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Semisynthesis of sperm whale myoglobin by fragment condensation

(peptide synthesis/reconstitution/heme protein)

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ABSTRACT **Reconstruction of the sperm whale myoglobin structure was accomplished by a series of aqueous condensations of suitably protected synthetic myoglobin fragments to a large fragment prepared from the native protein. Reaction of $N^{\alpha},N^{\epsilon}_{19}$-acetimidomyoglobin with 3-bromo-2-(2-nitrophenylsulfenyl)skatole (BNPS-skatole) yielded the fragment corresponding to residues 15–153. The covalent structure was reformed by sequential coupling of the *N*-hydroxysuccinimide esters of *o*-nitrophenylsulfenyl-L-tryptophan (residue 14) and selectively protected peptides corresponding to residues 1–5 and 6–13, which were synthesized by the solid-phase method and removed from the resin by methoxide-catalyzed methanolysis. A mixed aqueous solvent system containing methanol and *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine/trifluoroacetic acid buffer (Quadrol) solubilized the peptide and protein fragments during the condensations. Replacement of the heme moiety and immunoaffinity chromatography made possible the isolation and purification of the reconstructed native molecule. The development of this nondestructive synthetic procedure allows investigation of the structural and functional significance of individual residues by isotopic enrichment or selective amino acid substitutions.**

Studies using selective, consecutive removal and substitution of the NH_2-terminal residues of Mb by specific degradation and resynthesis (1, 2) have indicated the subtle contribution of the NH_2-terminal charge and the uncharged side chains of residues 1 and 2 to the conformation and stability of the native molecule (3). To examine further the effects of synthetic sequence alteration with respect to stability, conformation, and electrostatic and hydrophobic interactions, a method for creating internal sequence variations must be developed. A promising approach is that of semisynthesis, a technique whereby the natural product is selectively cleaved and a fragment is isolated that is suitable for rebuilding the native structure or an analog.

Previous attempts at partial synthesis with Mb have utilized tryptic or CNBr fragments in which citraconyl or maleyl protecting groups were used on the ϵ-NH_2 groups (4, 5). The current strategy exploits the oxidative lability of the tryptophans at positions 7 and 14 of the Mb sequence by the use of 3-bromo-2-(nitrophenylsulfenyl)skatole (BNPS-skatole; skatole = 3-methylindole) to effect cleavage, yielding the fragment corresponding to residues 15–153 (designated fragment 15–153) in which the ϵ-NH_2 groups have been protected previously by reaction with methyl acetimidate (1). Sequential coupling of the *N*-hydroxysuccinimide (HOSu) esters of *o*-nitrophenylsulfenyl-L-tryptophan (NPS-Trp; residue 14) and the selectively protected peptides corresponding to residues 1–5 and 6–13 (designated peptides 1–5 and 6–13) regenerated the native sequence. Isolation of reconstructed Mb capable of correctly positioning the heme moiety was accomplished by passage through successive affinity columns on which the native semisynthetic protein was selectively bound by anti-Mb antibodies isolated from goat antiserum and by a monoclonal antibody that preferentially recognizes Mb residues 4, 79, and possibly 12 (6).

The development of this nondestructive synthetic procedure allows isotopic enrichment or selective amino acid substitutions to investigate the structural and functional significance of individual residues within the Mb A-helix in intramolecular interactions and in interactions of the molecule with various anti-Mb antibodies.

MATERIALS AND METHODS

Preparation of Mb and Fragment 15–153. Sperm whale myoglobin was isolated, and the major component band IV (7) was purified (8). Preparation of acetimidomyoglobin and its reaction with tetrahydrophthalic anhydride to isolate the $N^{\alpha},N^{\epsilon}_{19}$-acetimidyl derivative follow the procedure of DiMarchi *et al.* (1). Upon removal of the heme (9), the apoprotein was dissolved (3%) in 50% (vol/vol) acetic acid/6 M urea, and a 180-fold molar excess of phenol was added. The solution was chilled to 4°C, and BNPS-skatole was added in a 10-fold molar excess for each tryptophan. After reaction for 48 hr at 4°C, the reaction was stopped by dialysis against water. Reagent not removed by precipitation and centrifugation was eliminated by elution of the cleavage products on a Sephadex G-25 column (100 × 3 cm) equilibrated with 8 M urea/0.1 M acetic acid. The methionine sulfoxide produced during the cleavage reaction was converted to methionine by incubation of the protein with 0.725 M 2-mercaptoethanol for 62 hr at 37°C (10). The yield of fragment 15–153 was determined by automated Edman degradation of the unfractionated products, described below.

