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RED CELL GLUTATHIONE REDUCTASE: MECHANISM OF ACTION OF INHIBITORS*

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SUMMARY

1. The mechanism of action of 3 inhibitors of human red cell glutathione reductase (NADPH:oxidized glutathione oxidoreductase, EC 1.6.4.2), hexavalent chromium (Cr(VI)), *N*-ethylmaleimide, and 2,5-dinitrobenzoic acid was studied in relation to the two forms of the enzyme, an active form with flavin adenine dinucleotide (FAD) and an inactive form without FAD.

2. *N*-Ethylmaleimide (1 mM optimal) inhibits glutathione reductase activity both in the active form and in the inactive form to the same extent in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The inhibition of the active form of glutathione reductase by *N*-ethylmaleimide is completely reversible, but the effect of *N*-ethylmaleimide on the inactive form is irreversible.

3. Hexavalent chromium (0.5 mM optimal) inhibited almost irreversibly 90% of glutathione reductase activity in the active form associated with FAD in the presence of NADPH. However, hexavalent chromium appears to be incapable of inhibiting the inactive form of glutathione reductase without FAD. Sulfhydryl group(s) of the enzyme protein of glutathione reductase are essential for irreversible inhibition of the active form by Cr(VI).

4. 2,5-Dinitrobenzoic acid (4 mM optimal) irreversibly inhibited about 50% of glutathione reductase activity in both the active and the inactive forms to the same extent. Sulfhydryl group(s) or FAD appears to be unnecessary for inhibition of the enzymatic activity. 2,5-Dinitrobenzoic acid may bind irreversibly to some structure(s) of the enzyme protein other than sulfhydryl radicals.

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INTRODUCTION

Glutathione reductase (NADPH:oxidized glutathione oxidoreductase, EC 1.6.4.2) plays an important role in the protection of protein in red cells against oxidation. The structure and function of glutathione reductase have been investigated extensively, especially in relation to flavin metabolism[1-11]. It is now generally accepted that red cell glutathione reductase consists mainly of two portions, that is, an active form with flavin adenine dinucleotide (FAD) and an inactive form without FAD[2-9,11]. Two molecules of FAD and two polypeptide chains with sulfhydryl groups are present in each molecule of the purified holoenzyme[6-9]. Thus, glutathione reductase is a flavin enzyme with FAD as the prosthetic group and with sulfhydryl group(s) as the active center.

Studies on the relationship of the structure to the function of glutathione reductase have been performed using several inhibitors. Among them, *N*-ethylmaleimide is an alkylating agent that forms stable addition products with sulfhydryl compounds and reacts rather specifically with sulfhydryl groups[12]. The inhibitory effect of *N*-ethylmaleimide on glutathione reductase activity has been reported in yeast[5], in peas[3], and in human red cells[7,13]. Recently, Beutler and his co-workers[14] have suggested the formation of loose alkylating products of *N*-ethylmaleimide in relation to the reversible inhibition of *N*-ethylmaleimide on reduced glutathione. It is also of interest that inhibition of *N*-ethylmaleimide on purified red cell glutathione reductase requires the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH)[7]. Another inhibitor, hexavalent chromium (Cr(VI)), has been known as one of the specific inhibitors. Koutras and his co-workers[13,15] reported inhibition of glutathione reductase by Cr(VI) in intact red cells and in hemolysates, and noted that the presence of NADPH was required. In addition, 2,5-dinitrobenzoic acid has been reported to be a specific inhibitor of glutathione reductase[1,16].

However, the results cited above have been reported mainly for the active form of glutathione reductase. It is assumed that the enzyme protein of glutathione reductase may differ stoichiometrically between the active form with FAD and the inactive form without FAD, and that the cofactor, FAD, may play an important role in the conformational change of the enzyme protein of glutathione reductase. Thus, the inhibition by these inhibitors of two different forms of the enzyme protein of glutathione reductase has been of increasing interest.

In this paper, we have demonstrated the different mechanism of action of these inhibitors on the active and the inactive forms of glutathione reductase.

MATERIALS AND METHODS

Venous blood was obtained from normal human adults with heparin as anticoagulant. After the plasma and buffy coat were discarded, red cells were washed three times with cold 0.15 M saline solution, and resuspended in saline in a concentration of approx. 3 million cells/μl as previously described[17]. Hemolysates were prepared in a 1 to 10 dilution with water and then frozen and thawed three times.

