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MULTIPLE FORMS OF SULFMYOGLOBIN AS DETECTED BY ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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The proton nuclear magnetic resonance spectrum of sulfmyoglobin prepared in standard fashion reveals the presence of three forms, A, B, and C, with different chemical reactivity. Conditions for some interconversions of these forms are given. The ^{1}H NMR spectra of the different forms show similar patterns. It appears that the differences between forms involve chemical modification on the porphyrin periphery. The altered heme can be extracted from Fe^{III}(CN)sulfmyoglobin C to give a stable green substance.

Sulfhemoglobin is a non-functional form of the protein which can occur under physiological conditions (1). Sulfmyoglobin (SMb) is an analogous substance which is frequently used to facilitate the study of the structural modification of the protein (2-4). The method of sulfmyoglobin formation involves the sequential addition of hydrogen peroxide (to generate the ferryl $(FeO)^{2+}$ form), catalase (to remove excess peroxide), and sulfide to myoglobin (2) followed by chromatography to remove excess reagents. The heme is believed to be reduced to give a chlorin-type unit with possible sulfur incorporation (5,6), but the exact structure is not established. Removal of the modified heme from previous preparations has yielded an unstable green substance that rapidly converts to protoheme before allowing definitive characterization (4). Previous studies of sulfmyoglobin have presumed a single, although problematically purifiable, species. Here we present ^{1}H NMR data which allow for the detection of multiple forms of sulfmyoglobin which are created during the stan-

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Abbreviations: Mb, myoglobin; SMb, sulfmyoglobin; NMR, nuclear magnetic resonance; ppm, parts per million.

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dard method of preparation and meet former standards of purity. These forms display significant chemical differences. Moreover, one of these provides the first stable derivative which may facilitate full structural characterization. The identity of the species formed under a particular set of conditions and the ability to detect heterogeneity is essential for the evaluation of all physical data involved in structural characterization of these substances. As found for intermediates formed during the reconstitution of heme proteins, high field NMR spectroscopy is ideally suited for detecting entities which are not differentiated by optical spectroscopy (7).

METHODS

Sperm whale myoglobin was purchased from Sigma Chemical Co. and used as received. Solutions of Fe^{II} SMb (~4 mM in either H_2O or ^2H_2O) were prepared by the successive addition of hydrogen peroxide, catalase, and $(NH_4)_2S$ to sperm whale myoglobin (2). These preparations were monitored by optical spectroscopy. The ratio of A_{617}/A_{561} ranged from 2.9 to 3.2 and indicated a greater than 90% conversion occurred (2). To obtain the NMR spectrum shown in Figure 1, Trace I, the green Fe^{II} form was oxidized to the brown Fe^{III} form by the injection of 40 µl of 0.2 M $K_3Fe(CN)_6$ in ^2H_2O followed by the addition of 100 µl 1 M pH 6 potassium phosphate buffer in ^2H_2O and observed *in situ* without chromatography. To obtain the spectrum shown in Figure 1, Trace II, the green Fe^{II} form was subject to chromatography on Sephadex G-25 (1 x 36 cm) which was equilibrated with 0.1 M potassium phosphate buffer, pH 8.0, in H_2O. The green band was eluted with 0.1 M potassium phosphate buffer, collected, and concentrated through the use of ultrafiltration (Amicon YM-5 membrane). The sample was exchanged five times with a 0.1 M potassium phosphate pH 8.0 buffer in ^2H_2O using ultrafiltration. The sample was then oxidized to the brown Fe^{III} form as described above.

The spectra shown in Figure 2 were obtained from a sample of Fe^{II} SMb which was chromatographed on Sephadex G-25, oxidized by passage through a second column of Sephadex G-25 layered with $K_3Fe(CN)_6$ as described in the standard procedure (2), and then concentrated and exchanged with ^2H_2O as described above. Conversion to the cyano form was accomplished by the addition of 10 µl of 1.0 M KCN in ^2H_2O to the sample before NMR analysis.

Extraction into 2-butanone of the hemin from Fe^{III}(CN)SMb C was performed by the method of Teale (8).

360 MHz ^1H NMR spectra of the proteins were obtained on a Nicolet NTC-360 spectrometer. Typical spectra consisted of 1000 to 10000 transients of 8192 points using a 7 µs 90° pulse. The residual water signal was suppressed by a low power pulse. All chemical shifts are given in ppm from internal 2,2-dimethyl-2-silapentane-5-sulfonate.

