Isoelectric Focusing Purity Criteria and ^{1}H NMR Detectable Spectroscopic Heterogeneity in the Major Isolated Monomer Hemoglobins from *Glycera dibranchiata*†

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ABSTRACT: Three major monomeric hemoglobins have been isolated from the erythrocytes of *Glycera dibranchiata*. Their importance to structure–function studies of heme proteins lies in the fact that they have been shown to possess an exceptional amino acid substitution. In these proteins, the E-7 position is occupied by leucine rather than the more common distal histidine. This substitution alters the polarity of the heme ligand binding environment compared to myoglobin. Due to this, the *G. dibranchiata* monomer hemoglobins are attracting much attention. However, until now no purity criterion has been developed. Here we demonstrate that, for all of the *Glycera* monomer hemoglobins, multiple line patterns are shown on high-voltage isoelectric focusing (IEF) gels. Most of these lines are shown to be a consequence of heme-related phenomena and can be understood on the basis of changes in oxidation and ligation state of the heme iron. The multiple line pattern does not indicate significant impurities in the monomer hemoglobin preparations. Similar behavior is also demonstrated for horse heart myoglobin. The multiple line patterns on IEF gels disappear when gels of the apoproteins alone are focused. Single bands occur in this case for all of the monomer hemoglobins except component II, which displays two bands, one major and one minor. The minor band is found to be a modified apoprotein form. It is sensitive to apoprotein handling prior to focusing and depends upon whether the IEF gel is prefocused or not. From this analysis, IEF is shown to be a valuable purity criterion, and the purity of our monomer hemoglobin component II preparation is 97% one globin. Establishing the purity of the monomer component II hemoglobin is important for interpreting the hyperfine proton NMR shift pattern of the met(aquo) form of this component. The NMR results show that two types of spectroscopic heterogeneity are also present in component II, and these are unrelated to the protein purity. One of these is accounted for by heme isomerism, or positional reversal within the heme pocket. The second is due to the adoption of multiple positions by a phenylalanine ring at position CD1 in the globin II primary sequence.

Glycera dibranchiata, a marine annelid, possesses hemoglobin in nucleated erythrocytes. The total hemoglobin content of these cells is separable into polymeric and monomeric forms by gel filtration (Vinogradov et al., 1970), and the monomer fraction is further resolvable into five hemoglobins (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985a). The importance of this monomer hemoglobin fraction resides in the fact that a protein was crystallized from it which both sequencing (Imamura et al., 1972) and crystallography (Padlan & Love, 1974) agree has the exceptional replacement of histidine E-7 (distal histidine) by leucine. In view of the expected impact such an amino acid substitution is predicted to exert on the ligand binding and spectroscopic properties of these heme proteins, we have carried out studies of purification and characterized these proteins' behavior on isoelectric focusing (IEF) gels. This is the analysis required for the extensive kinetics and infrared, Raman, and NMR spectroscopy work that is now in progress.

Although other heme–globins with substitutions of the distal histidine are known, the *G. dibranchiata* monomer is unusual because, unlike virtually all other E-7 position mutant proteins in which the replacement is by a polar or charged amino acid (Romero-Herrera et al., 1981; Tucker et al., 1978; Mims et al., 1983), leucine is an amino acid with a nonpolar, completely hydrocarbon side chain. The ligand binding environment is therefore expected to be significantly different in the *G. dibranchiata* monomer hemoglobin, and initial spectroscopic studies of the CO ligated form have borne out this expectation (Satterlee, 1983, 1984). Our work in progress (J. Mintorovitch, D. Van Pelt, and J. D. Satterlee, unpublished results) also shows that ligand binding equilibria and kinetics for the individual *G. dibranchiata* proteins are significantly different than those reported for myoglobins and normal hemoglobins. Unusual kinetic properties have also been noted by others (Parkhurst et al., 1980), and all of these results point specifically to the E-7 substitution since there are extensive structural similarities between the *G. dibranchiata* monomer and vertebrate myoglobins and hemoglobin subunits (Imamura et al., 1972; Padlan & Love, 1974; Satterlee, 1984; Dickerson & Geis, 1983).

Despite the historical interest in the *G. dibranchiata* monomer fraction (Vinogradov et al., 1970; Imamura et al., 1972; Padlan & Love, 1974; Seamonds et al., 1971a,b, 1976; Weber et al., 1977; Harrington et al., 1978; Hoffman & Mangum, 1970; Seamonds & Forster, 1972; Seamonds, 1971; Satterlee, 1983) little high-resolution purification was carried out until recently (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985; Parkhurst et al., 1980). Still, however, we have remained the only group to have published high-

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resolution isoelectric focusing results of monomer hemoglobin preparations (Kandler & Satterlee, 1983; Kandler et al., 1984). In fact, it is widely known by those working with these proteins that IEF gels of even the purest holoprotein preparations display several lines. However, IEF analysis is critical to a true evaluation of the purity of preparations since electrophoresis does not distinguish even the presence of isoglobins in these preparations (Seamonds et al., 1971a, vide infra). Of further importance to the work presented here is the necessity to assay the purity of preparations in view of the presence of NMR-detectable multiple protein forms that are shown to be unrelated to the presence or absence of isoglobins in the preparations. Accordingly, this work presents an analysis of the behavior of both horse heart myoglobin and the *G. dibranchiata* monomer hemoglobin II in thin-layer, high-voltage polyacrylamide gel (PAG) isoelectric focusing and electrophoresis. Comparison of these results with proton NMR studies of the oxidized (met form) component II monomer hemoglobin is also made. Limited results for the monomer component III and component IV hemoglobins are presented as well.

