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Identification of a coenzyme A – glutathione disulfide (DSI), a modified coenzyme A disulfide (DSII), and a NADPH-dependent coenzyme A – glutathione disulfide reductase in *E. coli*[1]

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Loewen, P. C. (1977) Identification of a coenzyme A – glutathione disulfide (DSI), a modified coenzyme A disulfide (DSII), and a NADPH-dependent coenzyme A – glutathione disulfide reductase in *E. coli*. *Can. J. Biochem. 55*, 1019–1027

The nucleotides DSI and DSII induced during a slowdown in growth of *E. coli* have been characterized using chemical and biochemical analysis and by enzymic and alkaline fragmentation. DSI consists of coenzyme A and glutathione joined by a disulfide linkage. DSI could be isolated either containing Fe(III) with an $A_{250:260}$ ratio of 1.05 or not containing iron with an $A_{250:260}$ of 0.87. DSII (isolated in 10% the yield of DSI) is a coenzyme A disulfide dimer that also contains two molecules of glutamic acid. DSI was a substrate for NADPH-dependent CoAS-SG reductase (EC 1.6.4.6) which was present in crude extracts of *E. coli*. The specific activity of CoAS-SG reductase increased during growth from early log phase into stationary phase and during a shift from aerobic to anaerobic growth.

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Les deux nucléotides DSI et DSII induits durant un ralentissement de croissance chez *E. coli* sont caractérisés par analyse chimique et biochimique et par fragmentation enzymatique et fragmentation alcaline. Le DSI est formé du coenzyme A et du glutathion reliés par une liaison disulfure. Il peut être isolé contenant du Fe(III) avec un rapport $A_{250:260}$ de 1.05 ou ne contenant pas de fer avec un rapport $A_{250:260}$ de 0.87. Le DSII (isolé avec un rendement de 10% par rapport au DSI) est un dimère de coenzymes A disulfure qui contient aussi deux molécules d'acide glutamique. Le DSI est un substrat de la NADPH – CoAS-SG réductase (EC 1.6.4.6) présente dans les extraits bruts de *E. coli*. L'activité spécifique de la CoAS-SG réductase augmente depuis le début de la phase logarithmique de la croissance jusqu'à la phase stationnaire et lors du passage de la croissance aérobie à la croissance anaérobie.

[Traduit par le journal]

Introduction

The isolation and partial characterization of two nucleotides, labelled DSI and DSII, from *E. coli* were recently reported (1). The nucleotides were found to be present in low amounts throughout cell growth. During a shift from aerobic to anaerobic growth and during growth into stationary phase the level of DSI increased up to 10-fold. Purification of the nucleotides was effected by ion-exchange chromatography and gel filtration of formic acid extracts of the cell paste. Chromatographic properties and UV absorption spectra showed that neither DSI nor DSII was related to the "MS" compounds, ppGpp and pppGpp, previously described by Cashel (2).

ABBREVIATIONS: $A_{250:260}$, the ratio of ultraviolet absorbance at 250 and 260 nm; GSH, glutathione, reduced form; UV, ultraviolet; CoAS-SG, coenzyme A – glutathione mixed disulfide; GSSG, glutathione, oxidized form.

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A subsequent report (3) showed that both DSI and DSII were capable of inhibiting *E. coli* RNA polymerase (EC 2.7.7.6). The inhibitory activity of the nucleotides was unstable and the loss of the inhibitory activity could be correlated with a change in the ratio of UV absorbance at 250 and 260 nm from 1.05 to 0.87. No other change in the physical properties of the nucleotides was evident.

This work describes the further characterization of DSI and DSII. DSI is shown to be a coenzyme A – glutathione mixed disulfide capable of binding one molecule of Fe(III) ion. Binding of the Fe(III) ion is responsible for the increase in the $A_{250:260}$ spectral ratio. Although the same molecule has previously been isolated from yeast (4) and liver (5, 6), there has been no report of such a compound being isolated from procaryotes nor that CoAS-SG could bind Fe(III) ion. DSII is shown to be oxidized coenzyme A also containing two molecules of glutamic acid. DSII like DSI can bind iron.

Two enzymes capable of using CoAS-SG as a substrate have been reported in yeast (7), rat liver (8, 9), and bovine kidney (10). CoAS-SG reductase (NADPH: CoAS-S glutathione oxidoreductase; EC 1.6.4.6) from rat liver catalyzes a NADPH-dependent reduction of CoAS-SG to CoA and GSH (8). GSH–CoAS-SG transhydrogenase (EC 1.8.4.3) from kidney catalyzes a sulfhydryldisulfide exchange between CoAS-SG and GSH to form CoA and GSSG (10). This work describes the presence of a CoAS-SG reductase activity in crude extracts of *E. coli*. The specific activity of this reductase is shown to increase during growth into stationary phase and during a shift from aerobic to anaerobic growth. DSI is a substrate for the enzyme.

