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FINAL PROGRESS REPORT

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Title: Design & Synthesis of Oxygen-Binding Heme Peptides

Principal Investigator: M. Zouhair Atassi

Institution: Department of Biochemistry
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

Telephone of Principal Investigator: (713) 798-6050

FAX No. of Principal Investigator: (713) 796-8040

Signature of Principal Investigator: M. Zouhair Atassi

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A. FINAL TECHNICAL REPORT

SUMMARY OF PROGRESS REPORT

The goal of this work was to design and synthesize peptides that will form functional complexes with heme and thus have physiologically useful oxygen-binding properties. Several peptides which will mimic the natural environment of the heme group in myoglobin (Mb) and in the α- and β-subunits of hemoglobin (Hb) were proposed. The heme cavity of Mb and the α- and β-chains would be mimicked by the concept of surface-simulation synthesis into three peptides that incorporate the essential contact residues with apprcpriate spacing and directionality. Control peptides would also be made that have all the essential contact residues, but in a random order. Other designs were based on the fact that the heme group in the aforementioned proteins is sandwiched between helices E and F. Thus, one set of peptides would correspond to the E-F helical segment. In three other sets of peptides, parts of the E-F segment which form hair-pin-loops that fold away from the heme cavity, would be bypassed by appropriate spatial bridging. All these peptides were synthesized, purified and characterized. The free peptides, except for those peptides based on the surface-simulation design, were able to bind the heme group and form stable peptide-heme complexes. The binding of heme to peptides was studied by several methods and the relative affinities of the peptides to the heme were compared. Also, the changes in the conformation of the peptides as a result of heme binding were studied by circular dichroism.

In the second phase of the work, the oxygen-binding properties of all heme complexes were studied in detail to determine their utility under physiological conditions.

DETAILED PROGRESS REPORT

Design of the Peptides

The three-dimensional structures of Mb and of Hb are known in detail. Each of the Hb subunits and Mb carry a heme group which is responsible for oxygen-carrying properties. the physiological and biochemical differences between Mb and Hb in their oxygen-binding properties were described in our previous proposal to ONR. We will briefly repeat here only the main elements of peptide design.

I. Synthetic peptides corresponding to the E-F helical segments.

In Mb and the two Hb subunits, the heme group inserts in the space effected by the bend between the E and F helices. the penta and hexa coordinate ligands of the heme iron are occupied by histidine F8 (the 8th position in helix F) and, via a water bridge, by histidine E7. A peptide corresponding to the segment E-F will carry these two essential histidine residues as well as several of the other contacts. Such a segment will therefore be expected to form complexes with the heme group. The following three synthetic strategies were adopted:

(1) Synthesis of the entire segment E7 to F9. Three peptides were synthesized corresponding to the following sequences E7-to-F9 in Mb and the two Hb subunits. Since position 93 in the β-chain is cysteine it was also replaced by Ala in synthesis to avoid dimerisation. The structures of these peptides are:

Segment E7 to F9

Mb	64---94	31 residues
α-Chain	58---88	31 residues
β-Chain	63---93	31 residues

	64	70	75	80	85	90	94
Mb:	H.G.V.T.V.L.T.A.L.G.A.I.L.K.K.K.G.H.H.E.A.E.L.K.P.L.A.Q.S.H.A.						
	60	65	70	75	80	85	88
α-Chain:	H.G.K.K.V.A.D.A.L.T.A.L.V.A.H.V.D.D.M.P.N.A.L.S.A.L.S.D.L.H.A.						
	63	70	75	80	85	90	93
β-Chain (a):	H.G.K.K.V.L.G.A.F.S.D.G.L.A.H.L.D.N.L.K.G.T.F.A.T.L.S.G.L.H.C.						
(b):	----------A						

(2) Bypassing of the EF bend. In the 3-D structure of Mb and Hb, the EF bend is far from the heme group and does not make any contribution to the heme cavity. As it approaches the bend, the polypeptide chain goes out of the environment of the heme group at residue E14. Then the chain folds back so that residue F1 comes close (6.7Å) to residue E14.

(a) We made a peptide corresponding to the segment E4 to E14, then bridged the gap E14 to F1 by a glycine spacer and then continued the synthesis from F1 through FG5 as follows:

(E4---E14) ---6.7Å--- (F1---FG5)

Mb:	(61---71)	Gly (86---99)	26 residues
α-Chain:	(55---65)	Gly (80---93)	26 residues
β-Chain:	(60---70)	Gly (85---98)	26 residues

	61		65				71	86		90			95			99									
Mb:	L.	K.	K.	H.	G.	V.	T.	V.	L.	T.	A.	(G).	L.	K.	P.	L.	A.	Q.	S.	H.	A.	T.	K.	H.	K.	I.
	55						65	80			85				90		93									
α-Chain:	V.	K.	K.	H.	G.	K.	K.	V.	A.	D.	A.	(G).	L.	S.	A.	L.	S.	D.	L.	H.	A.	H.	K.	L.	R.	V.
	60						70	85			90				95		98									
β-Chain (a):	V.	K.	A.	H.	G.	K.	K.	V.	L.	G.	A.	(G).	F.	A.	T.	L.	S.	E.	L.	H.	C.	D.	K.	L.	H.	V.
(b):	----------																						A			

(b) In another set, we bridged the gap (6.8Å) between F1 and E18 (instead of E14):

(E4---E18) ---6.8Å--- (F1---FG5)

Mb:	(61---75)	Gly (86---99)	30 residues
α-Chain:	(55---69)	Gly (80---93)	30 residues
β-Chain:	(60---74)	Gly (85---98)	30 residues

61 65 70 75 86 99

Mb: L.K.K.H.G.V.T.V.L.T.A.L.G.A.I.(G).L.K.P.L.A.Q.S.H.A.T.K.H.K.I.

55 60 65 69 80 85 90 93

α-Chain: V.K.G.H.G.K.K.V.A.D.A.L.T.N.A.(G).L.S.A.L.S.D.L.H.A.H.K.L.R.V.

60 65 70 74 85 90 95 98

β-Chain (a): V.K.A.H.G.K.K.V.L.G.A.F.S.D.G.(G).F.A.T.L.S.E.L.H.C.D.K.L.H.V.

(b): ---A--------------

(c) The third set of peptides were designed by taking into account the fact that the five residues F9 through FG4 are also away from the sphere of influence of the heme group. Furthermore, they make a turn so that residue F8 comes to within 4.9Å from residue FG5. We, therefore, synthesized a third set of peptides in which F8 is directly linked to FG5 via a spacer as follows:

(E4---E14) ---6.7Å--- (F1---F8) ---4.9Å--- (FG5)

Mb:	(61---71) Gly (86---93)---(99)	21 residues
α-Chain:	(55---65) Gly (80---87)---(93)	21 residues
β-Chain:	(60---70) Gly (85---92)---(98)	21 residues

61 71 86 93 99

Mb: L.K.K.H.G.V.T.V.L.T.A.(G).L.K.P.L.A.Q.S.H--I

55 65 80 87 93

α-Chain: V.K.K.H.G.K.K.V.A.D.A.(G).L.S.A.L.S.D.L.H--V

60 70 85 90 98

β-Chain: V.K.A.H.G.K.K.V.L.G.A.(G).F.A.T.L.S.E.L.H--V

II. Surface-simulation synthesis of the heme cavity.

The amino acids constituting the contact residues in the heme cavity of Mb and the two Hb subunits are summarized in Fig. 1. Also, given in Fig. 1 are the spatial distances (C^{α}-to-C^{α} in Å) separating neighboring contact residues. From the x-ray coordinates and taking into account the participation of the

residues listed in Fig. 1, peptides were designed by surface-simulation synthesis to mimic the heme cavity in Mb, and the two Hb subunits. It should be emphasized that none of these peptides exists in the respective protein, but rather mimics the topographic arrangement of the residues in each heme cavity. The elements of the designs which are based on spatial and distance considerations, are shown in Fig. 1. Also, control peptides were prepared which incorporated all the contact residues in a random fashion.

Purification and characterization of the peptides

All the peptides described in Sections I and II have been synthesized, purified as described in the proposal and characterized. The amino acid compositions of the synthetic pure peptides are summarized in Tables 1-6.

Preparation and characterization of peptide-heme complexes

To study the ability of these peptides to bind the heme group we have devised a spectral titration method as described in Figure 2. When a peptide formed a complex with heme, then addition of heme to the peptide resulted in increase in absorbance at 420 nm relative to the absorbance of a buffer solution (or an unrelated peptide or a randomized sequence peptide that do not bind heme) that received similar aliquots of heme. It was found that each of the peptides in Section I was, in fact, able to bind heme with a 1-to-1 stoichiometry. The peptide-heme complexes were stable. For example, the heme could not be removed by gel filtration on sephadex. The complexes gave absorption spectra typical of heme proteins. An example is given in Figure 3 which shows the spectra of peptide β63-93 and its complex.

Another method was also devised to study peptide-heme binding. A solid-phase adsorbent of the heme was synthesized and was used in radiometric titration assays to bind ^{125}-I-labeled peptide. The assay was done in two ways. Constant amounts of labelled peptide were reacted with increasing amounts of adsorbent (see Figure 4, for example). Also, fixed amounts of adsorbent were titrated with increasing amounts of ^{125}I-labeled peptide (e.g., see Figure 5). These studies showed that each of the synthetic peptides was, in fact, able to bind to heme and did not bind to adsorbents of unrelated controls. The specificity of the binding of each peptide was confirmed by inhibition studies (Figures 6-10). The inhibition studies will also enable comparisons of the relative affinities of these peptides for the heme group.

The changes in peptide conformation a result of binding to the heme group were measured by circular dichroism (CD) studies on the free peptides and their heme complexes. Examples of the results are summarized in Figures 11-13. In each case, CD spectra of the peptide-heme complex showed higher negative ellipticity than exhibited by spectra of the free peptide. This indicated that, on binding to heme, a peptide assumes a more folded structure, perhaps as a result of wrapping around the heme group. It is noteworthy that the CD spectra of control peptides in which these sequences were randomized exhibited no changes whatsoever upon mixing of the peptide with heme (Figure 14), clearly indicating that the conformational changes observed in the heme-peptide complexes (Figures 11-13) were specific.

It should be noted that the surface-simulation peptides described in Section II and Figure 1 were completely insoluble in aqueous solvents and could not, therefore, form complexes with heme. The peptides were soluble in some organic solvents such as dimethylformadide and acetonitrile. However, their solution in these organic solvents were unable to form heme complexes. Conjugates of the peptides to soluble carriers (e.g. serum albumin, lysozyme or polylysine) were also insoluble and could not, therefore, form heme complexes. As a result of this physical property of the surface-simulation peptides, their utility could not be assessed.

Oxygen-Binding Properties of Peptide-Heme Complexes

The peptide-heme complexes were reduced by sodium dithionite and their continuous spectra determined in the region 400-700 nm. At the same time, myoglobin and hemoglobin solutions were treated in the same manner and their spectra determined. This served to confirm that the heme group was in fact reduced and that the two proteins were able to bind oxygen under there conditions since they showed typical oxyhemoglobin and oxymyoglobin spectra (Figures 15 and 16). On the other hand, the spectral studies indicated that the peptide-heme complexes did not give the typical oxygenation spectra (examples in Figures 17 and 18) and indicated that these compounds either did not bind oxygen or did so very poorly.

The oxygen-binding properties of the peptide-heme complexes were further studied in detail using a Hemox-Analyzer (we are grateful to Dr. Fran Ligler, Naval Research Laboratory, for making the instrument available for this work). The results, which are summarized in Figure 19, show that myoglobin and hemoglobin gave the expected oxygen-saturation curves. Myoglobin exhibited a hyperbolic oxygen-saturation curve, whereas hemoglobin showed a Bohr effect evidenced by an S-shaped saturation curve. On the other hand heme-peptide complexes showed little or no oxygen-binding capacity. Figure 19 also shows the oxygen-saturation curves for six of the peptide-heme complexes. The saturation curves for the remaining peptide complexes were essentially similar, both quantitatively and qualitatively to those given in Figure 19 and are therefore not shown in order to avoid overcrowding the diagram.

