Evaluation of the extent of heterogeneity in the *Glycera dibranchiata* monomer haemoglobin fraction by the use of n.m.r. and ion-exchange chromatography

Richard L. KANDLER,*† Ioannis CONSTANTINIDIS† and James D. SATTERLEE†§

*Department of Chemistry, Northern Illinois University, DeKalb, IL 60115, U.S.A., and †Department of Chemistry, University of New Mexico, Albuquerque, NM 87131, U.S.A.

(Received 30 July 1984/Accepted 16 October 1984)

The coelomic haemoglobin of *Glycera dibranchiata* is known to be separable into monomeric and higher- M_r fractions. Although exhibiting homogeneity with respect to M_r , the extent of haemoglobin heterogeneity for the monomer fraction has never been adequately assayed. In the present paper we demonstrate that there exists in the monomer haemoglobin fraction reproducibly detectable heterogeneity regardless of the presence or absence of proteinase inhibitors during the isolations. These results show that, considered on the same time scale as previous preparations used for amino acid sequencing, crystallography and kinetics, the monomer haemoglobin fraction is highly heterogeneous. Application of ion-exchange chromatography and ion-filtration methods resulted in the isolation of four resolvable haem protein components from the *Glycera* monomer haemoglobin fraction. Three of these components were isolated in sufficient quantity to employ proton n.m.r. as a successful analytical tool for discriminating the individual haemoglobins. These results are not surprising. Several previous studies indicated less extensive heterogeneity in the monomer fraction. Moreover, the ability of the *Glycera* monomer haemoglobin to bind oxygen at even quite low partial pressures has been attributed to functional diversity originating in multiple haemoglobin components. The present work reveals the extent of the haemoglobin heterogeneity. The results show that it is more extensive than previously believed. Examination of this monomer fraction is particularly important, since crystallography indicates that one of the components of the monomer fraction lacks the E-7 (distal) histidine residue. As a consequence, the identification of such extensive heterogeneity is important to many previously published ligand-binding studies.

In 1974, the crystal structure (Padlan & Love, 1974) of a *Glycera dibranchiata* monomer haemoglobin (Vinogradov *et al.*, 1970; Seamonds *et al.*, 1971; Weber *et al.*, 1977) revealed that this naturally occurring fully functional erythrocyte protein lacked the distal histidine residue at position E-7 in its primary sequence. This fact corroborated earlier amino acid analysis (Imamura *et al.*, 1972). In virtually all normal vertebrate haemoglobins and myoglobins an E-7 histidine residue is ubiquitous (Dayhoff, 1976; Antonini & Brunori, 1972). There are exceptions, of course. Not including the human mutant haemoglobin Zurich (E-7 His→Arg), which is abnormal (Tucker *et al.*,

1978), elephant myoglobin (Romero-Herrera *et al.*, 1981) and opossum haemoglobin are the best examples of apparently healthy species with E-7 replacements (E-7 His→Gln). Nevertheless, the *Glycera* monomer haemoglobin offers the only characterized haemoglobin, to date, in which the E-7 substitution (E-7 His→Leu) results in replacement by an amino acid with a totally hydrocarbon side chain. This obviates any interaction with the haem ligand-binding site that includes N or O heteroatoms, and has made the *Glycera* monomer haemoglobin an ideal protein for study of the effects of such a change on ligation.

The crystallographic and sequencing results on the *Glycera* monomer haemoglobin provided a picture of this invertebrate protein that indicated a close structural relationship with vertebrate

† Present address: Travenol Laboratories, Round Lake, IL 60073, U.S.A.

§ To whom correspondence should be addressed.

haemoglobins. The overall structure contains the myoglobin fold and is nearly superimposable on structures of myoglobins and haemoglobin chains (Padlan & Love, 1974; Dickerson & Geis, 1983). A comparison according to amino acid class shows that the correspondence between this monomer and its more evolutionarily advanced relatives is nearly as good as between myoglobins and haemoglobin chains, α - and β -subunits of the same haemoglobin, or cross-comparison between subunits from different species' haemoglobins (Imamura *et al.*, 1972; Padlan & Love, 1974; Dayhoff, 1976; Dickerson & Geis, 1983; Satterlee, 1984).

