Proton NMR study of the influence of heme vinyl groups on the formation of the isomeric forms of sulfmyoglobin

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Received 5 August 1986

The formation of sulfmyoglobin has been investigated for myoglobin reconstituted with hemins having vinyls replaced by hydrogens to determine the participation of the vinyl groups in the reaction processes. Green complexes are produced in all cases, proving that vinyls are not obligatory for the formation of sulfproteins. In the presence of the 4-vinyl group, the ^{1}H NMR spectra of the met-cyano derivatives indicate the formation of three green species; however, the most stable of these products is not formed in the absence of this group, confirming reaction of the 4-vinyl in this species. Two new red extractable sulfmyoglobin derivatives are formed in the absence of the 4-vinyl group.

Sulfmyoglobin *^{1}H-NMR* *Heme* *Vinyl group* *Myoglobin*

1. INTRODUCTION

Sulfmyoglobin is a physiologically inactive form of the protein which is formed in the laboratory by the successive addition of hydrogen peroxide and sulfide to give a green material [1]. Optical spectra suggest a chlorin-type structure with one pyrrole ring reduced [2]. Several structures have been proposed to account for the hemin modification in sulfmyoglobin [1], but the role of vinyl groups in sulfmyoglobin formation has not been satisfactorily addressed, and contradictory information about their involvement exists [3,4].

We have recently demonstrated the formation of three forms of sulfmyoglobin occurring under standard preparative procedures [5]. These three, designated S_AMb, S_BMb, and S_CMb in order of appearance, are all produced to some degree in the deoxy state, although chromatography facilitates the conversion of S_AMb to S_BMb, and long term storage at 4°C as the met-cyano derivative favors the formation of the more stable S_CMb [5]. This latter form has allowed extraction of the green pigment, which isotope labeling has revealed to possess a reacted 4-vinyl group on a saturated pyrrole B [6]. Although formation of sulfmyoglobin has been claimed for a protein reconstituted with hematohemin [7], its known reversion to protohemin has caused these results to be questioned [4]. In order to shed light on whether the vinyl groups participate in formation of S_AMb and/or S_BMb and to confirm the importance of the 4-vinyl group in S_CMb formation [6], we have investigated by optical and ^{1}H NMR spectroscopy the nature of sulfmyoglobins formed from sperm whale Mb reconstituted with hemins having either or both vinyls replaced by hydrogen.

The four hemins are native protohemin (R_2 = R_4 = vinyl), pemptohemin (R_2 = H, R_4 = vinyl), deuterohemin (R_2 = R_4 = H), and isopemptohemin (R_2 = vinyl, R_4 = H), shown in I of fig.1. The ^{1}H NMR spectra of the met-cyano state of the three forms of native protohemin-sulfmyoglobin yield characteristically different hyperfine shifts, par-

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Abbreviations: SulfMb, sulfmyoglobin; S_AMb, S_BMb, S_CMb, green forms of sulfmyoglobin; S_DMb, S_EMb, red forms of sulfmyoglobin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

ticularly for the lowfield heme methyls [5]. Since the heme methyl hyperfine shifts of the unreacted proteins have been shown to be very similar [8], we expect that comparable forms of sulfmyoglobin will lead to very similar heme resonance patterns for each of the three species.

2. MATERIALS AND METHODS

Sperm whale myoglobin was purchased from Sigma and used as received. Apo-Mb was prepared by the modified method of Teale [9] and reconstituted with the desired hemin [10]. Deutero-, pempto- and isopemptohemin were prepared by literature methods [11]. Sulfmyoglobin samples involving both in situ preparations (without chromatography) and chromatographed preparations were obtained as described [5] except that chromatographed samples were stored at 4°C in the metaquo form 5 h prior to conversion into the met-cyano form by the addition of 3 μl of 1 M potassium cyanide. Samples of the met-cyano form were stored at 4°C for varying periods to effect conversion to S_CMb, S_DMb or S_EMb.