In view of their poor ability to bind oxygen, it was concluded that the peptide-heme complexes will not be useful for blood-transfusion purposes.

APPENDIX

Table 1.

Amino acid sequences of the segment (E7 - F9) of Mb and the α and β chains of Hb.

Mb:	(64-94):	H-G-V-T-V-L-T-A-L-G-A-I-L-K-K-K-G-H-H-E-A-E-L-K-P-L-A-Q-S-H-A
α chain:	(58-88):	H-G-K-K-V-A-D-A-L-T-A-L-V-A-H-V-D-D-M-P-N-A-L-S-A-L-S-D-L-H-A
β chain:	(63-93):(A)	H-G-K-K-V-L-G-A-F-S-D-G-L-A-H-L-D-N-L-K-G-T-F-A-T-L-S-E-L-H-C
	(B)	____A

Amino acid composition of the synthetic peptides:

Peptide:	Mb (64-94)	α chain (58-88)	β chain (63-93) A	β chain (63-93) B
Asp/Asn	-	5.11(5)	2.98(3)	3.01(3)
Thr	2.03(2)	0.97(1)	2.19(2)	2.20(2)
Ser	0.98(1)	1.94(2)	2.27(2)	1.79(2)
Glu/Gln	3.14(3)	-	0.98(1)	1.12(1)
Pro	1.11(1)	0.99(1)	-	-
Gly	2.89(3)	0.89(1)	3.79(4)	4.33(4)
Ala	4.98(5)	7.21(7)	3.03(3)	3.72(4)
Cys	-	-	1.09(1)	-
Val	1.84(2)	3.01(3)	0.97(1)	1.02(1)
Met	-	1.04(1)	-	-
Ile	0.98(1)	-	-	-
Leu	5.24(5)	5.16(5)	6.09(6)	5.82(6)
Phe	-	-	2.13(2)	2.04(2)
His	2.26(4)	2.11(3)	2.59(3)	1.98(3)
Lys	4.03(4)	1.85(2)	2.69(3)	2.82(3)

Table 2.

Amino acid sequence of the peptides: (E4 - E14) G (F1-FG5) of Mb and the α and β chains of Hb

Mb: (61-71) G (86-99): L-K-K-H-G-V-T-V-L-T-A-(G)-L-K-P-L-A-Q-S-H-A-T-K-H-K-I

α chain: (55-65) G (80-93): V-K-G-H-G-K-K-V-A-D-A-(G)-L-S-A-L-S-D-L-H-A-H-K-L-R-V

β chain: (60-70) G (85-98): (A) V-K-A-H-G-K-K-V-L-G-A-(G)-F-A-T-L-S-E-L-H-C-D-K-L-H-V
(B) _____ A

Amino acid composition of the synthetic peptides:

Peptide:	Mb (61-71)G(86-99)	α chain (55-65)G(80-93)	β chain (60-70)G(85-98) A	β chain (60-70)G(85-98) B
Asp/Asn	-	2.20(2)	1.10(1)	1.17(1)
Thr	3.07(3)	-	1.00(1)	1.05(1)
Ser	0.94(1)	1.82(2)	1.01.(1)	0.93(1)
Glu/Gln	1.04(1)	-	1.08(1)	1.04(1)
Pro	1.02(1)	-	-	-
Gly	2.2(2)	2.44(3)	3.22(3)	3.04(3)
Ala	3.33(3)	3.93(4)	3.06(3)	3.12(3)
Cys	-	-	0.86(1)	-
Val	2.04(2)	3.02(3)	3.01(3)	3.90(4)
Ile	1.09(1)	-	-	-
Leu	3.92(4)	4.05.(4)	4.12(4)	3.88(4)
Phe	-	-	1.04(1)	1.02(1)
His	2.12(3)	1.60(3)	2.11(3)	2.06(3)
Lys	5.17(5)	3.87(4)	4.02(4)	4.04(4)
Arg	-	0.89(1)	-	-

Table 3

Amino acid sequence of the peptides (E4-E18)G(F1-FG5) of Mb and the α and β chains of Hb.

Mb: (61-75) G (86-99): L-K-K-H-G-V-T-V-L-T-A-L-G-A-I-(G)-L-K-P-L-A-Q-S-H-A-T-K-H-K-I

α chain: (55-69)G(80-93) V-K-G-H-G-K-K-V-A-D-A-L-T-N-A-(G)-L-S-A-L-S-D-L-H-A-H-K-L-R-V

β chain: (60-74)G(85-98)(A): V-K-A-H-G-K-K-V-L-G-A-F-S-D-G-(G)-F-A-T-L-S-E-L-H-C-D-K-L-H-V
(B) ——————————————————————————————————————— A

Amino acid composition of the synthetic peptides:

Peptide:	Mb. (61-71)G(86-99)	α chain (55-69)G(80-93)	β chain (60-74)G(85-98) A	β chain (60-74)G(85-98) B
Asp/Asn	-	3.20(3)	1.97(2)	2.22(2)
Thr	2.86 (3)	1.14(1)	0.99(1)	0.97(1)
Ser	1.02(1)	2.02(2)	1.99(2)	2.04(2)
Glu/Gln	1.17(1)	-	1.01(1)	1.04(1)
Pro	1.08(1)	-	-	-
Gly	3.20(3)	3.21(3)	4.12(4)	4.26(4)
Ala	4.08(4)	5.00(5)	3.27(3)	4.15(4)
Cys	-	-	0.99(1)	-
Val	1.92(2)	2.58(3)	2.97(3)	3.04(3)
Ile	1.73(2)	-	-	-
Leu	5.04(5)	5.38(5)	3.77(4)	4.02(4)
Phe	-	-	2.06(2)	2.07(2)
His	2.11(3)	1.72(3)	2.14(3)	1.96(3)
Lys	4.95(5)	4.11(4)	3.76(4)	3.91(4)
Arg	-	0.75(1)	-	-

Table 4

Amino acid sequence of the peptides (E4-E14) G (F1-F8) (FG5) of Mb and the β chain of Hb.

Mb: (61-71) G (86-93)(99): L-K-K-H-G-V-T-V-L-T-A-(G)-L-K-F-L-A-Q-S-H-I

β chain: (60-70) G (85-92)(98): V-K-A-H-G-K-K-V-L-G-A-(G)-F-A-T-L-S-E-L-H-V

Amino acid composition of the synthetic peptides:

Peptide:	Mb:(61-71)G(86-93)(99)	β chain (60-70)G(85-92)(98)
Thr:	2.11(2)	1.04(1)
Ser	.98(1)	0.99(1)
Glu/Gln	1.08(1)	1.10(1)
Pro	1.11(1)	-
Gly	2.09(2)	3.04(3)
Ala	2.14(2)	3.05(3)
Val	1.97(2)	3.01(3)
Ile	1.03(1)	-
Leu	4.09(4)	3.05(3)
Phe	-	1.00(1)
His	1.56(2)	1.43(2)
Lys	2.96(3)	2.87(3)

Table 5. Amino Acid Sequence of Randomized Peptides:

The following peptide corresponds to a randomized sequence of the region: Mb (61-71) G (86-93)-(99): H.F.K.S.F.H.S.F.S.V.S.G.E.T.V.F.E.V.T.E.A

The following peptide corresponds to a randomized sequence of the structure: Mb(61-71) G (85-99): K.A.H.E.V.P.S.I.K.S.A.S.P.K.S.L.T.I.H.E.P.T.S.K.S.A.

The following peptide corresponds to a randomized sequence of the structure: Mb (64-94): I.P.Q.E.K.S.V.I.T.P.G.E.P.I.P.H.E.P.E.V.A.K.S.L.L.H.Q.I.P.S.A.

Amino Acid Composition of the Synthetic Peptides:

Peptide	Randomized Mb(61-71)G(86-93)-(99)	Randomized Mb(61-71)G(85-99)	Randomized Mb(64-94)
Thr	2.05(2)	1.8(2)	1.02(1)
Ser	3.68(4)	6.04(6)	2.88(3)
Glu/Gln	3.25(3)	1.86(2)	6.12(6)
Pro	-	2.74(3)	6.08(6)
Gly	1.06(1)	-	0.92(1)
Ala	0.98(1)	2.73(3)	2.03(2)
Val	3.08(3)	1.12(1)	1.94(2)
Ile	-	1.1(2)	3.88(4)
Leu	-	0.88(1)	2.10(2)
Phe	3.89(4)	-	-
His	1.90(2)	1.62(2)	1.74(2)
Lys	1.03(1)	3.93(4)	2.05(2)

Table 6

Structure of the surface simulation peptides designed to mimic the heme cavity of Mb and the two Hb subunits.

SSP-Mb: L-S-H-I-P-(G)-Y-L-I-F-(G)-L-L-F-R-F-(G)-H-T-V-A-L

SSP-α chain: L-L-H-V-P-(G)-N-F-L-L-(G)-L-L-F-H-F-(G)-H-K-V-A-L

SSP-β chain: L-L-H-V-P-(G)-N-F-L-L-(G)-L-L-F-S-F-H-F-(G)-H-K-V-A-F

Amino acid composition of the synthetic peptides

Peptide:	SSP-Mb	SSP-α chain	SSP-β chain
Asp/Asn	-	1.12(1)	1.12(1)
Thr	1.17(1)	-	-
Ser	0.88(1)	-	0.85(1)
Pro	1.08(1)	0.97(1)	1.14(1)
Gly	3.02(3)	3.14(3)	3.07(3)
Ala	.98(1)	1.04(1)	1.05(1)
Val	1.07(1)	2.02(2)	2.06(2)
Ile	1.96(2)	-	-
Leu	5.01(5)	6.79(7)	5.82(6)
Phe	3.06(3)	2.73(3)	4.95(5)
Tyr	0.85(1)	-	-
His	1.69(2)	2.96(3)	1.75(2)
Lys	-	1.09(1)	1.07(1)
Arg	0.82(1)	-	-

Surface simulation synthesis of the heme cavity of Mb and the two Hb subunits

(A) Contact residues (Helix position and distances)	(B) Mb Residue No.	peptide	(C) α-Chain Residue No.	peptide	(D) β-Chain Residue No.	peptide
F4	Leu-89	Leu	Leu-88	Leu	Leu-88	Leu
5.0A		|		|	|
F7	Ser-92	Ser	Leu-86	Leu	Leu-91	Leu
F8	His-93	His	His-87	His	His-92	His
4.8A		|		|	|
FG5	Ile-99	Ile	Val-93	Val	Val-98	Val
(G1 or G2)	(G1,Pro-100)	Pro	(G2,Pro-100)	Pro	(G2,Pro-95)	Pro
5.7A		Gly		Gly		Gly
G4	Tyr-10	Tyr	Asn-97	Asn	Asn-102	Asn
G5	Leu-10	Leu	Phe-98	Phe	Phe-103	Phe
4.5A		|		|	|
G8	Ile-107	Ile	Leu-101	Leu	Leu-106	Leu
G7	Phe-106	Phe	Leu-100	Leu	Leu-105	Leu
		|		|	|
7.9A		Gly		Gly		Gly
B10	Leu-29	Leu	Leu-48	Leu	Leu-28	Leu
4.3A		|		|	|
CD7	Leu-49	Leu	Leu-48	Leu	Leu-48	Leu
4.9A		|		|	|
CD4	Phe-46	Phe	Phe-46	Phe	Phe-45	Phe
CD3	Arg-45	Arg	His-45	His	Ser-44	Ser
4.7A		|		|	|
CD1	Phe-43	Phe	Phe-43	Phe	Phe-42	Phe
		|		|	|
6.2A		Gly		Gly		Gly
E7	His-64	His	His-58	His	His-63	His
5.1A		|		|	|
E10	Thr-67	Thr	Lys-61	Lys	Lys-66	Lys
E11	Val-68	Val	Val-62	Val	Val-67	Val
5.1A		|		|	|
E14	Ala-71	Ala	Ala-65	Ala	Ala-70	Ala
E15	Leu-72	Leu	Leu-66	Leu	Phe-71	Phe

Figure 1. Design of the surface-simulation peptides mimicking the heme cavity in Mb and the α- and β-subunits of Hb. (A) This column identifies the contact residues in the heme cavity and the distances (from C^{α}-to-C^{α} in Å) separating appropriate residues. (B) The residues in Mb and the surface-simulation synthetic peptide designed to mimic the Mb cavity. (C) and (D) The residues and the two peptides designed to mimic the heme cavities in the α- and β-chains, respectively. The peptides are written with the N-terminus on the top and the C-terminus on the bottom. Residue in parentheses is not a contact residue but is instrumental in efficting a bend at the deep end of the cavity.