Synthesis of Peptides. The peptides corresponding to residues 1–5 (Val-Leu-Ser-Glu-Gly) and 6–13 (Glu-Trp-Gln-Leu-Val-Leu-His-Val) of the sperm whale Mb sequence were synthesized by standard solid-phase techniques (11). To retain the N^{α}-*t*-butoxycarbonyl (Boc) and β,γ-*t*-butyl protecting groups, the peptides were removed from the solid-phase resin by trans-

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Abbreviations: BNPS-skatole, 3-bromo-2-(2-nitrophenylsulfenyl)skatole; NPS-Trp, nitrophenylsulfenyl-modified tryptophan; Boc, *t*-butoxycarbonyl; HOSu, *N*-hydroxysuccinimide; NaCl/P_i, phosphate-buffered saline; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine/CF_3COOH buffer in *n*-propanol/H_2O, 3:4 (vol/vol), pH 9.0; oxindole derivatives, 2-hydroxytryptophan and 2,3-dihydroxytryptophan.

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esterification for 24 hr at 25°C in methanol, catalyzed by 0.5 equivalents of sodium methoxide (Aldrich). The resulting methyl ester peptides were saponified for 24 hr at 4°C to yield a free α-carboxyl group suitable for activation with HOSu (10).

Purification of peptide 6–13 was accomplished by gel filtration on an LH-20 column (100 × 2.5 cm) equilibrated with dimethylformamide. Peptide 1–5 was purified by high performance liquid chromatography on a reversed-phase C-18 column equilibrated with 0.1% CF_3COOH and eluted with a gradient (0–100%) of acetonitrile in 0.1% CF_3COOH. The purity of the peptides was confirmed by amino acid analysis after acid hydrolysis as described below.

The extent of racemization was determined by digestion with L-amino acid oxidase (12, 13) and quantitation of D-amino acids by automated analysis. Digestion of native sperm whale Mb served as a control for amino acids resistant to the enzyme and for racemization incurred during acid hydrolysis. The amount of side chain deprotection and deamidation was measured by reaction of the saponified peptide 6–13 with dicyclohexylcarbodiimide and glycine methyl ester. After removal of excess reactants by gel filtration, the peptide was acid-hydrolyzed, and the yield of glycine in excess of one equivalent was determined by automated analysis and was taken as the combined yield of deprotection and deamidation.

Fragment Condensation. The HOSu active esters of NPS-Trp (residue 14), N^{α}-Boc-γ-*t*-butyl-Glu^6–peptide 6–13, and N^{α}-Boc-β-*t*-butyl-Ser^3-γ-*t*-butyl-Glu^4–peptide 1–5 (in which Boc = *t*-butoxycarbonyl) were formed by the method of Anderson *et al.* (14). For each successive coupling, a 10- to 20-fold excess of the active ester species was dissolved in the minimal amount of methanol and added to a 3% aqueous solution of the apoprotein fragment. Solubility was enhanced by the use of *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine/CF_3COOH buffer (Quadrol) to adjust and maintain the condensation reaction pH at 7.2 (10). Couplings proceeded for 48 hr at 25°C and were ended by dialysis against water. Excess peptide noncovalently associated with the protein was removed by gel filtration of the coupling products on a Sephadex G-25 column (100 × 3 cm) equilibrated with 8 M urea/0.1 M acetic acid. After the couplings of residue 14 and peptide 6–13 and prior to N^{α} deprotection, the noncoupled fragments were terminated from further reaction by carbamylation with a 180-fold excess of KCNO in 0.1 ionic strength phosphate buffer (pH 6.5). The N^{α}-NPS group was removed from fragment 14–153 by treatment for 24 hr with 0.1 M 2-mercaptoethanol in H_2O, pH 7.0; the N^{α}-Boc and β,γ-*t*-butyl groups were removed from fragments 6–153 and 1–153 by treatment for 1 hr with 10 g of anhydrous CF_3COOH per 1 g of Mb (1560:1), with 100 μl of anisole per 1 g of Mb (16:1) and 200 mg of dithioerythritol/1 g of Mb (23:1) used as scavengers. (The numbers in parentheses are mole ratios.)

