Korn, E. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 588.
Lad, P. M., Hill, D. E., & Hammes, G. G. (1973) *Biochemistry 12*, 4303.
Lehrer, S. S., & Kerwar, G. (1972) *Biochemistry 11*, 1211.
Ling, K. H., Paetkau, V., Marcus, F., & Lardy, H. A. (1966) *Methods Enzymol. 9*, 425.
Liou, R.-S. (1979) Doctoral Dissertation, Oregon State University.
Marchesi, V. T., Furthmayr, H., & Tomita, M. (1976) *Annu. Rev. Biochem. 45*, 667.
Masters, C. J. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, 206.
Mueller, H. (1966) *Biochem. Z. 345*, 300.
Odeide, R., Dupuis, B., Guilloton, M., Savany, A., & Rosenberg, A. J. (1969) *Bull. Soc. Chim. Biol. 51*, 1199.
Otto, M., Jacobasch, G., & Rapoport, S. (1976) *Eur. J. Biochem. 65*, 201.
Paetkau, V. H., Younathan, E. S., & Lardy, H. A. (1968) *J. Mol. Biol. 33*, 721.
Pette, D. (1978) *Biochem. Soc. Trans. 6*, 9.
Richards, S., Higashi, T., & Uyeda, K. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2998.
Sigel, P., & Pette, D. (1969) *J. Histochem. Cytochem. 17*, 225.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.
Szent-Gyorgyi, A. (1947) in *Chemistry of Muscular Contraction*, Academic Press, New York.
Tornheim, K., & Lowenstein, J. M. (1976) *J. Biol. Chem. 251*, 7322.
Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 193.
Uyeda, K., Miyatake, A., Luby, L. J., & Richards, E. D., (1978) *J. Biol. Chem. 253*, 8319.
Walsh, T. P., Clarke, F. M., & Masters, C. J. (1977) *Biochem. J. 165*, 165.
Wilson, J. E. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, No. 6, 124.
Woods, E. F. (1967) *J. Biol. Chem. 242*, 2859.

Kinetics of Oxygen and Carbon Monoxide Binding to the Hemoglobins of *Glycera dibranchiata*†

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ABSTRACT: The monomeric hemoglobin fraction from *Glycera dibranchiata*, the blood worm, has been fractionated into four components. Rate constants and activation energies have been measured for O_2 and CO association and dissociation reactions for the three most abundant monomeric hemoglobins. Hemoglobin I appears to have the largest O_2 and CO association and O_2 dissociation rate constants yet reported for a hemoglobin. Hemoglobin II shows very similar kinetics. The values for M, the O_2–CO partition constant, for these two components appear to be the largest for any hemoglobin. The CO and O_2 association and O_2 dissociation rate constants for hemoglobin III, the most basic of the three major monomeric hemoglobins, are about one-sixth to one-eighth those for hemoglobins I and II, a striking example of functional heterogeneity. On the other hand, oxygen equilibrium constants are very similar for the three hemoglobins. It appears that hemoglobin II corresponds to that hemoglobin for which the sequence has been reported. The unusually rapid ligand kinetics are thus associated with the substitution of the distal histidine by leucine. In all cases, the monomer ligand kinetics were monophasic, in contrast to earlier results [Seamonds, B., McCray, J. A., Parkhurst, L. J., & Smith, P. D. (1976) *J. Biol. Chem. 251*, 2579]. The O_2 and CO association ligand kinetics for the polymer were at least biphasic. The rates for the faster component were similar to those for hemoglobin III of the monomer and about four times faster than the rates for the slower polymeric component. CO dissociation was monophasic for the polymer. Analysis of the O_2–CO relaxation data suggests that there is very little heterogeneity in the O_2 dissociation reaction for the polymeric component.

