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Immunological Measurements of Conformational Motility in Regions of the Myoglobin Molecule†

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ABSTRACT: The conformational motilities of three regions of the sperm whale myoglobin molecule and of an isolated peptide of myoglobin have been examined by measuring the equilibrium constant for the native ↔ nonnative transition. The immunological approach of Furie et al. (Furie, B., Schechter, A. N., Sachs, D., and Anfinsen, C. B. (1975), *J. Mol. Biol. 92,* 497–506) was used with convenient modifications. Antibodies specific to the nonnative conformations were used in assaying for competition between the radioactively labeled peptide and native myoglobin. Labeling was by ^{125}I iodination of the peptide or its 3-(4-hydroxyphenyl)propionyl derivative, and separation of the immune complex from the free peptide was either by ammonium sulfate precipitation or by centrifugation of the antibodies immobilized on Agarose beads. For the antigenic regions of the sequence (1–55), the measured conformational equilibrium constant was 840 ± 200 at 22 °C; the value for the C-terminal region (132–153) was 280 ± 120 at 25 °C, while that for the region (66–76) adjacent to the heme group was greater than 2.5×10^6. Measurements on the isolated peptide (132–153) indicated that 1% of the molecules adopt native-type folding in aqueous solution at 36 °C.

Globular proteins in solution undergo conformational fluctuations during which their structure alternates rapidly between native and nonnative conformations (Anfinsen and Scheraga, 1975). The native structure, which normally predominates, is in equilibrium with a population of nonnative or "denatured" states that are present in concentrations below the limit of detection of physiochemical techniques.

Several approaches have been introduced to measure the overall dynamics of a protein molecule. Hydrogen–deuterium exchange experiments on insulin by Linderstrøm-Lang (1955) gave the first evidence for conformational flexibility of a native protein. The extent of this flexibility was recently estimated by Nakanishi et al. (1972) using the hydrogen–deuterium exchange method on lysozyme at 20 °C. Only 1 molecule/3×10^5 was in a nonnative conformation. Upon addition of 6.5 M LiCl, however, the number increased to 1/83, although there was no detectable change in molar ellipticity at 222 nm. Other physical methods which have been used to measure conformational equilibria in proteins usually focus on a group of chromophores (as in circular dichroism, ultraviolet difference spectroscopy, nuclear magnetic resonance, or fluorescence), and provide averaged information about their state of unfolding or flexibility. In special cases, attention can be focused on a single chromophore or chain segment. In general, however, the amount of information is limited by the sensitivity of these methods; their utility for quantitative measurements of conformational equilibria has been largely in the analysis of data acquired from transition curves in the region representing 10–90% unfolding.

The use of proteolytic enzymes as probes of protein unfolding (Rupley and Scheraga, 1963; Ooi and Scheraga, 1964; Klee, 1967; Imoto et al., 1974; and Burgess et al., 1975) has given qualitative information about local chain flexibility, and some indication of the sequence of events during unfolding. Kinetic studies using small chemical probes specific for individual protein residues are capable, in principle, of providing much quantitative information about regions surrounding the modifiable residues. Indeed, Vas and Boross (1974) studied regional conformational changes around a partially buried cysteine residue (Cys-153) of glyceraldehyde-3-phosphate dehydrogenase by a kinetic analysis of its reaction with *p*-mercuribenzoate. They concluded that 1 molecule/37 in the carboxymethylated holoenzyme at 25 °C was in a nonnative conformation, while the carboxymethylated apoenzyme had 1 in 18. This approach is restricted in its application to regions of a protein possessing unique and specifically reactive residues, but has obvious potential for active site residues in enzymes.

A highly sensitive immunological approach, which allows quantitative evaluation of the motility or tightness of folding of antigenic regions within protein molecules, but without the

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limitations of the methods discussed above has been described by Furie et al. (1975). Using monospecific antibodies directed against an unstructured peptide excised from staphylococcal nuclease, they measured a conformational equilibrium constant (K_{conf}) for the nonnative $\rightleftharpoons$ native interconversion of the protein chain segment concerned. In this way, they estimated that a helix-rich 28-residue chain segment in the enzyme had 1 molecule/2900 in a nonnative conformation at 25 °C and pH 8.1, and that the addition of stabilizing ligands decreased the number of unfolded forms by a factor of 10. Previously, Sachs et al. (1972) had used antibodies to study the conformation of isolated antigenic polypeptides in solution. Their methods are based on the assumption that polypeptide fragments from proteins, even when apparently structureless by conventional physical criteria, exist in the form of an equilibrium between native-like and (many) nonnative conformations; further, that antibodies raised against the native protein will contain subpopulations which recognize only that fraction, however small, which has the native-type conformation.

These immunological approaches open the possibility of probing any chosen regions of a protein molecule by measuring the conformational equilibrium constants for these segments as long as they are immunogenic. It is reasonable to suppose that the motilities of such segments will vary according to the number of noncovalent interactions within the segment and between the segment and its immediate protein environment in the native state. In the present paper, we have applied the immunological approach with some convenient modifications to a study of the conformational equilibria in three regions of sperm whale myoglobin. The conformational equilibrium of a carboxyl-terminal fragment (residues 132–153) has been measured both for the free peptide and for this region within the native molecule. Similarly, two other regions of the native protein—an amino-terminal fragment (residues 1–55) and a small synthetic fragment (residues 66–76), surrounding the heme pocket—have been examined.

Materials and Methods

Preparaton and Purification of CNBr Peptides. Sperm whale myoglobin (Sigma, Lot 14C-0710) was digested with CNBr (5:2, w/w) in 70% formic acid under nitrogen according to the method of Taso et al. (1974). Reducing conditions were maintained by the addition of dithioerythritol (0.02%, w/v) as employed by Drapeau and Yanofsky (1967). After 24 h, the solution was diluted 1:10 with glass-distilled water and lyophilized. The initial peptide separation was carried out by gel filtration on a column (2 × 136 cm) of Sephadex G-50 (fine) equilibrated with formic acid–acetic acid–water (2:2:7). All peptide fractions were chromatographed twice to ensure complete removal of contaminating peptides. The purity of the peptides was indicated by the symmetry and clean separation of eluted peaks and assessed objectively by amino acid analysis. Peptide concentrations were determined by the method of Lowry et al. (1951) or spectrophotometrically using a molar extinction coefficient for tyrosine of 1500.

Synthesis, Purification, and Analysis of Peptides. Protected amino acid derivatives were purchased from the Protein Research Foundation, Minoh, Osaka, Japan.

Two peptides corresponding to residues (147–153), and (66–76), respectively, of sperm whale myoglobin, and the 3-(4-hydroxyphenyl)propionyl derivative of the second peptide were synthesized using the solid-phase procedure of Merrifield (1963). The syntheses were performed manually as described by Rosenblatt et al. (1976) in a reaction vessel consisting of a 50-ml plastic syringe barrel fitted with a porous polyethylene disk, a three-way Luer adaptor stopcock, and a Teflon-coated stirring paddle coupled to an overhead mechanical stirrer. The resin was a styrene–1% divinylbenzene copolymer containing 1.29 mequiv of chloromethyl groups/g (Bio-Rad Laboratories, Richmond, Calif.).

The first amino acid was esterified to the chloromethylated resin in ethyl acetate as described by Stewart and Young (1969). The extent of esterification was estimated by amino acid analysis as described by Scotchler et al. (1970). The amounts of glycine and L-leucine incorporated in the resin–*tert*-butoxycarbonyl amino acid complexes were 0.20 mM/g and 0.18 mM/g, respectively.