Reconstitution of Mb with Heme. The semisynthetic products were dissolved in water to a 1% concentration, and the pH was adjusted to 11.0 at 4°C (2). A stoichiometric amount of hematin dissolved in minimal 0.1 M NaOH was added dropwise and allowed to react 30 min before extensive dialysis against water. The stoichiometric amount of heme was determined prior to the reaction by automated Edman degradation of the unfractionated semisynthetic products to determine the quantity of reconstructed Mb in the sample.

Purification of Semisynthetic Mb. The crude semisynthetic aquoferrimyoglobin was converted to the more stable cyanoferri form and simultaneously prepared for affinity chromatography by dialysis against 1 mM KCN in phosphate-buffered saline NaCl/P_i, pH 7.4. The dialyzed and centrifuged semisynthetic Mb (70 nmol) was applied to a column (20 × 1.2 cm) of Sepharose 4B (Pharmacia) to which 250 nmol of goat anti-Mb antibody (for preparation, see ref. 15) was covalently attached. The column was washed at 10 ml/hr at 4°C until the optical absorbance of the eluate indicated no further passage of protein as monitored at 280 nm. The bound Mb was eluted with 0.1 M NH_4OAc, pH 10.5/1 mM KCN, collected and dialyzed against 1 mM KCN in NaCl/P_i, pH 7.4. Further purification of the semisynthetic Mb was attained by a second affinity chromatography on a column (14 × 1.2 cm) of Sepharose 4B to which was covalently attached 250 nmol of clone 3.4 monoclonal antibody, which binds preferentially to Mb residues 4, 79, and possibly 12 (6). The elution procedure was the same as above.

Determination of Cleavage and Coupling Yields. The mole fraction of nascent protein fragments within a sample of cleavage or coupling products was determined by subjecting the unfractionated products to several degradative cycles on the Beckman 890C sequencer directed by the Beckman fast protein Quadrol program (072172C); the NH_2-terminal residues were identified as described (1). The recovered amino acids were correlated with the fragment of origin by the known sequence of each protein fragment (15–153, 14–153, 6–153, and 1–153), and the mole fraction of each was calculated by dividing the amount of recovered NH_2-terminal residue originating from the fragment by the total amount of protein whose sequence was determined. The blocked NH_2 terminus of $N^{\alpha},N^{\varepsilon}_{19}$-apoacetimidylmyoglobin not cleaved by BNPS-skatole was resistant to Edman degradation and did not appear among the recovered amino acids (see Fig. 1).

Amino Acid Analysis. Protein and peptide hydrolysates were prepared by the procedure of Spackman *et al.* (16) and analyzed as reported (17).

UV and Visible Absorption Measurements. Spectra measured with a Perkin-Elmer 552 spectrometer were converted to protein concentrations by using $\varepsilon_{409} = 16.8 \times 10^4$ and $\varepsilon_{280} = 3.15 \times 10^4$ M^{-1} cm^{-1} for the aquoferri derivatives and $\varepsilon_{423} = 11.2 \times 10^4$ and $\varepsilon_{280} = 3.22 \times 10^4$ M^{-1} cm^{-1} for cyanoferrimyoglobin (18).

RESULTS

Characterization of the cleavage reaction and the Mb fragment it generated was done prior to the use of the fragment in semisynthesis. Production of Mb fragment 15–153 by cleavage of $N^{\alpha},N^{\varepsilon}_{19}$-apoacetimidylmyoglobin with BNPS-skatole occurred in 85% yield. Amino acid analysis of the cleavage products after digestion by methanesulfonic acid (19) indicated no tryptophan but did indicate the presence of oxindole derivatives (2-hydroxytryptophan and 2,3-dihydroxytryptophan) arising from the Mb in which the tryptophans were oxidized but not cleaved by the reagent.

The structural viability of fragment 15–153 resulting from the cleavage conditions and the effect of all other conditions applied to the nascent Mb throughout the semisynthesis were assessed by a series of control reactions. Samples of virgin apo-Mb were treated with the various solvents, temperatures, and reagents of the semisynthesis, omitting only the cleaving and coupling reagents; the yield of Mb that could be recovered upon replacement of the heme was determined as well as the A_{409}/A_{280} spectral ratio, a sensitive measure of structural integrity (20, 21). The data (Table 1) indicate (*i*) that the fragment condensation and deprotection conditions are within acceptable limits of tolerance by the molecule and (*ii*) that the apomyoglobin can, in fact, withstand numerous assaults by conditions far removed from the physiological environment and yet retain the ability to refold properly and to position the heme correctly when returned to nondenaturing conditions.