360 MHz ^{1}H NMR spectra were obtained on a Nicolet NTC-360 spectrometer. Typical spectra consisted of 1000–10000 transients of 8192 points using a 7 μs 90° pulse. The residual water signal was suppressed by a decoupler pulse. All chemical shifts are given in ppm from internal 2,2-dimethyl-2-silapentane-5-sulfonate. Optical spectra were observed at ambient temperature on a Hewlett-Packard 8450A UV/vis spectrophotometer using 1 cm light path quartz cells referenced against water. Composition of protein samples were determined by a computer fit of the ^{1}H NMR data.

3. RESULTS

Reaction of native and reconstituted Mbs with excess H_2O_2, followed by 1.5 equivalents of sulfide yields green pigments for native proto-, pempto-, deutero- and isopempto-hemin (optical spectra in II of fig.1). Immediate oxidation with ferricyanide and ligation with cyanide yields the ^{1}H NMR traces shown in figs 2-I, 2-IV, 3-I and 3-V, respectively. Except for some minor contamination with unreacted protein (whose peaks are labeled M, m), the NMR spectra are very similar (particularly the positions of obvious methyl peaks) and consistent

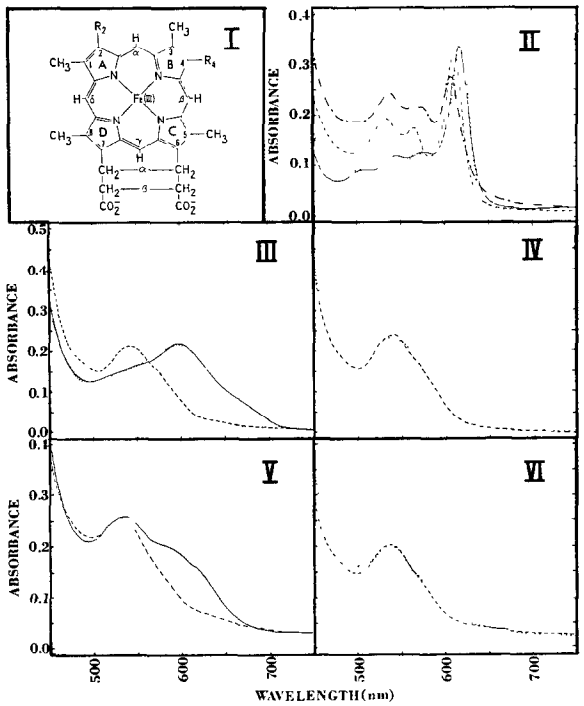

Fig.1. Structures of hemin derivatives (I) and optical spectra of sulfMb complexes (II–VI). (II) Deoxy sulfMb, pH 8.0, containing native protohemin (——, 93% conversion); deuterohemin (– – –, >80% conversion); isopemptohemin (···, >80% conversion); pemptohemin (-----, >60% conversion). (III) Met-cyano native protohemin-sulfMbs, pH 7.1, as 84% S_AMb, 9% S_BMb, 7% Mb (——); 75% S_CMb, 15% Mb, 10% other (···); and unreacted Mb (– – –). (IV) Met-cyano pemptohemin-sulfMb, pH 7.1, as 48% S_BMb, 20% S_CMb, 19% other, 13% Mb (···) and unreacted protein (– – –). (V) Met-cyano deuterohemin-sulfMb, pH 7.1, as 65% S_AMb, 7% S_BMb, 28% Mb (——); as 7% S_BMb, 23% S_DMb, 31% S_EMb, 39% Mb (– – –); the unreacted deuterohemin-Mb trace (– – –) is off-set slightly downward for clarity as it superimposes the trace for primarily S_DMb and S_EMb. (VI) Met-cyano isopemptohemin-sulfMb, pH 7.1, as 9% S_AMb, 2% S_BMb, 45% S_DMb, 5% S_EMb, 38% Mb (···) and as unreacted protein (– – –).

with the presence of one species. We designate this species S_AMb for each protein. Chromatography as the deoxy form followed by oxidation and CN^- ligation yields the NMR traces shown in figs 2-II, 2-V, 3-II and 3-VI, respectively, for the same four proteins. As found earlier for the native protein [5], the optical spectra are unaltered, although the

