Appl Biochem Biotechnol (2011) 164:1422–1430
DOI 10.1007/s12010-011-9222-2

Biochemical Studies on Hemoglobin Modified with Reactive Oxygen Species (ROS)

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Received: 20 October 2010 / Accepted: 1 March 2011 /
Published online: 18 March 2011

Abstract Hemoglobin is the iron-containing oxygen transporting metalloprotein in the red cells of blood in mammals and other animals. Hemoprotein-mediated oxidative stress is thought to play a major role in pathophysiology of cerebral hemorrhage, blast pressure injury, crush injury, myocardial ischemia reperfusion injury. Hemoglobin undergoes oxidation–reduction reactions that lead to both generation and consumption of highly reactive oxygen and nitrogen species. In the present study, hemoglobin molecule was treated with hydrogen peroxide and the modification so incurred was analyzed by UV spectra, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detection of carbonyl content. Our observations suggest that carbonyl content increases with increase in concentration of hydrogen peroxide. Production of hydroxyl radical was assessed by using benzoate degradation analysis. Our results was in tandem with the fact that hemoglobin on treatment with hydrogen peroxide rapidly generates free-radical species that can degrade benzoate to thiobarbituric acid reactive material which on reacting with thiobarbituric acid gives color. The increase in absorbance of ROS-modified hemoglobin at 532 nm shows the increase in benzoate degradation, which is a parameter of hydroxyl radical formation with increase in concentration of hydrogen peroxide. Modified hemoglobin was treated with catalase, mannitol, thiourea, glutathion, sodium benzoate and their effect were detected by spectroscopy and SDS-PAGE (12%). Substantial scavenging effect of aforementioned antioxidants reiterates the formation of hydroxyl radical. Catalase shows the maximum scavenging effect followed by thiourea and mannitol.

Keywords Hemoglobin · Reactive oxygen species · Hydrogen peroxide

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Introduction

Under normal physiological conditions about, 3% of the total body hemoglobin (Hb) undergoes autoxidation every day producing metHb and superoxide [5]. Preservation of red cell integrity and functions require that the antioxidant enzymes and metHb reductase are able to cope with the oxidative load. The level of oxidative stress may increase in the presence of destabilizing hemoglobin mutations or chain imbalance. Three systems in which oxidative stress has recently been highlighted are sickle cell disease, thalassemia, and hemoglobin C disease [14, 15]. Hydrogen peroxide (H_2O_2), produced by superoxide dismutation or direct enzymatic production (SOD, amine oxidase, glucose oxidase), can be generated by a variety of mammalian cells, including neutrophils, macrophages, vascular smooth muscle, and endothelial cells. Oxyhemoglobin react with hydrogen peroxide to catalyze the removal of hydrogen peroxide, without being consumed in the process (a property that qualifies Hb as a pseudoperoxidase enzyme), and to generate ferrylhemoglobins as a transient intermediate [3]. Clinical use of cell-free Hb as a blood substitute was limited by the significant in vivo toxicities observed in clinical trials. These toxicities present as dysregulation of hemostasis and include hypertension, bradycardia [12], mild prolongation of partial thromboplastin time [3], disseminated intravascular coagulation with resultant thrombosis and ischemic parenchymal damage [2]. We have shown in previous studies that heme degradation is a reliable indicator of the formation of reactive oxygen species (ROS) in red cells both in vitro and in vivo. This is based on the reaction of hydrogen peroxide with hemoglobin, which initiates a cascade of oxidative reactions, resulting in degradation of heme with the formation of two fluorescent heme degradation products and the release of iron [9]. This reaction occurs when either hemoglobin or red cells react with hydrogen peroxide, but also during hemoglobin autoxidation, during the storage of red cells and even in vivo.

In the present study, we have modified hemoglobin molecule with hydrogen peroxide. ROS-modified hemoglobin was ascertained by UV spectra, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and formation of carbonyl content. Benzoate degradation experiment was performed to confirm the formation of hydroxyl radical. We also studied the effect of different antioxidants on hydrogen peroxide mediated oxidation and hydroxyl radical production.

