Glutathionylspermidine metabolism in *Escherichia coli*

Keith SMITH, Adolfo BORGES,† Mark R. ARIYANAYAGAM and Alan H. FAIRLAMB*
Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WCIE 7HT, U.K.

Intracellular levels of glutathione and glutathionylspermidine conjugates have been measured throughout the growth phases of *Escherichia coli*. Glutathionylspermidine was present in mid-log-phase cells, and under stationary and anaerobic growth conditions accounted for 80% of the total glutathione content. N^1,N^8-bis(glutathionyl)spermidine (trypanothione) was undetectable under all growth conditions. The catalytic constant $k_{cat.}/K_m$ of recombinant *E. coli* glutathione reductase for glutathionylspermidine disulphide was approx. 11 000-fold lower than that for glutathione disulphide. The much higher catalytic constant for the mixed disulphide of glutathione and glutathionylspermidine (11% that of GSSG), suggests a possible explanation for the low turnover of trypanothione disulphide by *E. coli* glutathione reductase, given the apparent lack of a specific glutathionylspermidine disulphide reductase in *E. coli*.

INTRODUCTION

The tripeptide L-γ-glutamyl-L-cysteinylglycine (glutathione, GSH) is the principal low-molecular-mass thiol in most organisms [1,2]. Generally, redox balance is maintained by the NADPH dependent glutathione reductase (GR; EC 1.6.4.2) [3] resulting in a high ratio of free thiol to disulphide ([GSH]/[GSSG]). A novel glutathione–spermidine conjugate, N^1-monoglutathionylspermidine (GspdSH), was initially detected in *Escherichia coli* under stationary or anerobic growth conditions [4]. Subsequently, GspdSH and the N^1,N^8-bis(glutathionyl)spermidine conjugate, termed trypanothione, were identified in trypanosomatids, a group of flagellated protozoa which include many human pathogens [5,6]. In these organisms, dihydrotrypanothione ($T[SH]_2$) and GspdSH are regenerated from trypanothione disulphide ($T[S]_2$) and N^1-monoglutathionylspermidine disulphide ($[GspdS]_2$) respectively by trypanothione reductase (TR; EC 1.6.4.8) [7,8]. Although human GR and TR exhibit mutually exclusive substrate specificities [7], in contrast *E. coli* GR possess significant activity with $T[S]_2$ [9]. In the present investigation we have considered the possibility that the broader substrate specificity of *E. coli* GR may be related to the presence of GspdSH in *E. coli*. To this end, we have examined the levels of glutathione and glutathione–spermidine conjugates in *E. coli* in various phases of growth, and we have also investigated possible mechanisms for the reduction of $[GspdS]_2$.

MATERIALS AND METHODS

Materials

$T[S]_2$ and $[GspdS]_2$ were purchased from Bachem, GSH and GSSG from Sigma and NADPH from Boehringer. All other reagents were of the highest grade commercially available. The mixed disulphide of $[GspdS]_2$ and glutathione (GspdS–SG) was prepared by mixing equal volumes of 4.5 mM $[GspdS]_2$ and 3 mM GSH in 40 mM Hepps buffer, pH 7.4, containing 4 mM diethylenetriamine pentaacetic acid and incubating for 30 min at room temperature. The solution was acidified by the addition of an equal volume of 0.5 M acetic acid and small aliquots of the mixed disulphide were purified by FPLC on a Pharmacia Mono-S ion-exchange column. The column was fractionated with a decreasing linear gradient of acetic acid (0.5 M to 0 M) superimposed on an increasing linear gradient of ammonium acetate (0 M to 0.5 M). Fractions were analysed for amino compounds by ion-paired reverse-phase HPLC [10]. Fractions containing GspdS–SG were freeze-dried and resuspended in assay buffer just before use.

Thiol analysis

E. coli NTCC 10418 was grown in M9 minimal salts supplemented with 0.5% (w/v) glucose under aerobic or anaerobic conditions as follows. Cells were inoculated at 0.8×10^8 cells/ml and grown aerobically in shaken culture for up to 6 h. Cells grown under similar conditions for 4 h (8.11×10^9 cells/ml) were then transferred to air-tight containers for growth under non-shaking conditions for up to 6 h. Cell growth, usually monitored at 540 nm was correlated with a viable cell count determined by plating. Thiols were determined by HPLC after derivatization with monobromobimane as described previously [11]. Approx. 5×10^9 cells were derivatized before analysis.

