Determination of the Amounts and Oxidation States of Hemoglobins M Boston and M Saskatoon in Single Erythrocytes by Infrared Microspectroscopy*

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The reduced abnormal subunits of two M-type hemoglobins, Boston (His→Tyr) and Saskatoon (His→Tyr), have been determined in the presence of normal human hemoglobin A by measurement of C=O stretch bands in infrared spectra of carbon monoxide complexes. Use of an infrared microscope coupled to a Fourier transform infrared spectrometer of high sensitivity permitted measurements to be made on as small a hemoglobin mixture as is contained in a single erythrocyte. The abnormal subunits of both HbS exhibit bands near 1970 cm⁻¹ compared with bands near 1951 cm⁻¹ for the normal subunits. The increase in 1970 cm⁻¹ band intensity upon erythrocyte reduction with dithionite provided a measure of the extent of abnormal subunit oxidation; in cell suspensions about 60% of the abnormal subunits of Hb M Boston and 80% for Hb M Saskatoon remained reduced. The amount of Hb present as abnormal Hb averaged about 25% for Hb M Boston cells and about 50% for Hb M Saskatoon cells. However, the ratio of Hb M to Hb A in individual cells varied markedly, with the ratio expected to decrease as the cell ages. These results demonstrate the unique utility of infrared microspectroscopy for the study of differences in abnormal Hb status among individual erythrocytes.

Red blood cells of individuals with hemoglobin M disease contain mutant Hb molecules in which the ability of abnormal subunits to transport O₂ is compromised since they are unstable and unable to be maintained as a reduced species under physiological conditions (1–3). Clinically, the presence of Hb M is accompanied by chronic cyanosis caused by methemoglobinemia (4). Five M-type Hb variants have been identified, and two of them, namely Hb M Boston (α₂β²(58→Tyr)), and Hb M Saskatoon (α₂β²(63→Tyr)), have the distal histidine replaced by a tyrosine residue (5). The structural and functional properties of Hb M Boston and Hb M Saskatoon have been extensively studied (4–15). The x-ray crystallographic analysis of Hb M Boston in the natural valence hybrid (α₂β²(63→Tyr)) has been carried out by Pulsinelli et al. (16). The ferric iron atoms in the abnormal α subunits were found bonded to the tyrosines that replaced distal histidines. Both proteins were also studied by using various spectroscopic techniques including infrared (17), circular dichroism (18), nuclear magnetic resonance (19), electron paramagnetic resonance (20, 21), and resonance Raman (14, 22). A high frequency infrared C=O stretching band, which arises from the abnormal subunits, was observed near 1970 cm⁻¹ in both reduced Hb M Boston and Hb M Saskatoon carbonyls (17, 23).

A homozygous Hb M carrier has never been found, presumably because the absence of at least some Hb A is incompatible with fetal survival. The percentage of Hb M molecules in the total Hb molecule population for an individual heterozygous carrier was found to be about 20–30% for Hb M Boston (6, 7, 24) and 20–50% for Hb M Saskatoon (8, 21, 25, 26). However, little is known about the distribution and oxidation state of abnormal Hb molecules within individual erythrocytes. In order to fully understand the genetic expression, biological synthesis, degradation, and oxidation state of mutant Hb molecules in circulating red cells, it is important and highly desirable to study them in a single cell under physiologically relevant conditions. Recent developments in infrared instrumentation, in which a microscope with infrared optics is coupled to a Fourier transform infrared spectrophotometer, have greatly enhanced the sensitivity of infrared spectroscopy and enable us to study the individual cells under physiologically relevant conditions (27). In the present study, we have examined the IR spectra of erythrocytes that contain Hb M in single cells as well as in cell suspensions. Two discrete C=O stretch bands near 1970 and 1956 cm⁻¹ from CO bound to reduced heme iron were observed in both Hb M Boston- and Hb M Saskatoon-containing erythrocytes exposed to carbon monoxide. The proportion of functional (Fe²⁺) heme relative to oxidized heme in the abnormal subunits of Hb M molecules in individual erythrocytes varied widely. Hb M Boston erythrocytes averaged only about 25% of total Hb molecules as abnormal Hb, whereas Hb M Saskatoon cells contained about 50% Hb M and 50% Hb A. About 60% of abnormal α subunits of Hb M Boston and about 80% of abnormal β subunits of Hb M Saskatoon were fully reduced, as demonstrated by the ability to bind CO without addition of reductant.

