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Enhancing peptide antigenicity by helix stabilization

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Abstract The engineering of antigenic determinants on super secondary structures using de novo design approaches often involves synthesis of long peptide chains (35–80 residues long). This communication illustrates that the stabilization of secondary structure by rational design can also greatly enhance immunogenicity and antigenicity, but in much shorter peptide sequences (21 residues long). A peptide epitope the sequence of which has been derived from the C-terminus of the chicken riboflavin carrier protein (cRCP), H_2N-Tyr-His-Ala-Cys-Gln-Lys-Lys-Leu-Leu-Lys-Phe-Glu-Ala-Leu-Gln-Gln-Glu-Glu-Gly-Glu-Glu-OH, has been chosen for analysis. Helical conformations were induced in the peptide in aqueous trifluoroethanol. Analogs were designed to stabilize this conformation in water by either the introduction of appropriately spaced ion pairs or the strongly helix nucleating residue α-aminoisobutyric acid (Aib), substituted for Ala/Gly, thus affording a comparison of the helix stabilization strategies. Circular dichroism (CD) results demonstrate that all the designed analogs are appreciably more helical than the parent peptide in 50% aqueous trifluoroethanol. Peptide antisera were raised for all analogs in rabbits. The affinities of these antisera for the native protein antigen, determined using a chaotrope disrupted binding assay, correlated very well with the helix content determined by CD.

Key words: Peptide antigenicity; Helix stabilization; De novo design; Riboflavin carrier protein; Circular dichroism; Peptide conformation

1. Introduction

The observation that antibodies raised to short synthetic peptide fragments often cross react with the native protein antigens has stimulated a great deal of interest in the development of peptide vaccines [1–4]. Well-defined conformational states have been demonstrated for a wide variety of antigenic peptides in solution [5]. Transferred NOE [6–9] and crystallographic [10,11] studies of peptides complexed to Fab fragments of monoclonal antibodies have clearly demonstrated the formation of ordered backbone conformations in the bound states. The incorporation of specific stereochemical constraints into an antigenic peptide that stabilize conformational states recognized by antibodies, appears to provide an attractive approach to engineering improved immunological characteristics [12–15]. We describe in this report that helix stabilization in a 21 residue synthetic peptide fragment of chicken riboflavin carrier protein (cRCP) dramatically enhances the immunogenic properties of the peptide.

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Riboflavin carrier protein (RCP) from chicken egg is a 219 residue phosphoglycoprotein that is involved in transport and yolk deposition of the vitamin [16,17]. RCP is evolutionarily conserved and is critical for transplacental transport of the vitamin in pregnant mammals [18]. In vivo immunoneutralization of RCP has been shown to result in fetal resorption in rodents and curtailment of pregnancy in primates [19]. Antibodies to the C-terminal peptide of RCP are effective in neutralizing the protein in vivo. Preliminary conformational studies of the native C-terminal peptide (Y-21) suggested that helical conformations may be involved in determining peptide antigenicity [20]. Fig. 1 lists the sequence of the five 21 residue peptides examined in the present study. In all cases, the central segment of the peptide, QKKLLKFE, was left unaltered since this has been implicated by epitope mapping to be important in binding to monoclonal antibodies (unpublished). The following strategies were adopted to stabilize helical conformations in the designed analogs. The incorporation of i→i + 3/i + 4 salt bridges involving lysine and glutamic acid pairs gave the peptide HC-21 [21]. This peptide was further engineered to have an N-terminal negatively charged residue (glutamic acid) to neutralize the helix dipole to give the peptide EC-21 [22]. The peptide HE-21 was designed with an extra i→i + 3 ion pair near the N-terminus. In these three cases the design also abolishes the clustering of negative charges near the C-terminus, a feature which may further enhance helix stability. The last peptide analog YAib-21 has the strongly helix promoting α-aminoisobutyryl (Aib) residue as replacements for glycine and alanine residues [23–25].

2. Materials and methods

The peptides were synthesized on a LKB-Biolynx 4175 semi-automatic peptide synthesiser using Fmoc chemistry. Sidechain protecting groups were cleaved along with the peptide from the resin by treatment with 95% aqueous TFA for 3 h. Analytical HPLC on a Lichrosorb C18 column (4 × 250 mm, 5 μM particle size) showed the peptide to be >98% pure. Solvent systems used were solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile containing 0.1% TFA. A linear gradient of 5–40% in 40 min was used (flow rate 0.8 ml/min, detection at 226 nm, retention times ranged between 26–28 min). The peptide identity was confirmed by either sequencing or mass spectroscopy. All CD spectra were recorded on a JASCO-J-500A spectropolarimeter using a cell of path length 1 mm at ambient temperature. Peptide concentrations (≈0.22 μM) were determined by tyrosine absorbance at 275 nm (ε_{275} = 1,340 $M^{-1} \cdot cm^{-1}$). Peptides were conjugated to diphtheria toxoid (DT) through their N-terminal tyrosine residues using bis-diazotised benzidine [26]. Peptide antisera were raised by immunizing rabbits subcutaneously with 500 μg of peptide-DT conjugate emulsified with 2 volumes of complete Freund's adjuvant. Animals were boosted (250 μg) at 20 day intervals and test bleeds were collected ten days following a booster injection. Antibody titers were estimated by coating the respective peptides on to a 96-well polystyrene microtitre plates at concentration of 20 μg/ml in 0.1 M phosphate buffer, pH 7.2, contain-