RESULTS

Treatment of myoglobin under the conditions described by Berzofsky et al. (2) gives a solution of Fe^{II}SMb A. This solution is stable for periods of more than one day in the A state. However, in order to detect the various

forms of sulfmyoglobin, it is informative to oxidize the sample to the Fe^{III} state which produces ^1H NMR spectra with greater hyperfine shifts and greater resolution. Thus, oxidation of the sample of Fe^{II}SMb A with ferricyanide gives the ^1H NMR spectrum shown in Trace I of Figure 1. Peaks due to Fe^{III}SMb A, and resonances of unreacted Mb are labeled A and M, respectively. Resonances whose relative intensity indicates that they are methyl resonances are denoted by an asterisk. A different spectrum results when the same sample is subjected to chromatography on the G-25 Sephadex column in the Fe^{II} form, and then oxidized after concentration. That spectrum is shown in Trace II of Figure 1. New peaks due to a second form, Fe^{III}SMb B, are labeled B. A control sample, which was not subject to the chromatography, concentration, and ^2H_2O exchange process, did not show the peaks of Fe^{III}SMb B when oxidized after the same time interval necessary to perform these operations. This same new set of peaks is also formed when a sample of Fe^{III}SMb A is allowed to stand at pH

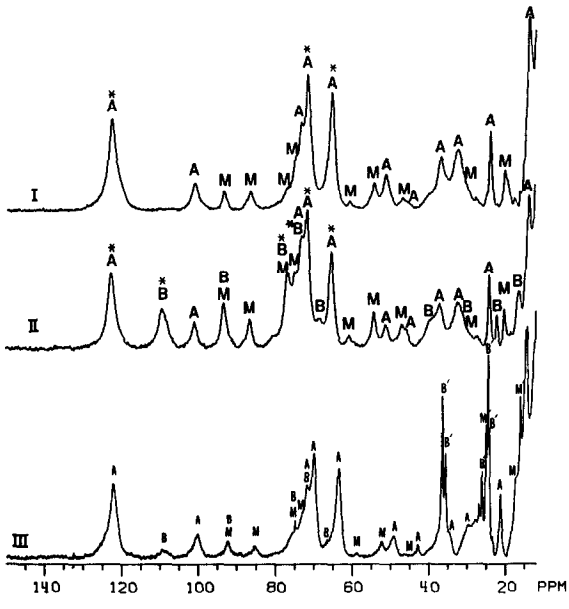

Figure 1. The 360 MHz ^1H NMR spectra of: I, Fe^{III}SMb A in ^2H_2O at pH = 7.02 and 20°C directly after preparation and oxidation with $K_3Fe(CN)_6$; II, a mixture of Fe^{III}SMb A and B produced upon chromatography of the Fe^{II} form through Sephadex G-25 followed by concentration, exchange with ^2H_2O and oxidation by $K_3Fe(CN)_6$ at pH = 7.02 and 20°C; III, the same sample as II after the addition of KCN, B' , M' designate the cyano forms of B and M, respectively.

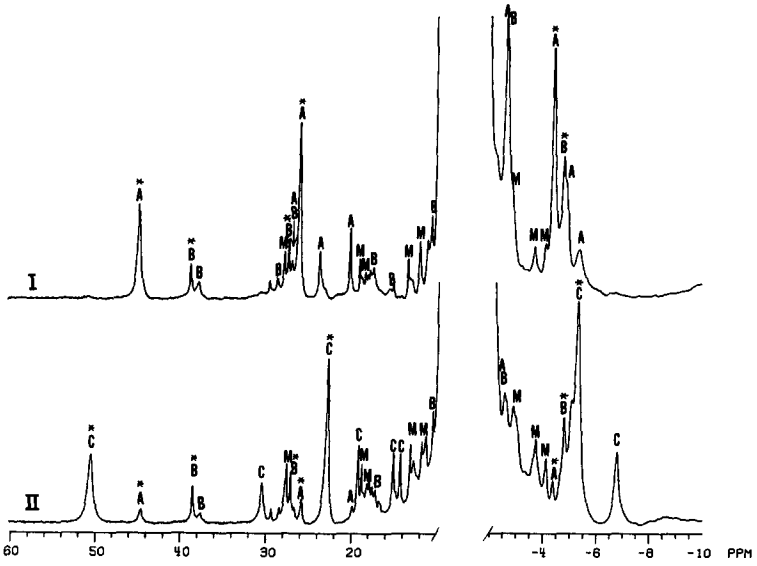

Figure 2. The 360 MHz ^1H NMR spectrum of a sample of Fe^{III}(CN)SMb in ^2H_2O at 20°C and pH = 6.68. Trace I shows the spectrum after the normal preparation procedure while Trace II shows the same sample after storage at 4°C for three months.