These fundamental structural similarities to vertebrate proteins and the concept of a single *Glycera dibranchiata* monomer haemoglobin stimulated a large amount of work on spectroscopy (Seamonds *et al.*, 1972; Harrington *et al.*, 1973, 1978; Satterlee, 1983; Kandler & Satterlee, 1983) and ligand binding (Hoffman & Mangum, 1970; Seamonds, 1971; Seamonds & Forster, 1972; Seamonds *et al.*, 1976; Parkhurst *et al.*, 1980). That work was carried out on isolations of the monomer haemoglobin fraction like that from which the crystals were grown. Low-resolution analytical methods (such as separation by M_r , ion-exchange chromatography where the protein pI values were not considered, with incompletely developed columns, and thick-slab low-voltage isoelectric focusing) (Vinogradov *et al.*, 1970; Seamonds *et al.*, 1971; Weber *et al.*, 1977; Harrington *et al.*, 1978) indicated minimal heterogeneity for the monomer. However, Parkhurst *et al.* (1980) more recently suggested a broader heterogeneity that was functional. Subsequently, we have detailed the isolation of four major haemoglobins from the *Glycera* monomer haemoglobin milieu (Kandler & Satterlee, 1983). We now present the spectroscopic results (n.m.r., u.v.-visible-region absorption) from large-scale isolations that, along with chromatography, indicate the true extent of this heterogeneity. Critical to our results here is the recognition that these proton-n.m.r. studies, carried out on the monomer haemoglobin fraction, are completed on the same time scale as other methods which report homogeneous preparations. Thus n.m.r. sensitivity detects extensive heterogeneity for the monomer haemoglobin fraction that can ultimately be resolved chromatographically.

Experimental

General

The *Glycera dibranchiata* monomer haemoglobin was collected as previously described (Kandler & Satterlee, 1983). Isolation of the monomer haemo-

globin fraction and all subsequent preparative manipulations were carried out at 2–4°C.

An important feature is that isolation carried out in the presence of proteinase inhibitors does not alter the results presented here and described below.

The following mixture of proteinase inhibitors was used: phenylmethanesulphonyl fluoride (10mM), iodoacetic acid (1mM), *N*-ethylmaleimide (5mM), benzamidinium hydrochloride (5mM), EDTA (10mM), ϵ -aminohexanoic acid (10mM), trypsin inhibitor (1mg/ml) and pepstatin (0.36mM) (Aoyagi *et al.*, 1971; Degema *et al.*, 1975; Pearson & Mason, 1977; Roughley & Barrett, 1977). In all processes up to the ion-exchange chromatography this mixture was employed.

After gel filtration, the monomer fraction was uniformly oxidized to the ferric state by using a 10% excess of $K_3Fe(CN)_6$ (Aldrich) followed by extensive dialysis against 100–500 times the protein volume of buffer. Complete conversion into the ferric state was confirmed by u.v.-visible-region spectroscopy.

Cation-exchange chromatography

The ferric haem proteins of the *Glycera dibranchiata* monomer haemoglobin fraction were fractionated by ion-exchange chromatography on CM-cellulose cation-exchanger (Whatman or Sigma) with either ionic-strength-gradient elution or isocratic elution. The dry resin was swollen, prepared and then equilibrated with 10mM- or 5mM-potassium phosphate buffer, pH 6.8, in accordance with the supplier's directions before application of the protein fraction. Elution profiles were created from u.v.-visible-region absorptions of the collected fractions, and ionic strength was measured with Yellow Springs or Barnstead conductivity meters and cells.

The monomer fraction haemoglobin bound with CO to maintain the ferrous state was also subjected to ion-exchange chromatography. Solutions were saturated with CO immediately after lysis, then carried through the separation procedure with the use of CO-saturated buffers. At the end these reduced haemoglobins were oxidized, as previously described, so that n.m.r. and isoelectric focusing could be carried out in order to compare ferrous and ferric isolations.

Anion-exchange chromatography

DEAE-cellulose (Whatman) anion-exchanger was equilibrated at pH 8.0 with 5mM-potassium phosphate buffer. Component I was equilibrated at pH 7.5 and loaded. A dilute yellow-green solution passed through the column with the buffer

front and was discarded. A brown-red band characteristic of haem protein was eluted from the column by using only the equilibrium buffer. A second brown-red band indicative of haem protein was eluted once the buffer had been adjusted to 0.2M-KCl.

