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Subunit interacting surfaces of human haemoglobin

Localization of the α-subunit–β-subunit interacting surfaces on the β-chain by a comprehensive synthetic strategy

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A synthetic approach is introduced for localization of subunit interacting surfaces in oligomeric proteins. It consists of studying the binding activity of consecutive uniform overlapping peptides encompassing an entire subunit to the other, radiolabelled, subunit. This permits the establishment of the full profile of peptides that bind the other intact subunit. This approach has been demonstrated with haemoglobin, and its application here with the β-chain peptides has enabled the localization on the β-chain of the submolecular regions responsible for its binding to α-chain in solution. There was good agreement between the binding surfaces found here in solution and those expected from the crystal structure. There were also, however, some significant differences in the levels of binding found in solution and those expected from the crystal. Peptide 21–35 possessed much higher binding activity than would be expected from its contribution to subunit association in the crystal. Conversely, other regions expected to possess considerable binding capacity for α-chain either showed low (peptides 111–125 and 121–135) or almost no binding (peptides 91–105 and 101–115) capacity. On the other hand, two interacting surfaces (within peptides 11–25 and 71–85) that make a contribution in solution do not appear to play a role in the crystal. It is concluded that the regions of subunit association in solution are close to, but not identical with, those in the crystal. The approach should serve as an effective method for localization of subunit interacting surfaces of unknown proteins, even those that can be isolated only in traces.

INTRODUCTION

Haemoglobin is one of the first proteins for which the three-dimensional structure was determined by X-ray crystallography (Perutz *et al.*, 1968; Fermi, 1975). The native molecule is a tetrameric protein composed of two non-identical pairs of subunits (i.e. two α-chains and two β-chains). The X-ray-crystallographic studies have revealed the mode of folding of each chain (i.e. its tertiary structure), and also the manner by which the chains interact to form the oligomer (i.e. the quaternary structure) in the crystal. The assembly of the chains in the tetramer involves well-defined surfaces that comprise specific amino acid residues on each subunit. Chemical approaches to subunit assembly in solution have yielded information that is mostly general and empirical, and have not enabled localization and definition of the sites involved in subunit interaction and assembly.

The present paper describes a synthetic approach for localization of subunit interacting surfaces in oligomeric proteins. It involves the synthesis of a series of consecutive overlapping peptides that account for an entire subunit of an oligomeric protein and then the determination of the binding activity of each of these peptides to the other subunit(s). Its application to the localization of the sites on the β-chain that bind the α-chain of HbA is reported here.

MATERIALS AND METHODS

Isolation and purification of HbA and its α- and β-subunits and synthesis and purification of the overlapping peptides that encompass the β-chain were described in an accompanying paper (Yoshioka & Atassi, 1986*a*). Each peptide consisted of 15 residues and overlapped both its two adjacent neighbours by five residues at each end. These synthetic β-peptides were: 1–15, 11–25, 21–35, 31–45, 41–55, 51–65, 61–75, 71–85, 81–95, 91–105, 101–115, 111–125, 121–135 and 131–146.

Radioiodination of the α-chain was carried out by a modification of the chloramine-T procedure (Hunter & Greenwood, 1962) with carrier-free [^{125}I]iodide (Amersham Corp., Arlington Heights, IL, U.S.A.). To avoid oxidation of the thiol groups on the isolated subunits by the oxidizing agent chloramine-T, the thiol groups were protected by dithiothreitol during the radiolabelling reaction. Thus labelling of the chain (25 μg) was carried out by the normal procedure, except that the solvent used was 0.1 M-sodium phosphate buffer, pH 8.0, made 0.1% with respect to dithiothreitol. Free $^{125}I^-$ was separated from the radiolabelled material on a column (0.5 cm × 15 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), which was eluted with 0.1% BSA solution in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2, containing 0.1% dithiothreitol.

Abbreviations used: HbA, human adult haemoglobin (major chromatographic component obtained by CM-cellulose chromatography; Atassi, 1964); BSA, bovine serum albumin.

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Precipitation with 10% (w/v) trichloroacetic acid showed that the label attached to the protein in the various preparations acounted for 95–97% of the total label in the preparations eluted from the column. The specific radioactivities of the labelled subunit preparations were 18–21 μCi/μg.