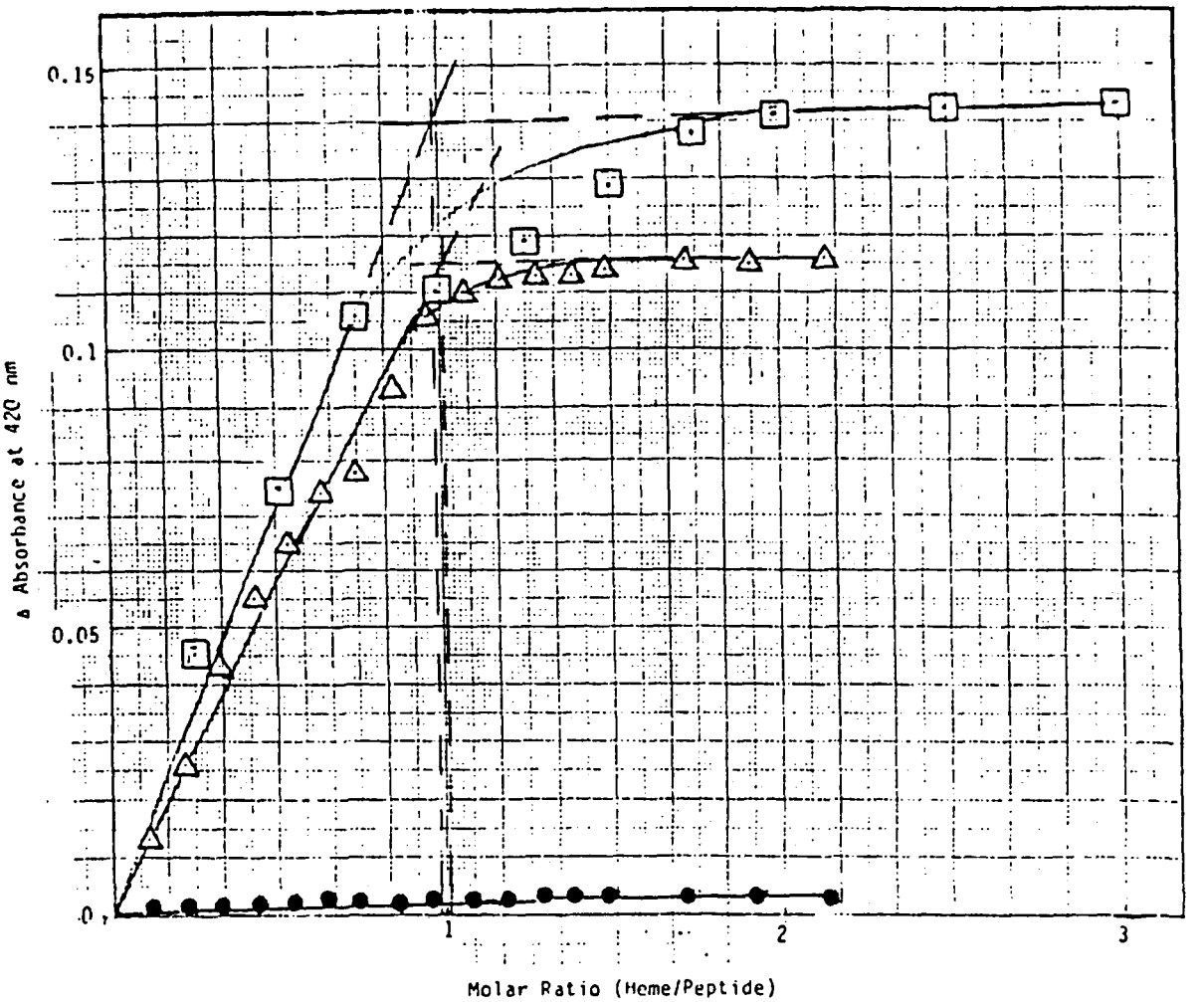

Figure 2. Spectral titration of peptide α58-88 (△), ApoMb (□), and nonsense peptide (●) with heme. Aliquots of heme solution (5 μg containing 1.887 x 10^{-3} μ mole) in 0.01M sodium phosphate buffer pH 7.2 were added to 1 ml of a solution of the peptide (17.711 x 10^{-3} μ mole) in 0.01M sodium phosphate buffer at pH 7.2 in a 1.5 ml cuvette with 1 cm light path. A similar aliquot of heme was added to a control cuvette containing 1 ml of buffer. The increase in the absorbance at 420 nm of the peptide solution relative to the buffer solution which was measured by zeroing the instrument with the latter is plotted here against the amount of heme added (in heme/peptide molar ratio) after correction for dilution. From the titration curve it can be calculated that one mole of peptide α 58-88 will bind 1.01 moles of heme. ApoMb (7.58 x 10^{-3} μ mole) was titrated by the same method as a control and was found to bind at equivalence, 0.99 moles of heme per mole of ApoMb. A peptide corresponding to a randomized sequence of Mb region 64-94, used also here as a control, did not bind heme. Controls of other unrelated proteins (e.g. hen lysozyme) gave titration curves that superimposed with the randomized peptide.

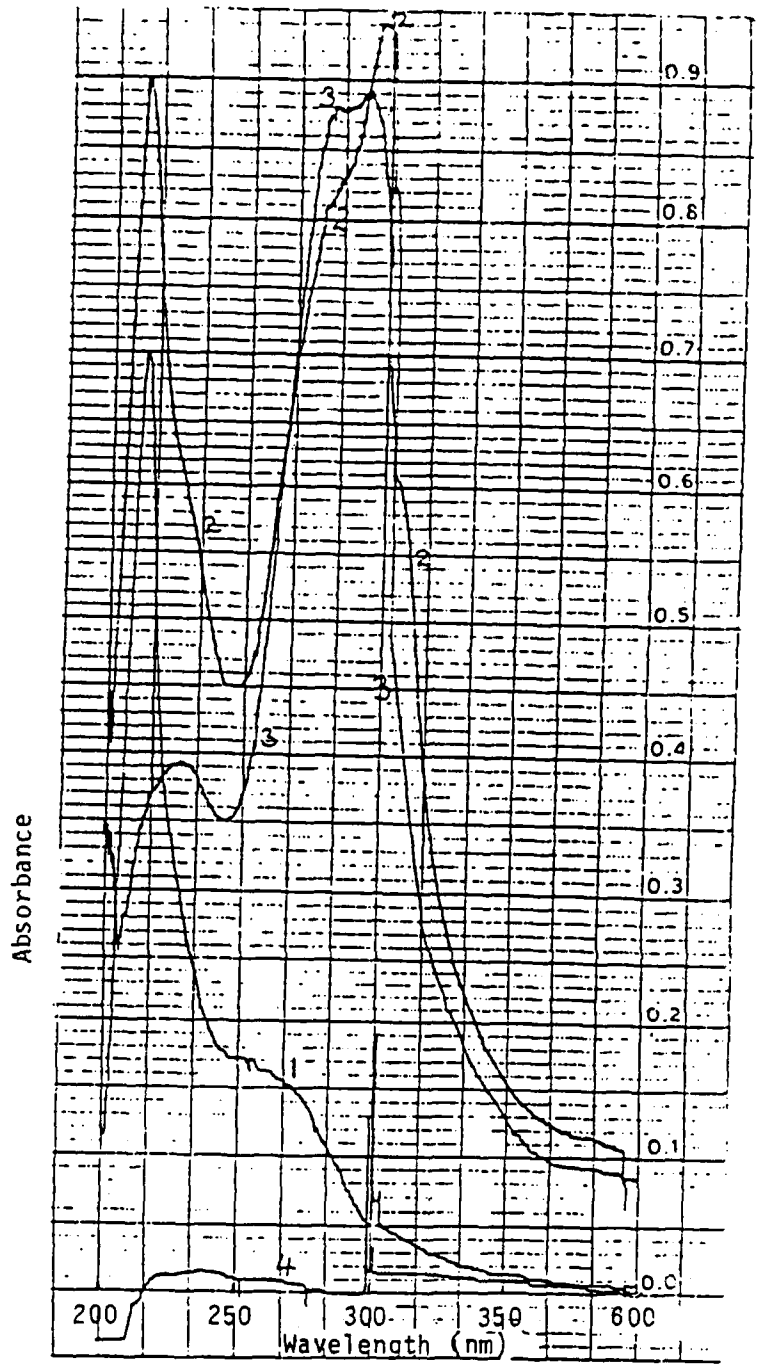

Figure 3. Absorption spectra of (1) peptide B 63-93, (2) heme complex of peptide B63-93, (3) ferriheme, and (4) buffer baseline. Spectra were determined in 0.01 M sodium phosphate buffer at pH 7.2. The heme peptide complex was prepared by mixing 130 µg of peptide with 22.5 µg of ferriheme followed by removal of excess heme on a column of Sephadex G15.

