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Fast-reacting Thiols in Rat Hemoglobins Can Intercept Damaging Species in Erythrocytes More Efficiently Than Glutathione*

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The *S*-conjugation rates of the free-reacting thiols present on each component of rat hemoglobin with 5,5-dithio-bis(2,2-nitrobenzoic acid) (DTNB) have been studied under a variety of conditions. On the basis of their reactivity with DTNB (0.5 mM), three classes of thiols have been defined as follows: fast reacting (fHbSH), with $t_{1/2}$ <100 ms; slow reacting (sHbSH), with $t_{1/2}$ 30–50 s; and very slow reacting (vsHbSH), with $t_{1/2}$ 180–270 s. Under paraphysiological conditions, fHbSH (identified with Cys-125β(H3)) conjugates with DTNB 100 times faster than glutathione and ~4000 times more rapidly than (v)sHbSH (Cys-13α(A11) and Cys-93β(F9)). Such characteristics of fHbSH reactivity that are independent of the quaternary state of hemoglobin are mainly due to the following: (i) its low p*K* (~6.9, the cysteinyl anion being stabilized by a hydrogen bond with Ser-123β(H1)) and (ii) the large exposure to the solvent (as measured by analysis of a model of the molecular surface) and make these thiols the kinetically preferred groups in rat erythrocytes for *S*-conjugation. In addition, because of the high cellular concentration (8 mM, *i.e.* four times that of glutathione), fHbSHs are expected to intercept damaging species in erythrocytes more efficiently than glutathione, thus adding a new physiopathological role (direct involvement in cellular strategies of antioxidant defense) to cysteinyl residues in proteins.

Human but not rat erythrocytes are reported (1) to be able to restore the cellular pool of GSH, which is strongly decreased after treatment with diazenedicarboxylic acid bis(*N*,*N*-dimethylamide), a thiol-oxidizing agent known by the trivial name diamide (2). Such a difference in redox behavior was attributed to a lower enzymatic capacity in reducing disulfides of rat red cells relative to the human ones (3). However, recent work in this laboratory demonstrated that the reversibility of such process can be observed also in rat erythrocytes, depending on diamide dose (4). Additional evidence (3) may suggest that these differences in behavior between rat and human erythrocytes could be related to diversities in reactivity of sulfhydryl groups of the corresponding hemoglobins.

Studies of the reactivities of the sulfhydryl groups of oxygen carriers (5–8) are mainly restricted to hemoglobins containing only two accessible thiols per tetrameric molecule (such as human hemoglobin), located at the (F9)93 position of each β subunit. Many sulfhydryl reagents can be bound to Cys-93β(F9), even in intact red blood cells (8). Nevertheless, despite its high intracellular level (about half the concentration of the hemoglobin tetramer (9)), glutathione is not significantly combined with this protein-bound thiol under normal conditions. GSSG in fact is reported (5–8) (i) to react very slowly with human hemoglobin *in vitro* and (ii) to spontaneously form with this protein adduct *in vivo* only under very special conditions and in small amounts (2–7% of the total hemoglobin), having been observed to date solely in hemolysates of patients on long term anti-epileptic therapy (10). On the contrary, evidence exists that rat erythrocytes produce large quantities of glutathione-protein mixed disulfides under any oxidative stress (4, 11–13), most of them being adducts with hemoglobin thiols (HbSSG).[1] In particular, treatment of rat red cells with *t*-BOOH leads to GSH oxidation with formation of both GSSG and HbSSG, followed by a recovery of the initial values within 60 min; on the other hand, addition of diamide to the same system only causes an increase in HbSSG with no formation of GSSG (4).

All these facts, as a whole, prompted us to investigate further some structural and functional properties of rat hemoglobin thiols. Many hemoglobin components (6–10) have been separated from hemolysates of adult rats (14–16), and two types of α subunits and four types of β subunits have been identified (17). The primary structures reveal the presence of 3 cysteinyl residues on the major α chain (All(13), G11(104), and G18(111)) and up to 2 on the β chain (F9(93) common to all 4 β subunits, and H3(125) in 3 out of 4 β subunits) (15–19). In the present study, the reactivities of cysteinyl residues in rat hemoglobin components have been characterized, and the differences have been interpreted in terms of molecular structure. The reported results are consistent with a major role played, in the transient adaptation of rat erythrocyte to oxidative stress, by thiols that do not show conformational-associated changes in their microenvironment.

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[1] The abbreviations used are: HbSSG, glutathione-hemoglobin mixed disulfide; *t*-BOOH, *tert*-butylhydroperoxide; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); HbSSHb, hemoglobin-hemoglobin disulfides; HbSH, reactive sulfhydryl groups on hemoglobin; fHbSH, fast reacting sulfhydryl groups on rat hemoglobin components; vsHbSH, slow reacting sulfhydryl groups on rat hemoglobin components; vsHbSH, very slow reacting sulfhydryl groups on rat hemoglobin components; (v)sHbSH, very slow reacting and/or slow reacting sulfhydryl groups on rat hemoglobin components; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—Glutathione (reduced and oxidized forms), nicotinamide adenine dinucleotide phosphate (reduced form), and glutathione reductase (EC 1.6.4.2) were purchased from Boehringer Mannheim Italia (Milano, Italy). Glutathione transferase (EC 2.5.1.18), *tert*-butylhydroperoxide, dithiothreitol, diamide, iodoacetamide, 1-chloro-2,4-dinitrobenzene, 5,5-dithio-bis(2-nitrobenzoic acid), and guanidine hydrochloride (recrystallized from methanol) were Sigma products (Sigma Chimica, Milano, Italy). Iodo[2-^{14}C]acetamide was obtained from Amersham Pharmacia Biotech Italia (Milano, Italy); cyanogen bromide was from Fluka Chimica (Milano, Italy), and trypsin (code TRTPCK) was from Worthington. The liquid chromatography solvents, HPLC-grade, were purchased from Carlo Erba Reagenti (Milano, Italy), and the sequence-grade chemicals were from Perkin Elmer Italia (Monza, Milano, Italy). All other reagents were of analytical grade purity.

Animals—Blood was collected from the abdominal aorta of male rats (Sprague-Dawley strain) fed *ad libitum*, anesthesized with chloral hydrate; Na_2EDTA was used to prevent blood clotting.

Erythrocyte Counts and Indices—Total number of erythrocytes per unit blood volume, average volume of red cells, and hemoglobin content per average red cell were determined by an Abbot Cell-dyn 300 apparatus.