Preparation of protein and peptide adsorbents was also done as given in an accompanying paper (Yoshioka & Atassi, 1986*a*). Quantitative radioimmunoadsorbent titrations, with a fixed amount of adsorbent and increasing amounts of ^{125}I-labelled α-chain, were carried out as previously described (Twining & Atassi, 1978, 1979). However, in the present studies it was found that the labelled subunits exhibited an unusually high non-specific binding to the glass tubes in which the binding studies were performed. Therefore, after the binding was complete, the adsorbents were transferred quantitatively to clean new tubes and their radioactivities counted on a γ-radiation counter. In order to correct for non-specific binding to the test adsorbents, negative control adsorbents were titrated with ^{125}I-labelled α-chain under exactly the same conditions. These control adsorbents were: uncoupled Sepharose and Sepharose conjugates of BSA and a 12-residue synthetic BSA peptide 168–179 (Atassi, 1982).

Finally, specificity of the binding to those adsorbents that bound ^{125}I-labelled α-chain was checked by inhibition studies with unlabelled α-chain and with control unrelated proteins (BSA and ovalbumin).

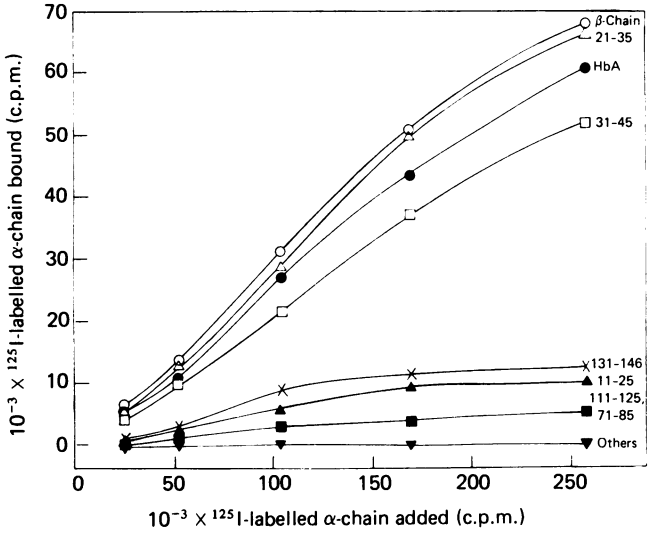

Fig. 1. Radiometric titration of ^{125}I-labelled α-chain with β-peptide adsorbents

Various amounts of radioiodinated α-chain were added to a fixed volume (25 μl of 1:1 suspension) of the following adsorbents: ○, β-chain; ●, HbA; △, peptide 21–35; □, peptide 31–45; X, peptide 131–146; ▲, peptide 11–25; ■, peptides 111–125 and 71–85 (their titration curves were almost superimposable); ▼, all other peptides and negative controls. For details see the text.

RESULTS

Conditions of binding to adsorbents

Initial binding studies with protein and peptide adsorbents and ^{125}I-labelled α-chain, which was labelled without any attempts to protect the thiol group of the α-chain from oxidation by chloramine-T, were not able to show any binding, even between the haemoglobin chains. Protection of the thiol groups with dithiothreitol afforded a labelled α-chain that was able to bind to β-chain adsorbents. This, in all likelihood, is due to protection of the thiol group, thus preventing its oxidation and the formation of an intersubunit disulphide bond. Also, other side chains, such as those of tryptophan, histidine, methionine and tyrosine, which may be subject to oxidation by chloramine-T (for review of oxidation reactions see Atassi, 1977), should also be protected in the presence of dithiothreitol. The labelled preparation, however, had an unusually high binding to the glass tubes. The obstacle was overcome by transferring the adsorbents, after binding and washing, to new glass tubes and counting their radioactivities. With this method, the non-specific binding to unrelated adsorbents was only 1.0–2.5% relative to the binding of labelled α-chain to β-chain adsorbents.

