Assignment of Selected Hyperfine Proton NMR Resonances in the Met Forms of *Glycera dibranchiata* Monomer Hemoglobins and Comparisons with Sperm Whale Metmyoglobin†

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ABSTRACT: This work indicates a high degree of purity for our preparations of all three of the primary *Glycera dibranchiata* monomer hemoglobins and details assignments of the heme methyl and vinyl protons in the hyperfine shift region of the ferric (aquo?) protein forms. The assignments were carried out by reconstituting the apoproteins of each component with selectively deuteriated hemes. The results indicate that even though the individual component preparations consist of essentially a single protein, the proton NMR spectra indicate spectroscopic heterogeneity. Evidence is presented for identification and classification of major and minor protein forms that are present in solutions of each component. Finally, in contrast to previous results, a detailed analysis of the proton hyperfine shift patterns of the major and minor forms of each component, in comparison to the major and minor forms of metmyoglobin, leads to the conclusion that the corresponding forms of the proteins from each species have strikingly similar heme–globin contacts and display nearly identical heme electronic structures and coordination numbers.

The erythrocytes of the marine annelid *Glycera dibranchiata* were originally shown to contain three major monomeric hemoglobins (components II, III, and IV) that could be easily isolated (Kandler & Satterlee, 1983; Kandler et al., 1984). Later work confirmed our original results and produced additional information about the spectroscopic uniqueness of these proteins (Cooke & Wright, 1985a,b; Constantinidis & Satterlee, 1987). The principal interest in these proteins originates from early work in which crystallography (Padlan & Love, 1974) and sequencing (Imamura et al., 1972) both indicated that the distal histidine (E-7) was missing in one of the monomer hemoglobins. In its place leucine was found.

The implications of this substitution seem to have been immediately realized. The overall three-dimensional structural resemblance of one of the *G. dibranchiata* hemoglobins to myoglobins (Padlan & Love, 1974) and their comparable sequence homologies (Imamura et al., 1972; Satterlee, 1984) made these proteins of immediate interest to workers studying heme–globin structure–function relationships (Seamonds et al., 1976; Seamonds & Forster, 1972; Seamonds, 1971; Weber et al., 1977; Parkhurst et al., 1980). The reason for this is that unlike myoglobins or hemoglobins that possess exceptional E-7 substitutions which are generally polar or charged amino acids (Romero-Herrera et al., 1981; Dene et al., 1980; Huber et al., 1971; Wollmer et al., 1971) the *G. dibranchiata* substitution is one that replaces the distal histidine by an amino acid with a completely hydrocarbon side chain (leucine).

Given the interest in these unique proteins, our goal has been biochemical, spectroscopic, and kinetics characterization of their behavior (Kandler & Satterlee, 1983; Kandler et al., 1984; Constantinidis & Satterlee, 1987; Satterlee, 1984; Mintorovitch & Satterlee, 1985, 1987). The previous results relevant to this work include our discovery that preparations of the monomer hemoglobin fraction contain three major monomeric hemoglobins (components II, III, IV) and that by our isolation and purification procedures component II consists of essentially a single globin. That work led us to identify a useful purity criterion for these proteins where none had previously existed (Constantinidis & Satterlee, 1987). Furthermore, proton NMR spectra of an analytically pure preparation of component II suggested the presence of two holoprotein forms that are unrelated to the extent of globin purity (Constantinidis & Satterlee, 1987). Our results were found to be consistent with an interpretation whereby the two spectroscopically detectable ferric component II forms (major and minor) were attributed to heme orientational isomerism within the intact heme pocket of the ferric holoprotein (met form). Although this phenomenon was previously demonstrated for the ferrous CO-ligated monomer hemoglobins (Cooke & Wright, 1985b), no evidence of its existence in the ferric forms had been presented. In our work the two forms were estimated by NMR results to be present in each component, as isolated, in the approximate ratio of 85% to 15%. This distribution has been constant throughout five different preparations.

Here we present results that confirm the presence of NMR-detectable major and minor forms in solutions of all of the monomeric methemoglobin components (II, III, IV). This is accomplished by making assignments of selected hyperfine proton resonances for all three monomer components and by analyzing the results with respect to metmyoglobin spectra. Hyperfine resonance assignments were accomplished by reconstituting each monomer hemoglobin component (II, III, IV) with several derivatives of protohemin IX in which various heme substituents were specifically deuterium labeled.

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These assignemnts are important data alone because they allow detailed comparisons to be made with sperm whale myoglobin, but they also confirm the subclassification of proton NMR spectra into major and minor sets of hyperfine resonances for analytically pure ferric state preparations of each of the isolated monomer hemoglobins. The data obtained here are compared to data for metmyoglobin with the result that strikingly similar shift patterns occur in corresponding forms of the proteins from each species. This correlation lends further support to the idea that the origin of the spectroscopic heterogeneity is heme reversal, and it establishes the essential similarity of heme–globin contacts for corresponding forms of the proteins from each species.

Experimental Procedures

Isolation and Purification. The three major monomer hemoglobin components were isolated and purified as previously described (Kandler et al., 1984; Constantinidis & Satterlee, 1987). The purified components were stored frozen as lyophilized powders following extensive dialysis in 0.01 M potassium phosphate buffer, pH 6.8. The details of extensive gel filtration, ion exchange, gel electrophoresis, and isoelectric focusing experiments that were carried out on components III and IV are identical with those described for component II (Constantinidis & Satterlee, 1987).

Proof of Purity. Previous work showed that isoelectric focusing carried out on the apoprotein (globin) was the best criterion of purity so far devised (Constantinidis & Satterlee, 1987). Component II apoprotein was found to focus as a single sharp line, whereas all holoprotein forms focused as multiple lines, for understandable reasons. The large amount of detailed work originally devoted to understanding monomer hemoglobin II was repeated for both components III and IV. The results were virtually identical and for that reason will not be elaborated here. The reader interested in detail can refer to the component II work (Constantinidis & Satterlee, 1987).