Glutathione reductase activity was assayed at optimal conditions by a slight modification of the method of Long and Carson[18] at 37 °C unless otherwise described specially. The assay system consisted of 2.2 ml of 0.177 M Tris–0.0435 M EDTA

buffer (pH 7.6), 0.5 ml of 0.0318 M oxidized glutathione (GSSG), and 0.1 ml of 8.7 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was recorded at 340 nm in the Gilford Automatic Recording Spectrophotometer Model 2000. Activity was expressed in international units, I.U. (μmoles NADPH oxidized per min per 10^{10} red cells).

Incubation experiments were performed mainly on intact red cells or hemolysates, due to the absence of a well-established procedure of purification of glutathione reductase, especially of the inactive FAD-free form. However, in some experiments, FAD-removed preparations of hemolysates were utilized.

In some preliminary experiments, stroma was removed by centrifuging hemolysates at 4 °C at 20 000 $\times$ g for 30 min. However, the removal of stroma from hemolysates did not significantly affect the enzymatic activity of glutathione reductase.

FAD, GSSG, and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo., or Calbiochem, Los Angeles, Calif. Other chemicals were reagent grade.

RESULTS

(1) Effect of FAD on activation of glutathione reductase activity in human adult red cells

Preliminary experiments indicated that maximum activation of glutathione reductase activity by FAD was achieved at a concentration of 1 μM FAD (no greater effect with 5 μM) after about 10 min of incubation, and was maintained for at least 24 h.

Mean glutathione reductase activity in red cells (and standard deviation) from 30 normal adults was 3.04 $\pm$ 0.36 I.U. (the active form). After addition of FAD to the hemolysates, activity increased to 4.38 $\pm$ 0.37 I.U., an increase of 1.34 $\pm$ 0.37 I.U. (the inactive form). Thus, in normal adults, 69.4% of glutathione reductase activity is in the active form, and 30.6% is in the inactive form.

(2) Experiments with N-ethylmaleimide

(a) *Reversibility of N-ethylmaleimide inhibition of the active form of glutathione reductase and irreversible inhibition of the inactive form.* After intact red cells were preincubated with 5.6 mM glucose and further incubated with *N*-ethylmaleimide (0, 0.7, 0.8, and 1.0 mM) at 37 °C for 15 min, the hemolysates in 0.177 M Tris–EDTA buffer (pH 7.6) were incubated in the presence of 8.7 mM NADPH at 37 °C for periods up to 12 h, and the glutathione reductase activity was determined.

Glutathione reductase activity that been inhibited in proportion to the various concentrations of *N*-ethylmaleimide returned spontaneously to the original level on incubation in the buffer for 12 h, as shown in Fig. 1. After that point, the mixtures were divided into two portions, and incubated at 37 °C in the presence or absence of 1 μM FAD for an additional 12 h. The glutathione reductase activity was determined at 0 time, 1, 3, and 12 h after the addition of FAD. A typical experiment and its results are shown in Fig. 1. FAD activated only that portion of the inactive form not inhibited by *N*-ethylmaleimide. The degree of FAD activation of glutathione reductase activity in the inactive form depended on the concentration of *N*-ethylmaleimide used.

(b) *Activation of glutathione reductase by FAD in "FAD-removed" hemolysates*

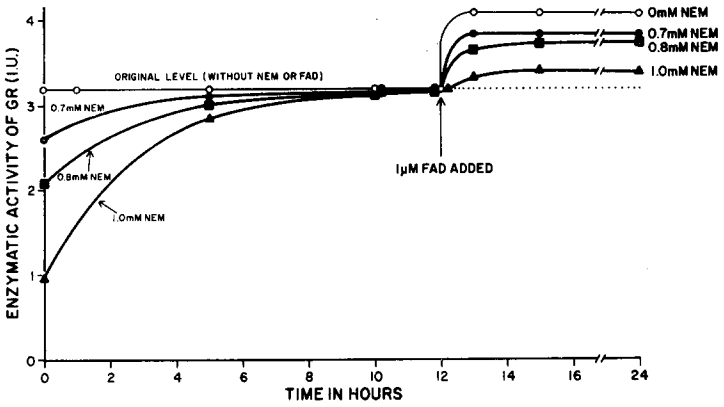

Fig. 1. Spontaneous reversibility of red cell glutathione reductase (GR) activity inhibited by *N*-ethylmaleimide (NEM) and activation of glutathione reductase by FAD. Red cell suspensions were preincubated with glucose and then incubated with 0, 0.7, 0.8, and 1.0 mM *N*-ethylmaleimide. Hemolysates were incubated up to 12 h and then further incubated with 1 μM FAD up to 24 h.