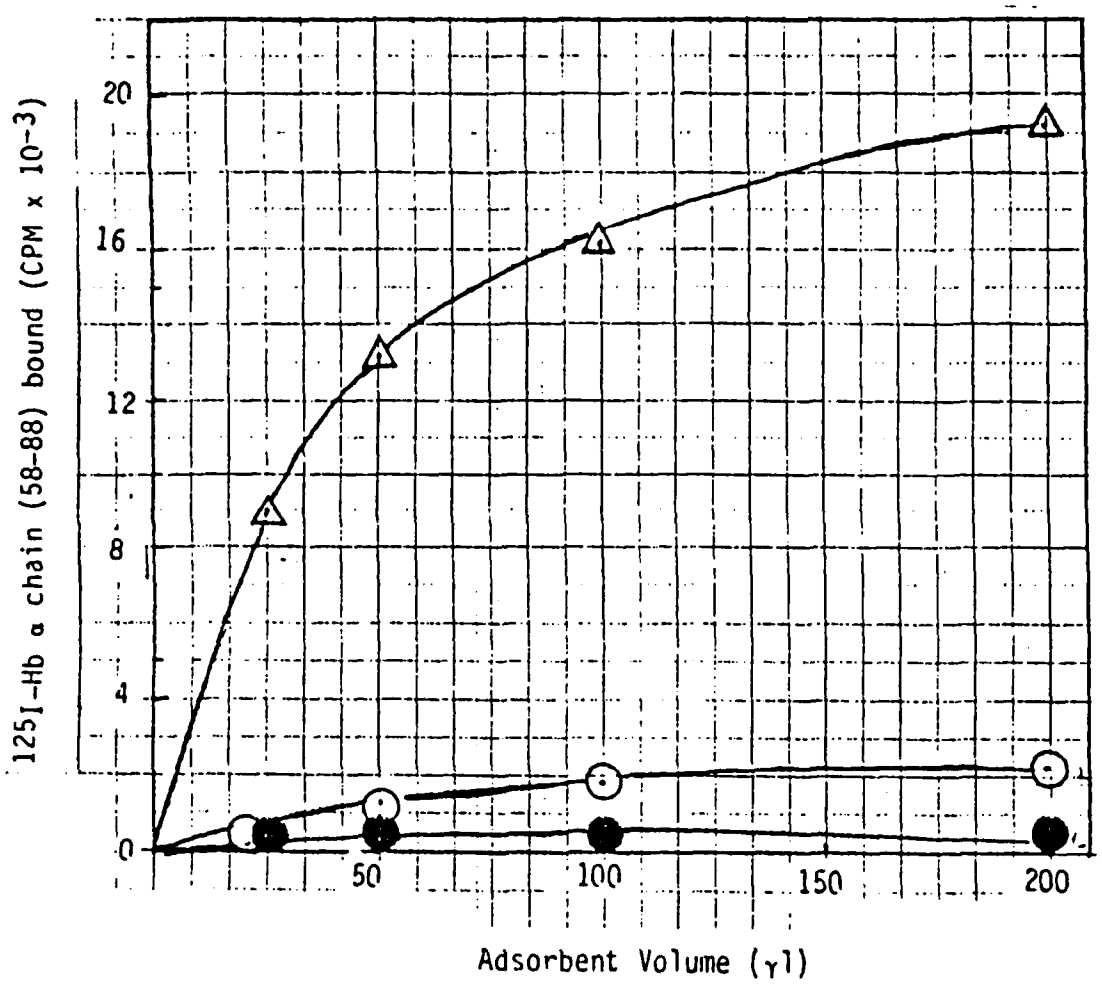

Figure 4. Radiometric titration of ^{125}I-labelled peptide α58-88 of Hb α chain with adsorbents of heme or unrelated proteins. Constant amounts of labelled peptide (1 x 10^5 cpm) were reacted with increasing amounts of adsorbent suspension (1:1 v/v) at 4°C for 16 hrs. Experiments were carried out in PBS containing 0.1 lysozyme in a reaction volume of 260 µl. After reaction, the adsorbent were washed on the centrifuge 5 times with PBS and then their radioactivity counted. (Δ) Binding of labelled peptide to heme; (o) binding to an adsorbent of goat IgG; (●) binding to an adsorbent of hen lysozyme.

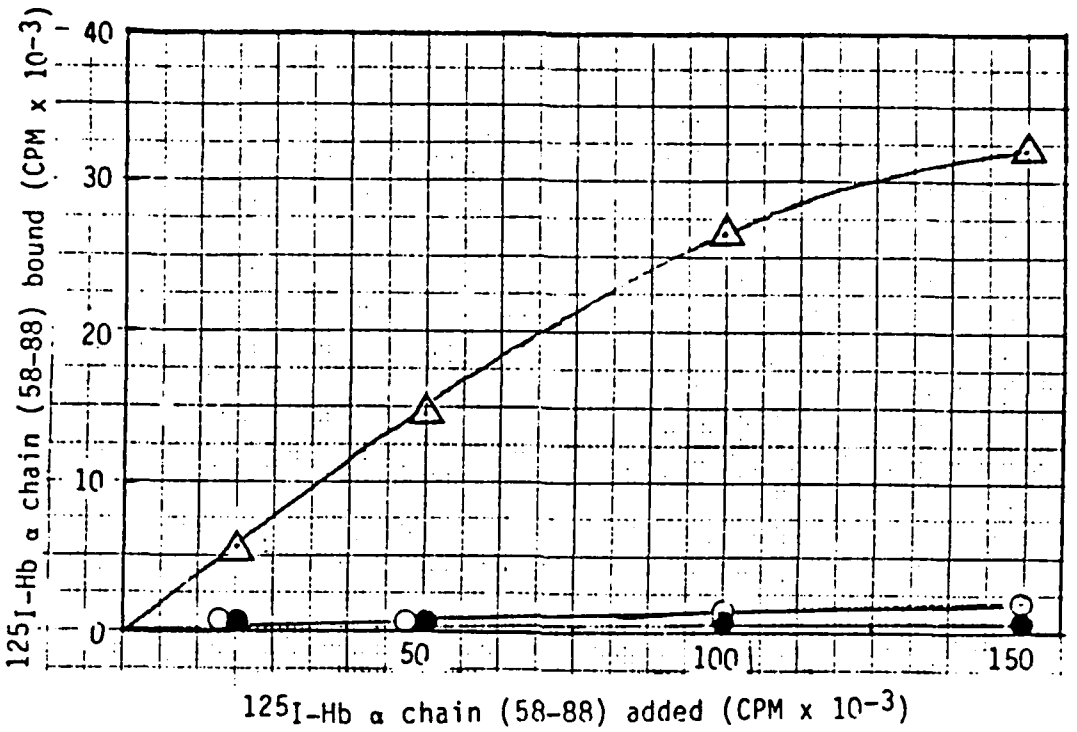

Figure 5. Titration of a fixed amount of adsorbents of heme or control proteins with increasing amounts of ^{125}I-labelled peptide α 58-88 of Hb α chain. Fixed amounts (5 µl) of adsorbent suspension (1:1, v/v) were titrated with increasing amounts of labelled peptide at 4°C for 16 hrs. Binding was done in PBS containing 0.1% lysozyme (reaction volume, 10 µl). After reaction, the tubes were washed on the centifuge 5 times with PBS and then counted. (Δ) Binding of labelled peptide to heme adsorbent; (o) binding to an adsorbent of goat IgG; (•) binding to an adsorbent of lysozyme.

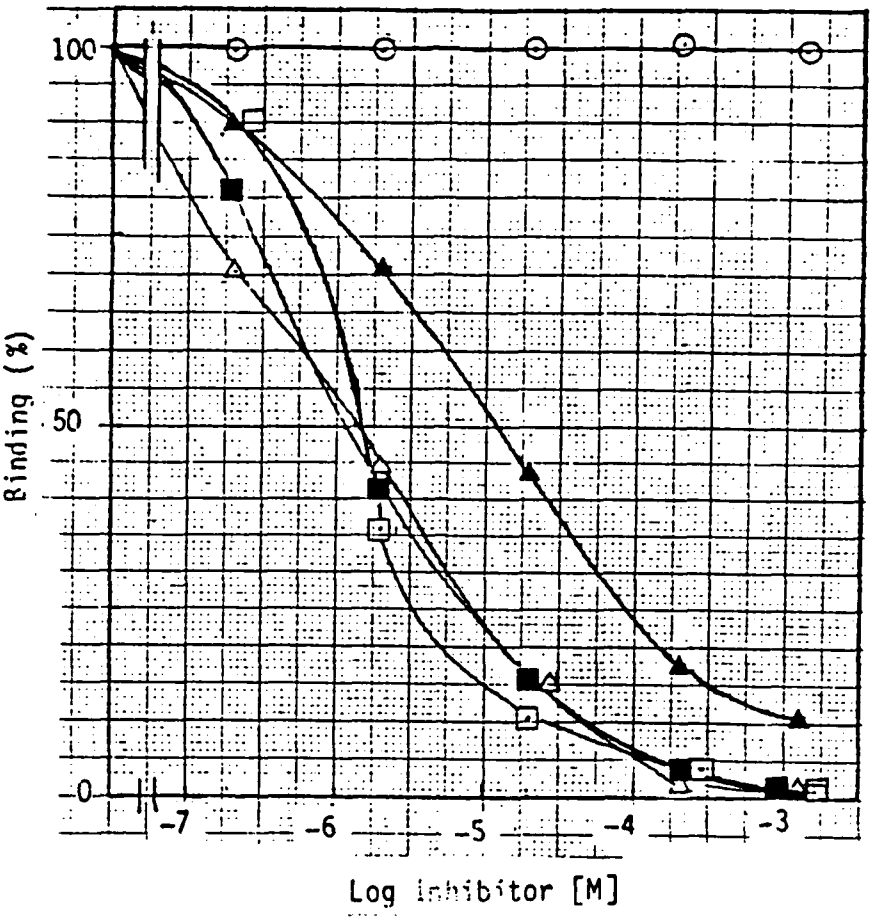

Figure 6. Inhibition of the binding of ^{125}I-labelled peptide α58-88 to heme adsorbent by unlabelled peptides (△) α58-88; (■) β63-93A; (□) β63-93B; (▲) Mb 64-94; and (o) a nonsense control peptide corresponding to a randomized sequence of Mb (64-94). Fixed amounts (5 µl) of heme adsorbent suspension (1:1, v/v) were mixed (3 hrs, room temperature) with increasing amounts of unlabelled peptide inhibitor. Labelled peptide α58-88 (1.5×10^5 cpm) was added to each tube and the tubes were mixed by gentle agitation for 16 hrs at 4°C afterwhich the adsorbents were washed on the centrifuge 5 times with PBS and then counted. Per cent inhibition is calculated relative to amount of label bound to uninhibited controls or controls to which unrelated peptides were added (mean = 29201 ± 901 cpm).

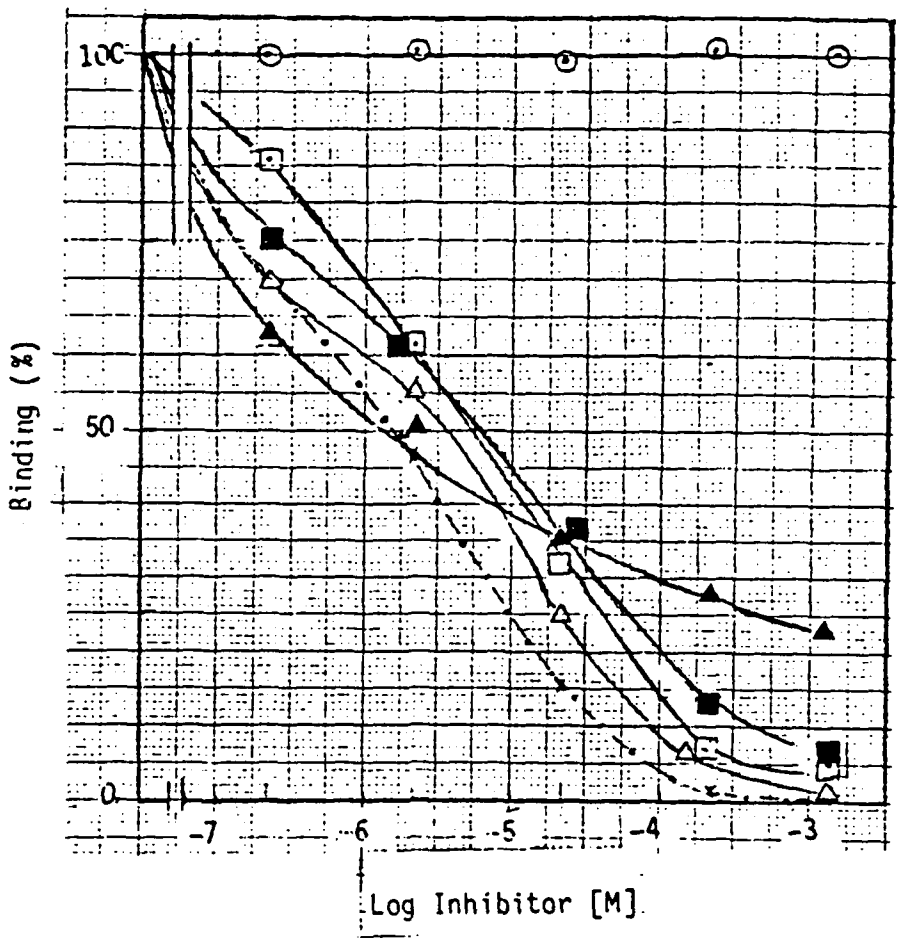

Figure 7. Inhibition of the binding of ^{125}I-labelled α58-88 to heme adsorbent by unlabelled peptides (▲) Mb (61-71) G (86-99); (△)α(55-65) G (80-93); (□)β(60-70) G (85-98)A; (■) β(60-70) G (85-98) B and (o) a nonsense peptide corresponding to randomized Mb(61-71) G (86-99). In addition, experiments were performed as described in Figure 5. Note that uninhibited controls bound 26670 ± 334 cpm.