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YHAC	QKKLLKFE	ALQQEEGEE	Y-21
YHAC	QKKLLKFE	ALEQEKGKE	HC-21
YHAE	QKKLLKFE	ALEQEKGKE	HE-21
YEAC	QKKLLKFE	ALEQEKGKE	EC-21
YHUC	QKKLLKFE	ULQQEEUEE	YAib-21

Fig. 1. Sequences of the peptides studied. The residues in the epitopic region have been boxed. The replacements made in the design of the analogs are shown in bold type. Single letter code used: A, alanine; C, *S*-acetamidomethyl cysteine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; Q, glutamine; U, α-aminoisobutyric acid; Y, tyrosine.

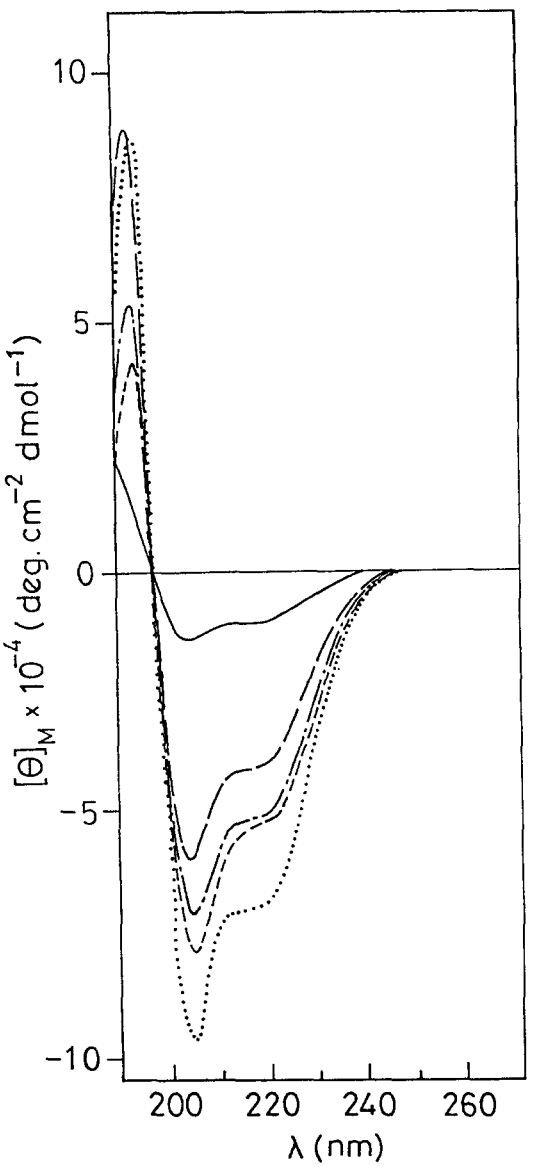

Fig. 2. Circular dichroism spectra of the peptides in 50% 2,2,2-trifluoroethanol–100 mM phosphate buffer (pH 7.0). Peptide concentrations were in the range of 0.22 mM. CD band intensities are plotted as molar ellipticities in deg·cm^2·dmol^{-1}. Y-21 (———), YAib-21 (----), HC-21 (– – –), EC-21 (–·–·–) and HE-21 (·····).