Seamonds (1969) and Seamonds et al. (1971a) have reported the occurrence of monomeric and polymeric hemoglobins in the erythrocytes of the bloodworm *Glycera dibranchiata*. Imamura et al. (1972) have reported the sequence of one of the monomeric hemoglobins, and Padlan & Love (1974) have determined the crystal structure of the same hemoglobin to 2.5-Å resolution. They noted that 79% of the 147 residues occur in helical regions and that the D helix is absent, as in the α chains of vertebrate hemoglobins. A feature of interest is the replacement of the distal histidine by leucine in the monomer sequence (Imamura et al., 1972). Seamonds et al. (1976) reported oxygen association (k') and both CO association (l') and dissociation (l) rate constants for the total monomeric fraction and noted that the association kinetics were at least biphasic. Since the biphasicity was not consistent with earlier electrophoretic results, the authors were led to propose equilibria among monomeric conformers, a suggestion supported by electron paramagnetic resonance (EPR) measurements (Seamonds et al., 1972). This lack of agreement between kinetic and electrophoretic results also prevented analysis of the O_2–CO replacement data so as to obtain dissociation rate constants (k) for oxygen, since the mechanism for the ligand binding reactions was unclear. In this paper, we report the isolation of four components from the monomeric fraction of *G. dibranchiata* hemoglobin and report oxygen and CO association and dissociation rate constants and activation energies for the three principal components. The same four

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rate constants are reported for the polymeric fraction.

Materials and Methods

(*1*) *Chemical Reagents.* All chemicals were reagent grade, except where noted. Buffers at pH 7 were 0.05 M potassium phosphate and at pH 9, 0.05 M sodium borate. Dithionite was Manox brand from Holdman and Harding, Miles Platting, Manchester, England. Gases were chemically pure from Matheson. Buffers were equilibrated with Ar, CO, and NO by bubbling for at least 15 min through an 18-gauge needle inserted in a 50-mL syringe containing buffer. All gases were passed through a water bubbler which preceded the buffer solutions. For NO, a second bubbler containing 2 M KOH preceded the buffer solutions. For oxygen solutions, air-equilibrated buffers were used. Activities of the gases were determined from solubility tables (Hodgman, 1960).

(*2*) *Glycera Hemoglobin Fractionation and Isolation.* Blood worms, *G. dibranchiata*, were purchased from Maine Bait Co., Newcastle ME.[1] One hundred worms were bled by incising the coelomic cavity. The blood was collected in a cold (4 °C) beaker. The cells were washed three times with 1.2% NaCl by centrifuging in a Sorvall RC-2B refrigerated centrifuge at 6000*g* for 15 min at 4 °C. The cells were lysed by freezing in liquid nitrogen and then thawing. A small amount of washed Celite and 0.5 mL of 2.5 M potassium phosphate buffer, pH 6.8, were added to the lysate. Cell debris was removed by centrifugation at 30000*g* for 25 min, and fatty material was removed by filtering through glass wool. The monomer and polymer fractions were separated on a 2.2 × 25 cm Sephadex G-100 column equilibrated with pH 6.8, 0.01 M potassium phosphate buffer saturated with carbon monoxide. The polymer fraction (first band from G-100) was not further fractionated. The monomer fraction (second band from G-100, collected in 35 mL) was applied to a 5 × 50 cm CM-Sephadex C-50 column previously equilibrated with 0.01 mM potassium phosphate buffer, pH 6.8, saturated with carbon monoxide, and the column was run at 4 °C. The flow rate was 45 mL/h, and, after approximately 8 h, four bands were observed on the column. Hemoglobins (Hbs) I and II (bottom and second band) were separated by ~5 cm; Hbs II and III were separated by ~10 cm; Hbs III and IV (top of column) were separated by ~3 cm. The column gel was carefully forced out of the column by gentle air pressure, and the bands were cut out. The proteins were eluted from the gel by repouring the gel into smaller columns and washing with 0.2 M potassium phosphate, pH 7.5. This also served to concentrate the proteins. Isoelectric points were determined by isoelectric focusing in gels as previously described (LaGow & Parkhurst, 1972; Steinmeier & Parkhurst, 1975).

