ical behavior. Clearly, however, more experimental results are needed before it can be definitely concluded that a large fraction of the total protein of the erythrocyte membrane is indeed organized into a few well-defined structural units under certain solvent conditions.

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Comparison of Human Hemoglobin A Carrying Glutathione as a Mixed Disulfide with the Naturally Occurring Human Hemoglobin A_3†

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ABSTRACT: A method for the quantitative preparation of a mixed disulfide between native human hemoglobin A and glutathione (Hb ASSG) without formation of methemoglobin is described. Properties of the uniformly modified Hb ASSG are presented. One glutathione molecule was found to be bound to the cysteinyl residue in position β-93 (F-9). The failure to find such a hemoglobin in normal hemolysates and in the blood from two patients with Heinz body anemia suggests that this compound does occur, if at all, in less than 0.1% of the total hemoglobin. The naturally occurring minor component of human blood, Hb A_3, with chromatographic and electrophoretic properties nearly identical with the ones of Hb ASSG, is not a mixed disulfide. Hb ASSG is converted *in vitro* to Hb A by GSH but not by glutathione reductase from human erythrocytes, when both are used at physiological concentrations.

In contrast to most cells, the mammalian reticulocyte loses its ability to synthesize proteins after being present for 1 or 2 days in circulating blood. Beyond the reticulocyte stage the human erythrocytes survive for over 100 days with their original protein complement and therefore appear to be a favorable system for studying the "aging" of protein molecules. Oxidative processes in erythrocytes are considered to be such aging phenomena. Indeed, hemoglobin A carrying a glutathione molecule as a mixed disulfide on the β chains (Hb ASSG)[1] has been found in undialyzed hemolysates and

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[1] Nonstandard abbreviations used are Hb ASSG, hemoglobin carrying as a mixed disulfide 1 mol of glutathione/mol of β chain; CM-Hb A, hemoglobin with the thiol groups at position β-93 carboxyamidomethylated; GSH, reduced glutathione; GSSG, oxidized glutathione; *p*-ClHgBzO, *p*-chloromercuribenzoate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).

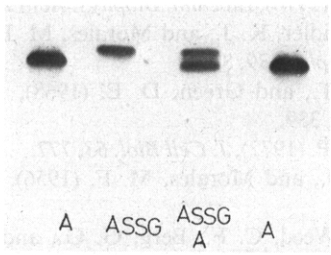

FIGURE 1: Starch gel electrophoresis of Hb A, Hb ASSG, and a mixture of both at pH 8.6 (Poulik, 1957).

red cells, both aged *in vitro* (Huisman and Dozy, 1962; Huisman *et al.*, 1966). However, on the presence of Hb ASSG in normal human blood conflicting data are reported (Muller, 1961; Boyd *et al.*, 1967; Dozy and Huisman, 1971; Srivastava *et al.*, 1972).

Although no *de novo* protein synthesis takes place in erythrocytes, the red cell still exhibits a very active enzymatic synthesis of GSH, which is present at concentrations of 2.3 mM or roughly half the concentration of the hemoglobin tetramer. It is concluded that GSH in the *in vivo* red cell is an essential component for the maintenance of Hb A in a physiologically active form. GSSG, on the other hand, is either continuously reduced by the glutathione reductase system or actively transported out of the erythrocyte (Srivastava and Beutler, 1969). The concentration of GSSG inside red cells was found to be 0.004 mM or less (Srivastava and Beutler, 1968).

For the present study a pure glutathione-labeled Hb A was synthesized under conditions preventing oxidation of the ferroheme and alterations of amino acid side chains. The structure and the properties of this artificially prepared Hb ASSG were examined. The derivative allowed an investigation of its interaction with GSH and glutathione reductase under conditions reflecting these of the red cell. For this purpose glutathione reductase from erythrocytes was purified in a simple procedure about 1000-fold. In order to detect naturally occurring Hb ASSG in human blood, radioactively labeled Hb ASSG was used as a tracer. Hb ASSG was also compared with the naturally occurring minor component of human blood, hereafter denoted as Hb A_3, which shows similar electrophoretic and chromatographic properties.

Materials and Methods

(*a*) *Chemicals.* GSH labeled with ^3H was obtained from New England Nuclear and with ^{35}S from Schwarz. GSH, GSSG, and *p*-ClHgBzO (recrystallized from ethyl acetate–hexene) were purchased from Fluka; yeast glutathione reductase (EC 1.6.4.2) and NADPH were from Boehringer.