Expression and purification of recombinant *E. coli* GR

The entire glutathione reductase coding region [12] was amplified from *E. coli* genomic DNA by the PCR using the oligomeric primers ECGRN (sense), 5′-CGCGC*GAATTC*AGGAGGCGT-AAAACGATGACTAAACACTATGATTACATCGCCATC-GGC-3′ and ECGRC (antisense), 5′-GCGC*CTGCAG*TT AAC-GCATTGTCACGAACTCTTCTGCCGCCGT-3′ (start and stop codons in bold). *Eco*R1 and *Pst*1 restriction sites (in italics) were included in ECGRN and ECGRC respectively to facilitate the cloning of the resultant fragment (approx. 1.4 kb) into the expression vector pBSTNAV [13] to produce the recombinant plasmid pBECGR. Additionally a ribosomal binding site and a

Abbreviations used: $T[SH]_2$, dihydrotrypanothione; $T[S]_2$, trypanothione disulphide; GspdSH, N^1-monoglutathionylspermidine; $[GspdS]_2$, N^1-monoglutathionylspermidine disulphide; GspdS–SG, glutathionylspermidine–glutathione mixed disulphide; GR, glutathione reductase (EC 1.6.4.2); TR, trypanothione reductase (EC 1.6.4.8).

* To whom correspondence should be addressed.
† Present address: Post-Grado en Biologia Aplicada, Escuela de Clenclas, Universidad de Oriente, P.O. Box 245, Cumana, Estado, Sucre, Venezuela.

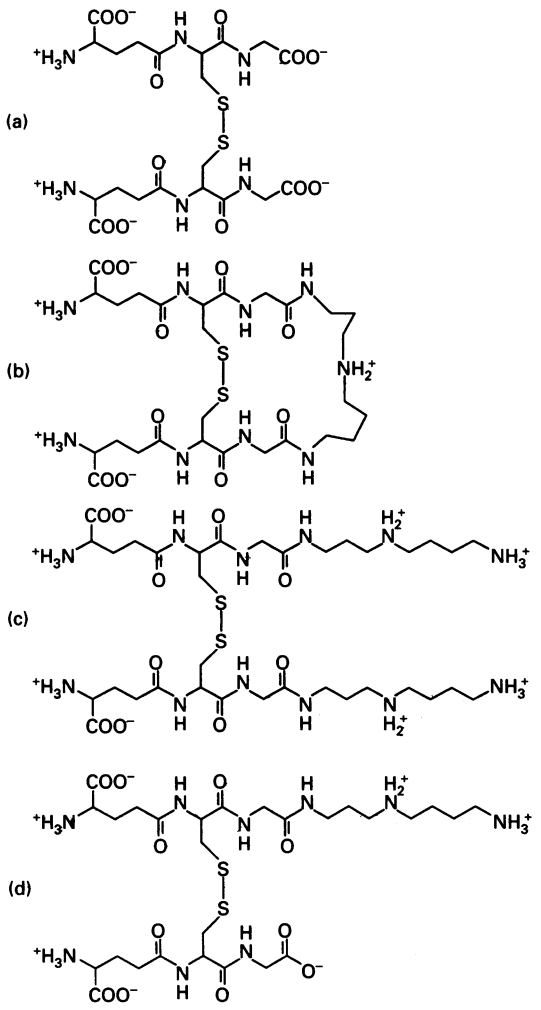

Figure 1 Structures of GSSG (a), T[S]$_2$ (b), [GspdS]$_2$ (c) and GspdS-SG (d)

translation separation element were included in ECGRN to optimize expression. JM109 harbouring pBECGR was grown up in LB supplemented with 50 μg/ml of ampicillin. Recombinant *E. coli* GR was purified as described by Perham and co-workers [14,15].