The optimum conditions for the fragment condensation steps

Table 1. Yield and spectral ratio of Mb reconstituted after control semisynthetic reactions

Sample conditions	Time, hr	°C	Yield, %	$\frac{A_{409}}{A_{280}}$
1. Sperm whale Mb	—	—	—	5.29
2. No reaction; reconstitution of fresh apomyoglobin	—	—	92	5.06
3. Cleavage to yield Mb(15–153)	48	4	90	5.02
4. Reduction of Met sulfoxide in Mb(15–153)	64	37	78	4.72
5. Fragment condensation	48	25	83	5.03
6. Deprotection with CF_3COOH	1	25	83	4.73
7. Sequential semisynthetic conditions	840	—	84	4.80

The effects of semisynthetic reaction conditions on the viability of the Mb structure were assessed by exposing samples of fresh apomyoglobin to the conditions of each reaction involving the major Mb fragment, omitting only the cleaving and coupling reagents. Samples 3–6 each tested the effect of a single set of conditions; sample 7 demonstrated the cumulative effect of all steps of the semisynthesis, including dialysis and lyophilization, conducted over a period of 5 wk in parallel with an authentic semisynthetic preparation.

were determined by trial couplings on an analytical scale. The yields of coupling residue 14, peptide 6–13, or peptide 1–5 to fragment 15–153 were 97%, 43%, and 63%, respectively. Samples of the products resulting from each of the peptide couplings were applied to a Sephadex G-50 superfine column equilibrated with 10% acetic acid and eluted at 15 ml/hr. The elution profiles (Fig. 1) show the distribution of products within each sample.

The ineffectiveness of gel filtration chromatography to separate large protein fragments differing in molecular weight by 15% or less and the propensity of apomyoglobin to aggregate in the mid-pH range and to bind to ion-exchange resins determined the current synthetic strategy wherein intermediate purifications were eliminated. The blocked amino terminus of $N^{\alpha},N^{\varepsilon}_{19}$-apoacetimidylmyoglobin that was not cleaved by BNPS-skatole prevented this species from participating in the fragment condensation, whereas carbamylation of noncoupled fragments after each condensation terminated any further reaction of these products.

Termination products, deletion or racemized sequences, branched chains, and other globin molecules were separated from the sound semisynthetic product by their inability to associate in a specific manner with the heme to form a stable Mb. Noncovalent interactions relate the heme to 11 residues in five of the globin helices that form the hydrophobic heme pocket. Reconstitution of the holoprotein occurs only when these helices are in a configuration similar to the native geometry; other conformations result in nonspecific binding of the heme to form