(*3*) *Kinetic Experiments.* The stopped-flow apparatus has been described previously (Görisch et al., 1976). For these experiments, it was used in the absorbance mode with a Jarrell-Ash quarter meter monochromator and a 150-W Osram Xe lamp. For the photolysis experiments, a Phase-R 2100 XBH dye laser (0.5-μs pulse, 585 nm) was used with rhodamine 6G as the lasing dye. Discharge energies from the Marx bank were kept below 35 J. The sample chamber for photolysis has been described previously (Boelts & Parkhurst, 1971). Temperatures were determined from the output of a calibrated thermistor located in the reaction curvette. The data-acquisition system was the same as that previously described (LaGow & Parkhurst, 1972) except that a Nova 2-10 with 32K of memory replaced the Super-Nova minicomputer. For the oxygen association kinetics for bands I and II of the monomer, data were obtained from photographs of the reaction traces on a storage oscilloscope. The four rate constants were measured in the following ways.

(a) CO association (l') was measured by following the recombination reaction after photolysis of HbCO. A small amount of dithionite was present to prevent the occurrence of a rapid recombination reaction from oxygen. The temperature ranged from 4 to 21 °C and the wavelength from 430 to 437 nm. Protein concentrations were 10 μM (monomer) and 10–100 μM (polymer) (heme basis). (b) Oxygen association (k') was measured by following the rapid phase of the reaction occurring after laser photolysis of HbCO in the presence of oxygen. The concentrations were O_2, 229–236 μM; CO, 77–100 μM; protein, 6–11 μM (monomer) and 10 μM (polymer). The temperature ranged from 3 to 20 °C, and the observing wavelengths were 431–437 nm. (c) Oxygen dissociation (k) was measured from the very slow phase (half-time of 5 ms–0.6 s) of the replacement reaction from experiment b, observed at 426 nm for the monomer and 425 nm for the polymer. The replacement reaction corresponds to a relaxation:

$$HbO_2 + CO \underset{k'}{\overset{k}{\rightleftharpoons}} Hb + CO + O_2 \underset{l}{\overset{l'}{\rightleftharpoons}} HbCO + O_2$$

Either from the smaller eigenvalue of the (rg) relaxation matrix (Castellan, 1963) or for Hb as a steady-state intermediate, by inspection (Gilbert, 1977), one obtains for the first-order decay constant, R

$$R = \frac{l'(CO)k + k'(O_2)l}{l'(CO) + k'(O_2)} \simeq \frac{l'(CO)k}{l'(CO) + k'(O_2)}$$

from which the constant k can be determined. Preliminary experiments in which HbO_2 was flowed against dithionite solutions in a stopped-flow apparatus had shown that the dissociation reaction was too fast to measure for hemoglobins I and II of the monomer and would not be determined reliably for the other proteins. (d) CO dissociation (l) was measured by flowing HbCO against a buffer half Ar and half NO saturated. The HbCO solution was 10 μM in protein (heme basis) and was ~10 μM in CO. It was obtained by diluting concentrated HbCO (over which had been blown a stream of water-saturated argon for 20 min to remove excess CO) into Ar-saturated buffer. The reaction was followed at 415 nm, and the temperature ranged from 6.2 to 21 °C.

(*4*) *Data Analysis.* All monomer kinetic data and the replacement–relaxation and CO dissociation data for the polymer were monophasic and were processed according to a one-exponential decay model using a Fletcher–Powell least-squares minimization program (Fletcher & Powell, 1963). In all cases, the parameter errors were small, the distributions of residuals as time series (Swed & Eisenhart, 1943) were satisfactory, and the sums of squares at the minimum of the response surface were small. Parameter errors from the variance–covariance matrix are given in the tables. These data could not be fit well by a two-exponential model since enormous parameter errors were obtained with virtually no improvement in the sums of squares. The polymer-association kinetic data were fit by a two-exponential model using the Fletcher–Powell minimization routine (Fletcher & Powell, 1963), and parameter standard deviations were obtained from the variance–covariance matrix. The errors in k and the equilibrium constants K (for oxygen binding), L (for CO binding), and M (the partition constant, K/L) were obtained from the errors in the rate constants using error-propagation theory (Benson, 1960). Activation energies were obtained by a linear least-squares fit

[1] The morphologically similar blood worm, *G. americana*, does not occur north of Buzzard's Bay, MA (Pettibone, 1963).