Assay of GR

Substrate-dependent oxidation of NADPH was monitored at 340 nm in 0.1 M potassium phosphate, pH 7.5, at a fixed NADPH concentration of 100 μM [16] and varying concentrations of disulphide substrates. The structures of the disulphide substrates used in this study are shown in Figure 1. Concentrations of T[S]$_2$, [GspdS]$_2$ and GspdS–SG were determined following complete reduction with NADPH and *Trypanosoma cruzi* TR; the concentration of GSSG was likewise determined following reduction with GR. Initial rate measurements were performed with disulphide substrate concentrations in the range 0.5–3 or 10 times the apparent K_m. Velocity/substrate concentration profiles were analysed as described previously [17]. Using [GspdS]$_2$ the apparent K_m was too large for a complete analysis, and in this case the $k_{cat.}/K_m$ was determined by varying both the substrate and enzyme concentrations. Protein determinations were performed using the Bradford method.

Assay for a specific [GspdS]$_2$ reductase in *E. coli*

E. coli (200 ml cultures), grown aerobically or following a shift to anaerobic non-shaking conditions for 6 h, were harvested by centrifugation. Cell pellets were resuspended in 4 ml of 0.1 M potassium phosphate, pH 7.5. Cell suspensions were sonicated with five pulses each of 30 s at an amplitude setting of approx. 22 μm on an MSE Soniprep 150 sonicator. Cell-free extracts were centrifuged at 25000 *g* for 30 min to yield cell-free extract supernatants, which were dialysed exhaustively against 0.1 M potassium phosphate buffer, pH 7.5. [GspdS]$_2$-dependent oxidation of NADPH or NADH by extracts of *E. coli* was monitored at 340 nm. Assay mixtures contained 200 μM [GspdS]$_2$, 100 μM cofactor and up to 680 μg of dialysed protein extract in a final volume of 1 ml.

RESULTS

In all phases of aerobic growth the levels of the principal intracellular thiol, glutathione, remained essentially unchanged (Figure 2a). Low levels of GspdSH (3% of total GSH) were

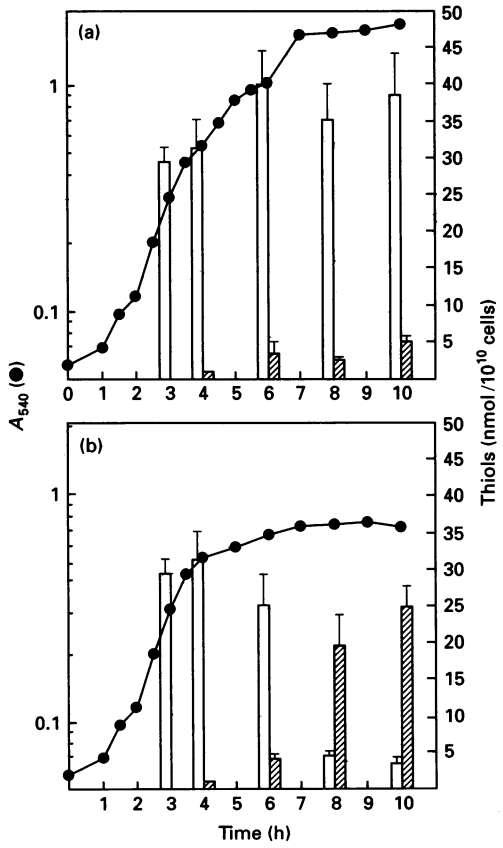

Figure 2 Glutathione and glutathionylspermidine levels in *E. coli* under aerobic (a) and anaerobic conditions (b)

Cell density was monitored by absorbance at 540 nm (●) (left-hand axis label) and the levels of GSH (□) and GspdSH (▩) represent the mean values (±S.E.M.) (right-hand axis label) for samples from three cultures at the specified sampling times (3, 4, 6, 8 or 10 h).

Table 1 Catalytic parameters of *E. coli* GR for various disulphide substrates

For $[GspdS]_2$ only the ratio $k_{cat.}/K_m$ could be determined (see Materials and methods section). nd, not determined.