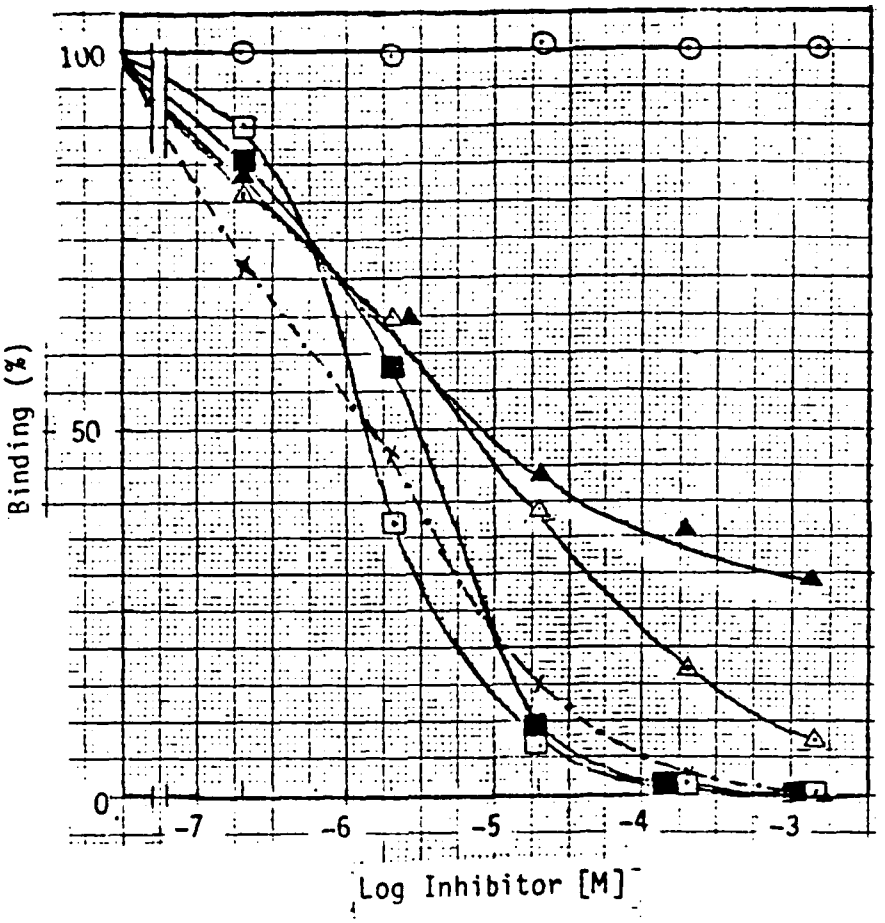

Figure 8. Inhibition of the binding of ^{125}I-labelled peptide α58-88 to heme adsorbent by unlabelled peptides (▲) Mb (61-75) G (86-99); (△) α(55-69) G (80-93); (□) β(60-74) G (85-98)A: (■) β (60-74) G (85-98) and (o) a nonsense peptide corresponding to a randomized sequence of Mb (64-94). The inhibition by unlabelled peptide α58-88 itself is shown (dashed line) for comparison. Inhibition experiments were done as described in Figure 5. Note that uninhibited controls bound 32607 ± 17 cpm.

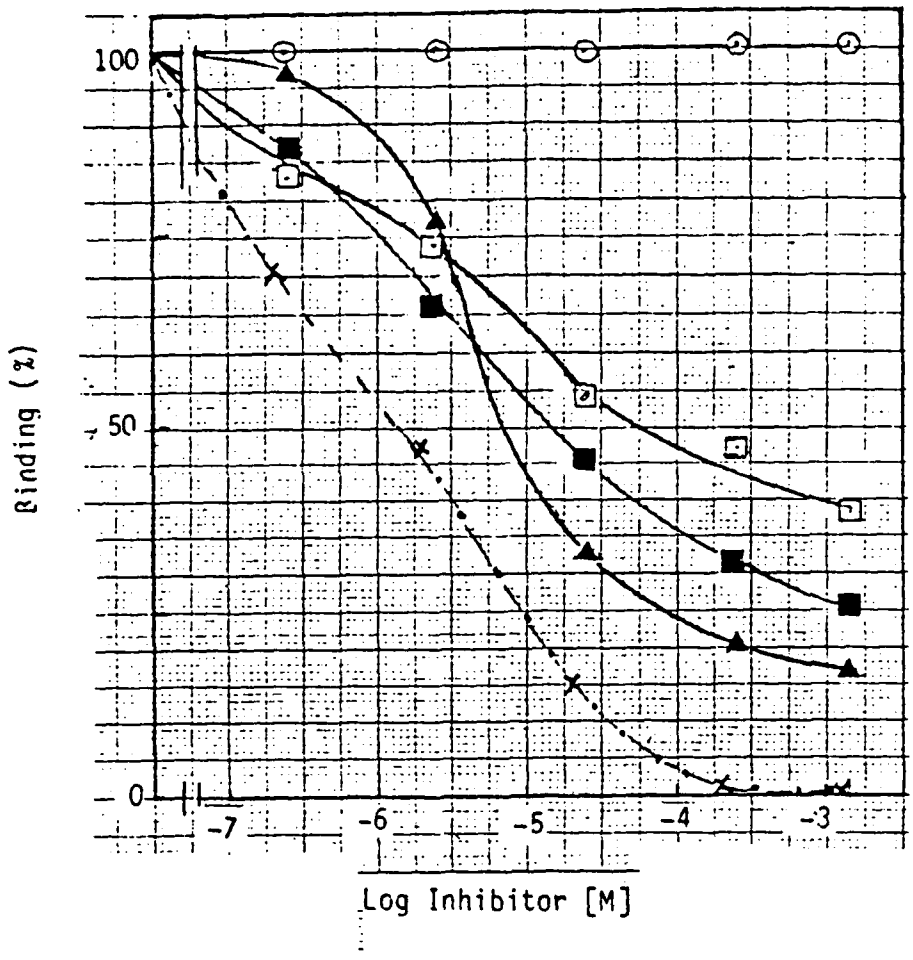

Figure 9. Inhibition of the binding of ^{125}I-labelled peptide α 58-88 to heme adsorbent by the unlabelled peptides (▲) Mb (61-71) G (86-93)-(99); (□) α (55-65) G (80-87)-(93) (■) β (60-70) G (85-92)-(98) and (o) a nonsense peptide control corresponding to a randomized sequence of Mb (61-71) G (86-93-99). The inhibition of unlabelled peptide α58-88 itself is shown (dashed line) for comparison. Note that uninhibited controls bound 34460 ± 840 cpm.

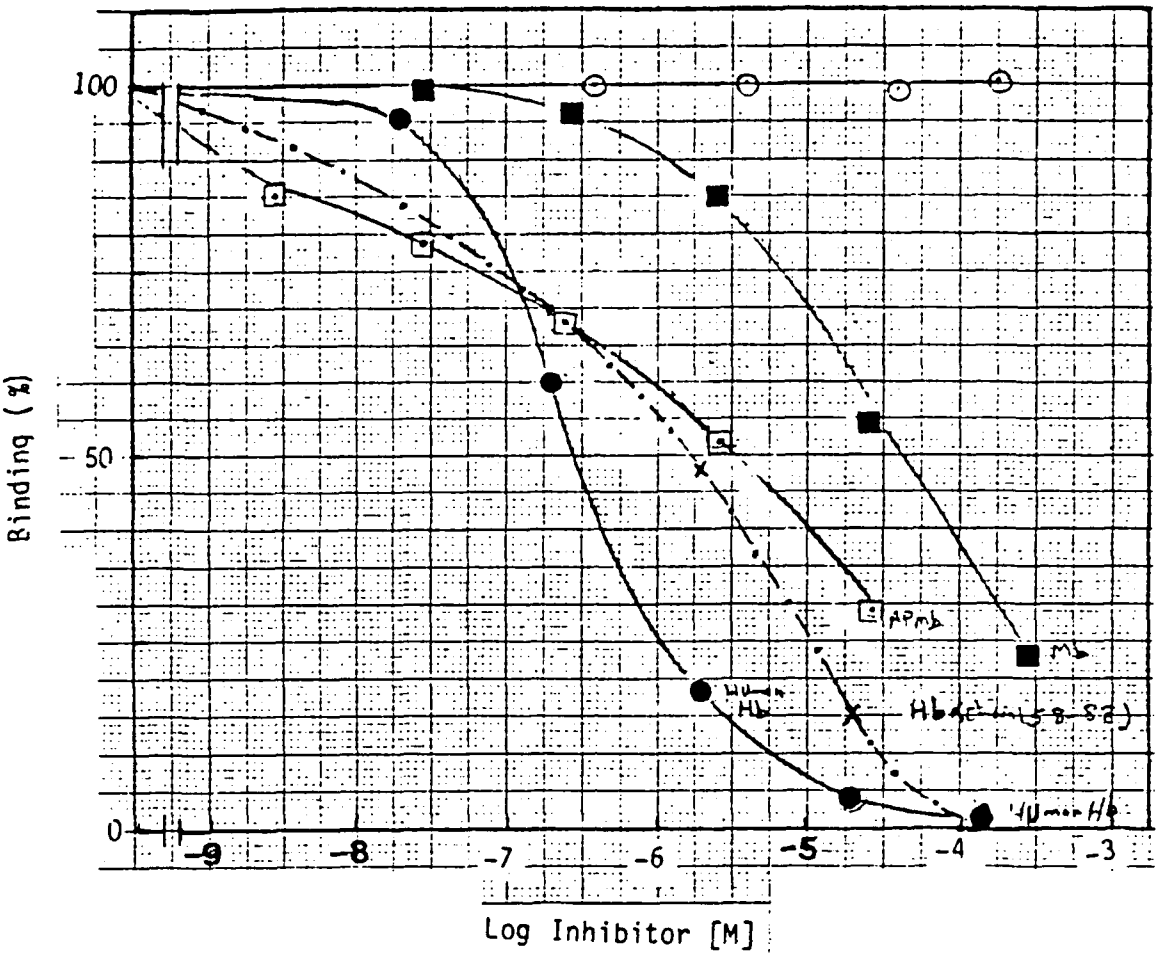

Figure 10. Inhibition of the binding of ^{125}I-labelled peptide α58-88 to heme adsorbent by unlabelled (■) Mb; (□) ApoMb; (●) human Hb and (o) a nonsense peptide corresponding to a randomized sequence of Mb (64-94). The inhibition by unlabelled peptide α58-88 itself is shown (dashed line) for comparison. Inhibition experiments were performed as described in the legend of Figure 5. Note that *uninhibited controls bound* 28867 ± 333 cpm.