Table I: Oxygen (k') and Carbon Monoxide (l') Association Rate Constants and Activation Energies for Monomeric *G. dibranchiata* Hemoglobins at 20 °C

protein	p*I*	% abundance[a]	pH	k' (μM^{-1} s^{-1})	E_a (kcal/mol)	l' (μM^{-1} s^{-1})	E_a (kcal/mol)
Hb I	6.84	41–43	7	190 ± 18	6.4 ± 2.0	22.4 ± 0.4	2.8 ± 0.4
			9	250 ± 22		26.8 ± 0.4	
Hb II	6.97	30–38	7	186 ± 18	5.6 ± 1.7	26.8 ± 0.6	3.2 ± 0.9
			9	238 ± 22		24.8 ± 0.4	
Hb III	7.25	21–27	7	39 ± 3	11.3 ± 1.6	2.15 ± 0.03	8.4 ± 0.7
			9	52 ± 4		3.00 ± 0.06	

[a] These figures are the ranges found in three preparations, 100 worms each. In a fourth preparation, 100 worms, the percentages were 21, 15, and 64 for Hbs I, II, and III, respectively. These latter percentages would give a percent fast component of 36 in O_2 and CO association kinetics, in excellent agreement with the findings of Seamonds et al. (1976). For these calculations, Hb IV, the most basic monomeric hemoglobin detected, is neglected. It usually contributed ~4% to the monomer fraction.

Table II: Oxygen and Carbon Monoxide Association Constants and Activation Energies for Polymeric *G. dibranchiata* Hemoglobin at 20 °C[a]

heme concn (μM)	pH	k'_1 (μM^{-1} s^{-1})	E_a (kcal/mol)	k'_2 (μM^{-1} s^{-1})	E_a (kcal/mol)	l'_1 (μM^{-1} s^{-1})	E_a (kcal/mol)	l'_2 (μM^{-1} s^{-1})	E_a (kcal/mol)
10	7	28 ± 4	12 ± 3	6.5 ± 0.6	10 ± 3	2.11 ± 0.05	4.0 ± 0.4	0.56 ± 0.01	5.6 ± 0.4
	9	47 ± 6		10.8 ± 0.5		2.46 ± 0.04		0.67 ± 0.01	
100	7					1.81 ± 0.14		0.59 ± 0.05	

[a] The faster component is denoted by a subscript 1 and the slower by subscript 2. The average fraction of the rapid component was 0.55 for both O_2 and CO association.

of the data to the Arrhenius equation, and the errors were obtained from the least-squares error estimate for the slope.