Experimental Procedures

The *G. dibranchiata* monomer hemoglobins were isolated and purified in the presence of protease inhibitors essentially as before (Kandler et al., 1984) with the exception that a stepwise ionic strength gradient was used in place of the continuous gradient previously employed. In half of all preparations, a second ammonium sulfate (Fisher) precipitation was employed, although its use did not affect the outcome of the results presented here. After isolation, the individual hemoglobins were dialyzed against either deionized, filtered water or 0.01 M phosphate buffer and either lyophilized or frozen for storage. All protein manipulations were carried out at 4 °C, and the pH was constantly monitored as previously described (Kandler & Satterlee, 1983; Kandler et al., 1984). Myoglobin (horse heart) was purchased from Sigma and used without further purification.

Apoprotein formation was achieved by the procedure of Teale (1959). A precooled (4 °C) protein solution was brought to pH 4.6 or lower with 0.1 M HCl. This aqueous phase was then extracted with 1.5 volumes of precooled methyl ethyl ketone. The apoprotein containing aqueous phase was extensively dialyzed at 4 °C, first against aqueous potassium bicarbonate (Antonini & Brunori, 1972) and then against 0.1 M potassium phosphate and 0.1 M KCl (Fisher), pH 6.8. The apoprotein that was to be used in IEF experiments was next dialyzed against 0.1 M glycine buffer, pH 7.4, 4 °C, for 12 h before the IEF experiments were performed. The incubation experiments with the *G. dibranchiata* apoproteins consisted of changing the pH of individual samples (in the glycine buffer) and allowing them to stand at room temperature (20 °C) for 1 h prior to beginning the IEF experiment.

Isoelectric focusing was carried out with a high-voltage (1500 V), thin-layer PAG-plate apparatus (LKB) as previously described (Kandler & Satterlee, 1983; Kandler et al., 1984). Precast gels (PAG plates; LKB) were used. Polyacrylamide gel electrophoresis was performed with a Bio-Rad Protean II vertical electrophoresis chamber and a 2000-V power supply. For isoelectric focusing, gels contained 5% acrylamide and were stained with Coomassie blue. For sodium dodecyl sulfate (SDS) gel electrophoresis, gels, containing 15% acrylamide, were prepared and run according to the procedure of Laemmli (1970). Seven milliliters of distilled water was mixed with 7.5 mL of 1.5 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.8, and 0.3 mL of 10% (w/v) sodium dodecyl sulfate. A 30% T solution of acrylamide/bis(acrylamide) was prepared, and 15 mL was added to the Tris-HCl/SDS solutions. The mixture was then degassed, with suction, for 15 min. Polymerization was initiated by the addition of 150 mL of freshly made 10% ammonium persulfate and 50 mL of *N,N,N′,N′*-tetramethylethylenediamine (Temed). This mixture was then cast and allowed to polymerize. The resulting 15% acrylamide gel is capable of separating protein with molecular weights ranging from 10000 to 70000. A stacking gel was used on top of the 15% acrylamide separation gel. The components of the stacking gel were as follows: 6.1 mL of distilled water mixed with 2.5 mL of 0.5 M Tris-HCl buffer, pH 6.8, 100 mL of 10% SDS, and 1.3 mL of a 30% T solution of acrylamide/bis(acrylamide). The mixture was degassed for 15 min, and then 50 μL of fresh 10% ammonium persulfate and 10 μL of Temed were added to initiate polymerizations. The gel was run under constant current conditions at 16 mA until the protein passed through the stacking gel into the separating gel. The current was then increased to 24 mA. The running time was between 4 and 5 h, depending upon the length of the gel. The experiment was stopped right before the tracking dye had run off the gel. Figure 2 illustrates the whole gel. The running buffer, pH 8.3, consisted of Tris base (15 g/L), glycine (72 g/L), and sodium dodecyl sulfate (5 g/L). The sample buffer consisted of 4.0 mL of distilled water, 1.0 mL of 0.5 M glycine buffer, pH 6.8, 0.8 mL of glycerol, 1.6 mL of 10% (w/v) sodium dodecyl sulfate, and 0.4 mL of 2 M β-mercaptoethanol. The sample was diluted to at least 1:4 with the sample buffer and heated at 95 °C for 4 min. Gels were scanned and integrated with a densitometer (Biomed Instruments). All IEF and electrophoresis experiments were repeated at least 3 times. The pH gradient was determined by placing a surface pH electrode (LKB) on the gel at several positions between the cathode and anode. The electrode was calibrated at 10 °C, which is the temperature at which the gels were maintained.

NMR spectra were obtained on a GE360 spectrometer operating at 361.068 MHz for protons using methods previously described (Kandler & Satterlee, 1983; Kandler et al., 1984). NMR solutions were made in 99.8% ^2H_2O (Merck), and solution pH is reported as the meter reading or pD. Resonance integrations were carried out with the GE software program NMRCAP. Shifts are reported as observed, referenced to the residual H^2HO peak at 4.6 ppm. Downfield shifts are shown as positive.

Results and Discussion

Background. Examination of IEF behavior is important because all of the isolated *G. dibranchiata* monomer hemoglobins display multiple lines on fully developed LKB precast IEF gels. This behavior is also exhibited by myoglobin (vide infra). Although this multiple line behavior was indicated in the only IEF gel figure yet published (Kandler & Satterlee, 1983), the actual complexity is belied by tabulated or descriptive reports of IEF experiments (Cooke & Wright, 1985a; Parkhurst et al., 1980; Weber et al., 1977; Harrington et al., 1978). Clearly, development of a purity criterion is essential to further work with these proteins and, as will become obvious later, to understanding the NMR-detected heterogeneity, which is shown to be a phenomenon not related to the globin homogeneity of the *Glycera* monomer hemoglobin preparations. We show here that the multiple line behavior is understandable on the basis of how the proteins and the IEF gels are manipulated and is due, in part, to the ligation and oxidation states of the heme in the protein. Figure 1 introduces this by showing the IEF pattern displayed by the unseparated