The *tert*-butoxycarbonyl group was used to protect the α-amino group of each amino acid during coupling. Side-chain protection was as follows: (a) the hydroxyl groups of tyrosine and threonine were protected with *O*-2,6-dichlorobenzyl and *O*-benzyl ether groups, respectively, (b) the carboxyl group of glutamic acid was protected with a benzyl ester group, and (c) the ϵ-amino group of lysine was protected by a benzyloxycarbonyl group.

Cleavage of the *tert*-butoxycarbonyl group by 30% trifluoroacetic acid in methylene chloride and neutralization of the trifluoroacetate salt was according to Gutte and Merrifield (1971). Coupling of the second residue to the resin was accomplished by adding a *tert*-butoxycarbonyl amino acid (2.7 equiv) and dicyclohexylcarbodiimide (2.7 equiv) in methylene chloride (10 ml/g of resin). The mixture was stirred for 120 min and then the peptide–resin complex was washed with methylene chloride (four times). L-Glutamine was coupled as its *p*-nitrophenyl ester in *N,N'*-dimethylformamide for 240 min. The active ester was prepared according to Stewart and Young (1969) and 7.5 equiv were used for coupling. Where required as an *N*-terminal group, 8 equiv of 3-(4-hydroxyphenyl)propionyl *N*-hydroxysuccinimide ester (Pierce Chemical Co.), an iodinatable acylating group developed by Bolton and Hunter (1973), were reacted with a portion of the peptide–resin complex in *N,N'*-dimethylformamide for 240 min.

The extent of coupling was monitored qualitatively by the fluorescamine test of Felix and Jimenez (1973), except that plastic 1-ml tuberculin syringe barrels fitted with polyethylene disks were used.

Cleavage of the peptides from the resin and simultaneous removal of side-chain protecting groups was by anhydrous HF at 0 °C for 60 min in the presence of redistilled anisole (1 ml/g of resin). Excess HF was removed by distillation under reduced pressure. The peptide–resin mixture was washed with ethyl acetate to remove anisole. The peptide was then extracted first with glacial acetic acid (twice) and then water (twice). The extracts were combined and lyophilized to yield the crude products: (147–153), viz., Lys-Glu-Leu-Gly-Tyr-Gln-Gly, 587 mg; (66–76), viz., Val-Thr-Val-Leu-Thr-Ala-Leu-Gly-Ala-Ile-Leu, 439 mg; and 3-(4-hydroxyphenyl)propionyl-(66–76), 36 mg.

Purification of peptide (147–153) was performed by chromatography on a Sephadex G-10 column (2.8 × 34 cm) in 0.1 M ammonium bicarbonate followed by SE-Sephadex (2.8 × 30 cm). For the second column, a salt gradient using two ammonium acetate buffers (pH 5.2) was used for elution (10 and 100 mM, respectively, 250 ml of each). The N-terminal residue was determined by the dansylation procedure of Gray (1967).

Peptide (66–76) and its 3-(4-hydroxyphenyl)propionyl derivative were used without further purification. The peptides

were examined by TLC,[1] high-voltage electrophoresis, amino acid analysis, carboxypeptidase A digestion, and Edman degradation. Stock solutions (1 mg/ml) were prepared in BBS[1] buffer containing 30% *N,N'*-dimethylformamide. The latter was necessary for complete solubilization and the alkaline pH was used to reverse possible N → O shifts involving threonine. These stock solutions were diluted with the aqueous buffer in the radioimmunoassays.

Thin-layer chromatography was carried out on silica gel GF-254 (Camag, Chemie Erzeugnisse und Adsorptionstecknik AG, Muttenz, Switzerland) containing 0.5% soluble starch on 0.3-mm coated plates in two solvent systems: butanol–acetic acid–water–pyridine (BAWP) 30:6:24:20, and butanol–acetic acid–water (BAW) 4:1:1.

The peptides, both isolated from myoglobin and synthesized, were characterized by amino acid analysis. The salt-free peptides (~50 nmol of each amino acid) were dissolved in HCl (6 M, 250 μl containing 1% phenol) and hydrolyzed at 110 °C for 24 h in evacuated sealed tubes. The analyses were performed with a Beckman 120 B amino acid analyzer.

The two synthetic peptides were examined for impurities by two-dimensional peptide mapping. The peptides (1 mg/cm) were electrophoresed at pH 3.5 on Whatman 3MM at 3 kV for 50 min. The strips were dried, cut, and sewn onto new sheets for descending chromatography at right angles in BAWP. After drying, the papers were stained with 1% cadmium–ninhydrin solution in acetone.

Peptide (66–76) was subjected to five cycles of the Edman degradation using a modification of the procedure outlined by Petersen et al. (1972). Six hundred nanomoles of peptide was suspended in coupling buffer (0.4 M dimethylallylamine in 1-propanol–water (60:40), 100 μl, adjusted to pH 9.5 with trifluoroacetic acid). Most of the peptide dissolved on standing overnight at room temperature. Phenyl isothiocyanate (10 μl) (Pierce Chemical Co., Sequanal grade) was added to the peptide solution, under nitrogen, and incubated at 60 °C. After 30 min, the solution was extracted twice with benzene (0.5 ml), lyophilized, and then warmed to 50 °C for 20 min in vacuo. Cleavage of the N-terminal residue was carried out by the addition of trifluoroacetic acid (50 μl) and incubating at 60 °C for 10 min under nitrogen. The thiazolinone derivative of the amino-terminal residue was extracted with redistilled diethyl ether (3 × 0.5 ml) after the addition of water (0.2 ml). Conversion of the thiazolinones to the phenylthiohydantoins was by the method of Edman and Begg (1967). The Pth-amino acids were dissolved in ethylene chloride (50 μl) and an aliquot (20 μl) was loaded onto aluminum-backed TLC plates containing the fluorescent indicator Kieselgel 60 F_{254} (Merck) adjacent to reference Pth standard mixtures. Chromatography was carried out in the solvent system of Inglis and Nicholls (1973). Carboxypeptidase A (Sigma Chemical Co., St. Louis, Mo., Lot 18B-2690) digestion of peptide 66–76 was carried out by the method of Ambler (1967).

Peptide Conjugation and Immunization. The peptides used to produce antibodies were conjugated to either bovine serum albumin or rabbit immunoglobulin G (isolated by precipitation with 35% saturated ammonium sulfate) by the carbodiimide method of Goodfriend et al. (1964). After dialysis against water (3 days at 4 °C), the peptide–protein conjugate (1 mg in 0.5 ml) was emulsified in Freund's complete adjuvant (0.5 ml) and injected intramuscularly at two sites in each of two rabbits at 2-week intervals. After four injections, the animals were bled from a marginal ear vein at 2-week intervals for three bleeds (50 ml/bleed). Specific antisera were prepared to CNBr peptides (1–55) and (132–153) and to synthetic peptide (66–76). Antiserum specific to native sperm whale myoglobin was raised in sheep by intramuscular injection according to the method of Hurrell et al. (1976). Antibody titers were estimated using the appropriate immunoadsorbent columns.

Peptide and Protein Labeling. Peptide (132–153) was labeled with ^{125}I by the chloramine-T method of Greenwood et al. (1963), as described by Hurrell et al. (1976). Holo sperm whale myoglobin was also labeled by this procedure. The labeled peptide or protein was freed of excess ^{125}I by gel filtration on Sephadex G-25, equilibrated with PBS buffer containing 0.25% bovine serum albumin. The specific activities of the fragment (132–153) and the holoprotein immediately after labeling were 2.11×10^5 cpm/nmol (1 mol of ^{125}I/1.6 mol of peptide) and 2.55×10^6 cpm/nmol (1 mol of ^{125}I/1 mol of protein), respectively.