Heme Reconstitutions. For reconstitutions the lyophilized hemoglobins were first dissolved in 0.1 M potassium phosphate buffer containing 0.1 M KCl (both Fisher), pH 6.8, and then treated with acidified butanone according to the method of Teale (1959). The aqueous apoprotein was dialyzed extensively, first against aqueous sodium bicarbonate (Fisher) and then against the potassium phosphate/KCl buffer. Next, the apoprotein solution was concentrated by pressure ultrafiltration, diluted with a D_2O solution of the potassium phosphate/KCl buffer, pD 6.8, and reconcentrated to approximately 1×10^{-3} M. This dilution/reconcentration step was repeated at least 3 times with the apoprotein solution. The appropriate specifically deuteriated hemin was dissolved in minimal dilute NaOD (MSD Isotopes), further diluted with D_2O (99.8%, MSD Isotopes), and added dropwise to the chilled, stirred globin solution. The reconstituted protein solution was then finally concentrated to 0.5 mL and used immediately for NMR experiments. This method is essentially identical with that previously used for cytochrome *c* peroxidase (Satterlee et al., 1983), and the protohemin IX derivatives have the same nomenclature as before but are abbreviated in this work simply by indicating the deuteriated substituent and its heme position (Figure 1). Thus, the heme in which the methyl group at position 1 is perdeuteriated is abbreviated in the figures as 1-CD_3; that in which the vinyl group at position 4 is perdeuteriated is designated 4-CD=CD_2, and so forth.

Protein Handling and Spectroscopy. All protein manipulations were carried out at 4 °C, and pH was constantly monitored throughout all manipulations by using a Beckman pH 60 meter and a Fisher combination electrode that was

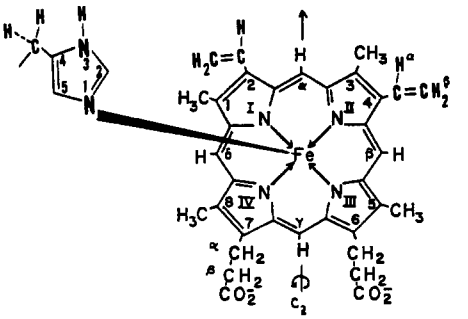

FIGURE 1: Protoheme IX with the nomenclature system used.

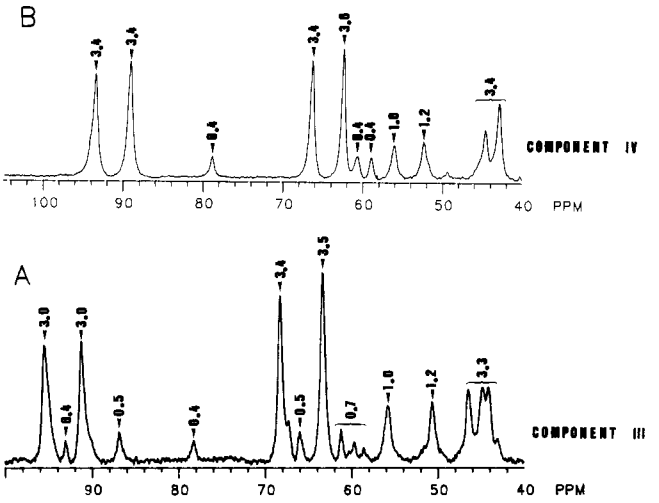

FIGURE 2: Downfield proton hyperfine shift region of *G. dibranchiata* monomer hemoglobin components III (A) and IV (B). Relative peak area integrations are shown above each resonance. Spectra were taken at 361 MHz, 22 °C. Solution conditions were 0.1 M potassium phosphate, 0.1 M KCl, pD 6.8.

standardized prior to each use. The protein solutions were concentrated in Amicon stirred ultrafiltration cells before NMR spectra were taken. For NMR use the buffer employed was deuteriated potassium phosphate (0.1 M) and KCl (0.1 M) in D_2O (99.8%, MSD Isotopes), pD (meter reading) 6.8. Sample concentrations ranged from 5×10^{-4} to 2.5×10^{-3} M. NMR spectroscopy was carried out by using a GE 360 spectrometer operating at 361.068 MHz, as previously described (Satterlee et al., 1983). Spectra are referenced to the residual internal water resonance that was assigned a value of 4.63 ppm, and downfield hyperfine shifts are reported as positive.

Results and Conclusions

General Features of the Proton NMR Spectrum. Proton NMR spectra of the *Glycera dibranchiata* unseparated monomer methemoglobin fraction and the three isolated monomer hemoglobin components have been previously published (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985a). Overall, the spectra display similar patterns that are characteristic of ferric heme proteins that are in predominantly high-spin iron ion orbital ground states (Satterlee, 1986, 1987; La Mar, 1979). The hyperfine resonances exhibit dramatic temperature dependences consistent with their paramagnetic origin (I. Constantinidis and J. D. Satterlee, unpublished results).

Cursory inspection of these spectra would suggest, by comparison with other heme proteins (Satterlee, 1986, 1987; La Mar, 1979), that the higher intensity resonances between 60 and 100 ppm (Figure 2) be assigned to the four heme methyl resonances, that the smaller resonances between 10 and 60

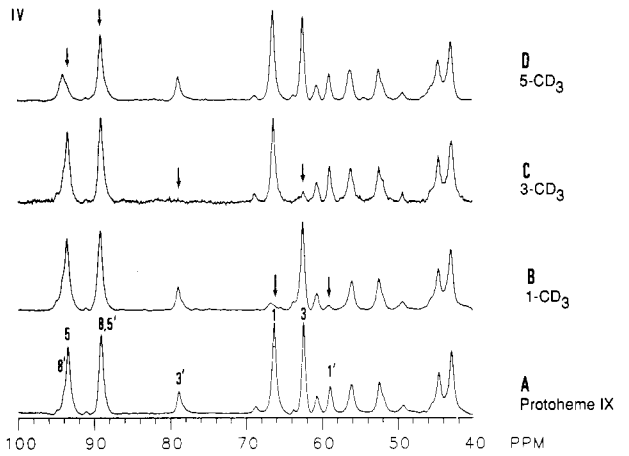

FIGURE 3: Heme methyl resonance assignments for *G. dibranchiata* component IV monomer methemoglobin by reconstitutions with (A) protoheme IX, (B) 1-CD_3-protoheme IX, (C) 3-CD_3-protoheme IX, and (D) 5-CD_3-protoheme IX. Primed labels indicate assignments in the minor protein form; unprimed labels are assignments in the major protein form. Solution conditions identical with those of Figure 2. Arrows indicate resonances exhibiting reduced intensity.