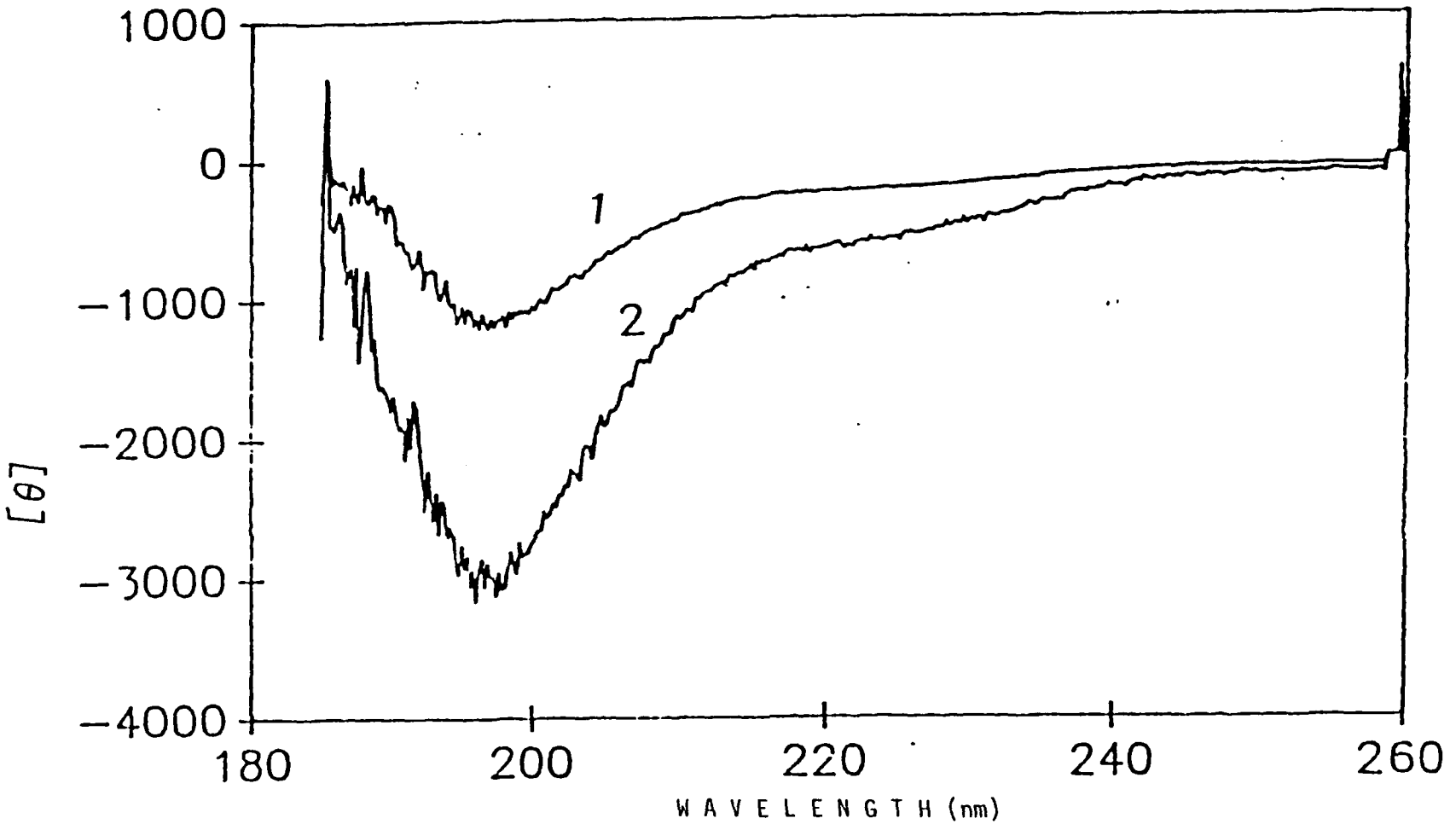

Figure 11. Circular dichroism (CD) spectra of (1) peptide Mb 64-94 and (2) the heme complexs of peptide Mb 64-94. The heme complex was prepared as described in the legend of Figure 3. Values are expressed in mean residue elliplicity [θ] and represent a computer averaging of 5 scans. Spectra were performed in a 5 mm cuvette on solutions containing 100 µg of peptide per ml of 0.01M phosphate buffer at pH 7.2.

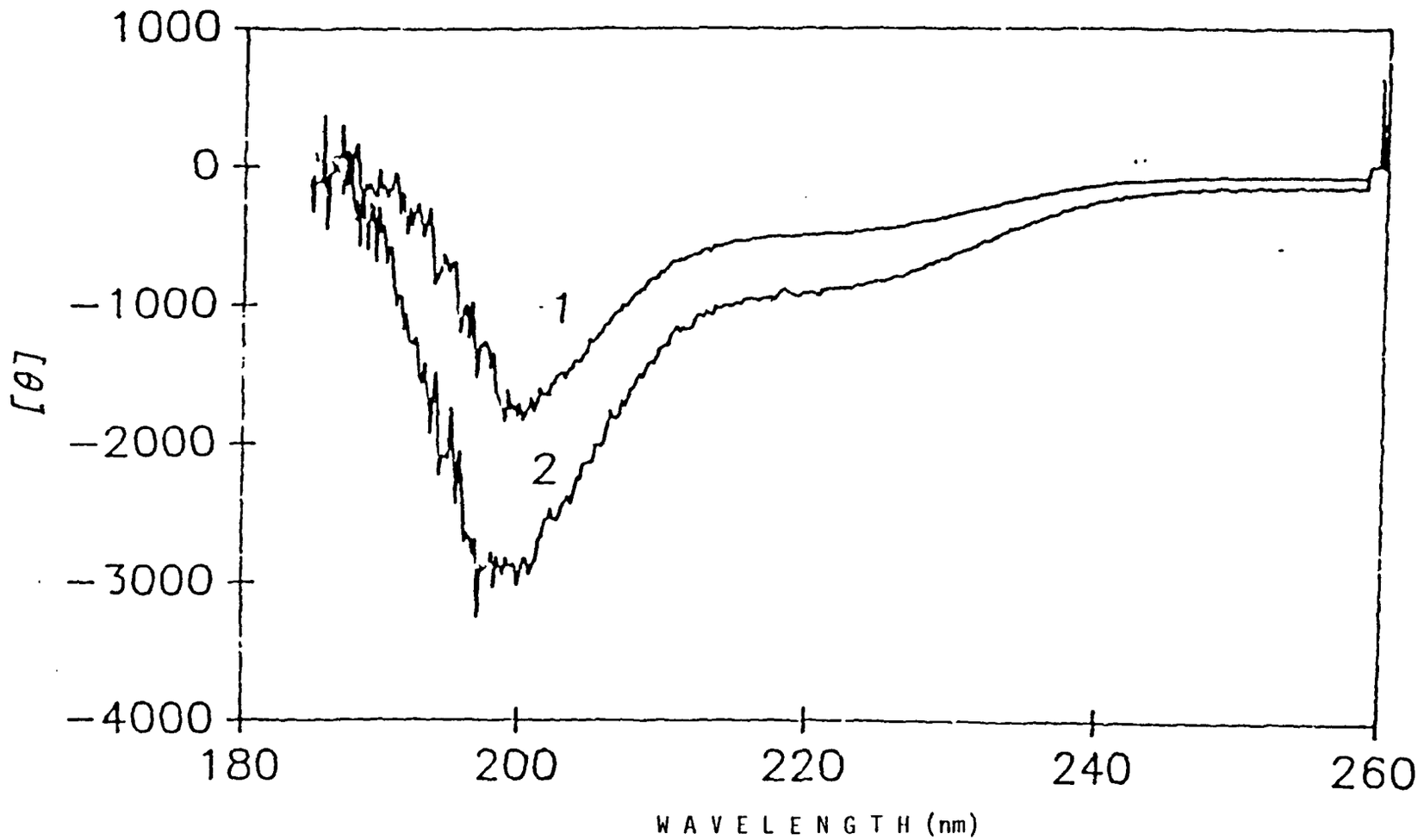

Figure 12. CD spectra of (1) peptide α58-88 and (2) its heme complex. Details are as in Figure 11.

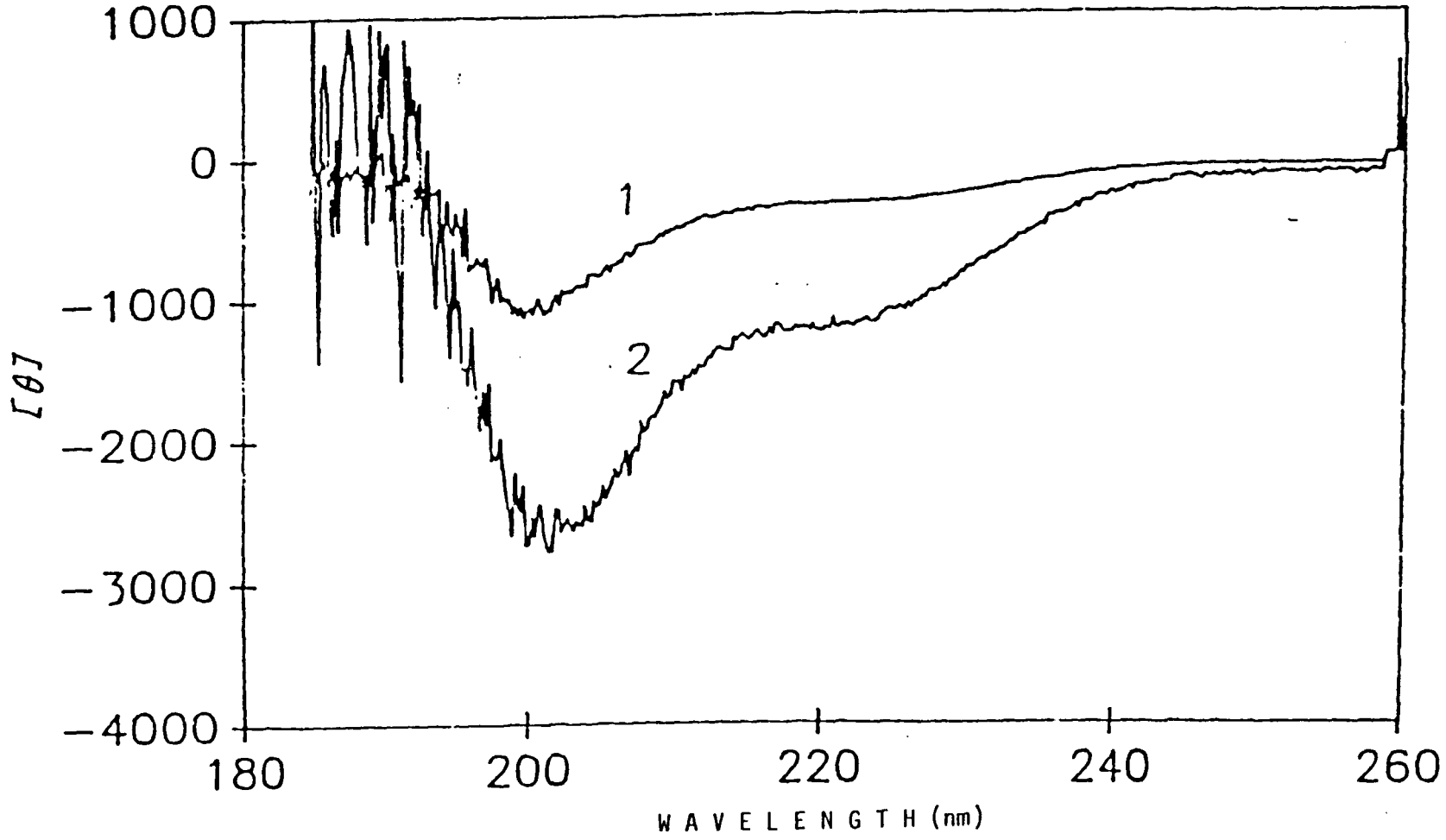

Figure 13. CD spectra of (1) peptide β63-93 and (2) its heme complex. Details are as in Figure 11.

