Glutathione Metabolism of the Erythrocyte

THE ENZYMIC CLEAVAGE OF GLUTATHIONE-HAEMOGLOBIN PREPARATIONS BY GLUTATHIONE REDUCTASE

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A complex of haemoglobin and GSH was prepared by incubating haemoglobin with GSH and acetylphenylhydrazine. GSH could be released from the crude preparation by incubation with NADPH. However, when the haemoglobin preparation was separated from glutathione reductase by DEAE-Sephadex chromatography, NADPH no longer released GSH. Rather, the addition of a combination of either partially purified human erythrocyte or crystalline glutathione reductase and NADPH was required to release GSH from the haemoglobin-GSH complex. This complex is commonly believed to represent a mixed disulphide of GSH and the cysteine- β -93 thiol group. This interpretation was supported by the finding that prior alkylation of available haemoglobin thiol groups prevented the formation of the complex. By using haemoglobin- ^{35}S -GSH complex as a substrate, it was shown that GSH itself released the radioactivity from the complex only very slowly. In contrast, the release of ^{35}S -GSH was very rapid in the presence of NADPH and glutathione reductase. This suggests that the cleavage of the haemoglobin-GSH complex is not mediated by GSH with cyclic reduction of GSSG formed, but rather proceeds enzymically through glutathione reductase.

Allen & Jandl (1961) and Huisman & Dozy (1962) have shown that normal adult haemoglobin can form complexes with GSH. Their formation may be of importance both in normal erythrocyte ageing and in the pathogenesis of drug-induced haemolytic anaemia. It has been suggested by several workers (Allen & Jandl, 1961; Huisman & Dozy, 1962; Jacob, Brain, Dacie, Carrell & Lehmann, 1968b; Jacob, Brain & Dacie, 1968a) that these complexes are mixed disulphides of GSH and cysteine- β -93 of haemoglobin. The nearness of the readily accessible thiol group of cysteine-93 of the β chain to the proximal histidine residue, to which haem is attached, and the apparent participation of disulphides in Heinz-body formation have focused attention on the role of such haemoglobin-GSH complexes in haemolytic disease (Jacob *et al.* 1968a,b). However, the fate of complexes of GSH and haemoglobin has received only scant attention.

In the present study we have investigated the capacity of the erythrocyte to break such bonds once they have been formed. These studies indicate that although GSH itself has little capacity to break GSH-haemoglobin complexes, enzymic cleavage by glutathione reductase is accomplished with high efficiency.

MATERIALS AND METHODS

NADH, NADPH, GSH, GSSG, 5,5'-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, yeast glutathione reductase (100 units/mg of protein) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. ^{35}S -GSH (11.2 mCi/mmol) was obtained from Schwartz Bio-Research Inc., Orangeburg, N.Y., U.S.A. Crystalline human haemoglobin ('100% pure') was purchased from Calbiochem, Los Angeles, Calif., U.S.A. However, it was found to be contaminated with glutathione reductase. DEAE-Sephadex A-50 (particle size 40–120 μm) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

GSH and GSSG determinations. GSH was determined in a metaphosphoric acid filtrate by using the 5,5'-dithiobis(2-nitrobenzoic acid) method (Beutler, Duron & Kelly, 1963). GSSG was determined enzymically in a trichloroacetic acid filtrate with NADPH and glutathione reductase after prior alkylation of GSH by *N*-ethylmaleimide (Srivastava & Beutler, 1968).

Haemoglobin determinations. Haemoglobin was measured by using the cyanmethaemoglobin technique.

Glutathione reductase assays. Glutathione reductase activity was determined by incubation of samples of haemolysate or haemoglobin-GSH complexes, before or after fractionation on DEAE-Sephadex columns, with 1.22 mM-GSSG and 3.0 mM-NADPH for 2 h at 37°C. The

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increase in GSH concentration during the incubation period was determined by the 5,5'-dithiobis (2-nitrobenzoic acid) method (Beutler *et al.* 1963).

Purification of erythrocyte glutathione reductase. Glutathione reductase from normal erythrocytes was partially purified by ammonium sulphate fractionation as described by Beutler & Yeh (1963). Further purification of the enzyme was achieved by DEAE-Sephadex chromatography and by heating the enzyme-rich fraction at 55°C for 10 min. This resulted in a 1500-fold purification (S. K. Srivastava & E. Beutler, unpublished work).