Results and Discussion

The monomer component was fractionated into four hemoglobins. The p*I*'s and abundances of the three major components are listed in Table I. The abundances were found to vary considerably, even though pooled blood from 100 worms was used for every preparation. Weber et al. (1977) recently reported two major and several minor monomeric Hbs isolated from *G. dibranchiata* by isoelectric focusing. Their band III elution profile (Weber et al., 1977, Figure 3) appears heterogeneous, and the fraction was eluted over a pH range consistent with its being a mixture of Hbs I and II (our notation). Their band II has a p*I* corresponding to that of our Hb III, and our minor Hb IV appears to correspond to their band I. From the association data (Table I) it is clear that Hbs I and II together must account for the rapid phase reported earlier by Seamonds et al. (1976) for association kinetics, yet, in that study, they comprised only ~35% of the total; in these studies, the percent was more typically ~75 (see footnote *a*, Table I). In all cases, the isolated three major fractions showed monophasic kinetics. Ultracentrifuge studies (Seamonds, 1969; Seamonds et al., 1971a) suggest that the polymer may be a hexadecamer. Seamonds (1969) obtained a value of 0.94 ± 0.02 for the Hill number for oxygen equilibrium. From our kinetic data, which assumes a noncooperative model, the heterogeneity results in a theoretical value of 0.90 for the Hill number for both oxygen and CO binding at both pH 7 and pH 9. At pH 8 in Tris buffer, Mizukami & Vinogradov (1972) obtained values for the Hill number of 1.1–1.2 for the polymeric hemoglobin, 30–100 μM in heme. Weber et al. (1977) found a value for n of 1.2 for the polymeric fraction, 0.5 mM in heme. Similar results showing very low cooperativity were reported by Harrington et al. (1978). We noted (Table II) a small dependence of l' on polymer concentration, perhaps suggesting weak cooperative interactions. No Hb* or quickly reacting form of the polymer was detectable in laser-photolysis experiments in which the photolysis varied from 15% to 98%. If the polymer were cooperative and one could treat the system in terms of R and T states with heterogeneity of subunits, our l' and k' correspond to overall association constants starting from the T state of the protein (assuming the R to T transition following photolysis to be much faster than ligand binding at both pH 7 and pH 9, a valid assumption, since flow and flash results agree for CO binding). The constants k and l, determined by replacement reactions, would correspond to rate constants for the R state of a cooperative polymeric hemoglobin. Owing to the lack of strong evidence for significant cooperativity, however, we treated the polymer as a heterogeneous noncooperative protein [see Tanford (1961)].

Table III: Dissociation Rate Constants and Activation Energies for *G. dibranchiata* Monomeric (A) and Polymeric (B) Hemoglobins at 20 °C

(A) Monomeric Hemoglobins

protein	pH	k (s^{-1})	E_a (kcal/mol)	l (s^{-1})	E_a (kcal/mol)
Hb I	7	2800 ± 260	19 ± 1.5	0.055 ± 0.001	1.8 ± 0.3
	9	2650 ± 250		0.056 ± 0.001	
Hb II	7	1800 ± 175	18 ± 1.6	0.042 ± 0.0008	7.8 ± 0.3
	9	2350 ± 200		0.034 ± 0.0006	
Hb III	7	385 ± 25	21.5 ± 1.5	0.022 ± 0.0002	5.4 ± 0.3
	9	440 ± 30		0.017 ± 0.0003	

(B) Polymeric Hemoglobins

pH	k_1 (s^{-1})	E_a (kcal/mol)	k_2 (s^{-1})	E_a (kcal/mol)	l (s^{-1})	E_a (kcal/mol)
7	105 ± 15	16 ± 2	91 ± 9	13.2 ± 1.4	0.054 ± 0.001	8.7 ± 0.4
9	121 ± 16		103 ± 6		0.054 ± 0.001	

The values for the rate constant l for the monomer (Table III) are not very different from that found for whale myoglobin (LaGow & Parkhurst, 1972), nor is there much difference among the three monomeric components. The rate constants k' and l' were previously measured for the fast phases of both *Glycera* and *Chironomus* monomeric hemoglobins, and the constants for *Glycera* were found to be somewhat the larger (Rumen & McCray, 1973; Seamonds et al., 1976). We have found that the individual monomeric hemoglobins from *Glycera* show monophasic kinetics; thus, the conformer model previously proposed (Seamonds et al., 1976) for the monomer kinetics is no longer tenable. The oxygen dissociation rate constant (k) for Hb I (2800 s^{-1}, 20 °C) is even larger than

Table IV: Calculated Equilibrium Constants and ΔH° Values for *G. dibranchiata* Hemoglobins at 20 °C[a]

protein	pH	K (μM)	ΔH° (kcal/mol)	L (nM)	ΔH° (kcal/mol)	M	ΔH° (kcal/mol)
Hb I	7	14.7 ± 1.9	−12.67 ± 2.5	2.45 ± 0.07	1.0 ± 0.5	6000 ± 800	−13.6 ± 2.5
	9	10.6 ± 1.4		2.09 ± 0.05		5100 ± 650	
Hb II	7	9.7 ± 1.3	−12.4 ± 2.3	1.57 ± 0.05	−4.6 ± 0.9	6200 ± 850	7.8 ± 2.5
	9	9.9 ± 0.8		1.37 ± 0.04		7200 ± 650	
Hb III	7	9.9 ± 1.0	−10.2 ± 2.2	10.2 ± 0.2	3.0 ± 0.8	970 ± 100	−13.2 ± 2.3
	9	8.4 ± 0.8		5.67 ± 0.16		1500 ± 160	
polymer	7	6.8 ± 0.8		46 ± 1		148 ± 14	
	9	4.6 ± 0.4		39 ± 1		118 ± 10	