Materials and Methods

Chemicals

Common nucleotides, nucleosides, and amino acids were obtained from either Sigma or P-L Biochemicals. Radioactively labelled amino acids and nucleosides and $^{32}P_i$ were obtained from New England Nuclear. Authentic CoAS-SG and CoA dimer were prepared as described by Dyar and Wilken (8). 4-Phosphopantetheine-glutathione mixed disulfide, 3′,5′-ADP, and 4-phosphopantethine were isolated by DEAE-A25 Sephadex ion-exchange chromatography after nucleoside pyrophosphatase (EC 3.6.1.1) cleavage of CoAS-SG and oxidized CoA respectively.

UV Spectra

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

Enzymes

All enzymes were purchased from Sigma. Nucleotide pyrophosphatase reactions were carried out in 50 m*M* Tris pH 7.6, 10 m*M* $MgCl_2$, and 100 μg/ml of enzyme for 1 h at 37°C. Bacterial alkaline phosphatase (EC 3.1.3.1) reactions were carried out in 50 m*M* Tris pH 7.6 and 50 μg/ml of enzyme at 37°C for 1 h. Carboxypeptidase A (EC 3.4.12.2) digests were carried out in 50 m*M* Tris pH 7.6 and 100 μg/ml of enzyme at 37°C for 1 h.

Chromatography

Thin-layer chromatography of amino acid mixtures was performed on cellulose sheets (Brinkmann) in isopropanol:water: formic acid (80:20:4) in the first dimension and *n*-propanol:water (7:3) in the second dimension. The amino acids were located using a ninhydrin spray. Nucleoside and purine analyses were carried out on cellulose sheets in two dimensions as previously described (1, 11). Adenine had R_f values in the first and second dimensions of 0.79 and 0.22 respectively while adenosine had R_f values of 0.70 and 0.19. Descending paper chromatography was carried out using Schleicher and Schuell orange ribbon C paper in a solvent containing ammonium sulfate: 0.1 *M* sodium phosphate pH 7.0: *n*-propanol (12 g: 20 ml: 0.4 ml). Sulfhydryl- and disulfide-containing compounds were located using a sodium azide – iodine spray (12). All Sephadex gels were obtained from Sigma.

Purification of DSI and DSII

The purification of DSI and DSII was carried out as previously described (1). Chilling the stationary-phase *E. coli* cultures drastically reduced the amount of DSI and DSII recovered. Therefore, centrifugation was at room temperature. Routinely 1.0 to 1.5 μmol of DSI and 0.1 to 0.2 μmol of DSII were obtained from an 8-ℓ culture. A 1 × 100 cm column of G50 Sephadex could be used in place of the second DEAE-A25 Sephadex column, eliminating the G10 Sephadex desalting column (Fig. 1). Alternatively, G50 Sephadex could be used as a fourth purification step.

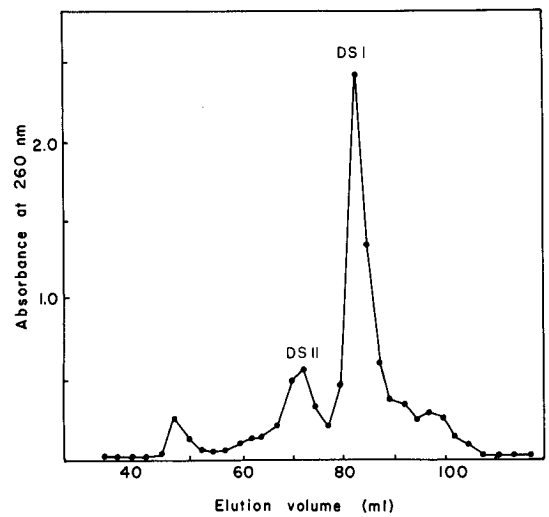

FIG. 1. G50 Sephadex separation of the components in peak D (1) from A25 DEAE Sephadex. Peak D, eluted from A25 DEAE Sephadex with triethylammonium bicarbonate pH 8.0 as previously described (1) was concentrated to dryness, dissolved in 1 ml of 50 m*M* Tris pH 7.6, and loaded on a 1 × 100 cm G50 Sephadex column. The column was eluted with 50 m*M* triethylammonium bicarbonate pH 8.0 collecting fractions of 1.2 ml. Absorbance at 260 nm was determined using a Gilford model 2400 spectrophotometer.

