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# The Conformational Restriction of Synthetic Peptides, Including a Malaria Peptide, for Use as Immunogens

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# The conformational restriction of synthetic peptides, including a malaria peptide, for use as immunogens

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A new strategy is advanced for the conformational restriction of peptidyl immunogens. Our approach is to replace putative amide–amide hydrogen bonds with covalent hydrogen-bond mimics. Because on average every other amino acid in a protein engages in this bond, the syntheses of diversely shaped peptides can be contemplated. Synthetic methods for introducing a potential hydrogen-bond mimic into a peptide with α-helical potential is reported and the structural consequences are discussed.

The replacement of the hydrogen bond with a chemical link will modify as well as shape the peptide. To explore the consequences of these changes, a potential synthetic vaccine for malaria, the repeating tetrapeptide Asn-Pro-Asn-Ala, was conformationally restricted. Antibodies to the shaped malarial peptide showed a strong cross reaction with *Plasmodium falciparum* sporozoites.

## Introduction

Short peptides are largely disordered structures in water (Creighton 1983) and generally lack the biological activity of the ordered cognate sequences in the protein (Komoriya *et al.* 1984). It was therefore surprising to find that synthetic peptides induce antibodies that bind well to proteins (Lerner 1984). The results were unanticipated, because antibodies to disordered structures were not expected to bind to protein surfaces, which are presumably ordered.

As more information has become available, it has become clear that anti-peptide antibodies show activities that range from those which complex proteins quite strongly to those which bind surface amino acids poorly or not at all (Gariépy *et al.* 1986). This had led to studies that attempt to identify the requirements for a binding reaction. Evidence in favour of two possible mechanisms has emerged. First, small populations of peptides may be partly or even completely ordered in solution (Bierzynski *et al.* 1982; Dyson *et al.* 1985). As potential folding intermediates that may ultimately be retained in the final protein structure, short peptides could give rise to antibodies with binding pockets complementary to protein surfaces. Secondly, antibody binding pockets or the epitopes to which they bind may be able to adjust their surfaces for a complementary match without an undue expenditure of energy (Tainer *et al.* 1985).

The observation of anti-peptide antibody–protein union has thus forced us to re-examine both peptide and protein structure. Although we have learned that some peptides are partly structured in water and we are more appreciative of mobile protein surfaces, the earlier notion that antibody–protein interactions involved the interaction of rigid, complementary surfaces may still be operative in many instances (Amit *et al.* 1986). This could prove problematic in the

case of synthetic vaccines directed to specific neutralizing epitopes, which may not prove adaptable to binding pockets formed against disordered peptides.

One approach to improving the effectiveness of a synthetic peptide for use as a vaccine would be to restrict its conformation to that assumed by the cognate sequence in the protein. Early experiments by Arnon *et al.* (1971) identified this as a workable strategy by showing that a lysozyme peptide cyclized by disulphide-bond formation was an effective immunogen. However, disulphide bonds are infrequent in proteins, and even when they can be employed in peptides they do not guarantee a native structure.

## Covalent hydrogen-bond mimics

A general strategy for shaping peptides is clearly needed. The problem, however, is daunting considering the countless number of structures present in proteins. None the less, an examination of the features responsible for protein structure shows the hydrogen bond to be a common element in these diverse structures (Baker & Hubbard 1984). The most frequent type of hydrogen bond is that formed between amide groups (figure 1). On an average, every other amino acid in a protein is hydrogen bonded in this manner. Of greater interest is the fact that different patterns of hydrogen bonds define different structures. For example, α-helices are characterized by a series of hydrogen bonds from the amide hydrogens of the $(i+4)$ amino acids to the carbonyl oxygens of the $i$ amino acids, whereas β-sheets show intrastrand amide–amide hydrogen bonds separated by a variable number of amino acids.

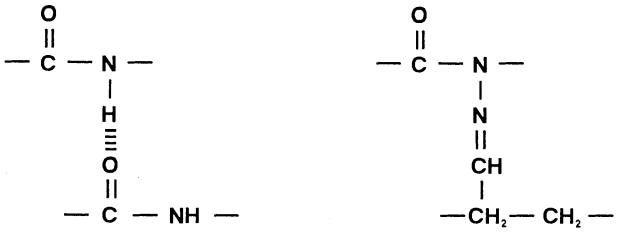


Figure 1. A comparison of the amide–amide hydrogen bond with the hydrazone–ethane covalent mimic.