GSH, GSSG, and HbSSG Determinations—Samples were deproteinized by addition of 4 volumes of a solution containing 6.25% (w/v) trichloroacetic acid. After centrifugation (3 min at 14,000 rpm), GSH and GSSG were determined in the clear supernatant. GSH was measured by either an enzymatic (at pH 7.0) or colorimetric (at pH 7.4) assay (20, 21). In the first case, sample aliquots and 1-chloro-2,4-dinitrobenzene (0.2 mM final concentration) were added to the cuvette containing 0.1 M phosphate buffer, pH 7.0, and the reaction was started with 0.25 units of glutathione transferase (final concentration, one enzymatic unit conjugates 1.0 μmol of 1-chloro-2,4-dinitrobenzene with reduced glutathione per min at pH 6.5 at 25 °C) (readings at 340 nm, ϵ = 9.6 mM^{-1} cm^{-1}; end of reaction at 4–6 min). Colorimetric determination of GSH was performed by Ellman's method, slightly modified (22), adding DTNB as the last reagent (0.1 mM final concentration, end of reaction at 2 min). GSSG was assayed enzymatically by the procedure of Di Simplicio *et al.* (21). To determine HbSSG, acid-precipitated proteins were thoroughly washed with the precipitating solution until no trace of soluble GSH or GSSG was detected. Alkaline pH (7.5 for rat samples; 12.0 for human samples) was then restored by adding 0.1 M phosphate buffer, pH 7.8, or an appropriate amount of 0.125 N NaOH. The pellets were resuspended by stirring with a glass rod. Under these conditions, GSH is released via an SH/SS exchange reaction as described previously by Rossi *et al.* (22). The amount of GSH released was then determined enzymatically in the supernatant (see above).

Hemoglobin Preparation—Hemoglobin was prepared from stroma-free hemolysate by crystallization as described by Condò *et al.* (23). Crystals were then dissolved in 25 mM Tris acetate buffer, pH 8.8, and stored under nitrogen. The iron oxidation state was checked spectrophotometrically on the basis of the absorbance ratio at 576/541 nm, taking 1.14 as normal value. In all experiments, the concentration of the ferric derivative was monitored as reported by Giardina and Amiconi (24).

Purification of Rat Hemoglobin Components—Isolation and purification of the rat hemoglobin components were carried out by chromatofocusing on a MonoP column (0.5 × 20 cm) (Amersham Pharmacia Biotech, Sweden). Hemoglobin solution, dialyzed against 0.025 M Tris acetate buffer at pH 8.3, was applied to the column equilibrated with the same buffer. Elution was achieved at 4 °C by application of a Polybuffer 96 exchanger (1 ml/min) previously adjusted at pH 6.5 with 1 M acetic acid. Nine main peaks (A to I) were resolved. Homogeneity of the fractions was checked by polyacrylamide gel electrophoresis analysis. In general, components C, E, and F were the main ones. In particular, the relative abundance of the hemoglobin components was as follows: isoform A, 0.03–0.05; isoform B, 0.025–0.035; isoform C, 0.08–0.12; isoform D, 0.02–0.03; isoform E, 0.22–0.28; isoform F, 0.43–0.51; isoform G, 0.012–0.022; isoform H, 0.018–0.028; and isoform I, 0.02–0.05.

Hemoglobin Thiol Titration—Exposed sulfhydryl groups (HbSH) of hemoglobin were assayed by a modification of Ellman's method (22) (conditions, 0.1 M phosphate buffer pH 7.4; DTNB, 0.2 mM final concentration). Monitoring traces were recorded by a stopped-flow apparatus (Applied Photophysics, UK) at room temperature and read at 450 nm; at this wavelength the HbSH concentration was calculated taking ϵ = 7.0 mM^{-1} cm^{-1}.

Cysteine Labeling—In a typical experiment, a purified rat hemoglobin component, *e.g.* isoform F (1 mg, *i.e.* 15 nmol in tetramer or 90 nmol in free-reacting thiols) in 0.4 ml of 0.1 M Tris-HCl, pH 8.0, was incubated for 5 min with 25 nmol of iodo[2-^{14}C]acetamide plus 5 nmol of cold iodoacetamide. The sample was then divided into two 0.2-ml aliquots as follows: to the first aliquot was added 100 mg of guanidine and 100 μg of dithiothreitol and after 30 min 5 μmol of iodoacetamide; the sample was left for 0.5 h at room temperature and then dialyzed against a large volume of deionized water. The second aliquot was treated in the same way, but with the addition of 10 nmol of iodo[2-^{14}C]acetamide in order to label all free-reacting cysteines. After lyophilization, the two samples were separately digested each with 10 μg of trypsin, and the peptide mixtures were fractionated by HPLC using a Beckman System Gold chromatographer, on a macroporous reversed-phase column (Aquapore RP-300, 4.6 × 250 mm (Perkin-Elmer, Rome, Italy)), eluted with a 50-min linear gradient from 0 to 50% acetonitrile/isopropyl alcohol (4:1) in 0.2% (by volume) trifluoroacetic acid, at a flow rate of 1 ml/min. Elution of the peptides was monitored by a diode array detector (Beckman model 168) at 200 and 280 nm. Peaks were collected manually, and 10% aliquots were counted for radioactivity. Radiolabeled peptides were subjected to automated Edman degradation on an Applied Biosystems 476A protein sequencer. Samples (0.1–0.5 nmol) were loaded onto polyvinylidene difluoride membranes, coated with 2 μl of Polybrene (100 mg/ml, 50% methanol), and run on the sequencer with a Blott cartridge using an optimized gas phase fast program. In parallel, the very same hemoglobin component (about 8 nmol in tetramer or 48 nmol in free-reacting thiols) was also incubated in 0.1 M Tris-HCl, pH 8.0, with 5 nmol of iodo[2-^{14}C]acetamide for 30 s in a syringe. Then the mixture was applied to a Sep-Pak cartridge (Millipore, Milford, MA), washed with 4 ml of water, 4 ml of 0.2% (by volume) trifluoroacetic acid, and eluted with 2 ml of acetonitrile/isopropyl alcohol (4:1) in 0.2% trifluoroacetic acid. After lyophilization, the hemoglobin chains were separated (see below), and a 10% aliquot was counted for radioactivity. Cyanogen bromide cleavage was obtained by adding few crystals of the reagent to 20 μg of β chain dissolved in 70% formic acid.

Separation and Sequence Analysis of the Hemoglobin Chains—Hemoglobin chains were separated by HPLC under the conditions described above (see "Cysteine Labeling"). The reversed phase column was eluted with a 40-min linear gradient from 30 to 50% of the same solvent systems as above. Amino acid composition, sequence analysis, and peptide maps of chains from the various hemoglobin components were performed as described previously (25).

Electrophoretic Search for Hemoglobin Polymerization through Disulfides (HbSSHb)—Rat erythrocytes were separated by centrifugation of the whole blood at 2,800 rpm for 10 min. The plasma and buffy coat were removed, and then the red cells were washed three times with 10 times their volume of isotonic phosphate-buffered saline, pH 7.4, supplemented with 10 mM D-glucose. After removal of the supernatant from the final wash, erythrocytes (2 ml, 40% v/v suspension in the washing solution) were incubated with 2 mM (final concentration) diamide or *t*-BOOH. A 200-μl aliquot of cell suspension was hemolyzed by adding 50 volumes of 10 mM phosphate, pH 7.4, at various times (5, 15, and 30 min). After centrifugation (12,000 × *g* for 15 min) the supernatant was diluted 1:1 and subjected to analysis by SDS (0.1% (w/v)) polyacrylamide (12%) gel electrophoresis, according to the method of Laemmli (26), using a sample buffer without β-mercaptoethanol and in the presence of 2 mM *N*-ethylmaleimide. Chemicals for gel electrophoresis were from Bio-Rad (Segrate, MI, Italy). A Bio-Rad model 220 (Bio-Rad) slab gel apparatus was used.