MATERIALS AND METHODS

Cell Preparation—Freshly drawn blood from individual donors with Hb M disease was saturated with CO by passing CO gas over the top of a blood sample for 30 min. The cell sample for the single cell infrared (SCIR) analysis was prepared as described previously (23, 27). Hbs M Boston and M Saskatoon were isolated and purified as described previously (28).

Measurement of the CO-IR Spectrum of a Single Erythrocyte Exposed to CO—The CO-IR spectra were measured by placing the cell suspens-

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1 The abbreviation used is: SCIR, single cell infrared spectroscopy.
**RESULTS**

**CO-IR Spectra of Hb M Boston-containing Erythrocytes**—Fig. 1 shows infrared spectra of CO-saturated Hb M Boston-containing single erythrocytes, cell suspension, and reduced purified Hb M Boston and Hb A carbonyls. In order to determine the oxidation state of abnormal subunits of Hb M molecules under circulating conditions, the blood sample was saturated with CO gas when freshly drawn and then stored under CO to prevent further autoxidation. Two discrete C–O stretch bands were clearly observed in about 40% of the single erythrocytes examined by SCIR as they were in cell suspensions and solutions of purified Hb M Boston. The unusual C–O stretch band near 1970 cm\(^{-1}\) has been assigned to the normal \(\alpha\) subunits of Hb M Boston (His\(^{67}\) \(\rightarrow\) Tyr); the band near 1951 cm\(^{-1}\) can be assigned to the normal \(\beta\) subunits of Hb M Boston and the \(\alpha\) and \(\beta\) subunits of Hb A (17, 23, 29). The large separation between the C–O stretch frequencies of abnormal \(\alpha\)-CO and normal \(\beta\)-CO (19 cm\(^{-1}\)) of Hb M Boston permits quantitative as well as qualitative study of the mutant subunits in single erythrocytes as well as in cell suspensions. The intensity of the 1970 cm\(^{-1}\) band varied widely among individual erythrocytes. The most intense 1970 cm\(^{-1}\) band found for a single erythrocyte represented about 15% of total integrated band intensity.

The percentage of total Hb that is Hb M Boston can be estimated from the integrated intensity of C–O stretch bands that remains after subtraction of the C–O band caused by Hb A under fully reduced conditions (Fig. 2A). The integrated intensity under the 1970 cm\(^{-1}\) band of a typical CO-saturated fully reduced cell suspension amounted to 12.5% of the total integrated C–O band area, which indicates that 25% of the Hb present is Hb M Boston. Since CO molecules bind only to reduced heme iron (Fe\(^{2+}\)), the percentages of both reduced and oxidized Hb M Boston molecules under physiological conditions can be obtained by comparing the CO-IR spectra of cell suspensions saturated with CO as freshly drawn with spectra after sodium dithionite reduction. This approach revealed about 60% of abnormal \(\alpha\) subunits of Hb M Boston to be in the functional reduced form (Fe\(^{2+}\)) and about 40% in the non-functional oxidized form (Fe\(^{3+}\)). This result corresponds to about 5% methHb.
Infrared spectroscopy has been very useful in studies of the structure and reactions of hemoglobin (23). Earlier studies were limited to the purified proteins (29, 30) and cell suspensions (31, 32). Recent developments in infrared microspectroscopy make possible the extension of these studies to the individual erythrocyte (27). In this way information on differences between erythrocytes in areas such as mutant gene expression, abnormal protein distribution over a cell population, the oxidation state of subunits, and the binding of infrared-active ligands such as CO can be obtained.