ppm (Figure 2) be assigned to individual protons of heme substituents, and that the resonances in the upfield hyperfine shift region [−5 to −20 ppm; not shown; see Kandler et al. (1984)] are attributable to neighboring amino acid side-chain protons as well as to the heme. However, this simplistic type of correlation is not adequate to fully describe these spectra for two reasons: (1) The areas of the individual downfield hyperfine resonances do not integrate to whole-number ratios as would be expected for a protein with only a single solution form. This was shown initially in spectra of the component II methemoglobin (Constantinidis & Satterlee, 1987). The spectra of components III and IV (Figure 2) indicate that this also occurs for them. Figure 2 shows the relative integrations of the resonance areas (above each peak) in the downfield hyperfine shift region. The relative areas have been normalized to the resonance near 56 ppm (Figure 2). This peak was chosen because it appears in each spectrum, and results presented later in this paper reveal that this peak consists of only a single vinyl proton. It can be clearly seen that the other resonances display intensities that are not integral multiples of this resonance intensity. This indicates that the spectrum shows peaks which consist of multiple overlapping resonances (integrations >1) and resonances due to the presence of a minor form (integrations <1). (2) Knowledge of the hyperfine proton shift patterns of heme proteins (La Mar, 1979; Satterlee, 1986, 1987) suggests that 12 resonances are predicted to occur in the shift region from 40 to 100 ppm. In Figure 2 component III shows 19 resonances (counting shoulders), and component IV shows 13 resonances (with shoulders) in this region.

These observations provided the basis for our initial suggestion that spectra of these proteins, as we isolate them, should be subclassified into overlapping contributions from major and minor protein forms. The following hyperfine resonance assignments emphasize this conclusion as well as provide valuable assignments.

Heme Methyl Assignments. Figure 3 shows the individual heme methyl assignments for monomer methemoglobin IV. Figure 3A shows a portion of the downfield hyperfine shift region displayed by the protein that has been reconstituted with protohemin IX, a procedure that was followed for all reconstitutions in order to assess the effect of the reconstitution procedure. In parts B–D of Figure 3 spectra are shown that are the consequence of reconstituting the apoprotein with heme in which the methyls at positons 1 (B), 3 (C), and 5 (D) (refer to Figure 1) were perdeuteriated. In comparison to the spectrum of Figure 3A one or more peaks in each of the subsequent spectra disappear or exhibit reduced relative intensity, indicating their assignment. For example, in Figure 3B two resonances are seen to diminish in relative intensity in comparison to the spectrum in Figure 3A. These are the large resonance that displays a shift at 66.3 ppm and a smaller resonance at 58.9 ppm. The conclusion drawn here is that the larger resonance belongs to the heme 1-methyl group in the major form of the protein and the smaller resonance belongs to the corresponding heme in the minor form (labeled 1′). Thus, direct, simultaneous assignments for each form may be obtained in the absence of extensive resonance overlap.

In the case of the heme 3-methyl resonance, comparison of spectra 3A and 3C indicate that the resonance at 62.4 ppm corresponds to the 3-CH_3 protons in the major form and that the resonance at 78.8 ppm corresponds to the 3-CH_3 group in the minor form (3′). One can proceed in a similar fashion for the 5-CH_3 group by comparing Figures 3A and 3D. The 5-CH_3 in the major form is assigned to the large resonance at 93.4 ppm, but no obvious smaller resonance, which would correspond to the 5′-CH_3 (minor form), disappears. Note, however, that the large resonance at 89.0 ppm exhibits a reduced intensity relative to the 1-CH_3 and 3-CH_3 resonances in this spectrum. This suggests that the 5′ resonance contributes to the intensity of the resonance at 89.0 ppm. The heme 8-CH_3 group (major form) is also assigned to the 89.0 ppm resonance from its relative intensity (Figure 2) and because it is the only large remaining resonance that could correspond to a methyl group. The protoheme IX derivative in which the 8-CH_3 group is perdeuteriated was unavailable for these experiments, rendering it impossible to unambiguously make this assignment in the minor protein form; however, Figure 3D suggests that the 8′-CH_3 is attributable to the low-field shoulder on the 5-CH_3 resonance (93.4 ppm). In Figure 3D this resonance, with a shift of 93.9 ppm, appears to maintain intensity that approximately corresponds to the intensity of the 3′-CH_3 resonance at 78.8 ppm, even allowing for incomplete deuteriation of the 5-methyl position in the major form (see below). Additional evidence for assigning the 93.9 ppm shoulder to a methyl resonance in the minor form is demonstrated under Heme Vinyl Assignments. These results indicate that the relative ordering of the hyperfine shifts for the heme methyl resonances in monomer methemoglobin IV are 5 (most downfield) > 8 > 1 > 3 (most upfield), whereas in the minor form it appears to be 8′ > 5′ > 3′ > 1′.

In this manner it can be recognized that the downfield hyperfine shift pattern for methemoglobin IV consists of two sets of overlapping subspectra. In most instances the resonances of the substituent groups do not completely disappear (e.g., Figure 3B,D) as might be expected. The reason for this is that the extent of deuteriation varies between individual hemes and, also, that the two protein forms exhibit overlapping resonances. This effect occasionally complicates assignments and may even preclude some. Moreover, each reconstitution represents separate samples of differing concentration, so that the spectra in Figures 2–8 do not possess uniform signal-to-noise ratios. It is for these reasons that relative intensity decreases are the useful indices. The results of all assignments for all three hemoglobins are given in Table I.