Binding of ^{125}I-labelled α-chain to protein and peptide adsorbents

Binding of ^{125}I-labelled α-chain to various adsorbents was carried out with a fixed amount of adsorbent and various amounts of labelled α-chains. The results of such a titration experiment are shown in Fig. 1. Table 1 summarizes the maximum binding values obtained with 25 μl of adsorbent suspension (1:1, v/v) and 260000 c.p.m. of labelled α-chain. As expected, both HbA and β-chain bound labelled α-chain. It was, however, remarkable that many of the peptides were able to bind α-chain. The highest binding activity was exhibited by peptides 21–35 and 31–45. In fact, the binding activity of peptide 21–35 closely resembled that of the β-chain (Fig. 1 and Table 1). Considerable binding activity also resided in peptides 131–146, 11–25, 111–125, 71–85 and 121–135. The other peptides possessed either very slight or no binding activity. Non-specific binding by unrelated adsorbents was 1–1.5% relative to label bound by the β-chain (Table 1). Binding to β-chain and to peptides was completely inhibited by preincubation of their adsorbents (5 μl) with free unlabelled β-chain (100 μg) before the addition of labelled α-chain. No inhibition was obtained with unrelated proteins, thus confirming the specificity of the binding to β-chain and its active peptides.

DISCUSSION

Haemoglobin is a tetrameric protein molecule comprised of two subunits identical in pairs (two α-subunits and two β-subunits). Each of the subunits carries a haem group, the prosthetic group responsible for carrying O_2. Haemoglobin has been extensively studied in terms of its primary structure (Braunitzer *et al.*, 1961; Hill & Konigsberg, 1962), its three-dimensional structure (Perutz *et al.*, 1968; Fermi, 1975; Perutz & Fermi, 1981) and its O_2-binding properties.

Because of this detailed knowledge, the protein serves as an excellent model for protein chain folding and assembly. However, our understanding is, by necessity, derived from a static model based on molecular packing within the crystal. But in solution, unlike the crystal, constraints on molecular translational movements and

Table 1. Binding of ^{125}I-labelled α-chain to haemoglobin, β-chain and synthetic β-peptides

A fixed amount of each adsorbent (25 μl) as a suspension (1:1, v/v) in 0.1% BSA in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2, was titrated with increasing amounts of ^{125}I-labelled α-chains (from 25000 to 260000 c.p.m.). After binding and washing, the adsorbents were transferred to new tubes and their radioactivities counted. The results, which summarize binding values when 2.6×10^5 c.p.m. of labelled α-chain is used, are given before and after correction for non-specific binding to uncoupled Sepharose and adsorbents of BSA and synthetic BSA peptide. The values represent the average of triplicate analyses, which varied ±1.2% or less.

Protein or peptide adsorbent	^{125}I-labelled α-chain bound (c.p.m.)	
	Uncorrected values	Corrected values
HbA	61727	60830
β-Chain	68920	68020
1–15	900	0
11–25	11460	10560
21–35	67250	66350
31–45	53400	52500
41–55	1320	420
51–65	890	0
61–75	1180	280
71–85	5280	4383
81–95	905	0
91–105	1170	270
101–115	1470	570
111–125	6070	5170
121–135	3100	2200
131–146	13570	12670
Uncoupled Sepharose	802	
BSA	1020	
BSA peptide	820	

Table 2. Contact residues on the β-chain residing in the α–β interfaces and the β-chain peptides expected to bind α-chain

The identity of the β-chain contact residues that are involved in the β_1–α_1 and β_2–α_1 interactions were obtained from Fermi (1975).

β-Chain contact residues and β-peptides expected to bind α-chain			
β-Chain residues in subunit contacts		β-Chain peptides expected to bind α-chain*	No. of 'contact' residues on peptide
β_1–α_1	β_2–α_1		
Arg-30			
Val-33			
Val-34	Val-34		
Tyr-35	Tyr-35	Peptide 21–35	6
	Pro-36		
	Trp-37		
	Arg-40		
	Ala-43	Peptide 31–45	10
	His-97		
	Val-98		
	Asp-99		
	Glu-101		
	Leu-105	Peptide 91–105	5
Asn-108			
Cys-112			
Ala-115		Peptide 101–115	5
His-116			
Gly-119			
Phe-122			
Thr-123			
Pro-124			
Pro-125		Peptides 111–125	8
Gln-127			
Ala-128			
Gln-131		Peptide 121–135	7
	Tyr-145		
	His-146	(Peptide 131–146?)	3

* This would be the expectation if each of the contact surfaces (comprising a group of contact residues on the β-chain) were sufficient to give a fruitful stable complex.

segmental motility are less rigid. For an oligomeric protein, the molecular freedom in solution could affect subunit interaction and assembly. Comparisons of solution and X-ray-crystallographic structures have employed a variety of physical or chemical approaches that enable description of gross conformational and hydrodynamic parameters or interaction or exposure of a very limited number of residues. The results are frequently empirical and the interpretations are subjective.