Disulphide	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ (relative)
GSSG	65 ± 7	32920 ± 1050	8.44×10^6	100
$T[S]_2$	2780 ± 350	5400 ± 620	3.2×10^4	0.4
$[Gspd]_2$	$\gg 6000$	nd	760	0.009
GspdS–SG	430 ± 50	24350 ± 3160	9.44×10^5	11.2

present in mid-log cells (8.11×10^9 cells/ml) but in stationary-phase cells GspdSH accounted for approx. 11% of the total intracellular GSH. On transfer from aerobic to anaerobic conditions the levels of GSH and GspdSH underwent pronounced alteration. Within 4 h GspdSH was the principal thiol, accounting for 80% of total GSH. *E. coli* appears to be unable to convert GspdSH to $T[SH]_2$ by the addition of a second glutathione molecule, since this dithiol was undetectable under all growth conditions.

Levels of GR in extracts of *E. coli* grown under aerobic and anaerobic conditions were 0.06 and 0.05 units $\cdot min^{-1} \cdot mg^{-1}$ respectively. These levels are comparable with those reported for *E. coli* strain SG5 [16]. In contrast, there was no detectable $[GspdS]_2$-dependent oxidation of NADPH, or indeed NADH, by extracts of *E. coli*. This suggests that there is no specific $[GspdS]_2$ reductase in *E. coli* and raises the question as to how this disulphide is reduced *in vivo*.

The amplified PCR fragment containing the entire *E. coli* GR gene was ligated directionally into the expression vector in which the expression of GR was under the control of the strong constitutive *lpp* promoter. When transformed into JM109 cells, the construct was found to express GR at extremely high levels, accounting for as much as 50% of the total soluble protein. Unlike other high-level expression systems described for *E. coli* GR [15], it was not necessary to reconstitute inactive apoenzyme by the addition of excess FAD to the cell-free extract. With such high levels of expression, representing approx. 3000-fold amplification, approx. 50 mg of homogeneous *E. coli* GR was obtained from one litre of cells using the procedure described [15]. The *E. coli* GR migrated on SDS/PAGE with a M_r of 49500, in agreement with the derived amino acid sequence (results not shown). The substrate specificity of the recombinant protein was studied in detail (Table 1). Recombinant GR was fully active as assessed by a $k_{cat.}/K_m$ of 8.4×10^6 $M^{-1} \cdot s^{-1}$ for GSSG. Furthermore the kinetic parameters of the enzyme for $T[S]_2$ were also in reasonable agreement with those reported previously [9]. With $[GspdS]_2$ as substrate, the enzyme velocity–substrate profile was linear with $[GspdS]_2$ concentrations up to 6 mM and a complete kinetic analysis could not be performed. However the specificity constant ($k_{cat.}/K_m$) was determined to be approx. 11000-fold lower than that of GSSG, suggesting that this disulphide is not a physiological substrate for GR.

Given that both GSH and GspdSH are present in *E. coli*, the mixed disulphide of these two thiols was prepared and tested as a substrate. For such studies, it is essential that the mixed disulphide is free of contaminating GSSG. The mixed disulphide was analytically pure as judged by HPLC analysis (Figure 3). This mixed disulphide was found to be a reasonable substrate with a $k_{cat.}$ 73% of that observed with GSSG but with a K_m 6.6-fold higher.

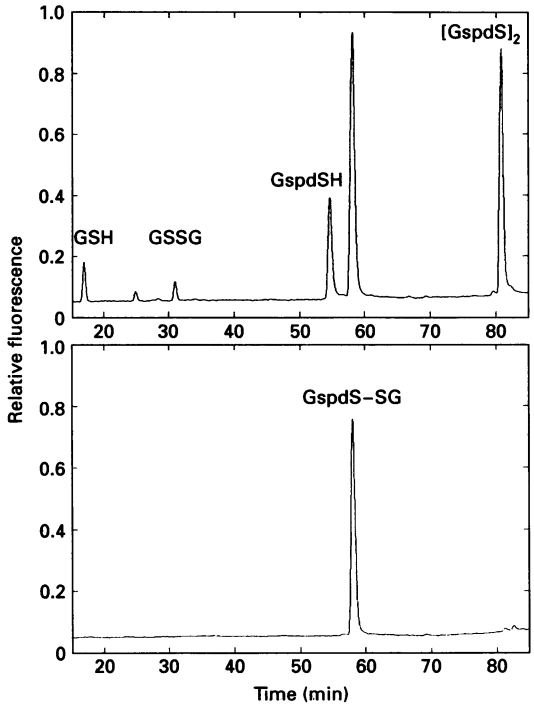

Figure 3 Analysis of reduced, oxidized and mixed disulphide forms of glutathione and glutathionylspermidine by reverse-phase HPLC