The assignments for methemoglobin component III are shown in Figure 4. This figure is in a format identical with that of Figure 3, and the results obtained show that the ordering of the heme methyl assignments for the major form of

Table I: Observed Shifts and Assignments for Selected Heme Resonances of the Two Forms of the Three Principal *G. dibranchiata* Monomer Methemoglobin Components

assignment	shifts for components[a] II	III	IV
major form			
8	91.8	91.4	89.0
	91.0		
5	94.9	95.7	93.4
3	63.9	63.4	62.4
1	68.8	68.3	66.3
	67.7		
4-α	45.2	44.1	42.8
2-α	56.3	55.9	56.1
minor form			
8′	(94.6)	87.0	93.9
5′	(91.8)	93.3	(89.0)
3′	78.2	78.5	78.8
1′	(59.0)	59.8	58.9
2-α′		58.5	

[a] Shifts reported in ppm from residual HDO, which was assigned a value of 4.63 ppm. Tabulated values are at 22 °C, pD 6.8, 0.1 M potassium phosphate–0.1 M KCl–D_2O solution. Parentheses indicate uncertainty of ±0.3 ppm in precise shift due to peak overlaps.

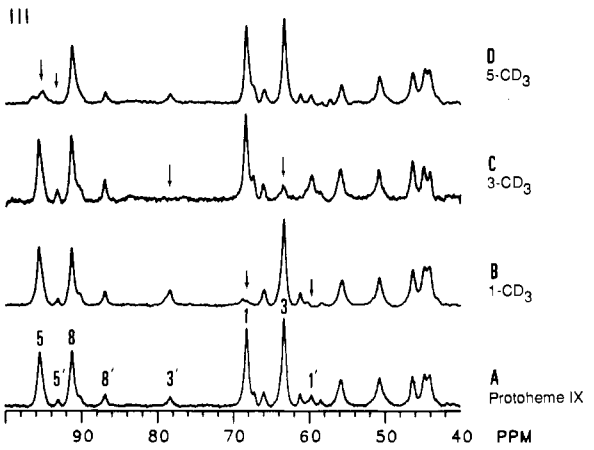

FIGURE 4: Heme methyl resonance assignments for the component III monomer methemoglobin using the hemes described in Figure 3. Conditions identical with those of Figure 2.

component III is identical with that of component IV (5 > 8 > 1 > 3), although the individual resonance positions are different. For the component III minor form the spectrum differs from the component IV minor form spectrum. This is evident in the resolved small resonances at 93.3 and 87.0 ppm, which have no counterparts in the spectrum of component IV (Figure 3). One of these (93.3 ppm) disappears when the 5-CD_3 heme is used and is thus assigned to the 5′-methyl group. The resonance at 87 ppm is tentatively assigned to the 8′-methyl group because it is unaffected by these reconstitutions and because it possesses intensity equivalent to the 3′- and 1′-methyl resonances.

Heme methyl assignments for methemoglobin component II are shown in Figure 5. This figure differs from Figures 3 and 4 in that Figure 5B depicts the results of reconstituting apoglobin II with a protoheme IX derivative in which the 1-CH_3 and 3-CH_3 were simultaneously perdeuteriated. This heme was used in place of the 1-CD_3 derivative. It can be readily seen from comparison of the resonance intensities in the region from 62 to 70 ppm (Figure 5A–C) that the extent of deuteriation of the 3-methyl group in the 1,3-CD_3 heme (Figure 5B) is less than it is in the 3-CD_3 heme (Figure 5C).

The heme methyl assignments for monomer methemoglobin II follow the same logic applied to the other components. The resonances of the major form heme methyls are assigned di-

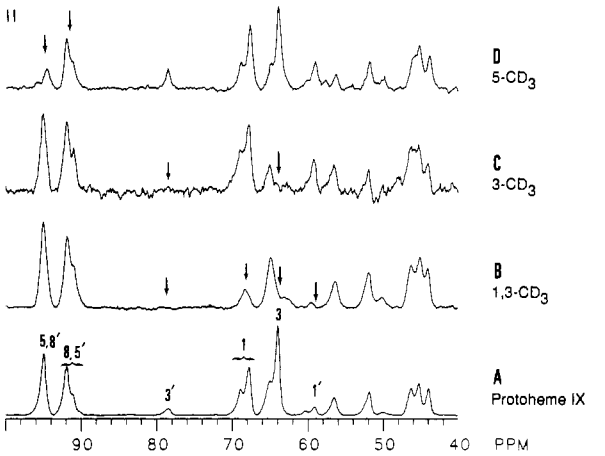

FIGURE 5: Heme methyl resonance assignments for component II monomer methemoglobin by reconstitutions with (A) protoheme IX, (B) 1,3-CD_3-protoheme IX, (C) 3-CD_3-protoheme IX, and (D) 5-CD_3-protoheme IX. Primes indicate minor-form resonances, unprimed major form. Arrows indicate resonances exhibiting reduced intensity. Conditions identical with those of Figure 2.

rectly from intensity decreases. Assignment of the minor form heme methyls follows the same reasoning as applied to component IV (Figure 3). The residual intensity that appears slightly upfield from 94.8 ppm when the 5-CH_3 group is deuteriated (5-CD_3, Figure 5D) is assigned to the 8′-CH_3. The relative partial intensity reduction of the 8-CH_3 resonance (91.8 ppm), compared to the 1- and 3-CH_3 resonances, when the 5-CD_3 heme is used (Figure 5D) indicates the overlap of the 8- and 5′-methyl resonances just as observed for component IV. Consequently, for component II the heme methyl pattern is ordered identically with that for component IV, for both major and minor protein forms.

There is one additional complicating feature of the monomer methemoglobin II spectra shown in Figure 5, namely, the shoulders on the 1-CH_3 resonance (downfield shoulder) and the 8-CH_3 resonance (upfield shoulder). Relative integrations revealed that the shoulders must be included with each major resonance in order to generate an intensity comparable to three protons (Constantinidis & Satterlee, 1987). Preliminary variable-temperature work shows that each shoulder coalesces with its overlapping major resonance as the temperature is raised (I. Constantinidis and J. D. Satterlee, unpublished data).

On the basis of this information we concluded that the two shoulders must originate from the larger resonances with which they respectively overlap. The resolved splitting was attributed to variable positioning of a phenylalanine that appears in the monomer hemoglobin structure at a position closely related to that observed for phe CD1 in myoglobin (Padlan & Love, 1974). Variable positioning would create different magnetic environments for the heme methyl groups (1, 8) that are on the same side of the heme and positioned in proximity to this Phe. The spectrum in Figure 5B supports this conclusion by revealing that both the larger resonance of the 1-CH_3 (67.6 ppm) and its associated shoulder (68.8 ppm) lose relative intensity upon deuteriation of the heme 1-methyl. The residual intensity at that position is due to incomplete 1-CH_3 deuteriation (as shown in Figure 3 and 4) and to overlap with a resonance of the protein minor form that has been detected in variable-temperature experiments.