3-(4-Hydroxyphenyl)propionyl (66–76) was labeled by the chloramine-T method (see above) to a specific activity of 3.50×10^5 cpm/nmol (1 mol of ^{125}I/1.05 mol of peptide).

Fragment 1–55 was labeled with ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl *N*-hydroxysuccinimide ester (Pierce Chemical Co.) as described by Bolton and Hunter (1973) and freed of excess reagent by gel filtration on a Sephadex G-25 column equilibrated with PBS buffer containing 0.1% gelatin. The concentration of the labeled peptide was calculated from the amount of unlabeled peptide required to cause 50% inhibition of binding of the labeled peptide in a solid-phase radioimmunoassay. The labeled peptide had a specific activity of 1.23×10^5 cpm/nmol.

Assessment of Immunoreactivity of Labeled Peptide (132–153). Anti-$(myoglobin)_N$ serum was added in increasing dilutions to fixed amounts (285 ng) of ^{125}I-labeled (132–153), incubated at 30 °C for 20.5 h, and precipitated at 4 °C with an equal volume of saturated ammonium sulfate. After centrifugation, the precipitates were washed twice with 50% saturated ammonium sulfate and counted. Each sample was in triplicate. The whole procedure was repeated with a fivefold dilution of the ^{125}I-labeled (132–153) using BBS buffer only in one case and a fivefold dilution with unlabeled peptide (132–153) in the other.

Coupling of Polypeptides to Sepharose 4B. Peptides (132–153), (1–55), and (147–153), as well as the specific anti-$(1–55)_N$ and anti-$(1–55)_R$ antibody populations, isolated as described below, were coupled to Sepharose 4B (Pharmacia) using the CNBr activation method of Cuatrecasas and Anfinsen (1972). Peptide (66–76) was coupled in 50% dimethylformamide in BBS buffer, according to Cuatrecasas (1970). The amounts of peptides coupled were 1 μmol/ml of packed Sepharose and 15 μg/ml of immunoglobins. The efficiency of coupling was usually greater than 98%, as measured either spectrophotometrically or by fluorescamine assay as described by Udenfriend et al. (1972).

Immunoadsorption of Antibodies. (a) Anti-$(1–55)_R$, Anti-$(66–76)_R$, and Anti-$(132–153)_R$. Hyperimmune rabbit

[1] Abbreviations used are: BBS buffer, borate–saline (0.15 M NaCl–0.1 M boric acid–0.025 M $Na_2B_4O_7$, pH 8.5); PBS buffer, phosphate–saline (0.15 M NaCl–0.015 M Na_2HPO_4–0.004 M KH_2PO_4, pH 7.4); RIA buffer, radioimmunoassay buffer, that is BBS buffer containing 0.25% bovine serum albumin and 0.5% Tween 20; CD, circular dichroism; TLC, thin-layer chromatography; UV, ultraviolet; (x–y), a polypeptide fragment of the protein whose amino terminal residue is the *x*th and whose carboxyl terminal residue is the *y*th of the protein; anti-$(x–y)_N$ indicates antibody populations isolated from hyperimmune sera prepared against native myoglobin and anti-$(x–y)_R$ indicates antibody isolated from hyperimmune sera prepared against peptide (x–y); nomenclatures used by Furie et al. (1975).

serum (10 ml), prepared by immunization with conjugated (1–55), (66–76), and (132–153), as outlined previously, were applied to columns containing approximately 5 ml (packed volume) of Sepharose–(1–55), Sepharose–(66–76), and Sepharose–(132–153), respectively. Each column was washed with 20–30 bed volumes of BBS buffer to elute nonabsorbed protein. The specific antibodies were then eluted with acetic acid (0.5 M) and immediately neutralized to pH 8.5 with KOH. The concentration of immunoglobin was estimated by the absorption at 280 nm assuming the $E_{1cm}^{1\%}$ 13.5 of Stevenson and Dorrington (1970). Whole preimmunization serum, to act as carrier, diluent, and stabilizer, was then added to a final concentration of one-tenth normal.

(b) Anti-$(1–55)_N$ and Anti-$(132–153)_N$. Hyperimmune sheep serum (5 ml), prepared against sperm whale myoglobin, was applied to columns of Sepharose–(1–55) and Sepharose–(132–153), respectively. The specific antibodies were eluted, neutralized, quantitated, and diluted as described above.

(c) Anti-$(132–146)_R$. Hyperimmune rabbit serum (10 ml), prepared against (132–153), was applied to Sepharose–(147–153) and the column was washed with BBS buffer. The nonbound serum and washings were collected and reapplied to a Sepharose–(132–153) column. The specific antibodies were eluted, neutralized, quantitated, and diluted, as above.

Estimation of Myoglobin Concentration. Concentrations were estimated by a variant of the pyridine–hemochrome method (de Duve, 1948) using ϵ_M^{556} 3.46×10^4 (Paul et al., 1953), criteria for complete reaction being $A_{556}/A_{538} \geq 3.5$ with a peak at exactly 556 nm.

Determination of K_{ass} for anti-$(132–153)_N$. The association constant for the binding of anti-$(132–153)_N$ to the native determinant in sperm whale myoglobin was measured by the ammonium sulfate precipitation technique described by Stupp et al. (1969) and Steward and Petty (1972). The intrinsic association constant and heterogeneity index were obtained from a Sips plot of the binding data (Sips, 1948), after determining the anti-$(132–153)_N$ concentration by the method of Pinckard and Weir (1973).

Determination of K_{conf} for Myoglobin Regions. The conformational equilibrium constants for myoglobin regions (66–76) and (132–153) were obtained by measuring the inhibition by myoglobin of complex formation between the radioactively labeled fragment and antibodies specific to these fragments in the nonnative conformation. Constant aliquots of labeled peptide of known specific activity were incubated with varying concentrations of specific antibody and of native myoglobin at constant temperature in polystyrene tubes (Camalec V. H. A., Melbourne). Each estimation was carried out in triplicate for a given set of concentrations. Separation of free and complexed fragments was carried out by precipitation of the immune complexes by the addition of an equal volume of saturated ammonium sulfate at 4 °C. After standing at 4 °C for 20 min, the precipitates were collected by centrifugation (10 000*g*, 10 min) and washed twice with 50% saturated ammonium sulfate. Complete collection of the radioactive complex was ensured by the use of a one-tenth dilution of normal serum as the diluent for specific antibodies collected by immunoadsorption.[2] The presence of this serum seems both to prevent aggregation and denaturation of the specific antibodies isolated and to act as a carrier in the precipitation step. A correction was made for nonspecific adsorption of labeled peptide onto the precipitate by determining the binding of the labeled peptide to a one-tenth diluted normal serum. A counting standard containing only labeled peptide was included in each experiment to correct the specific activity for radioactive decay. Precipitates containing ^{125}I were counted without further manipulation in a Packard 5110 Auto-Gamma counter. Specific details for each peptide are as follows.

(a) Peptide ^{125}I-Labeled (132–153). Labeled peptide (1.26×10^{-10} mol) and increasing amounts of myoglobin (0–8.3×10^{-6} mol) were incubated with anti-$(132–153)_R$ (38 μg) in a total volume of 450 μl. The K_{conf} for the region (132–146) was obtained using the anti-$(132–146)_R$ remaining after passing anti-$(132–153)_R$ through a Sepharose-(147–153) column.

(b) Peptide ^{125}I-Labeled 3-(4-Hydroxyphenyl)propionyl-(66–76). Labeled peptide (1.43×10^{-10} mol) and myoglobin (0, 5.07×10^{-8}, and 2.54×10^{-7} mol) were incubated with anti-$(66–76)_R$ (25 μg) in a total volume of 300 μl.

