^{13}C Nuclear Magnetic Resonance Studies of the Binding of Isocyanides to Various Hemoglobins and Myoglobins†

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ABSTRACT: Interactions between ethyl and isopropyl isocyanides and various hemoglobins and myoglobins have been studied by ^{13}C nuclear magnetic resonance. The results indicate that the chemical shift of the bound isocyanide depends on the structure of the hemoglobin subunit or myoglobin. The resonances exhibited by isocyanides bound to myoglobin are sensitive to pH in contrast to the situation with rabbit and human hemoglobins. β subunits of opossum, rabbit, and human hemoglobins show a significantly greater preferential affinity for CO relative to EIC than do α subunits which have allowed the assignment of resonances. Rabbit, human, and opossum hemoglobin subunits bind ethyl isocyanide without observable preferences and an excess of DPG does not appear to affect this random order of ligation. In contrast, an excess of IHP seems to cause preferential ligation of the α subunits in these hemoglobins. The results have been used to gain insights into the differing characteristics of the ligand binding pockets of these various hemoglobins.

A fundamental question in the allosteric cooperativity displayed by hemoglobin ligation is the detailed molecular mechanism by which ligand binding to one subunit is able to affect the ligand affinity of other subunits. Based on X-ray crystallographic studies, Perutz (1970) proposed that ligation causes the iron atom to move from a position out of the porphyrin plane on the proximal side (toward histidine F8) to a position in the plane. This movement acts like a trigger to initiate structural changes within the liganded subunit which are transmitted to adjacent subunits, thereby affecting their affinity for ligand. Perutz (1970) also suggested that, in the T state of the molecule (unliganded), the α chains have a higher affinity than the β chains because of the steric hindrance, caused by valine (E11) in the binding pocket of β chain in the T state, to the approach of ligand to the iron atom.

Huestis & Raftery (1972a–c, 1973, 1975) observed that ligands such as *n*-butyl isocyanide, oxygen, and carbon monoxide bind randomly to subunits of human hemoglobin labeled with a trifluoroacetonyl group on cysteine β93 (Huestis & Raftery, 1978). In this case, addition of organic phosphates (DPG, IHP)[1] results in preferential binding of these ligands by the α chains. Olson & Gibson (1970–1972) have utilized stopped flow techniques to show that *n*-butyl isocyanide binds randomly to hemoglobin subunits while, in contrast to the work of Huestis & Raftery, addition of organic phosphates leads to kinetically faster binding of *n*-butyl isocyanide by the β chains. A higher affinity of the β subunits for BIC in the presence of IHP has been reported by Lindstrom et al. (1971) using proton magnetic resonance techniques.

We have used ^{13}C nuclear magnetic resonance to study the binding of ligands such as ethyl and isopropyl isocyanides to various hemoglobins (human, New Zealand white rabbit, opossum) and myoglobins (sperm whale, horse). We have investigated the thermodynamic affinities of the unliganded subunits for EIC, the thermodynamic competition between EIC and carbon monoxide for binding to the α and β subunits, and the Hill coefficients for binding of these isocyanides to the various hemoglobins. In addition, we sought to identify the origin of the chemical shift differences displayed by isocyanides bound to the various heme proteins.

Isocyanides are useful as probes of the binding site because of the extreme sensitivity of the terminal carbon of these molecules to changes in the environment as well as to changes in hybridization of the nitrogen as a result of movement of the alkyl substituent (Stephany et al., 1974). Moreover, these compounds, being considerably bulkier than O_2 or CO, can serve as sensitive steric probes of the ligand binding site (Anderson et al., 1970; Talbot et al., 1971; Brunori et al., 1972). Preliminary results of binding of [^{13}C]ethyl isocyanide to myoglobin and human adult and fetal hemoglobin have been reported in which chemical shifts alone were used to provisionally assign the observed absorptions to ligand bound to the various subunits (Mansuy et al., 1976).

Materials and Methods

Whole blood from human, rabbit (New Zealand white), and opossum was freshly drawn and citrated. Red cells were separated from the plasma by centrifugation at 2500*g* and were subsequently washed twice with 0.15 M sodium chloride. The red cells were lysed with 2 volumes of distilled water or 0.001 M Tris buffer (pH 7.4), and the stromata were removed by centrifugation at 30 000*g* for 30 min. The hemoglobin was then exhaustively dialyzed against 0.15 M sodium chloride. This dialysis removes organic phosphates (Bunn et al., 1971). Hemoglobin solutions were concentrated by ultrafiltration (Amicon PM-10 membrane) to a final concentration of 2–4 mM.

Sperm whale and horse (skeletal) myoglobins were purchased from Sigma Chemical Co. Myoglobin samples were prepared in 0.1 M phosphate buffer, pH 7.0, and a small amount of dithionite was added to reduce any metmyoglobin which might form. The final concentration of myoglobin usually was 3–4 mM. NMR samples were prepared by the addition of isocyanides to deoxyhemoglobins and myoglobins in aqueous solutions which had been deoxygenated and to

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[1] Abbreviations used: DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Me_4Si, tetramethylsilane; PMB, *p*-mercuribenzoate; EIC, ethyl isocyanide; IPIC, isopropyl isocyanide; BIC, *n*-butyl isocyanide; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