Chemicals

All buffers were dissolved in distilled deionized (Barnstead PCS) water or glass-distilled deuterium oxide (Merck). Potassium phosphate salts (mono-, di-, and tri-basic) and KCl were from Fisher and were Certified ACS grade. Solution pH was regulated with HCl and KOH (Mallinckrodt) or, in the case of n.m.r. samples in $^2\text{H}_2\text{O}$, with ^2HCl and NaO^2H (Merck).

Isoelectric focusing and n.m.r. spectroscopy

These were carried out as previously described (Kandler & Satterlee, 1983).

Results and discussion

Separations with the use of ferric protein

As described in the Experimental section, two types of isolations were carried out. For ease in handling the protein, which is autoxidizable during the 5-day period necessary for isolation, we chose to isolate the oxidized (ferric) protein except when reproducing previous results with ferrous-CO forms (see below). In fact, we have already indicated (Kandler & Satterlee, 1983) that this proclivity for autoxidation is likely to have contributed to confusing results in a prior *Glycera dibranchiata* haemoglobin isolation attempt (Parkhurst *et al.*, 1980).

Fig. 1 presents the results of cation-exchange chromatography carried out with the oxidized monomer haemoglobin fraction on a CM-cellulose column. The elution profile in Fig. 1(a) is the result of an increasing ionic-strength-gradient elution and shows that four major bands are resolved. This Figure indicates the degree of reproducibility achievable in these separations when it is compared with previously reported results for separations carried out under slightly different ionic-strength conditions (Fig. 1 in Kandler & Satterlee, 1983). Fig. 1(b) is the profile of an isocratic elution of the ferric monomer fraction. The data in Fig. 1 are typical of ten isolations carried out with an ionic-strength gradient and two isolations in which isocratic elution was employed. Two of the ionic-strength-gradient columns were run in the presence of a mixture of proteinase inhibitors, which did not affect the elution profiles. The percentages of the components in these isolations, averaged over all isolations, are: I ($8.4 \pm 1.5\%$), II ($12.9 \pm 3.4\%$), III ($40.6 \pm 2.6\%$) and IV

Fig. 1. Column chromatography of the unseparated monomer haemoglobin fraction on CM-cellulose

Proteins and cellulose were equilibrated in 10mM-potassium phosphate, pH6.8, and separations were carried out at 2–4°C. (a) A gradient elution profile. The gradient was applied at fraction 130; 25ml fractions were collected. Roman numerals indicate the major haem protein bands that were eluted. (b) An isocratic elution profile.

($38.1 \pm 5.0\%$), as determined by graphical integration.

The elution profiles indicated that the first component isolated from the CM-cellulose columns was heterogeneous. The pH and ionic strength of the column and sample solution were adjusted so that the protein of interest was eluted just after the non-interacting proteins at the buffer front of the column. Purification was thereby effected both by pH-regulated differences in the protein interaction with the ion-exchange resin as well as by binding competition (Kirkegaard *et al.*, 1982). When the *Glycera dibranchiata* unseparated monomer haemoglobin fraction components were equilibrated in 10mM-KCl/10mM-potassium phosphate buffer, pH6.8, and applied to the CM-cellulose columns, the haemoglobins distributed themselves with the following mobilities. Those with isoelectric points close to pH6.8 were eluted with the buffer front. Those with higher pI values were eluted more slowly, and those with the highest pI values (near 7.2) required application of ionic-

strength gradients or large volumes of elution buffer for removal. Since component I was eluted near the front, further purification was carried out by anion-exchange chromatography.

One measure of relative haem protein purity that can be applied to these components is comparison of the Soret-band-maximum intensity with the intensity of the absorption band near 280 nm (Yonetani & Ray, 1965; Yonetani *et al.*, 1966; Wittenberg & Wittenberg, 1981). This is the A_{393}/A_{278} ratio and reflects the haem content (A_{393} , Soret absorption) relative to the content of aromatic amino acids (A_{278} absorption). Where the charge-separated monomer components cannot be differentiated by size (Seamonds *et al.*, 1971; Harrington *et al.*, 1978), the ratio can be used to estimate the freedom from contaminating protein of each isolated component. These haemoglobins are contained in nucleated erythrocytes, and this may result in nucleic acid contamination of the monomer haemoglobin fraction (Hoffman & Mangum, 1970). Because nucleic acids also absorb near 275 nm, the A_{393}/A_{278} ratio cannot differentiate between non-haem protein and nucleic acid contamination, but it indicates such impurities, and nucleic acid contamination can be monitored by observing the absorption intensity at 260 nm.