(c) Peptide ^{125}I-Labeled 3-(4-Hydroxyphenyl)propionyl-(1–55). The K_{conf} for region (1–55) was determined using a solid-phase radioimmunoassay. Sepharose-anti-$(1–55)_R$ was suspended in RIA buffer (Wide, 1969) to form a 1:1 (v/v) slurry. Labeled peptide (1–55) (1.18×10^{-10} mol) was added to the Sepharose-anti-$(1–55)_R$ slurry (100 μl) and incubated with varying amounts of myoglobin (0–9.18×10^{-8} mol) in triplicate. The total incubation volume was 160 μl. After 2.5 h shaking at constant temperature, two drops (approximately 100 μl) of a 1:1 (v/v) slurry of underivatized Sepharose 4B in the above buffer was added as a carrier to each tube, followed by more buffer (2.0 ml). The tubes were centrifuged (10 000*g*, 5 min) at room temperature, aspirated, and then washed twice with the same buffer (2.0 ml). Nonspecific binding of the labeled fragment was corrected for by replacing the Sepharose-anti-$(1–55)_R$ with Sepharose–immunoglobulin G (nonspecific (15 μg/ml) in one set of tubes.

Calculations of the K_{conf} from the binding inhibition data were carried out using eq 7 of the model developed by Furie et al. (1975):

$$K_{conf} = \frac{[\text{anti-(pep)}_R \cdot \text{pep*}][\text{protein}_N]}{[\text{anti-(pep)}_R \cdot \text{protein}_R][\text{pep*}]} \quad (1)$$

where the asterisk indicates the presence of ^{125}I.

Each term in eq 1 can be determined, directly or by difference, from the inhibition of formation of the anti-$(pep)_R$·pep* complex by the competing protein. [Anti-$(pep)_R$·pep*] is the quantity measured in the precipitate of the radioimmunoassay. $[\text{Protein}]_N$ is the amount of protein (e.g., myoglobin) added as competitor, after correction for the amount bound by anti-$(pep)_R$ (usually negligible). [Anti-$(pep)_R$·protein_R] is calculated by difference between the amount of labeled fragment in the immune complex with and without competing protein. [Pep*] is the amount of peptide added (determined from a counting standard) minus the amount bound specifically in [anti-$(pep)_R$·pep*].

Determination of K_{conf} for Isolated Peptide (132–153). The K_{conf} for peptide (132–153) in solution was measured by a modification of the method of Sachs et al. (1972). Anti-$(132–153)_N$ (31 μg) was incubated with different amounts of ^{125}I-labeled (132–153) (1.42×10^{-11} to 1.12×10^{-10} mol) in a total volume of 400 μl for 24 h. Separation of the bound and unbound labeled fragment was by the ammonium sulfate precipitation method previously described. K_{conf} was calculated using eq 7 of Sachs et al. (1972):

[2] As a matter of interest and caution, it has been noted that Sepharose–Mb_N columns adsorb protein nonspecifically from preimmunization serum added to anti-Mb_N serum and that such protein is eluted with the specific anti-Mb_N antibodies.

TABLE I: Amino Acid Compositions of Isolated and Synthetic Peptides from Sperm Whale Myoglobin.

	(1-55)		(132-153)		(147-153)		(66-76)	
	Found	Theory	Found	Theory	Found	Theory	Found	Theory
Lys	5.0	5	3.9	4	1.0	1		
His	3.8	4						
Arg	1.8	2	1.0	1				
Asp	2.9	3	2.0	2				
Thr[a]	1.9	2					1.4	2
Ser[a]	1.8	2						
Glu	8.7	9	3.1	3	2.0	2		
Pro	0.9	1						
Gly	3.1	3	2.0	2	2.0	2	1.0	1
Ala	4.1	4	3.1	3			1.8	2
Val	4.2[b]	5					1.6	2
Ile	2.0	2	0.9	1			1.0	1
Leu	6.7	7	3.0	3	0.9	1	2.8	3
Tyr[a]	0	0	1.9	2	0.9	1		
Phe	2.9	3	1.0	1				
Trp	nd	2						
Met	nd	1						

[a] Not corrected for destruction during hydrolysis. [b] Also found to be low by Epand and Scheraga (1968).

$$K_{conf} = \frac{[\text{anti-(pep)}_N \cdot (\text{pep})_N]}{K_{ass}[\text{anti-(pep)}_N][(\text{pep})_T - \text{anti-(pep)}_N \cdot (\text{pep})_N] - [\text{anti-(pep)}_N \cdot (\text{pep})_N]} \quad (2)$$

where $(pep)_T$ is the total peptide concentration.

The K_{ass} for anti-$(132-153)_N$ binding to myoglobin was calculated as previously described. [Anti-$(pep)_N$·$(pep)_N$] is the quantity measured in the radioimmunoassay. [Anti-$(pep)_N$] is the equilibrium antibody concentration determined as the difference between the total amount of antibody added and the amount of antibody in the [anti-$(pep)_N$·$(pep)_N$] complex. [$(Pep)_T$-anti$(pep)_N$·$(pep)_N$] is the concentration of free peptide determined as the difference between the total amount of peptide added and the amount of peptide in the [anti-$(pep)_N$·$(pep)_N$] complex.

Circular Dichroism (CD) Spectra. CD measurements on myoglobin fragments (1-55) and (132-153), as well as peptide (147-153), were made in BBS buffer as outlined by Nicola et al. (1975).

Results

CNBr Cleavage of Myoglobin. An elution profile for the peptides obtained from the CNBr digest and separated by gel filtration on a Sephadex G-50 (fine) column is shown in Figure 1. Peaks C and D, containing peptides (1-55) and (132-153), respectively, were passed through the column a second time to ensure removal of contaminating peptides. The amino acid composition of each peptide (Table I) agreed with those found by other authors (Edmundson, 1963; Epand and Scheraga, 1968).

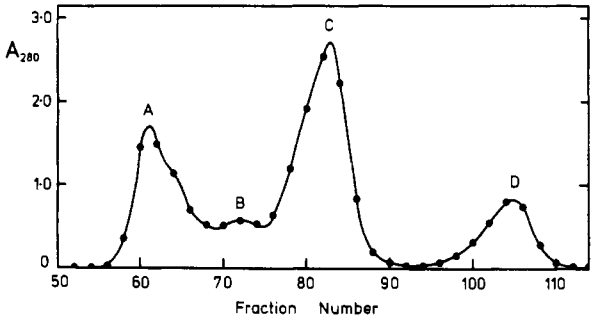

FIGURE 1: A typical elution profile for the separation of CNBr digestion products of sperm whale myoglobin on a Sephadex G-50 (fine) column (2 × 136 cm) equilibrated and eluted with formic acid-acetic acid-water (2:2:7). Elution was carried out at 0.75 ml/min and 1.4-ml fractions were collected. Peak A contained undigested protein and partially digested peptides (1-131) and (56-153). Peak B contained peptide (56-153) with some contaminating material from peaks A and C. Peak C contained peptide (1-55) with a small amount of peptide (56-131). Peak D contained essentially homogeneous peptide (132-153).

Fractionation and Purity of Peptide (147-153). The fractionation of synthetic peptide (147-153), using SE-Sephadex and eluting with an acetate gradient at pH 5.2, gave the elution profile shown in Figure 2. Fractionation of 40 mg of crude peptide mixture yielded 31.5 mg of the pure heptapeptide (78.6% yield) in peak B.