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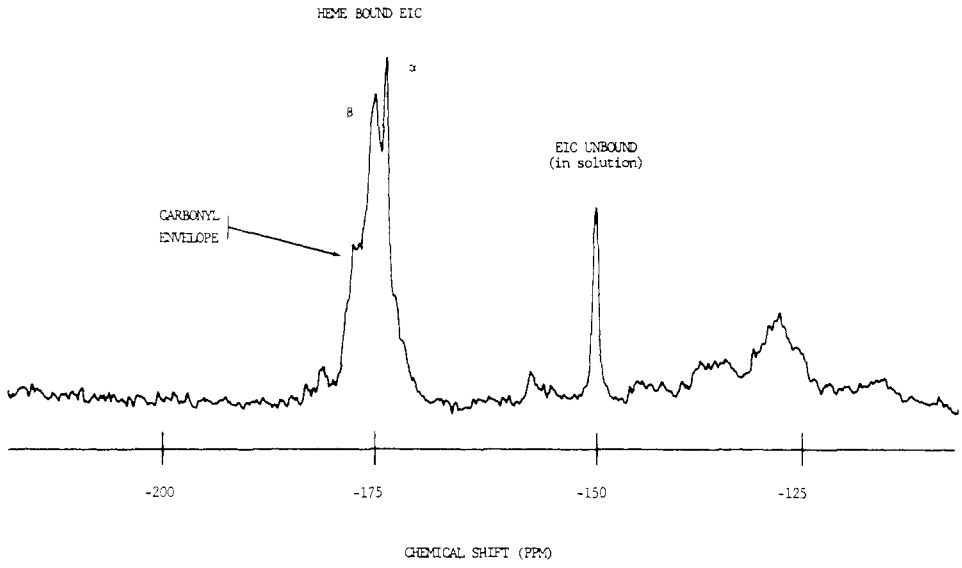

FIGURE 1: The downfield region in the carbon-13 spectrum of human Hb-[$^{13}C^0$]EIC in the presence of excess EIC showing the resonances due to free and bound EIC. The conditions under which this spectrum was obtained were: 2.75 mM Hb in 0.1 M Tris buffer, pH 7.2. This spectrum required 20 000 transients taken over a spectral width of 5 kHz with 0.8 s between pulses. Chemical shifts are reported downfield from Me_4Si.

which a small amount of dithionite had been added.

Separated α and β chains were prepared as the *p*-hydroxymercuribenzoate (PMB) derivatives by the method of Geraci et al. (1969). Mercury free α and β chains were prepared by the method of Winterhalter & Colosimo (1971).

Partial saturation studies of hemoglobins with ^{13}C-enriched EIC were carried out in a sealed NMR tube with a 1-mm path length cuvette fused to one end. The NMR tube was extensively flushed with water-saturated nitrogen before deoxyhemoglobin was introduced via syringe through the septum. The sample was then extensively flushed with nitrogen in the NMR tube to ensure complete deoxygenation. Sample deoxygenation was determined by measuring absorbances at 670 and 730 nm. An A_{670}/A_{730} ratio of 2.29 or better signified complete deoxygenation (Benesch et al., 1965). Small aliquots of an aqueous solution of ^{13}C-enriched EIC, which had been flushed with nitrogen, were then injected into the NMR tubes through the septum and the extent of ligation was monitored at 650 nm. The concentration of ^{13}C-enriched EIC was determined by the assay method of Anderson et al. (1970). NMR spectra were recorded after each addition of EIC. The state of ligation of the hemoglobin samples did not change during a determination of the ^{13}C NMR spectrum.

The amount of methemoglobin present was assayed in each sample before and after the determination of the CMR spectrum by removing a 10-μL aliquot of the sample, dissolving it in 10 mL of 0.15 M sodium chloride, and mixing it extensively with atmospheric air. Absorbance measurements at 415, 420, and 430 nm were used to calculate met-, oxy-, and deoxyhemoglobin concentrations (Benesch et al., 1965). Samples prepared from freshly drawn blood typically contained no more than 4% methemoglobin. Also, the methemoglobin in a sample did not change appreciably during a partial saturation experiment.

Hill coefficients for the binding of EIC and IPIC to various hemoglobins were determined in sealed cuvettes using a method similar to that described by Anderson et al. (1970). The 1-cm path length cuvettes were extensively flushed with nitrogen and then sealed with a rubber septum. Three milliliters of deoxygenated hemoglobin solution, 10^{-4} to 10^{-6} M, were injected into the cuvette and the same volume of nitrogen withdrawn. A small amount of dithionite was added to ensure deoxygenation. Optical density measurements at 555 nm were used to calculate the hemoglobin concentrations (Anderson et al., 1970). Small aliquots (usually 0.5–1 μL) of a standard isocyanide solution were added and the state of ligation of the hemoglobin was monitored at 540 nm. The concentration of free isocyanide was determined from the degree of ligation of the hemoglobin and from the known amount of isocyanide injected.

Unenriched isocyanides were prepared by the method of Jackson & McKusick (1955). The ^{13}C-enriched isocyanides (enriched at the C^0 carbon) were prepared by somewhat modified procedures. ^{13}C-enriched sodium cyanide or potassium cyanide (90–92% enrichment) was purchased from Merck, Sharpe and Dohme and converted to silver cyanide. The enriched silver cyanide was dried and reacted with either ethyl or isopropyl iodide at 130 °C. After 4–6 h, or when the reaction mixture had turned to a brownish syrup, it was cooled and aqueous sodium cyanide was added. The isocyanide was steam distilled and the first milliliter or two of distillate was collected. ^{13}C NMR indicated that enriched isocyanide was only present in the aqueous layer.

Stock solutions containing 53.6 mM IPIC and 67.3 mM EIC were prepared by dissolving either 100 mL of fresh IPIC or 100 mL of fresh EIC in 20 mL of degassed water. Stock solutions were then used to determine the Hill coefficients.

IHP was obtained as the sodium salt from Sigma Chemical Co. Solutions of IHP were prepared by dissolving a certain amount of the salt in water and then adjusting the pH with 1 N HCl. DPG was obtained as the pentacyclohexylammonium salt from Sigma Chemical Co. Solutions of DPG were prepared by dissolving the commercial DPG in water; the pentacyclohexylammonium ion was then removed by adding this solution to Dowex 50 W-X8 in the H^+ form. The pH of the solution recovered from this cation exchange resin was then adjusted with 1 M NaOH to 7.0. A 5× excess of IHP or DPG was added to hemoglobin samples for NMR experiments involving these organic phosphates.