[a] The equilibrium constants were calculated from rate constants: $K = k/k'$, $L = l/l'$, and $M = K/L$. For the polymer, a noncooperative two-component model was assumed, with a fraction of the high-affinity form = 0.55, and $l_1 = l_2 = l$ observed (Table III). For the polymer, the calculated K and L correspond to concentrations of ligand for half-saturation.

that reported by Sawicki & Gibson (1977) for the β chains in T-state human hemoglobin and appears to be the largest oxygen dissociation constant yet reported for a hemoglobin. The equilibrium constants derived from kinetic data are given in Table IV. Seamonds (1969) obtained values for K of 8.8 ± 0.7 and 8.9 ± 0.5 μM for monomer (total) and polymer (75 μM in heme), respectively, at 20 °C from tonometric measurements. The variation in the composition of the monomer fraction prevents a direct comparison with the value for K of Seamonds (1969). The values for K for Hbs II and III, however, are within experimental error of the value reported by Seamonds (1969) for the monomer. Both our K and ΔH° values (Table IV) calculated from kinetic data are in very good agreement with the values reported by Weber et al. (1977) from direct equilibrium measurements. The agreement in the K values suggests that the k obtained by relaxation is appropriate and that a more complex mechanism need not be invoked for relaxation in the 273–300 K region. The values for k' reported here are also in good agreement with those reported earlier (from laser photolysis of HbO_2) for the two phases of the monomer fraction (Seamonds et al., 1976), suggesting that if there are conformational differences between HbCO and HbO_2, relaxation of the protein to the Hb form is rapid with respect to the rate of ligand binding. In addition to the extremely large values of the constants k', l', and k for Hb I, it should be noted that the values of M for this hemoglobin and Hb II are apparently the largest yet found for a hemoglobin. It was noted earlier (Seamonds et al., 1976) that the monomer is readily contaminated by traces of CO, which can lead to difficulty in interpreting oxygen kinetic data.

Of the four rate constants, the largest activation energies are found for oxygen dissociation. From Tables I–III it can be noted that the activation energies are quite low for Hbs I and II, except for oxygen dissociation. For the association reactions, Hb III and the polymer fraction have, in general, the higher activation energies. For oxygen dissociation, the polymeric components appear to have the lowest activation energies. For Hbs II and III and the polymer, the activation energies for CO dissociation are very similar. Hbs I and II are quite similar, except for CO dissociation at low temperature and the very low activation energy of Hb I for CO dissociation.

