Biochemical and Biophysical Research Communications **275,** 517–523 (2000)
doi:10.1006/bbrc.2000.3326, available online at http://www.idealibrary.com on IDEAL®

Highly Reactive Cysteine Residues in Rodent Hemoglobins

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Received June 24, 2000

Two hemoglobins with cysteine residues highly reactive toward electrophiles have been identified and characterized. Cys-125β of guinea pig hemoglobin has a low pK_a and forms conjugates with electrophiles more quickly than glutathione and several orders of magnitude more quickly than other protein thiols. This cysteine is capable of intercepting benzoquinone, a known carcinogenic metabolite, before other protein nucleophiles can be modified. Cys-13β of mouse hemoglobin was observed to conjugate with electrophiles as quickly as glutathione. The structural basis of reactivity is different in the two hemoglobins and is analyzed in terms of hydrogen-bonding, solvent accessibility, and helix-dipole contributions. Complementing a previously characterized highly reactive cysteine in rat hemoglobin, identification of these cysteines suggests that the reactivity of these hemoglobins could represent a common function as a detoxification sink against carcinogens.

Key Words: **hemoglobin; cysteine; thiol; conjugation; 5,5-dithio-bis(2-nitrobenzoic acid); carcinogens; benzoquinone.**

Cysteine residues with increased nucleophilic character are commonly found as catalytic residues in the active sites of enzymes such as the thioredoxin family (1), viral cysteine proteases (2), papain (3), and rhodanese (4). Irreversible conjugation with electrophiles, however, would inactivate these enzymes. Using 5,5-dithio-bis(2-nitrobenzoic acid), DTNB, as a model electrophile, several "fast-reacting" thiol groups not directly involved in enzymatic catalysis have been reported. Mammalian 3-phosphoglycerate kinase contains two thiols that react with a second order rate constant of 6.4×10^2 to 1.1×10^3 $M^{-1}s^{-1}$ (5). While these cysteine residues are not directly involved in the catalytic mechanism, cysteine conjugation inhibits enzyme activity, possibly through the prevention of conformational changes required for catalysis (6, 7). The fast-reacting thiols of rabbit aldolase, also not directly involved in catalysis (8), only react with a second order rate constant of 13.0 to 2.62×10^2 $M^{-1}s^{-1}$ (9, 10).

Rat hemoglobin has been shown to possess a cysteine residue, Cys-125β, whose reaction with DTNB has a rate constant of 2.94×10^4 $M^{-1}s^{-1}$ (11). The oxygen binding properties of hemoglobin were not affected by conjugation. This thiol reacts with DTNB faster than any other previously characterized protein thiol and an order of magnitude more quickly than glutathione. Protein thiols have been implicated as part of the cellular antioxidant defense mechanism (12), and this highly reactive cysteine is involved in that mechanism. Increased nucleophilic character, however, also suggests that these thiol groups could potentially play a role in cellular defense mechanisms against electrophilic carcinogens. The presence of a highly reactive cysteine in most cells could be problematic given possible interactions with endogenous electrophiles without the selectivity imposed by an active site. Mammalian erythrocytes, however, provide a unique environment with no organelles and very little protein other than hemoglobin. Given the high concentration of hemoglobin in erythrocytes, it would make an effective detoxification sink for exogenous electrophilic species. This spatial separation would prevent electrophiles from forming potentially mutagenic or toxic DNA and protein adducts.

Observing only one example of a highly reactive cysteine in which conjugation does not affect another protein function still suggests that this is a case of unique chemical reactivity. Identification of hemoglobins with the same reactivity in other species would support the argument that such a group of proteins represent a motif with a specific physiological function. Human hemoglobin does not contain a highly reactive cysteine, but hemoglobins from two rodents are shown here to possess such reactivity.

MATERIALS AND METHODS

Materials. Human ferrous hemoglobin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade quality.