Chemical Analysis

Ribose, phosphate, uronic acid, and hexose analyses were carried out as described (13). The sodium nitroprusside test for disulfide and sulfhydryl groups was carried out as described (12). Free sulfhydryl groups were quantitated after sodium amalgam treatment to reduce any disulfide bonds. Sodium amalgam (0.5% sodium) was prepared under toluene and mixed with equal volumes of nucleotide solution at 37°C. Aliquots were removed, added to 5,5′-dithiobis (2-nitrobenzoic acid) at pH 7.6 and the absorbance at 412 nm was measured. The molar absorption coefficient of 13 600 was assumed for 5-thio-2-nitrobenzoic acid. Analysis for thiol esters and phosphate carboxylate anhydrides was carried out as described (14).

Iron analysis was carried out by a modification of the method of Suzuki and Silver (15). Samples containing 5–40 nmol (1–20 μl) of iron were treated with 20 μl of 10% perchloric acid for 10 min at room temperature. Then 1 ml of water, 0.1 ml of saturated ammonium acetate, 10 μl of 10% ascorbic acid, and 50 μl of 10 m*M* 2,2′-dipyridyl were added. After 30 min at room temperature, the absorbance was measured at 520 nm. The assay was linear between 5 and 40 nmol of iron.

Amino Acid Analysis

Acid hydrolysis for amino acid analysis was carried out in sealed tubes at 105°C with 6 *N* HCl for 16 h without the complete evacuation of oxygen. Samples were analyzed both by thin-layer chromatography and on a Beckman model 121 amino acid analyzer operated by the Department of Plant Science, University of Manitoba.

Enzyme Assays

Both CoAS-SG reductase and GSSG reductase (EC 1.6.4.2) were assayed as described by Dyar and Wilken (8). Cultures of *E. coli* B were grown in LB medium (5 g yeast extract, 10 g tryptone, and 5 g sodium chloride per litre) with vigorous aeration. Samples of 150–500 ml were withdrawn at various times, centrifuged at room temperature, and the pellets immediately frozen at −76°C. Cell growth was followed using a Klett–Summerson colorimeter with a blue filter. The pellets were resuspended in 10 m*M* Tris pH 7.6, 10 m*M* $MgCl_2$, and 50 m*M* NaCl followed by sonication and centrifugation of cell debris. The supernatants were assayed for CoAS-SG reductase, GSSG reductase, and protein concentration (16).

Results

DSI

Chemical Analysis

DSI was found to contain the expected components of coenzyme A and glutathione. The amino acid composition was determined using both two-dimensional thin-layer chromatography and an amino acid analyzer (Table 1). Only glycine, glutamic acid, and cysteic acid from glutathione as well as β-alanine and taurine from the pantetheine portion of CoA were found in significant amounts. DSI could be pulse labelled with ^{14}C-labelled glutamic acid, glutamine, glycine, and cystine but not threonine while five times more [^3H]adenosine than [^3H]guanosine was incorporated.

TABLE 1. Amino acid analyzer results

Amino acid	DSI	CoA	DSII
Cysteic acid	0.85	Tr	0.02
Taurine	0.71	1.00	0.84
Aspartic acid	0.04	0.01	0.03
Threonine	0.01	0.01	0.02
Glutamic acid	1.01	0.03	1.02
Glycine	1.06	0.34	0.32
Alanine	0.01	0.02	0.03
Leucine	0.02	Tr	0.02
β-Alanine	0.61	1.00	0.61

NOTE: DSI (120 nmol), CoA (105 nmol), and DSII (52 nmol; 104 nmol of adenosine) were hydrolyzed and the amino acid compositions determined as described in Materials and Methods. The values are given as the amino acid:adenosine ratio using CoA as the standard. The DSII analyzed had been prepared using four purification steps.

The phosphate to ribose to adenosine ratio was 3.3 to 1.0 to 1.0 (Table 2). This ratio was consistent with the structure of CoAS-SG except for the reproducibly high phosphate content. No impurities were evident in preparations of DSI using ion-exchange chromatography, gel filtration, or paper chromatography suggesting that a consistent error was made in the phosphate analysis of DSI. Subsequent data suggested an alternate explanation. Similar high phosphate analyses for CoAS-SG have been reported in preparations from yeast (4; 3.07–3.32) and bovine liver (5, 17; 3.03–3.32).

The low sulfur quantitation of 1.5 sulfur atoms per adenosine was consistent with the alkali lability of the cysteine sulfhydryl group in glutathione (18). Sodium amalgam used in the sulfur assay to reduce the disulfide bonds produced a very alkaline solution in which the glutathione sulfhydryl, once formed, would be quite labile. Only after sodium cyanide treatment would DSI react with the nitroprusside reagent. This was indicative of a disulfide linkage.