of N-ethylmaleimide-treated red cells. In these experiments, hemolysates were freed of FAD by acid treatment (pH 3.0) according to the method of Icen[7] for removal of the prosthetic group from the flavin enzyme. Red cells were incubated with *N*-ethylmaleimide (0, 0.7 or 1.0 mM). After washing the red cells with cold 0.15 M saline, hemolysates of *N*-ethylmaleimide-treated red cells were prepared. The stroma-free hemolysates were subjected to pH 3.0 at 4 °C for 2 h in saturated $(NH_4)_2SO_4$. Thereafter, the precipitate was collected by centrifugation at 1000 $\times$ *g* for 30 min at 4 °C, and redissolved by the addition of 0.2 M Tris–HCl buffer (pH 8.0). An aliquot was added to 0.177 M Tris–0.0435 M EDTA buffer (pH 7.6); this was divided into two equal parts. FAD in a final concentration of 1 μM was added to one part, and water, as the control, was added to the other part. The mixtures were incubated at 37 °C up to 14 h, and glutathione reductase activity was determined.

Results are shown in Fig. 2. Glutathione reductase activity inhibited by the

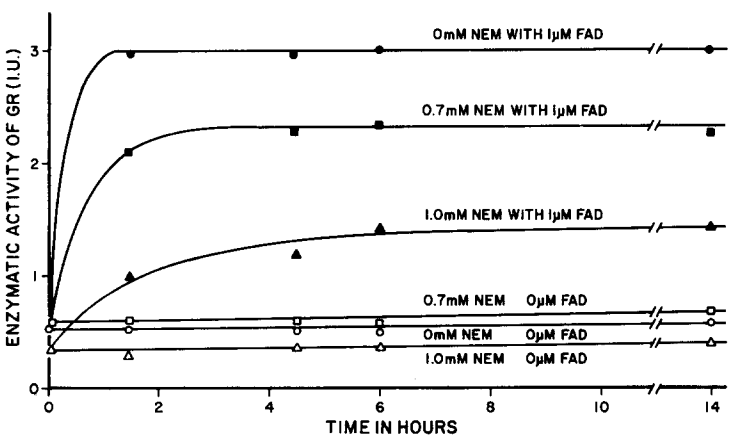

Fig. 2. Activation of "FAD-removed" glutathione reductase (GR) by FAD in red cells treated with *N*-ethylmaleimide (NEM) (0, 0.7, and 1.0 mM). Glutathione reductase in *N*-ethylmaleimide treated hemolysates was removed of FAD, and then incubated with 1 μM FAD up to 14 h.

lower concentration of *N*-ethylmaleimide (0.7 mM) was activated from 0.57 to 2.25 I.U. after incubation with FAD. With the higher concentration of *N*-ethylmaleimide (1.0 mM), the enzymatic activity was activated only to 1.47 I.U. in comparison to the maximum level (3.01 I.U.) when no *N*-ethylmaleimide was used.

(3) Experiments with hexavalent chromium

(a) *Activation of glutathione reductase by FAD in hemolysates of chromated intact red cells.* In experiments on the inhibitory effect of hexavalent chromium (Cr(VI)) on glutathione reductase activity, the freshly prepared red cell suspension was preincubated with 5.6 mM glucose, and further incubated with Na_2CrO_4 (0, 1.25, 2.5, 5, or 25 μg as elementary Cr/ml of red cell suspension) according to the method of Koutras *et al.*[15]. After Cr(VI) was reduced by adding ascorbic acid (0.2 mg/μg of elementary Cr), hemolysates prepared from washed red cells were incubated at 37 °C up to 6 h with Tris–EDTA buffer containing 1 μM FAD.

Typical results on red cells from three individuals are shown in Fig. 3. FAD activated glutathione reductase activity from 3.12 I.U. (the active form) to 5.08 I.U. The calculated amount of glutathione reductase activity activated by FAD was 1.96 I.U. (the inactive form). Cr(VI) (2.5 μg/ml) decreased the activity in intact red cells from 3.12 to 1.39 I.U., but FAD activated glutathione reductase activity in the hemolysate prepared from chromated red cells from 1.39 to 3.36 I.U. The calculated amount of glutathione reductase activity activated by FAD in this experiment was