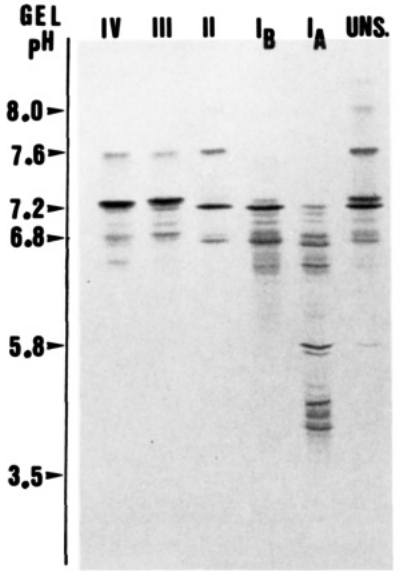

FIGURE 1: PAG-plate isoelectric focusing gel of the unseparated monomer hemoglobin plus the two minor (I_A and I_B) and three major (II–IV) isolated *Glycera* hemoglobin monomer components. The samples were fully oxidized before they were placed on the gel. The gel was not prefocused. In the component II lane, note the weak band at pH 8.0.

monomer fraction plus the five isolated monomer hemoglobin components labeled IA, IB, and II–IV. These are the individual fractions that are isolated from the annelid erythrocytes by a combination of gel filtration and ion-exchange chromatography (Kandler & Satterlee, 1983; Kandler et al., 1984). By amount, IA and IB constitute very minor components, with II–IV being the major components. Since only components II–IV are objects of spectroscopic and kinetic studies, attention will be focused solely on them at present. Component II exhibits four sets of lines between pI = 6.8 and pI = 8.0. Components III and IV exhibit bands in positions essentially identical with those of the three lower pI bands of component II. The two major band areas that lie between pI = 6.8 and pI = 7.2 in all components were previously shown to originate from the reduced (lower pI bands) and oxidized (high pI bands) protein forms (Kandler & Satterlee, 1983). Myoglobin exhibits similar behavior on these gels as shown in Figures 3–5 and Tables I and II. As previously indicated for the *G. dibranchiata* components, and now also demonstrated for myoglobin, the characteristic banding pattern on these gels for the holoproteins is one doublet (major and minor) of bands near pI = 6.8 and one near pI = 7.2 (Figure 1). Elucidating the source of these bands for the *G. dibranchiata* proteins is the emphasis of this work. It is a complicated task, and we choose to focus primarily on component II for the balance of this presentation because it is the most complicated. Our approach will be to assign bands that are due to apoprotein formation and then to show that the remaining bands are due to heme-related phenomena. First, we demonstrate that the IEF behavior is not due to molecular weight heterogeneity (i.e., isoglobins) in our samples.

Proof of Molecular Weight Homogeneity. As demonstrated in Figure 2 for monomer hemoglobin component II, SDS gel electrophoresis using 15% polyacrylamide gels reveals that only a single species of uniform molecular weight is present in our preparations. A 15% polyacrylamide gel would resolve different molecular weight species in the molecular weight range of this protein (16 000). IEF gels of the same component II preparation, however, display multiple lines. Our conclusion is that the individual bands shown for component II on IEF gels correspond to essentially identical molecular weight entities, at least within the resolution of our electrophoresis experiments. Exactly identical results (not shown) occur for monomer hemoglobin components III and IV. Furthermore, mixtures of components II–IV run on these same gels develop as only one electrophoretic band, indicating the essentially identical molecular weights of the three monomer hemoglobins.

Table I: Integrated Relative Intensities Indicated as Percentages from the Nonprefocused IEF Gel Shown in Figure 4

	bands						
position on gel	A	B	C	D	E	F	G
Myoglobin Holoprotein							
3.5	1	0	75	22	1	1	<1
4.2	1	0	70	27	1	1	<1
5.0	1	0	80	17	1	1	<1
Glycera Holoprotein							
3.5	2	7	1	65	10	15	
4.2	2	4	2	66	9	17	
5.0	2	2	1	66	10	19	

Glycera Apoprotein

		bands		
solution pH	position on gel	A	B	C
3.5	3.5	18	80	2
	4.2	19	80	1
	5.0	19	80	1
4.2	3.5	15	84	1
	4.2	21	78	1
	5.0	22	77	1
6.6	3.5	22	77	1
	4.2	23	76	1
	5.0	19	80	1
7.8	3.5	18	80	2
	4.2	19	80	1
	5.0	18	81	1
8.3	3.5	13	86	1
	4.2	21	78	1
	5.0	20	79	1

Table II: Integrated Relative Intensities Indicated as Percentages from the Prefocused IEF Gel Shown in Figure 5

	bands						
position on gel	A	B	C	D	E	F	G
Myoglobin Holoprotein							
3.5	2	13	48	25	7	3	2
4.2	1	0	50	35	10	2	2
5.0	1	0	61	24	10	2	2
Glycera Holoprotein							
3.5	10	18	3	41	14	14	
5.0	5	5	5	52	13	20	

Glycera Apoprotein

		bands		
solution pH	position on gel	A	B	C
3.5	3.5	9	66	25
	4.2	25	66	9
	5.0	28	69	3
4.2	3.5	8	68	24
	4.2	20	78	2
	5.0	26	73	1
4.5	3.5	13	57	30
	4.2	20	75	5
	5.0	23	72	5
6.6	3.5	8	58	34
	4.2	11	88	1
	5.0	26	72	2
7.8	3.5	9	68	23
	4.2	17	81	2
	5.0	19	80	1
8.3	3.5	8	88	4
	4.2	19	79	2
	5.0	25	72	3

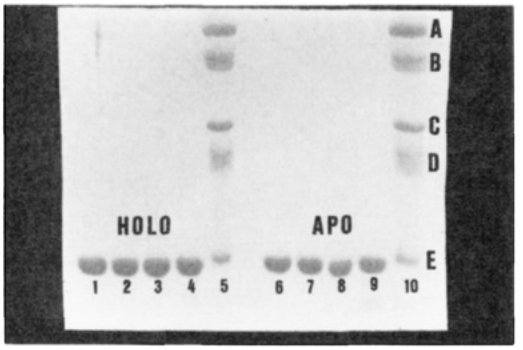

FIGURE 2: SDS gel electrophoresis of major component II. Lanes 5 and 10 are the molecular weight markers: (A) bovine albumin (66K); (B) egg albumin (45K); (C) carbonic anhydrase (29K); (D) bovine pancreas trypsinogen (24K); (E) α-lactoglobulin (14.2K). Lanes 1–4 are *Glycera* monomer hemoglobin component II at pH 3, 5, 7, and 8, respectively. Lanes 6–9 are the apo form of *Glycera* hemoglobin component II at pH 3, 5, 7, and 8, respectively.