(*b*) *Hemoglobins.* Hb A was isolated from the blood of healthy males according to the procedure of Winterhalter and Huehns (1964). All experiments with native hemoglobins were performed at 4°, unless otherwise stated. Hemoglobin in the CO form was converted to the oxy derivative by dialysis against oxygen-saturated buffer. Ferric cyanide derivatives were made by incubating in the presence of excess KCN the hemoglobin in 0.01 M sodium phosphate buffer (pH 7.0) at room temperature with a 1.2 molar excess of potassium ferricyanide over heme. After complete oxidation small molecular reaction products were removed by exhaustive dialysis. Further experiments with this derivative were carried out in the presence of 1.5 mM KCN. Hemoglobin concentrations are given as millimolar dimers (mol wt 32,200) and determined spectrophotometrically (Antonini and Brunori, 1971).

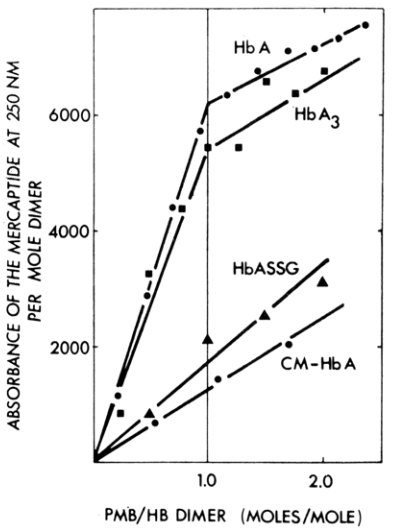

FIGURE 2: Thiol group titrations of Hb A, Hb ASSG, Hb A_3, and CM-Hb A (ferric cyanide forms) with *p*-ClHgBzO (Boyer, 1954). *p*-ClHgBzO was added in aliquots to 0.005 mM hemoglobin in 0.01 M sodium phosphate (pH 7.0).

Hb ASSG was obtained by incubating native Hb A (0.5 mM) as either the CO or the ferric cyanide from with a large excess of GSSG (100 mM) for 15 hr at 4° in 0.5 M Tris-HCl (pH 8.5). The derivative was freed from unreacted glutathione by gel filtration on a column of Sephadex G-25, equilibrated with 0.01 M sodium phosphate (pH 7.0). In order to synthesize radioactive Hb ASSG, the GSSG used was labeled by preincubation with radioactive GSH for 15 hr in 0.5 M Tris-HCl (pH 8.5) at 4°. In a typical experiment the final specific radioactivity of GSSG was 2.7×10^5 dpm of $^3H/\mu$mol.

Hb A_3 was purified from fresh hemolysates on a 3.2×50 cm column of CM-Sephadex equilibrated with 0.05 M sodium phosphate (pH 6.0). The column was developed with a 1000-ml gradient from 0 to 0.2 M NaCl in 0.05 M sodium phosphate (pH 6.0). Further purification was achieved by chromatography on a 2.0×30 cm column of DEAE-Sephadex, equilibrated with 0.05 M Tris-HCl (pH 7.9), which was developed with a 500-ml gradient of 0.05 M Tris-HCl from pH 7.9 to 7.0.

Native hemoglobin chains were separated according to Bucci and Fronticelli (1965) and oxygen equilibria were done according to Rossi-Fanelli and Antonini (1958). Molecular weights were determined by gel filtration on a 1×145 cm column of Sephadex G-100, equilibrated with CO-saturated 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. Thiol group titrations of the hemoglobins were performed either with *p*-ClHgBzO according to Boyer (1954) or with Nbs_2 according to Ellman (1959). For determinations of the thiol content in denatured protein, sodium dodecyl sulfate was added to a final concentration of 0.4%. The Nbs_2 reaction was followed spectrophotometrically at 450 nm and the sulfhydryl content calculated on the basis of the extinction coefficient of thionitrobenzoate ϵ_{450} 7200 M^{-1} cm^{-1}. Globin was prepared by precipitation with acetone–HCl (Winterhalter and Huehns, 1964). Radioactivity assays were performed in a scintillator composed of 50 ml of hydroxide of Hyamine 10X (Packard), 200 ml of methylcellosolve, and 750 ml of toluene containing 3 g of 2,5-diphenyloxazole, and 80 mg of 1,4-bis-