Preparation of haemoglobin-GSH complex from human erythrocytes. Venous blood from normal donors was collected in heparinized tubes (2000 U.S.P. units of heparin/10 ml of blood) and was centrifuged at 1000g for 30 min at 4°C. Plasma and buffy coat were aspirated and the erythrocytes were washed twice by suspending them in 5–7 vol. of 0.15 M-potassium phosphate buffer, pH 7.4–0.154 M-NaCl (1:9, v/v). Washed packed erythrocytes (1 ml), 1.0 ml of phosphate buffer-NaCl, 10–30 μ mol of GSH neutralized to pH 7.0 and 3.0 ml of water were frozen and thawed once. Haemolysate (4 ml) was transferred to tubes containing 5 mg of acetylphenylhydrazine and incubated in a Dubnoff shaker at 37°C for 2 h. The tubes were shaken at about 100 oscillations/min. In some experiments 3.0 μ mol of [35 S]GSH (2.5 μ Ci) were also added/ml of erythrocytes. At the end of the incubation period the contents of the tubes were dialysed for 48 h against 1 litre of 10-times-diluted phosphate buffer-NaCl. The dialysis liquid was changed four times. Dialysed haemolysate was designated as MD1 and the dialysed haemolysate where [35 S]GSH was used as MD 35 S. Samples of preparations MD1 and MD 35 S were taken for determination of GSH, GSSG, haemoglobin and glutathione reductase.

Fractionation of haemolysate and preparation MD1. DEAE-Sephadex A-50 was equilibrated with 0.05 M-tris-HCl buffer, pH 7.9. A column (45 cm $\times$ 2.5 cm) was packed to 25 cm and washed with 0.05 M-tris-HCl buffer, pH 7.9, until the pH of the eluate was 7.9. A portion (16 ml) of preparation MD1 dialysed overnight against 1 litre of 10-times-diluted phosphate buffer-NaCl without any change of dialysis liquid, or 16 ml of 25% haemolysate dialysed against 1 litre of 0.05 M-tris-HCl buffer, pH 7.9, was absorbed on a column at a flow rate of about 0.05 ml/min. In some experiments 16 ml of a 5% (w/v) solution of crystalline human haemoglobin was applied to the column. The column was washed with 400 ml of 0.05 M-tris-HCl buffer, pH 7.9. Haemoglobin was eluted with a linear pH gradient from pH 7.9 to pH 7.4 by using 500 ml of 0.05 M-tris-HCl buffer, pH 7.9 and 500 ml of the same buffer, pH 7.4. Haemoglobin was eluted after about 400 ml of elution buffer had passed through the column. Fractions (50 ml) were collected and freeze-dried. The dried powder of the haemoglobin-GSH complex was dissolved in water and dialysed for 2 h against two changes of 1 litre of water at 4°C. Each fraction was assayed for acid-soluble GSH and GSSG. About six fractions in each experiment were devoid of free GSH, GSSG and glutathione reductase activity. The preparation of haemoglobin-GSH complex that was found to be free from glutathione reductase activity, acid-soluble GSH and GSSG was designated as MD2. These fractions were pooled and used for further experiments.

Fractions of haemoglobin obtained either from the haemolysate or from crystalline haemoglobin that were found to be free from glutathione reductase activity, GSH and GSSG were freeze-dried, dissolved in water and dialysed against water for 2 h. The dialysed fractions were treated with GSH in the presence of acetylphenylhydrazine. Several haemoglobin fractions free from glutathione reductase were precipitated during the preparation. This might be attributed to oxidation of thiol groups other than those of cysteine- β -93, which will unfold the haemoglobin molecule (Jacob *et al.* 1968b). This mixed-disulphide preparation was dialysed for 4 days against 10-times-diluted phosphate buffer-NaCl solution. Samples of the preparation were tested for acid-soluble GSH and GSSG, but none was found. This preparation was designated as MD3.

Preparation and fractionation of GSH-haemoglobin complex from rat erythrocytes. Rat erythrocytes react uniquely when subjected to H₂O₂ diffusion in that the GSH lost cannot be recovered as GSSG (Srivastava & Beutler, 1969). Twice-washed erythrocytes (1 ml) were suspended in 3 ml of phosphate buffer-NaCl solution with 0.014 M-glucose and 1.3 μ M-Na₂CrO₄ and placed in the main compartment of a Warburg flask. Hydrogen peroxide (0.2 ml; 30%) was placed in the centre well and the flask was incubated in a Dubnoff shaker at 37°C at 100 oscillations/min. At the end of the incubation period the contents of the main compartment were centrifuged and GSH and GSSG were determined in the supernatant and erythrocytes. Practically no GSSG was found in the erythrocytes and the recovery of total glutathione in the erythrocytes and supernatant was only about 20%. Thus about 80% of the glutathione could have complexed with proteins, mainly haemoglobin.