Material and Methods

Chemicals

Hemoglobin was purchased from Sigma chemicals, USA. Hydrogen peroxide was from Merk. Acrylamide, APS, bis-acrylamide, formaldehyde, guanidine hydrochloride, SDS, sodium acetate, sodium carbonate, TCA, Tris and 2, 4-DNPH (dinitrophenylhydrazine) were from SRL, India. Acetic acid, disodium hydrogen phosphate, ethanol, glycerol, mannitol, methanol, sodium benzoate, sodium chloride, sodium hydroperoxide, sodium dihydrogen phosphate, and thiourea were purchased from Rankem, India. Agarose Catalase, EDTA and TEMED were from Himedia, India and all other chemicals used were of highest purity and of analytical grade.

Modification of Hemoglobin with Hydrogen Peroxide

Hemoglobin (250 μg/ml) was incubated with various concentrations of H_2O_2 in PBS (pH 7.4). The reaction was performed for 2 h at 37°C. UV spectra were plotted on Systronic

double beam spectrophotometer-119 between 200 and 700 nm. Percent hypochromicity and variation in λmax were calculated.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed by the Tris–Glycine system [6], using slab apparatus (Genei, India). We have performed 12% SDS-PAGE. Protein samples were prepared to give final concentration of 0.0625 M Tris–HCl (pH 6.8) and 10% (*v*/*v*) glycerol and trace of bromophenol blue as tracking dye. Protein bands were detected by staining the gels with 0.1% Coomassie Brilliant Blue R–250 in 40% isopropanol and 10% acetic acid and destaining was carried out with 10% of glacial acetic acid.

Silver Staining Procedure

After electrophoresis, the protein bands were fixed by immersing the gel in a mixture of 40% methanol and 13.5% formaldehyde for 15 min with instant shaking. The gel was washed with distilled water twice and transferred to 0.02% $Na_2S_2O_3$ solution for 2 min, then gel was again rinsed twice with distilled water. This was followed by incubation with 0.1% silver nitrate solution for 20 min, the gel was rinsed with distilled water briefly immersed in developer solution (3% sodium carbonate solution containing 0.5% formaldehyde and 0.02% $Na_2S_2O_3$) for 15 min or until properly stained. The reaction was stopped by transferring the gel to stopper solution (25% isopropanol solution containing 10% acetic acid) for 5 min. The gel was washed twice with distilled water and finally stored in distilled water [8].

Determination of Carbonyl Content

Carbonyl contents of native and ROS-modified hemoglobin were analyzed [7]. The reaction mixture containing 15 μM native Hb and ROS-Hb, 0.5 ml of 10 mM 2,4-DNPH/2.5 M HCl was added and thoroughly mixed. After addition of 250 μM TCA (20%) and centrifugation the pellet was collected and washed three times with 1 ml ethanol/ethyl acetate (1:1) mixture. The pellet was then dissolved in 1 ml of 6 M guanidine solution and incubated at 30°C for 15 min. After centrifugation, the supernatant was collected and carbonyl contents were estimated from the absorbance at 370 nm using a molar absorption coefficient at 22,000 M^{-1} cm^{-1}. Samples were spectrophotometrically analyzed against a blank of 1 ml of guanidine solution (6 M). Protein content was also measured [4].

Benzoate Degradation Assay for Hydroxyl Radical

Benzoate can be degraded by hydroxyl radicals to release thiobarbituric acid reactive material, which can be measured spectrophotometrically [11]. A 0.2 ml portion of 10 mM benzoate, 0.2 ml of protein sample (14.5 μM), 0.1 ml of iron complex and 0.1 ml of appropriate inhibitor were mixed, and the reaction was started by the addition of 0.1 ml of 5 mM hydrogen peroxide. Samples were incubated for 1 h at 37°C. Then 0.2 ml of thiobarbituric acid (1% *w*/*v*) in 50 mM NaOH was added to each tube followed by 0.2 ml of 2.8% (*w*/*v*) trichloroacetic acid. The tubes and their contents were heated for 10 min at 100°C to develop the pink colored chromogen. Benzoate samples were measured spectrophotometrically at 532 nm against a blank which did not contain hemoglobin samples.

Assay of Superoxide Radicals

Superoxide radical was detected by the reduction of nitroblue tetrazolium (NBT) [10]. A typical assay was performed in total volume of 3.0 ml PBS pH 7.4, 33 mM NBT, 0.1 mM EDTA and 0.06% triton X-100. The reaction was started by addition of native hemoglobin and hemoglobin samples treated with H_2O_2. The reaction mixture was measured spectrophotometrically at 560 nm against a blank, which did not contain hemoglobin samples.