The u.v.-visible-region-spectrum characteristics of each of the ion-exchange-isolated components is presented in Table 1. All components except component I gave virtually identical absorption maxima and A_{393}/A_{278} ratios. Component I was subsequently chromatographed on DEAE-cellulose at pH 8.0. Two haem protein bands were separated, and the absorption intensity at 260 nm characteristic of nucleic acids was removed from the protein spectra by this procedure (Fig. 2 and Table 1).

N.m.r. characterization

The evidence presented in Table 1 shows that u.v.-visible-region spectroscopy cannot discriminate between the chromatographically isolated

Fig. 2. U.v.-visible-region spectrum of the haem proteins that comprise the CM-cellulose-isolated component I. This further resolution was achieved on a DEAE-cellulose column. —, Component I total, before the DEAE-cellulose chromatography; ---, haem protein component that was eluted from the column with the wash buffer; — · —, protein that bound strongly to the DEAE-cellulose column and could be removed with a 0.2M-KCl step gradient. Conditions: 5mM-potassium phosphate buffer, pH 8.0, 23°C.

Table 1. U.v.-visible-region spectroscopic characteristics of *Glycra* monomer haemoglobin compounds. The reproducibility of the $\lambda_{\max}$ values is ± 1 nm, due to peak broadness. The accuracy of A_{393}/A_{278} measurements was estimated to be ± 0.1 .

Glycra component	$\lambda_{\max}$ (nm)					A_{393}/A_{278}
Total monomer	278	393	639			2.42
I* (total)	260 280 322 407 504 624					1.15
I† A	276	417 544 (610)§				1.50
I‡ B	278	407 500 620				2.19¶
II	278	393 504 639				2.93
III	278	393 504 639				2.95
IV	278	393 504 639				2.99
V	278	393 504 639				2.50

* Unseparated component I as it was eluted from the CM-cellulose column.

† Protein from component I that was eluted from the subsequent DEAE-cellulose column with a 0.2M-KCl step gradient.

‡ Protein from component I that was eluted from the subsequent DEAE-cellulose column with the wash buffer.

§ Estimated absorbance maximum without deconvolution from neighbouring broad peaks; accuracy ± 5 nm.

|| A_{417}/A_{278} .

¶ A_{407}/A_{278} .

components, except for component I proteins. Our previous experience with high-spin oxidized haem proteins (Satterlee & Erman, 1980; Satterlee *et al.*, 1983), and the preliminary work that we have completed on the *Glycera dibranchiata* monomer haemoglobin fraction (Kandler & Satterlee, 1983), suggested that the proton-n.m.r. hyperfine shifts could be useful analytically. Consequently, we have compared the proton-n.m.r. hyperfine-shift region of the unseparated *Glycera dibranchiata* monomer haemoglobin fraction with its ion-exchange-separated major components (II, III and IV). We have not yet isolated enough of the two component I haemoglobins, or of the minor component V, to obtain reasonable n.m.r. spectra. Fig. 3 and Table 2 show these data. The components shown in this Figure correspond to the isoelectric-focusing-analysed components (Kandler & Satterlee, 1983).

The spectra presented in Fig. 3 show the proton-n.m.r. region outside the diamagnetic (0–10 p.p.m.) spectrum. It is in this area that many of the haem peripheral-group protons appear by vir-

tue of the paramagnetism of the ferric protoporphyrin IX moiety. Deuterium labelling of several high-spin ferric haem proteins revealed that the four haem methyl groups resonate between 60 and 100 p.p.m. downfield from the reference (Satterlee *et al.*, 1983; LaMar, 1979).

The spectrum in Fig. 3(a), of the unseparated monomer haemoglobin fraction, exhibits 12 large, sometimes overlapping, peaks in the haem methyl-group region (peaks A–L at 60–100 p.p.m.). These have been previously analysed in terms of contributions from three non-identical sets of resonances (Kandler & Satterlee, 1983). A normal pure ferric haem protein would have four major resonances in this region, attributable to each of the four haem methyl-group substituents (cf. LaMar, 1979; Satterlee *et al.*, 1983). This spectrum occurs when the preparations are subjected to n.m.r. spectroscopy within 20 h from erythrocyte lysis, indicating that preparations used by other workers, where at least this amount of time was required for preparation, are likely to contain this same degree of heterogeneity. Identical spectra are obtained

Fig. 3. Proton-n.m.r. spectra of the upfield and downfield hyperfine-shift regions of (a) unseparated *Glycera* monomer haemoglobin in the presence of ten proteinase inhibitors, taken at 20 h after erythrocyte lysis, (b) component IV, (c) component III and (d) component II

The spectra are of the ferric form of each, in 0.1 M-potassium phosphate buffer, pH 6.9 ± 0.3 , taken at a proton frequency of 360 MHz. The haem methyl region is defined by labels A–L. This Figure and Table 1 show that the heterogeneity detected in the unseparated fraction (a) can be resolved into contributions from the major haem proteins that compose the monomer fraction [(b)–(d)].