Heme Vinyl Assignments. Heme vinyl proton assignments were attempted by using the following protoheme IX derivatives: (1) 4-position vinyl perdeuteriated (4-$C^{\alpha}D{=}C^{\beta}D_2$); (2) α protons of the 2- and 4-position vinyls deuteriated (2,4-$C^{\alpha}D{=}C^{\beta}H_2$); and (3) the β protons of both 2- and 4-vinyl

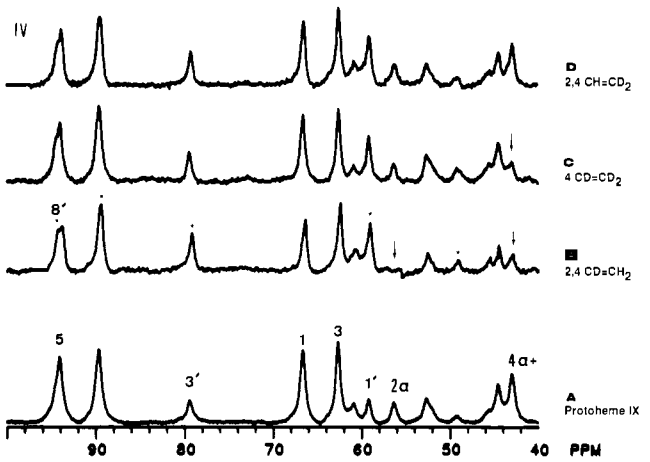

FIGURE 6: Heme vinyl proton assignments for component IV monomer methemoglobin by heme reconsitutions with (A) protoheme IX, (B) 2,4-$C^{\alpha}D$-protoheme, (C) 4-vinyl perdeuteriated protoheme IX, and (D) 2,4-$C^{\beta}D_2$-protoheme IX. Arrows indicate reduced intensity. Primed label indicates resonance of minor form. Asterisks indicate positions of minor-form resonances demonstrating increased intensity in spectra B–D, as discussed in the text. Other conditions identical with those of Figure 2.

positions deuteriated (2,4-$C^{\alpha}H{=}C^{\beta}D_2$). For monomer methemoglobin component IV the spectra that are the results of these reconstitutions are shown in Figure 6. Again, Figure 6A shows the spectrum resulting from reconstituting the apoprotein with protohemin IX, whereas spectra 6B and 6D show the results of reconstitutions with the three isotopically labeled hemes. Notice here that the 4α peak contains intensity contributions from more than a single proton, as indicated by its relative intensity in Figure 6A and the residual intensity at its position in Figure 6C.

The assignments may be made by comparing each of the spectra in Figure 6B–D with Figure 6A; however, for component IV use of the vinyl deuterium-labeled hemes caused the major/minor protein ratio to change in favor of increased minor form. This can be illustrated by noting how the *relative* intensities of the minor-form resonances at 78.8 (3′-CH_3) and 58.9 ppm (1′-CH_3) are increased compared to any of the major-form heme methyl resonances, such as the 1-CH_3 resonance at 66.3 ppm. The positions containing other minor-form resonances that display increased relative intensity are marked by asterisks. The origin of this sensitivity of major/minor ratio is not understood at this time, but it does not compromise our ability to make the vinyl proton assignments in the major form of component IV. This reconstitution sensitivity was only demonstrated by component IV. The spectra in Figures 7 and 8 (components III and II, respectively) do not display this reconstitution dependence. Proceeding, Figure 6B indicates the assignment of the resonances at 42.8 and 56.1 ppm to the pair of vinyl α protons on the basis of the fact that their intensity decreases as a consequence of vinyl α position deuteriation *and* that these are known from Figure 6A to both be single proton resonances of the major protein form. The ambiguity in the individual identities of these resonances is resolved in Figure 6C, which shows clearly the decrease in relative intensity experienced selectively by the resonance at 42.8 ppm (relative to that at 56.1 ppm) when the 4-vinyl perdeuteriated heme is used. The resonance at 42.8 ppm is therefore assigned to the 4-vinyl α proton, whereas the resonance at 56.1 ppm is assigned to the 2-vinyl α proton.

One advantage gained by the sensitivity of the major/minor ratio to these reconstitutions for monomer hemoglobin IV is that it presents evidence supporting the assignment (suggested

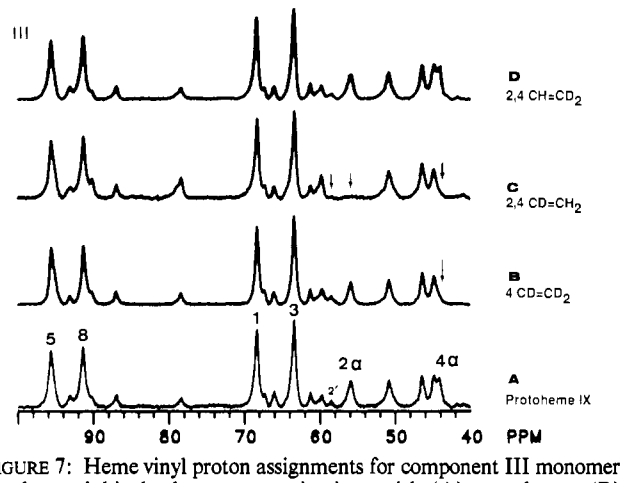

FIGURE 7: Heme vinyl proton assignments for component III monomer methemoglobin by heme reconstitutions with (A) protoheme, (B) 4-vinyl perdeuteriated, (C), 2,4-$C^{\alpha}D$-protoheme, and (D) 2,4-$C^{\beta}D_2$-protoheme. All other conditions identical with those described in Figure 2.