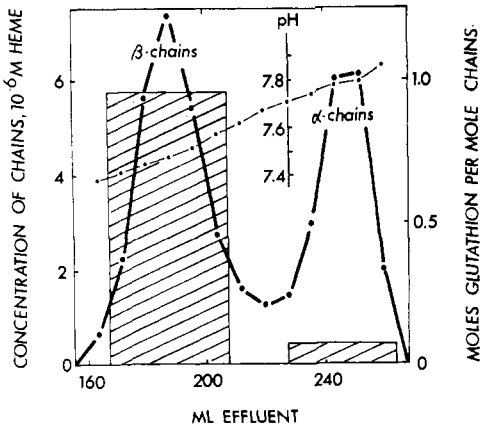

FIGURE 3: Separation of α and β subunits from tritiated Hb ASSG (CO form) according to Bucci and Fonticelli (1965). The chains were applied to a 0.9 × 30 cm column of CM-Sephadex, equilibrated with 0.01 M sodum phosphate (pH 6.8) containing CO and 0.1 mM EDTA. The column was developed with a 500-ml gradient from 0.01 M sodium phosphate (pH 7.0) to 0.02 M Na_2HPO_4, containing CO and 0.1 mM EDTA. The specific radioactivity of the pooled chains is given as equivalents of glutathione per mole of subunits (hatched area).

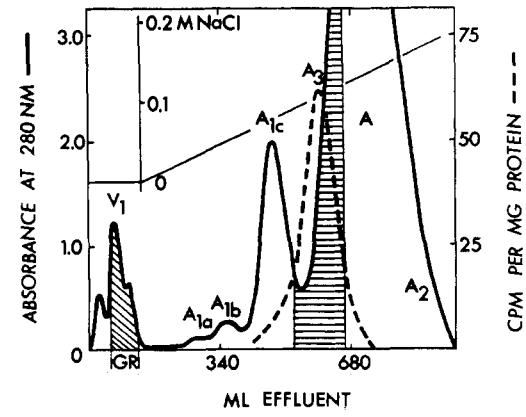

FIGURE 4: Chromatography of fresh hemolysate on a CM-Sephadex column in the presence of radioactively labeled Hb ASSG. Hemolysate (30 μmol) containing 0.7 μmol of ^{35}S-labeled Hb ASSG (125,000 cpm) was chromatographed as described under Materials and Methods. Peak V_1 represents the nonhemoglobin proteins of the erythrocyte and contains the glutathione reductase (GR). Hb A_{1a}, A_{1b}, and A_{1c} denote minor hemoglobin fractions. Hb A_3 is eluted with the tracer Hb ASSG between Hb A_{1c} and the main hemoglobin A.

[2-(5-phenyloxazolyl)]benzene. Samples of up to 0.4 ml were mixed with 10 ml of scintillation fluid and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3380, equipped with an Absolute Activity Analyzer.

(*c*) *Purification of Erythrocyte Glutathione Reductase.* Washed packed red cells were lysed with a fivefold volume of 0.01 M sodium phosphate (pH 6.0) and freed of solids by centrifugation. The supernatant was dialyzed against 0.05 M sodium phosphate (pH 6.0) containing EDTA and mercaptoethanol, both 0.5 mM. Subsequently, the hemolysate was applied to a CM-Sephadex column and chromatographed under conditions identical with those for the purification of Hb A_3 (see above). The fractions containing glutathione reductase were pooled, concentrated by ultrafiltration, and further fractioned on a 1.8 × 150 cm column of Sephadex G-200, equilibrated with 0.05 M sodium phosphate (pH 7.0) containing EDTA and mercaptoethanol, both 0.5 mM. The enzymatic activities of the yeast and the erythrocyte enzyme were assayed according to Scott *et al.* (1963) in 0.05 M sodium phosphate (pH 6.8) containing 0.9 mM GSSG, 1 mM EDTA, and 0.2 mM NADPH.