Since the subunit interacting surfaces between the HbA subunits are well characterized, it was decided to examine here whether continuous regions of the polypeptide chain, residing at the subunit interface, would possess enough binding activity to be able to associate with the other subunit. The primary structures of the constituent subunits of a large number of oligomeric proteins are now known. Many can be isolated only in minute quantities and, further, the three-dimensional structures of only a few of these proteins have been determined. Any approach that will enable the localization and synthesis of the subunit interacting surfaces would be extremely valuable in studying structure–activity relationships, even before knowledge of the X-ray-crystallographic structure. Application of this approach to HbA is demonstrated in the present paper, in which we have examined the ability of radiolabelled α-chain to bind to a panel of overlapping peptides representing the entire β-chain.

It is apparent from the present work that ^{125}I-labelled α-chain bound specifically to some of the β-chain peptides (Fig. 1 and Table 1). The 'contact' residues on the β-chain that are involved in the α–β association (Fermi, 1975) are summarized in Table 2. From this, the β-peptides expected to bind to α-chain (if segments of the contact surfaces are able to mediate a fruitful complex) can be derived (Table 2) and are shown schematically in Fig. 2. It can be seen that the β peptides 21–35, 31–45, 91–105, 101–115, 111–125, 121–135 and possibly 131–146 would be expected to bind α-chain. From the number of essential 'contact' residues on each of these peptides, the peptides would be expected to exhibit activity, in decreasing order, as follows: peptide 31–45 (ten contacts) > peptide 111–125 (eight contacts) > peptide 121–135 (seven contacts) > peptide 21–35 (six con-

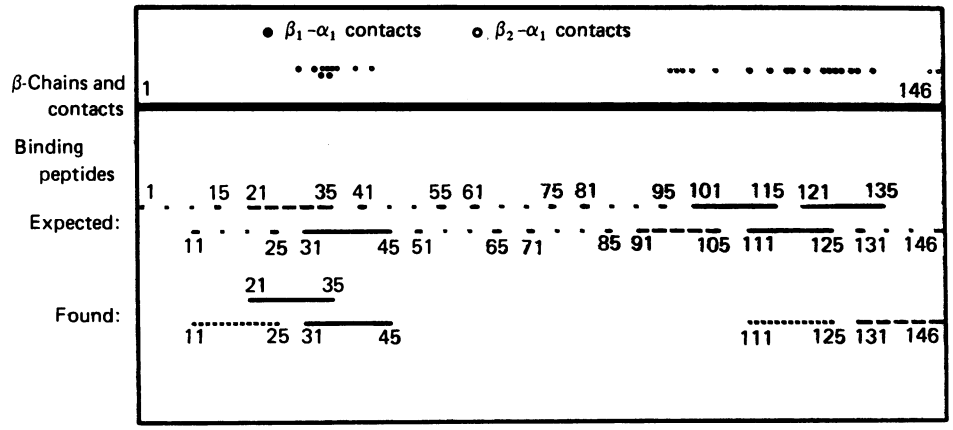

Fig. 2. Schematic diagram summarizing the positions of the subunit contact residues on the β-chain and comparing the expected and found binding activities of the overlapping β-peptides

●, β_1–α_1 contacts; ○, β_2–α_1 contacts; both being placed at appropriate positions to scale along the polypeptide chain. —— High binding activity; ---, moderate binding activity; ------, low binding activity; - - -, no binding.

tacts) > peptides 91–105 and 101–115 (five contacts each) > peptide 131–146 (three contacts). Indeed, it was found that five of these peptides bound α-chain. However, the order of peptide activities was quite different from that expected simply on the basis of the number of contact residues. The order of activities found experimentally for the peptides was: peptide 21–35 > peptide 31–45 ≫ peptide 131– 146 ≫ peptide 111–125 ≫ 121–135; surprisingly, peptides 91–105 and 101–115 did not bind α-chain. In addition, unexpected binding activities were found in peptides 11–25 and 71–85. These latter two peptides carry no contact residues that are indicated from the X-ray-crystallographic structure of the tetramer. Fig. 2 gives a convenient schematic presentation of the foregoing analysis.