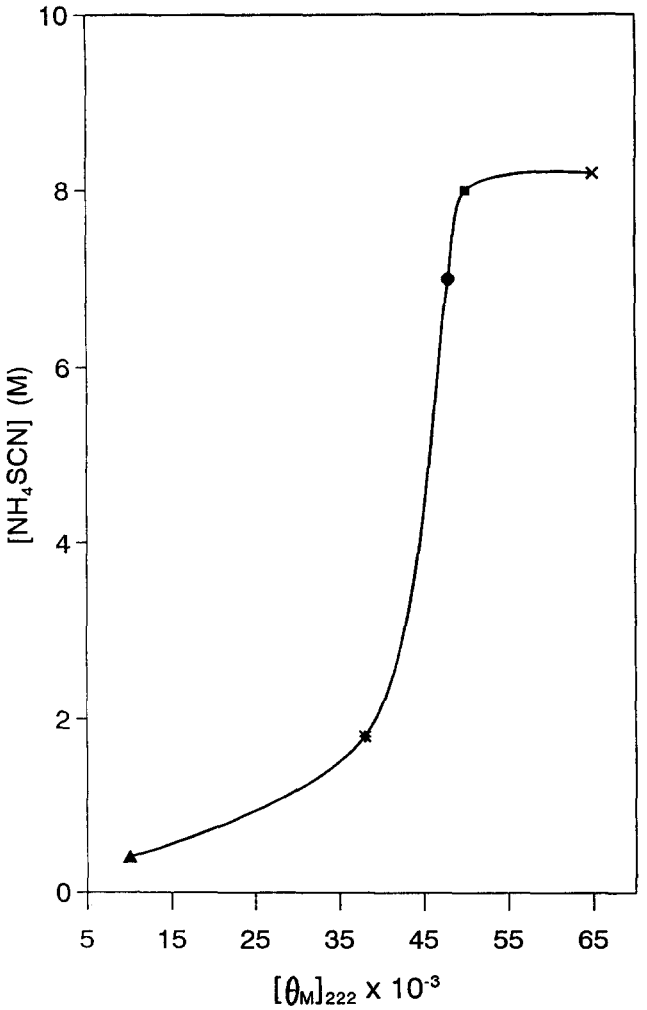

Fig. 3. Plot of the molar ellipticity at 222 nm ($[\theta_{222}]_M$/deg·cm^2·dmol^{-1}) as monitored by circular dichroism versus the relative affinity indices of the antisera. Resistance to thiocyanate elution in a variation of the standard ELISA [15] was utilized as a measure of affinity, the index representing the molar concentration of NH_4SCN required to bring about 50% reduction in initial absorbance. ▲, Y-21; ■, YAib-21; *, HC-21; ●, EC-21; ×, HE-21.

ing 150 mM NaCl for 2 h. The unoccupied sites were blocked with 0.1 M phosphate buffer containing 150 mM NaCl and 0.5% BSA for 1 h, followed by incubation with antipeptide antisera appropriately diluted in the same buffer for 2 h at 37°C. Thereafter anti rabbit IgG conjugated to alkaline phosphatase (1 : 1,000 dilution) was added. The binding of the antibody was quantitated using *p*-nitrophenylphosphate (1 mg/ml) in 1 M diethanolamine-HCl buffer, pH 9.8, containing 0.5 mM $MgCl_2$ as substrate. Relative affinities of the peptide antibodies to the native protein were estimated by the method of Macdonald et al. using thiocyanate elution [27].

3. Results and discussion

Fig. 2 shows the far UV circular dichroic (CD) spectra of all the five peptides in 50% aqueous 2,2,2-trifluoroethanol (TFE). Enhanced helicity as determined by the intensity of the 208 and 222 nm bands is observed for all the designed analogs as compared to the native Y-21. The synthetic peptides did not show appreciable helicity in aqueous buffers but required addition of at least 20% TFE to induce stable secondary structures. Poly-

clonal antisera were raised to peptide-diphtheria toxoid conjugates [26] in rabbits and their cross reactivity evaluated against the native protein and various peptide antigens. Fig. 3 shows a plot of molar ellipticity at 222 nm versus the affinities of their antisera to the native protein antigen, as estimated using a chaotropic agent ammoniumthiocyanate [27]. A very good correlation is observed between the helicity of the peptide antigen and the relative affinities of the derived antibody for the native protein, with similar affinities being observed for the analogs with the highest helical content. Besides, the antibody titers were higher in the case of designed analogs suggesting enhancement of both immunogenicity and antigenicity. A further point of interest is that antibodies raised to the native, flexible antigenic peptide (Y-21) bind weakly to the conformationally constrained antigen (YAib-21). In contrast, antibodies raised to the more ordered peptide YAib-21 bind strongly to the flexible native peptide Y-21 (manuscript in preparation). Both sets of antibodies bind to the native protein. These results suggest that induced fit of the flexible antigen at the antibody combining sites may be operative [8].

The above results conclusively demonstrate that the presentation of the epitope on a helical scaffolding has greatly enhanced the affinity of the corresponding antibodies to the native protein antigen. In the past, attempts to engineer antigenic determinants have focussed mainly on super secondary structures [12–15]. While these strategies have their own merits, one drawback lies in the fact that the designed sequences are quite long (often in the range of 35–80 residues). The present strategy however, emphasizes the stabilization of relatively short peptide fragments. Thus the speed and ease of such syntheses together with simple design principles should greatly simplify the construction of conformationally defined epitopes.

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