Results

(*a*) *Hemoglobin ASSG.* Hb ASSG was prepared in the carbomonoxy, the ferric cyanide, and the oxy forms as described under Materials and Methods. No significant spectral differences in comparison with Hb A could be observed in either of these derivatives. In the CO form Hb ASSG and Hb A behaved identically upon molecular weight determinations on Sephadex G-100. On the other hand, Hb ASSG showed a slightly higher anodic mobility on starch gel electrophoresis at pH 8.6 (Figure 1). Titration of Hb ASSG with *p*-ClHgBzO revealed the absence of the reactive thiol group (Figure 2); the response to *p*-ClHgBzO was found to be similar to that of Hb A carboxyamidomethylated at sulfhydryl group β-93 (Guidotti and Königsberg, 1963; Birchmeier *et al.*, 1972). Nbs_2 titrations of native and denatured Hb ASSG revealed 0 and 1.7 mol of thiol groups/mol of dimer, respectively. Control experiments with Hb A showed 0.9 and 2.6 equiv, respectively. Furthermore, ^{3}H-labeled Hb ASSG revealed 1.2 mol of radioactive glutathione bound/mol of hemoglobin dimer. Most of the radioactivity was associated with the β chains (Figure 3). Incubation of Hb ASSG with excess mercaptoethanol resulted in quantitative conversion to a protein having the electrophoretic properties of Hb A. The oxygen affinity of Hb ASSG measured in 0.2 M sodium phosphate was found to be 4–10 times higher than that of Hb A, depending strongly on pH (K. H. Winterhalter, manuscript in preparation). The *n* value from the Hill equation was decreased to approximately 1.7. No difference between Hb ASSG and Hb A in attachment to thiol groups insolubilized on Sephadex G-100 (Tyuma *et al.*, 1966) could be detected in either the oxy, carbomonoxy, or ferric cyanide form.

(*b*) *Hemoglobin A_3.* In order to detect a naturally occurring Hb ASSG a fresh hemolysate mixed with radioactively labeled Hb ASSG as a tracer was subjected to chromatography on CM-Sephadex (Figure 4). The radioactivity eluted prior to Hb A but at higher salt concentration than necessary for the elution of Hb A_{1c} (Holmquist and Schroeder, 1966; Bookchin and Gallop, 1968). Further purification of the tracer on DEAE-Sephadex (Figure 5) resulted in the isolation of a naturally occurring hemoglobin fraction, traditionally referred to as Hb A_3. In all further experiments Hb A_3 was isolated from fresh hemolysates without the addition of tracer material. On starch gel electrophoresis at pH 8.6 Hb A_3 showed an anodic mobility nearly identical with the one of

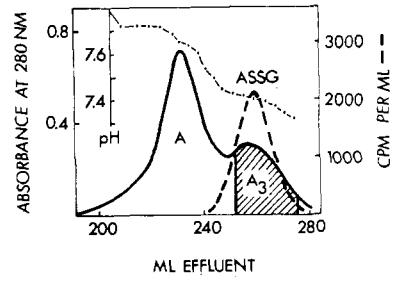

FIGURE 5: Chromatography of the Hb A_3-containing pool from CM-Sephadex (*cf.* Figure 4) on DEAE-Sephadex. Hemoglobin (0.8 μmol) was chromatographed in parallel with 0.6 μmol of tritiated Hb ASSG (see Materials and Methods).

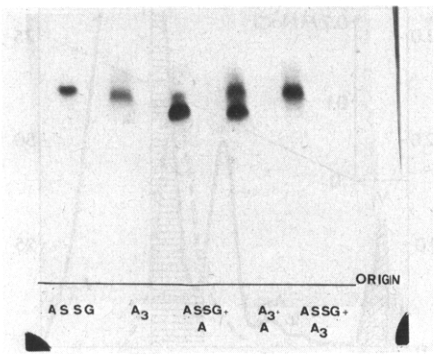

FIGURE 6: Starch gel electrophoresis of Hb A, Hb ASSG, and Hb A_3 at pH 8.6 (Poulik, 1957).

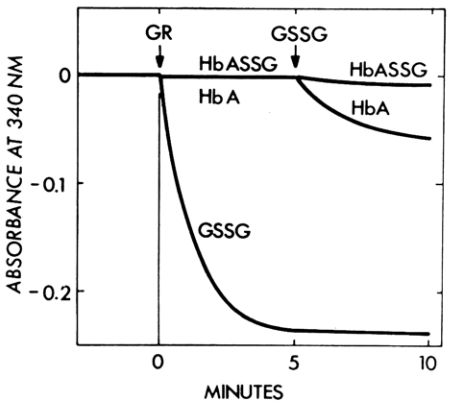

FIGURE 7: Spectrophotometric assay of the reaction of Hb ASSG (oxy form) with glutathione reductase from erythrocytes. In three experiments either 0.045 mM Hb ASSG, Hb A, or GSSG was incubated at 21° in 0.05 M Tris-HCl–1.25 mM EDTA (pH 7.5), with 0.13 mM NADPH in both reference and sample cuvets. The difference spectra were recorded at 340 nm. At $t = 0$, glutathione reductase was added to the sample cuvet in a final concentration of 0.047 IU/ml. At $t = 5$ min, 0.05 mM GSSG was added to the hemoglobin samples.