Kinetic Experiments—Samples of hemoglobin (0.1–0.3 mg/ml) in 0.2 mM Tris acetate buffer, pH 8.6, were rapidly mixed with equal volumes of a solution containing 1 mM DTNB in 0.2 M buffers (sodium acetate, pH 5.0–6.0, sodium/potassium phosphate, pH 6.0–8.0, and Tris acetate, pH 8.0–9.0), by means of an Applied Photophysics MV 17 stopped-flow apparatus: tracings were recorded at 450 nm. Data were fitted to two (*e.g.* isoform B) or three (isoforms D, E, and F) exponentials, imposing the same amplitude for each kinetic process. The kinetics of carbon monoxide binding to hemoglobin isoforms with free and glutathione-blocked sulfhydryl groups were measured with the same stopped-flow apparatus. Photolysis experiments were carried out using an instrument developed in our laboratory. Briefly, the 5-ns pulse of a Q-switched Quanta Systems Nd-YAG HYL101 laser equipped with a frequency doubler (maximal output, 200 mJ at 532 nm) was focused onto a 4-face fluorescence cuvette containing the hemoglobin component equilibrated with 1 atm of carbon monoxide in the gas phase; oxygen was removed by addition of a few grains of sodium dithionite. The transmittance of the sample was probed continuously by a 300-watt lamp as light source, a Spex Minimate monochromator, and a Hamamatsu R1398 photomultiplier tube. The light beam for observa-

TABLE I
Kinetics of formation of oxidized glutathione (GSSG) and mixed disulfides (HbSSG) after addition of diamide (2 mM, final concentration) to 2-ml blood sample (hematocrit = 0.4) from rat or from man at room temperature

Values are expressed in nmol/ml.

Time	Rat erythrocytes			Human erythrocytes		
	GSH	GSSG	HbSSG	GSH	GSSG	HbSSG
min						
0	740	66	150	950	55	81
1	0	54	842	0	550	69
2	0	53	843	0	527	70
3	0	52	882	0	531	71
15	0	42	819	0	520	74
30	22	37	837	62	507	80
60	35	43	855	389	375	81
120	32	45	836	791	125	85

tion was oriented at 90° from the laser beam. The current generated by the photomultiplier was displayed with a Tektronix TDS 360 digital oscilloscope triggered by the laser Q-switch and output as a MS-DOS ASCII file. In a typical experiment, the optical changes caused by 32–128 laser shots were averaged, in order to improve the signal to noise ratio. The energy of the laser pulse was usually kept to 20 mJ or less, and the degree of photolysis ranged between 50 and 10%. The standard deviation for replicate determinations of rate constants is approximately 30 to 60% of the determined value, independently of pH and hemoglobin species.

Oxygen Binding Equilibria—Values of $p_{½}$ (the oxygen partial pressure required to half-saturate the hemes) and n (the empirical Hill coefficient, a measure of the cooperativity among the hemes) for oxygen association to hemoglobin were determined at 20 °C from light absorbance changes accompanying the oxygen binding by a tonometric method (24).

Measurement of Glucose-6P-Dehydrogenase (EC 1.1.1.49) and Glutathione Reductase Activities—Enzymatic determinations were carried out at room temperature on hemolysate, previously passed through a Sephadex G25 column, according to the standard methods (27). Catalytic parameters were obtained by fitting experimental data to the Michaelis-Menten equation by Sigmaplot software (Jandel Scientific, version 2.01).

Prediction of Three-dimensional Structure of Isoform F—The three-dimensional models of the rat isoform F subunits were based on the structural template of human ligated and unligated hemoglobin, whose atomic coordinates were recovered from the protein (crystallographic) data distribution tape (Protein Data Base, codes 1hho and 3hhb, respectively; see Ref. 28). The models were built with the HOMOLOGY and DISCOVER modules in the package Insight II (MSI, Inc.) running on a Silicon Graphics Indigo 2 Extreme Graphics. Conformation of the substituted side chains was manually adjusted to remove steric strains, and the model was finally refined with 100 cycles of energy minimization. Stereochemical properties, potential H bonds, solvent accessibility surface, and secondary structures were calculated with the programs PROCHECK (29), HBPLUS (30), NACCESS (31), and DSSP (32), respectively. Drawing were produced with MIDAS (33) and MOLSCRIPT (34).

RESULTS

Effects of Diamide Treatment on Intraerythrocyte Thiols—Treatment of human and rat blood with diamide evidenced remarkable differences in the network of intraerythrocyte metabolic correlations (see Table I). In particular, HbSSG, but not GSSG, was produced after drug addition to rat erythrocytes; on the other hand, only GSSG, but not HbSSG, concentration increase occurred in human erythrocytes after the same treatment. Moreover, the latter cells, but not rat erythrocytes, were able to restore (within 2 h incubation at 37 °C) control values of GSH and HbSSG (see Table I).

Even though HbSSHb might form in rat erythrocytes, negligible or no association of hemoglobin tetramers through disulfide bridges occurred under the experimental conditions chosen, at least as measured (4) by gel electrophoresis experiments (see Fig. 1); on the contrary, abnormal human hemoglobin Porto Alegre (Ser-9β(A6) → Cys) spontaneously forms disulfide polymers that can already be detected after 3 days of shelf storage (35). Therefore, it appeared interesting to investigate the molecular bases that limit the polymerization of rat hemoglobin. The formation of disulfides from thiol groups only requires the presence of an electron acceptor, such as oxygen; the chemical nature of bond formation (possibly depending on metal ions that transiently bind to the thiols (36)), however, is not completely understood, even though sulfenic acid as electrophilic intermediate seems to occur. Since (i) it is impossible to measure the reduction potential (E'_0) value of the hypothetical disulfide bond in HbSSHb; (ii) E'_0 values differ greatly for these bonds, even if they are in the same sequence motif (37–39), and (iii) disulfide bonds exist in a cell only if the overall reduction potential value in the cytosol allows (40); the redox buffer capacity of rat erythrocytes was set to zero. Thus, rat blood was incubated with diamide at a concentration so high to generate HbSSG irreversibly (see *e.g.* Table I); this event was taken as an index of complete consumption of GSH (the chemical species that usually restores the control values of HbSH and GSSG). Because HbSSHb was not observed in rat erythrocytes even under the strongest (5 mM diamide) oxidative stress, the factors that prevent its formation were considered not strictly chemical. Fig. 2 reports computer-generated models of complexes between two α/β dimers of hemoglobin Porto Alegre (*panel A*) and of rat isoform F (*panel B*), oriented in such a way to present their potentially reactive thiols (Cys-125β(H3) and Cys-9β(A6), respectively) at the least distance compatible with steric hindrance. These thiols can approach each other 2.5 Å in the abnormal human protein and no less than 4 Å in rat hemoglobin. This model-based evidence suggests that the absence of polymerization into rat erythrocytes treated with oxidants could mainly be a result of steric factors.

