella*. After incubation at 37° for 15 min, the enzyme reaction was terminated by the addition of 0.017 ml of 3 M trichloroacetic acid, followed by centrifugation to remove the denaturated protein. To determine the amount of (^{14}C)γ-glutamylcysteine formed, 20 μl portions of the supernatant were subjected to electrophoresis on Whatman 3 MM paper strips for 16 hr at 6.5 V/cm and 4° in a buffer consisting of acetic acid/pyridine/water (75:15:2000), pH 3.9. Under these conditions (^{14}C)glutamic acid migrates 9–13 cm in the direction of the positive electrode while (^{14}C)γ-glutamylcysteine and its oxidized form [(^{14}C)diglutamylcysteine] move 14–20 cm in the same direction, as determined by comigration of reference amounts of glutamic acid, γ-glutamylcysteine and glutamylcystine. The paper strips were dried, cut into 1 cm sections and the amount of radiolabel determined in a toluene-based scintillation fluid using a Beckman liquid scintillation counter.

Assay of GSH synthetase. The same method was used as that described by Apontoweil and Berends [10], which itself is based on the method of Mooz and Meister [16] for crude extracts, with some modification. The formation of (^{14}C)GSH from γ-glutamylcysteine and (^{14}C)glycine was determined in a mixture (0.5 ml, final pH 8.5) containing: 2.5 μmol ATP, 5 μmol phosphoenolpyruvate, 0.004 mg pyruvate kinase, 2.5 μmol γ-glutamylcysteine, 7.5 μmol (^{14}C)glycine (sp. act. 60 μCi/mmol), 5 μmol $MgSO_4$, 50 μmol KCl, 0.05 ml 1 M diethanolamine/HCl buffer pH 9.05, 0.5 mg bovine serum albumin and 0.1 ml cell-free extract of *E. coli* or 0.2 ml of an extract of *Salmonella*. After incubation at 37° for 30 min, the reaction was stopped by addition of 0.017 ml 3 M trichloroacetic acid followed by centrifugation. The supernatant was analyzed by paper electrophoresis as described above. Electrophoresis was carried out for 2 hr at 10.6 V/cm and room temperature. (^{14}C)GSH moved 1–4 cm towards the anode and (^{14}C)glycine 0–2 cm in the opposite direction.

Determination of intracellular GSH content. The fluorometric method described by Hissin and Hilf [17] was used to estimate the endogenous GSH content of the bacteria, with small modifications. To 0.1 ml of bacterial extract 0.02 ml of 25% trichloroacetic acid were added, followed by centrifugation to remove the precipitated protein. Aliquots (0.04 ml) of the resulting supernatant fractions were diluted with 2 ml of the 0.1 M phosphate–0.005 M EDTA buffer (pH 8.0). The final reaction mixture (2.0 ml, pH 8.0) contained 0.1 ml of the diluted supernatant, 1.8 ml of phosphate–EDTA buffer and 0.1 ml of the *o*-phthalaldehyde (OPT)

Table 2. Specific activities of γ-glutamylcysteine synthetase and GSH synthetase in extracts of *E. coli* and *Salmonella typhimurium*

Strains	γ-Glutamylcysteine synthetase		GSH synthetase	
	Specific activity	Relative specific activity	Specific activity	Relative specific activity
AB1157	0.043	100	0.33	100
821	0.004	9	0.25	76
830	0.052	121	<0.003	<1
343/408	0.079	100	0.25	100
343/408/NGR-2	<0.001	<1	0.27	108
343/408/NGR-9	0.004	5	0.32	128
TA1535	0.065	100	0.36	100
TA1535/NG-19	<0.001	<2	0.25	69
TA100	0.078	100	0.35	100
TA100/NG-57	<0.001	<1	0.34	97
TA100/NG-54	0.003	4	0.32	91
TA100/NG-11	<0.001	<1	0.30	86

Assays for the determination of γ-glutamylcysteine synthetase and GSH synthetase activities were performed as described in Materials and Methods. Specific activity is given in μmol peptide formed per mg protein in 15 min (γ-glutamylcysteine synthetase) or 30 min (GSH synthetase). Relative specific activity is expressed in percentage of the specific activity of the parent strain. The values shown are the means of 2 separate experiments. The protein concentration of the bacterial extracts was determined according to the method described by Lowry *et al.* [15].

solution, containing 0.1 mg OPT. After incubation at room temperature for 15 min, the fluorescence of the reaction mixture was measured at 420 nm (excitation at 350 nm).