The peptide was taken to be homogeneous, since only one ninhydrin-positive spot was seen after peptide mapping. Thin-layer chromatography also revealed only one ninhydrin-positive spot at a loading of 5 μl (10 mg/ml): R_f 0.06(BAWP) and R_f 0.06(BAW). Dansylation and subsequent hydrolysis revealed only a single N-terminal amino acid, namely, lysine, the expected residue. The amino acid compo-

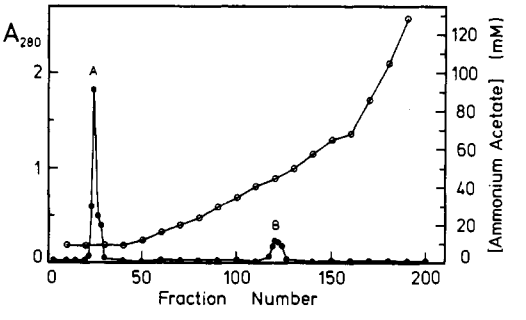

FIGURE 2: Elution profile of crude peptide (147-153) on SE-Sephadex. Chromatography was at pH 5.2 using an ammonium acetate gradient (○), collecting fractions of 2.1 ml. Peak A emerged at the void volume and contained both ninhydrin-positive and -negative material. Peak B contained homogeneous peptide (147-153).

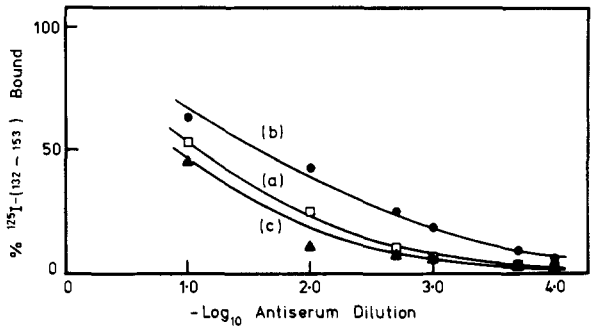

FIGURE 3: The effect of radioiodination on the immunoreactivity of peptide (132–153). Curve a shows the percentage of ^{125}I-labeled (132–153) peptide precipitated by ammonium sulfate as immune complex, plotted as a function of the concentration of anti-(myoglobin)$_N$ serum. Curve b shows the effect of diluting the ^{125}I-labeled (132–153) fivefold using BBS buffer. In curve c, the fivefold dilution is with unlabeled (132–153) of the same concentration as the radioactive peptide.

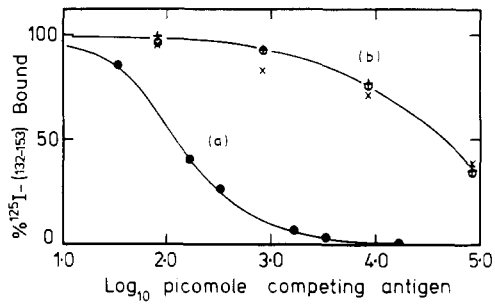

FIGURE 4: Competitive binding curves for anti-(132–153)$_R$ and ^{125}I-labeled (132–153) in the presence of (a) unlabeled (132–153), incubated for (●) 20 h and (b) native myoglobin, incubated for (+) 2.5 h, (○) 8.0 h, and (X) 20.5 h, all at 25 °C. Binding in the absence of competitor corresponds to 100% ^{125}I-labeled (132–153) bound.

sition is shown in Table I.

The far-UV CD spectrum of this peptide resembled closely that for a 100% random coil, using the models of Greenfield and Fasman (1969), indicating that this peptide is essentially structureless.

Purity of Peptide (66–76). High-voltage electrophoresis at pH 3.5 of the HF-cleaved peptide revealed four ninhydrin-positive spots. The major spot, estimated to be 70% of the total, was located at the origin. The other spots (one representing ~20% and two approximately 5% each) migrated towards the cathode. On thin-layer chromatography, in BAWP, the crude peptide migrated as a streak with R_f 0.44–0.65 and in BAW two discrete ninhydrin-positive spots were seen at R_f 0.29 (major) and R_f 0.38 (minor). The amino acid composition of the crude material is shown in Table I.

Manual sequence determination on peptide (66–76) proved to be very difficult due to the hydrophobicity of the peptide. Unequivocal identification was achieved for the first five residues from the amino terminus. These confirmed the sequence Val-Thr-Val-Leu-Thr for the major peptide in the mixture. Although no unexpected residues were found, minor truncated peptides would not be detected because of the presence of overlapping residues and the repeating occurrence of valine and threonine. Carboxypeptidase A digestion showed only two residues, in order from the C-terminus, leucine and isoleucine. No other amino acid was detected. This agrees with the sequence of myoglobin region (66–76) viz. Val-Thr-Val-Leu-Thr-Ala-Leu-Gly-Ile-Leu. The CD spectrum of this peptide was not determined because of its insolubility in aqueous buffer.

Effect of Iodination on Immunoreactivity of Peptide (132–153). The percentage of ^{125}I-labeled (132–153) peptide precipitated by ammonium sulfate as immune complex, plotted as a function of anti-(myoglobin)$_N$ serum dilution, is shown in curve a of Figure 3. The upper curve (b) shows the effect of diluting the ^{125}I-labeled (132–153) fivefold with BBS buffer. The displacement of curve b from curve a gives an indication of the simple dilution effect. In the lower curve (c), the fivefold dilution of the labeled peptide was carried out with BBS buffer containing peptide at the same concentration as the initial solution. If radioiodination had no effect on antigenicity, curves a and c would be superimposable. Although the displacement of curve c from curve a is only as great as the size of the error bars, it, nevertheless, seems significant, since all points in c are lower than in a.

Determination of K_{conf} for Region (132–153) of Myoglobin. Inhibition of ^{125}I-labeled (132–153) binding to anti-(132–153)$_R$ by unlabeled (132–153) and by native myoglobin at 25 °C is shown in Figure 4. Inhibition (say 50%) by myoglobin is less efficient than by the peptide by nearly three orders of magnitude and is independent of time after 2.5-h incubation. The K_{conf} values determined from the data of Figure 4 are tabulated in Table II, together with K_{conf} values determined for other concentrations of antibody and fragment at 25 and at 5 °C. Within experimental error, the values show no systematic variation as the concentrations of myoglobin, peptide, and antibody were varied, suggesting that a true equilibrium constant with a mean value of 246 at 25 °C was being measured. Region (132–153) of sperm whale myoglobin encompasses most of the H helix (H8-H24) and the C-terminal pentapeptide (149–153), which has no ordered structure (Kendrew et al., 1960; Perutz et al., 1965). Circular dichroic measurements in the far-ultraviolet region indicate that this peptide (132–153) has little α-helical structure, when excised from the protein (Epand and Scheraga, 1968; Hermans and Puett, 1971; Hurrell, Smith, Minasian, and Leach, unpublished).

The K_{conf} for the region (132–146), where *all* the residues are in an α-helical conformation in the native protein, was determined using antibodies specific to the nonnative conformations of this region only. The results are shown in Table III and suggest that the K_{conf} for this α-helical region is not significantly different from that for the C-terminal region (132–153) as a whole. One might have expected that the purely helical region would show a greater rigidity (larger K_{conf}) once the nonhelical tail was removed. This expectation would assume, however, that the helical segment and the tail segment each had discrete antigenic determinants. This is not the case. There is only one determinant, namely (145–151) (Atassi, 1975) and it resides partly in both segments. Immunoadsorption of anti-(132–153)$_R$ showed that only 8% of these antibodies were directed towards (147–153). This is further borne out in Figure 5 where the inhibition of ^{125}I-labeled (132–153) binding to anti-(132–153)$_R$ by peptide (147–153) is compared to the inhibition by peptide (132–153). The poor efficiency of peptide (147–153) as an inhibitor is a reflection of the absence of antibodies to this C-terminal region of peptide (132–153).