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	5 15	122 142
Human	PEEKSAVTALW	FTPPVQAAYQKVVAGVANALA
Rat β1	DAEKAAVNGLW	FSPCAQAAFQKVVAGVASALA
Rat β2	DAEKAAVNGLW	FTPCAQAAFQKVVAGVASALA
Rat β3	DAEKAAVNGLW	FTPCAQAAFQKVVAGVASALA
Rat β4	DAEKAAVNGLW	FTPSAQAAFQKVVAGVASALA
Guinea Pig	AAEKSAILDLW	FTPCTQAAFQKVTAGVANALA
Mouse $Hbb^{d/p}$ major	DAEKAAVSCLW	FTPAAQAAFQKVVAGVATALA
Mouse $Hbb^{d/p}$ minor	DAEKSAVSCLW	FTPAAQAAFQKVVAGVATALA
Mouse Hbb^{s}	DAEKAAVSGLW	FTPATQAAFQKVTAGVAAALA

FIG. 1. Multiple sequence alignment of the A and H helixes of the β chains of hemoglobins used in this study. Highly reactive cysteines are underlined.

Search for hemoglobins with highly reactive cysteines. A pattern-hit initiated similarity search was performed using PHI-Blast (13) (http://www.ncbi.nlm.nih.gov/blast/psiblast.cgi?Jform=1). The sequences of the β1 and β2 chains of rat hemoglobin (14) were manually entered as templates and the patterns SPC and TPC, respectively, were used as seeds. A second search involved downloading all sequences found using the search query of "hemoglobin [TI] OR globin [TI]' AND beta [TI]" in Batch Entrez (http://www.ncbi.nlm.nih.gov:80/Entrez/batch.html). Sequences were aligned in groups of 50 to 100 using ClustalW (15) and the alignments were manually searched for cysteine residues in position 125β.

Hemoglobin preparation. Guinea pig hemolysates and purified hemoglobin were kindly provided by Professor Billy W. Day (University of Pittsburgh). Blood was obtained from Hartley guinea pigs by venous puncture into tubes containing heparin as an anticoagulant. Erythrocytes were isolated via centrifugation, washed with phosphate buffered saline by centrifugation, and lysed via freezing. Sprague-Dawley [Crl:CD(SD)] rats were anesthesized with a ketamine/xylazine/acepromazine cocktail. C57BL/6J and DBA/2J mice were anesthesized with chloral hydrate. Rat and mouse blood were obtained by cardiac puncture into tubes containing EDTA as an anticoagulant. Erythrocytes were washed with isotonic saline via centrifugation and lysed in hypotonic water. For all samples, cell debris was removed via centrifugation at 10,000*g* for 30 min at 4°C and dialyzed in 10 mM sodium/potassium phosphate, pH 7.0, overnight. The final hemoglobin concentration was determined based on the absorbance of the α-band such that absorbance $\times$ dilution $\times$ 1.14 = mg/mL (16). Oxidation to ferric hemoglobin was monitored based on the ratio of absorbance of the α and β bands. Samples with significant amounts of methemoglobin were discarded.

Highly reactive cysteine assays. All assays were performed on a Perkins Elmer Lambda 11 UV/Vis Spectrophotometer at 4°C. Hemoglobin, 6.8 μM, was incubated in 100 mM sodium/potassium phosphate, pH 7.0. After recording the initial absorbance due to heme, DTNB, from a 34 mM stock solution in ethanol, was added to 340 μM and the production of 5-thio-2-nitrobenzoic acid was monitored at 450 nm using an extinction coefficient of 6.51 $mM^{-1}cm^{-1}$ (17). Standard monitoring of the reaction at 412 nm was not possible due to the high heme absorbance in the Soret region. Benzoquinone conjugation assays were performed by incubating specific concentrations of *para*-benzoquinone with hemoglobin for one minute before the addition of DTNB. The difference in the amount of highly reactive cysteines relative to a standard assay determined the amount of cysteine residues blocked via conjugation with benzoquinone. Benzoquinone concentrations were determined by assaying the solution in hexane using an extinction coefficient of 24.3 $mM^{-1}cm^{-1}$ at 239.8 nm (18).

Stopped-flow kinetics. Kinetic experiments were performed on a SFM-4 stopped-flow apparatus (Bio-Logic, Claix, France) with a TC100/10 1 cm path length cuvette at room temperature. Under the chosen mixing conditions, the estimated dead time was 2.3 ms. Solutions of 0.5 to 1.7 μM hemoglobin in a 100 mM buffer were rapidly mixed with equal volumes of 680 μM DTNB in the same buffer. Various solutions were used to buffer a given pH: sodium acetate, pH 5.0; sodium/potassium phosphate, pH 6.0 to 8.0; and Tris–HCl, pH 9.0. The absorbance change at 450 nm was monitored. Each experiment consisted of the average of five traces with each trace containing 1200 to 2000 data points. Traces were fit to a second order exponential equation in which the magnitude of both exponentials were constrained as equal. Statistical analysis and data fitting were performed with SigmaPlot 5.0 (SPSS Inc., Chicago, Illinois).