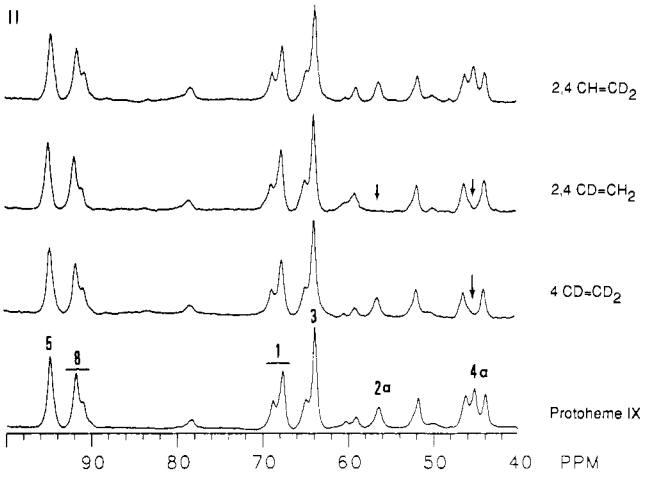

FIGURE 8: Heme vinyl proton asignments for component II monomer methemoglobin by heme reconstitutions. All data identical with those described in Figure 7.

above) for the minor form 8′-CH_3 resonance. Assignment of this resonance, which appears as a shoulder on the 5-methyl peak, to a heme methyl group (Figures 3B and 4), is borne out by the data of Figure 6 in the following way. For component IV in Figure 6A a poorly resolved shoulder is indicated on the downfield side of the resonance assigned to the heme 5-CH_3 resonance at 93.4 ppm (major form). This shoulder is more prominent in the subsequent spectra 6B,C in which the relative amount of the protein minor form is increased. It is, for example, nearly 50% of the total protein in Figure 6B on the basis of relative intensity comparison. The relative intensities of this shoulder in the spectra of Figure 6 parallel that of the 3′-CH_3, thereby labeling it a methyl group in the minor form. Reasoning from the pattern demonstrated for component IV, we propose its assignment to the 8′-CH_3 group of the minor protein form.

Figures 7 and 8 present similar spectra that lead to heme α vinyl assignments for monomer hemoglobin components III and II, respectively. These two components do not demonstrate the major/minor ratio reconstitution dependence that component IV did. Just as for the heme methyl assignments, the pattern of resonances for all of the components is similar, but the actual shift positions are not identical. Only in the case of component III was it possible to unambiguously arrive at a vinyl proton assignment in the minor form (2α' at 58.5 ppm; Figure 7C). This is due to the increased resolution caused by

the slightly different minor-form shift pattern in the component III spectrum compared to those of components II and IV (noted above) and its constant major/minor form ratio.

Normally in proteins such as these at least some of the vinyl β proton resonances are resolved in the spectral region upfield from 0 ppm and consequently are amenable to assignment by this method (Satterlee, 1986, 1987). However, no intensity decreases were observed in this work in any hyperfine resonance region of the proton spectrum as a result of reconstituting globins of any of the components with protoheme IX derivatives in which the β vinyl positions were deuteriated. This is puzzling but may be due to the fact that these resonances lie under the bulk of proton resonances which occur in the region between 0 and 10 ppm and, thus, would not be detectable when this method is used.

Discussion

Elucidating the solution properties of the *Glycera dibranchiata* monomer hemoglobins is important for several reasons. First, these proteins are good variants for comparison to myoglobin. Second, they offer the potential for comparatively easy genetic engineering since they occur in nucleated erythrocytes. In fact, recent work in our laboratory has emphasized cloning the three *G. dibranchiata* globin genes (P. Simons and J. D. Satterlee, unpublished work). Consequently, it is important to compare the solution properties of the monomer hemoglobins with the corresponding myoglobin forms, particularly since the recent definition of purity criteria (Constantinidis & Satterlee, 1987) will allow easy analysis of future gene products.

Discussion of the results presented here must take place within the context of previous results. We have focused on the ferric forms of the protein in attempting a methodical approach to establishing the purity of preparations and understanding the solution properties of the monomer hemoglobins. Cooke and Wright (1985a) corroborated our earlier results (Kandler & Satterlee, 1983; Kandler et al., 1984) and have studied both the ferric and ferrous (Cooke & Wright, 1985b) forms of their preparations of the monomer hemoglobins. For the ferric forms their data compare favorably, but in a limited way, with our previously published results (Kandler & Satterlee, 1983; Kandler et al., 1984). The primary reason for this is that their spectra cover less of the spectral regions compared to the figures presented here and elsewhere (Kandler et al., 1984; Constantinidis & Satterlee, 1987). Furthermore, their isolation procedure differs from ours, and the purity of their preparations has yet to be established, particularly since their stated observation of single lines upon isoelectric focusing the holoproteins (Cooke & Wright, 1985a,b) is puzzling in view of our extensive isoelectric focusing study that showed multiple-line behavior (Constantinidis & Satterlee, 1987). In the ferrous CO forms of the monomer hemoglobins Cooke and Wright (1985b) have identified one of the most interesting properties of these proteins, the presence of heme reversal. In their work they identified the fact that the heme orientation in the *G. dibranchiata* monomer hemoglobins (ferrous CO form) is 180° reversed (by rotation about the heme α–γ axis; Figure 1) from the heme orientation in sperm whale myoglobin. We have shown that this is also the case for the ferric CN hemoglobins (Mintorovitch & Satterlee, 1987, 1985; Mintorovitch and Satterlee, unpublished results; Santucci et al., 1987).