RESULTS

Table 2 gives the values for γ-glutamylcysteine synthetase and GSH synthetase in extracts of *E. coli* strains 343/408 and AB1157 and *Salmonella* strains TA1535 and TA100 and their respective GSH-deficient derivatives, as listed in Table 1. The specific activity of γ-glutamylcysteine synthetase found in the extracts of *E. coli* strains 343/408 and AB1157 and *Salmonella* strains TA1535 and TA100 is on the average 0.066 μmol γ-glutamylcysteine/mg protein/15 min. The activities found in the GSH-strains are strongly reduced, except for *gshB* mutant 830, as shown in Table 2. These results indicate that the GSH-mutants 821 (*gshA*), 343/408/NGR-2 and -9 as well as TA100/NG-11, -54, -57 and TA1535/NG-19 are defective in γ-glutamylcysteine synthetase. No reduction in the activity was observed in the *gshB* mutant 830. The specific activity of GSH synthetase found in the GSH^+ parent strains used is on the average 0.32 μmol GSH/mg protein/30 min, as fol-

Table 3. Cellular glutathione content of *E. coli* and *Salmonella typhimurium* strains differing in GSH^- synthesizing activity

Strains	GSH content (nmol GSH/mg protein)	Percentage of the parent strain
AB1157	67.4 ± 14.5	100
821	22.9 ± 11.5	34
830	71.9 ± 17.8	107
343/408	27.8 ± 6.6	100
343/408/NGR-2	8.4 ± 5.7	30
343/408/NGR-9	13.2 ± 4.2	47
TA1535	41.7 ± 11.9	100
TA1535/NG-19	12.7 ± 6.0	30
TA100	24.4 ± 4.6	100
TA100/NG-57	7.8 ± 2.8	32
TA100/NG-54	6.2 ± 1.0	25
TA100/NG-11	7.3 ± 2.9	30

Cell-free extracts were prepared from overnight cultures grown in nutrient broth to stationary phase, as described in Materials and Methods. The GSH concentration of the extracts was determined by the fluorometric method described by Hissin and Hilf [17]. The GSH content is the average of 3 separate determinations ± SEM. The protein concentration of the bacterial extracts was determined according to Lowry *et al.* [15].

lows from Table 2. The activities found in the GSH^- derivatives are comparable to those observed in the GSH^+ parent strains, except for *gshB* mutant 830, as shown in Table 2. 830 showed no measurable GSH synthetase activity in cell extracts. From the results obtained with the enzyme assays it can thus be concluded that the present GSH^- deficient mutant strains of *E. coli* K12/343/408 and *Salmonella* TA1535 and TA100 are *gshA* mutants.

The GSH content of the strains used, as determined by the fluorometric assay, are given in Table 3. The GSH levels found in the GSH^- mutant strains of *E. coli* and *Salmonella* are on the average 34% of those found in the corresponding GSH^+ parent strains, with the exception of *gshB* mutant 830. As Table 3 shows, the GSH level found in that strain is approximately equal to that of the GSH^+ parent AB1157. This result was unexpected since cell-free extracts 830 exhibited no measurable GSH synthetase activity (see also [7]). On the other hand, it has been reported that *gshB* mutants accumulate γ-glutamylcysteine (or its oxidized form) at rather high concentrations [7, 9]. This led us to expect that γ-glutamylcysteine interferes with the measurement of GSH. Figure 1 shows two calibration curves established with known amounts of GSH (Fig. 1a) or γ-glutamylcysteine (Fig. 1b) with OPT, at pH 8.0. They show that the fluorescence intensity of equimolar quantities of these compounds exposed to OPT, under similar conditions (pH 8.0), is approximately equal. It can, therefore, be concluded that the fluorometric method using OPT for the determination of GSH is equally sensitive to γ-glutamylcysteine present in the extracts.

DISCUSSION

The present results show that the GSH-deficient derivatives of *E. coli* 343/408 (NGR-2 and NGR-9), of *Salmonella* TA1535 (NG-19) and of *Salmonella* TA100 (NG-57, NG-54 and NG-11) are *gshA* mutants, in analogy to previously described mutants of *E. coli* [7, 8]. It can also be concluded that the GSH depletion observed in these strains results from a decreased synthesis of GSH, since these mutants appear to be deficient in γ-glutamylcysteine synthetase. Whether the method used to isolate the GSH-mutants, namely selection for resistance to *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine [1–3], is directly related with the finding that all the presently characterized mutants are *gshA* mutants remains to be determined. The average value of the specific activity of γ-glutamylcysteine synthetase found in the extracts of *E. coli* strains 343/408 and AB1157 and *Salmonella* strains TA1535 and TA100 is a factor of 3.5 lower than the value reported by Apontoweil and Berends [10]. They determined the γ-glutamylcysteine synthetase and GSH synthetase activity in AB1157. The average value of the specific activity of GSH synthetase found in the GSH^+ parent strains used does agree, however, with the value published by Apontoweil and Berends [10]. We used the same procedure, i.e. 15 min for γ-glutamylcysteine and 30 min for GSH, as these authors did for characterizing their GSH^- mutants (see [7] and [10]). They showed that the reaction catalyzed by γ-glutamylcysteine synthetase is not linear with time, while that catalyzed by GSH synthetase is reasonably linear with time for up to 30 min. They also showed