Except for a few cases, synthetic peptides show no evidence of intramolecular hydrogen bonding in water. Because the amide–amide hydrogen bond is of similar length ($2.0 \pm 0.2$ Å†) and angle ($155° \pm 15°$, N–H···O) in diverse structures we reasoned that different protein structures might be mimicked by replacing putative amide–amide hydrogen bonds in peptides with covalent replacements (Arrhenius *et al.* 1987). This approach, which seeks to reduce a very complex problem to a manageable level, has both potential strengths and limitations. Its strength is that a single chemistry might be employed at different sites in the peptide chain for the generation of a large number of differently shaped peptides. Its potential limitation derives from the fact that there is no perfect covalent hydrogen-bond mimic. Any covalent substitute will differ in bond length, angles, charge and size from the hydrogen bond and effects of these changes on structure are difficult to predict at present.

After examining space-filling molecular models of potential hydrogen-bond mimics, we

† 1 Å = $10^{-10}$ m = $10^{-1}$ nm.

decided to focus on the hydrazone–ethane link (figure 1). The centrepiece of this mimic is a carbon–nitrogen double bond, which replaces the hydrogen bond. Associated changes are required and a hydrocarbon link replaces an amide bond. The covalent mimic is readily substituted into Corey–Pauling–Koltun (CPK) space-filling molecular models of α-helical and antiparallel β-sheet structures. Model building, however, provides no guarantee that the predicted structure will be formed. In fact there are no reliable predictive methods at present (Rose *et al.* 1985). One must simply make a best guess, synthesize the molecule, determine its structure and improve the structure with further syntheses if necessary.

As an initial synthetic target we decided to replace the $(i+4) \rightarrow i$ hydrogen bond in one turn of an α-helix (A, figure 2) with the hydrazone–ethane link. A series of peptides incorporating this link (B, C, D, figure 2) have now been synthesized. These compounds were made by first linking modified peptides to give a precyclization intermediate (figure 3). Cyclization was achieved in the presence of boron trifluoride etherate. Protection of the amide adjacent to the dimethylacetal with a methyl or t-butyloxycarbonyl (t-BOC) group prevents a competing cyclization reaction. When an unprotected Leu is substituted in this adjacent position, the competing reaction opens up a pathway leading to the dimer. The cyclized monomeric intermediate is then converted to final products by using standard reactions. The primary structures of compounds B–D were confirmed by high-resolution mass spectroscopy and proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy. The full details of the synthesis of these compounds will be reported later.

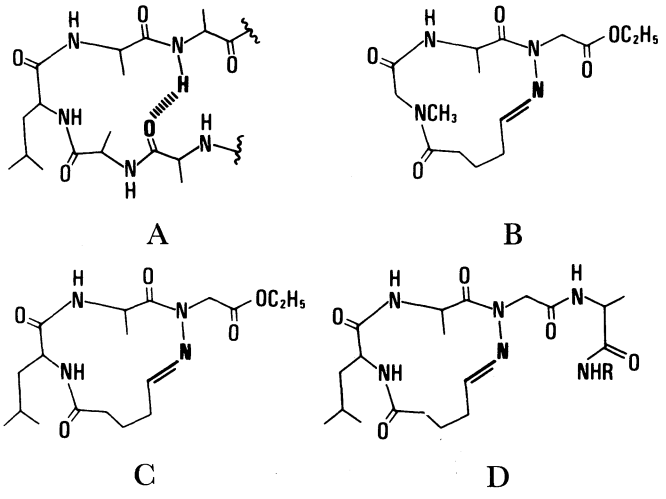


FIGURE 2. Comparison of a hydrogen-bonded turn of an α-helix (A) with peptides incorporating the hydrazone–ethane mimic; B, sarcosine analogue; C, leucine analogue; D, leucine analogue with a peptide extension.