Hb ASSG (Figure 6). On the other hand, thiol group titration with *p*-ClHgBzO revealed the presence of 1 mol of reactive thiol groups/mol of dimer (Figure 2). Nbs_2 titrations of native and denatured Hb A_3 showed 1.0 and 2.5 equiv of thiol groups/dimer, respectively.

In addition, the electrophoretic properties of Hb A_3 were not influenced by treatment with mercaptoethanol. The quantity of Hb A_3 was approximated as 1% of the total hemoglobin, measured in either fresh hemolysates or in hemolysates converted to the ferric cyanide form.

From fresh hemolysates of several healthy subjects Hb A_3 was isolated as previously described and tested electrophoretically for the presence of naturally occurring Hb ASSG. The isolated fractions were incubated with mercaptoethanol under conditions sufficient for the reduction of Hb ASSG to Hb A. No reducible hemoglobin could be detected by this procedure indicating the absence of Hb ASSG. In addition, two hemolysates from patients with Heinz body anemia as a consequence of phenacetin abuse did not contain Hb ASSG at concentrations higher than the minimal detection limit (0.1% of the total hemoglobin).

(*c*) *Isolation of Glutathione Reductase from Erythrocytes.* The first step in the purification of erythrocyte glutathione reductase on CM-Sephadex resulted in a separation of the enzyme from the hemoglobin (Figure 4). Chromatography on Sephadex G-200 yielded an enzyme approximately 1000-fold purified. The results of the purification are summarized in Table I. The final preparation of the enzyme was specifically NADPH dependent and showed no oxidation activity in the absence of GSSG.

(*d*) *Reaction of Hb ASSG with either GSH or Glutathione Reductase.* As shown in Figure 7, Hb ASSG was not reduced by glutathione reductase from erythrocytes at concentrations at which GSSG is cleaved rapidly.[2] The same result was observed with the yeast enzyme. Even in concentrations of the enzyme up to twice as high as normally present in the red cell (2.1 IU/ml) no reduction of Hb ASSG could be observed electrophoretically. In contrast to this observation, Hb A was formed readily upon incubation of Hb ASSG with GSH at concentrations as normally encountered in erythrocytes (2.3 mM). When radioactively labeled Hb ASSG was treated with glutathione reductase from erythrocytes (2.1 IU/ml) and NADPH, the specific radioactivity of the hemoglobin did not change during 24 hr (Figure 8). However, if radioactive Hb ASSG was incubated with labeled GSH at erythrocytic concentrations, a rapid decrease of the protein bound radioactivity was observed. Under aerobic conditions an end value of only ~55% reduction is reached, presumably because of oxidation of GSH to GSSG (Allen and Jandl, 1961). In order to avoid decrease of the radioactivity of Hb ASSG by simple exchange of bound and free glutathione, the GSH used in this experiment was labeled with the same specific radioactivity as the Hb ASSG dimers. The reaction of Hb ASSG with either GSH or glutathione reductase in this experiment was further monitored by starch gel electrophoresis and yielded the same results.

TABLE I: Purification of Glutathione Reductase from Human Erythrocytes.

	Sp Act. (IU/mg of Protein)	Yield (%)
Hemolysate	0.0042[b]	100
After chromatography on CM-Sephadex[a]	0.21[c]	40
After chromatography on Sephadex G-200	4.0[d]	25

[a] The chromatography on CM-Sephadex is illustrated in Figure 4. [b] Determination of protein concentration on the basis of the absorbance of Hb at 540 nm. [c] Determination of protein concentration with Biuret on the basis of albumin. [d] Determination of protein concentration with Biuret and amino acid analysis.