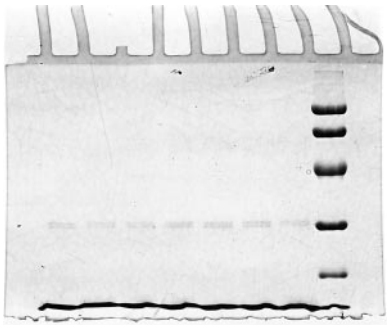

FIG. 1. **Electrophoretic pattern of rat hemolysate from treated red blood cells.** Rat erythrocytes (40% (v/v)) in isotonic phosphate-buffered saline, pH 7, supplemented with 10 mM D-glucose, were treated with 2 mM (final concentration) diamide or *t*-BOOH for different times at room temperature. Samples were then hemolyzed and treated with Laemmli's solution without mercaptoethanol and in the presence of 2 mM *N*-ethylmaleimide. *Lanes* (from *left* to *right*): *lane 1*, hemolysate in 2 mM diamide, 5-min incubation; *lane 2*, hemolysate in 2 mM diamide, 15 min; *lane 3*, hemolysate in 2 mM diamide, 30 min; *lane 4*, hemolysate in 2 mM *t*-BOOH, 5 min; *lane 5*, hemolysate in 2 mM *t*-BOOH, 15 min; *lane 6*, hemolysate in 2 mM *t*-BOOH, 30 min; *lane 7*, control hemolysate; *lane 8*, molecular mass standards (from *top* to *bottom*: 94, 67, 43, 30, 20.1, 14.4 kDa).

S-Thiolation of Hemoglobin Components in Rat Erythrocytes Treated with Oxidants—Titratable sulfhydryl groups in proteins react with DTNB with different rate constants, depending on local environmental factors (41, 42). The time course of DTNB combination with sulfhydryl groups of rat hemoglobin components (see below) was described by either two or three pseudo first-order processes of equal amplitude, whose rate constants fell within one of the three classes of thiols defined as follows in kinetic terms (at a DTNB concentration of 0.5 mM): fast reacting HbSH (fHbSH) with a $t_{½}$ value <100 ms; slow

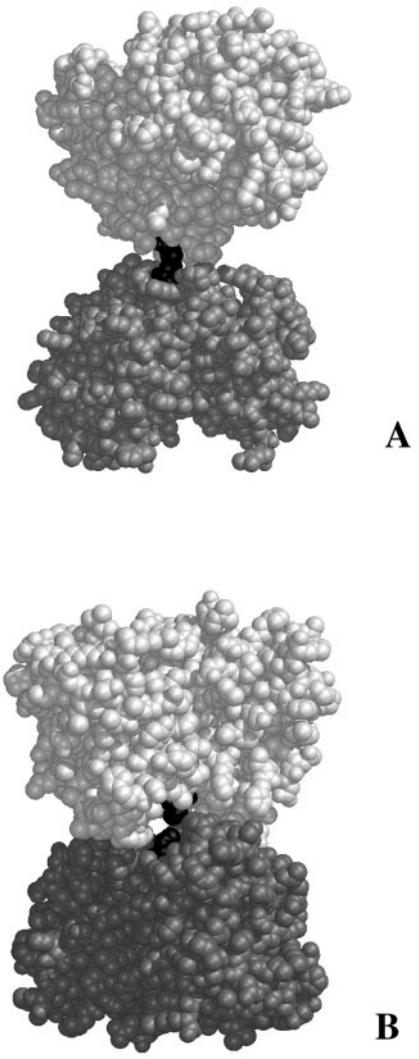

FIG. 2. **Space-filling models of two α/β dimers of human hemoglobin mutant Porto Alegre (Ser-9β(A6) → Cys) (*panel A*) and isoform F of rat hemoglobin (*panel B*), which were manually docked to present one in front of the other potentially reactive thiols (Cys-125β(H3) and Cys-9β(A6), respectively).** In both cases, the two dimers are colored with *light* and *dark gray*, and cysteines with *black*.

reacting HbSH (sHbSH) with $t_{1/2}$ = 30–50 s; and very slowly reacting HbSH (vsHbSH) with $t_{1/2}$ = 180–270 s; on the other hand, thiols in human hemoglobin (Cys-93β(F9)) reacted homogeneously with a $t_{1/2}$ value of about 30 s, a value corresponding closely to sHbSH of the rat protein. Since all kinetic processes described above induced similar or identical optical changes and were second-order with respect to DTNB concentration, they were assigned to the reaction of a couple of cysteinyl residues per tetramer; accordingly, the end point of the thiol reaction traces allowed the estimation of 4 or 6 titratable Cys/tetramer on rat hemoglobin (depending on the component, see below) whereas, as expected, only two were calculated on the human protein.

Nine main peaks (A to I) were resolved by chromatofocusing between pH 8.3 and pH 6.5 (Fig. 3, *panel A*) of rat hemoglobin from blood of control animals. All peaks (see Table II) showed two slow and two very slow reacting thiols per tetramer; however, isoforms D–G also contained fast reacting thiols as follows: two per tetramer were present in D–F isoforms, whereas in the components G one fHbSH/tetramer and one HbSSG were determined. The latter result was in line with the evidence that after treatment of rat hemolysate with dithiothreitol the peak corresponding to the G component disappeared and peak F increased (Fig. 3, *panel B*) (meaning that isoform G corresponded to the component F with one thiol blocked by glutathione, this reactant being identified by colorimetric and HPLC procedure (22)).

The ability of some HbSH to produce HbSSG after diamide or *t*-BOOH treatment was analyzed in more detail using both erythrocytes and purified hemoglobins (human and rat). In the presence of GSH, diamide was reported (3, 4) to form mixed disulfides with hemoglobin via a two-step reaction, *i.e.* (HbSH + diamide → HbS-diamide) followed by (HbS-diamide + GSH → HbSSG + diamide (reduced form)). As shown (see Figs. 3, *panels C* and *D*, 4, *panel A*, and 5, *panel A*), under these conditions only fHbSH of rat hemoglobin was able to produce HbSSG. On the other hand, treatment with *t*-BOOH formed GSSG that in turn produced mixed disulfides through a thiol/disulfide exchange (3, 4), according to the following scheme: HbSH + GSSG → HbSSG + GSH. As shown in Figs. 4, *panel B* and 5, *panel B*, both HbSSG production and HbSH depletion were evident only with rat hemoglobin. All hemoglobin isoforms were separated by chromatofocusing after *t*-BOOH (not shown) or diamide treatment of rat hemolysate (Fig. 3). Similar results were obtained with erythrocytes. With both oxidants a marked increase in peak G area, a decrease in concentration of isoforms D–F, and no changes in peak areas of A–C, H, and I components were found. Furthermore, in both cases HbSSG formation determined the appearance of new peaks retarded with respect to the original components (in particular, see Fig. 3, *panel C*), since the glutathione-hemoglobin adduct is more acidic relative to the unreacted protein. In summary, thiol titration with DTNB demonstrated that (i) fast reacting HbSH (present only in rat hemoglobin) was depleted after oxidant addition, and (ii) neither rat slow reacting HbSH nor human HbSH were grossly affected by the treatment with diamide. These observations, as a whole, indicate that only isoforms of rat hemoglobin with fHbSH are able to produce disulfides either via thiol/disulfide exchange (after *t*-BOOH treatment) or via diamide intermediate (after diamide addition).