Effect of Various Scavengers

Various free-radical scavengers were added in experiments of hydrogen peroxide-mediated hemoglobin modification. The different concentrations of these scavengers were used. The scavenging effects were analyzed by spectral and PAGE studies.

Results

Modification of Human Hemoglobin by H_2O_2

Hypochromicity represents the modification of hemoglobin. Hemoglobin shows maximum hypochromicity (93.7%) at 31 mM concentration of H_2O_2 (Fig. 1). Hemoglobin shows maximum modification after incubation for 2 h.

Polyacrylamide Gel Electrophoresis

Two sharp bands were obtained for native Hb molecules while diminished bands of lower intensity were obtained for modified hemoglobin. Molecular weight of both bands was analyzed by standard protein molecular weight marker and it was found to be

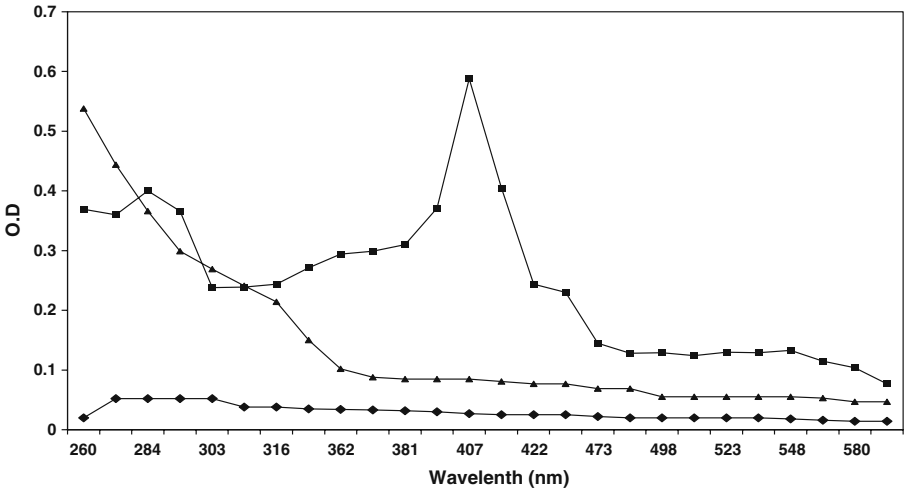

Fig. 1 UV spectra of native hemoglobin (*black square*), H_2O_2 modified hemoglobin (*black triangle*), and H_2O_2 alone (*black diamond*)

approximately 31 and 14.5 KDa (Figs. 2 and 7). Silver staining was performed to detect the fragmented hemoglobin.

Measurement of Carbonyl Content

The increase in absorption at 370 nm shows increased carbonyl content in modified Hb. The carbonyl content at 31 mM H_2O_2 was found to be maximum, i.e., 12.8 nmol/mg of protein (Fig. 3).

Detection of Superoxide by NBT Test

No increase in absorbance was found at 560 nm after addition of NBT in modified hemoglobin. Thus, one can infer that no superoxide radical was formed after modification of hemoglobin with H_2O_2.

Detection of Hydroxyl Radical by Benzoate Degradation

The increase in absorbance at 532 nm shows the increase in benzoate degradation, which is a parameter of hydroxyl radical formation. With increase in concentration of H_2O_2,