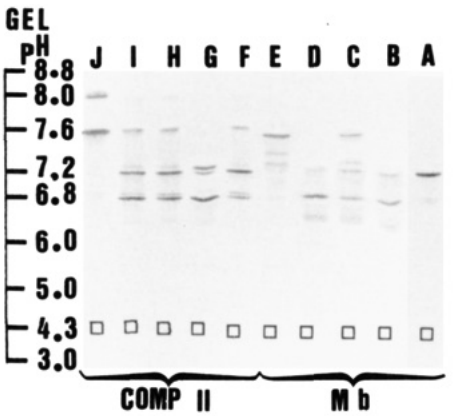

FIGURE 3: PAG-plate isoelectric focusing gel of horse heart myoglobin and *Glycera* monomer hemoglobin component II. Lanes A–E are myoglobins: (A) metaquo-Mb; (B) met-CN-Mb; (C) oxy-Mb; (D) CO-Mb; (E) apo-Mb. Lanes F–J are *Glycera* component II: (F) metaquo-Hb; (G) met-CN-Hb; (H) oxy-Hb; (I) CO-Hb; (J) apo-Hb. The gel was not prefocused, and the protein solutions were kept at pH 7.0.

Identification of IEF Bands. Turning again to component II, we note that it displays four IEF band areas (Figure 1). We have determined that the two single bands with higher isoelectric points (7.6, 8.0) originate from the apoprotein. This conclusion was drawn from the results of gels like that presented in Figure 3. This figure illustrates the IEF pattern demonstrated by *G. dibranchiata* monomer hemoglobin component II and myoglobin, each in the apo form (lanes E, J) and in the following holoprotein forms: ferric (lanes A, F), ferric cyanide (lanes B, G), ferrous oxygenated (lanes C, H), and ferrous carbon monoxide (lanes D, I). Clearly assignable are the apoprotein (globin) bands for each protein (lanes E, J) at approximately pI = 7.6. Further, in *G. dibranchiata* component II apoprotein (lane J), the higher pI band (p$I \approx$ 8.0) is also present (~23%) and is likely to be a slightly degraded apoglobin form, perhaps as a consequence of deamidation (vide infra).

Besides identifying the IEF band(s) due to apoprotein, the importance of Figure 3 is that it demonstrates the essential similarity in IEF behavior of horse heart myoglobin and the *G. dibranchiata* monomer hemoglobin II. Isoelectric focusing band patterns are nearly identical for both types of proteins. For example, the same type dual-band pattern is observed in the holoprotein lanes of both myoglobin (lanes A–D) and the *Glycera* monomer component II (lanes F–I). For both of these proteins, observation of an IEF band area with a pI near 7.2 (Figure 3) and another near pI = 6.8 is characteristic of the globins with heme incorporated. This pattern suggests that the simultaneous observation of p$I \approx$ 6.8 and 7.2 IEF bands in the holoprotein forms is due to a heme-related phenomenon and not to the presence of multiple holoproteins. If that were the case, similar patterns would be expected to occur for the apoprotein, and they are not found.

In order to test this hypothesis, we attempted to determine whether the net charge at the heme site played a role in determining the dual pI IEF pattern (Figure 3, lanes A–D, F–I). The heme site net charge is formally calculated from the net charge on the heme iron and its ligands as follows: heme = −2; iron = +2 (ferrous) or +3 (ferric, met); proximal histidine, CO, O_2 = 0; CN^- = −1. The results were obtained by integrating the gel band intensities with optical scanner tracings of each lane (A–D, F–I). These analyses were repeated in several gels such as that shown in Figure 3. Relative percentages of the band(s) near pI = 7.2 and those near pI = 6.8 were calculated from densitometer scans. The results show that the relative percentage of each band depends upon the formal net charge at the heme site. The trend is identical for either myoglobin or the *G. dibranchiata* monomer hemoglobins (all components). For example, the nature of this dependence can be visually detected in Figure 3 for the *Glycera* component II hemoglobin by comparing lanes G, H, and I against lane F. In G–I, it is clear that the pI = 6.8 region contains more protein than the pI = 7.2 region. These lanes contain ligated holoprotein forms in which the heme is formally neutral. In contrast, for lane F, the pI = 7.2 region contains more protein than does the pI = 6.8 region. This lane contains met-(aquo)hemoglobin, in which case the heme carries a net +1 charge. Similar results are quantitated by densitometer integrations for myoglobin, although it may be difficult to visually confirm this from the reproduction of the gel in Figure 3 (other results not shown). Thus, the behavior of both of these heme protein types on PAG-plate IEF gels is that the more neutral the net charge on the heme center (ferrous oxy and carbonmonoxy; ferric cyanide) the greater the percentage of intensity in the pI = 6.8 region, irrespective of whether the protein is myoglobin or the *Glycera* monomer hemoglobins.