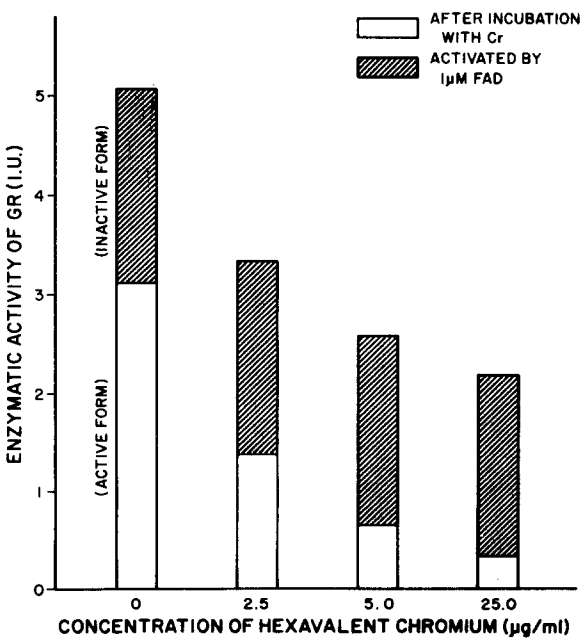

Fig. 3. Activation of glutathione reductase (GR) activity by FAD in red cells incubated with hexavalent chromium (Cr(VI)). Red cells were incubated with Cr(VI) (0, 2.5, 5.0, and 25 μg/ml). Glutathione reductase activity after incubation with Cr(VI) is shown as an open area in columns. The shaded area depicts the amount of glutathione reductase activity activated by FAD in the hemolysates of chromated red cells.

1.97 I.U. This value is equivalent to the amount (1.96 I.U.) activated by FAD in intact red cells not incubated with Cr(VI). Similar results were obtained with other concentrations of Cr(VI), as shown in Fig. 3. The same results were obtained in the hemolysates incubated with Cr(VI) in the presence of 8.7 mM NADPH (data not shown).

(b) *Activation of glutathione reductase in "FAD-removed" hemolysates of non-chromated and chromated red cells.* Samples with little or no enzymatic activity of glutathione reductase were prepared by acid treatment[7] from hemolysates of the chromated and non-chromated intact red cells, as described previously. After removing FAD, aliquots were added to Tris–EDTA buffer; this was divided into two equal parts. The mixtures were incubated at 37 °C for 6 h with or without 1 μM FAD. Activity of glutathione reductase was determined at 0 time, and after 6 h of incubation. Results of typical experiments are shown in Table I. After incubation with Cr(VI), glutathione reductase activity was markedly decreased from 2.54 to 0.34 I.U. in the hemolysate prepared from red cells incubated with Cr(VI). After acid treatment to remove FAD, glutathione reductase activity decreased from 0.34 (average) to 0.22 I.U. (average) in the chromated red cells, and from 2.54 to 0.34 I.U. in red cells untreated with Cr(VI). After "FAD-removed" enzymes were incubated with FAD, glutathione reductase activity was increased from 0.34 (average) to 3.62 I.U. (average) in red cells untreated with Cr(VI). These values were close enough to the total glutathione reductase activity (3.95 I.U.), that is, the sum of glutathione reductase activity in the active form and the inactive form, though glutathione

TABLE I

ACTIVATION OF FAD-REMOVED GLUTATHIONEREDUCTASE BY FAD IN CHROMATED AND NON-CHROMATED RED CELLS

After FAD was removed from the hemolysates of the chromated red cells (25 μg Cr/ml) by acid treatment (pH 3.0), aliquots were incubated with 0.177 M Tris–EDTA buffer (pH 7.6) with or without 1 μM FAD. Glutathione reductase activity was determined before and after 6 h of incubation with FAD, and before and after 45 min of incubation with Cr(VI). The expected level (F) of the FAD-removed glutathione reductase after incubation with Cr(VI) first, and then with FAD, was calculated as the sum of the level (C) of glutathione reductase after incubation with Cr(VI) (0.34 I.U.) and the amount (G) activated by FAD (1.41 I.U.) representing the amount of the inactive form. In the FAD-removed glutathione reductase from red cells not treated with Cr(VI), 3.95 I.U. (B) represents the total activity of the enzyme originally obtained after incubation with FAD. Amount activated by FAD (G) was calculated by subtracting (A) from (B). Amount inhibited by Cr(VI) (H) was calculated by subtracting (C) from (A).