NMR spectra were obtained on a Varian Associates XL-100-15 spectrometer equipped with a 620i computer (16K memory) operating in the FT mode. Except for the special tubes used in the partial saturation work, NMR samples were prepared in 12- or 18-mm round-bottomed tubes with a 5-mm

TABLE I: $^{13}C^0$ Chemical Shifts for [$^{13}C^0$]EIC and IPIC Bound to Native Myoglobins and Hemoglobins[a]

source	[$^{13}C^0$]EIC	[$^{13}C^0$]IPIC
myoglobin		
sperm whale	173.4 (pH 7.5)	173.4 (pH 7.46)[b]
horse	171.7 (pH 7.5)	168.5 (pH 7.42)
hemoglobin		
human adult		
α subunit	173.85	177.27 (pH 7.42)
α-PMB	173.40	
α-SH	173.48	
β subunit	175.04	177.99 (pH 7.42)
β-PMB	173.73	
β-SH (tetramer)	171.57	
rabbit		
α subunit	169.27	170.37
β subunit	173.81	176.88
trifluoroacetonyl-label at β93		
$-\alpha$ subunit	169.27	
$-\beta$ subunit	173.81	
opossum		
α subunit	173.71	
β subunit	177.66	

[a] Myoglobin samples were run in 0.1 M sodium phosphate. Hemoglobin samples were run in 0.15 M NaCl. [b] Chemical shifts which have pH values in parentheses are pH sensitive. The chemical shift values given are those observed at that particular pH.

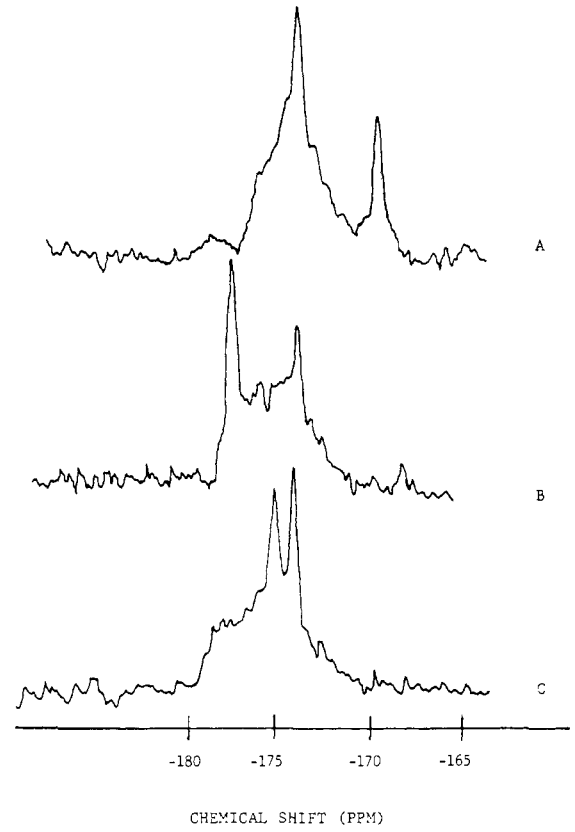

FIGURE 2: Representative spectra of [$^{13}C^0$]EIC bound to rabbit (A), opossum (B), and human (C) hemoglobins in the presence of excess EIC. A required 17 289 scans and a protein concentration of 3 mM in tetramer. B required 15 922 scans and 3 mM tetramer. Figure 2C required 10 366 scans and 4 mM in tetramer. All samples were in 0.15 M NaCl, pH ~7.0, and the respective ^{13}C spectra were not run in sealed tubes. Chemical shifts are downfield from Me_4Si.

capillary containing deuterium oxide inserted concentrically as a field frequency lock. All samples were run at a probe temperature of 34 °C using a 90° radiofrequency ^{13}C excitation pulse of 55 μs with spectral widths of 2.5 or 5 kHz and an acquisition time of 0.8 s. Proton noise decoupling with a bandwidth of 1.5 kHz centered on water was used throughout.

Results

Figures 1 and 2 show representative spectra of ^{13}C-enriched EIC bound to various hemoglobins. Table I collects the chemical shift data for ^{13}C-enriched EIC and IPIC bound to various myoglobins and hemoglobins. In all cases, the spectra are characterized by slow exchange of the ligand between the solution and bound form as demonstrated in Figure 1 by the simultaneous presence of free and bound EIC resonances. Similar spectra were observed for IPIC binding, with the free IPIC resonance appearing at −148.7 ppm from Me_4Si.

Ethyl isocyanide, when bound to various hemoglobins, exhibits two distinct peaks in the region of 175 ppm downfield from Me_4Si. The carbonyl resonances of the protein also occur in this region which hinders good quantitative analysis of the early stages of ligation in some of the partial saturation experiments.

Identification of the two resonances exhibited by [$^{13}C^0$]EIC bound to subunits of human, rabbit, and opossum hemoglobin was accomplished by an equilibrium exchange experiment utilizing ^{13}CO. For example, human hemoglobin saturated with [$^{13}C^0$]EIC exhibits resonances at 175.04 and 173.85 ppm from Me_4Si. Addition of ^{13}CO leads to a decrease in the intensity of the resonance at 175.04 ppm and the concomitant appearance of a new resonance at 206.19 ppm which is known to correspond to ^{13}CO bound to the β subunit (Moon & Richards, 1974). Accordingly, the resonance at 175.04 ppm downfield from Me_4Si represents the [$^{13}C^0$]EIC bound to the β subunit and the resonance at 173.85 corresponds to [$^{13}C^0$]EIC bound to the α subunit. Where such ^{13}CO-[$^{13}C^0$]EIC exchange experiments were performed (opossum, rabbit, and human hemoglobins), the downfield resonance of the hemoglobin saturated with EIC represented the isocyanide bound to the β subunit. The signals corresponding to ^{13}CO bound to the α and β subunits of these hemoglobins have been previously determined (Moon & Richards, 1974; Moon et al., 1977). In all these cases, the resonance corresponding to EIC bound to the α subunit did not decrease in intensity until all the EIC was displaced from the β chain by ^{13}CO.