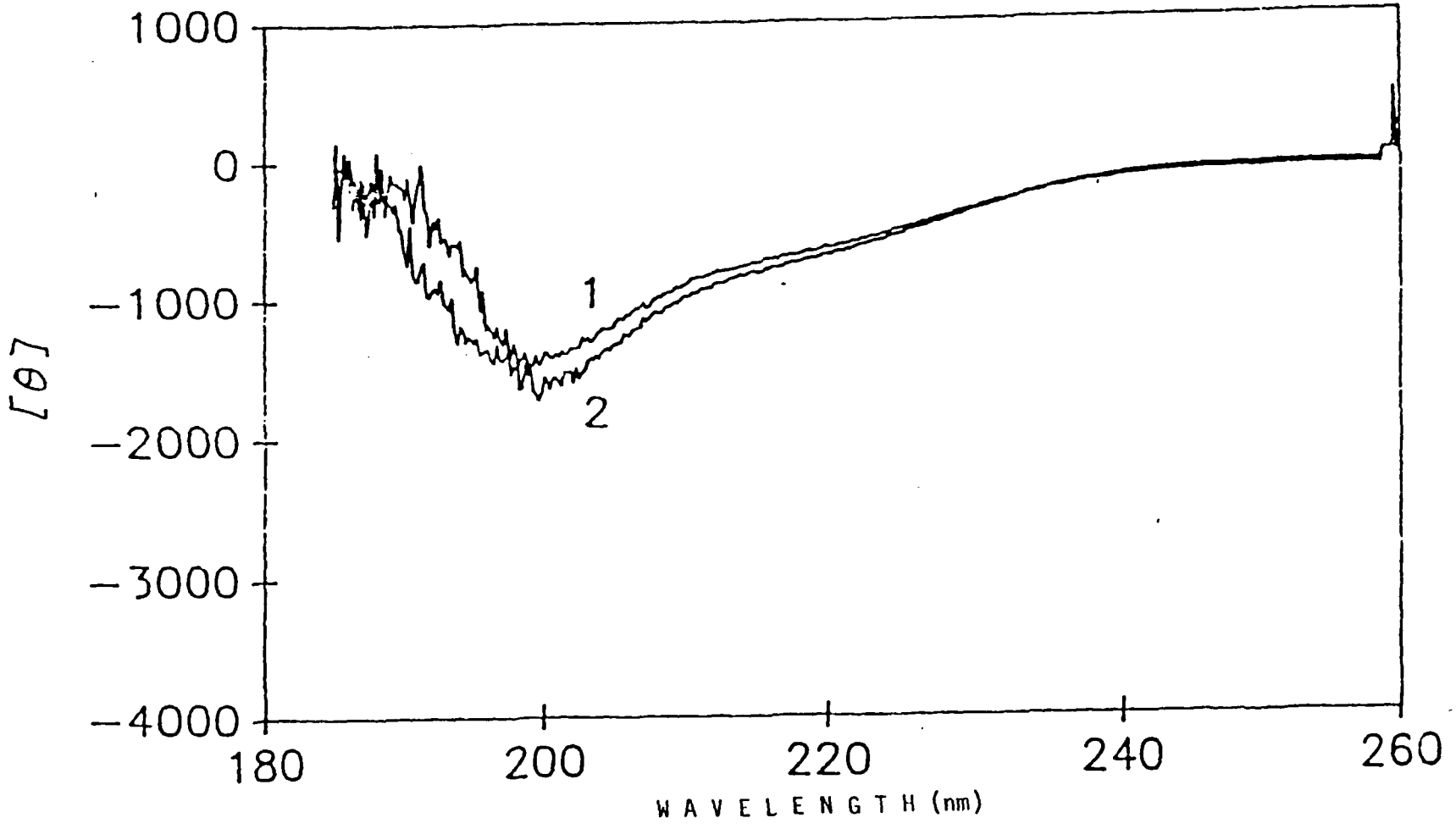

Figure 14. CD spectra of (1) a nonsense peptide corresponding to a randomized sequence of Mb 64-94 and (2) after mixing heme and chromatography as described in Figure 11.

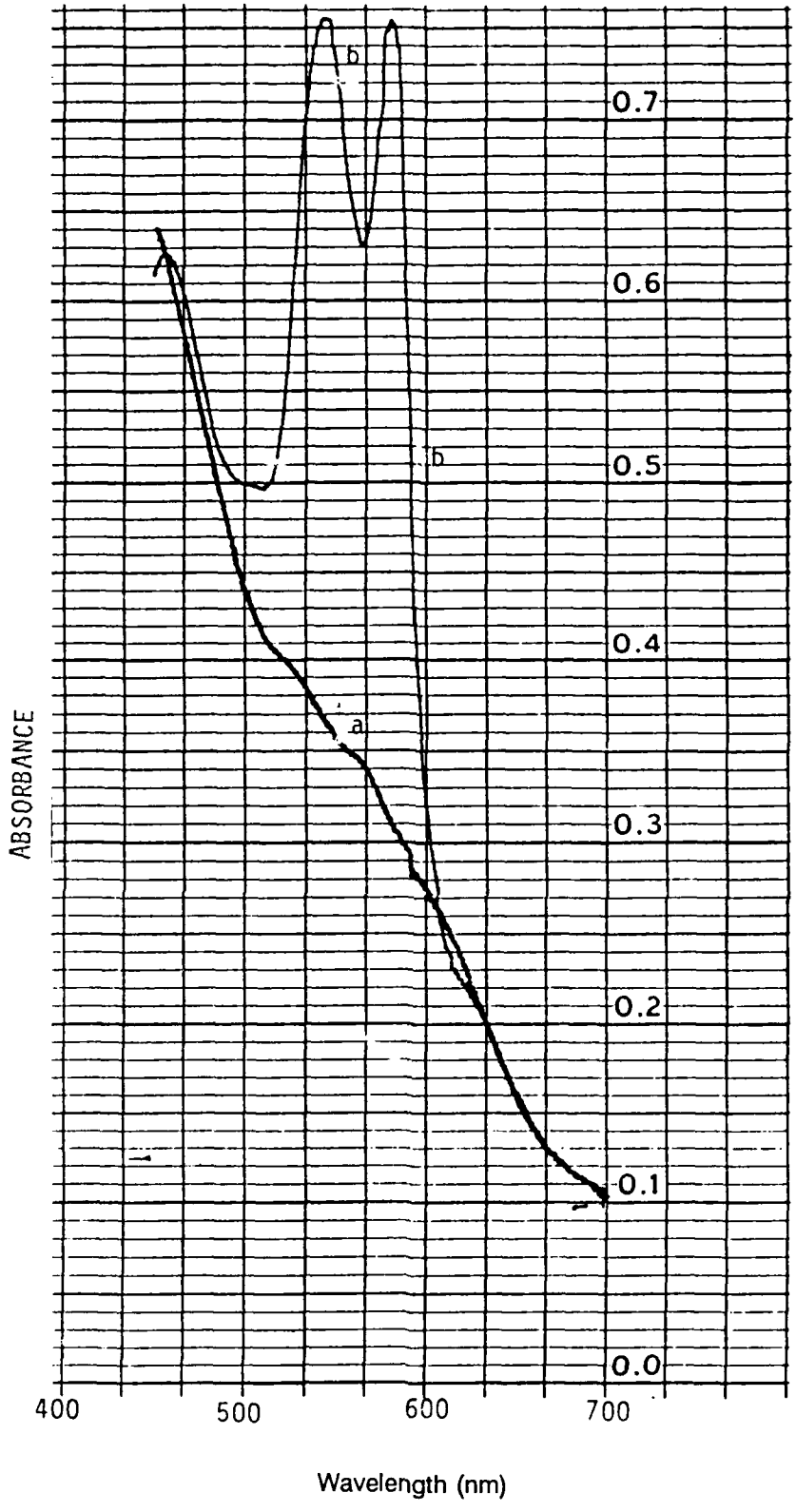

Figure 15. Continuous spectra of human Hb before (a), and after (b), reduction by sodium dithionite. Spectra were done in the range 400-700 nm on protein solutions (4 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.0. Reduction of the heme group was done by the addition of 3 mg of $Na_2S_20_4$ to 3 ml of Hb solution which was then saturated with oxygen before the spectrum was taken.

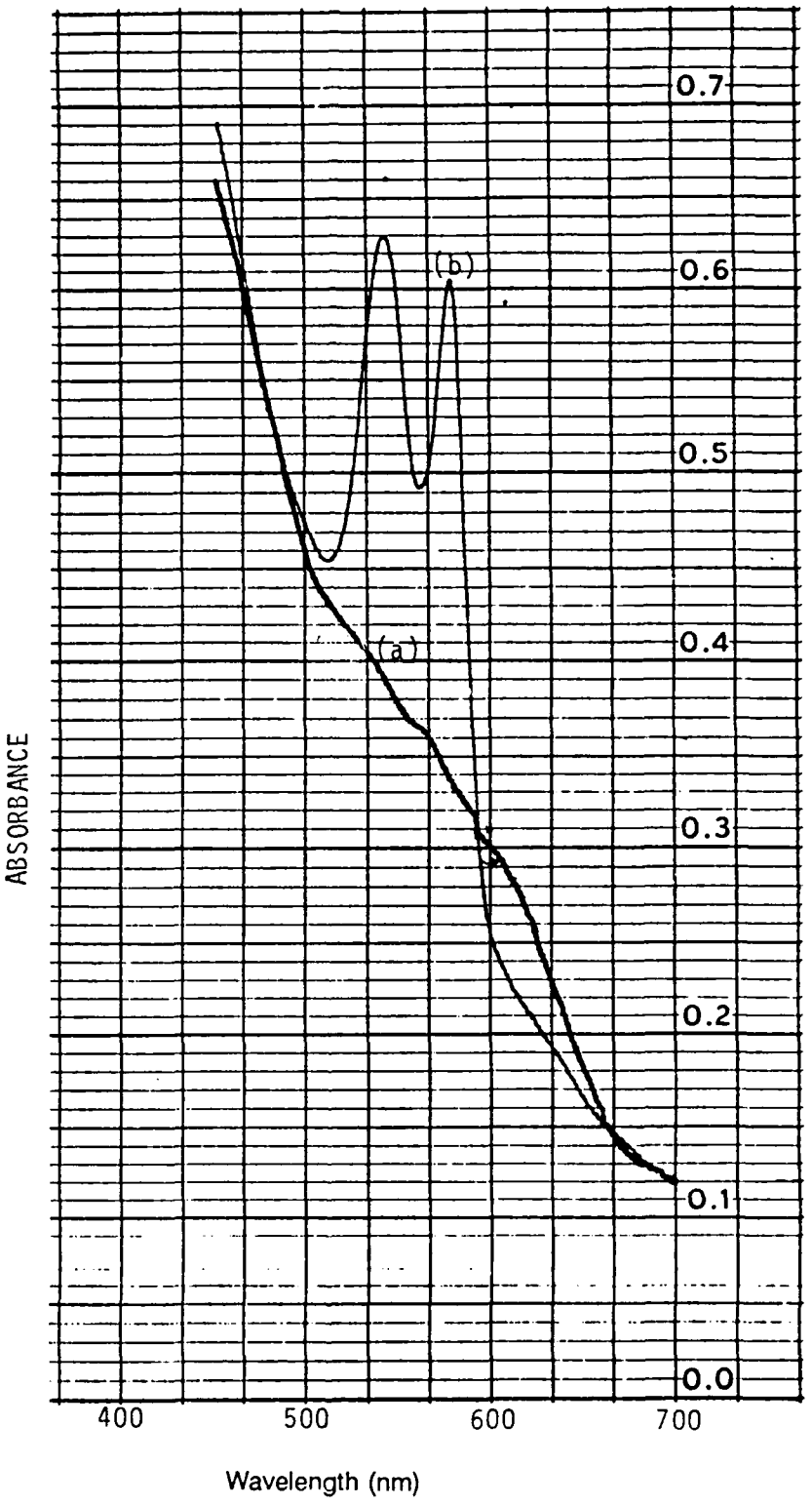

Figure 16. Continuous spectra of myoglobin before (a), and after (b), reduction by sodium dithionite. Experiment was carried out as described in the legend to Figure 15.