Identification of Fast Reacting Cysteinyl Residue—All cysteine-containing tryptic peptides were identified in the samples radiolabeled under denaturating conditions, by sequence analysis. After treatment with iodo[2-^{14}C]acetamide and addition of cold iodoacetamide, the identification of the radiolabeled peptides was made on the basis of their chromatographic behavior. The specific radioactivity of each peptide indicated that Cys-93β(F9) and Cys-125β(H3) reacted faster than Cys-13α(A11).

In order to discriminate the fastest reacting sulfhydryl group between Cys-93β(F9) and Cys-125β(H3), a second labeling experiment was performed using a lower reagent/thiol ratio and a shorter incubation time. Radioactivity measurements of the anilinothiazolinone derivatives collected at each cycle of automated Edman degradation of a sample of labeled β chain cleaved at methionines with CNBr revealed that, under these conditions, only in the test tube corresponding to the derivatives of Cys-125β(H3) (cycle 16) was there a significant increase in radioactivity.

Next, the samples that did not contain a fast reacting cysteinyl residue (such as isoforms A–C) were treated with 4-vinylpyridine under denaturating conditions, then cleaved with CNBr, and lastly subjected to sequence analysis. The phenylthiohydantoin derivatives of the fragments expected from CNBr cleavage were clearly identified during the automated Edman degradation of the peptide mixture. In cycle 16, corresponding to position β125 of the pertinent CNBr fragment, no pyridylethylcysteinyl derivative was detected, but a serine residue was present, as expected on the basis of sequences (17).

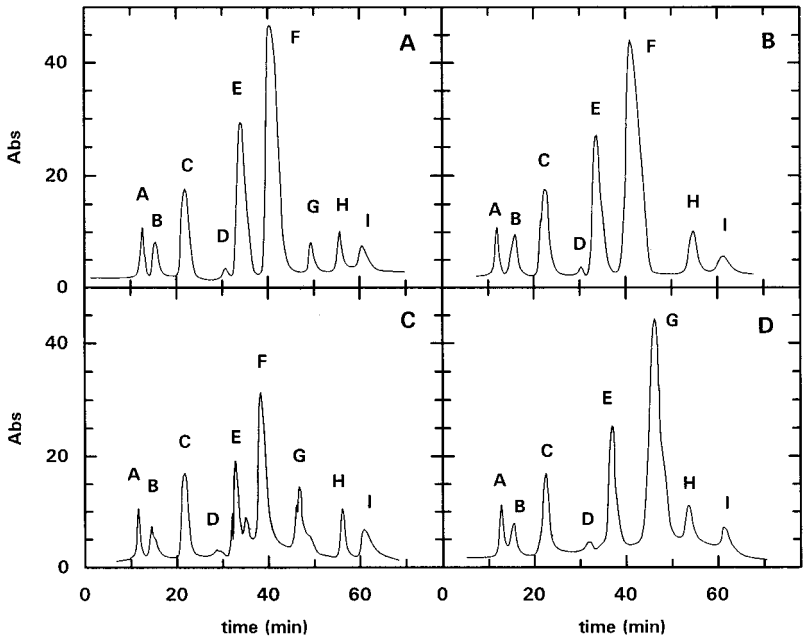

FIG. 3. **Elution profiles (between pH 8.3 and 6.5) of rat hemolysate, *i.e.* total hemoglobin components.** *Panel A*, control hemolysate (4 mg/ml); *panel B*, hemolysate treated with 1 mM dithiothreitol; *panel C*, hemolysate (4 mg/ml) treated with 30 μM diamide plus 40 μM GSH; *panel D*, hemolysate (4 mg/ml) treated with 175 μM diamide plus 200 μM GSH.

TABLE II
Association rate constants of DTNB to the oxyderivative of various isoforms of rat hemoglobin and human hemoglobin in 0.1 M phosphate buffer, pH 7

Experimental conditions in the reaction vessel are as follows: [Hb] = 6.8 μM in heme; [DTNB] = 0.34 mM; 20 °C. k_f, k_s, and k_{vs} refer, respectively, to second-order rate constants with fast reacting, slow reacting, and very slow reacting thiols of the investigated isoform; F_{G2} symbolizes the component F with the two fast reacting sulfydryls blocked by glutathione; F* indicates the component G after treatment with dithiothreitol.

Protein	k_f	k_s	k_{vs}
	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$
Rat hemoglobin			
A[a]		37.6	6.72
B		38.2	6.68
C		36.3	6.81
D	2.81×10^4	35.3	6.76
E	2.81×10^4	36.4	6.47
F	2.94×10^4	38.2	7.06
F_{G2}		38.7	6.86
F*	2.87×10^4	37.7	6.86
G	2.85×10^4	38.1	6.99
H		36.9	6.81
Human hemoglobin		30.2	

[a] Isoform A, for example.

Therefore, the *S*-thiolation rate of rat HbSH increases as follows: Cys-13α(A11) < Cys-93β(F9) < Cys-125β(H3).

Functional Properties of Rat Hemoglobin Isoforms—It was shown (41, 42) that the thiolate anion form of the sulfhydryl group preferentially reacts with DTNB. Therefore, the reactivity of thiols at each pH will depend on the fractional population of the thiolate anion.

The fast reacting sulfhydryls in rat oxyhemoglobin showed an ample effect of pH, such that the rate constant observed at pH 8 was more than five times higher than that observed at pH 6. The pH dependence of the observed thiolation rate constant (k) of Cys-125β(H3) can be given by Equation 1.

$$k = \frac{K \cdot k_{s^-}}{[H^+] + K} + \frac{[H^+] \cdot k_{SH}}{[H^+] + K} \qquad \text{(Eq. 1)}$$

where K represents the acid dissociation constant for the thiol group, and k_{s^-} and k_{SH} indicate the rate constants for the reaction of DTNB with the thiolate anion and the protonated sulfhydryl, respectively. Allowing for experimental error (±10%), values of the rate constants for the dissociated ($3.9 \times 10^4\ M^{-1}\ s^{-1}$) and protonated (practically zero) fHbSH can be calculated from Equation 1. The undissociated Cys-125β(H3) therefore makes no significant contribution to the observed rate.

The deprotonation profile of fHbSH is representative of only one ionization (see Fig. 6, *panel B*); this finding precludes the possibility of important contributions to the reactivity from other ionizable groups on the protein, because this would have appeared as an additional inflection point in the pH profile (as observed, for instance, in dog hemoglobin (43)). This conclusion was reinforced by the three-dimensional model of rat isoform F, which does not predict the formation of any salt bridge that could restrict access to Cys-125β(H3).