Also shown in Figure 5 is the inhibition of ^{125}I-labeled (132–153) binding to anti-(132–153)$_N$ by peptide (147–153). The low efficiency of inhibition is the same as that observed for the anti-(132–153)$_R$. This shows that region (147–153) is only weakly antigenic in the native protein.

TABLE II: K_{conf} of Myoglobin Region (132–153) (Using Specific Antibodies Which Bind to Sepharose-(132–153)).

Concn of Peptide (132–153) ($M \times 10^7$)	Amount of anti-$(132–153)_R$ (μg)[a]	Concn of Myoglobin (M)	Temp (°C)	Time of Inc. (h)	K_{conf}	Av K_{conf} (±SD)
2.80	37.5	1.84×10^{-4}	25	2.5	431	
		1.84×10^{-5}			357	
		1.84×10^{-6}			153	
		1.84×10^{-7}			166	277 ± 120
2.80	37.5	1.84×10^{-4}	25	8.0	419	
		1.84×10^{-5}			191	
		1.84×10^{-6}			142	
		1.84×10^{-7}			214	242 ± 106
2.80	37.5	1.84×10^{-4}	25	20.5	349	
		1.84×10^{-5}			153	
		1.84×10^{-6}			457	
		1.84×10^{-7}			172	283 ± 126
2.80	15.1	1.84×10^{-4}	25	2.5	254	
		1.84×10^{-5}			110	
		1.84×10^{-6}			191	
		1.84×10^{-7}			123	170 ± 58
0.46	31.7	1.84×10^{-4}	25	2.5	88	
		1.84×10^{-5}			243	
		1.84×10^{-6}			348	
		1.84×10^{-7}			358	260 ± 109
2.80	31.7	1.84×10^{-4}	5	2.5	945	
		1.84×10^{-5}			623	
		1.84×10^{-6}			552	
		1.84×10^{-7}			387	627 ± 203

[a] Under the conditions used, 37.5 μg corresponds to approximately 5×10^{-7} M antibody.

TABLE III: K_{conf} of Myoglobin Region (132–146) (Using the Population of Anti-$(132–153)_R$ Antibodies Which Did Not Bind to Sepharose(147–153)).[a]

Concn of Myoglobin (M)	K_{conf}	Av K_{conf} ±SD
1.84×10^{-4}	155	
1.84×10^{-5}	107	
1.84×10^{-6}	163	147 ± 25

[a] Peptide (132–153) was 2.8×10^{-7} M and anti-$(132–146)_R$ was 17.6 μg. Incubation was for 2.5 h at 25 °C.

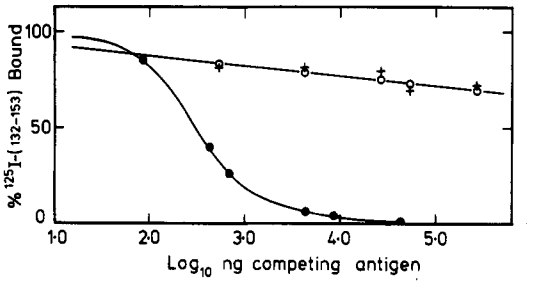

FIGURE 5: Competitive binding curves for anti-$(132–153)_R$ and ^{125}I-labeled (132–153) in the presence of (●) unlabeled (132–153) and (○) (147–153), also (+) anti-$(132–153)_N$ and ^{125}I-labeled (132–153) in the presence of (147–153). Incubation was for 2.5 h at 25 °C. Binding in the absence of competitor corresponds to 100% of ^{125}I-labeled (132–153) bound.

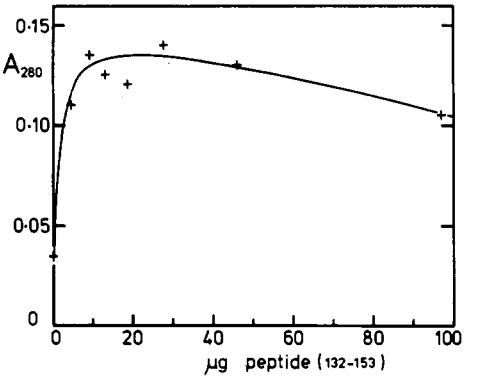

FIGURE 6: Quantitative precipitation reaction for anti-$(132–153)_R$ titrated with peptide (132–153).

When Furie et al. (1975) calculated K_{conf} of staphylococcal nuclease, they used antibodies prepared against a large peptide fragment of the protein and selected from this a subpopulation which was monospecific for a smaller region of the native protein, by immunoadsorption on a Sepharose-nuclease column followed by selective adsorption on a Sepharose-peptide column. In our experiments, the antibodies used to calculate K_{conf} for the myoglobin region (132–153) were isolated by passage only through a Sepharose-(132–153) column. These were not monospecific as assessed by their precipitability in the presence of peptide (see Figure 6). Although the peptide (132–153) must therefore contain at least two antigenic determinants, the native myoglobin has only one determinant in this region (Atassi, 1975). Our anti-$(132–153)_R$ contains antibodies to a determinant which is largely "immunosilent" in the native molecule, probably as a result of its relative inaccessibility. This is substantiated by immunoadsorption of anti-$(132–153)_R$ on a Sepharose-myoglobin column. As shown

TABLE IV: Immunoadsorption of Anti-$(132-153)_R$ by Sepharose-Antigen Columns (In Each Case, 2111 μg of Immunoglobulin Was Applied to the Column).

Sepharose-Antigen	Antibody Prep	Immunoglobulin Bound (μg)	Immunoglobulin Bound Recovery (%)
Peptide (132-153)	Anti-$(132-153)_R$ (whole serum)	2111	100
Native myoglobin	Anti-$(132-153)_R$ (whole serum)	1267	60
Native myoglobin	[a] Specific anti-$(132-153)_R$	1149	54

[a] Selected from anti-$(132-153)_R$ whole serum by immunoadsorption on a Sepharose-(132-153) column.

TABLE V: K_{conf} of Myoglobin Region (132-153) (Using the Specific Antibodies Which Bind to Sepharose-(132-153) and to Sepharose-Myoglobin).[a]

Concn of Fragment ($M \times 10^7$)	Concn of Myoglobin (M)	K_{conf}	Av K_{conf} ±SD
2.48	5.07×10^{-4}	144	
2.46	5.07×10^{-4}	200	
2.47	1.66×10^{-4}	61	
2.41	5.07×10^{-5}	49	
2.32	5.07×10^{-5}	86	
2.37	1.66×10^{-5}	46	
2.21	5.07×10^{-6}	45	
2.06	5.07×10^{-6}	44	
2.06	1.66×10^{-6}	43	
1.85	5.07×10^{-7}	44	76.2 ± 74.9

[a] 31 μg of anti-$(132-153)_R$ was used throughout. Incubation was for 2.5 h at 25 °C.