Compounds were detected by post-column derivatization with fluorescamine (see the Materials and methods section). Trace (**a**), reaction mix of $[GspdS]_2$ and GSH after 30 min at room temperature, containing GSH, GSSG, GspdSH, $[GspdS]_2$ and GspdS–SG. Trace (**b**), GspdS–SG following purification by cation-exchange on FPLC

DISCUSSION

Although glutathionylspermidine was originally discovered in *E. coli* grown to stationary phase over 20 years ago [4], there have been few further studies on this interesting aspect of thiol-polyamine metabolism in this organism and no reports on the presence of this metabolite in any other bacteria. We have quantified the level of GspdSH and its precursor, GSH, throughout the cell-cycle and also demonstrated the lack of the bis(glutathionyl)spermidine conjugate (trypanothione) in this prokaryote. An ATP-dependent ligase, glutathionylspermidine synthetase, has been partially purified from *E. coli* [18]. In the insect trypanosomatid, *Crithidia fasciculata*, trypanothione biosynthesis proceeds through the concerted action of two ATP-dependent ligases, glutathionylspermidine synthetase and trypanothione synthetase [19]. Trypanothione synthetase, which catalyses the addition of the second glutathione molecule to the free primary amine of glutathionylspermidine, would thus appear to be absent from *E. coli*.

In previous studies, levels of GspdSH have been expressed in terms of percentage of total spermidine, itself reported as μmol/g wet weight of cells [4], and so it is difficult to make a direct comparison with the present study. However, the levels of GSH reported here are comparable with those reported for other strains of *E. coli* [20]. The present data show that under anaerobic and stationary-phase conditions, 80% of the total GSH is in the form of GspdSH (Figure 2). These findings on GspdSH are in agreement with previous work [4,21], although it is now clear

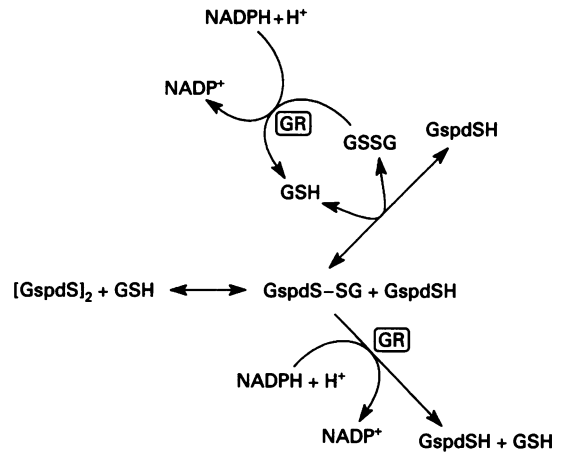

Figure 4 Possible mechanism for the reduction of $[GSpdS]_2$ in *E. coli*

$[GspdS]_2$ and GSH undergo thiol exchange (possibly enzyme mediated) to form the mixed disulphide GspdS–SG. This may undergo further thiol exchange with GSH to regenerate the second GspdSH molecule. Alternatively, reduction of the mixed disulphide may be mediated by GR.

that GspdSH is also present, albeit at low levels, in mid-log *E. coli* cells.

It is not clear why *E. coli* synthesizes GspdSH, although it has been suggested that this might be one way in which the levels of the precursor metabolites, spermidine and glutathione, might be regulated within the cell [4]. Another possibility that merits further investigation is that GspdSH may be a bettter DNA-protectant against radical- or oxidant-induced damage than GSH, as has been suggested for $T[SH]_2$ [22]. Thus, although both GSH [20] and spermidine [23] appear to be dispensible for cell growth, at least under certain conditions, the accumulation of GspdSH under stationary-phase conditions may reflect an important response to certain environmental stresses.