Table 2. Observed haem methyl-proton hyperfine shifts for the *Glycera dibranchiata* monomer haemoglobin components. The estimated accuracy of the observed shifts is ± 0.1 p.p.m. due to line broadness; all shifts are reported at 18°C. Oxidized isolation refers to the isolation of ferric haemoglobin on a CM-cellulose column; all components were in 0.1 M-potassium phosphate buffer. The reduced isolation refers to the isolation of ferrous-CO haemoglobin on a CM-cellulose column; all components were in 0.1 M-potassium phosphate buffer.

Component	Observed shifts (p.p.m.)			
Oxidized isolation				
II*	95.7	92.6	69.5	64.6
III†	96.2	92.3	69.2	64.2
IV‡	94.0	89.7	66.9	62.9
Reduced isolation				
Band II†	95.6	92.5	69.5	64.3
III†	96.3	92.2	69.0	64.0
Band IV‡	94.0	89.8	66.9	62.8
Unseparated monomer	96.0 (2)§	94.3 (1)§	69.3 (2)§	67.2 (1)§
	92.2 (2)§	89.9 (1)§	62.2 (2)§	63.0 (1)§

* pD7.50, where pD refers to meter reading in $^2\text{H}_2\text{O}$.

† pD6.50.

‡ pD6.57.

§ Number in parentheses refers to the minimum number of overlapping resonances that contribute to this line (see Fig. 3 and Kandler & Satterlee, 1983).

regardless of use of proteinase inhibitors. From Figs. 3(b), 3(c) and 3(d) it is clear that the major proton-n.m.r. peaks in the spectra of components II–IV account for most of the detectable heterogeneity in the proton-n.m.r. spectrum of the unseparated haemoglobin.

A principal conclusion to be derived from this Figure is that components II, III and IV are the major proteins found in the *Glycera dibranchiata* monomer haemoglobin fraction. They account for all of the different major isotropic shift positions found in the spectrum of the unseparated haemoglobin.

Separations with reduced protein

We have previously (Kandler & Satterlee, 1983) sought to reconcile the more extensive heterogeneity that we have found for the *Glycera dibranchiata* monomer haemoglobin fraction with earlier attempts by Weber *et al.* (1977), Harrington *et al.* (1978) and Parkhurst *et al.* (1980) that indicated less-extensive heterogeneity. Previous attempts at *Glycera dibranchiata* isolations utilized the protein in the ferrous form (Weber *et al.*, 1977; Harrington *et al.*, 1978; Parkhurst *et al.*, 1980). Because the isoelectric points of the monomer proteins differ by 0.4 pI unit in the ferric and the ferrous states (Kandler & Satterlee, 1983), the slightest neglect of diligence in keeping the proteins in a reducing environment will cause mixtures of oxidation and ligation states within the monomer haemoglobin fraction. The result will be a confusing isolation of mixtures of monomer haemoglobin fraction components. One characteristic of this problem is an inability to reproduce the

Fig. 4. Elution profile of the ferrous CO-ligated *Glycera* monomer haemoglobin fraction carried out on CM-cellulose. Column conditions were 10 mM-potassium phosphate buffer, pH 6.8. Four haem protein bands were resolved. The roman numerals indicate the composition of these bands in terms of the isolated ferric components from the higher-resolution columns (Fig. 1) as determined from n.m.r. spectroscopy (Fig. 3 and Table 2).

relative amounts of components isolated under seemingly identical conditions, but at different times, as noted by others (Parkhurst *et al.*, 1980).

To further understand the differences between ferric and ferrous isolations, we carried out two isolations of the ferrous CO-ligated monomer haemoglobin fraction as described by Weber *et al.* (1977). A typical elution profile is shown in Fig. 4

and is virtually identical with that shown by Weber *et al.* (1977).