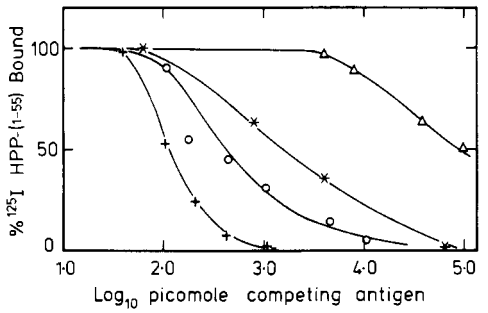

FIGURE 7: Competitive binding curves for Sepharose-anti-$(1-55)_R$ and ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-(1-55) in the presence of (+) (1-55) and (Δ) myoglobin. Also for Sepharose-anti-$(1-55)_N$ and ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-(1-55) in the presence of (*) (1-55) and (○) myoglobin, incubation for 3.5 h at 22 °C. Binding in the absence of competitor corresponds to 100% of ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-(1-55) bound. In the ordinate label, HPP means ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl.

in Table IV, approximately 43% of the anti-$(132-153)_R$ isolated by adsorption on Sepharose-(132-153) will not bind to Sepharose-myoglobin. K_{conf} values determined using the population of anti-$(132-153)_R$ which *can* bind to Sepharose-myoglobin (Table V) are slightly, but significantly, lower than those determined using the whole antibody population. The somewhat lower K_{conf} values obtained by this procedure apply to antibodies to determinants accessible only in the native or "near-native" states; that is, they probably exclude more "extreme" conformations accessible to the native molecule in solution but not in the immobilized state.

TABLE VI: K_{conf} of Myoglobin Region (1-55) (Using Sepharose-Anti-$(1-55)_R$ in Solid-Phase Radioimmunoassays).[a]

Concn of Myoglobin (M)	Temp (°C)	K_{conf}	Av K_{conf} ± SD
5.69×10^{-4}	5	2213	
4.90×10^{-5}		2348	
2.45×10^{-5}		2029	2197 ± 131
5.69×10^{-4}	22	1228	
2.45×10^{-4}		635	
2.45×10^{-4}		860	
4.90×10^{-5}		661	
4.90×10^{-5}		833	
2.45×10^{-5}		692	
2.45×10^{-5}		986	842 ± 196
5.69×10^{-4}	37	350	
2.45×10^{-4}		620	
2.45×10^{-5}		253	
2.45×10^{-6}		291	379 ± 144

[a] Peptide (1-55) was 7.38×10^{-7} M and 13 μg of anti-$(1-55)_R$ was used in each case. Incubation was for 3.5 h.

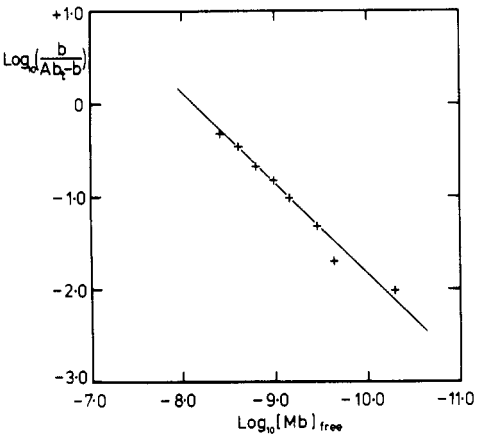

FIGURE 8: Sips plot of anti-$(132-153)_N$ binding to [^{125}I]myoglobin. Ab_t is the concentration of antibody binding sites (2.38×10^{-8} M) and b is the concentration of specifically bound [^{125}I]myoglobin; incubation was for 20 h at 37 °C.

Determination of K_{conf} for Region (1-55). The K_{conf} values of region (1-55) of myoglobin, at three different temperatures, are shown in Table VI. The inhibition of ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-(1-55) binding to both anti-$(1-55)_R$ and anti-$(1-55)_N$ by peptide (1-55) and myoglobin at 37 °C is seen in Figure 7. The peptide is three orders of magnitude more efficient as an inhibitor of binding to anti-$(1-55)_R$ than is myoglobin. In contrast, myoglobin is more efficient than the peptide in inhibiting binding to anti-$(1-55)_N$.

Determination of K_{conf} for Region (66-76). No inhibition of ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-(66-76) binding to anti-$(66-76)_R$ by myoglobin could be detected. The minimum inhibition that can be experimentally determined is ±100 cpm in 50 000 total counts. This limit of detection establishes the lower limit of K_{conf} for this region as 2.5×10^6. K_{conf} is inversely related to the concentration of peptide free in solution.

TABLE VII: K_{conf} of Isolated Peptide (132–153) (Using Anti-$(132–153)_N$ Isolated from Anti-Myoglobin Serum Using Sepharose-(132–153)).[a]

Concn of Peptide (132–153) (M)	K_{conf}	Av K_{conf} ± SD
2.78×10^{-7}	11.4×10^{-3}	
1.87×10^{-7}	6.4×10^{-3}	
1.43×10^{-7}	11.6×10^{-3}	
7.15×10^{-8}	8.7×10^{-3}	
3.52×10^{-8}	9.3×10^{-3}	$(9.5 \pm 1.9) \times 10^{-3}$

[a] The concentration of anti-$(132–153)_N$ was 3.84×10^{-7} M throughout. Incubation was for 2.5 h at 36 °C.

Thus, any overestimation of the concentration of peptide brought about by contaminating peptides would be expected to increase this lower limit for K_{conf}.

Determination of K_{ass} for Anti-$(132–153)_N$ with Myoglobin. The K_{ass} for myoglobin binding to anti-$(132–153)_N$ at 35 °C was determined from a Sips plot of [^{125}I]myoglobin binding to isolated antibodies (Figure 8). The K_{ass} obtained was 1.5×10^8 l. mol^{-1}. The heterogeneity index was 1.02, suggesting that the isolated antibody population was of essentially homogeneous affinity.

K_{conf} of Peptide (132–153). The K_{conf} of peptide (132–153) in solution was determined at 35 °C using four peptide concentrations. The values obtained are shown in Table VII. The values indicate that about 1% of the peptide was present in the native conformation.

Discussion

The development of an immunological approach to the measurement of conformational equilibria first for peptides (Sachs et al., 1972) and later for proteins (Furie et al., 1975) has opened up new possibilities for measuring conformational motility within localized regions of these molecules. The limitations and assumptions, both in technique and interpretation, were well recognized by these authors. The method as applied to proteins is limited to those regions which, when excised from the protein, are immunogenic. Furie et al. also chose regions with a single determinant so that the antibody population elicited by the excised peptide was monospecific and gave soluble complexes with these antibodies.

The analytical problem of estimating the concentration of the peptide–antibody complex and of the free peptide in an equilibrium mixture of antibody, peptide, and competing native protein was solved by Furie et al. (1975) using peptide which had a ^{14}C label at its N-terminal group, precipitating free and combined antibody with rabbit anti-goat immunoglobulin, and measuring the radioactivity in the precipitate.

In the present work, we have chosen to avoid the double-antibody procedure, which requires prolonged incubation at 4 °C and we have "frozen" the equilibrium using ammonium sulfate precipitation of the immune complexes. Reequilibration during the early stages of *any* precipitation technique cannot be precluded but several studies have shown (Farr, 1958; Seppälä, 1975) that, using ammonium sulfate, there is negligible dissociation of antibody complexes in nonhaptenic systems. Our time studies show that there is no reequilibration after 2.5 h.

N-terminal labeling procedures, such as that used by Furie et al. (1974), undoubtedly avoid the dangers of chemically modifying the antibody-combining affinity at or near the antigenic sites being studied. Rather than ^{14}C carbamylation, however, which produces rather low specific activities, we have used ^{125}I which was introduced either *via* the iodinatable acylating reagent of Bolton and Hunter (1973) or by direct, but mild, iodination of the peptide tyrosines (1 mol/mol). The convenience of the iodination method, the high specific activities obtainable, and the convenience of γ counting make this an attractive procedure. In the case of myoglobin, the antigenic determinants are known (Atassi, 1975). The determinant in peptide (132–153) carries a tyrosine at each end and both are stated to be involved in the peptide's antigenicity, as judged by Atassi's nitration experiments. On the other hand, Crumpton et al. (1970) have shown that the replacement of tyrosine-151 by either phenylalanine or *p*-methoxyphenylalanine has no effect on the antigenicity of peptide (147–153). It is, nevertheless, possible that iodination procedures, however mild, might affect the binding affinity and, therefore, the observed K_{conf} for this particular peptide. Figure 3, which illustrates the effect of radioiodination on the immunoreactivity of peptide (132–153), does indicate a small but significant loss of antigenicity. The K_{conf} values we have reported using this peptide are therefore probably less reliable (presumably low) than the values using the other two peptides where N-terminal labeling was used.