Cellular levels of GSH and GspdSH are dependent on their relative rates of biosynthesis and turnover. Moreover, ATP-dependent glutathione-S-conjugate export carrier proteins have been identified in many eukaryotic and plant cells [24,25]. GSSG appears to be an endogenous substrate for these pumps, and although there have been no reports of this class of transporter protein in *E. coli*, we cannot preclude the possibility that such pumps also contribute to the modulation of cellular levels of GSH and GspdSH throughout the various growth phases of *E. coli*. Indeed it would be most interesting to determine whether *E. coli* and trypanosomatids have specific efflux pumps for glutathionylspermidine conjugates.

The efficient catalysis of $T[S]_2$ by *E. coli* GR reported by others [9] is noteworthy, given the apparent tight specificities of the human GR and trypanosomatid TRs for their eponymous substrates [7]. Given that we could not detect trypanothione in *E. coli*, we considered initially the possibility that the related, albeit bulkier and more positively charged glutathione–spermidine disulphide conjugate, $[GspdS]_2$, might be a physiological substrate for *E. coli* GR and that this might also fortuitously account for the efficient reduction of $T[S]_2$. However, the reduction of $[GspdS]_2$ was bearly discernable. Given the apparent lack of a specific $[GspdS]_2$ reductase in *E. coli*, this then raised the question as to how GspdSH might be regenerated from the disulphide form. In the scheme considered in Figure 4 it is proposed that $[GsdpS]_2$ undergoes thiol exchange with GSH to form the mixed disulphide; indeed this non-enzymic reaction was employed in this study to prepare the disulphide. The kinetic properties of the mixed disulphide are sufficiently good to suggest that it may be a physiological substrate for GR. However there also remains the possibility that the disulphide undergoes further thiol-exchange with GSH to regenerate the second GspdSH molecule. Clearly, the relative importance of these reactions depends on the intracellular concentrations of the disulphides and on the rates of non-enzymic and possibly enzyme catalysed thiol-disulphide exchange reactions. Intriguingly mutants of *E. coli* that are GR-deficient are able to maintain a normal glutathione redox state [26]. It would be of interest to quantify the levels of GspdSH in these strains, given that it represents a potential reductant of GSSG (Figure 4).

The free glycine carboxylates in GSSG are covalently linked to spermidine in trypanothione disulphide (Figure 1). Three of the five residues in human GR that interact with these regions of the substrate GSSG are different in *E. coli* GR [27,28]. These exchanges of Arg-37, Ile-113 and Asn-117 for Asn-21, Ser-98 and Val-102 respectively in *E.coli* GR may contribute to the more relaxed substrate specificity of the latter enzyme. Site-directed mutagenesis studies have revealed that a single N21R mutation in *E. coli* GR does not generate an enzyme with the kinetic properties of the human GR [9]. X-ray crystallographic studies on the *E. coli* enzyme [28] have revealed that the presence of Ile-95 versus Leu-110 in human GR displaces the polypeptide backbone in the vicinity of the conserved active-site residue Tyr-99 by 2.5 Å (1 Å = 0.1 nm), as in trypanothione reductase [29–31].

Despite all these changes the catalytic constant $k_{cat.}/K_m$ of *E. coli* GR for $T[S]_2$ is still only 1 % of that observed for TRs [6] and the enzyme is virtually inactive against $[GspdS]_2$. The present work suggests that the broad specificity of the *E. coli* enzyme may be a consequence of the ability of the enzyme to reduce acyclic GspdS–SG, which is structurally very similar to $T[S]_2$ (Figure 1) but which has one free glycine carboxylate and a flexible spermidine moiety. We are currently using a crystallographic approach to further our understanding of the interaction of GR with peptide disulphides of physiological significance.

Note added in proof (received 3 October 1995)

The purification, cloning, overexpression and characterization of a bifunctional *E. coli* glutathionylspermidine synthetase/amidase has recently been reported [32].