The resonances resulting from [$^{13}C^0$]EIC bound to the α subunit of the different hemoglobins generally occur in the region of 173.5 ppm downfield from Me_4Si; rabbit hemoglobin is an exception (169.27 ppm; see Table I). In contrast, the resonances of [$^{13}C^0$]EIC bound to the β subunits of the various hemoglobins occur over a region of 177 to 173 ppm downfield from Me_4Si.

Studies on the PMB derivatives of the isolated subunits of adult hemoglobin yielded results that differ significantly from those observed for EIC bound to these subunits in an intact tetramer. Ligand bound to mercury free subunits shows, for the α(SH) subunit, a shift very close to that both for the intact tetramer and for the α(PMB) derivative (see Table I). For the β(SH) subunit (which exists as a tetramer) a shift is observed 1.68 ppm upfield from that of EIC bound to β(PMB) and 3.47 ppm upfield from that of EIC bound to the β subunit of the native tetramer. Derivatization of cysteine β93 (Huestis & Raftery, 1972a, 1978) with a trifluoroacetonyl group in the rabbit hemoglobin tetramer does not change the chemical

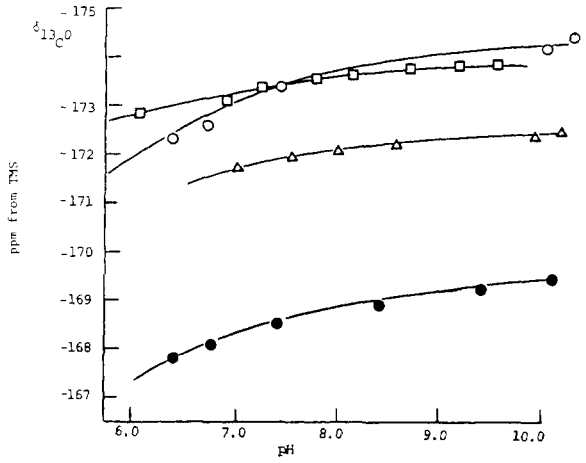

FIGURE 3: The pH dependence of the [$^{13}C^0$]EIC chemical shifts of sperm whale myoglobin (□) and horse myoglobin (△). The pH dependence for the [$^{13}C^0$]IPIC chemical shifts of sperm whale (○) and horse (●) myoglobin are also given. Chemical shifts are downfield from Me_4Si.

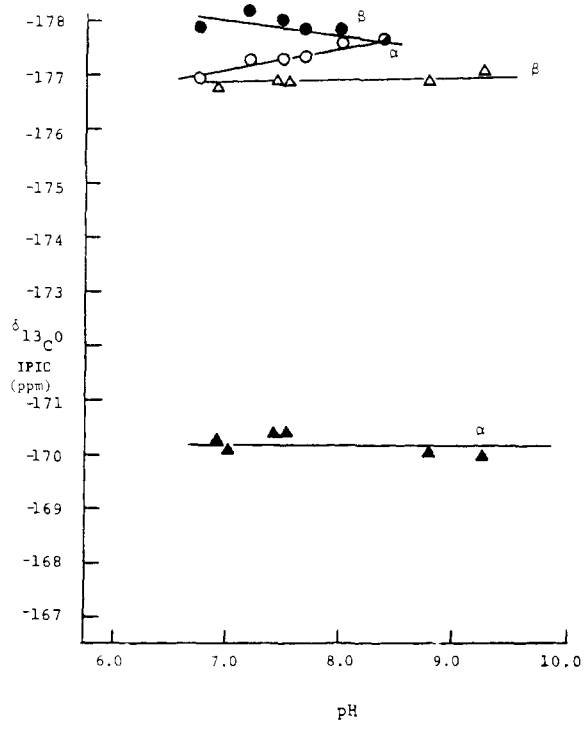

FIGURE 4: The pH dependence for the [$^{13}C^0$]IPIC chemical shifts of the β (●) and α (○) subunits of adult human hemoglobin as well as those for the α (▲) and β (△) subunits of rabbit hemoglobin. Chemical shifts are downfield from Me_4Si.

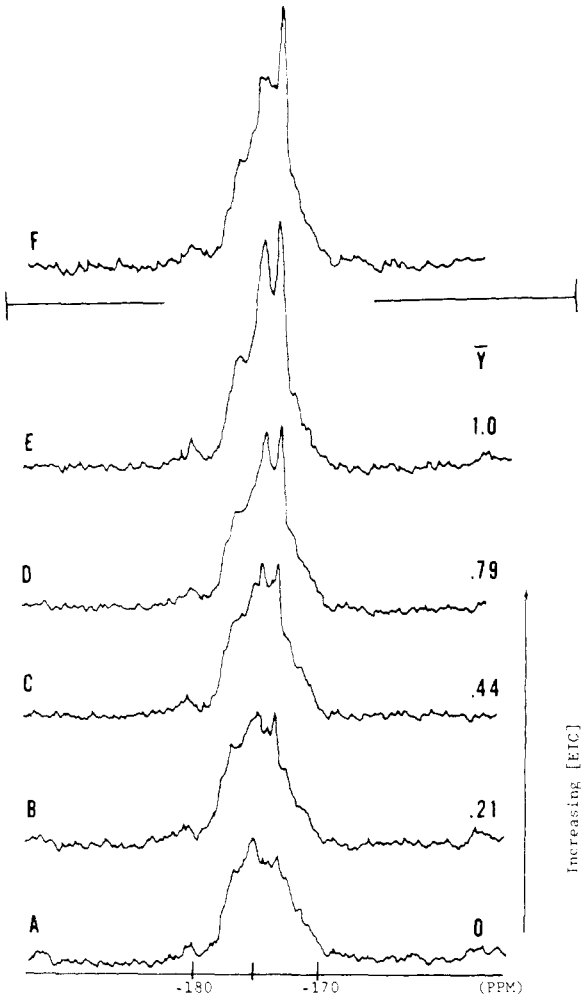

FIGURE 5: Partial saturation studies of $^{13}C^0$-enriched EIC with human hemoglobin. Spectra A through E represent the saturation of stripped 2.75 mM Hb in 0.1 M Tris, pH 7.2, in the absence of IHP. Spectrum F is spectrum E with 10.0 mM IHP added (pH 7.2). These spectra were obtained using an 18-mm diameter sample tube which required 20 000 transients.

shifts of EIC bound to either the α or β subunit.