The magnitude of such effects is unlikely to be large, since peptide (147–153), which contains one of the two tyrosines, competes only weakly with peptide (132–153) whether the latter is iodinated, as in our experiments, or not, as in the experiments of Koketsu and Atassi (1973) or of Crumpton (1967). If iodination attenuated the antigenicity of peptide (132–153) appreciably this would enhance the ability of unlabeled (147–153) to compete with it.

Furie et al. (1975) have stressed that the region of staphylococcal nuclease chosen (99–126) for their investigation of K_{conf} was univalent with respect to antigenicity. In myoglobin, our fragments (132–153) and (1–55) comply with this requirement (Atassi, 1975). On the other hand, the populations of antibodies to the excised fragments were more heterogeneous in the present experiments than in those of Furie et al. In the latter case, antiserum to each peptide was first passed down an affinity column of the intact native protein. This isolated only those antibodies which recognized determinants normally accessible in the native protein. Only after this "screening" were the antibodies further purified on a Sepharose–peptide column. In our experiments with region (132–153) in myoglobin, we have found (Table IV) that the "screening" step, indeed, removes some 40–45% of the total anti-$(132–153)_R$ antibodies. The antibodies remaining, when used to measure K_{conf}, do lead to a somewhat lower K_{conf} value (Table V) than when the whole anti-$(132–153)_R$ population is used (Table II). The decision as to which of the two antibody populations provides a K_{conf} which more truly reflects segmental motility is, however, by no means clear cut. Antibodies to the *most* inaccessible determinants of a protein do not appear to participate in K_{conf} measurements. Thus, antibodies to peptide $(66–76)_R$—a segment of the E helix of myoglobin in which 5 of the 11 residues are inaccessible to solvent in the crystalline state (Perutz et al., 1965)—showed no measurable binding to native myoglobin. The standard free energy of unfolding for this region must be at least −8.7 kcal mol^{-1}—a value considerably more negative than those for the N-terminal (1–55) and C-terminal (132–153) regions which were −3.9 and −3.3 kcal mol^{-1} at 295 and 298 K, respectively. The

overall $\Delta G°$ for the unfolding of myoglobin is estimated to be -13.6 kcal mol^{-1} at 298 K (Puett et al., 1973). Such values no doubt relate to the most tightly folded regions of the molecule and K_{conf} values for such regions would not be accessible using the present methods. It seems most likely that the anti-$(132-153)_R$ antibodies removed by the native protein immunoadsorbent are not only those which are directed towards determinants which are totally inaccessible in the native protein but also some which are directed towards "native-like" conformations. If this were not so, K_{conf} would be unaffected by their removal. There is a good reason for not removing such populations of antibodies, since they probably represent conformations which are accessible to the protein molecule in solution but not when immobilized on a Sepharose column. It is well known that proteins are stabilized when adsorbed on immunoadsorbents and this is probably because conformational fluctuations are "damped" in the adsorbed (or crystalline) state.

The strategem of measuring the K_{conf} of myoglobin region (1–55) with antibodies covalently bound to a Sepharose support arose from necessity but may well prove in the future to be a method of choice, particularly for synthetic peptides. This peptide aggregates in solution and precipitates nonspecifically when any precipitant is added to isolate the immune complexes. The hydrophilic matrix support of Sepharose 4B, used in the presence of a nonionic detergent (Tween 20), overcame this problem.

It is interesting that the K_{conf} values obtained for region (132–146), a region entirely α helical in myoglobin, are the same as those for the full C-terminal region (132–153). The seven terminal residues are largely nonhelical. The reason appears to be that the antibody population raised to the (132–153) peptide is the same as that raised to the (132–146) peptide. The (147–153) region is low in antigenicity not only in the nonnative but also in the native state (Figure 5); this is unexpected in view of the fact that five of the seven residues comprising the C-terminal antigenic determinant of native myoglobin reside in the (147–153) sequence (Atassi, 1975).

It remains to comment on the conformational equilibrium constants measured for the isolated peptide (132–153) of myoglobin. Anfinsen and Scheraga (1975) have suggested that the observed value of 1 in 5000 (K_{conf} is 2×10^{-4}) for isolated nuclease peptides (Sachs et al., 1972) is significantly higher than one would expect for an unstructured or random state. In the present case, the argument is even more compelling. In myoglobin, where the minimum size of a determinant is known to be six residues, and assuming a five-state model for each residue, an observed value of K_{conf} of 6×10^{-4} would be equivalent to an unstructured peptide. The observed value of about 10^{-2} at 36 °C (Table VII) suggests a significant degree of folding, even though this peptide shows little evidence of secondary structure by circular dichroic measurements and such physical data cannot be readily interpreted in terms of residual native folding (Epand and Scheraga, 1968; Hurrell et al., unpublished).

The significantly higher degree of structure for the myoglobin (132–153) peptide compared with that for the staphylococcal nuclease peptides is presumably due to the juxtaposition of more helix-inducing residues in the former. In the native protein, 17 of the 22 residues take up the α-helical conformation (Perutz et al., 1965). The propensity of isolated CNBr peptides from myoglobin to take up the α-helical conformation in structure-inducing solvents has been well documented (Epand and Scheraga, 1968; Atassi and Singhal, 1970; Hermans and Puett, 1971).

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Isolation of Type III Collagen from Human Adult Parenchymal Lung Tissue†

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ABSTRACT: The isolation and characterization of type III collagen from adult human lung parenchyma are described. The identity of this molecule as type III collagen has been established on the basis of (a) demonstration of intramolecular disulfide cross-links in the helical portion of the molecule, (b) amino acid analysis characteristic for type III collagen, and (c) composition and size of isolated cyanogen bromide peptides α1(III)-CB3, α1(III)-CB5, and α1(III)-CB8. The molecular weight of lung α1(III) was determined as 93 000 by Agarose chromatography, but its electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels was slower than that of type I α chains which also have a molecular weight of 93 000.

The lung parenchyma is biochemically and morphologically a complex structure. Approximately 40 different cell types are held together in a complicated supporting structure composed of collagen, elastin, and ground substance (Hance and Crystal, 1975a,b). Collagen is the most abundant structural protein in the lung representing 20-30% of the dry weight of adult human lung (Bradley et al., 1975). It is responsible for the maintenance of airway and vascular stability and contributes significantly to lung recoil. Collagen is fundamental to normal lung structure and function in certain disease states including the fibrotic lung disorders (Hance and Crystal, 1975a,b).

The insolubility of lung collagen has frustrated direct attempts at its characterization (Bradley et al., 1975; Hurst and Baker, 1975). Bradley et al. (1975) were able to extract less than 10% of the total collagen present in lung tissue from 12-17-week-old human fetuses and were unable to extract intact collagen molecules from adult human lung. The latter, it should be noted, has on the average 11 times more collagen per unit lung mass than the fetal tissue (Bradley et al., 1975). The collagen extracted from fetal lung contained α and β chains with chromatographic, molecular weight, and compositional properties similar to those reported for type I collagen extracted from human dermis.

Short-term cultures of lung minces prepared from 3-week-old rabbit lungs have been used to study collagen synthesis by peripheral lung, bronchial tree (first through seventh

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