No thiol ester or phosphate–carboxylate anhydride linkages were evident and no carbohydrate other than ribose was found. DSI eluted from G50 Sephadex (Fig. 1) in the same volume as authentic CoAS-SG. ATP, ADP oxidized CoA, and CoAS-SG were used as elution standards.

Enzymic and Alkaline Hydrolysis

Nucleotide pyrophosphatase cleavage of DSI produced two products separable by paper and ion-exchange chromatography (Fig. 2*a* and Table 3). Peak 2*a*-B (peak B in Fig. 2*a*) was identified as 3′,5′-ADP on the basis of chromatographic and chemical analysis (Tables 2 and 3). Peak 2*a*-A had the same chromatographic mobility and chemical composition as 4-phosphopantetheine-glutathione, a product from the cleavage of CoAS-SG by nucleotide pyrophosphatase.

Alkaline hydrolysis of DSI occurred in two stages. After 1 h, two products were formed (Fig. 2*b*). Peak

TABLE 2. Analysis of various samples for phosphate, ribose, sulfur, and amino acids

Sample	Adenosine	Phosphate	Ribose	Sulfur	Amino acids
1. DSI ($A_{250:260}$, 0.87)	3.4 (1.0)	11.4 (3.4)	3.6 (1.1)	5.1 (1.5)	1–5
2. DSI ($A_{250:260}$, 1.03)	2.9 (1.0)	9.5 (3.3)	2.9 (1.0)	4.5 (1.5)	1–5
3. Peak 2*a*-A	—	2.8	0.1	2.5	1–5
4. Peak 2*a*-B	1.8 (1.0)	4.0 (2.2)	2.0 (1.1)	0.1 (—)	—
5. Peak 2*b*-A	—	1.5	—	0.2	3–5
6. Peak 2*b*-B	1.1 (1.0)	3.2 (2.9)	1.2 (1.1)	1.2 (1.1)	1, 2
7. Peak 2*c*-A	—	2.7	—	0.4	1–5
8. Peak 2*c*-B	2.5 (1.0)	5.3 (2.1)	2.7 (1.1)	0.1 (—)	—
9. Peak 2*d*-A	—	0.7	—	*n.a.*	3–5
10. Peak 2*d*-B	1.2 (1.0)	3.4 (2.8)	1.3 (1.1)	*n.a.*	1, 2
11. Dephospho-DSI	2.3 (1.0)	4.5 (2.0)	2.3 (1.0)	3.4 (1.5)	1–5
12. DSII	1.2 (1.0)	3.5 (2.9)	1.3 (1.1)	1.1 (0.9)	1, 2, 3
13. Peak 4*a*-A	—	1.9	0.1	1.8	1, 2
14. Peak 4*a*-B	1.5 (1.0)	3.1 (2.1)	1.6 (1.1)	0.1 (—)	—
15. Peak 4*b*-A	—	1.8	—	0.2	1, 2
16. Peak 4*b*-B	1.7 (1.0)	3.6 (2.1)	1.8 (1.1)	0.1 (—)	—

NOTE: Microanalyses were carried out as described in Materials and Methods. The molar absorption coefficient at 260 nm for adenosine was taken as being 15 300. The amino acids were designated as: 1, β-alanine; 2, taurine; 3, glutamic acid; 4, glycine; and 5 cysteic acid. The numbers in parentheses are the molar ratios. The values are expressed as nanomoles per microlitre. Refer to Figs. 2 and 4 for the source of samples 3–10 and 13–16. Dashes indicate that the component was not present while *n.a.* indicates that no assay was performed.