Discussion

In a previous study we have shown that some of the unstable β variants of hemoglobin have the tendency to lose heme from the β chains, resulting in increased reactivity of 1 equiv of thiol groups/hemoglobin dimer (Jacob and Winter-

[2] It could be shown that in the presence of both Hb ASSG and Hb A at concentrations comparable to GSSG the activity of the reductase was diminished. This phenomenon was not further investigated but it was found to be reversed by higher GSSG concentrations.

halter, 1970). In a further investigation we demonstrated that an artificially altered hemoglobin A carrying heme only on the α chains exhibits approximately a tenfold increased reactivity of the sulfhydryl group in position 93 of the heme-depleted β chain (Birchmeier *et al.*, 1972). On the other hand, *in vitro* experiments suggested that a hemoglobin–glutathione mixed disulfide could be a naturally occurring aging product of Hb A (Huisman and Dozy, 1962; Huisman *et al.*, 1966). On the basis of the postulated alteration near the β-heme pocket in Hb ASSG, the natural breakdown of this compound was also thought to involve heme loss from the β chains, followed by an increased reactivity of the mixed disulfide toward thiol groups of the erythrocyte membrane. Such a breakdown mechanism was previously proposed for the β-variant Hb Köln ($\beta^{98\text{-Val}\rightarrow\text{Met}}$) which shows in comparison to Hb A an increased tendency to combine with glutathione and to form inclusions (Heinz bodies) in circulating red cells (Jacob *et al.*, 1968).

The present study was designed to reinvestigate the structure of Hb ASSG and its possible role in the erythrocyte. For this purpose Hb ASSG was synthesized from Hb A by incubation with excess GSSG. The bound glutathione in artificially produced Hb ASSG was restricted to a 1:1 ratio with the β chains. This association blocked the normally reactive sulfhydryl group in position β-93. Furthermore, the dissociation behavior of the Hb ASSG tetramer was not altered when compared with Hb A by gel filtration, indicating that the formation of the mixed disulfide could not have occurred at the internal sulfhydryl groups in positions β-112 and α-104 which both are involved in subunit contacts (Perutz *et al.*, 1968). It is therefore concluded that the mixed disulfide is located on the thiol group in position β-93 (F-9). In contrast to methods given previously, the synthesis used in the present study allowed a preparation of Hb ASSG in the ferro form without formation of methemoglobin.

In an attempt to find a naturally occurring Hb ASSG, radioactively labeled Hb ASSG was added to normal hemolysate and the mixture subjected to column chromatographies. Subsequently, a hemoglobin with chromatographic and electrophoretic properties identical with the ones of Hb ASSG was isolated in a yield of 1% from both normal and abnormal hemolysates. However, this protein had in contrast to artificially prepared Hb ASSG one titratable thiol group per dimer and could not be converted to Hb A by treatment with mercaptoethanol. These findings show that the isolated protein, referred to as Hb A_3 (Muller, 1961; Boyd *et al.*, 1967) and as a fraction of Hb A_1 (Huisman *et al.*, 1966), is not the mixed disulfide. On chromatography of both the native and the apoprotein subunits of the hemoglobin, which is designated as Hb A_3 in the present study, two different α and two different β chains could be observed in equal amounts. For one α and one β chain identical behavior with the components of normal Hb A was observed (preliminary results from our laboratory).

Since Hb ASSG was absent in both normal and abnormal hemolysates, it appears that this compound is either an aging product occurring only *in vitro* or is too unstable in the erythrocyte to build up to a detectable level. For a breakdown of the Hb ASSG formed in circulating erythrocytes different models are proposed (Allen and Jandl, 1961; Srivastava and Beutler, 1970; Rifkind, 1972). Rapid denaturation and intraerythrocytic precipitation seem unlikely in view of the observed high stability of Hb ASSG, which is comparable to that of Hb A at 4°; neither could we demonstrate the presumed instability of Hb ASSG upon incubation with thiol-carrying

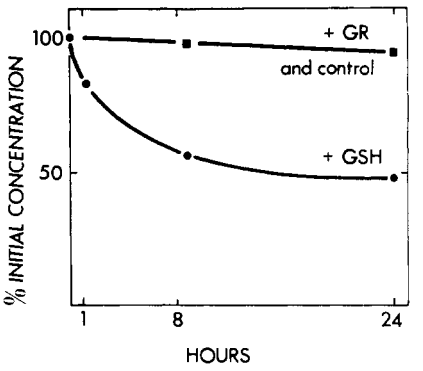

FIGURE 8: Reduction of Hb ASSG (oxy form) by GSH and glutathione reductase from erythrocytes. Tritiated Hb ASSG (0.028 mM) (160,000 dpm/μmol) was incubated at 21° in 0.05 M Tris-HCl–1.25 mM EDTA (pH 7.5) with: (●) 2.3 mM [^{3}H]GSH (160,000 dpm/μmol); (■) either 2.1 IU/ml of glutathione reductase plus 0.3 mM NADPH, 2.1 IU/ml of glutathione reductase alone, or buffer (control). After the indicated time aliquots of the incubation mixture were removed and the globin precipitated in a 100-fold volume of acetone–HCl. The acetone precipitable radioactivity is given as per cent of the initial value. After 24 hr a further aliquot was analyzed by starch gel electrophoresis.