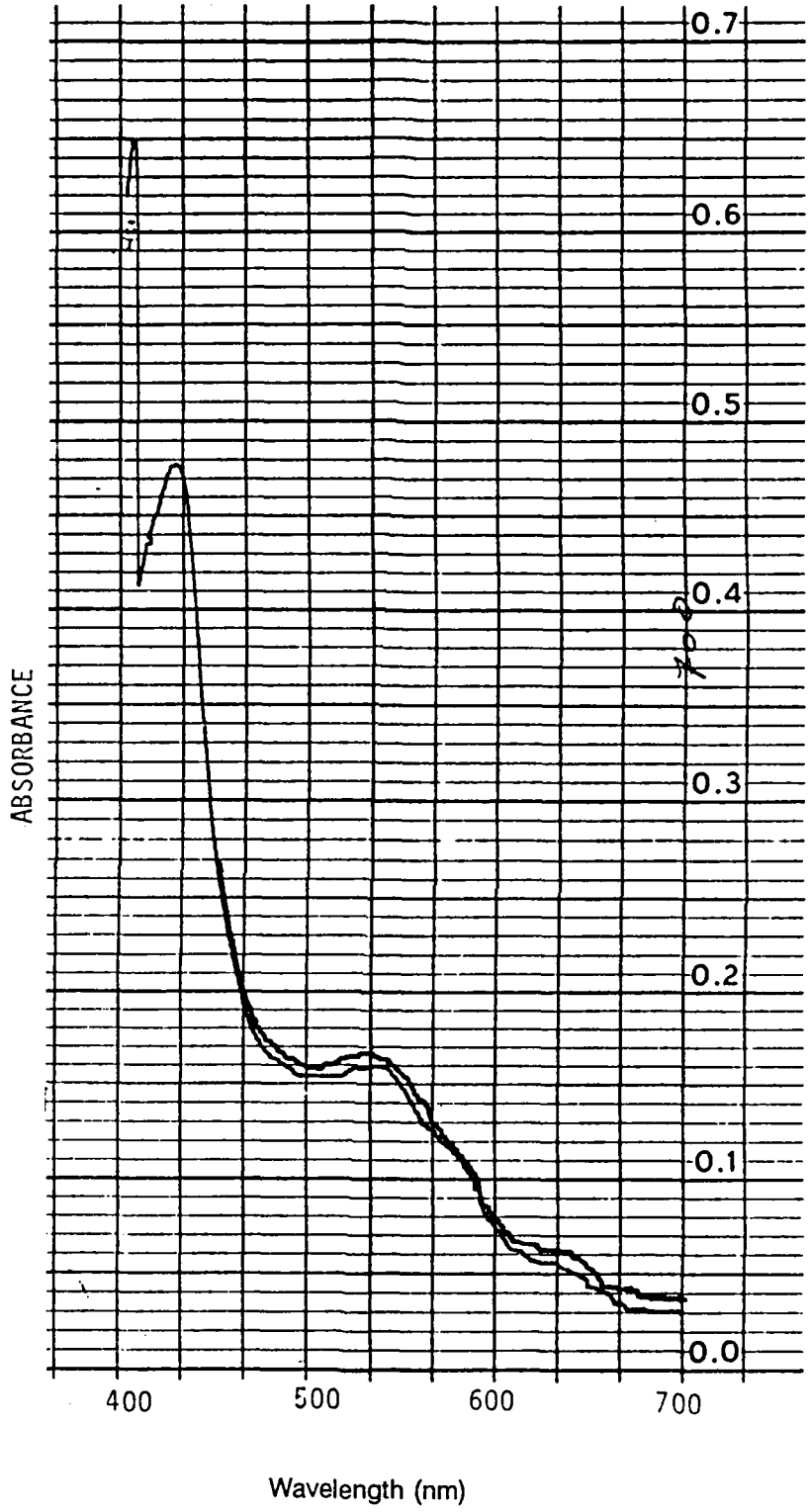

Figure 17. Continuous spectra of heme complexes of peptide α58-88 before and after reduction of the heme by sodium dithionite. Spectra were done in the range 400-700 nm. Experiments were carried out as described in Figure 15.

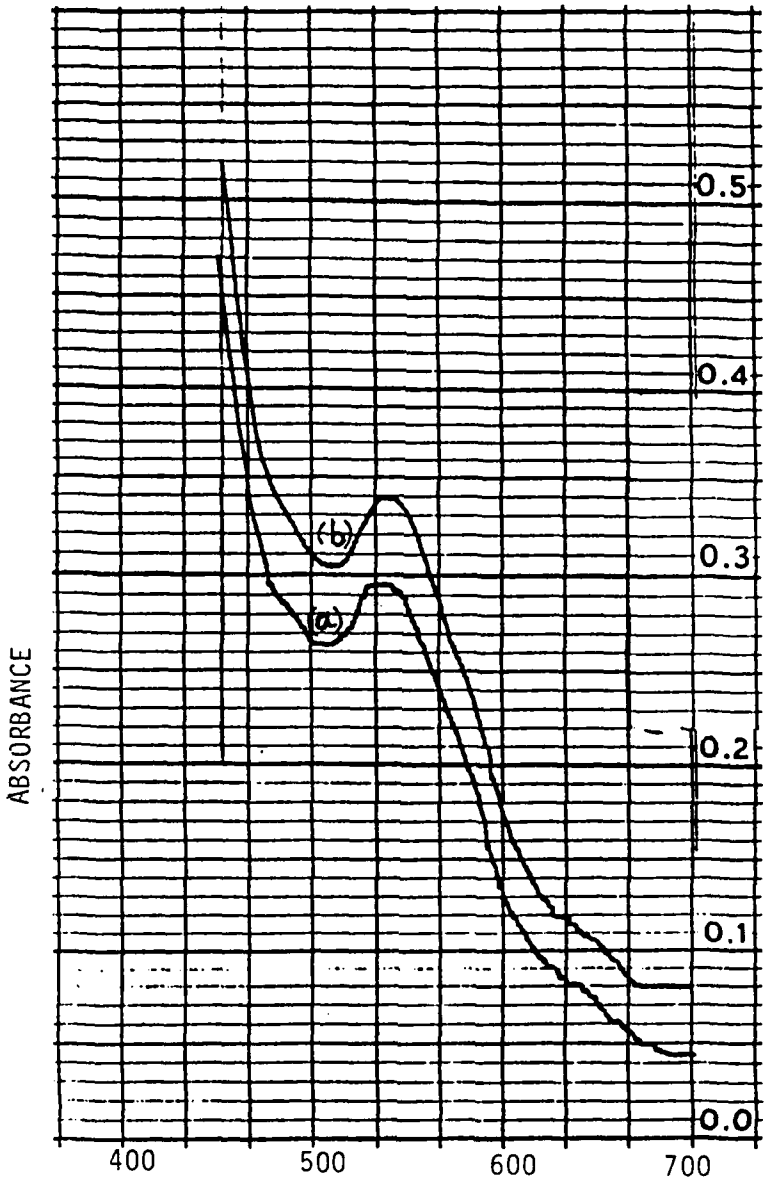

Figure 18. Continuous spectra in the range 400-700 nm of heme complexes of peptide β-63-93 before (a) and after (b) reduction of the heme group by sodium dithionite. Experiments were carried out as described in Figure 15.

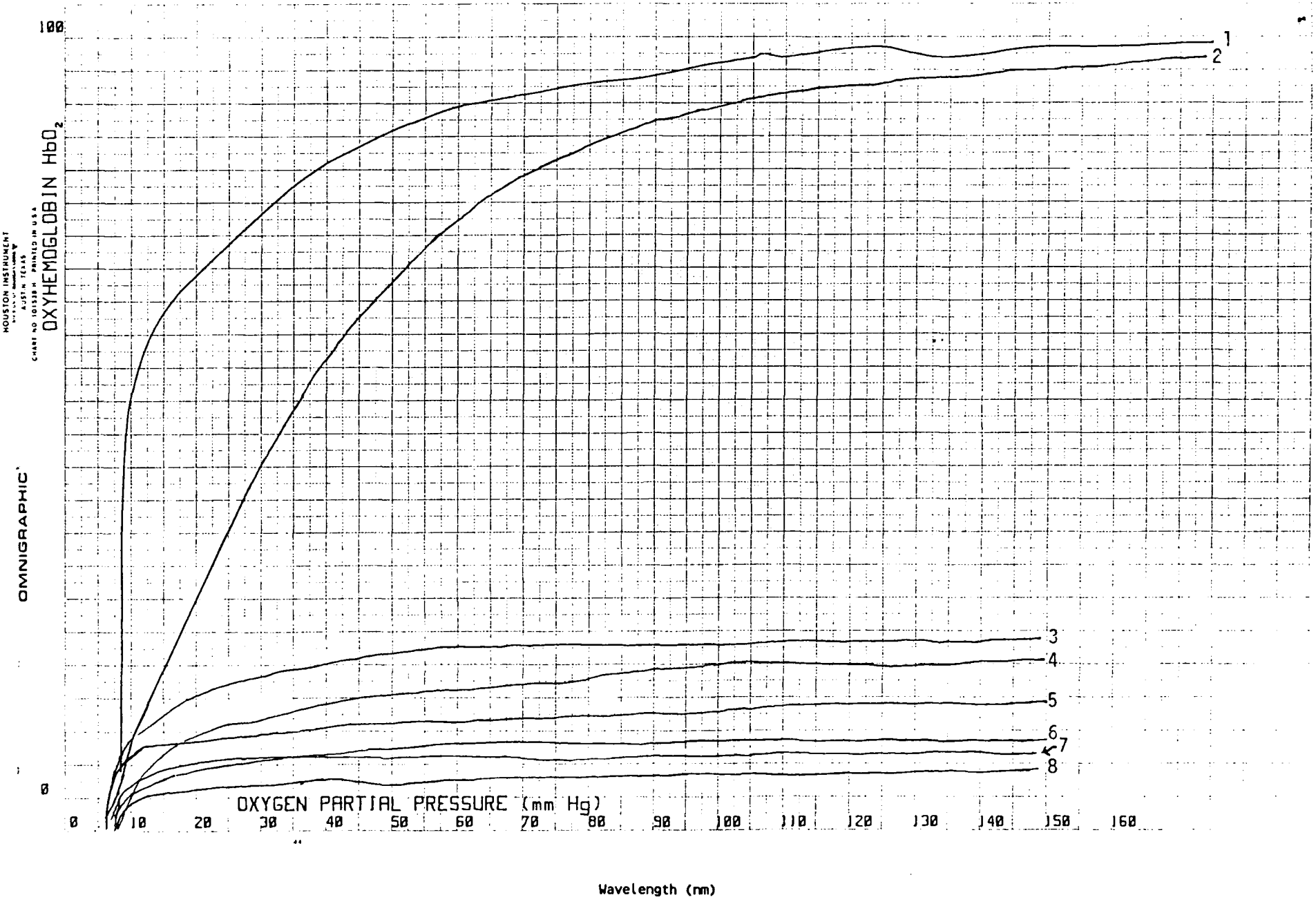

Figure 19. Oxygen binding curves of Hb, Mb and peptide-heme complexes. The figure shows per cent of oxygenated form as a function of the partial pressure of O_2. Solutions (4 mg/3 ml) were in 0.1 M potassium phosphate buffer, pH 7.0 and were reduced as described in Figure 15. Oxygen-binding was determined in a Hemox-Analyzer at 37°C. **(1)** Mb; **(2)** Hb; **(3)** peptide Mb (60-70) G(85-98); **(4)** peptide Mb 64-94; **(5)** peptide α58-88; **(6)** peptide α(55-65)G(80-87)-(93); **(7)** peptide Mb (61-71) G(86-93)-99; **(8)** peptide α(55-65)G(80-93).

B. FINAL REPORT OF INVENTIONS AND SUBCONTRACTORS

Patents

In view of the inability of the peptide-heme complexes to bind oxygen, it was concluded that these compounds will not have any physiological utility in blood transfusion. Therefore, a patent application on the compounds was not relevant or useful. No applications for patent were made.

Subcontracts

No subcontracts were involved in this work.

C. FINAL PROPERTY INVENTORY

Equipment Purchased on Navy Contract N0014-85-K-0585

Date Purchased	Vendor	Item	BCM Tag No.	Serial No.	Cost
09/85	Gilson Medical	Microfraction collector	054413	128J5731	1185.91
		Microfraction collector	054415	128J5735	1185.91
		Microfraction collector	054558	128J5736	1185.91
08/87	Sergeant Welch	Pump w/out base	059129	121247	609.00
12/88	LKB	GammaMaster gamma counter	063976	2770401	23995.00
12/88	McDuff	Norge frost free freezer	064006	EP949776	1083.96
		Norge frost free freezer	064007	EP949778	1083.96