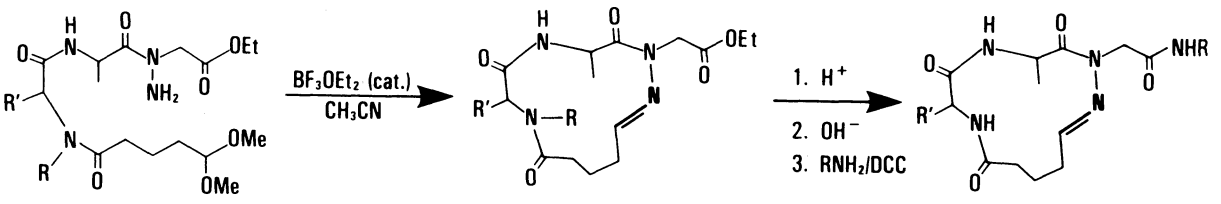


FIGURE 3. Synthetic scheme for inserting the hydrazone–ethane hydrogen bond mimic into peptides.




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Conformational analysis was done by using various NMR studies including nuclear Overhauser enhancement (NOE) experiments. NOE experiments provide through-space information by identifying protons that are within 4.5 Å of each other, although in small peptides the distance is generally shorter (Bruch *et al.* 1985). Previously, we had reported (Arrhenius *et al.* 1987) NMR results that suggested that the sarcosine analogue (B, figure 2) was a mixture of at least two conformers in chloroform $d_1$, probably owing to *cis*/*trans* isomerization at the *N*-methylated amide bond. Sarcosine has now been replaced by Leu to give C (figure 2), which shows only one set of NMR signals in different organic solvents including chloroform $d_1$ as would be expected for a *trans* Leu peptide bond. When C was mixed with trifluoroethanol $d_3$, the Leu amide proton exchanged at a much more rapid rate than the Ala amide proton, suggesting that C exists as a stable conformer in this solvent. As with B, strong NOEs were observed between the hydrazone proton and the *N*-aminoglycine α-protons in chloroform $d_1$, providing evidence for a *trans* hydrazone link. Significantly, weak NOEs between the amide protons, which are required of a helical peptide (Wagner *et al.* 1986), were observed in several solvents including chloroform $d_1$. However, the coupling constant between the α-proton and amide proton ($J_{\alpha n}$) for Leu (8.4 c.p.s.) and Ala (9.1 c.p.s.) in chloroform $d_1$ deviates from that predicted for an ideal α-helix (4.2 c.p.s.).

The NMR experiments supported a relaxed α-helical structure for C in which the pitch has been reduced or lost. Perhaps this is what would occur if one turn of a helix was excised from a protein and lost all but one of its hydrogen-bonding partners. We are extending the *C*-terminal end of the Leu analogue with a polypeptide (D, figure 2) to determine whether it might serve as a nucleation site for helix formation.

The replacement of a hydrogen bond with a covalent mimic will chemically modify the peptide as well as restrict its conformation. This could affect immunogenicity in a number of ways. For example, although conformational restriction may induce antibodies with binding pockets more complementary to protein surfaces, a cross reaction is involved and some binding energy would be expected to be lost if the binding pocket were directed against the covalent modification (Eisen 1980). On the other hand, increased titres might result indirectly from a lowered susceptibility of the modified peptide to proteolysis and/or in response to the increased 'foreignness' of the modified peptide (Schechter & Sela 1967). To explore these different possibilities, we have conformationally restricted a potential synthetic vaccine for malaria.

## Conformationally restricted malarial peptides

*Plasmodium falciparum* is the most vicious form of malaria, infecting over 100 million people annually. The sporozoite form of malaria is injected into the bloodstream of the human by the female *Anopheles* mosquito. It travels rapidly to the liver where it begins a cycle of infection. The surface of the sporozoite is covered with a circumsporozoite (CS) protein that induces antibodies to a repeating tetrapeptide sequence, Asn-Ala-Asn-Pro, which is believed to play a role in protective immunity (Zavala *et al.* 1985). Both a synthetic peptide (Herrington *et al.* 1987) and recombinant vaccine (Ballou *et al.* 1987) based on this repeating sequence have been tested in humans and found to protect some, but not all, vaccinees from sporozoite infections. The repeating peptide seemed an excellent target for exploring the effects of conformational restriction.