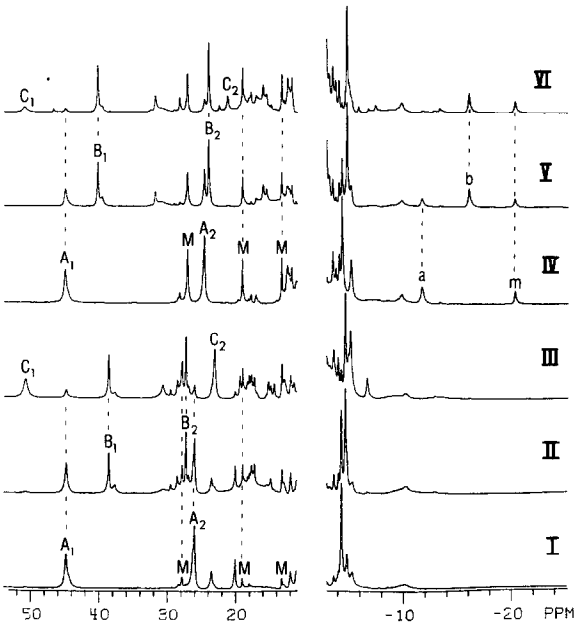

Fig.2. 360 MHz ^{1}H NMR spectra of met-cyano complexes of native (I–III) and pemptohemin-(IV–VI) sulfMbs. (I) Initially formed S_AMb, pH 7.1. (II) SulfMb, pH 7.1, following chromatography. The sample was equilibrated an additional 4 h at 20°C prior to the addition of cyanide. (III) Sample of II after two months at 4°C. (IV) Initially formed pemptohemin-S_AMb, pH 7.1. (V) SulfMb, following chromatography, pH 7.1. (VI) Sample of V after two months at 4°C. Prominent peaks for S_AMb, S_BMb, S_CMb and unreacted Mb are labeled A, B, C, M (methyls) and a, b, c, m (single protons), respectively.

NMR traces reveal partial conversion of the S_AMb form to a second form we designate S_BMb. The degree of conversion is variable among the four proteins, with deuterohemin and isopemptohemin generally yielding only limited conversion.

Upon allowing the chromatographed samples to equilibrate as the met-cyano complexes at 4°C, monitoring by ^{1}H NMR reveals formation of a third green pigment for native protohemin (fig.2-III) and pemptohemin (fig.2-VI), which we designate S_CMb, and for which the extracted prosthetic group yielded the reacted 4-vinyl for protohemin [6]. Further equilibration favors complete conversion to S_CMb. Equilibration at 4°C of deuterohemin- and isopemptohemin-reconstituted samples, on the other hand, fails to yield a species with ^{1}H NMR spectral features similar to S_CMb. However, both proteins yield a new species (designated S_DMb) with NMR spectral characteristics very similar to but not identical to that of the unreacted proteins (fig.3-III, -VII). Further equilibration yields a fourth dominant species designated S_EMb (fig.3-IV, -VIII). The samples containing only S_DMb and S_EMb are not green but red in color, with optical spectra very similar to but not identical to that of the unreacted proteins (fig.1-V, -VI). The extraction [9] of the red 'sulfhemin' prosthetic group from a mixture of S_DMb and S_EMb for the deuterohemin-reconstituted protein, followed by incorporation into fresh apo-Mb, regenerated a spectrum like in fig.3-IV, indicating that the deuterohemin and not the protein is modified.