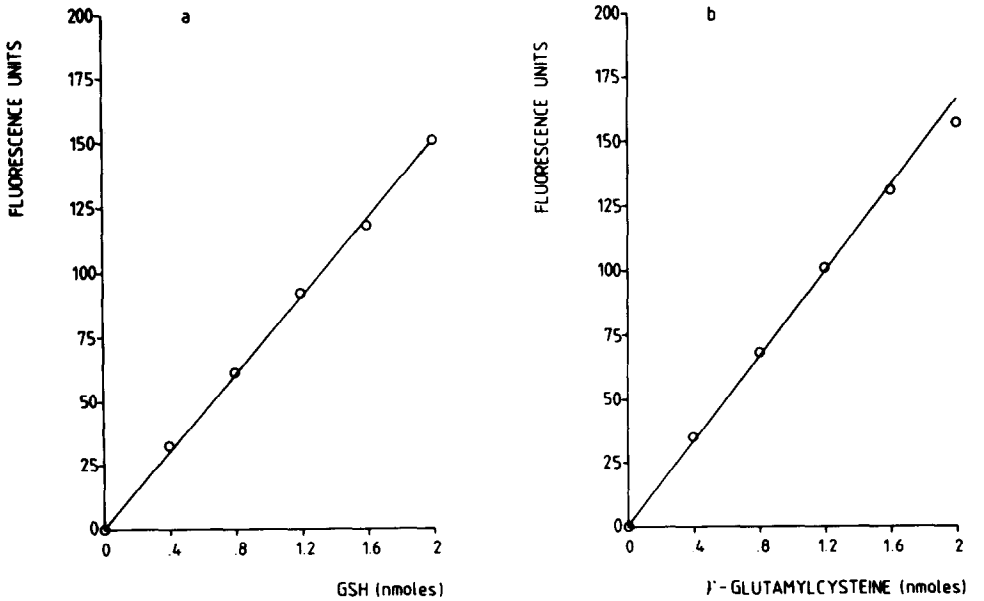

Fig. 1. Fluorescence of reaction mixtures containing OPT and GSH or γ-glutamylcysteine (b). GSH and γ-glutamylcysteine were dissolved in phosphate–EDTA buffer, pH 8.0. Various amounts of these solutions were mixed with 100 μl of an OPT solution and adjusted to 2 ml with buffer. After incubation for 15 min at room temperature, the fluorescence intensity was measured at 420 nm (excitation at 350 nm). The experimental points represent the values of 1 representative experiment.

that the initial velocity of γ-glutamylcysteine synthesis is approximately 2-fold of that of the reaction catalyzed by GSH synthetase. It is possible that our conditions for assaying γ-glutamylcysteine synthetase have not been optimal. Since, on the other hand, only the ratios of the activities were important for our results, no further attempts were made to optimize these conditions. The relative specific activities of γ-glutamylcysteine synthetase and GSH synthetase found in *gshA* mutant 821 and *gshB* mutant 830 are in agreement with data published by Apontweil and Berends [7]. This confirms the suitability of the presently applied method.

Our present results showed that the *gshA* mutants of *E. coli* strains 343/408 and AB1157 and *Salmonella* strains TA1535 and TA100 contain about 34% of the normal endogenous level of GSH. This corroborates previous data published by Kerklaan *et al.* [2, 3] in *Salmonella* strains. On the other hand, Apontoweil and Berends [7] found no detectable levels of GSH in *gshA* mutant 821. This discrepancy may be explained by the fact that we used nutrient broth as growth medium, in contrast to the minimal growth medium used by Apontoweil and Berends [7]. As we showed in previous experiments, external GSH is actively taken up by (stationary) GSH^- cells of *E. coli* and *Salmonella* [1–3], but it is not likely that reduced GSH will be present in significant amounts in nutrient broth. Loewen [18] has demonstrated that the intracellular pool of GSH in *E. coli* increases with increasing amounts of cysteine in the growth medium. As mentioned earlier, the fluorometric method used to measure the GSH content of the bacteria is also sensitive to γ-glutamylcysteine. It should also be noted that we determined, in general, lower activities of γ-glutamylcysteine synthetase than did Apontoweil and Berends (see above). This possible underestimation may be taken as an indication that part of the observed GSH contents of *gshA* mutants still arose through residual activity of γ-glutamylsynthetase.

In conclusion, the present results demonstrate that *gshA* mutants of *E. coli* and *Salmonella* can be readily isolated through their resistance to *N*-alkyl-*N'*-nitro-*N*-nitrosoguanidines. That these findings may also be applicable to other micro organisms is indicated by the report on the isolation of several GSH^- mutants of *Saccharomyces cerevisiae* which exhibit resistance to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and which all belong to one complementation group [19]. It is also very likely that the reduced GSH levels observed in the mutants used in the present study are not due to an increased excretion of GSH into the medium, although export of GSH outside bacterial cells has recently been shown by Owens and Hartman [20] to occur in *E. coli* and *Salmonella*. Nevertheless, this possibility remains to be investigated as an additional factor leading to decreased GSH levels in the mutants described in this paper.

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