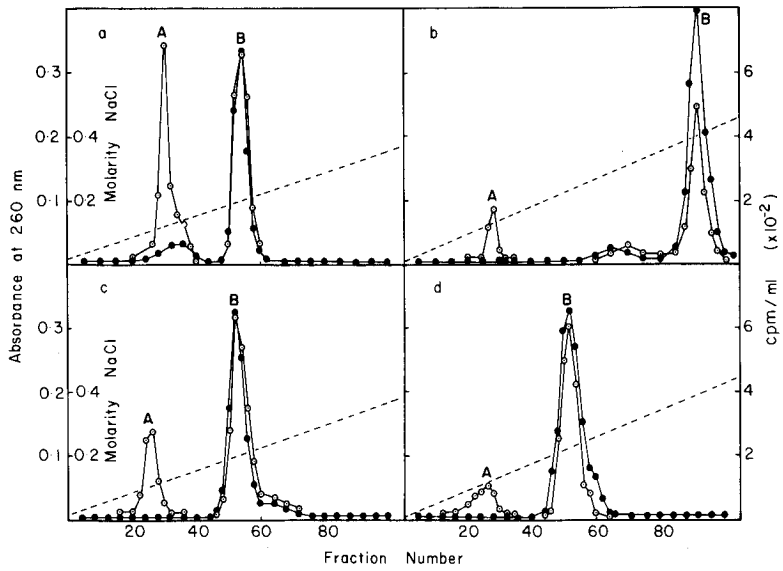

FIG. 2. A25 DEAE Sephadex ion-exchange separation of various DSI digest mixtures. All samples, containing 670 nmol of digested [^{32}P]DSI, were loaded on a 1 × 15 cm column of A25 DEAE Sephadex equilibrated with 0.01 *M* NaCl in 50 m*M* Tris pH 7.6. A linear gradient of 200 ml 0.01 *M* NaCl and 200 ml 0.5 *M* NaCl in 50 m*M* Tris pH 7.6 was run collecting 4-ml fractions. Radioactivity was determined by the Cherenkov method (30). All peaks were subsequently pooled concentrated and desalted using a 1 × 100 cm column of G10 Sephadex eluted with 40 m*M* triethylammonium bicarbonate pH 8.0. Absorbance (●); ^{32}P (○). (*a*) DSI incubated with nucleotide pyrophosphatase as described in Materials and Methods. (*b*) DSI incubated for 1 h at 37°C with 0.3 *M* KOH followed by neutralization with 1 *M* KH_2PO_4. (*c*) DSI incubated for 16 h at 37°C with 0.3 *M* KOH followed by neutralization with 1 *M* KH_2PO_4. (*d*) DSI incubated for 2 h at room temperature with 10 m*M* dithiothreitol. This column was equilibrated with 0.5 m*M* dithiothreitol.

TABLE 3. Chromatographic properties

	Eluting salt concentration from DEAE-A25 Sephadex	R_f after paper chromatography
Authentic samples		
2′-AMP	—	0.13
3′-AMP	—	0.18
5′-AMP	0.17	0.25
5′-ADP	0.20	0.32
5′-ATP	0.23	0.36
Adenosine-5′-tetraphosphate	—	0.39
CoA	0.22	0.14
Oxidized CoA	0.43	0.02
CoAS-SG	0.30	0.14
Pantethine	—	0.05
Glutathione	—	0.74
Glutamic acid	0.07	0.85
GSSG	—	0.52
3′, 5′-ADP	0.20	0.28
4-Phosphopantetheine–glutathione	0.11	0.49
4-Phosphopantethine	0.14	0.27
Pi	0.14	0.95
Experimental samples		
DSI	0.30	0.14
DSII	0.41	0.02
Dephospho-DSI	0.23	0.12
Peak 2*a*-A	0.10	0.50
Peak 2*a*-B	0.20	0.28
Peak 2*b*-A	0.11	0.47
Peak 2*b*-B	0.40	0.02
Peak 2*c*-A	0.10	0.45
Peak 2*c*-B	0.20	0.28
Peak 2*d*-A	0.10	0.72
Peak 2*d*-B	0.22	0.14
Peak 4*a*-A	0.15	0.27
Peak 4*a*-B	0.20	0.28
Peak 4*b*-A	0.10	0.45
Peak 4*b*-B	0.20	0.28

2*b*-B had the same chemical composition and chromatographic properties as authentic oxidized CoA (Tables 2 and 3). Peak 2*b*-A was not identified but contained the component amino acids of glutathione and ^{32}P-labelled material. After 16 h, two different products were formed (Fig. 2*c*). Peak 2*c*-B was identified as 3′,5′-ADP on the basis of chromatographic data and chemical analysis (Tables 2 and 3). Peak 2*c*-A was not identified but contained all five of the amino acids originally present in DSI. Alkaline hydrolysis of CoAS-SG for 16 h also produced 3′,5′-ADP and peak 2*c*-A.

Dithiothreitol caused the cleavage of DSI into two products separable by ion-exchange chromatography. Peak 2*d*-B was identified as coenzyme A. Sulfur was not assayed because of contaminating dithiothreitol. Peak 2*d*-A contained the components of glutathione and migrated like glutathione on paper but contained some organically bound phosphate.

Bacterial alkaline phosphatase released P_i from DSI to produce a P_i to nucleotide ratio of 1.4 to 2.0 (838 to 1148 cpm). Dephospho-DSI was purified by A_{25} Sephadex ion-exchange chromatography (not shown) and its component analysis is contained in Table 2.

Carboxypeptidase A released glycine from both DSI and peak 2*a*-A while hot aqueous treatment of both DSI and peak 2*a*-A released glutamic acid. Both reactions are consistent with the presence of glutathione in DSI and peak 2*a*-A.