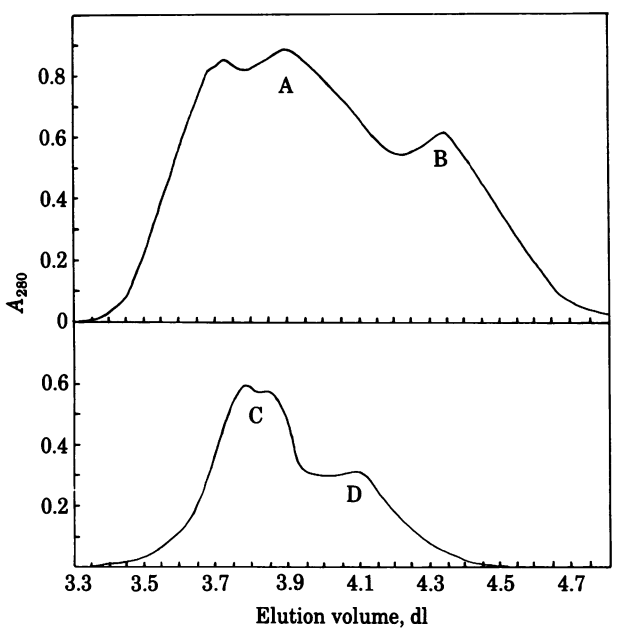

FIG. 1. Analytical fractionation of coupling products by gel filtration. Samples were loaded on a Sephadex G-50 superfine column (2.6 × 200 cm) equilibrated with 10% acetic acid and eluted at 15 ml/hr. Fractions were identified by NH_2-terminal sequence analysis. (*Upper*) Products (115 mg) resulting from the coupling of peptide 6–13 to Mb(14–153). Fractions: A, Mb(6–153); B, Mb(14–153). (*Lower*) Products (40 mg) resulting from the coupling of peptide 1–5 to Mb(6–153). Fractions: C, Mb(1–153) + Mb(6–153); D, Mb(14–153). The discrepancy in elution positions between the two samples arises from differences in the quantity and viscosity of each sample at loading.

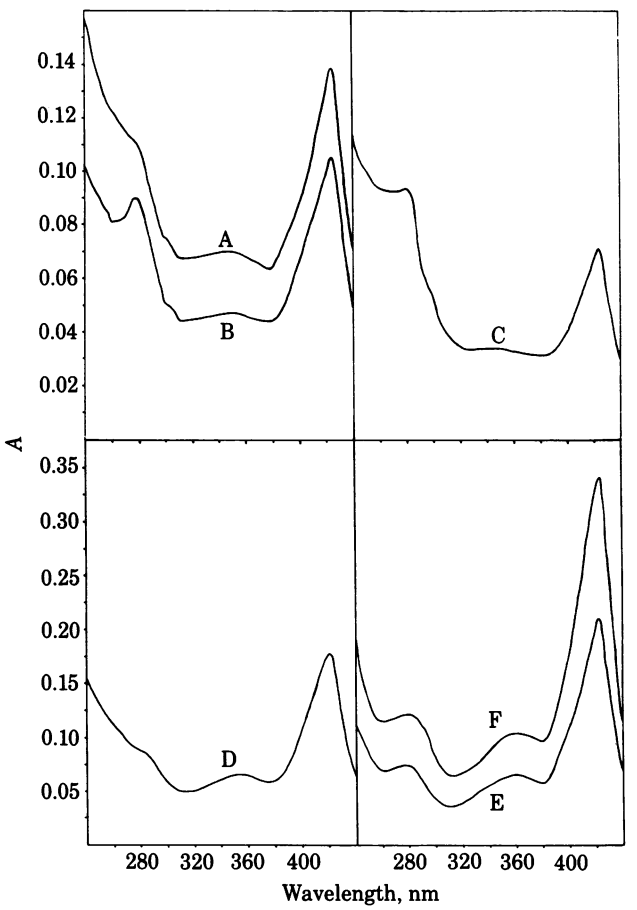

FIG. 2. UV–visible absorbance spectra of semisynthetic cyanoferrimyoglobin. Spectra: A, crude semisynthetic Mb in $NaCl/P_i$ containing 1 mM KCN (pH 7.4); B, partially purified semisynthetic Mb after immunoaffinity chromatography with polyclonal goat antibodies, in $NaCl/P_i$ containing 1 mM KCN (pH 7.8); C, material not recognized by clone 3.4 antibody and removed from the monoclonal immunoaffinity column by a wash of $NaCl/P_i$ containing 1 mM KCN (pH 7.4) (A_{423}/A_{280} = 0.76); D, Mb with altered tryptophan, which was bound by clone 3.4 antibody but selectively removed from the monoclonal column by elution with 0.1 M NH_4OAc/1 mM KCN, pH 10.2; E, semisynthetic Mb bound by clone 3.4 antibody and released by elution with 0.1 M NH_4OAc/1 mM KCN, pH 10.5 (A_{423}/A_{280} = 2.84); F, natural N^{ε}_{19}-acetimidylmyoglobin bound by clone 3.4 antibody in a separate experiment and released from the monoclonal column by elution with 0.1 M NH_4OAc/1 mM KCN, pH 10.5 (A_{423}/A_{280} = 2.88).

hemochromogen molecules, which typically exhibit a spectral ratio of A_{409}/A_{280} in the range 1–2 and which may be removed by precipitation.