As with many potential neutralizing epitopes, the native conformation assumed by the




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tetrapeptide repeat on the sporozoite surface is unknown. None the less, the relatively simple repeating unit invites proposals. Chou–Fasman (1974) analysis indicates that the frame-shifted repeat, Asn-Pro-Asn-Ala, has a strong preference for a reverse-turn conformation. Other proposals have been made on the basis of more complex calculations (Gibson & Scheraga 1986; Brooks *et al.* 1987) but no agreement on structure has been reached. On the other hand, high-resolution NMR studies of acetyl-(Asn-Ala-Asn-Pro)$_3$-$NH_2$ and acetyl-(Asn-Pro-Asn-Ala)$_3$-$NH_2$ suggest that a fraction of the peptide forms reverse turns or helical structures in water (Satterthwait *et al.* 1987).

An examination of space-filling molecular models of Asn-Pro-Asn-Ala in a reverse-turn conformation revealed that the sidechains of Asn can readily form an amide–amide hydrogen bond. In accordance with our strategy we replaced the hydrogen bond with a covalent link in an attempt to restrict the conformation. Because sidechain carboxamide groups rather than main-chain amides were to be joined, a more convenient ethylene bridge can be used.

Three series of peptides (table 1) were synthesized by utilizing the techniques of solid-phase peptide synthesis. Included were native peptides, X-peptides, which incorporate β-*N*-methyl-L-Asn in place of L-Asn, and the shaped peptides where the Asn sidechains are linked with an ethylene bridge. Each peptide is a dodecamer, consisting of three tetrapeptide repeats, and thus corresponds to the synthetic vaccine. The X-peptides contain the chemical elements of the ethylene bridge but are not conformationally restricted. By comparing the response to X-peptides and the shaped peptides in immunological assays, it is possible to distinguish steric effects from conformational effects. Two shaped peptides were synthesized with asparagine joined around proline (peptide A) and around alanine (peptide B) (figure 4). Peptide A was designed to give a series of conformationally restricted reverse turns and peptide B provides an alternative structure for comparative purposes. The synthesis of the shaped peptides has been briefly described (Satterthwait *et al.* 1988). Although we have not completed our structural investigations of peptide A, NMR experiments clearly indicate that each cyclic unit has a rigid structure in water.

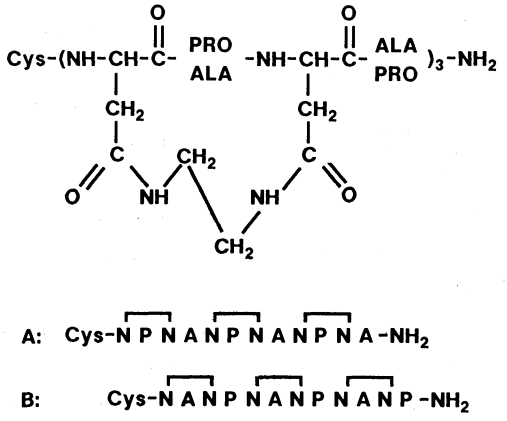


FIGURE 4. Conformationally restricted malarial peptides. The cross bars symbolize an ethylene bridge that links asparagine sidechains.

Polyclonal rabbit antisera were raised against both native and shaped cysteinyl-peptides linked to keyhole lymphet haemocyanin (KLH) with m-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBS) by using standard procedures (Lerner *et al.* 1981). Titres determined

Table 1. Synthetic immunogens

(N = L-asparagine; P = L-proline; A = L-alanine; X = β-*N*-methyl-L-asparagine; the cross bar symbolizes an ethylene bridge that links together asparagine sidechains as shown in figure 4.)