We have found that the C^0 resonances of EIC bound to various hemoglobins and isolated chains are insensitive to pH changes between 7 to 10. In contrast, the chemical shifts of [$^{13}C^0$]EIC and IPIC bound to horse and sperm whale myoglobins are extremely sensitive to pH (Figure 3). Though the shifts of the terminal carbons of IPIC and EIC liganded to sperm whale myoglobin fall in approximately the same region, their shifts show somewhat different pH dependencies. When bound to sperm whale myoglobin, the chemical shift of [$^{13}C^0$]EIC moves 0.96 ppm downfield as the pH is varied from 6.1 to 9.6, whereas the resonance of [$^{13}C^0$]IPIC shifts 1.84 ppm downfield as the pH is varied from 6.4 to 0.1. The chemical shifts of the C^0 carbons of EIC and IPIC bound to horse myoglobin differ surprisingly from the shifts of these substances bound to sperm whale myoglobin. Whereas the chemical shifts of the C^0 carbon EIC and IPIC bound to sperm whale myoglobin fall in approximately the same region, those for EIC and IPIC bound to horse myoglobin differ by 3 ppm. Again, when bound to horse myoglobin, EIC is less sensitive to changes in pH (a shift of 0.74 ppm downfield as the pH is varied from 7 to 10) than is IPIC (a shift of 1.62 ppm downfield as the pH is varied from 6.5 to 10.1).

^{13}C-enriched IPIC bound to adult human and rabbit hemoglobin gave results strikingly different from those observed with EIC. Whereas IPIC bound to rabbit hemoglobin shows no pH dependence, when bound to human hemoglobin an increase in pH causes an upfield shift for IPIC bound to β subunits and a downfield shift for IPIC bound to α subunits; at pH 8.40 the two resonances overlap and exhibit a chemical shift 177.59 ppm downfield from Me_4Si (Figure 4).

Since the resonances for EIC and IPIC bound to myoglobins were well separated from the envelope of protein carbonyl carbons, we could determine their spin-lattice relaxation times (T_1). T_1's for the $^{13}C^0$ carbons of the variously bound EIC and IPIC are in the vicinity of 0.5 s which differs significantly from the T_1 values for EIC in water (37 s), EIC in CCl_4 (36.8 s), and IPIC in CCl_4 (48.2 s). These changes are attributable to in-

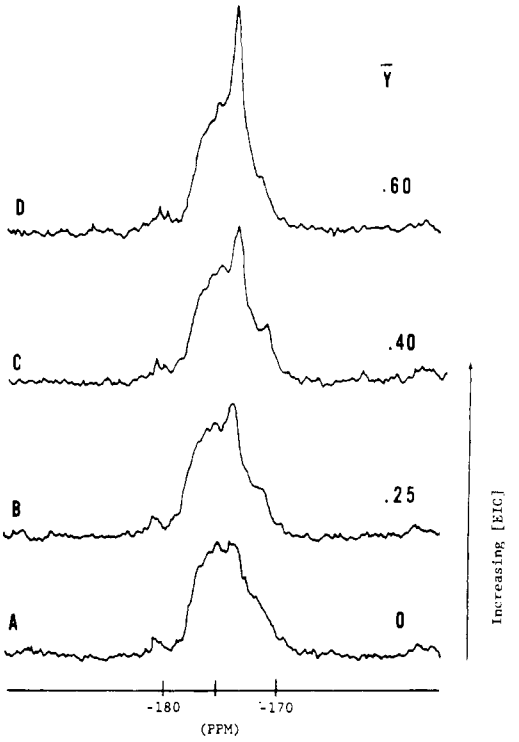

FIGURE 6: Partial saturation studies of $^{13}C^{0}$-enriched EIC with human hemoglobin. Spectra A through D represent the saturation of stripped 2.75 mM Hb in 0.1 M Tris, pH 7.2 and 10.0 mM IHP (pH 7.2). These spectra were obtained using an 18-mm diameter sample tube and required 20 000 transients.

crease in correlation time in going from free to protein bound forms.

Figure 5 illustrates results during a typical saturation experiment of human hemoglobin with EIC. (Because of the intensity of the protein carbonyl resonance envelope in this region, the relative order of ligation of α and β subunits below $\bar{Y} = 0.2$ cannot be inferred from these studies.) However, the results summarized in Figure 5 suggest that the ligation is random in human adult hemoglobin above $\bar{Y} = 0.2$. In our studies, addition of DPG did not noticeably affect the order of ligation. In contrast, IHP seems to suppress EIC binding to β subunits, as illustrated in Figure 5 by the disappearance of the β-EIC resonance when IHP is added to a sample. This is further demonstrated by the absence of a β-EIC peak when progressive saturation of Hb with EIC is carried out in the presence of 10^{-2} M IHP (Figure 6). The addition of these organic phosphates (DPG, IHP) did not affect the chemical shift of EIC bound to the α or β subunit of rabbit, opossum, or human hemoglobin.

Table II summarizes the results of determinations of the Hill coefficients for the binding of EIC and IPIC by human, rabbit, and opossum hemoglobin in 0.1 M Tris, 0.05 M NaCl, pH 7.0. The values of 2.4 ± 0.1 for EIC and 2.3 ± 0.1 for IPIC binding to human hemoglobin agree with those in the literature (Anderson et al., 1970).