Nucleoside Analysis and Spectral Data

After complete digestion of DSI with bacterial alkaline phosphatase and nucleotide pyrophos-

phatase, the resulting nucleoside cochromatographed in two dimensions with authentic adenosine. Acid hydrolysis produced a purine base which cochromatographed with adenine.

Some preparations of DSI exhibited UV absorption spectra which were not consistent with the presence of adenosine (1) while other batches exhibited an adenosinelike spectrum. In addition the nonadenosine spectrum ($A_{250:260}$ of 1.03) could change to that of adenosine ($A_{250:260}$ of 0.87; Fig. 3) following first-order kinetics with a rate constant of 0.017 h^{-1} at 4°C. Batches of DSI which exhibited an $A_{250:260}$ ratio greater than 1.00 were found to contain 0.72–0.85 iron atoms per adenosine. The iron was shown to be in the Fe(III) oxidation state by the lack of reaction when ascorbic acid was omitted from the iron assay mixture. Batches of DSI with $A_{250:260}$ ratios of 0.87 (adenosinelike) had fewer than 0.06 iron atoms per adenosine. The ribose, phosphate, sulfur, and amino acid compositions of DSI were the same regardless of the spectral ratio (Table 2).

Addition of Fe(III) ion to a solution of DSI with an $A_{250:260}$ ratio of 0.87 caused an immediate increase in the spectral ratio to 0.94–1.05. This DSI–Fe(III) complex could be isolated by G10 or G50 Sephadex gel filtration and contained 1.09 iron atoms per adenosine. Fe(II) caused a similar but slower change in the spectral ratio of DSI but a DSI–Fe(II) complex could not be isolated. The following metal ions were found to have no effect upon the spectrum of DSI: Zn(II), Ca(II), Mn(II), Mg(II), Cu(II), and Co(II).

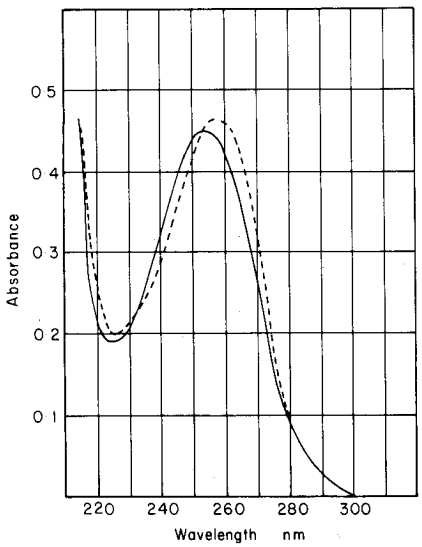

FIG. 3. UV spectra of DSI. The solid line is the spectrum in 50 m*M* Tris pH 7.6 of DSI with an $A_{250:260}$ of 1.03. The broken line is the spectrum in 50 m*M* Tris pH 7.6 of the same batch of DSI after incubation in the same buffer at 4°C for 6 days until the $A_{250:260}$ was 0.87. The spectrum of coenzyme A is superimposable upon the broken line.

The spectra of ATP and CoA were not affected by Fe(III) solutions but oxidized CoA, CoAS-SG, and DSII had their $A_{250:260}$ ratios increased (Table 4).

CoAS-SG Reductase Levels in E. coli

The enzyme CoAS-SG reductase has been identified in rat liver and yeast as one of the enzymes responsible for the metabolism of CoAS-SG. During growth of *E. coli* from early log to stationary phase, the specific activity of CoAS-SG reductase increased sixfold from 0.49 to 3.23 mU/mg (Table 5). The specific activity of GSSG reductase increased just twofold from 60.6 to 124.5 mU/mg during the same period of growth. A shift from aerobic to anaerobic growth also resulted in a transient twofold increase in CoAS-SG reductase levels while GSSG reductase levels remained unchanged. These fluctuations in CoAS-SG reductase levels are very similar to the fluctuations in DSI levels reported earlier (1). DSI either with or without Fe(III) ion could replace CoAS-SG in the enzyme assay with no change in enzyme activity. The reverse of this reaction, that is the formation of CoAS-SG from GSH and CoA in the crude extracts, was not evident using the conversion of $NADP^+$ to NADPH for the assay.