Molecular modeling and analysis. The sequences of the guinea pig β chain and Hbb^{d} major allelic variant of the mouse β chain, Swiss-Prot accession codes P02095 and P02088, respectively, were submitted to Swiss-Model (19, 20) (http://www.expasy.ch/swissmod) using the first approach mode. Solvent accessible surface areas of the cysteine residues were calculated using the dms subroutine of Midas (21).

RESULTS AND DISCUSSION

Database searches and identification of hemoglobins with highly reactive cysteine residues. The first highly reactive cysteine residue in hemoglobin was identified

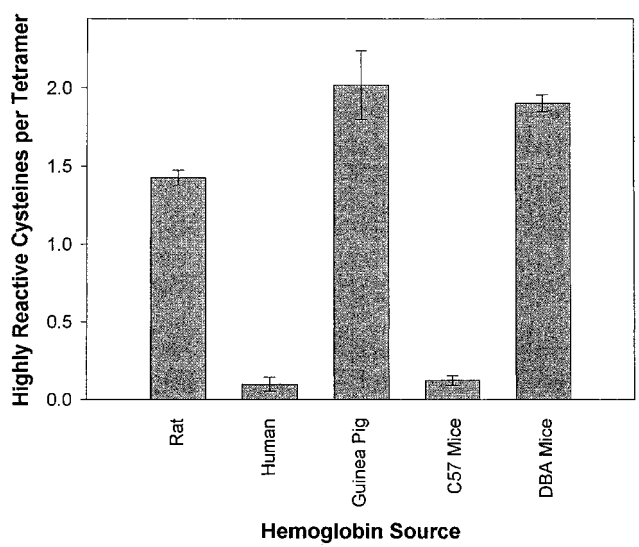

FIG. 2. Highly reactive cysteine assays of hemoglobins from various sources. Error bars represent one standard variation.