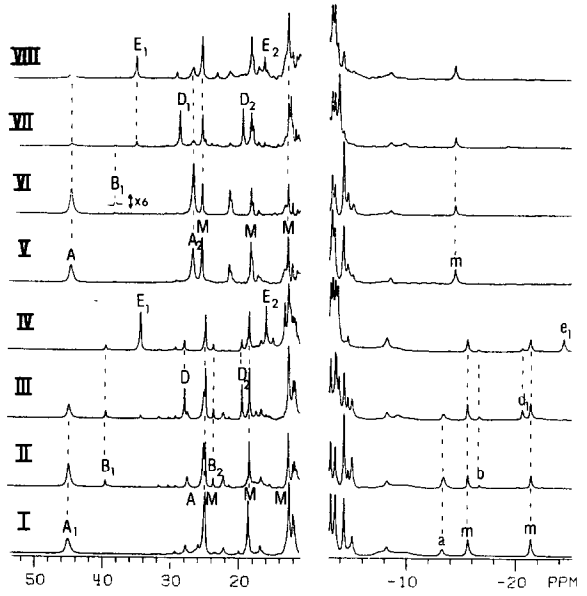

Fig.3. 360 MHz ^{1}H NMR spectra of met-cyano complexes of deuterohemin-(I–IV) and isopemptohemin-(V–VIII) sulfMbs. (I) Initially formed S_AMb, pH 8.0. (II) SulfMb following chromatography, pH 6.8. (III) Sample of II after 7 days at 4°C. (IV) Sample of II after 4 months at 4°C. (V) Initially formed S_AMb, pH 7.1. (VI) SulfMb following chromatography, pH 7.1. (VII) Sample of VI after 1 month at 4°C. (VIII) Sample of V following 3 days at 22°C. Prominent peaks for S_AMb, S_BMb, S_DMb and S_EMb and unreacted Mb are labeled A, B, D, E, M (methyls) and a, b, d, e, m (single protons), respectively.

4. DISCUSSION

The preparation of green pigments for all four hemins demonstrates unequivocally that vinyl groups are not a prerequisite to sulfmyoglobin formation. The initial green products formed for each of the four hemins (S_AMb) have very similar NMR spectra. In particular, the low-field heme methyl (peak A_1) exhibits a distinct shift of 44.8 ± 0.1 ppm for all four samples. Similarly, the second species (S_BMb) also displays very similar shifts (i.e. peak B_1 at 39.3 ± 0.9 ppm). The common optical and ^1H NMR spectral features among the four S_AMb and S_BMb complexes containing the different hemins argue strongly for essentially the same structure of the pyrrole macrocycle for the four hemins in either sulfmyoglobin form. This further confirms the remarkable structural diversity for sulfmyoglobin in that the above conclusion dictates that neither S_AMb nor S_BMb for native protohemin possesses the cyclic thioether resulting from reaction at the 4-vinyl group, as found in S_CMb [6].

The unique role of the 4-vinyl group in forming the most stable green sulfmyoglobin derivative is evidenced by the fact that only native protohemin and pemptohemin with 4-vinyl groups yield S_CMb, with characteristic low-field heme methyl shift C_1 at 50.8 ± 0.1 ppm for the met-cyano complex. The other two hemins fail to yield any evidence for a stable green pigment analogous to S_CMb, but instead yield two other red forms. Thus both isopemptohemin and deuterohemin yield an intermediate, S_DMb, with characteristic low-field methyl shift D_1 at 28.3 ± 0.3 ppm, and a terminal product, S_EMb, with low-field methyl shift E_1 at 34.2 ± 0.2 ppm. The fact that S_DMb and S_EMb are red and possess optical spectra very similar to native protein dictates that, in contrast to the green pigments, the macrocycle retains full conjugation. Thus we have demonstrated here that sulfmyoglobins can be formed where only peripheral substituents are modified.

Both deuterohemin-S_DMb and -S_EMb exhibit methyl shift patterns similar to those for the unreacted proteins [12], except that they display only a single narrow one-proton peak in the upfield region where the 2-H and 4-H peaks appear in the unreacted protein. For isopemptohemin-S_DMb and -S_EMb, no such upfield signal is observed. Thus these high-field one-proton peaks, d_1 and e_1 in the former protein, must originate in 2-H, and the 4-H resonance is missing in both complexes of both proteins. The absence of the 4-H peak at its characteristic position suggests that this site has been modified in the formation of the red sulfmyoglobins. Therefore, the same pyrrole (B) is reacted in both green and red sulfmyoglobins.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Institutes of Health, GM 26226 and HL-22252.

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