TABLE II: Hill Coefficients for the Binding of Isocyanides to Various Hemoglobins.

isocyanide	hemoglobin	Hill coefficient
ethyl	adult human	2.4 ± 0.1
ethyl	rabbit	2.5 ± 0.1
ethyl	opossum	2.2 ± 0.1
isopropyl	adult human	2.3 ± 0.1
isopropyl	rabbit	2.6 ± 0.1
isopropyl	opossum	2.1 ± 0.1

Discussion

The functional nonequivalence of the α and β subunits of hemoglobin has been demonstrated by a number of techniques. Kinetic differences in the affinities of the α and β subunits in binding ligands such as nitric oxide, *n*-butyl isocyanide, and oxygen have been reported (Henry & Cassoly, 1973; Olson & Gibson, 1972; Gibson, 1973). Environmental differences experienced by CO bound to α or β subunits have been observed (Moon & Richards, 1972b, 1974; Matwiyoff & Needham, 1972; Matwiyoff et al., 1973; Vergamini et al., 1973). Differences in protein conformation surrounding the heme in the α and β subunits of carboxyhemoglobins (Lindstrom et al., 1972) have been established by ^{1}H NMR techniques. Moreover, the chemical shifts of the protons of the methyl groups of the α and β hemes have been shown to be nonequivalent in the ^{1}H NMR spectrum of deoxyhemoglobin by several groups (Lindstrom et al., 1971; Lindstrom & Ho, 1972; Johnson & Ho, 1974; Huang & Redfield, 1976). Thus, ample precedence exists for these results which show that EIC and IPIC experience quite different environments when bound to the α or β subunit.

Environmental Influences on Chemical Shifts for EIC Bound to Hemoglobins. Changes in the $C^{0}{=}N{-}C^{1}$ bond angle of isocyanides have a profound effect on the chemical shift of the C^{0} carbon (Stephany et al., 1974) because such changes in bond angle cause rehybridization at the nitrogen with resulting changes in the hybridization of C^{0}. Thus, steric interactions between alkyl groups of the isocyanide ligand and amino acid side chains which cause changes in the $C^{0}{=}N{-}C^{1}$ bond angle of the bound isocyanide can give rise to a wide range of chemical shifts for C^{0}.

In each case examined [$^{13}C^{0}$]EIC bound to hemoglobins shows two resonances in the vicinity of 175 ppm downfield from Me_4Si. (In solution, the C^{0} resonance of EIC itself appears about 150 ppm downfield from Me_4Si.) Experiments using ^{13}CO in competition with [$^{13}C^{0}$]EIC show that in rabbit, human, and opossum hemoglobins the low-field resonance corresponds to [$^{13}C^{0}$]EIC bound to the β subunit while the higher field resonance arises from [$^{13}C^{0}$]EIC bound to the α subunit. (These assignments agree with those provisionally proposed, but not established, by Mansuy et al. (1976).)

Previous results of ^{13}CO bound to various hemoglobins (Moon & Richards, 1972b, 1974; Moon et al., 1977) show that ^{13}CO bound to the β subunits of various hemoglobins exhibits resonances which have essentially the same chemical shift; in contrast ^{13}CO bound to the α chains of these hemoglobins exhibit resonances which fall over a wider range. With [$^{13}C^{0}$]EIC, the chemical shift range is much greater than with ^{13}CO. In these cases, [$^{13}C^{0}$]EIC when bound to β subunits gives resonances which fall over a range of 4 ppm; when bound to α subunits, the [$^{13}C^{0}$]EIC resonance is nearly uniform in all species examined except rabbit where it appears shifted upfield 4.6 ppm. What changes in hemoglobin structure can account for these widely differing chemical shifts?

Brunori et al. (1972) have suggested that large ligands such as isocyanides are forced to bend away from valine (E-11), phenylalanine (CD-1), and histidine (E-7) toward the methine bridge between pyroles 2 and 3 of the heme. In this region the alkyl group of the isocyanide can contact other residues (B-10 and B-8) and may also be influenced by more distant residues (B-13, B-9, and G-12).

TABLE III: Short Contacts of EIC and IPIC Bound to Myoglobin and Hemoglobin.[a]

	sperm whale	horse	human α	rabbit α	opossum α	human β	rabbit β	
E-11	Val	Val	Val	Val	Ile	Val	Val	close contacts
B-10	Leu	Leu	Leu	Val	Leu	Leu	Leu	close contacts
G-8	Ile	Ile	Leu	Leu	Leu	Leu	Leu	close contacts
G-12	Ile	Ile	Leu	Leu	Leu	Leu	Leu	
B-9	Ile	Val	Ala	Ala	Ala	Ala	Ala	distant contacts
B-13	Leu	Leu	Met	Met	Thr	Leu	Leu	distant contacts
CD-1	Phe	Phe	Phe	Phe	Phe	Phe	Phe	distant contacts

[a] Amino acid sequences obtained from Waterman & Stenzel (1974), Dayhoff (1969, 1972) and Antonini & Brunori (1971, p 82).

Amino acid substitutions in the contact regions of the various hemoglobin subunits could be one reason for the differences observed in the chemical shift of bound EIC (see Table III). The α-EIC of rabbit hemoglobin exhibits the largest chemical shift deviation, appearing 4.6 ppm upfield from human α-EIC. This is most likely a manifestation of substitutions at B-10 (Leu → Val), CD-6 (Leu → Phe), and CD-7 (Thr → Ser) (von Ehrenstein, 1966; Dayhoff, 1972) in rabbit α chains relative to the human adult α chain (Braunitzer et al., 1961; Dayhoff, 1969). Thus, the variation of the chemical shifts of EIC bound to the α subunits of these hemoglobins may reflect these amino acid substitutions (Moon & Richards, 1974).