Concentration of Cr(VI) (μg/ml)	*Glutathione reductase activity (I.U.)*							
	(A) Original level	*(B) After incubation with FAD*	*(C) After incubation with Cr(VI)*	*(D) After removing FAD*	*(E) After further incubation with FAD*	*(F) Expected after incubation with FAD*	*(G) Amount activated by FAD*	*(H) Amount inhibited by Cr(VI)*
0	2.54	3.95					1.41	
25	2.54		0.34					2.20
25			0.34	0.21	1.68	1.75		
25			0.34	0.22	1.70	1.75		
0	2.54			0.34	3.60	3.95		
0	2.54			0.33	3.63	3.95		

reductase activity was not recovered completely after acid treatment and subsequent incubation with FAD. However, the activity of the "FAD-removed" glutathione reductase in chromated red cells was only partially restored with FAD from 0.22 to 1.69 I.U. This latter value is nearly equal to the sum of the enzymatic activity of the inactive form (1.41 I.U.) and that of the active form not inhibited by Cr(VI) (0.34 I.U.).

(c) *Effect of preincubation of intact red cells with Cr(VI) on the inhibition of glutathione reductase activity by N-ethylmaleimide.* Red cells incubated with 5.6 mM glucose were divided into two aliquots in the presence or absence of Na_2CrO_4 (25 μg Cr(VI)/ml). After incubation of red cells with Cr(VI), the Cr(VI)-treated, and the Cr(VI)-untreated red cells were further divided into two aliquots, and one aliquot was then treated with 1 mM *N*-ethylmaleimide as already described. Hemolysates were prepared from (1) the Cr(VI)- and then *N*-ethylmaleimide-treated, (2) the Cr(VI) alone-treated, (3) the *N*-ethylmaleimide alone-treated, and (4) control red cells. For each of these four preparations, the hemolysate was added to the Tris–EDTA buffer, and incubated at 37 °C for up to 27 h. Glutathione reductase activity was determined in each of these four preparations.

Results are shown in the left half of Fig. 4. Inhibition of glutathione reductase activity by *N*-ethylmaleimide was spontaneously reversible as reported previously. On the other hand, the spontaneous restoration of the enzymatic activity in chromated red cells was considerably less than that in the *N*-ethylmaleimide-treated red cells. When chromated glutathione reductase was further incubated with *N*-ethylmaleimide the inhibition of glutathione reductase activity was maintained with only minimal reversion during the incubation. These results were quite similar to those seen in red cells incubated with Cr(VI) alone.

(d) *Effect of preincubation of intact red cells with N-ethylmaleimide on the inhi-*

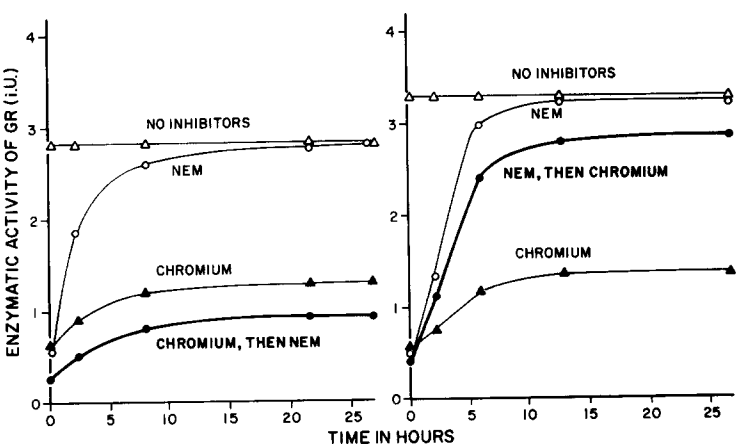

Fig 4. Effect of hexavalent chromium (Cr(VI)) on sulfhydryl groups of glutathione reductase (GR) (left half). See text for details of procedures. Red cells were incubated with Na_2CrO_4 (25μg/ml) and then further incubated with 5 mM *N*-ethylmaleimide (NEM). Some red cells were incubated only with Cr(VI), or only with *N*-ethylmaleimide. Hemolysates were incubated up to 27 h. Glutathione reductase activity in hemolysates was determined. Right half: Procedures for incubation of red cells were the same as mentioned above except for reversing the order of addition of inhibitors, that is, *N*-ethylmaleimide initially and then Cr(VI).

bition of glutathione reductase activity by Cr(VI). The same experiments, except for the reversal of the order of addition of *N*-ethylmaleimide and Cr(VI) were carried out in the presence of 8.7 mM NADPH.