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REFERENCES

1 Meister, A. and Anderson, M. E. (1983) Annu. Rev. Biochem. **52**, 711–760
2 Dolphin, D., Poulson, R. and Avramovic, O., eds. (1989) Glutathione: Chemical, Biochemical and Medical Aspects, Parts A and B, Wiley Interscience, New York
3 Ghisla, S. and Massey, V. (1989) Eur. J. Biochem. **181**, 1–17
4 Tabor, H. and Tabor, C. W. (1975) J. Biol. Chem. **250**, 2648–2654
5 Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T. and Cerami, A. (1985) Science **227**, 1485–1487
6 Fairlamb, A. H. and Cerami, A. (1992) Annu. Rev. Microbiol. **46**, 695–729
7 Shames, S. L., Fairlamb, A. H., Cerami, A. and Walsh, C. T. (1986) Biochemistry **25**, 3519–3526
8 Henderson, G. B., Fairlamb, A. H., Ulrich, P. and Cerami, A. (1987) Biochemistry **26**, 3023–3027
9 Henderson, G. B., Murgolo, N. J., Kuriyan, J. et al. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8769–8773
10 Fairlamb, A. H., Henderson, G. B., Bacchi, C. J. and Cerami, A. (1987) Mol. Biochem. Parasitol. **24**, 185–191

11 Shim, H. and Fairlamb, A. H. (1988) J. Gen. Microbiol. **134**, 807–817
12 Greer, S. and Perham, R. N. (1986) Biochemistry **25**, 2736–2742
13 Meinnel, T., Mechulam, Y. and Fayat, G. (1988) Nucleic Acids Res. **16**, 8095–8096
14 Berry, A., Scrutton, N. S. and Perham, R. N. (1989) Biochemistry **28**, 1264–1269
15 Deonarain, M. P., Berry, A., Scrutton, N. S. and Perham, R. N. (1989) Biochemistry **28**, 9602–9607
16 Scrutton, N. S., Berry, A. and Perham, R. N. (1987) Biochem. J. **245**, 875–880
17 El-Waer, A., Douglas, K. T., Smith, K. and Fairlamb, A. H. (1991) Anal. Biochem. **198**, 212–216
18 Tabor, H. and Tabor, C. W. (1971) Methods Enzymol. **17B**, 815–817
19 Smith, K., Nadeau, K., Walsh, C. T. and Fairlamb, A. H. (1992) Protein Sci. **1**, 874–883
20 Greenberg, J. T. and Demple, B. (1986) J. Bacteriol. **168**, 1026–1029
21 Tabor, H. and Tabor, C. W. (1983) Methods Enzymol. **94**, 434–437
22 Awad, S., Henderson, G. B., Cerami, A. and Held, K. D. (1992) Int. J. Radiat. Biol. **62**, 401–407
23 Xie, Q. W., Tabor, C. W. and Tabor, H. (1993) Gene **126**, 115–117
24 Martinoia, E., Grill, E., Tommasini, R., Kreuz, K. and Amrhein, N. (1993) Nature (London) **364**, 247–249
25 Muller, M., Meijer, C., Zaman, G. J. R. et al. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 13033–13037
26 Tuggle, C. K. and Fuchs, J. A. (1985) J. Bacteriol. **162**, 448–450
27 Aboagye-Kwarteng, T., Smith, K. and Fairlamb, A. H. (1992) Mol. Microbiol. **6**, 3089–3099
28 Mittl, P. R. E. and Schulz, G. E. (1994) Protein Sci. **3**, 799–809
29 Kuriyan, J., Kong, X.-P., Krishna, T. S. R. et al. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8764–8767
30 Hunter, W. N., Bailey, S., Habash, J. et al. (1992) J. Mol. Biol. **227**, 322–333
31 Bailey, S., Smith, K., Fairlamb, A. H. and Hunter, W. N. (1993) Eur. J. Biochem. **213**, 67–75
32 Bollinger, J. M., Jr., Kwon, D. S., Huisman, G. W., Kolter, R. and Walsh, C. T. (1995) J. Biol. Chem. **270**, 14031–14041

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