An overview of the data presented here suggests that the *G. dibranchiata* monomer hemoglobins differ in some ways from sperm whale myoglobin but, as shown later, not to the extent previously supposed (Cooke & Wright, 1985a,b). For

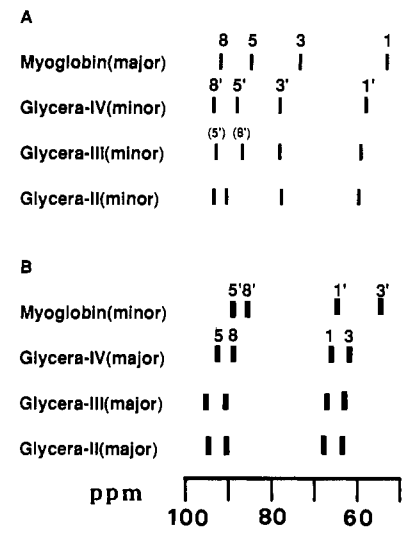

FIGURE 9: Schematic proper comparisons of observed heme methyl proton shift patterns for the respective major and minor forms of sperm whale metmyoglobin and *G. dibranchiata* monomer methemoglobin components II–IV. The myoglobin shifts are reported at 25 °C, pD 7.5; see text for reference. The *Glycera* shifts are reported at 22 °C, pD 6.8.

example, in myoglobin the equilibrium amount of the NMR-detectable minor form is very small (Jue et al., 1983). Although major amounts of the NMR-detectable metmyoglobin minor form can be created by reconstitution, the protein quickly reverts almost entirely to the equilibrium major form. In contrast, substantial amounts of an NMR-detectable minor form occur, apparently at equilibrium, in solutions of the *G. dibranchiata* monomer methemoglobins. In this respect the *G. dibranchiata* proteins behave like the monomer methemoglobins from *Chironomus thummi thummi* (La Mar et al., 1980a,b, 1983), which correlates with the fact that both the *G. dibranchiata* and *C. thummi thummi* monomer hemoglobins share the property of having unusual E-helix environments.

In appearance the proton NMR spectra of the major forms of the individual monomer methemoglobins (Figure 2 and Kandler et al., 1984) resemble each other in the following manner. The 5- and 8-CH_3 resonances are grouped as a pair and occur farther downfield than the 1- and 3-CH_3 resonances, which are also grouped as a pair. There is a striking symmetry of the resonance spacing here that is not observed in spectra of the metmyoglobin major form (but is observed in its minor form; see below and Figure 9), where the methyl peaks are unevenly spread. In order to interpret this difference, it must be noted that the source of the inequivalence in heme methyl proton shifts in ferric heme proteins has been inferred from work on model complexes (Budd et al., 1979). There it was concluded that heme–globin contacts could cause the observed in-plane asymmetry of the heme electronic structure necessary to account for the observed contact proton shifts typical of an orbitally nondegenerate ferriheme ground state.

The present problem is one of comparing the resonance patterns for proteins from two species, each of which displays major and minor forms. In the case of metmyoglobin the two forms were shown to differ in the orientation of the heme within the heme pocket (La Mar et al., 1980; Jue et al., 1983). The difference between the two metmyoglobin forms is that the heme is rotated by 180° about the α–γ meso axis. From data (Cooke & Wright, 1985b; Mintorovitch & Satterlee, 1987; Santucci et al., 1987; Mintorovitch and Satterlee, unpublished results) on other forms of the *G. dibranchiata* monomer components, heme reversal is also indicated as the source of major and minor forms, but the heme orientation

Table II: Average Shifts and Shift Spread for the Heme Methyl Resonances of *G. dibranchiata* Monomer Methemoglobins and Sperm Whale Metmyoglobin

	Glycera components[a]			
	II	III	IV	Mb[b]
av methyl shift				
major form	79.8	79.7	77.8	74.0 (minor)
minor form	80.9	79.6	80.1	75.8 (major)
shift spread				
major form	31.0	32.3	31.1	34.8 (minor)
minor form	35.6	33.5	35.0	38.5 (major)
asymmetry parameter[c]				
major form	0.39	0.41	0.40	0.47 (minor)
minor form	0.44	0.42	0.44	0.51 (major)

[a] Shifts and shift spread reported in ppm at 22 °C, pD 6.8, 0.1 M potassium phosphate buffer, 0.1 M KCl, D_2O solution referenced to HDO assigned a value of 4.63 ppm. [b] Shifts for myoglobin (Mb) as reported by Davis (1982) in ppm at 25 °C, pD 6.5, 0.2 M NaCl, D_2O solution referenced to HDO assigned a value of 4.76 ppm. Note that for comparison purposes the major form of Mb is compared to the minor forms of the monomer methemoglobins, and conversely, as discussed in the text. The smaller average shift displayed by Mb is due, in part, to the higher temperature for which shifts are reported. [c] Asymmetry parameter defined as (shift spread)/(average shift) as reported by Budd et al. (1979).

of the major form in each of the *G. dibranchiata* monomer methemoglobins is 180° reversed from the major form of myoglobin. Therefore, comparing the major forms of the two species' proteins, as previously done (Cooke & Wright, 1985a,b), is not valid since the heme–globin contacts would be a priori different due to the differing heme orientations.

Rather, the most appropriate comparisons rely on heme methyl assignments in both major (La Mar et al., 1980) and minor (Jue et al., 1983; Davis, 1982) metmyoglobin forms, remembering that the minor-form heme is rotated by 180° compared to the major form (La Mar et al., 1983; Jue et al., 1983). Thus, the metmyoglobin major form and the *G. dibranchiata* monomer methemoglobins minor forms should have the same heme orientation, and conversely. The appropriate resonance pattern comparisons are (A) the minor form of each *G. dibranchiata* component vs the major myoglobin form and (B) the major form of each *G. dibranchiata* component vs the minor myoglobin form.

These observed shift comparisons are shown schematically in Figure 9. The metmyoglobin shifts used in this diagram were taken at a temperature 3 °C higher than the monomer methemoglobin data and due to the temperature dependence of hyperfine resonances (La Mar, 1979; Satterlee, 1986, 1987) exhibit an overall upfield bias (smaller shifts). Nevertheless, in both cases (Figure 9A,B) the pattern similarities are remarkable. They indicate similar heme–globin contacts and coordination numbers for both species' proteins, which produce similar patterns of electron delocalization about the heme (La Mar, 1979). This observation confirms previous comparisons of the heme contacts between these two types of proteins, based upon primary sequences (Satterlee, 1984; Padlan & Love, 1974), that independently led to the idea of similar heme–protein interactions (contacts) for myoglobin and the monomer hemoglobins, apart from the E-7 substitution. The data and analysis presented here (Figure 9) also support the concept that the major and minor forms of the *G. dibranchiata* monomer methemoglobins are attributable to rotationally different heme orientations due to the demonstrated parallels with metmyoglobin. Another important conclusion that emerges from this analysis is that, contrary to previous conclusions (Cooke & Wright, 1985a) when the correct comparisons are made, the heme electronic structures, indicated by the observed shift patterns of the individual monomer methemoglobins, are all extremely similar to those of the corresponding forms of metmyoglobin.