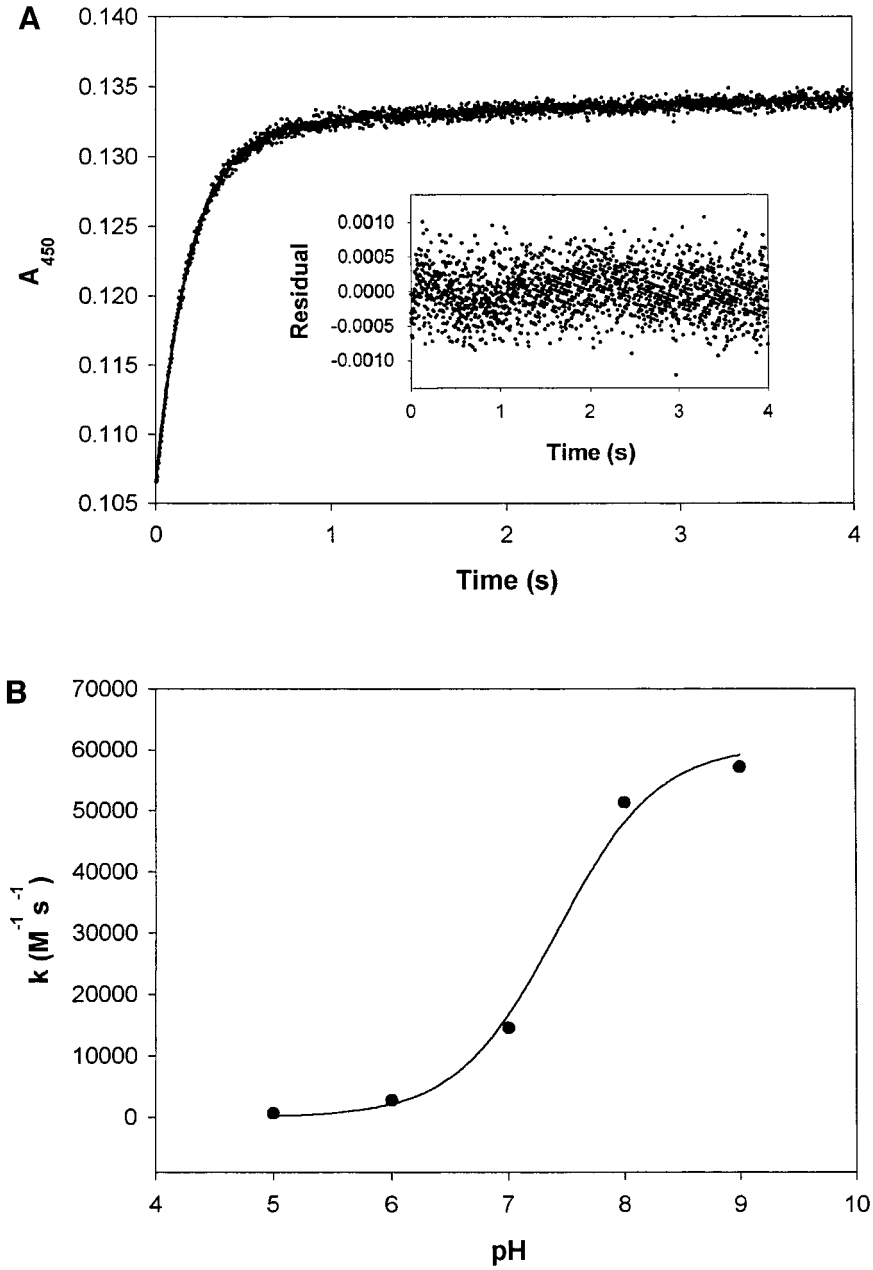

FIG. 3. (A) Stopped-flow kinetic trace of the cysteine conjugation reaction between guinea pig hemoglobin and DTNB in 100 mM sodium/potassium phosphate, pH 7.0, room temperature. Residuals to the second order exponential fit are shown in the inset. (B) pH dependence of the second order rate constant.

in rats. Sprague-Dawley rats have been shown to possess four β chain variants (14). The proposed mechanism for the structural basis of the fast-reacting thiol in rat hemoglobin involved a hydrogen bonding interaction between Ser-123β and the thiolate anion of Cys-125β (11). Ser-123β, however, is only found on the β1 variant and is replaced by threonine in the other variants (Fig. 1). The hydrogen bonding interaction, however, can be presumed to be conserved in this mutation. This case was used as a model on which to base a systematic search for other highly reactive cysteines.

Hemoglobins from other species which could possibly possess such a motif were identified using PHI-Blast (13). PHI-Blast performs a standard homology search and then filters the results for only proteins that possess a user entered pattern which can be found on the template protein. The β1 and β2 chains of rat hemoglobin were used as templates for searches that looked for homologous proteins with the hydrogen bonding motifs of SPC and TPC respectively. These two searches yielded eight unique sequences with eighteen different gi numbers: 546056, 223027, 984679, 546057,

92361, 122514, 2144723, 55823, 395939, 204570, 122529, 92360, 56256, 56260, 395941, 395945, 122581, and 70439. Of the proteins identified in the search, all but one were variants of rat hemoglobin. The protein with gi numbers 122581 and 70439, identical sequences from different databases, corresponded to hemoglobin from guinea pig (22).

The PHI-Blast search was limited in that it was not feasible to search for hemoglobins which contain cysteine in position 125β while residues 123β and 124β diverge from the observed motif. A cysteine residue at position 125β might be highly reactive without a hydrogen bond donor at position 123β if the hydrogen bonding motif is not the only cause of the reactivity. To overcome this limitation, a Batch Entrez query for proteins with words "hemoglobin" and "beta", or "globin" and "beta", was used to download the sequences of 897 proteins. In screening for a cysteine residue at position 125β, every sequence identified by the PHI-Blast search, except for gi 223027, was identified again and no novel hits were found.