**Distribution of Hb M Molecules among Erythrocytes**—In the present study, the erythrocytes from donors with Hb M Boston or Hb M Saskatoon disease have been studied individually as well as collectively by using Fourier transform infrared spectroscopy. For Hb M Boston-containing erythrocytes, an average of about 25% of Hb molecules in the cell population are found to be M-type Hb on the basis of the infrared spectrum of CO-saturated fully reduced erythrocyte suspension. The C–O stretch band at 1970 cm⁻¹ from the normal α subunits of Hb M Boston was observed only in two-fifths of the erythrocytes that were examined by SCIR. However, it is not likely that the remaining cells truly contain no mutant Hb. There are several possible explanations for the absence of an abnormal α-CO band in some of the erythrocytes examined by SCIR. First, the abnormal α subunits of Hb M Boston in those cells may be essentially all in the oxidized form (Fe³⁺), which is resistant to enzymatic reduction by metHb reductases (28). Second, the 1970 cm⁻¹ band may be too weak to be clearly detected in view of the limited sensitivity and signal-to-noise ratio of infrared microspectroscopy. Third, the inherent instability of the Hb M may have resulted in modifications in protein structure that prevent CO binding. The variations in band intensity of the 1970 cm⁻¹ band of single cells may reflect differences in cell age; the abnormal Hb M molecules are expected to be synthesized as reduced species in newly matured erythrocytes. Because of exposure to oxidizing conditions during the erythrocyte life span Hb M molecules will be gradually oxidized and will remain so because of their resistance to enzymic reductions (28). Therefore, it is not surprising that, as found, the most intense 1970 cm⁻¹ bands observed in some erythrocytes are actually more intense than that observed in a fully reduced cell suspension.

For Hb M Saskatoon-containing erythrocytes, an average of about 50% of the Hb molecules are found to be the M-type Hb. The C–O stretch band at 1970 cm⁻¹ from abnormal β subunits has been observed in almost all of the erythrocytes examined by SCIR. However, the intensities of these bands varied widely among individual cells. The most intense 1970 cm⁻¹ band observed in a single erythrocyte represented ~30% of integrated intensity of the CO-IR bands (suggesting a Hb M/Hb A ratio of 3/2), which is much more intense than the ~25% of total intensity observed in a reduced cell suspension. It should be noted that the single Hb M Saskatoon-containing erythrocytes were examined at a resolution of 8 cm⁻¹ rather than the 4 cm⁻¹ resolution used to examine the cell suspension, thereby making the single cell determination less accurate. However, these findings may indicate that the mutant Hb M Saskatoon gene can be expressed at a higher rate than Hb A in the cells.

**Oxidation State of Hb M Molecules in Circulation**—Treatment of a freshly drawn blood sample with CO gas allows CO molecules to replace the O₂ ligands from heme iron (Fe⁺⁺) of hemoglobins and to prevent further autoxidation. Therefore, the oxidation state of Hb M molecules in circulating erythrocytes can be determined from CO-IR spectra. About 5% metHb was detected in both Hb M Boston- and Hb M Saskatoon-containing blood samples, and almost all of the metHb comes...
from the abnormal subunits. Two major metHb reductase systems have been found in human erythrocytes: NADH-dependent metHb reductase identified as NADH-cytochrome b5 reductase (33, 34) and NADPH-dependent metHb reductase identified as NADPH-flavin reductase (35). The most important pathway of metHb reduction utilizes NADH-cytochrome b5 reductase for the transfer of electrons from NADH to heme irons (36). The level of metHb in circulating blood of normal adults is maintained under 0.6% of total Hbs by the metHb reductases (4, 36, 37). The reduction of Hb M Boston, Hb M Saskatoon, and other M-type Hbs by various metHb reductases purified from human erythrocytes and by chemical reductants has been extensively studied (28, 38). It has been found that metHb M Saskatoon can be reduced by NADH-cytochrome b5 reductase at a rate comparable with that of metHb A (28). On the other hand, no enzymatic reduction of metHb M Boston was observed in the presence of NADH-cytochrome b5 reductase (38). The results of our present study show that about 80% of Hb M Saskatoon molecules in erythrocytes are maintained as reduced species that are able to bind CO ligand, whereas only 60% of Hb M Boston molecules in the cells exist as reduced species. The higher percentage of mutant Hb usually found in the patients with Hb M Saskatoon appears related to the greater ability of the erythrocyte to keep this type of Hb mutation in a stable reduced state.

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