A 25% frozen-and-thawed haemolysate of rat erythrocytes subjected to H₂O₂ diffusion was prepared and dialysed against 1 litre of water for 1 h. Haemolysate (16 ml) was absorbed on DEAE-Sephadex columns equilibrated at pH 7.9 as indicated above. After the absorption of haemoglobin on the column, about 600 ml of 0.05 M-tris-HCl buffer, pH 7.9, was passed through the column. Haemoglobin fractions were eluted with 0.05 M-tris-HCl buffer, pH 7.5. The fractions rich in haemoglobin were freeze-dried and the resulting haemoglobin was dissolved in water and designated as MDR. No acid-soluble GSH or GSSG were detected in this solution. However, preparation MDR was very slightly contaminated with glutathione reductase activity.

Cleavage of GSH-haemoglobin preparations. Unless otherwise indicated the complexes of GSH and haemoglobin prepared from human or rat erythrocytes or purified human haemoglobin were subjected to reductive cleavage in the presence of glutathione reductase and reduced nicotinamide nucleotides. A preparation representing 0.47–1.9 μ mol of haemoglobin β -chain was incubated for 2 h at 37°C with 1.0–4.0 μ mol of NADPH, and 17 units of glutathione reductase (yeast) in a total volume of 1.4 ml containing 30 μ mol of potassium phosphate buffer, pH 7.4. In each experiment control mixtures lacking glutathione reductase and NADPH were also studied. At the end of the incubation period proteins were precipitated with 3.0 ml of metaphosphoric acid reagent and GSH was determined by the 5,5'-dithiobis-(2-nitrobenzoic acid) method (Beutler *et al.* 1963).

Table 1. *Cleavage of GSH from human haemoglobin-GSH complexes*

Preparations MD1, MD2 and MD3 were obtained as described in the Materials and Methods section. Each 1.4 ml of the incubation system contained: preparation MD1 (representing 1.9 μmol of haemoglobin β -chains) or preparation MD2 (representing 0.47 μmol of haemoglobin β -chains) or preparation MD3 (representing 0.56 μmol of haemoglobin β -chains) or preparation MD3* (Expt. 4), prepared from crystalline haemoglobin (representing 0.82 μmol of haemoglobin β -chains); 0.2 ml of 0.15 M-potassium phosphate buffer, pH 7.4; and, where indicated, NADPH (2 μmol), NADH (2 μmol), yeast or human glutathione reductase (17 units). The tubes were incubated for 2 h at 37°C. A 1500-fold purified human glutathione reductase was used for splitting GSH from the mixed disulphide in Expt. 4. At the end of the incubation period proteins were precipitated with metaphosphoric acid and GSH was determined in the acid filtrate by the 5,5'-dithiobis(2-nitrobenzoic acid) method (Beutler *et al.* 1963). Abbreviations: ND, not determined; Hb, haemoglobin.

Expt. no.	Additions to haemoglobin-GSH complex				GSH released					
	Glutathione reductase				MD1		MD2		MD3	
	Human erythrocyte				$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$		$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$		$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$	
	NADPH	NADH	Yeast	Human erythrocyte	$(\mu\text{mol}/\text{ml})$	$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$	$(\mu\text{mol}/\text{ml})$	$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$	$(\mu\text{mol}/\text{ml})$	$(\mu\text{mol}/\mu\text{mol of Hb } \beta\text{-chain})$
1	—	—	—	—	0	0	0	0	0	0
2	+	—	—	—	0.244	0.128	0	0	0	0
3	+	—	+	—	0.247	0.129	0.066	0.141	0.058	0.104
4	+	—	—	+	ND	ND	ND	ND	0.106*	0.129*
5	+	—	—	+	ND	ND	0.073	0.155	ND	ND
6	—	—	+	+	ND	ND	0	0	ND	ND
7	—	+	—	—	ND	ND	0	0	ND	ND
8	—	+	+	—	ND	ND	0.066	0.141	ND	ND
9	—	+	—	+	ND	ND	0.071	0.151	ND	ND