In addition to all of this, Figures 1 and 3 show two additional characteristics of the behavior of these proteins on IEF gels that are not easily explainable. (1) Despite extensive attempts, we have never been successful in completely eliminating either the band(s) at p$I \approx$ 7.2 or the band(s) at p$I \approx$ 6.8. Every gel we have attempted shows both bands, although relative amounts of each vary. This is observed for both myoglobin and the *G. dibranchiata* monomer hemoglobins (components II–IV). Even in experiments in which apoprotein and holoprotein forms were formed from the same preparation and run on the gels simultaneously, we observed the dual-band holoprotein pattern but essentially a single band for the apoprotein. We conclude that the major dual-band (p$I \approx$ 6.8 and 7.2) holoprotein pattern cannot be due to the presence of isoglobins or impurities. Rather, it must be due to heme-related chemistry that occurs on the gel during the IEF experiment. In view of the sensitivity of IEF band protein content displayed on the gels to the net heme charge, illustrated above, we believe variable ampholine binding to be the most likely source of this effect. Specific possibilities include ampholine binding directly to the heme, ampholine-mediated heme ligand displacement, or even redox chemistry. At this point, we are not in a position to further identify this effect, aside from emphasizing that it is a consequence of the heme and is seen only in the holoproteins and not in the apoproteins. (2) Some of the dual-band

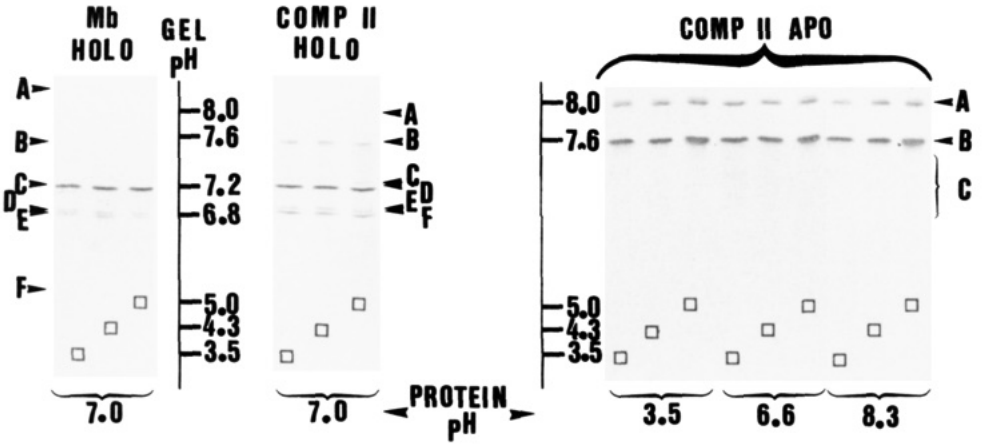

FIGURE 4: PAG-plate isoelectric focusing gel of holomyoglobin, *G. dibranchiata* component II holoprotein, and the *G. dibranchiata* apoprotein. The gel was not prefocused, and the protein application point is indicated by a □. The pH of the solutions in which the proteins were maintained prior to gel application is shown in the bottom of the figure, and the letters next to the bands mark their positions for comparison with Table I, which indicates the relative integrated amounts of each band. The axes labeling pH are those measured after focusing.

areas at p*I* = 7.2 and 6.8 (Figures 1 and 3) exhibit further splitting. As an example, note the obvious presence of two bands (major and minor) in the p*I* = 7.2 area of the gel in Figure 3 on lane G. Again, such division into two species with slightly different isoelectric points is not seen in the apoprotein gels of any of the *Glycera* monomere hemoglobin components (II–IV). For component II apoprotein (Figure 3, lane J), the major apoprotein band appears at p*I* = 7.6. The smaller band at about p*I* = 8.0 cannot be a manifestation of this splitting for two reasons. (a) The p*I* difference of the two bands in the apoprotein lane (J, Figure 3) is much different than the slight difference in p*I*'s exhibited within the holoprotein band area at p*I* = 7.2. (b) Later in this discussion we show that, for the apoprotein (lane J), the p*I* = 8.0 band can be attributed to degradation of the apoprotein.

Since this phenomenon is observed only in the heme-containing holoproteins, we conclude that the phenomenon must be heme related (as described above) and not due to isozymes. The small percentage of the minor band in each of these pairs (C vs D, E vs F in Figures 4 and 5 *Glycera* holo lanes and Tables I and II) suggests an additional possibility, namely, that the minor bands in the C, D and E, F pairs (Figures 4 and 5; Tables I and II) correlate to the minor form detected by NMR spectroscopy (vide infra), which is due to a reversed heme orientation within the intact holoprotein. Such heme reversal could result in changed heme–globin contacts by altering hydrogen bonding and/or electrostatic interactions between heme and globin in the holoproteins of the two different heme orientational isomers. Such an effect could produce the fractional protein charge differences required to account for the small isoelectric point changes such as those demonstrated by IEF bands C, D and E, F.

Finally, it is clear from Figures 1 and 3 that only *G. dibranchiata* monomer hemoglobin component II demonstrates appreciable apoglobin formation when the hologlobin is subjected to IEF. This indicates that Hb II may have a looser heme crevice than do the other monomer hemoglobin components and myoglobin.