The β subunits, unlike the α subunits, exhibit no amino acid substitutions in the critical regions mentioned above (Table III). The differences observed for the chemical shifts of [$^{13}C^0$]EIC bound to the various β subunits might then reflect differences in the tertiary structure of the binding site which may be caused by amino acid substitutions elsewhere in the molecule. In particular, changes in the B, G, and E helices, which are thought to contact the ligand directly, could be responsible and might reflect substitutions in the hinge regions of these helices. However, although the various α chains show a considerable number of substitutions in this region, no similar substitutions in the hinge regions of the β chain have been observed (Dayhoff, 1969, 1972; Waterman & Stenzel, 1974; Antonini & Brunori, 1971, p 64). These chemical shift changes for ligand bound to the β subunit might, therefore, reflect changes in the hinge regions of the α chain which have been transmitted to the β chain through the α–β subunit contacts which occur at the B, G, and H helices (Antonini & Brunori, 1971, p 82; see below).

The resonances of [$^{13}C^0$]EIC bound to isolated human α (PMB) subunits, at 173.40 ppm, and α (SH) subunits, at 173.48 ppm downfield from Me_4Si fall near the α resonance in the intact tetramer at 173.85 ppm. In contrast, the resonances for [$^{13}C^0$]EIC bound to the isolated β (PMB) at 173.73 ppm and to the β (SH) tetramer (Yip et al., 1972) at 171.57 ppm are considerably removed from that for the β resonance in the $\alpha_2\beta_2$ tetramer at 175.04 ppm. These differences observed between [$^{13}C^0$]EIC bound to $\alpha_2\beta_2$ tetramer, β (SH) tetramer, and β (PMB) monomer can be ascribed to changes in the region of the G helix. As EIC points toward the B and G helices, any change in the orientation of the G helices as a consequence of formation of the PMB derivative at Cys-G14 (β) could affect the environment of a large ligand (such as EIC). This may also account for the difference in the β (SH) tetramer which involves contacts of the G helices (Neer, 1970) and, moreover, emphasizes the point made in the previous paragraph that changes in the α subunits may be transmitted to the β subunits via the B, G, and H helices.

A smaller ligand, such as CO, may not reflect these alterations in the G helix which can explain the earlier observation (Moon & Richards, 1974) that the chemical shift is essentially the same for ^{13}CO bound either to the $\alpha_2\beta_2$ tetramer or to the β (PMB) monomer. Alternately, the attachment of a PMB group to Cys-β93 (F9) could perturb the binding of the proximal histidine (F8) to the heme and thereby alter the chemical shift of an attached ligand. However, this does not appear to be an important effect because the chemical shift of ^{13}CO when bound to the β (PMB) monomer is essentially the same as when bound to the β subunit of the $\alpha_2\beta_2$ tetramer and one would expect effects transmitted from His (F8) through the iron to the ligand to be largely electronic and not strongly dependent on the steric requirements of the ligand. Moreover, if PMB binding to Cys-β-93 (F9) were responsible for the observed shift differences, one might expect similar variations when Cys-F9 is labeled with trifluoroacetone but the data of Table I indicate that this is not observed.

The interactions of IPIC with rabbit and human hemoglobin reflect the differing ability of these heme proteins to accommodate large ligands. The chemical shifts of IPIC and of EIC bound to both subunits of rabbit hemoglobin exhibit no pH dependence; the C^0 resonance of EIC bound to human adult hemoglobin also exhibits no pH dependence. In contrast, the C^0 resonance of IPIC bound to adult human hemoglobin exhibits a significant pH dependence (Figure 4). At pH 7.0 the [$^{13}C^0$]IPIC α and [$^{13}C^0$]IPIC β resonances are 0.90 ppm apart, whereas at higher pH (8.4) they coalesce into a single absorption. A pH dependence has also been reported for the affinity of human hemoglobin for *n*-butyl isocyanide (Olson & Gibson, 1973).

Environmental Influences on Chemical Shifts for EIC and IPIC Bound to Myoglobins. Binding of EIC to sperm whale and horse myoglobin gives results somewhat different from those obtained for binding of EIC to hemoglobins. For example, the chemical shifts of the resonances of [$^{13}C^0$]EIC bound to these myoglobins are dependent on pH over a range of pH 6 to 10. (For ^{13}CO bound to dolphin myoglobin a pH dependence between pH 5 and 7 has been reported by Moon et al., 1977.) In contrast, no pH dependence above pH 6.5 is observed for either EIC or CO bound to human, rabbit, or opossum hemoglobins. The chemical shift of ^{13}CO bound to dolphin myoglobins exhibits only a small dependence at low pH; the much larger pH dependence of the EIC bound to horse and sperm whale myoglobin may reflect an amplification of the effect seen with CO by virtue of the larger alkyl isocyanide molecule. Along these same lines, the $^{13}C^0$ resonances of IPIC bound to the two myoglobins exhibit a greater pH dependence than do the $^{13}C^0$ resonances of bound EIC (Figure 3). As IPIC is a slightly larger molecule than EIC, IPIC might more sensitively reflect the steric properties of the binding pocket.

Hill Coefficients. The Hill coefficients we have obtained for the binding of EIC and IPIC to human hemoglobin agree with those of Brunori et al. (1972). Hill coefficients for binding

of isocyanides to opossum hemoglobins are slightly lower than those for human hemoglobin and could reflect the substitution of glutamine for histidine at E7. The Hill coefficient for binding IPIC to rabbit hemoglobin somewhat exceeds those for binding to opossum and human hemoglobins; this behavior may be a manifestation of the greater flexibility of rabbit hemoglobin (Moon & Richards, 1972a) in which the rabbit subunits can more easily accommodate larger ligands without energetically unfavorable changes in tertiary structure.

Relative Affinities: Carbon Monoxide vs. Isocyanides. Hemoglobin has a higher affinity by a factor of 2000 for CO than for EIC (Brunori et al., 1972). In this work, the displacement of EIC by CO preferentially from the β subunits (to give $(\alpha\text{-EIC})_2(\beta\text{-CO})_2$ almost exclusively) shows that the β chains have a significantly higher affinity for CO relative to EIC than do the α chains.