A typical result is shown in the right half of Fig. 4. The reversibility of *N*-ethylmaleimide inhibition of the enzyme and the rather stable inhibition of glutathione reductase activity by Cr(VI) were observed as in the previous experiment. However, when red cells preincubated with *N*-ethylmaleimide were further incubated with Cr(VI), the inhibition of glutathione reductase activity was spontaneously reversible to nearly the same degree as that in red cells incubated with *N*-ethylmaleimide alone.

(4) Experiments with 2,5-dinitrobenzoic acid

(a) *Inhibition of glutathione reductase by 2,5-dinitrobenzoic acid and activation by FAD.* Red cells were incubated with 2,5-dinitrobenzoic acid (0, 1, 2 or 3 mM). After washing the 2,5-dinitrobenzoic acid-treated red cells, hemolysates were further incubated with the buffer containing 1 μM FAD. Glutathione reductase activity in the hemolysates was determined at 0 time, and after 12 h of incubation.

The results of typical experiments are shown in Fig. 5. Glutathione reductase activity (the active form) was inhibited according to various concentrations of the inhibitor. The maximum degree of glutathione reductase inhibition was approx. 50% in a concentration of 3 mM 2,5-dinitrobenzoic acid. After addition of FAD to the hemolysate, FAD activated glutathione reductase activity in red cells treated with the inhibitor. However, the amount of glutathione reductase activated by FAD in red cells treated with 2,5-dinitrobenzoic acid was inversely proportional to the con-

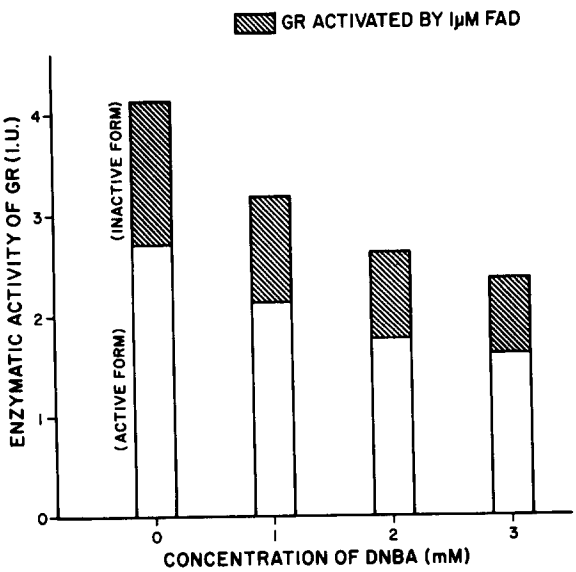

Fig. 5. Activation of glutathione reductase (GR) activity by FAD in red cells treated with 2,5-dinitrobenzoic acid (DNBA). Open columns represent the active form of glutathione reductase in red cells incubated with 2,5-dinitrobenzoic acid (0, 1, 2, and 3 mM). The shaded area shows the enzymatic activity activated by FAD.

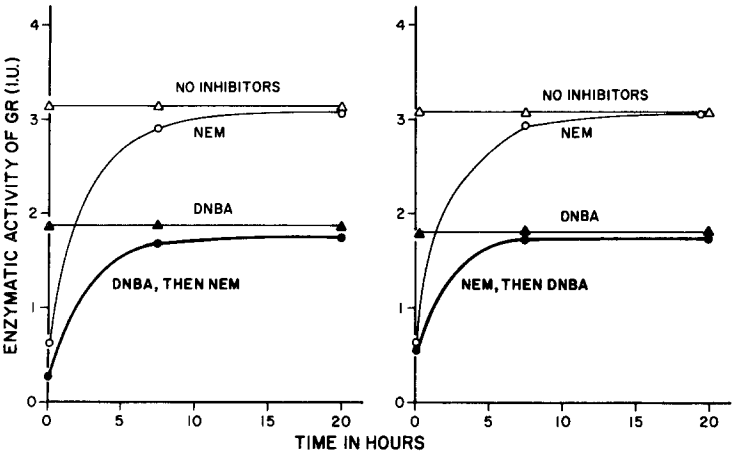

Fig. 6. Effect of 2,5-dinitrobenzoic acid (DNBA) on sulfhydryl group of glutathione reductase. See text for details of procedures. Left half: Red cells were incubated with 4 mM 2,5-dinitrobenzoic acid first, and then with 1 mM *N*-ethylmaleimide (NEM). Results are shown for red cells with *N*-ethylmaleimide alone, with 2,5-dinitrobenzoic acid alone, with 2,5-dinitrobenzoic acid and then *N*-ethylmaleimide, and without inhibitors. Right half: Red cells were incubated with *N*-ethylmaleimide first and then with 2,5-dinitrobenzoic acid.

centration of the inhibitor used. Thus, 2,5-dinitrobenzoic acid inhibited glutathione reductase activity in both the active and the inactive forms to a similar degree according to its concentration. FAD activated only that portion of glutathione reductase in the inactive form that was not affected by the inhibitor.