6 or 7 for periods of several hours. Thus in the Fe^{III} state there is conversion from the A form to the B form, while the total SMb content to that of Mb remains constant.

These two forms of sulfmyoglobin can also be observed in Fe^{III} samples which have been treated with potassium cyanide to form the low-spin cyanide complexes. Trace I of Figure 2 shows the ^1H NMR spectrum of a mixture of Fe^{III}(CN)SMb A and B obtained by the standard preparative procedure (2). Peaks of the two forms are labeled A and B as before. On standing in the Fe^{III} cyano form, this mixture of sulfmyoglobin is converted into a third form, Fe^{III}(CN)SMb C. Trace II of Figure 2 shows the ^1H NMR spectrum of the same samples used to record Trace I after storage at 4°C for three months. Form C is now dominant, and form B is now more abundant than A. However, originally form A was more abundant than B, and form C was almost nonexistent. Form C can also be formed more conveniently by warming a sample of Fe^{III}(CN)SMb A to 40°C, and samples consisting almost entirely of the C form can be prepared in that way. Fe^{III}(CN)SMbC is also formed when in situ samples of Fe(II) aquo

SMb are allowed to stand for three days at 25°C and then rapidly oxidized and treated with cyanide.

The low field resonances of Fe^{III}(CN)SMb A shown in Figure 2 correspond to the resonances of the major form of Fe^{III}(CN)SMb detected by Timkovich & Vavra (9).

There are distinct chemical differences between these forms. When treated with a substoichiometric amount of cyanide, a mixture of Fe^{III}SMb A and B showed preferential binding of the cyanide to the B form. The results are shown in Figure 2. Trace II shows a spectrum of the samples of Fe^{III}SMb A and B before the addition of KCN while Trace III shows the spectrum of the same sample immediately after the addition of a limited amount of cyanide. The peaks of Fe^{III}SMb B have nearly completely disappeared while those of Fe^{III}-(CN)SMb B have appeared.

Acidification of SMbFe^{III}(CN) C followed by aerobic extraction with 2-butanone yields a green extract (λ_{max} 490, 612, 754) similar to Berzofsky's extract (5), but which is optically stable for hours at 20°C, and for days in the dark at 4°C. Under similar extraction conditions the green pigment of Fe^{III}(CN)SMb A undergoes a rapid conversion to Fe^{III} protoporphyrin IX. The ^{1}H NMR spectrum of the green extract from Fe^{III}(CN)SMb C after solvent evaporation, dissolution into dimethyl sulfoxide-d_6, and addition of potassium cyanide in ^2H_2O has methyl resonances at 42, 20.5, -1.5 and -5.3 ppm and shows considerable similarity to that of Fe^{III}(CN)SMb C. This suggests that the modified heme has remained intact upon extraction.

The various forms of sulfmyoglobin detected here do not appear to be related to the phenomenon of heme rotational disorder encountered in many proteins (10). In one relevant experiment we compared the behavior of a normal myoglobin sample with one that contained a 50/50 mixture of the two rotationally disordered forms. In both cases only SMb A was detected in the freshly prepared sample.

DISCUSSION

These results clearly establish a new degree of complexity to the nature of sulfmyoglobin and its structure and reactivity. Considerable care needs to be taken in interpreting previously reported observations on these substances since it is hard to reconstruct the composition of the samples from existing data. This is particularly difficult since the electronic spectra of the samples which produced Traces I and II of Figure 1 showed no detectable differences other than the slight increase in the deoxy absorbance ratio after chromotography (2). Likewise the optical spectra of the samples used in recording Traces I and II of Figure 2 were essentially identical. Since the conversions reported here occur over a broad pH range (6-9), at temperatures from -20 to 40°C, and at varying concentration of protein and catalase, observations made on stored protein require monitoring by ^{1}H NMR spectroscopy in order to ascertain their composition. From the differences in chemical stability of the extracted green "heme" it appears that the variation responsible for the differences between these forms resides in the nature of alterations at the porphyrin periphery.

The hyperfine shift patterns of the A, B, and C forms in both aquo and cyano states show similar patterns. Moreover, each has a larger spread of heme methyl resonances compared to the native protein in the same state. This is consistent with chemical attack on the porphyrin skeleton as demonstrated by NMR studies on model systems (11,12).

Further studies designed to elucidate the chemical nature of the modified heme are in progress.

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