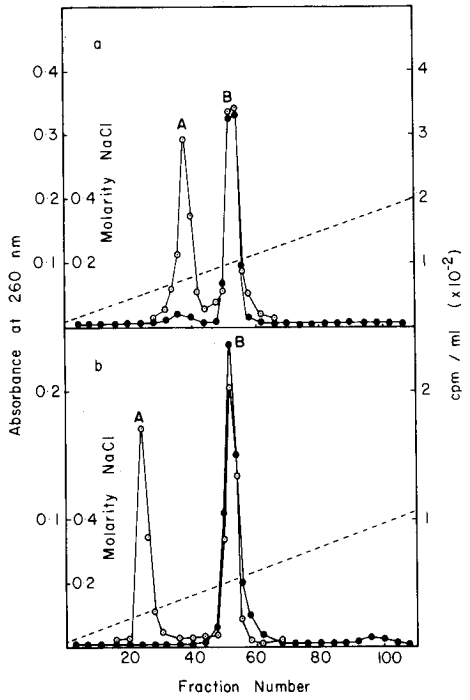

FIG. 4. A25 DEAE Sephadex ion-exchange separation of [^{32}P]DSII digest mixtures. All samples containing 670 nmol of digested DSII were separated as described in the legend to Fig. 2. (*a*) DSII incubated with nucleotide pyrophosphatase. (*b*) DSII incubated for 16 h at 37°C with 0.3 *M* KOH followed by neutralization with 1 *M* KH_2PO_4.

TABLE 4. Spectral ratios and iron content of DSI and related nucleotides

	No metal added		0.1 m*M* $Fe_2(SO_4)_3$	A		0.1 m*M* $FeSO_4$	B	
Compound	$A_{250:260}$	Fe:Ad	$A_{250:260}$	$A_{250:260}$	Fe:Ad	$A_{250:260}$	$A_{250:260}$	Fe:Ad
DSI	1.02	0.72	—	—	—	—	—	—
DSI	0.87	0.06	0.94–1.05	1.01	1.09	0.98	0.87	0.24
CoAS-SG	0.83	—	0.93–1.00	0.97	0.62	0.88	—	—
CoA	0.84	—	0.85	—	—	0.84	—	—
DSII	0.98	0.84	—	—	—	—	—	—
DSII	0.87	0.07	0.95–1.03	1.04	0.94	0.94	0.87	0.11
CoA dimer	0.83	—	0.94–1.01	0.86	0.11	0.88	—	—

NOTE: Columns under A contain the analysis of nucleotides purified by G50 Sephadex (Fig. 1) from the Fe(III)–nucleotide mixture analyzed in the previous column. Columns under B contain analysis of nucleotides purified by G50 Sephadex from the Fe(II)–nucleotide mixture analyzed in the previous column.

DSII

Chemical Analysis

Chemical analysis of DSII showed that it contained all of the components of coenzyme A and also glutamic acid. The glutamic acid was present in DSII even after two ion-exchange chromatography steps and two Sephadex gel filtration steps on G10 and G50. The phosphate to ribose to adenosine to sulfur ratio was 2.9 to 1.1 to 1.0 to 0.9 and the amino acids present were β-alanine, taurine, and glutamic acid (Tables 2 and 3). Sodium cyanide treatment was necessary for DSII to produce a color reaction with nitroprusside reagent, a fact indicative of a disulfide linkage. No evidence for any carbohydrate other than ribose was found.

Enzymic and Alkaline Hydrolysis

Nucleotide pyrophosphatase cleavage of DSII produced two products. The UV absorbing product peak 4*a*-B was identified as 3′,5′-ADP. The chromatographic properties of peak 4*a*-A were identical to those of 4-phosphopantethine produced by nucleotide pyrophosphatase cleavage of authentic oxidized CoA. Peak 4*a*-A contained a 1:1 ratio of phosphate to sulfur and contained β-alanine and taurine. Neither product contained glutamic acid which would elute from the column at 0.07 *M* NaCl.

Alkaline hydrolysis of DSII for 16 h produced two product peaks (Fig. 4*b*). Peak 4*b*-B was identified as 3′,5′-ADP. Peak 4*b*-A was not identified but contained taurine, β-alanine, phosphate, and sulfur. Neither product contained glutamic acid and alkaline hydrolysis of authentic oxidized CoA produced the same products.

Treatment of DSII with bacterial alkaline phosphatase produced P_i and nucleotide in a phosphate ratio of 1.0 to 1.9 (741 to 1131 cpm).

Nucleoside Analysis and Spectral Data

Combined treatment of DSII with bacterial alkaline phosphatase and nucleotide pyrophosphatase produced a nucleoside which cochromatographed with authentic adenosine. Acid hydrolysis produced a purine base which cochromatographed with authentic adenine. The UV spectral data for DSII was similar to that reported for DSI (1). The addition of Fe(III) ion to a solution of DSII with an $A_{250:260}$ ratio of 0.87 resulted in an increase in the ratio to 1.02. This complex could then be isolated by G10 or G50 Sephadex gel filtration (Table 4). Fe(II) ion also increased the $A_{250:260}$ ratio but an isolatable complex was not formed. DSII isolated from *E. coli* with an $A_{250:260}$ ratio of 0.98 was found to contain 0.84 iron atoms per adenosine. The location of the glutamic residues and their role, if any, in the binding of iron were not determined.