Assays and kinetic measurements. A simple assay using conventional spectrophotometry was developed to screen for hemoglobins with highly reactive cysteines. A similar protocol has been used to measure the kinetics of reactivity between slow-reacting thiol groups of hemoglobin, such as human Cys-93β, and DTNB (17). The entire course of a reaction involving slow-reacting thiols with second order rate constants of 10 to 100 $M^{-1}s^{-1}$ can be easily observed. Fast-reacting thiols with rate constants on the order of 10^4 $M^{-1}s^{-1}$ react completely during the manual mixing time required and appear as an initial burst. Quantitation of this burst can determine the stoichiometric amount of highly-reactive cysteines per tetramer given a known amount of hemoglobin. All assays were performed at low temperature to slow down the reactivity of the slow-reacting thiols in order to eliminate overestimation of the amount highly reactive cysteine present. These burst-phase assays were performed on guinea pig hemoglobin and two other hemoglobins whose reactivities are known (Fig. 2). As expected, human hemoglobin did not show any highly-reactive cysteines. Previously determined quantitation of isoforms with fast-reacting thiols in Sprague-Dawley rats (14) predicts the presence of 1.36 to 1.68 highly reactive cysteines per tetramer. The result of approximately 1.4 highly reactive cysteines per tetramer correlates with this prediction. Guinea pig hemoglobin demonstrated approximately 2.0 highly reactive cysteines per tetramer. Stopped-flow kinetics (Fig. 3A) established a second order rate constant of 1.46×10^4 $M^{-1}s^{-1}$.

The pH dependence of cysteine reactivity in guinea pigs was also examined (Fig. 3B). The species which reacts with DTNB is the thiolate anion (17), so the pH dependent rate data was fit into the following equation

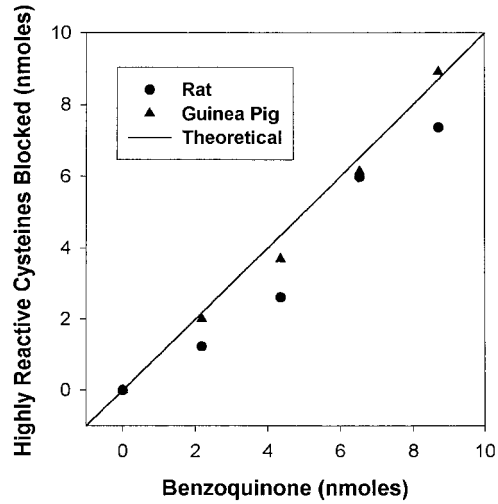

FIG. 4. Stoichiometric relationship between the amount of benzoquinone incubated with hemoglobin and the amount of highly reactive cysteine residues blocked from conjugation with DTNB. The diagonal represents the theoretical 1:1 ratio.

which treats the reaction rate between the protonated thiol and DTNB as zero:

$$k_{obs} = \frac{K_a \times k_{s^-}}{K_a + [H^+]} \qquad [1]$$

k_{obs} is the observed apparent rate constant of the reaction; K_a is the acid dissociation constant of the cysteine residue; and k_{s^-} is the rate constant of the reaction between the thiolate and DTNB. Fitting the data yielded a pK_a of 7.42 and a k_{s^-} of 6.07×10^4 $M^{-1}s^{-1}$. All of this data is similar to the behavior of the analogous cysteine from rat Hb (11): k_{obs}, 2.94×10^4 $M^{-1}s^{-1}$; pK_a, 6.89; and k_{s^-}, 3.9×10^4.

Benzoquinone conjugation assays. Benzoquinone is a metabolite of benzene (23), a known carcinogen, and is thought to play a role in its toxicity (24). Quinones even by themselves are thought to be toxic through adduct formation and other quinone mediated processes (25). Benzoquinone DNA adducts are potentially mutagenic (26) and hemoglobin adducts, *S*-(2,5-dihydroxyphenyl) conjugates which form via a Michael Addition (27), have been found *in vivo* (28). Assays incubating highly reactive cysteines with benzoquinone *in vitro* show that the electrophile quantitatively conjugates with hemoglobin leading to an instantaneous 1:1 decrease in the amount of such residues (Fig. 4). This suggests that benzoquinone forms adducts with these residues before reacting with any other nucleophile on the protein such as Cys-93β and the N-terminal valine, both of which play potential roles in the allosteric and regulatory properties of hemoglobin. This high reactivity could explain the high background