A pH dependence similar to that of fHbSH was also monitored for reduced glutathione (Fig. 6, *panel A*), whose reactivity is known not to be influenced by electrical charges on the molecule (41, 42). Since fHbSH had an apparent pK equal to 6.89 (see Fig. 6, *panel B*), it is not surprising that it reacted faster than glutathione whose higher pK (equal to 8.9, under the very same conditions; see Fig. 6, *panel A*) should generate a much smaller fractional population of anionic form of thiols at every pH value between 6 and 8. Moreover, since under intraerythrocyte conditions (pH ~7.2) fHbSH is in the anionic form, *i.e.* more nucleophilic and hence more reactive, for ~60% (see Fig. 6, *panel B*), it conjugates with electrophilic compounds much faster than (v)sHbSH which is dissociated for only a few percent (see Fig. 6, *panels C* and *D*). In particular, around neutrality the rate constant of (v)sHbSH with DTNB was approximately lower by a factor of 5–10 than that of sHbSH and 4000-fold lower than that of fHbSH (Table II). These considerations apply to glutathione too; in fact, at pH 7.2 the value of *S*-thiolation rate constant, as calculated from Equation 1, for fHbSH ($2.7 \times 10^4\ M^{-1}\ s^{-1}$) is 1 order of magnitude higher than that for glutathione ($2.6 \times 10^3\ M^{-1}\ s^{-1}$).

Such large differences in rate constants among the three kinds of hemoglobin thiols suggest that, in addition to the ionization state, accessibility could make a contribution to the observed reaction rate (see below). By analogy with older observations (36, 44, 45), these reactive thiols were assumed to correspond to partially buried cysteinyl residues. The ratio

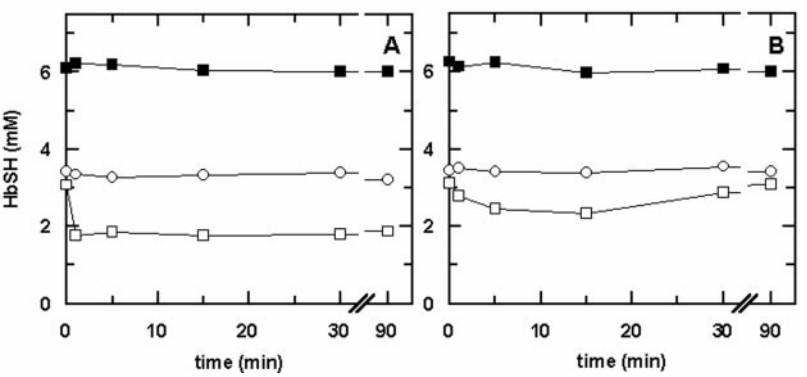

FIG. 4. **Comparison of time courses relative to HbSH level changes in rat (■, □) and human (○) blood (hematocrit, 0.4) incubated with 2 mM diamide (*panel A*) or 2 mM *t*-BOOH (*panel B*) at room temperature.** After treatment, erythrocytes were washed with isotonic saline and hemolyzed; then the solution was passed through a Sephadex G25 column and HbSH determined. ■, rat (v)sHbSH; ○, human HbSH; □, rat fHbSH.

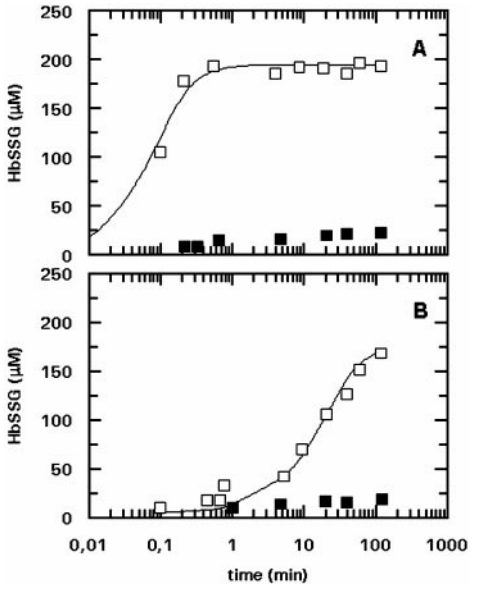

FIG. 5. **Comparison of time courses relative to HbSSG concentration changes in rat (□) and human (■) hemoglobin (0.5 mM in heme, in 0.1 M phosphate, pH 7.0), incubated at room temperature with 1 mM diamide plus 1 mM GSH (*panel A*) or with 1 mM GSSG (*panel B*).**

between the rate constants reported in Table II strongly suggests that the slow and very slow reacting thiols should have a similar reactive nature (*i.e.* they should be similarly embedded into the protein molecule and similarly protonated), whereas the fast reacting thiols are expected to stand out as a peculiar feature of some rat hemoglobin isoforms and cannot easily and simply be interpreted as less buried (see below).

As a further step of this analysis, the rate constants of DTNB combination with the deoxyhemoglobin derivative were measured. It is well known that the reactivity of the only titratable couple of thiols in human tetrameric hemoglobin (Cys-93β(F9)) depends on the quaternary conformation, being slower in the deoxy state (44, 45). Since DTNB is reduced by dithionite, studying its reaction with unligated hemoglobin demands that oxygen be removed by evacuation and equilibration with nitrogen. However, under these conditions some contamination with oxygen occurs frequently, and therefore a systematic check for the presence of the oxygenated derivative was done by recording the optical change induced by mixing with dithionite; the amount of oxyhemoglobin in the samples never did exceed 10%. Under these conditions small deviations from the expected two or three exponentials were observed; nevertheless, the data unequivocally showed that fast thiols were insensitive to the ligation state of the heme iron and the allosteric conformation of the protein (*e.g.* at pH 7, k for fHbSH thiolation of rat isoform F was 2.94×10^4 M^{-1} s^{-1} and 2.91×10^4 M^{-1} s^{-1} for oxy and deoxy derivatives, respectively; see also Table II).

A necessary complement to the results described so far was the analysis of the functional properties of rat hemoglobin isoforms in the absence and in the presence of reagents (glutathione or DTNB) bound to fHbSH. The oxygen binding isotherm of rat isoform F (2 fHbSH/tetramer) was demonstrated to be superimposable to that of the same hemoglobin component with the fHbSH blocked by glutathione (isoform F_{G2} in Table III; see also Fig. 7). Moreover, the overall rates of carbon monoxide combination with T state (as measured by stopped flow) and that with R state (by partial photolysis) of rat isoform F were insensitive to the presence of glutathione bound to fHbSH (Table III). These results unequivocally demonstrate that, in rat isoform F, fHbSH is not functionally linked to the hemes nor does it alter the ligand affinity at hemes.

Structural Bases of the High Reactivity of fHbSH Relative to (v)sHbSH—It is generally accepted that the major factors influencing pK values in protein ionizable groups are the interactions with other net charges or dipoles present on the macromolecule surface (46). Therefore, the physical nature of the potential interactions stabilizing the thiolate anion of fHbSH was investigated by molecular graphics. A positively charged group in the proximity of Cys-125β(H3) would be the most logical candidate, but none was apparent on the model of the oxy as well as deoxy derivative built on the structural template of human hemoglobin. However, in the model (see Fig. 8, *panel A*) the anion of Cys-125β(H3) is predicted to form a hydrogen bond with Ser-123β(H1) (2.9 Å distance between sulfur and oxygen atoms in both oxy and deoxy derivative), which therefore appears to be the most likely candidate to explain the low pK value of fHbSH. This interaction in fact can substantially contribute to the charge stabilization of the thiolate form in fHbSH. In its turn, Fig. 8, *panel B*, helps to make it clear why Cys-13α(A11) is the least reactive among the free cysteinyl residues in rat hemoglobins. In fact, this thiol is presumably H-bonded with His-113α(GH1) of the same chain (see Fig. 8, *panel B*), the distance between sulfur and nitrogen atoms being 3.5 and 4.1 Å in the deoxy and oxy form, respectively. Such an interaction is expected to stabilize the protonated form of Cys-13α(A11) and accordingly to decrease its reactivity at neutral pH in comparison to Cys-93β(F9), which is not involved in any interaction with net charges or polar groups on the protein surface. As a whole, these structural data are consistent with, and help to explain in rational terms, the functional behavior of the three classes of thiols.