(b) *Effect of preincubation of glutathione reductase with 2,5-dinitrobenzoic acid or N-ethylmaleimide on the subsequent inhibition of the enzyme by N-ethylmaleimide or 2,5-dinitrobenzoic acid.* The same experiments, as described in procedures of Cr(VI) experiments, were performed, and glutathione reductase activity was determined in the following samples: (1) control, (2) the 2,5-dinitrobenzoic acid alone-treated, (3) the *N*-ethylmaleimide alone-treated, (4) the *N*-ethylmaleimide- and then 2,5-dinitrobenzoic acid-treated, and (5) the 2,5-dinitrobenzoic acid- and then *N*-ethylmaleimide-treated.

The effect of preincubation of glutathione reductase with 2,5-dinitrobenzoic acid on the inhibition of the enzyme by *N*-ethylmaleimide is shown in the left half of Fig. 6. Inhibition of glutathione reductase by *N*-ethylmaleimide was reversible, as shown previously. 2,5-Dinitrobenzoic acid inhibition of glutathione reductase was irreversible and stable. When glutathione reductase in red cells pretreated with 2,5-dinitrobenzoic acid was further incubated with *N*-ethylmaleimide, spontaneous reversibility was not observed except for the effect of *N*-ethylmaleimide. When glutathione reductase preincubated with *N*-ethylmaleimide was further incubated with 2,5-dinitrobenzoic acid, the inhibition of the enzyme was spontaneously reversible only to nearly the same level as that obtained in red cells treated with 2,5-dinitrobenzoic acid alone, as shown in the right half of Fig. 6.

DISCUSSION

A well-known inhibitor, *N*-ethylmaleimide, has been used extensively to study

the metabolic significance of sulfhydryl group(s) in biological materials. Concerning the inhibitory effect of *N*-ethylmaleimide on glutathione reductase activity, Mapson and Isherwood[3] have reported that the inhibition produced by *N*-ethylmaleimide, once established, was irreversible, for neither prolonged dialysis nor treatment with high concentrations of reduced or oxidized glutathione reversed it. Furthermore, Icen[7] concluded that alkylation of glutathione reductase protein by *N*-ethylmaleimide is irreversible and that glutathione reductase inhibited by *N*-ethylmaleimide could not be reactivated by British Anti-Lewisite, EDTA, reduced glutathione, L-cysteine, or 2-mercaptoethanol.

Results of the present experiments with *N*-ethylmaleimide indicate that, in the presence of NADPH, *N*-ethylmaleimide inhibited the sulfhydryl group(s) of the enzyme protein not only in the active form but also in the inactive form to the same extent. However, glutathione reductase in the active form inhibited by *N*-ethylmaleimide is spontaneously reversible to the original level. In this respect, it has been reported that *N*-ethylmaleimide activity depends on the presence of NADPH[3,7]. Thus, one should keep in mind an insufficient NADPH level to maintain *N*-ethylmaleimide inhibition, even on addition of NADPH to the incubation system. In the present results, however, the addition of FAD to the incubation mixture after the complete restoration of the active form of *N*-ethylmaleimide-treated glutathione reductase did not activate the inactive form which had been inhibited by *N*-ethylmaleimide initially. Therefore, *N*-ethylmaleimide inhibition of glutathione reductase should be related to the structural difference between the active and the inactive forms of glutathione reductase, rather than the effect of an insufficient amount of NADPH. Glutathione reductase activity in the inactive form in red cells incubated with *N*-ethylmaleimide was only partially activated by FAD; the degree of activation was inversely related to the concentration of *N*-ethylmaleimide used. Thus, *N*-ethylmaleimide appears to bind irreversibly to the sulfhydryl radicals of the apoenzyme not associated with FAD.