Addition of heme to semisynthetic products and precipitation of hemochromogen by dialysis against 1 mM KCN in NaCl/P_i, pH 7.4, yielded the semisynthetic cyanoferrimyoglobin characterized by spectrum A in Fig. 2. Forty-six percent of the sample was bound by the polyclonal goat antibody column and was eluted at pH 10.5 (Fig. 2, spectrum B). The partially purified sample was applied to the monoclonal antibody column and washed extensively with KCN in NaCl/P_i, pH 7.4, to remove molecules not recognized by the antibody, amounting to 17% of the sample (Fig. 2, spectrum C). Mb with altered tryptophan, constituting 37% of the sample, was selectively removed from the column by elution with 1 mM KCN/0.1 M NH_4OAc, pH 10.2 (Fig. 2, spectrum D). Subsequent elution with 1 mM KCN/0.1 M NH_4OAc, pH 10.5, released the remaining 46% of the sample, the desired semisynthetic fraction (Fig. 2, spectrum E). The absorption spectrum of the reconstituted Mb paralleled that of natural N^{ε}_{19}-acetimidylmyoglobin under the same conditions (Fig. 2, spectrum F). Bisoxindolylalanine^{7,14}-Mb, treated by the identical affinity chromatographic procedures, yielded the spectra shown in Fig. 3. The shoulder at 250–260 nm in the absorbance profile, characteristic of oxindole derivatives (22), was absent from the spectrum of the purified semisynthetic Mb, indicating that the less stable Mb derivatives with oxidized tryptophan residues were eliminated and only Mb with newly incorporated, intact tryptophan was present.

Amino acid analysis of the semisynthetic Mb gave (theoretical values in parentheses) aspartic acid, 8.2 (8); threonine, 3.7 (5); serine, 4.5 (6); glutamic acid, 19.0 (19); glycine, 11.5 (11); alanine, 17.4 (17); methionine, 1.8 (2); isoleucine, 7.7 (9); leucine, 17.9 (18); tyrosine, 2.7 (3); phenylalanine, 5.8 (6); arginine, 4.3 (4); and tryptophan, destroyed. The values for threonine and serine are low because of destruction during hydrolysis; the low value for isoleucine is due to the acid-resistant Ile-Ile moiety; valine was eluted coincident with a buffer-change peak; and histidine was not sufficiently resolved from the acetimidolysine peak to permit quantitation.

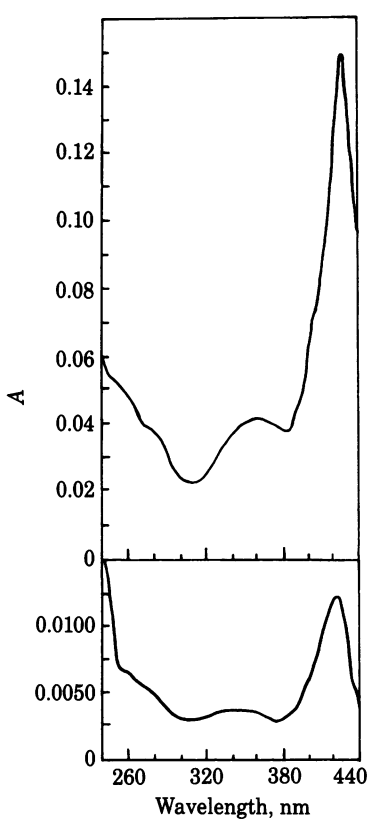

FIG. 3. UV–visible absorbance spectra of bisoxindolylalanine^{7,14}-Mb. (*Upper*) Purified cyanoferribisoxindolylalanine^{7,14}-Mb in NaCl/P_i containing 1 mM KCN (pH 7.4). (*Lower*) Cyanoferribisoxindolylalanine^{7,14}-Mb bound by clone 3.4 antibody and released from the monoclonal column by elution with 0.1 M NH_4OAc/1 mM KCN, pH 10.5.

As a result of two complete semisynthetic efforts, the yield of reconstructed Mb represented by spectrum E in Fig. 2 averaged 220 μg (13 nmol). The maximum theoretical yield of 4%, a function of the combined yields of the major synthetic steps shown in Table 2, does not include losses due to racemization and handling, nor to the complicating presence of multiple Mb fragments during the reintroduction of heme to the reconstructed globin sequence (23). The dilute solutions encountered in the latter stages of the synthesis incurred further losses and prevented collection of additional data.

DISCUSSION

By sequentially attaching synthetic peptides to a large natural fragment of Mb, the original sequence was regenerated and reassociated with heme to produce a semisynthetic Mb that assumes a conformation closely resembling the native molecule.