Additional factors are expected to modulate rat HbSH reactivity toward DTNB. Even though local conformational fluctuations are more useful for the interpretation of chemical data, it is interesting that static-accessible surface to solvent (47) shows a parallel trend to the thiolate fraction of each HbSH class. Thus, Cys-125β(H3) (70% static accessibility) is 10 to 15 times more exposed to the solvent than Cys-93β(F9) and of

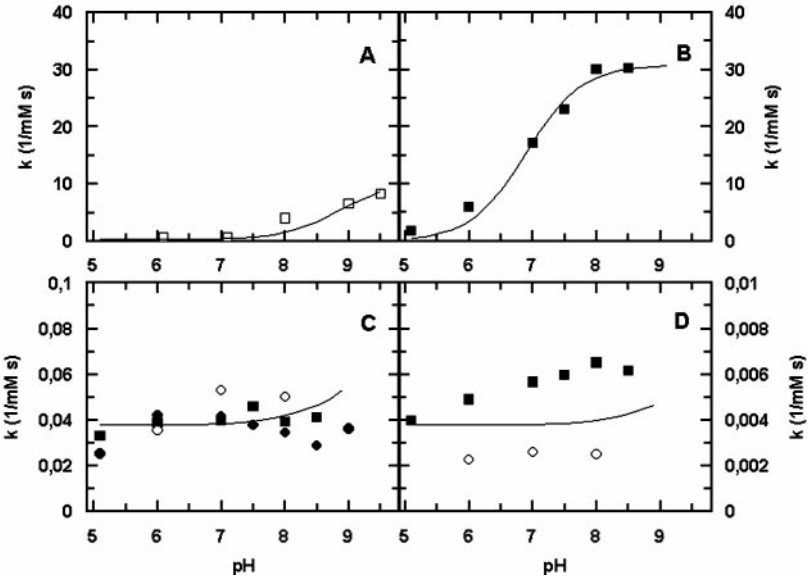

FIG. 6. **Dependence on pH of the second-order rate constant (*k*) for the reaction between DTNB and thiols present on glutathione (*panel A*), rat hemoglobin components (■, isoform F; ○, isoform C), and human hemoglobin (●).** *Panel B*, reports data of fHbSH; *panel C*, reports those of sHbSH, and *panel D* reports those of (v)HbSH (values in *panel D* were multiplied for 10). Experimental conditions in the reaction vessel are as follows: hemoglobin concentration, 6.5 μM in heme; DTNB concentration, 0.8 mM; temperature, 20 °C; 0.2 M buffers (acetate, pH 4.0–6.0; phosphate, pH 6.0–8.0; Tris acetate, pH 8.0–9.0).

TABLE III

Oxygen equilibrium ($p_{1/2}$ and n) and carbon monoxide kinetic (k_R and k_T) parameters for rat and human hemoglobins at 20 °C

Experimental conditions were as follows: (i) equilibria were carried out in 0.1 M HEPES, pH 7, and 20 μM concentration in heme; (ii) kinetic data were collected in 0.1 M Tris/HCl, pH 7.4, and 5 μM in heme (value in parentheses was obtained by photolysis experiments). The symbol F_{G2} represents isoform F with the two fHbSH (i.e. Cys-125β(H3)) blocked with glutathione. $p_{1/2}$ is the oxygen partial pressure required to yield half-saturation of hemes, and n the Hill empirical constant (24); k_R and k_T represent the overall rate constants for carbon monoxide combination with R and T quaternary state of hemoglobin, respectively.

Protein	$p_{1/2}$	n	$k_R \times 10^{-6}$	$k_T \times 10^{-6}$
	torr		$M^{-1}\ s^{-1}$	
Rat isoform F	2.62	2.51	5.4 (5.2)	0.33
Rat isoform G	2.56	2.58		0.30
Rat isoform F_{G2}	2.62	2.51	5.3	0.31
Human hemoglobin	2.00	2.60	5.1	0.12

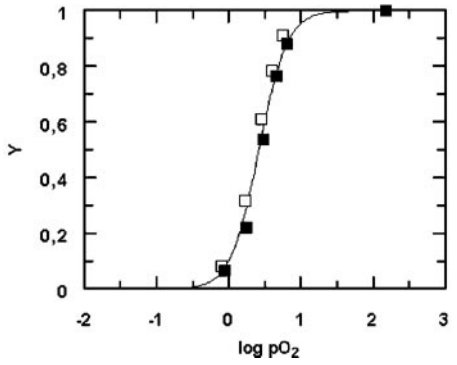

FIG. 7. **Oxygen binding curve of isoform F (4 mg/ml) with fHbSH free (■) and blocked with glutathione (□) in 0.1 M HEPES, pH 7.4, and 20 °C. *Y* is the fraction of hemes occupied with oxygen, and log pO_2 is the logarithm of the oxygen partial pressure (proportional to the oxygen chemical potential).**

Cys-13α(A11), respectively, indicating that, in addition to the ionization state, steric factors contribute to the low reactivity of (v)sHbSH toward DTNB.

Probing Some Aspects of Reducing Equivalent Transfer to Disulfide in Rat Erythrocytes—It is well known that NADPH is the primary nucleotide that channels reducing equivalents to GSSG and/or other oxidants (48). Therefore, the availability of NADPH should be the ultimate determinant of cellular thiol redox status following an oxidative stress. In the intact erythrocyte the supply of NADPH depends on the pentose phosphate pathway, *i.e.* on glucose-6-phosphate dehydrogenase activity (49). The factors controlling the interconversion of thiols and disulfides are not fully understood (50). In any case, glutathione reductase reduces the disulfide bond in GSSG and therefore contributes to the cellular mechanism for protection and repair under oxidative stress; even though this enzyme itself contains a thiol group, it is not very susceptible to oxidants (51). Therefore, some catalytic parameters (see Table IV) of these two enzymes from rat and human erythrocytes were determined. Whereas intracellular glucose-6-P-dehydrogenase activity was comparable for both animals, glutathione reductase appeared to work less efficiently in rat erythrocytes, the fraction of enzyme bound in any form whatsoever to NADPH and/or GSSG (as measured by K_m) being smaller in rat red blood cells relative to the human ones (at similar intracellular concentration of these substrates).