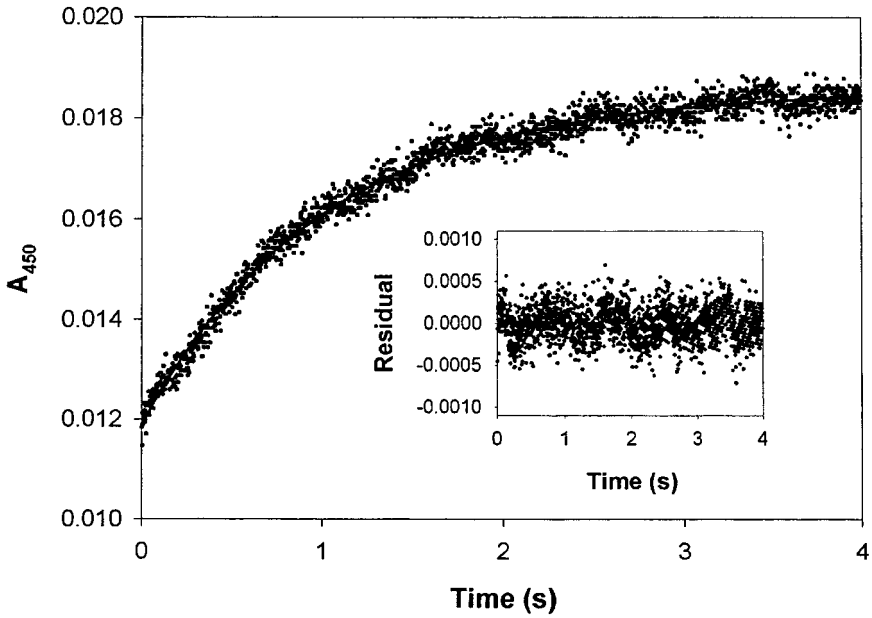

FIG. 5. Stopped-flow kinetic trace of the cysteine conjugation reaction between mouse hemoglobin and DTNB in 100 mM sodium/potassium phosphate, pH 7.0, room temperature. Residuals to the second order exponential fit are shown in the inset.

levels of hemoglobin adducts, presumably due to the endogenous formation and dietary consumption of phenolic compounds (28). Independent of its specific role in benzene metabolism, benzoquinone serves as a model for electrophilic, carcinogenic metabolites in general. Interception of these metabolites can prevent DNA adduct formation and modification of other proteins.

Mouse hemoglobin reactivity. Due to absence of cysteine at position 125β, mouse hemoglobin was initially thought to contain no highly reactive cysteines, but hemoglobin from the DBA strain of inbred mice contained a highly reactive cysteine while hemoglobin from the C57 strain did not contain any such reactivity (Fig. 2). Mouse hemoglobin β chains are expressed in either a single or diffuse manner (29). C57 mice express single-type hemoglobin in the form of two identical gene products which are the encoded by the β^s and β^t genes of the *Hbb*^{s} allelic complex (30, 31). DBA mice express diffuse-type hemoglobin with multiple gene products (29). Two diffuse allelic complexes are known. The *Hbb*^{d} allele consists of two gene products and codes for a major and minor chain (32, 33). A closely related *Hbb*^{p} allele also exists, but the gene products of that allele vary from the *Hbb*^{d} allelic products only in positions 22β and 23β of the minor chain (34). Identification of the DBA β chains as resulting from either the

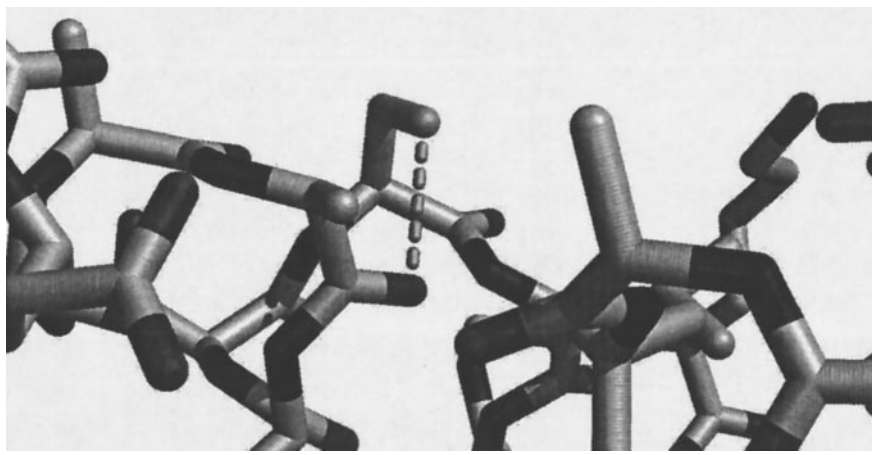

FIG. 6. Molecular model of the putative hydrogen bond between the thiol of Cys-13β and the carbonyl of Ala-10β on the surface of the protein. The hydrogen bond is denoted by a dotted line.