Order of Ligation. A large number of experiments, both kinetic and thermodynamic, have been carried out to determine the sequence of steps between unliganded and completely liganded hemoglobin. Using stopped-flow techniques, Olson & Gibson (1970–1973) found that BIC does not bind preferentially to either subunit in the absence of organic phosphates. However, addition of either DPG or IHP cause BIC to bind more rapidly to the β subunit. Work of Lindstrom et al. (1971) using ^{1}H NMR shows preferential binding in the presence of organic phosphates to β subunits. More recent results by Olson & Binger (1976) indicate that the β chain also exhibits a higher kinetic affinity for small isocyanides in the absence of organic phosphate. Studies of isocyanide binding by EPR observation of acquisition of NO as a ligand by subunits which have not previously acquired isocyanide also suggested preferential binding of isocyanides to β subunits (Reisberg et al., 1976).

Using hemoglobin labeled with bromotrifluoroacetone at Cys-β93, Huestis & Raftery (1972a, 1975, 1978) also found the BIC binds randomly in the absence of organic phosphates but in this case, in contrast to other work, addition of organic phosphates was interpreted as causing preferential binding to the α subunit.

Organic phosphates, such as DPG and IHP, are known to bind between the β chains of deoxyhemoglobin and stabilize the T state. Using X-ray evidence, Perutz (1970) suggested that α chains have a higher affinity for ligand when hemoglobin is in the T state. In the R state, the situation is reversed and the β chains have a higher ligand affinity, a proposal which is supported by the observation of a slightly higher affinity for ligand by separated β chains than by separated α chains (Talbot et al., 1971; Antonini & Brunori, 1971, p 314). Thus, by stabilizing the T state of the hemoglobin tetramer, the presence of IHP would be expected to encourage preferential ligation of the α chains, a prediction which seems to be substantiated by this work on the binding of EIC.

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Purification of an Atypical Mast Cell Protease and Its Levels in Developing Rats†

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ABSTRACT: A chymotrypsin-like serine protease produced by atypical mast cells in gut mucosa of rats was purified by a method involving affinity adsorption chromatography on potato chymotrypsin inhibitor I immobilized on Sepharose. The protease was shown by chemical, physical, enzymatic, and immunologic criteria to be identical with a protease isolated from small intestine by a method previously described (Katunuma, N., et al. (1975) *Eur. J. Biochem. 52*, 37–50). The levels of the protease in small intestine were determined in developing rats by a radial immunodiffusion assay. Protease levels were relatively low in suckling rats, but rapidly increased approximately fivefold 2 to 3 weeks after weaning and thereafter decreased to adult levels. The atypical mast cell population of small intestine followed a similar pattern. There was a maturation of existing cells as well as an increase in their numbers prior to the period of rapid protease production. A dramatic increase in the immunoglobulin A levels coincident with the increase in both protease and mast cell contents of the small intestine suggests that the enzyme and the cell may be involved in the first active immune response at the mucosal surface. The levels of ornithine aminotransferase, purported to be a substrate of the protease in vivo, were disproportionately low compared with those of the protease in adult rats and showed no correlation to the protease levels in developing rats. These observations are inconsistent with the concept that the protease initiates the degradation of several intracellular pyridoxal phosphate dependent enzymes in vivo.

Katunuma & co-workers (1971) isolated from rat small intestine a chymotrypsin-like serine protease of restricted specificity and proposed that this protease initiated the degradation of several intracellular pyridoxal phosphate dependent enzymes such as ornithine aminotransferase (OAT[1]) (Katunuma et al., 1971, 1975). The apo forms of these enzymes are susceptible to the action of the protease, but not the holoenzymes nor the apo forms of enzymes requiring other cofactors. Because of its limited and apparently selective action, the protease was termed "group-specific protease".

Recently, it was determined by immunofluorescent methods that the protease is located in "atypical" mast cells of the intestinal mucosa (Woodbury et al., 1978a). Such cells, containing the protease, also were observed beneath the epithelium of the bronchioles in lung. The enzyme was shown to be similar to, but distinct from, the previously known chymotrypsin-like protease of rat peritoneal mast cells (Benditt & Arase, 1959). To distinguish it from the newly discovered enzyme, the latter protease is designated rat mast cell protease I (RMCP I), and the present one rat mast cell protease II (RMCP II).

The cell-specific location of RMCP II suggests that it is a secretory enzyme. In light of this, it is appropriate to reconsider the proposal that the protease plays a role in the degradation of intracellular enzymes. In the current study, we have examined the quantitative relationship of the protease to its putative substrate, ornithine aminotransferase in both adult and developing rats. In addition, a new method employing affinity adsorption chromatography is described which permits rapid and efficient purification of the protease.

Experimental Section

Materials

A small amount (10 mg) of the protease was prepared from the thoroughly washed small intestines of 50 adult, female, outbred white rats (Tyler Laboratory, Bellevue, Wash.) as previously described (Katunuma et al., 1975) with the exceptions that benzamidine (5 mM) was added to all buffers and DEAE-Sephacel was substituted for DEAE-cellulose.

Ornithine aminotransferase was purified from the livers of 50 rats maintained on a high protein (60% casein) diet for 7 days (Peraino et al., 1969).

Chicken ovoinhibitor and human α_1-antitrypsin were gifts from H. Ako (University of Hawaii). Soybean, pancreatic and

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[1] Abbreviations used: OAT, ornithine aminotransferase; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; P.I.-1, potato inhibitor 1; Tos-Lys-CH_2Cl, *N*-α-*p*-tosyl-1-lysine chloromethyl ketone; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TosArgOMe, tosylarginine methyl ester; BzTyrOEt, benzoyltyrosine ethyl ester; DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; Pth, phenylthiohydantoin.

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