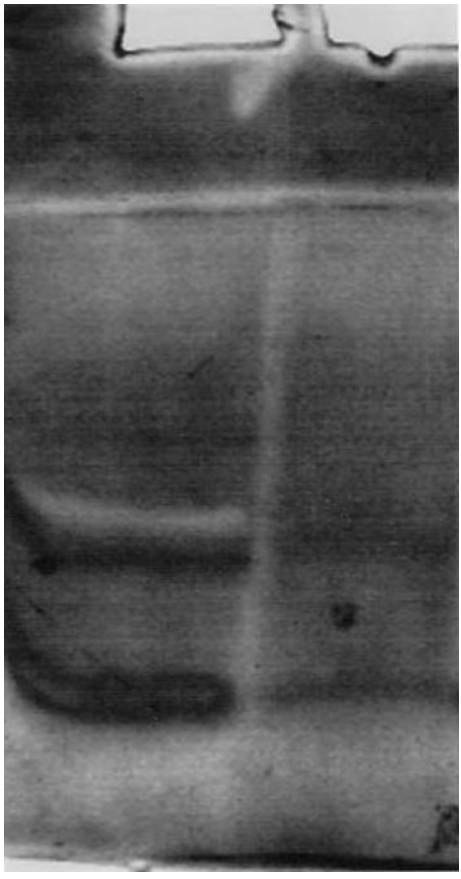

Fig. 2 Twelve percent SDS-PAGE of native and modified Hb with silver staining. *a* Native Hb and *b* modified Hb

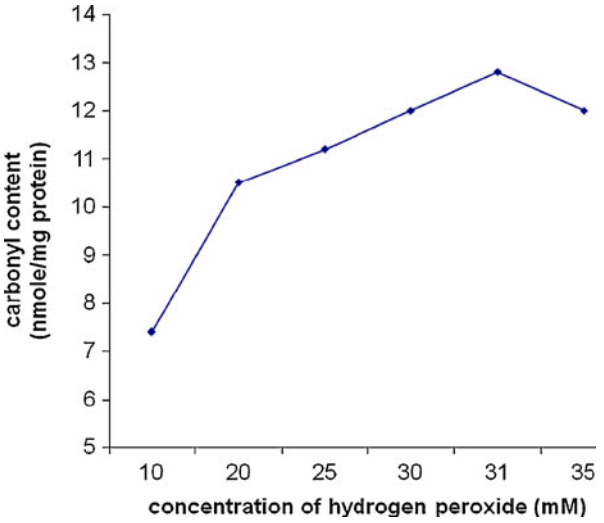

Fig. 3 Plot of carbonyl content versus varied concentrations of H_2O_2

hydroxyl radical increases and it showed threshold at 31 mM concentration (Fig. 4). Generation of hydroxyl radical were further substantiated by quenching with mannitol, thiourea, and catalase in which thiourea shows maximum scavenging effect as shown in Fig. 5.

Effect of Various Antioxidants on Protein Modification

Modified hemoglobin was treated with catalase, mannitol, thiourea, glutathione, and sodium benzoate and their effects were detected by spectroscopy and SDS-PAGE (12%). Catalase shows maximum scavenging effect followed by thiourea and mannitol as shown in Fig. 6. Effect of thiourea on modified hemoglobin was detected by 12% reducing SDS-PAGE. Marked difference in bands of thiourea-treated modified hemoglobin shows substantial scavenging effect (Fig. 7).

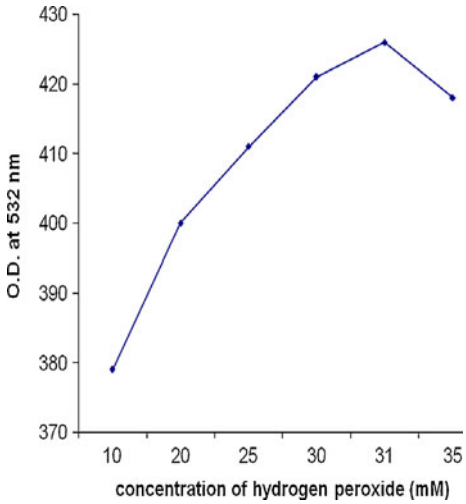

Fig. 4 Benzoate degradation profile at varied concentration of H_2O_2

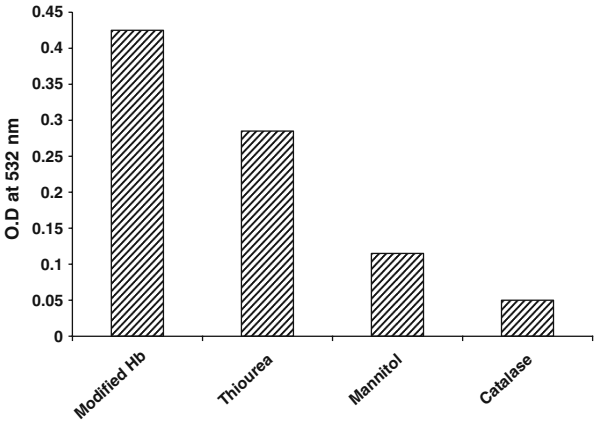

Fig. 5 Effect of various scavengers on hydroxyl radical generation by benzoate degradation method