*Origin of the p*I *= 8.0 Band in Component II.* An additional phenomenon was encountered during attempts to determine the origin of the highest p*I* band (p*I* ≈ 8.0) in hemoglobin component II. This effect is shown in Figures 4 and 5. Tables I and II indicate important differences in IEF patterns of the apoprotein that depend upon two things: (1) whether or not the IEF gel is prefocused for a period prior to apoprotein application and (2) the precise point of apoprotein application

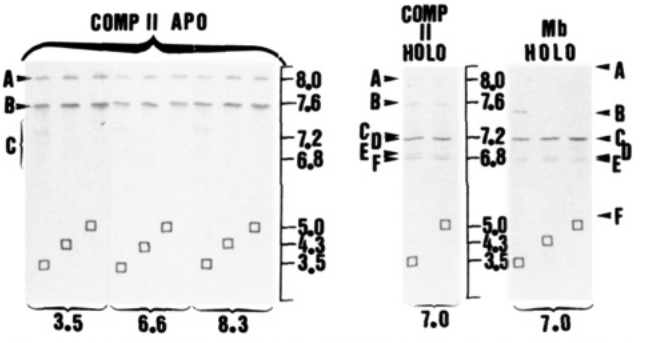

FIGURE 5: PAG-plate isoelectric focusing gel of the same proteins described in the Figure 4 caption. The gel was prefocused, and the protein application point is indicated by a □. The pH of the solutions in which the proteins were maintained prior to gel application is shown in the bottom of the figure, and the letters next to the bands mark their positions for comparison with Table II, which indicates the relative integrated amounts of each band.

on prefocused IEF PAG-plate gels. In the previous figures (1 and 3) only nonprefocused gels were employed. As shown in Figure 4 and Table I, component II apoproteins that were simply maintained in solution at different pH's (in glycine buffer) for 1 h prior to focusing on a nonprefocused gel show very little difference in relative integrated intensities of the p*I* = 7.6 (apo) band and the p*I* = 8.0 band. Furthermore, the amount of the p*I* = 8.0 band can be minimized by minimizing the time beteen apoprotein formation and IEF experiments and by using nonprefocused gels. This band is only detected for the component II hemoglobin in either apo or holo forms. For the *Glycera* component II monomer hemoglobin (holoprotein), a small percentage shows up on gels as the p*I* = 8.0 band (<3%). This band is a consequence of the greater extent of apoprotein formation in the component II monomer hemoglobin (holoprotein) than in the component III and IV monomer hemoglobins (holoproteins) and myoglobin.

In these experiments, apoprotein solutions were made up in 0.1 M glycine buffer and then titrated to the specific pH with HCl or NaOH. The pH's used were 3.5, 4.2, 6.6, 7.8, and 8.3. Of all these, only pH's 3.5, 6.6, and 8.3 are shown in Figures 4 and 5. After 1 h, these solutions were applied to either nonprefocused IEF gels (Figure 4) or prefocused IEF gels (Figure 5).

Gel prefocusing establishes the pH gradient on the gel so that placement of the protein will be at different initial pH (Figure 5, open squares indicate protein placement). For a nonprefocused gel, the gel pH is uniform and the different

protein loading positions indicated as open squares on the gel in Figure 4 have identical pH values. Thus, in Figure 4, the pH axis only indicates the gel pH gradient after focusing is completed. In Figure 5, similarly incubated solutions of monomer hemoglobin II, which were applied to a prefocused PAG-plate gel, show that formation of the pI = 8.0 band depends upon the pH of the initial application position, further establishing the point that chemical processes take place on the gel. Table II shows the average relative integrations for each lane found in three duplicate experiments. In order to generate enough of the pI = 8.0 band for accurate, reproducible integrations, the apoprotein solutions were again maintained in solution for 1 h prior to application to the prefocused gel. Therefore, the appearance of significant amounts of the highest pI band, corresponding to a chemically altered globin of different pI than the major globin band, is a consequence of the handling and the IEF procedure. We emphasize that this is a phenomenon associated only with handling the apoprotein of component II. It illustrates the fact that standard IEF procedures may produce artifacts on gels that are unrelated to the presence of isoglobins.

Under the optimum conditions of isoelectric focusing, namely, careful protein preparation and handling (i.e., no incubation time) and application to a nonprefocused gel, the purity of the *G. dibranchiata* monomer hemoglobins may be estimated by band integrations of isoelectric focused *apoproteins*. For monomer hemoglobin component II, the intact apoglobin is shown to focus as one line (p$I \approx 7.6$) that integrates as 97% of the total protein.

Two conclusions can be drawn from this. First, our isolation and purification procedures for monomer hemoglobin component II (Kandler & Satterlee, 1983; Kandler et al., 1984) can yield essentially pure protein. Second, the more complicated band pattern observed on IEF gels of the holoproteins is related not to purity or globin modification as implied by others (Cooke & Wright, 1985a) but rather to the presence of the heme group in the globin.

Summary of IEF Conclusions. In summary, this analytical IEF procedure has been shown to affect the holoprotein band pattern for both myoglobin and the *G. dibranchiata* major monomer hemoglobins in an understandable way. Moreover, careful protein handling indicates, from gels of the apoprotein, that the *G. dibranchiata* component II monomer hemoglobin has been isolated in at least 97% purity. These results establish the basic homogeneity of our samples and are essential to a precise understanding of the proton NMR spectrum of the component II monomer hemoglobin. We illustrate this in the following with a detailed analysis of the met(aquo) component II hemoglobin proton NMR hyperfine spectrum.

NMR Detection of Multiple Protein Forms Due to Two Heme Orientations. Proton NMR spectroscopy of the ferric (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985a) and ferrous (Cooke & Wright, 1985b) forms of the *G. dibranchiata* monomer hemoglobins has illustrated its usefulness as a sensitive bioanalytical tool. In this respect, it exceeds the capability of any other spectroscopic method that has so far been applied to the monomer hemoglobins. Since we first used it to fortify the concept that distinct, multiple hemoglobins were, indeed, present in the *G. dibranchiata* monomer hemoglobin fraction, our NMR work in progress has begun to yield specific heme resonance assignments for the ferric and ferric cyanide protein forms (I. Constantinidis, R. K. Pandey, H.-K. Leung, J. D. Satterlee, and K. M. Smith, unpublished results; J. Mintorovitch, R. K. Pandey, H.-K. Leung, J. D. Satterlee, and K. M. Smith, unpublished results). From the preliminary resonance assignments that we now possess, combined with the isoelectric focusing results, we are presently in a position to interpret the proton NMR spectra of the *G. dibranchiata* component II ferric holoprotein.