TABLE 1

Solvent Accessibility of Cysteine Residues

Residue	Side chain torsion angle (χ_1: N–C_α–C_β–S)	Side chain solvent accessible surface area (Å)	Sulfur atom solvent accessible surface area (Å)
Guinea Pig Cys-125β	63.7	42.1	25.6
Guinea Pig Cys-93β	−178.0	21.4	16.8
Mouse Cys-13β	64.9	33.7	19.5
Mouse Cys-93β	−178.1	22.1	17.5

Hbb^d or *Hbb*^{p} is not necessary as the gene products do not vary in terms of cysteine composition. Examination of the sequence alignment suggests that a polymorphism introducing a cysteine at position 13β may be responsible for the reactivity of mouse hemoglobin (Fig. 1) since there are no other mutations involving a cysteine residue between the two alleles.

The highly reactive cysteine of mouse hemoglobin shows slightly different properties compared to the other highly reactive cysteines identified. The rate constant for the reaction with DTNB is only 3.26×10^3 $M^{-1}s^{-1}$ (Fig. 5). Although not as fast as the highly reactive cysteines from guinea pig and rat, this cysteine is as reactive as glutathione which reacts with a rate constant of 2.6×10^3 $M^{-1}s^{-1}$ (11).

Molecular basis of reactivity. While the highly reactive cysteine in guinea pig hemoglobin is analogous to the previously characterized cysteine of rat hemoglobin, the reactivity of Cys-13β of mouse hemoglobin is novel. Homology modeling based on known structures of other hemoglobins yielded a theoretical model of the mouse hemoglobin β chain. Examination of the local environment around Cys-13β suggests a possible mechanism for its reactivity (Fig. 6). The cysteine thiol is within 2.81 Å of the Ala-10β carbonyl group at a 118° angle, C_β–S–O, in the proper geometry for hydrogen bonding. The majority of unmodified cysteine residues in proteins not involved in covalent bonds or metal coordination are hydrogen bond donors, predominantly to carbonyl groups (35, 36). This increases electron density around the sulfur atom and the more thiolate-like nature of the cysteine residue could explain its increased reactivity.

One important characteristic shared by highly reactive cysteines is solvent accessibility (Table 1). Highly reactive cysteine residues and side chains are generally more exposed to the solvent, and hence more accessible to electrophiles, than slow-reacting thiols.

The decreased pK_a of guinea pig Cys-125β is approximately one unit below the normal cysteine pK_a of 8.5 to 9.0 found in denatured peptides (1). Relative to normal cysteine residues, the lowered pK_a increases the concentration of the reactive thiolate species. The donation of a hydrogen bond by Ser-123β to the thiolate, and hence stabilization of the anion, has been proposed to explain the decreased pK_a of Cys-125β in rat hemoglobin (11). This explanation does not seem harmonious with model studies since all solvent exposed cysteines can receive hydrogen bonds from water, but not all solvent exposed cysteines have a pK_a around 7.0 to 7.5. With no positively charged groups in the local environment of Cys-125β, the effect of the helix dipole could account for the lowered pK_a. Cys-125β is located at the N-terminus of helix H. Helix dipole theory suggests that a thiolate anion can be stabilized by the positive charge of the macrodipole. Studies with model helix peptides have shown that cysteine residues at the N-terminus of a helix have a pK_a of approximately 7.5 (1), within the same range as the pKa observed for Cys-125β.

This study has observed highly reactive cysteine residues in two new species. Both utilize different structural motifs to obtain this reactivity. Given the characterized properties and methods of screening, other proteins with highly reactive cysteines could potentially be identified. The observation of such residues outside of the active site of enzymes is novel and such reactivity could potentially play a physiological role. In the case of hemoglobin, highly reactive cysteine residues can act as detoxification sinks via conjugation with carcinogenic electrophiles.

ACKNOWLEDGMENTS

I thank Arthur Glasfeld for helpful discussions and critical reading of the manuscript; Billy W. Day (University of Pittsburgh) for guinea pig hemolysates and hemoglobin; Michael I. Schimerlik (Oregon State University) and the Nucleic Acid and Protein Core Facility at the Oregon State University Environmental Health Sciences Center for technical assistance with the stopped-flow kinetics experiments; Kristine Sawicki for mouse blood samples; and the Department of Comparative Medicine of the Oregon Health Sciences University for rat blood samples.

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