Isoelectric-focusing measurements indicated that in the reduced CO-ligated state the various monomer haemoglobin components display pI values between 6.4 and 6.8 (Kandler & Satterlee, 1983). Therefore, performing ion-exchange separation of CO-haemoglobins on a CM-cellulose column equilibrated at pH 6.8, as in the present work and that by Weber *et al.* (1977), results in a loss of resolution relative to separating the oxidized components. The ferrous CO-haemoglobins are too negatively charged to pH 6.8 to be resolved on a CM-cellulose column. Fig. 4 shows that the elution profile contains one major asymmetric band near fraction number 80 (band B) with smaller bands centred at fractions 50 (band A), 140 (band C) and 165 (band D). We performed analytical isoelectric focusing on bands B and C as a means for assaying purity. Band C focused identically with reduced component IV previously described (Kandler & Satterlee, 1983), but band B was more complicated, focusing in the region of reduced components II and III.

In this case, the proton-n.m.r. hyperfine shifts allowed assignments to be made for the ferric forms of the reduced haemoglobins, which were isolated in the ferrous CO-ligated state, then oxidized to the ferric state for n.m.r. purposes. It is clear (Table 2) that the band correlation from the elution profile for the CO-haemoglobin components corresponds to the ferric isolated components as follows. Band C corresponds to component IV. For band B, the tailing end (fractions 88–98) corresponds predominantly to component III, and fractions 78–84 correspond to component II.

Summarizing remarks

These results, combined with those reported previously (Kandler & Satterlee, 1983), rationalize the differences with previous isolations (Weber *et al.*, 1977) and indicate that, under conditions of protein preparation essentially identical with those reported for the original amino acid-sequencing results (Imamura *et al.*, 1972) and X-ray crystallography (Padlan & Love, 1974), a high degree of haem protein heterogeneity exists for the *Glycera dibranchiata* monomer haemoglobin fraction. Significant heterogeneity can be resolved even at 20 h after lysis and even when the protein has been maintained in a mixture of eight proteinase inhibitors. We conclude that this heterogeneity is real and characteristic of the monomer haemoglobins of the annelid.

The consequence of this work is the conclusion that previous isolations (Seamonds *et al.*, 1971, 1976; Seamonds & Forster, 1972; Mizukami & Vinogradov, 1973; Weber *et al.*, 1977; Parkhurst *et*

al., 1980) contained heterogeneity essentially identical with that encountered in the present investigation. That work has obvious relevance to the physiology of *Glycera dibranchiata*, because the annelid's haemoglobins are known to transport oxygen under a variety of oxygen partial pressures (Hoffman & Mangum, 1970). However, until all of the monomer haemoglobin components have been characterized relative to the replacement of histidine E-7 by leucine, caution must be exercised when attributing physical measurements to this substitution.

The extent of the heterogeneity documented in the present paper is not, in retrospect, surprising. The very earliest attention that the *Glycera dibranchiata* monomer haemoglobins received indicated major heterogeneity, although it essentially remained undocumented. There are two sequencing reports that are different, even though both groups of workers received crystalline material from the same crystallography group. Li & Riggs (1971) have reported the sequence of the first 45 residues starting from the N-terminal end of a monomer haemoglobin crystallized by Padlan & Love (1974), whereas Imamura *et al.* (1972) have furnished the complete sequence of a crystallized monomer haemoglobin also received from Padlan & Love (1974). These sequences differ in eight positions when the first 45 amino acids are compared. The fact that two different proteins could be crystallized, even though the details of their origins remain obscure, indicated a heterogeneity in this fraction that is significant.

This research was supported by U.S. Public Health Service N.I.H. Grant AM30912 (to J. D. S.) and in part by N.I.H. Biomedical Research Support Grant (2-507-RR7185-04) to the University of New Mexico. Some of the n.m.r. spectra were obtained at the Purdue University Biochemical Magnetic Resonance Laboratory (P.U.B.M.R.L.), which is supported by N.I.H. Grant RR01077 to Purdue University. We further thank Dr. Milo Westler and Professor John Markley for making P.U.B.M.R.L. instrumentation so readily available to us. J. D. S. is a Fellow of the Alfred P. Sloan Foundation.