Table 2. Yields of the major semisynthetic reactions*

Reactions	Yield, %
Myoglobin	100
27% ↓ acetimidation	
N^{α},N^{ε}_{19}-Acetimidylmyoglobin	27
99% ↓ Heme removal	
N^{α},N^{ε}_{19}-Acetimidylapomyoglobin	27
85% ↓ BNPS-skatole cleavage	
90% ↓ 2-mercaptoethanol reduction	
N^{ε}_{19}-Acetimidyl-15–153 fragment	20
97% ↓ NPS-Trp-OSu	
N^{α}-NPS-N^{ε}_{19}-acetimidyl-14–153 fragment	20
99% ↓ carbamylation	
97% ↓ 2-mercaptoethanol deprotection	
N^{ε}_{19}-Acetimidyl-14–153 fragment	19
43% ↓ 6–13-OSu peptide	
N^{α}-Boc-N^{ε}_{19}-acetimidyl-6–153 fragment	8
99% ↓ carbamylation	
99% ↓ CF_3COOH deprotection	
N^{ε}_{19}-Acetimidyl-6–153 fragment	8
63% ↓ 1–5-OSu peptide	
N^{α}-Boc-N^{ε}_{19}-acetimidyl-1–153 fragment	5
99% ↓ CF_3COOH deprotection	
84% ↓ heme replacement	
N^{ε}_{19}-Acetimidylmyoglobin	4

* Percentages on the left denote the completeness of the specified reaction; percentages on the right denote the cumulative yield of desired product.

The strong Soret absorbance of the reconstructed Mb indicates correct positioning of the heme moiety within the hydrophobic globin heme pocket, and the envelope of the spectrum in the region of 280 nm and below exhibits the undisturbed structure of the reincorporated tryptophans. The high spectral ratio A_{423}/A_{280} (Fig. 2, spectrum E), nearly identical to natural N^{ϵ}_{19}-acetimidylmyoglobin under the same conditions (Fig. 2, spectrum F), demonstrates the close tertiary structural similarity of the semisynthetic Mb with the native molecule (2).

Further testimony to the correct placement of specific residues in space comes from the recognition and selective elution of the semisynthetic Mb from the monoclonal antibody column. The specificity of this antibody is directed toward the interaction between the side chains of glutamic acid at position 4 and lysine at position 79 of the sperm whale Mb sequence; myoglobins from species in which the carboxylate group is displaced 1.4 Å by substitution of an aspartic acid at position 4 are bound with an affinity 1/1,000 that of those with glutamic acid at that site (6) and, in fact, are not retained by the affinity chromatographic conditions used to purify the semisynthetic preparation. The recognition by this monoclonal antibody and its high affinity for the semisynthetic Mb argues that the reconstructed NH_2-terminal portion of the molecule has assumed the correct α-helical conformation and is precisely oriented to allow juxtaposition of the Glu^4-γ-COO^- with the ϵ-NH_3^+ of Lys^{79}. The selective retention of the semisynthetic Mb by the monoclonal antibody, under conditions in which Mb with oxidized tryptophan is released, further establishes that the three-dimensional structure of the semisynthetic Mb does not deviate significantly from the native structure (24).

The development of this semisynthesis procedure allows investigation of a broad range of residues within the Mb A-helix, spanning positions 3–18 of the sequence. Examination of the electrostatic stabilization of the molecule conferred by charge-pair interactions indicates that 4 of these 11 essential structural features have one partner resident in the A-helix (25). One of these charge pairs is primarily responsible for the weakened anchoring of the A-helix to other parts of the molecule in the acid-unfolding process prior to denaturation (26). The contribution of other charge pairs in maintaining the structural integrity of the molecule during the unfolding process now may be assessed by isotopic enrichment or selective amino acid substitutions made possible by this technique of semisynthesis. Further questions regarding the sites of antigenic determinants recognized by monoclonal antibodies to Mb (6, 27) and the nature of the high-affinity complementarity between the two globular proteins may be approached by sequence alteration in semisynthesis.

The techniques engendered by this synthesis extend the fragment condensation methodology to include large, minimally protected protein fragments soluble only in aqueous systems and eliminate the need for ineffective, loss-incurring intermediate purifications. The methods may find general application in the semisynthesis of other proteins neither aided by stabilizing intramolecular disulfide bonds nor capable of noncovalent interactions between cleavage fragments.

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