From these findings it can be concluded that the energy of binding is not necessarily dependent on the number of 'contact' residues in a binding site. For example, peptide 21–35 (six contact residues) had the highest binding activity even though other peptides had higher numbers of contact residues. Similarly, the considerable level of binding activity exhibited by peptides 131–146 (three contact residues) was extremely surprising, since, normally, such numbers of contacts would not be expected to be sufficient for forming a fruitful complex. Thus it is either that three contact residues can indeed muster enough binding energy for complex-formation or, alternatively, that peptide 131–146 possesses more than three contact residues. This would require that some slight differences exist between the crystal and solution structures, at least in terms of the subunit interfaces. Indeed, a similar conclusion can be inferred from the unexpected binding activities of peptides 11–25 and 71–85, since no contact residues on the β-chain reside within these two regions in the X-ray-crystallographic structure (Fermi, 1975).

It is relevant to consider at this point whether radioiodination of the α-chain will adversely affect its binding capacity to some of the β-peptides. This can be reasonably analysed in relation to α–β interface contacts known from the X-ray-crystallographic structure (Fermi, 1975). In the α-chain of HbA tyrosine residues 42 and 140 reside in the α_1-β_2 interface. α_1-Tyr-42 hydrogen-bonds to β_2-Asp-99 and is close to β_2-Arg-40. Thus iodination of α_1-Tyr-42 might adversely affect binding of α-chain to β-peptides 91–105 and 31–45. β-Peptide 31–45 (ten contacts) binds labelled α-chain very well (Table 1), but peptide 91–105 (five contacts) exhibits very low binding capacity. Whether the low binding activity of this peptide is the result of iodination of Tyr-42 on the α-chain is uncertain. However, because of its low number of contacts, only low binding activity would be expected for β-peptide 91–105. This is similar to the behaviour of β-peptide 101–115, which also has five contact residues (see Tables 1 and 2). In the X-ray-crystallographic structure α_1-Tyr-140 comes in contact with β_2-Pro-36 and β_2-Trp-37. Iodination of α_1-Tyr-140 might be expected to affect adversely binding of β-peptide 31–45 (ten contacts). However, since the labelled α-chain bound very well to this peptide, the effect of iodination of α_1-Tyr-140, if it did take place, need not be considered as a factor in the present analysis. Since the α-chain was labelled lightly in the presence of dithiothreitol and since labelling might be somewhat random, it can be concluded that radioiodination of the α-chain did not significantly distort the outcome of the results and would not account for the differences observed.

The unexpected lack of binding activities of peptides 91–105 and 101–115 and the low activity of peptides 121–135 and 111–125 may be attributed to loss of essential contact residues on the peptides in coupling to the adsorbents and/or unfavourable conformational states of the peptides. However, since similar adsorbents of these peptides have been shown in the preceding papers to bind anti-HbA antibodies (Yoshioka & Atassi, 1986*a*) and haptoglobin (peptide 121–135; Yoshioka & Atassi, 1986*b*), it is more likely that the subunit-binding energy inherent in these regions is low.

In conclusion, a comprehensive synthetic approach has been applied here for the localization, on the β-chain of HbA, of the regions that bind to the α-chain. The studies have shown that the binding energy is not dependent on the number of contact residues within a binding surface. The β–α contact surfaces in the crystal and in solution are similar, with some slight differences. At least two unexpected contact surfaces operate in solution that do

not exist in the crystal. The binding energy resides overwhelmingly within about one-fifth of the β-chain (residues 11–45). This approach should prove useful for studying and localizing subunit interacting surfaces in oligomeric proteins, especially these whose X-ray-crystallographic structures are not known and those that can be isolated only in traces (e.g. membrane receptors). Synthetic peptides that represent regions of high binding energy may be useful for interference with subunit association.

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