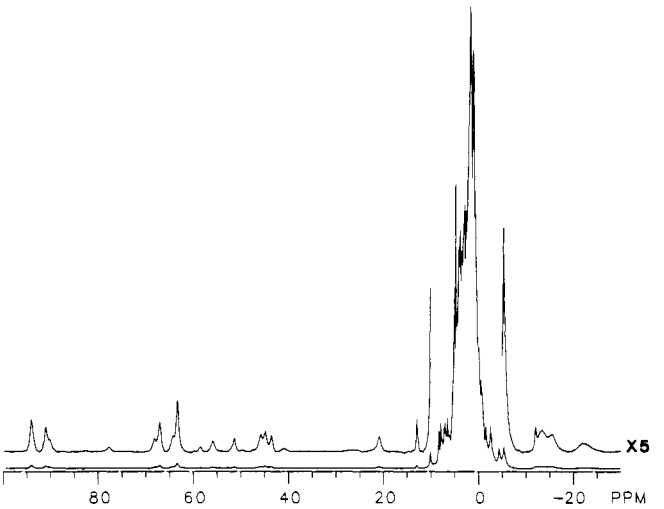

FIGURE 6: 361-MHz proton NMR spectrum of the *Glycera* monomer hemoglobin component II (pH 6.8; 0.1 M potassium phosphate buffer, 0.1 M KCl; 22 °C; [Hb] = 0.002 M).

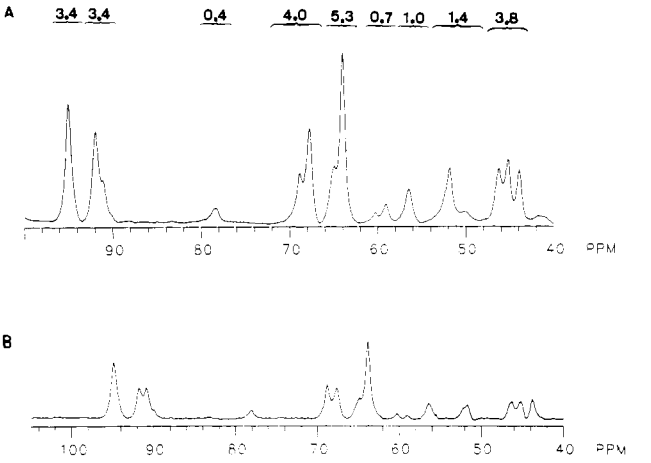

FIGURE 7: 361-MHz proton NMR spectrum of the downfield hyperfine-shift region of *Glycera* monomer hemoglobin component II for two different preparations (A and B). The numbers over the bracketed peak areas in (A) correspond to the number of protons under the assigned peaks, nomalized to the single-proton resonance at 56.4 ppm. Solution conditions: 0.1 M potassium phosphate, 0.1 M KCl; pH 6.8; 22 °C; [Hb] = 0.002 M.

Figure 6 shows the proton NMR spectrum at 361 MHz of monomer component II. In many respects it resembles the spectrum of other monomeric ferric heme proteins (La Mar et al., 1980a; Satterlee et al., 1983). For example, in the far downfield hyperfine-shifted region (60–100 ppm) four major resonances occur. A large body of past experience suggests that these resonances (La Mar et al., 1980a; Satterlee et al., 1983; Satterlee, 1986) should correspond to heme methyl protons. Several small-intensity resonances, corresponding to single proton resonances, also are resolved in the hyperfine-shift region outside the 0–10 ppm envelope.

Upon closer inspection (Figure 7), it becomes clear that the total number of resonances observed exceeds the maximum possible number of resonances which would be resolved in the event that only a single protein form were present. As indicated for monomer hemoglobin component II in Figure 7A, the downfield region (10–100 ppm) exhibits at least 16 resolved resonances of varying intensity. At maximum, 12 resonances

should appear in this region for a pure ferric heme protein form (Satterlee, 1986, 1987). Furthermore, Figure 7A reveals that the relative integrated intensities are not whole number ratios, as would be expected for a single pure protein form. Thus, the relative peak integrations shown in Figure 7A for monomer hemoglobin II are calculated by arbitrarily assigning the most symmetrical and apparently homogeneous resonance in the downfield region (at 56.4 ppm) a unit-integrated intensity, corresponding to a single proton.

In combination with the isoelectric focusing data shown earlier, these results lead to the conclusion that pure holoprotein preparations of the monomer component II hemoglobin yield at least two NMR-detectable protein forms. This type of phenomenon may, in principle, be a consequence of a silent point mutation in the globin sequence as shown for the *Diphyllobothrium dendriticum* monomer hemoglobin (LeComte et al., 1984), multiple positions for single amino acid side chains near the heme group (Burns & La Mar, 1979, 1981), or heme-centered rotational isomerism (Budd et al., 1979; La Mar et al., 1980b). Previous work on the ferrous CO-ligated form of all major components suggests different heme rotational orientations as the source of the minor resonances (Cooke & Wright, 1985b). For the ferric protein forms, spectral heterogeneity has also been observed for the cyanide-ligated monomer hemoglobins (Mintorovitch & Satterlee, (1987). Previous spectra reported for the ferric (aquo) monomer hemoglobins (Cooke & Wright, 1985a) failed to detect the heterogeneity that we report here due to their lower quality.

With the preliminary assignment (Constantinidis et al., unpublished results) of the small resonance near 78.2 ppm (Figures 6 and 7) to the heme 3-CH_3 group in the minor form for all of the isolated components, it becomes clear that the nonintegral relative intensities shown explicitly in Figure 7 are due to overlap between resonances of the two forms. This result will become clearer as additional assignments are obtained. For component II, the percentage of the minor form that is due to this phenomenon (which affects all hyperfine resonances) has been determined from resonance-integrated intensities to be approximately 10%.