Isoelectric focusing was carried out on a sample of monomeric *G. dibranchiata* hemoglobin provided by Professor Austen Riggs. This was one of two identical crystalline preparations sent from the laboratory of Professor Warner E. Love in 1970. The sequence of *G. dibranchiata* monomeric hemoglobin (Imamura et al., 1972) was determined on the major fraction from one of these samples. The sample sent to us gave isoelectric patterns consistent with an assignment of 5% Hb I, 95% Hb II, and no detectable Hb III. Both fractions showed CO kinetics identical with those for our Hbs I and II. We conclude that the published sequence for the monomeric hemoglobin is very likely that of Hb II. In the sequence that has been reported for *Glycera* (Imamura et al., 1972), the distal histidine (58) is replaced by a leucine, and an aspartate residue (57) is also near the site of ligand binding. The unusually large values for rate constants k, k', and l' for Hb II thus appear to be associated with substitution of the distal histidine. The strikingly different kinetics for Hb III lend considerable interest to a sequence determination for the heme cavity region in that hemoglobin. In Hb Norfolk ($\alpha_2{}^{57}\beta_2$ Gly → Asp) the aspartyl residue is adjacent to the distal histidine. Both Hb Norfolk (Bailey et al., 1968; Beetlestone et al., 1976) and *Glycera* monomeric hemoglobin show reduced affinity (Seamonds et al., 1971b) for the binding of anions in the ferric state. An EPR study (Ikeda-Saito et al., 1977) comparing the pH dependences of Co-Mb and Co-Hb (*Glycera*)[2] suggests the importance of distal imidazole–O_2 interaction in the former compound. The presence of a partial negative charge on bound oxygen and the occurrence of the two substitutions at sites 57 and 58 in *Glycera* hemoglobin should result in a reduced energy barrier for oxygen dissociation. The observed activation energy (19 kcal/mol) is, however, quite large for a hemoglobin. In kinetic studies with model heme compounds, Chang & Traylor (1975) noted the sensitivity of k (oxygen dissociation) to solvent (heme cavity) polarity and proximal ligand basicity. They obtained a value of 2500 s^{-1} for k for a substituted pyridine–heme–O_2 complex in toluene, a value very close to that obtained for Hbs I and II in this study. The rate constant decreased to 150 s^{-1} for the same complex in phosphate buffer and 2% cetyltrimethylammonium bromide. Preliminary collaborative work with the University of Illinois group[3] has shown that the last barrier to ligand binding in the heme cavity, before the Fe site itself, is missing from Hb I but not from Hb III. The data reported here suggest that the barrier can affect the three rate constants k', l', and k but has little influence on l.

References

Bailey, J. E., Beetlestone, J. G., & Irvine, D. H. (1968) *J. Chem. Soc. A*, 2913.

Beetlestone, J. G., Adeosun, O. S., Goddard, J. E., Kushimo, J. B., Ogunlesi, M. M., Ogunmola, G. B., Okonjo, K. O., & Seamonds, B. (1976) *J. Chem. Soc., Dalton Trans. 13*, 1251.

Benson, S. W. (1960) *The Foundations of Chemical Kinetics*, p 87, McGraw-Hill, New York.

[2] Abbreviations used: Co-Mb, whale oxymyoglobin with Fe in the heme replaced by cobalt; Co-Hb (*Glycera*), a *Glycera* monomeric oxyhemoglobin with Fe replaced by cobalt.

[3] Professors Hans Frauenfelder and Laura Eisenstein and Dr. A. Reynolds and colleagues.

Boelts, K. J., & Parkhurst, L. J. (1971) *Biochem. Biophys. Res. Commun. 43*, 637.
Castellan, G. W. (1963) *Ber. Bunsenges. Phys. Chem. 67*, 898.
Chang, C. K., & Traylor, T. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1166.
Fletcher, R., & Powell, M. J. D. (1963) *Comput. J. 6*, 163.
Gilbert, H. F. (1977) *J. Chem. Educ. 54*, 492.
Görisch, H., Goss, D. J., & Parkhurst, L. J. (1976) *Biochemistry 15*, 5743.
Harrington, J. P., Suarez, G., Borgese, T. A., & Nagel, R. L. (1978) *J. Biol. Chem. 253*, 6820.
Hodgman, C. D., Ed. (1960) *Handbook of Chemistry and Physics*, 41st ed., pp 1706–1707, Chemical Rubber Co., Cleveland, OH.
Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J., & Yonetani, T. (1977) *J. Biol. Chem. 252*, 4882.
Imamura, T., Baldwin, T. O., & Riggs, A. (1972) *J. Biol. Chem. 247*, 2785.
LaGow, J., & Parkhurst, L. J. (1972) *Biochemistry 11*, 4520.
Mizukami, H., & Vinogradov, S. N. (1972) *Biochim. Biophys. Acta 285*, 314.
Padlan, E. A., & Love, W. E. (1974) *J. Biol. Chem. 249*, 4067.
Pettibone, M. H. (1963) *Bull.—U.S. Natl. Mus. 227* (part 1), 215, 218.
Rumen, N. M., & McCray, J. A. (1973) *Biophys. J. 13*, 28a.
Sawicki, C. A., & Gibson, Q. H. (1977) *J. Biol. Chem. 252*, 7538.
Seamonds, B. (1969) Ph.D. Dissertation, University of Pennsylvania, Philadelphia, PA.
Seamonds, B., Forster, R. E., & Gottlieb, A. J. (1971a) *J. Biol. Chem. 246*, 1700.
Seamonds, B., Forster, R. E., & George, P. (1971b) *J. Biol. Chem. 246*, 5391.
Seamonds, B., Blumberg, W. E., & Peisach, J. (1972) *Biochim. Biophys. Acta 263*, 507.
Seamonds, B., McCray, J. A., Parkhurst, L. J., & Smith, P. D. (1976) *J. Biol. Chem. 251*, 2579.
Steinmeier, R. C., & Parkhurst, L. J. (1975) *Biochemistry 14*, 1564.
Swed, F. S., & Eisenhart, C. (1973) *Ann. Math. Stat. 14*, 66.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, p 539, Wiley, New York.
Weber, R. E., Sullivan, B., Bonaventura, J., & Bonaventura, C. (1977) *Comp. Biochem. Physiol. B 58*, 183.