In comparing the heme coordination numbers of the two species' proteins, we rely on indicators developed in a study of heme model systems (Budd et al., 1979). In the absence of resolved heme meso protons, that work showed that the average heme methyl shift, the spread of the heme methyl resonances, and the asymmetry parameter (see Table II) all correlated with the heme coordination number and the formal number of unpaired electrons associated with the ferric heme. Such data are gathered in Table II, where nearly identical values are observed for the major and minor forms of the monomer methemoglobins and metmyoglobin, at comparable temperatures. This strongly suggests that the proteins from both species have similar iron ion orbital ground states and heme iron ion coordination numbers.

Summary

The results of heme methyl and vinyl proton NMR assignments for the three principal monomer methemoglobins from *G. dibranchiata* have led to the following conclusions. (1) Our preparations of monomer component hemoglobins III and IV contain only one globin, indicating high purity for these preparations. (2) Analytically pure preparations of all three monomer methemoglobins consist of major and minor forms. (3) The proper comparison of the shift patterns for the major and minor forms of each *G. dibranchiata* component methemoglobin with the spectra of the two forms of sperm whale metmyoglobin provide compelling evidence that these forms are due to heme-related heterogeneity (orientational isomerism) within the globin matrix. (4) Comparison of the shift patterns with metmyoglobin support the identification of the heme isomerism as orientations differing by a 180° rotation about the heme α–γ meso axis. (5) Comparison of the protein forms that correspond to similar heme orientations in each species leads to the conclusion of overall similar heme–globin contacts and similar heme electronic structures. (6) This indicates a similar heme environment, for the proteins of each species, except for the well-documented E-7 substitution. (7) The monomer methemoglobins are best described as high-spin ferric proteins with heme coordination numbers remarkably similar to those of metmyoglobin.

References

Budd, D. L., La Mar, G. N., Langry, K. C., Smith, K. M., & Nayyir-Mazhir, R. (1979) *J. Am. Chem. Soc. 101*, 6091–6096.

Carson, S. D., Constantinidis, I., Satterlee, J. D., & Ondrias, M. R. (1985) *J. Biol. Chem. 260*, 8741–8745.

Constantinidis, I. C., & Satterlee, J. D. (1987) *Biochemistry 26*, 7779–7786.

Cooke, R. M., & Wright, P. E. (1985a) *Biochim. Biophys. Acta 832*, 357–364.

Cooke, R. M., & Wright, P. E. (1985b) *Biochim. Biophys. Acta 832*, 365–372.

Davis, N. L. (1982) Ph.D. Dissertation, University of California, Davis.

Dene, H., Goodman, M., & Romero-Herrera, A. E. (1980) *Proc. R. Soc. London, B 207*, 111–127.

Huber, R., Epp, O., Steigmann, W., & Formanek, H. (1971) *Eur. J. Biochem. 19*, 42–50.

Imamura, T., Baldwin, T. O., & Riggs, A. (1972) *J. Biol. Chem. 247*, 2785–2797.

Jue, T., Krishnamoorthi, R., & La Mar, G. N. (1983) *J. Am. Chem. Soc. 105*, 5101–5103.

Kandler, R. L., & Satterlee, J. D. (1983) *Comp. Biochem. Physiol., B: Comp. Biochem. B75*, 449–503.
Kandler, R. L., Constantinidis, I. C., & Satterlee, J. D. (1984) *Biochem. J. 225*, 131–138.
La Mar, G. N. (1979) *Biol. Appl. Magn. Reson. 1979*, 309–343.
La Mar, G. N., Budd, D. L., Smith, K. M., & Langry, K. C. (1980a) *J. Am. Chem. Soc.* 102, 1822–1827.
La Mar, G. N., Smith, K. M., Gersonde, K., Sick, H., & Overkamp, M. (1980b) *J. Biol. Chem. 255*, 66–70.
La Mar, G. N., Davis, N. L., Parish, D., & Smith, K. M. (1983) *J. Mol. Biol. 168*, 887–896.
Mintorovitch, J., & Satterlee, J. D. (1985) *Rev. Port. Quim. 27*, 199–200.
Mintorovitch, J., & Satterlee, J. D. (1987) *Recl. Trav. Chim. Pays-Bas 106*, 271.
Padlan, E. A., & Love, W. E. (1974) *J. Biol. Chem. 249*, 4067–4078.
Parkhurst, L. J., Sima, P., & Goss, D. J. (1980) *Biochemistry 19*, 2688–2692.
Romero-Herrera, A. E., Goodman, M., Dene, H., Bartnicki, D. E., & Mizukami, H. (1981) *J. Mol. Evol. 17*, 140–147.
Santucci, R., Mintorovitch, J., Constantinidis, I., Satterlee, J. D., & Ascoli, F. (1988) *Biochim. Biophys. Acta* (in press).
Satterlee, J. D. (1984) *Biochim. Biophys. Acta 791*, 384–394.
Satterlee, J. D. (1986) *Annu. Rep. NMR Spectrosc. 17*, 79–178.
Satterlee, J. D. (1987) *Met. Ions Biol. Syst. 21*, 121–185.
Satterlee, J. D., Erman, J. E., La Mar, G. N., Smith, K. M., & Langry, K. C. (1983) *Biochim. Biophys. Acta 743*, 246–255.
Seamonds, B. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, T., & Mildvan, A. S., Eds.) Vol. 2, pp 317–320, Academic, New York.
Seamonds, B., & Forster, R. E. (1972) *Am. J. Physiol. 223*, 734–738.
Seamonds, B., McCray, J. A., Parkhurst, L. J., & Smith, P. D. (1976) *J. Biol. Chem. 254*, 2579–2583.
Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543–546.
Weber, R. E., Sullivan, B., Boneventura, J., & Bonaventura, C. (1977) *Comp. Biochem. Physiol. 583*, 183–187.
Wollmer, A., Steffens, G., & Buse, G. (1977) *Eur. J. Biochem. 72*, 207–212.