References

- Antonini, E. & Brunori, M. (1972) *Hemoglobins and Myoglobins in their Reaction with Ligands*, pp. 55–96, Elsevier, Amsterdam
- Aoyagi, T., Kunitomo, S., Morishima, H., Takeuchi, T. & Umezawa, H. (1971) *J. Antibiot.* **24**, 687–694
- Dayhoff, M. O. (1976) *Atlas of Protein Sequence and Structure*, vol. 5, pp. 191–195, National Biomedical Research Foundation, Washington
- Degama, T. R., Hascall, V. C. & Dzigwiatkowski, D. (1975) *J. Biol. Chem.* **250**, 6151–6159

- Dickerson, R. E. & Geis, I. (1983) *Hemoglobins*, Benjamin-Cummings, Menlo Park
- Harrington, J. P., Pandolfelli, E. R. & Herskovits, T. T. (1973) *Biochim. Biophys. Acta* **328**, 61–73
- Harrington, J. P., Suarez, G., Borgese, T. A. & Nogel, R. L. (1978) *J. Biol. Chem.* **253**, 6820–6825
- Hoffman, R. J. & Mangum, C. P. (1970) *Comp. Biochem. Physiol. B* **36**, 211–228
- Imamura, T., Baldwin, T. O. & Riggs, A. (1972) *J. Biol. Chem.* **247**, 2785–2797
- Kandler, R. L. & Satterlee, J. D. (1983) *Comp. Biochem. Physiol. B* **75**, 499–503
- Kirkegaard, L. H., Johnson, T. J. A. & Bock, R. M. (1972) *Anal. Biochem.* **50**, 122–138
- LaMar, G. N. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., ed.), pp. 309–343, Academic Press, New York
- LaMar, G. N., Smith, K. M., Gersonde, K., Sick, H. & Overkamp, M. (1980) *J. Biol. Chem.* **255**, 66–70
- Li, S. L. & Riggs, A. F. (1971) *Biochim. Biophys. Acta* **236**, 208–210
- Mizukami, H. & Vinogradov, S. N. (1973) *Biochem. Biophys. Acta* **285**, 314–319
- Padlan, E. A. & Love, W. E. (1974) *J. Biol. Chem.* **249**, 4067–4078
- Parkhurst, L. J., Sima, P. & Gross, D. J. (1980) *Biochemistry* **19**, 2688–2692
- Pearson, J. P. & Mason, R. M. (1977) *Biochim. Biophys. Acta* **498**, 176–188
- Romero-Herrera, H. E., Goodman, M., Dene, H., Bartnicki, D. E. & Migukami, H. (1981) *J. Mol. Evol.* **17**, 140–147
- Roughley, P. J. & Barrett, A. J. (1977) *Biochem. J.* **167**, 629–637
- Satterlee, J. D. (1983) *Inorg. Chim. Acta* **79**, 170–172
- Satterlee, J. D. (1984) *Biochim. Biophys. Acta* in the press
- Satterlee, J. D. & Erman, J. E. (1980) *Arch. Biochem. Biophys.* **202**, 608–616
- Satterlee, J. D., Erman, J. E., LaMar, 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., eds.), vol. 2, pp. 317–320, Academic Press, New York
- Seamonds, B. & Forster, R. E. (1972) *Am. J. Physiol.* **223**, 734–738
- Seamonds, B., Forster, R. E. & Gottlieb, A. J. (1971) *J. Biol. Chem.* **246**, 1700–1705
- Seamonds, B., Blumberg, W. E. & Peisach, J. (1972) *Biochim. Biophys. Acta* **263**, 507–514
- Seamonds, B., McCray, J. A., Parkhurst, L. J. & Smith, P. D. (1976) *J. Biol. Chem.* **254**, 6533–6538
- Tucker, P. W., Phillips, S. E. V., Perutz, M. F. & Houtchens, R. A. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., ed.), pp. 1–15, Academic Press, New York
- Vinogradov, S. N., Machlik, C. A. & Chao, L. L. (1970) *J. Biol. Chem.* **245**, 6533–6538
- Weber, R. E., Sullivan, B., Bonaventura, J. & Bonaventura, C. (1977) *Comp. Biochem. Physiol. B* **58**, 183–187
- Wittenberg, J. B. & Wittenberg, B. A. (1981) *Methods Enzymol.* **76**, 29–42
- Yonetani, T. & Ray, G. S. (1965) *J. Biol. Chem.* **240**, 4503–4508
- Yonetani, T., Chance, B. & Kajiwar, S. (1966) *J. Biol. Chem.* **241**, 2981–2982