TABLE 5. CoAS-SG reductase and GSSG reductase levels in crude extracts of *E. coli* during growth into stationary phase (A) and during a shift from aerobic to anaerobic growth (B)

	Time*	Klett turbidity value	CoAS-SG reductase, mU/mg	GSSG reductase, mU/mg
(A)	1.75	47	0.46	60.6
	2.25	82	0.55	74.5
	3.00	143	1.00	88.8
	3.50	209	1.50	109.0
	4.50	365	1.58	114.5
	5.25	495	2.54	112.5
	6.00	550	2.75	119.6
	6.50	573	3.16	120.0
	7.00	574	3.25	124.5
	8.00	574	3.20	121.6
(B)	−20	103	1.01	48.6
	0	152	1.08	50.0
	7.5	152	1.46	49.7
	15.0	158	1.82	51.8
	22.5	164	2.00	54.3
	30.0	175	1.86	54.7
	37.5	185	1.62	47.8
	45.0	194	1.32	51.3

*In (A) time is expressed in hours after beginning of growth. In (B) time is expressed in minutes relative to the shift from aerobic to anaerobic growth.

Discussion

These experiments describe the structural characterization of two nucleotides DSI and DSII from *E. coli*. The major nucleotide, DSI, was shown to be a coenzyme A – glutathione mixed disulfide which was capable of binding one molecule of Fe(III) ion. Such a mixed disulfide has previously been isolated from yeast (4) and liver (5, 6) with a higher than expected phosphate content (3.03–3.32 phosphates per adenosine). The same high phosphate content was found in four batches of DSI and may therefore have resulted from a consistent error in determining the adenosine concentration. However, the phosphate analysis of DSII did not present this problem, nor did the analysis of dephospho-DSI. The phosphatase and dithiothreitol cleavage results suggested the alternative explanation of an impurity. A contaminant would have to be a non-UV-absorbing sulfhydryl-sensitive compound or a more highly phosphorylated CoAS-SG but neither paper electrophoresis nor ion-exchange chromatography using a very shallow salt gradient showed any impurity.

At least two parts of the CoAS-SG molecule must be involved in the iron complex. The spectral shift suggested that the adenine ring played a role or was in close proximity to the binding site. Since the iron bound only to a disulfide form of coenzyme A, the pantetheine–glutathione chain in DSI must also be involved. Unlike the dimer and polymer chelates frequently formed with ferric ion (19), the DSI–iron complex was isolated as a monomer. Other cations such as silver and mercury have been shown to interact directly with the purine and pyrimidine rings of nucleosides causing a shift of the absorption peaks to longer wavelengths (20, 21). The nature of the CoAS-SG–iron complex appears to be unique and is being studied further.

The levels of DSI in *E. coli* increased up to 10-fold during growth into stationary phase and during a shift from aerobic to anaerobic growth. The mixed disulfide may therefore be a storage form of the two components protecting them from degradation under certain unfavorable growth conditions. Stadtman and Kornberg made a similar suggestion when the total CoA pool from commercial brewers yeast was found in the form of CoAS-SG (4). However, the low levels of CoAS-SG in rat liver led Dyar and Wilken to conclude that it was not a storage form in the liver (8).

A NADPH-dependent CoAS-SG reductase was identified in liver and yeast as one of two enzymes which could use CoAS-SG as a substrate (7–10). The levels of this enzyme in *E. coli* fluctuate parallel to the levels of DSI during growth into stationary phase and during a shift from aerobic to anaerobic growth. The role of this reductase may be to replenish the CoA pool from CoAS-SG formed either chemically or enzymatically during cell growth. A control mechanism must be involved, however, because large amounts of CoAS-SG accumulate in vivo at the same time that the reductase levels are high.

DSI and DSII have been found to inhibit DNA-dependent *E. coli* RNA polymerase in vitro and the inhibitory activity was related to the size of the $A_{250:260}$ ratio. DSI with an $A_{250:260}$ ratio of 1.00 or greater inhibited the polymerase up to 90% while DSI with an $A_{250:260}$ ratio of 0.87 did not affect the polymerase (3). It has since been shown that the isolated DSI–iron complex can decrease the polymerase activity by 90% whereas, separately, Fe(III) and DSI (with an $A_{250:260}$ ratio of 0.87) had no effect (W. Bees and P.C.L., unpublished observations). Several nucleotides have been implicated in regulatory roles involving transcription and sporulation (2, 22–29). The mixed disulfide, CoAS-SG, may be another such nucleotide which has its regulatory function controlled by the environmental availability of iron.

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