Amino Acid Reorientation as the Source of a Second NMR-Detectable Heterogeneity. Another phenomenon observed only for component II is that the pairs of resonances centered at 91 and 68 ppm exhibit variable relative (within each pair) integrated intensities that are uncorrelated with the amount of minor (heme-reversed) form present. This effect is localized only to these two resonances and is illustrated in Figure 7. The relative intensities of the 91.8–91.0 ppm resonances and the 67.7–68.8 ppm resonances for component II vary dramatically for these two spectra (Figure 7A,B), whereas the amount of the heme-reversed minor form present, as judged by the relative intensity of the 78.2 ppm resonance, varies hardly at all. In all cases so far encountered, the relative integrated intensities of the combined 91.8 + 91.0 ppm resonances and 67.7 + 68.8 ppm resonances remain constant. The sum of each split pair integrated intensity (Figure 7A) closely approximates that of a methyl group. Moreover, past heme protein experience supports the assignment of each split pair to a single heme methyl group. This is further substantiated by variable-temperature NMR experiments (not shown) which reveal that, at higher temperatures, each of these two split peaks coalesces into a single resonance. Therefore, we must conclude that there is an additional process present in monomer hemoglobin component II which establishes pairwise magnetically inequivalent environments for a pair of heme methyl protons. This is the source of the splitting that has been observed in all isolations of monomer hemoglobin II so far completed in this laboratory. It is also observed in spectra of isolations performed elsewhere (Cooke & Wright, 1985a). Furthermore, these results show that it is unrelated to the amount of minor protein isomer present. Without complete resonance assignments, a firm conclusion regarding the source of this effect is impossible. However, if the CD1 phenylalanine, which is thought to lie near heme pyrroles I and IV in the component II structure (Cooke & Wright, 1985b), could adopt two inequivalent orientations, such as phenylalanine-82 does in horse cytochrome *c* (Burns & La Mar, 1979), this result would be explained on the same basis as for cytochrome *c* (Burns & La Mar, 1979). The two phenylalanine positions create the two different magnetic environments for the heme methyl groups on pyrroles I and IV. This also implies that the two split-resonance pairs observed here would be ambiguously assigned to the heme 1-CH_3 and 8-CH_3 groups and that, in solution, the Phe CD1 position relative to the heme in monomer hemoglobin component II is somewhat different than the crystal structure indicates.

Summary

The complicated PAG-plate isoelectric focusing behavior of the major isolatable holoproteins of the *Glycera dibranchiata* monomer hemoglobin fraction is attributable to heme-related phenomena. The results are understandable by comparison studies with horse heart myoglobin and lead to several conclusions. (1) The isolated monomer hemoglobin component II is highly purified by our isolation methods (97–99% pure) as judged by apoprotein IEF. (2) The component II monomer hemoglobin (holoprotein) shows a proclivity for apoprotein formation during PAG-plate IEF. (3) A degraded form of the apoprotein occurs as a function of globin handling and placement (of the apoprotein only) on prefocused PAG-plates. Use of unfocused gels is therefore recommended. (4) Proton NMR spectroscopy detects two types of heme-related heterogeneity. One type corresponds to a minor protein form that is probably attributable to the heme possessing two distinct rotational orientations relative to the fixed heme iron ion–proximal histidine bond. This minor form is observed in all three major monomeric *G. dibranchiata* hemoglobins. (5) The second type of spectroscopic heterogeneity is probably due to multiple rotational positions occupied by the side chain of the near-heme amino acid phenylalanine CD1 and is observed only in component II. (6) Neither of these two *spectroscopic heterogeneities* is related to the globin purity of our monomer hemoglobin preparations. Thus, this work is the first to delineate the purity of *G. dibranchiata* monomer hemoglobin component preparations. It is the initial step necessary as a foundation for ongoing studies of these unique proteins.

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Interaction of One-Chain and Two-Chain Tissue Plasminogen Activator with Intact and Plasmin-Degraded Fibrin

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ABSTRACT: Tissue-type plasminogen activator (t-PA) plays a central role in fibrinolysis in vivo. Although it is known to bind to fibrin, the dissociation constant (K_d) and number of moles bound per mole of fibrin monomer (n) have never been measured directly. In this study, the binding of both the one-chain form and the two-chain form of recombinant, human t-PA to fibrin was measured. Although more one-chain t-PA than two-chain t-PA is bound to fibrin, the K_d's and n's were within experimental error of each other. Significantly more t-PA is bound to clots made from fibrinogen which has been digested with plasmin than to clots made from intact fibrinogen. The additional binding was shown to be due to the formation of new set(s) of binding site(s) with dissociation constants that are 2–4 orders of magnitude tighter than the binding site present on clots made from intact fibrinogen. ϵ-Aminocaproic acid was capable of competing for the loose binding site present on both intact and degraded fibrin but had little effect on the binding of t-PA to the new site(s) formed by plasmin digestion. This increase in binding caused by plasmin-mediated proteolysis of fibrin suggests a possible mechanism for a positive regulation capable of accelerating fibrinolysis.

Human tissue-type plasminogen activator (t-PA) is a serine protease that plays a key role in the in vivo dissolution of fibrin (Collen, 1980; Verstraete & Collen, 1986). t-PA is initially synthesized as a single-chain form (Pennica et al., 1983). This species can be hydrolyzed at the Arg-275–Ile-276 peptide bond to form a two-chain form of t-PA in which the two chains are connected by a single disulfide bond (Pennica et al., 1983; Rijken & Collen, 1981). The hydrolysis at Arg-275 has been shown to be catalyzed by plasmin (Wallen et al., 1981), factor Xa, and tissue kallikrein (Ichinose et al., 1984). Two-chain t-PA is more active than one-chain t-PA with small molecular weight substrates (Ranby et al., 1982; Rijken et al., 1982; Tate et al., 1987) and with plasminogen in the absence of fibrin (Tate et al., 1987). At physiological concentrations of plas-

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