$Cys\text{-}(NPNA)_3\text{-}NH_2$
$Cys\text{-}(NANP)_3\text{-}NH_2$
$Cys\text{-}(XPXA)_3\text{-}NH_2$
$Cys\text{-}(XAXP)_3\text{-}NH_2$
$Cys\text{-}(\overline{NPNA})_3\text{-}NH_2$
$Cys\text{-}(\overline{NANP})_3\text{-}NH_2$

Table 2. Immune response as shown by titres

(X = β-*N*-methyl-L-asparagine; the cross bar symbolizes an ethylene bridge that links asparagine sidechains as shown in figure 4.)

| | antibody | |
|---|---|---|
| antigen | anti$(NPNA)_3\text{-}NH_2$ | anti$(\overline{NPNA})_3\text{-}NH_2$ |
| Cys $(NPNA)_3\text{-}NH_2$ | 5120 | 640 |
| Cys $(NANP)_3\text{-}NH_2$ | 2560 | 80 |
| Cys $(XPXA)_3\text{-}NH_2$ | 10 | 10024 |
| Cys $(XAXP)_3\text{-}NH_2$ | < 10 | 2560 |
| Cys $(\overline{NPNA})_3\text{-}NH_2$ | < 10 | 40096 |
| Cys $(\overline{NANP})_3\text{-}NH_2$ | < 10 | 20 |
| recombinant cs Protein | 5120 | 5120 |
| glutaraldehyde-fixed sporozoite | 10270 | 5120 |
| living sporozoite | | 2560 |

with enzyme-linked immunosorbent assays (ELISAs) of antisera from the best responders to native peptide and peptide A are compared in table 2.

A major difference between the responses to the native and shaped peptides is apparent in the cross reactions. Although the anti-peptide antiserum shows little or no reaction with the chemically modified peptides, the converse is observed; antibodies to the shaped peptide cross react with native sequences. These results can easily be accounted for, because the chemically modified X-peptides and shaped peptides are larger than the native sequences and should not be readily accommodated by the smaller binding pockets of the anti-peptide antibodies. On the other hand, the binding pockets formed against shaped peptide A are presumably larger, which could account for the cross reaction with the smaller native sequences.

Interestingly, although the peptide A antiserum cross reacts with both X-peptides demonstrating an affinity for the frame-shifted sequence, it shows little or no reaction with peptide B, suggesting that the anti-peptide A antibodies are conformationally specific. Of greater significance, however, is the observation that the antipeptide A antiserum reacts strongly with the cs protein and the glutaraldehyde-fixed sporozoite. To confirm that the anti-peptide A serum cross reacts with the native form of the cs protein it was titrated against living sporozoites with an immunofluorescent assays. A strong reaction was observed. This important result demonstrated for the first time that chemically modified, conformationally restricted peptides can serve as effective immunogens.




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## Discussion

Conformation is believed to play an important role in the immune response to native proteins. Because synthetic peptides are largely disordered structures in water, attempts have been made to restrict their conformation. Previous efforts to shape immunogens made use of Cys residues to form disulphide bonds (Arnon *et al.* 1971). Although disulphide-bond formation provides a natural way to restrict conformation, it is of limited use when the large number of different structures found in proteins is considered.

An alternative approach is to restrict peptide conformation chemically. Our strategy is based on the replacement of the amide–amide hydrogen bond with a covalent mimic. Because on average every other amino acid in a protein engages in an amide–amide hydrogen bond, a much more diverse set of structures can be imagined. However, the hydrogen bond is chemically and structurally unique and no replacement will prove an exact match. It remains to be seen whether a hydrogen-bond mimic can structure peptides. The chemistry we have developed for substituting a hydrazone–hydrocarbon link for the hydrogen bond now allows us to explore this possibility.

A separate question is whether chemically restricted peptides can serve as effective immunogens. Chemically modified amino acids are required and there is no guarantee that antibodies against chemically modified peptides will react with native peptidyl sequences. However, the shaped malarial peptide A proved to be an effective immunogen, generating conformationally specific antibodies that show a strong cross reaction with live sporozoites. Thus for malaria, we are in a position to explore more fully the indirect and direct consequences of using chemically shaped peptides as immunogens.

We acknowledge Beverly Hays for her technical assistance. Table 2 is reproduced with permission from *Vaccine* (Satterthwait *et al.* 1988). Work on the malarial peptides was initiated with a grant from the Rockefeller Foundation and continued with support from the MacArthur Foundation. This is publication no. 5510-MB from the Research Institute of Scripps Clinic.

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