DISCUSSION

Since amino groups generally remain protonated below pH 10, the major nucleophilic functionality available in biological systems are thiols, which, accordingly, are the chemical groups that provide strong defense against electrophilic species. One of the objectives of the present research was to characterize in kinetic terms the reactivity of rat hemoglobin thiols under conditions mimicking the physiological ones. Therefore, this study was limited to free reactive thiols, whereas the so-called masked sulfhydryls, *i.e.* Cys-104α(G11) and Cys-111α(G18), which are located at the $\alpha_1\beta_1$ interface and whose reactivity is controlled by the tetramer dissociation beyond the dimer stage (52), were neglected.

The peculiar reactivity of fHbSH present on most of the rat hemoglobin components is clearly responsible for the formation of HbSSG after oxidative stress by diamide. Thiols in proteins are integral to a number of cellular functions, including protein folding, enzyme catalysis, and metabolic regulation (36, 53). The reported findings clearly suggest an involvement of fHbSH as a direct moderator of oxidative stress at least in rat erythrocytes. Red cells are regularly subjected to high oxygen tension and are among the first body cells exposed to exogenous oxidative substances that are ingested, inhaled, or injected. The importance of an antioxidant function in erythrocytes is well known and is shown, *e.g.* by the massive oxidant-induced hemolysis seen in subjects with a marked deficiency of glucose-6-phosphate dehydrogenase (54).

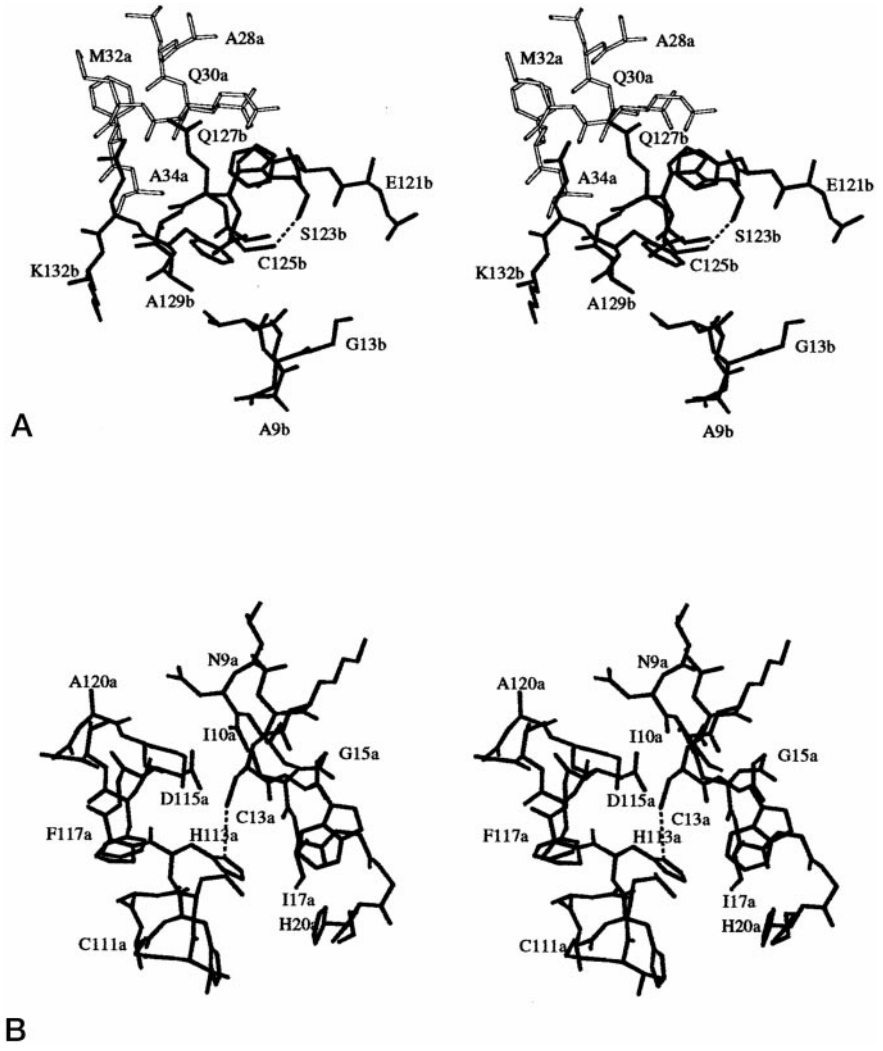

FIG. 8. **Stereo drawing of predicted interactions between Cys-125β(H3) and Ser-123β(H1) (*panel A*) and between Cys-13α(A11) and His-113α(GH1) (*panel B*) in the model of rat hemoglobin (isoform F).** Residues in α subunit in *panel A* are linked by *open bonds*. Amino acids are labeled with *one-letter code*. Potential hydrogen bonds are represented with a *dashed line*.

TABLE IV
Comparison (n 5) of some catalytic parameters of two enzymes in rat and human erythrocytes at pH 7.2 and 25 °C
G6P symbolizes glucose 6-phosphate.

Enzyme	Erythrocytes	
	Rat	Man
Glutathione reductase		
Overall activity (μM/min)	159 ± 12	425 ± 57
K_m for NADPH (μM)	41.9	12.5
K_m for GSSG (μM)	58.7	36.2
Glucose-6-P-dehydrogenase		
Overall activity (μM/min)	900 ± 113	992 ± 155
K_m for G6P (μM)	24.3	12.6
K_m for $NADP^+$ (μM)	6.2	4.9

The possible contribution of fHbSH to the intracellular detoxifying mechanism appears to be in line with the definition of the term antioxidant (55, 56) for both the intrinsic chemical properties and the intraerythrocyte concentration. However, this thiol system seems to be suited to resist acute episodes of oxidant fluxes but not the prolonged ones, because its efficiency is somewhat impaired by the long recovery time (see Table I). Since the reactivity of Cys-125β(H3) is not linked to the oxygenation nor to the quaternary state of hemoglobin (see Table III), fHbSH are expected to quickly produce *S*-conjugates in both the arterial and venous blood; in other words, flow in the blood circulation is expected not to induce a cycle (speeding up and slowing down) in the reactivity of fHbSH, as described for the highly conserved Cys-93β(F9) (and possibly true also for Cys-13α(A11)) that, being oxygen-linked, modulates arterial-venous differences in intraerythrocyte *S*-nitrosothiols (57, 58). Moreover, the *S*-conjugation capacity of fHbSH is very high. In fact, from the mean red cell volume (61.6 fl), the mean erythrocyte hemoglobin concentration (21.1 pg), the molecular mass of tetrameric hemoglobin (65 kDa), the fraction of isoforms carrying fHbSH (0.76), and the number of fast reacting cysteinyl residues per tetramer (two), an intracellular level of fHbSH equal to 8 mM can be calculated; this value, which is four times that of glutathione (2 mM), is great enough to force the conclusion that fHbSH works as a buffer system able to trap a large amount of attacking (electrophilic) species in a very short time. Finally, in the particular case investigated it can be also speculated that the efficient nonenzymatic antioxidant defense offered by fHbSH is able to compensate for the poor catalytic activity of glutathione reductase in rat erythrocytes (see Table III).

In conclusion and from a more general point of view, the reported results emphasize an additional function of some kinds of protein cysteinyl residues, *i.e.* a direct detoxification role by conjugation more efficient than low molecular weight thiols, such as glutathione.

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