Sephadex particles. Alternatively, a rapid reduction of the mixed disulfide of Hb ASSG by GSH used in concentrations comparable to the ones inside red cells could be demonstrated. In contrast, no reduction was observed by the action of glutathione reductase either from erythrocyte or yeast, consistent with the high specificity observed in the action of these enzymes. Results conflicting with our findings were recently reported by Srivastava and Beutler (1970). These authors detected an activity of glutathione reductase toward hemolysates containing small amounts of artificially produced Hb ASSG. However, their method of preparation of Hb ASSG differed from ours in that a stronger oxidant was used yielding presumably a product contaminated with methemoglobin or denatured material. On the basis of our results, we would like to propose that if Hb ASSG exists at all in the erythrocyte, it must have a short half-life as long as high concentrations of GSH are maintained in the cells.

Acknowledgments

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Interaction of Actin with Spin-Labeled Heavy Meromyosin in the Presence of Nucleotides†

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ABSTRACT: It has previously been shown that the mobility of spin labels attached to the fast reacting thiol groups of myosin (or its proteolytic subfragments) is decreased by the binding of F-actin and increased by nucleotide binding. In the present study the spin-labeling technique has been used to study the nature of the complex which exists when heavy meromyosin (HMM), F-actin, and nucleotide are simultaneously present. When actin is added to the spin-labeled HMM·ADP complex there is a decrease in spin-label mobility. This effect is dependent upon the actin concentration; saturation occurs at approximately 2 mol of actin monomer/mol of HMM. At low ADP concentrations the spectral effect of actin can be interpreted as a release of ADP from the nucleotide binding site and reversal of the nucleotide-induced conformational change. At high concentrations of ADP there is evidence for formation of a ternary actin·HMM·ADP complex in which the spectral change associated with nucleotide binding is reduced. In the presence of sufficient nucleoside triphosphate actin does not alter the mobility of the spin labels relative to that observed for the HMM·triphosphate complex alone. During ATP hydrolysis actin has no effect on the mobility of spin labels attached to HMM. However, spin-labeled HMM which has been affinity labeled with a 6-SH derivative of ATP (the resulting complex may mimic the predominant steady-state species) interacts weakly with actin as determined by spin-label mobility and viscosity.

The interaction of the thick filament protein (myosin) with the thin filament protein (actin) is a central event in the contractile cycle. Such interaction provides the mechanically continuous system requisite for tension development and also results in an acceleration of the energy yielding reaction of contraction, the myosin-catalyzed hydrolysis of ATP. From the work of Bárány and Bárány (1959) it is clear that the sites on myosin which bind nucleotide are distinct from those which bind actin. However, it is quite apparent that interaction between these sites occurs: ATP dissociates actomyosin; actin decreases the affinity of myosin for ADP (Kiely and Martonosi, 1969). Knowledge of this interaction on a molecular level is essential for a full understanding of the contractile mechanism.

The spin-labeling technique (Stone *et al.*, 1965; McConnell and McFarland, 1970) has proved useful for probing the conformation of discrete areas of the myosin molecule during interaction with nucleotides and actin. The SH_1 groups of myosin (whose modification results in an acceleration of the Ca^{2+}-moderated ATPase activity) are readily labeled with a paramagnetic derivative of iodoacetamide (Quinlivan *et al.*, 1969). Electron paramagnetic resonance (epr) spectra of the spin-labeled myosin indicate strong immobilization of the attached labels. Addition of actin causes a further small reduction in the mobility of the myosin-bound labels (Stone *et al.*, 1968; Seidel *et al.*, 1971; Tokiwa, 1971). Addition of nucleotides (in the absence of actin) has an opposite effect. The mobility of the labels is moderately increased when myosin is complexed with ADP or pyrophosphate (Seidel *et al.*, 1970; Stone, 1970) and markedly increased during steady-state hydrolysis of MgATP (Seidel and Gergely, 1971).

In the present study we have used the spin-labeling technique to study the nature of the complex which exists when

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