Discussion

Hemoprotein-mediated oxidative stress is thought to play a major role in pathophysiology of cerebral hemorrhage, blast pressure injury, crush injury, and myocardial ischemia reperfusion injury. Heme-mediated oxidative injury or dysregulation of the vasculature is also an important concern with the use of oxygen carrying blood substitute based on modified form of hemoglobin. The mechanism of cytotoxicity and vascular dysfunction are thought to involve the interaction with reactive oxygen and nitrogen species [4].

Our results indicated that in experimental conditions oxidation of hemoglobin is maximum which is measured by percent hypochromicity and decrease in absorbance at 415, 541, and 577 nm. We have also observed the effect of increasing time on hemoglobin oxidation, which indicates that the oxidation of hemoglobin is starts instantly with the addition of H_2O_2.

Hemoglobin in presence of H_2O_2 produces carbonyls. Our data indicated that carbonyl content increases with increase in concentration of H_2O_2. Reaction of hemoglobin with

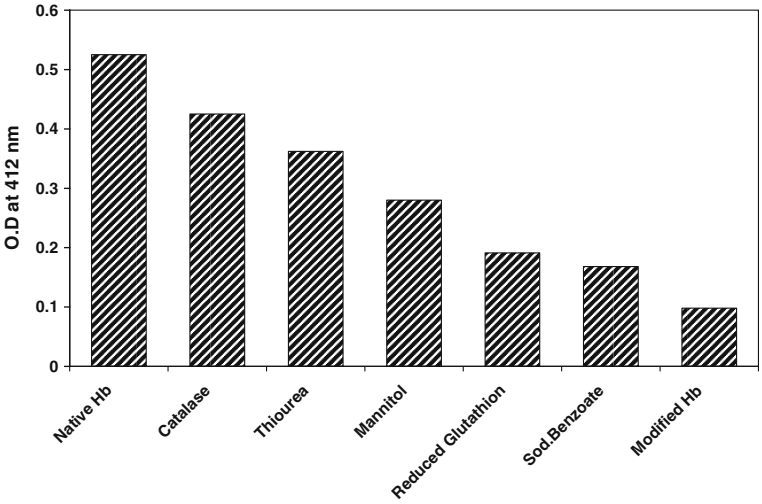

Fig. 6 Effect of various scavengers on hemoglobin modified with H_2O_2

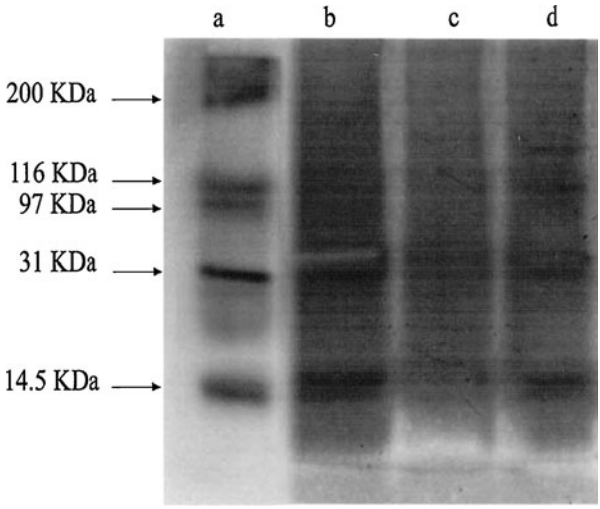

Fig. 7 Twelve percent SDS-PAGE of thiourea-treated modified Hb with silver staining. *a* MW marker, *b* native hb, *c* modified hb, and *d* modified hb after thiourea treatment

H_2O_2 generates a free radical which oxidizes amino acid residues at or near the cation binding site introducing carbonyl groups, which provides a moiety for quantification with DNPH. Such oxidative modification is an index of oxidative stress and may be significant in several physiological and pathological conditions. Previous studies have shown carbonyl formation in glycated hemoglobin [13].