RESULTS

Cleavage of human GSH-haemoglobin preparations. Preliminary studies, in which [^{35}S]GSH was used, indicated that in various experiments 0.08–0.16 μmol of GSH were bound/ μmol of haemoglobin β -chain. The preparation of GSH and human haemoglobin prepared by using acetylphenylhydrazine, GSH and haemolysate, i.e. MD1, contained no acid-soluble GSH and GSSG but contained a great deal of glutathione reductase activity. Large amounts of GSH were liberated from preparation MD1 incubated with NADPH or NADPH + yeast glutathione reductase (Table 1). In contrast, when preparation MD2 or MD3 was incubated with NADPH alone, no GSH was liberated, but when they were incubated in the presence of both NADPH and glutathione reductase, either from yeast or from human erythrocytes, large amounts of GSH were liberated. Incubation of the mixed disulphide, prepared by using crystalline human haemoglobin, with a 1500-fold purified human glutathione reductase and NADPH liberated large amounts of GSH. In some experiments NADH was used as hydrogen donor for glutathione reductase instead of NADPH and was also found to be effective in bringing about the release of GSH. This is not surprising, since erythrocyte glutathione

reductase is active with either nicotinamide nucleotide when GSSG serves as substrate.

The haemolysate prepared from human erythrocytes was preincubated for 10 min with 0.03 μmol of NADPH/mg of haemoglobin at 37°C, and then for 20 min with 1.05 μmol of *N*-ethylmaleimide/ μmol of titratable thiols. After extensive dialysis of this preparation it was treated in the manner described in the Materials and Methods section for the preparation of MD1. The preparation thus obtained was passed through a Sephadex G-50 column and the haemoglobin fractions free from *N*-ethylmaleimide, GSH and GSSG were treated in the usual manner with NADPH and glutathione reductase. No GSH was released.

Cleavage of rat GSH-haemoglobin preparations. Haemoglobin prepared by subjecting rat erythrocytes to oxidative stress in the presence of hydrogen peroxide was freed of GSH and GSSG by fractionation on DEAE-Sephadex columns. However, the preparation contained very small amounts of glutathione reductase activity. NADPH alone reduced the haemoglobin-GSH complex very slowly to liberate GSH. However, NADPH and yeast glutathione reductase reduced the haemoglobin-GSH complex much more rapidly.

Cleavage of haemoglobin-[^{35}S]GSH complex by added GSH. Various amounts of GSH were added

Fig. 1. Cleavage of [^{35}S]GSH-haemoglobin preparation by GSH. Preparation MD ^{35}S was obtained by incubation of haemolysate with [^{35}S]GSH in the presence of acetylphenylhydrazine and was dialysed for 4 days as described in the Materials and Methods section. It contained $0.09\text{ }\mu\text{mol}$ of glutathione/ μmol of haemoglobin β -chain. In a 1.6 ml system preparation MD ^{35}S ($1.91\text{ }\mu\text{mol}$ of haemoglobin β chain) was incubated with: ●, nothing added; ○, GSH ($0.064\text{ }\mu\text{mol}$); ▲, GSH ($0.64\text{ }\mu\text{mol}$); △, NADPH ($1.0\text{ }\mu\text{mol}$); ■, NADPH ($10.0\text{ }\mu\text{mol}$). At various time-intervals 0.1 ml samples were withdrawn into tubes containing 0.5 ml of 12% (w/v) trichloroacetic acid. The tubes were centrifuged and 0.1 ml of the clear supernatant was counted for radioactivity in a gas-flow counter. The percentage of radioactivity released from preparation MD ^{35}S was calculated as:

$$\frac{\text{Total radioactivity released from MD}^{35}\text{S} \times 100}{\text{Total radioactivity present in MD}^{35}\text{S}}$$

to the dialysed MD ^{35}S preparation and the mixture was incubated at 37°C . At appropriate intervals the proteins were precipitated with trichloroacetic acid and the radioactivity of the supernatant was counted in a gas-flow counter. The addition to the medium of quantities of GSH equal to the maximum that could be released from the preparation did not release appreciable radioactivity from the haemoglobin-[^{35}S]GSH complex (Fig. 1). Only massive amounts of GSH released radioactivity. This is attributed to an exchange reaction between unlabelled GSH and labelled glutathione bound to the haemoglobin preparation, since total glutathione, i.e. GSH + GSSG, in the supernatant did not increase after incubation with GSH. However, the rate of release of radioactive GSH from the preparation when large amounts of GSH were added was much slower than the rate when the mixed disulphide was incubated with NADPH and glutathione reductase. Here the release of radioactivity

was initially almost instantaneous. The late decline in the amount of acid-soluble radioactivity present in the incubation mixture could be attributed to the new formation of GSH-haemoglobin complexes once the supply of NADPH had been exhausted.