Recently, the reversibility of inhibition of reduced glutathione by *N*-ethylmaleimide has been reported by Beutler and his co-workers[14]. They suggested the formation of alkylating compounds of *N*-ethylmaleimide binding loosely to the sulfhydryl radical of reduced glutathione. Our data agree with these results but only in reference to the active form of glutathione reductase, and suggest that the mode of binding of *N*-ethylmaleimide to the inactive form of glutathione reductase might be different from that postulated by Beutler *et al.*[14]. If indeed it is the case that *N*-ethylmaleimide binds as loosely to the inactive form of the enzyme as to reduced glutathione, FAD should completely activate the inactive form, even that part inhibited by *N*-ethylmaleimide. In this regard, the possibility that the different mode of binding of *N*-ethylmaleimide to the enzyme protein of glutathione reductase might be related to its conformational change must also be kept in mind.

Concerning the mode of action of hexavalent chromium (Cr(VI)) in relation to the two forms of glutathione reductase, our data indicate that FAD did not activate the active form of glutathione reductase inhibited by Cr(VI), and that FAD activated only the inactive form of glutathione reductase in chromated red cells and in hemolysates and 'FAD-removed" preparations incubated with Cr(VI). The "FAD-removed" enzyme preparation of chromated red cells demonstrated only partial restoration of glutathione reductase activity even after incubation with FAD, in

contrast to the almost complete restoration of the activity in red cells not treated with Cr(VI). One may possibly explain this assuming that FAD could not be removed by acid treatment from glutathione reductase in the hemolysate of chromated red cells because Cr(VI) might be much more strongly bound to the enzyme protein–FAD complex. However, another possibility is that Cr(VI) may be bound simultaneously to the enzyme–FAD complex and the sulfhydryl radical(s) of the enzyme. Then, even if FAD were removed from the chromated glutathione reductase, Cr(VI) may still be bound to the sulfhydryl radical(s) of the "FAD-removed" glutathione reductase. Therefore, after incubation with FAD, the enzymatic activity of the "FAD-removed" glutathione reductase can not be restored. In this respect, Mertz[19] reported that chromium may react with sulfhydryl groups and facilitate or initiate the formation of new disulfide links. The assumption that Cr(VI) may bind to sulfhydryl radicals of glutathione reductase is supported by the results of our series of experiments. The sulfhydryl group of the enzyme was blocked by preincubation with *N*-ethylmaleimide. Then, the *N*-ethylmaleimide-treated glutathione reductase was further incubated with Cr(VI). The remarkable restoration of activity of the enzyme probably indicates that Cr(VI) was not bound to the enzyme, because sulfhydryl radicals of the enzyme were initially bound by *N*-ethylmaleimide. Thus, glutathione reductase activity was restored since the active form inhibited by *N*-ethylmaleimide is reversible spontaneously. On the other hand, there was only minimal restoration of the activity in red cells preincubated with Cr(VI) and then incubated with *N*-ethylmaleimide or incubated with Cr(VI) alone.

It may be interpreted that hexavalent chromium requires FAD and sulfhydryl group(s) at the same time for the inhibition of glutathione reductase In addition, there is another possibility that FAD may act as an allosteric effector in the conformational change of the enzyme with FAD, and that Cr(VI) can bind irreversibly to sulfhydryl group(s), possibly the active site of glutathione reductase conformed by association with FAD.

The mode of action of 2,5-dinitrobenzoic acid on glutathione reductase in partially purified enzyme preparations of rat red cells has been reported by Buzard and his co-workers[1]. They have described that the inhibition of glutathione reductase by 2,5-dinitrobenzoic acid does not appear to involve NADPH or sulfhydryl groups, and also that it is mixed or noncompetitive with respect to flavin adenine dinucleotide. Recently, Chan and his co-workers[16] have reported that 2,5-dinitrobenzoic acid is a fairly specific inhibitor of red cell glutathione reductase. However, the presence of the inactive form of the enzyme was not considered in these studies.

We have found that 2,5-dinitrobenzoic acid inhibits the active form and the inactive form of red cell glutathione reductase to a similar degree. Furthermore, the inhibition was not affected by the presence of *N*-ethylmaleimide. These results suggest that the inhibitor appears to act independently of FAD and sulfhydryl groups of the enzyme and to bind some other portion(s) of the enzyme.

In conclusion, the mechanism of inhibition of glutathione reductase by *N*-ethylmaleimide, Cr(VI) and 2,5-dinitrobenzoic acid is related to the two forms of the enzyme, with or without FAD. Speculations on the mechanism of action of these inhibitors must, of course, be cautious because the experiments in the present paper were performed on crude glutathione reductase preparations due to difficulties in purifying the inactive FAD-free form of glutathione reductase. However, in spite of

these limitations, these studies help to elucidate the relationship of the structure of glutathione reductase to its function, and may serve as a useful model for further investigations.

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