Superoxide radical is known to cause reduction of NBT as a result absorbance of reaction mixture increases [16]. But NBT assay of superoxide radical in our case does not show any increase in absorbance thereby indicating that the generation of reactive oxygen species by Hb in experimental conditions was shadowed by the formation of metHb and its release of iron ions. These findings suggest that superoxide radicals produced from modified Hb may further react with water to form hydrogen peroxide and so used for further reactions. So, superoxide does not play a critical role in hemoglobin modification on treatment with H_2O_2 in experimental conditions.

Previous studies have shown that H_2O_2 generate hydroxyl radical on treatment with heme which degrade deoxyribose or benzoate with the release of thiobarbituric acid reactive material which on reacting with thiobarbituric acid give color to reaction mixture [11]. Our results suggest that hemoglobin on treatment with H_2O_2 rapidly generates reactive species that can degrade benzoate to thiobarbituric acid reactive material which on reacting with thiobarbituric acid give color. Treatment of modified hemoglobin with scavengers like thiourea, mannitol, sodium benzoate, and reduced glutathione causes reduction of hemoglobin modification as shown by decrease in hydroxyl radical content. This antioxidant treatment confirmed the result that hemoglobin on treatment with H_2O_2 leads to the generation of hydroxyl radicals which are scavenged by known hydroxyl radical scavengers. Met-hemoglobin produced from autooxidation and modification with H_2O_2 readily converted to ferryl heme in presence of hydroxyl radicals and hydrogen peroxide.

Now it has been suggested that the reaction of hemoglobin with H_2O_2 can cause heme degradation and the iron is released via ferrylhemoglobin which is a strong oxidizing agent that is believed to mediate the peroxidation of lipids, proteins, carbohydrates, and nucleic acids [1].

Catalase responsible for the majority of H_2O_2 removal in experiment with red blood cells is known by previous studies [16]. Our observations also support these findings; in

each system, we examined catalase shows the 96% inhibition of oxidation caused by H_2O_2. Thus, free Hb is toxic. The ability of Hb to generate reactive oxidant species and iron ions in the presence of H_2O_2 provides a logical explanation of this toxicity. H_2O_2 decomposes heme and release iron ions from the oxidized protein these then react with H_2O_2 to form hydroxyl radical which may account for the damaging effects of free hemoglobin in the brain, the eye, and at sites of inflammation.

Acknowledgment We are very thankful to S.B.S.P.G. Institute of Biomedical Sciences and Research for providing the working facilities.

References

1. Alayash, A. I., & Cashon, R. E. (1995). *Molecular Medicine Today, 1*, 122.
2. Everse, J., & Hsia, N. (1997). *Free Radical Biology & Medicine, 22*, 1075–1099.
3. Giulivi, C., & Davies, K. J. (1990). *The Journal of Biological Chemistry, 265*, 19453–19460.
4. Goldman, D. W., Breyer, R. J., Yeh, D., Brockner-Ryan Beth, A., & Alayash, A. I. (1998). *American Journal of Physiology-Heart and Circulatory Physiology, 275*, 1046.
5. Jaffe, E. R., & Neurmann, G. (1964). *Nature, 202*, 607–608.
6. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
7. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., et al. (1990). *Methods in Enzymology, 186*, 464–478.
8. Merril, C. R., Dunau, M. L., & Goldman, D. (1981). *Analytical Biochemistry, 110*, 201.
9. Nagababu, E., & Rifkind, J. M. (2004). *Antioxidants Redox Signaling, 6*, 967–978.
10. Nakayama, T., Kimura, T., Kodama, M., & Nagata, C. (1983). *Carcinogenesis, 4*, 765–769.
11. Puppo, A., & Halliwell, B. (1988). *The Biochemical Journal, 249*, 185–190.
12. Savitsky, J. P., Doczi, J., Black, J., & Arnold, J. D. (1978). *Clinical Pharmacology and Therapeutics, 23*, 73–80.
13. Sen, S., Kar, M., Roy, A., & Chakraborti, A. S. (2005). *Biophysical Chemistry, 113*, 289.
14. Shikama, K. (1998). *Chemical Reviews, 98*, 1357–1374.
15. Steinberg, B. G., Higgs, D. R., & Nagel, R. L. (2001). *Disorders of hemoglobin: Genetics, pathophysiology and clinical management*. UK: Cambridge University Press.
16. Winterbourn, C. C., & Stern, A. (1987). *Journal of Clinical Investigation, 80*, 1486.