DISCUSSION

Erythrocytes contain large amounts of GSH. Although not all of the functions of this tripeptide are fully understood, it is believed that one role of GSH is to protect haemoglobin and other thiol-group-containing proteins from denaturation under oxidative stress (Allen & Jandl, 1961; Beutler, 1957). Indeed, the erythrocytes of individuals with a hereditary lack of GSH are unusually sensitive to the haemolytic effect of oxidant drugs (Prins, Oort, Loos, Zürcher & Beckers, 1966).

When erythrocytes are exposed to oxidative stress GSH is oxidized to GSSG. This may occur through interaction of the oxidative compound with haemoglobin to form peroxides, with the subsequent oxidation of GSH through the mediation of glutathione peroxidase (Mills, 1957; Cohen & Hochstein, 1964). Alternatively, free GS $^{\cdot}$ radicals may form and may combine with one another to form GSSG (Kosower, Vanderhoff, Kosower & Huang, 1965). It has been proposed that the formation of mixed disulphides may occur when some of these free radicals combine with the thiol group at the β -93 position of haemoglobin. Definitive proof that the binding of GSH to haemoglobin occurs at the β -93 position has not yet been obtained. However, prior treatment of haemoglobin with *N*-ethylmaleimide, which has been shown to alkylate cysteine- β -93 of haemoglobin (Riggs, 1959; Riggs & Wells, 1960) prevents the formation of a GSH-haemoglobin complex that can be split by NADPH and glutathione reductase. Thus, it is reasonable to suppose that the complex formed does represent the disulphide of GSH and cysteine- β -93 of haemoglobin. Only with further oxidative stress is the haemoglobin molecule unfolded to expose the remaining thiol groups and to subject them to oxidation (Jacob *et al.* 1968*b*). However, it seems reasonable as a working hypothesis to consider the haemoglobin-GSH complexes we have studied to be such mixed disulphides. The fate of haemoglobin that has formed such a complex with glutathione, probably at the β -93 position, has not, to our knowledge, been studied previously. The present investigations reveal that glutathione that has become bound to human or rat haemoglobin after being exposed to the oxidative stress of incubation with acetylphenylhydrazine or hydrogen peroxide is rapidly released by incubation with NADPH or NADH. However, the reduced nicotinamide nucleotides lose their capacity to release glutathione

from erythrocyte mixed disulphides when the glutathione reductase activity is removed from the haemolysate by column chromatography. Addition of either purified yeast glutathione reductase or the partially purified human erythrocyte enzyme restores the capacity of nicotinamide nucleotides to release GSH from haemoglobin. The possibility that the active enzyme is not glutathione reductase, but rather another enzyme contaminating the human erythrocyte glutathione reductase preparation, is rendered very unlikely by the fact that highly purified yeast glutathione reductase has the same capacity to cleave the mixed disulphide as does the less highly purified erythrocyte preparation.

The possibility has been considered that glutathione reductase actually acts on traces of GSSG that have escaped dialysis and that the GSH formed reduces a GSH-haemoglobin mixed disulphide (Hb-S-SG) and is continuously regenerated by glutathione reductase:

Such a sequence of events has been proposed (Pihl, Eldgarn & Bremer, 1957) for the reduction of *NN'*-diacetylcystamine, cystamine, *NN'*-tetramethylecystamine, *NN'*-tetraethylecystamine, cystine and homocystine by GSH. However, this possibility is ruled out conclusively by the demonstration that the release of [³⁵S]GSH from the mixed disulphide by GSH proceeds at an extremely slow rate, even when amounts of GSH much greater than the maximum that could be released are added. We propose that the release of GSH from the haemoglobin-GSH mixed disulphide proceeds in the single reaction catalysed by glutathione reductase:

Although glutathione reductase has been regarded as a relatively specific enzyme, it has been shown that in addition to GSSG it can catalyse the reduction of dihydrolipoic acid (Scott, Duncan & Ekstrand, 1963) and possibly of the mixed disulphide of GSH and dithionitrobenzoic acid (Owens & Belcher, 1965; Tietze, 1969). The action of glutathione reductase on mixed disulphides of

haemoglobin and GSH may be of greater physiological importance. It is likely that this mechanism permits the erythrocyte to live out its normal lifespan of 120 days in the face of frequent oxidative stress, without the accumulation of large amounts of denatured haemoglobin.

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