Basement Membrane Collagens. Cyanogen Bromide Peptides of the D Chain from Porcine Kidney†

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ABSTRACT: An α chain size collagenous component, designated as the D chain, was isolated from the pepsin digest of porcine kidney cortices. Reduction of the D chain with 2-mercaptoethanol and carboxymethylation with iodoacetic acid resulted in the formation of two smaller components of 75 000 and 15 000 molecular weights. The 75 000 and 15 000 molecular weight components were separated by molecular sieve chromatography on a column of agarose A-5M. The 75 000 molecular weight component was cleaved with CNBr in 70% formic acid. The resulting CNBr peptides were isolated by a combination of ion exchange and molecular sieve chromatography and were characterized for amino acid contents and molecular weights. A total of seven CNBr peptides were obtained, which together accounted for the amino acid content of the intact 75 000 molecular weight component.

Collagenous components of basement membrane have been found to be distinct from interstitial types I, II, and III collagens in several respects, including the amino acid composition and the primary structure. To date, the molecular organization and the chain composition of the collagenous component(s) of basement membranes remain controversial. Earlier studies on the material isolated by pepsin solubilization of anterior lens capsule, renal glomerular basement membrane, and descemet's membrane (Kefalides, 1971, 1972; Dehm & Kefalides, 1978) suggested that their structures contained a single type of collagen chains, type IV. The idea that basement membrane collagen may be composed of three identical chains, $[\alpha 1(IV)]_3$, is further supported by biosynthetic studies on procollagen in organ cultures of parietal yolk sac endoderm (Minor et al., 1976) and of lens capsule (Heathcote et al., 1978). However, the above proposed structure is not in agreement with the investigations reported from several laboratories (Hudson & Spiro, 1972; Daniles & Chu, 1975; Sato & Spiro, 1976; Freytag et al., 1976) suggesting the heterogeneity of collagenous component in glomerular basement membrane. Several other investigators (Bailey et al., 1979; Glanville et al., 1979; Kresina & Miller, 1979; Sage et al., 1979) reported on the presence of at least two types of genetically distinct chains, designated C and D chains,[1] in placenta, a tissue rich in basement membrane. Our laboratory also described isolation of the C and D chains from anterior lens capsule and glomerular basement membrane (Dixit, 1978, 1979; Dixit & Kang, 1979). The results of biosynthetic investigations on the basement membrane of murine tumor (Timpl et al., 1978) and on amniotic fluid cell culture (Crouch & Bornstein, 1978) have shown the presence of two electro-

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[1] The nomenclature of the C and D chains presented in this paper corresponds to that used by Kresina & Miller (1979). The recently described C chain from placenta (Sage & Bornstein, 1979) is different from the C chain referred to in this paper.

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