Delineation and Conformational Analysis of Two Synthetic Peptide Models of Antigenic Sites on Rodent Cytochrome *c*†

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ABSTRACT: Two regions of rodent cytochrome *c*, one within the first four residues of the molecule, which is N-acetylated, and one at a β bend around residue 44, are known to be immunogenic and antigenic in rabbits. Using sequential peptide synthesis, we have determined the residues required for linear synthetic peptides within these sequences to bind to antibody raised in rabbits to intact rat cytochrome *c*. The residues that were important in binding the N-terminal peptides were *N*-acetylglycine at position 1 and valine at position 3. The smallest peptide sequence around residue 44 that would bind to antibodies was Gln-Ala-Ala-Gly-Phe. A theoretical conformational analysis of these peptides showed that the amino-terminal tetrapeptide adopts a wide statistical ensemble of conformational states and that the addition of residues beyond 41 and 45 in the other sequence does not appear to stabilize longer peptides in the native β-bend conformation. Thus, the antigenicity conferred by Phe-46 and Gln-42 in this peptide is most likely due to the direct interaction of the side chains of these residues with the antibody binding site. The demonstration here that native conformation is not essential for antigenic peptides to bind to antibodies raised against the whole protein indicates that the association energy between antigen and antibody can be sufficient to induce conformation in conformationally flexible peptides. This supports the concept that anti-protein and anti-peptide antibodies may invoke conformational changes in cross-reactive protein antigens and may explain why longer peptides, which may adopt stable nonnative secondary structure, often do not bind to antibodies raised to the whole molecule.

The recognition of local surface conformation is fundamental to the biological function of proteins such as enzymes, hormone receptors, and antibodies. The specificity of the immunoglobulin binding site for localized surface recognition sites (epitopes) in proteins has provided the protein chemist with a library of unique probes of surface structure for proteins with which to study biological recognition at the molecular level. Information about the surface conformation of proteins can be obtained by studying the immunochemistry of well-characterized proteins for which the native structure and sequence are known, preferably for a number of species. Cytochrome *c* (cyt *c*)[1] is such a protein since there is a wealth of structural data at both the primary (Carlson et al., 1977; Margoliash et al., 1961; Needleman & Margoliash, 1966; Borden & Margoliash, 1976) and tertiary level (Dickerson et al., 1971; Swanson et al., 1977; Takano et al., 1977) that has been published for this molecule.

Studies on the immunological cross-reactivities of families of proteins from different species with sequence homologies have demonstrated the importance of evolutionarily variant residues in the immunogenicity of these molecules. Such studies have demonstrated that a single amino acid substitution can render immunogenic an area on the surface of protein molecules such as lysozyme (White et al., 1978; Prager & Wilson, 1971; Prager et al., 1972; Ibrahimi et al., 1979; Fujio et al., 1968; Takagachi et al., 1980), myoglobin (Hurrel et al., 1977; East et al., 1980, 1982; Leach, 1983), and cytochrome *c* (Urbanski & Margoliash, 1977a; Jemmerson & Margoliash, 1979a,b). Urbanski & Margoliash (1977a,b), on the basis of such fine specificity studies, have identified three potential sites on rodent cyt *c* that are immunogenic in rabbits. One of these sites occurs in the intact molecule as the $i + 1$ position of a β bend at sequence position 44 which is an evolutionarily variant residue within mammalian species, being valine in rabbits, alanine in rodents, and proline in ruminants. Using a synthetic peptide fragment that represents the sequence from residues 42 to 50 in intact bovine cyt *c*, Atassi has shown that this region is reactive with rabbit anti-bovine cyt *c* antibodies (Atassi, 1981). Recent work from this laboratory (Jemmerson et al., 1985) has identified a fourth site in an evolutionarily conserved region of mammalian cyt *c* within the first four residues of the molecule that is both immunogenic and antigenic in rabbits. A peptide, conjugated to bovine γ globulin, that contains this region was shown to elicit antibodies in rabits that recognize not only the immunizing peptide but also two other peptides which contain the same four amino-terminal residues as well as native rabbit cyt *c*. Furthermore, antisera raised in rabbits immunized with rodent cyt *c* included antibodies that reacted with synthetic peptides containing this evolutionarily conserved region (Jemmerson et al., 1985).

In this paper, we have further defined the residues essential for antibody binding for two of the antigenic regions described above for rat cyt *c*, i.e., the amino-terminal tetrapeptide and the β-bend region around residue 44, using a procedure in which sequential peptide synthesis is followed by radioimmunoassay while the peptide is still attached to the solid phase support (Smith et al., 1977). We found that the smallest peptide sequence around residue 44 that would bind to antibodies was Gln-Ala-Ala-Gly-Phe which is the sequence from residues 42 to 46 in rat cyt *c*. The reactivity of the amino-terminal tetrapeptide was largely determined by the N-acetylated glycyl residue at position 1; N-acetylation occurs naturally as a posttranslational modification in vertebrate

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[1] Abbreviations: cyt *c*, cytochrome *c*; FMOC, fluorenylmethyloxycarbonyl; *t*-Boc, *tert*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; BGG, bovine γ-globulin; BSA, bovine serum albumin; CDI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; RIA, radioimmunoassay.

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cytochromes *c* (Margoliash, 1972). However, the reactivity of the peptide increased with increasing chain length and was significantly enhanced by valine in position 3.

Having determined the length and sequence necessary for these peptides to bind to anti-rat cyt *c* antibodies, we have used conformational energy calculations to determine what role, if any, conformation plays in the antigenicity of these peptides. The conformational space of the shorter of the two peptides has been defined, and we have also investigated the possibility that the enhanced antigenicity of longer peptides from the β-bend region of cyt *c* is due to the stabilization of native structure in the peptide. The calculations show that the antigenic tetrapeptide from the amino-terminal region of cyt *c* is conformationally flexible with no unique low-energy minima in its conformational space. In addition, the antigenicities of the peptides from the region around Ala-44-Gly-45 are not related to different propensities of the peptides to adopt the conformations they would have in the native molecule; we conclude, therefore, that the additional amino acid residues convey antigenicity by direct interaction with the antibody binding site.

The demonstration here that peptide fragments of five residues and smaller can bind to antibody raised to whole proteins is in contrast to the findings of other workers (Green et al., 1982; Neurath et al., 1982; Delbende et al., 1983) using larger peptide fragments which, often, will not bind to antibodies against native molecules. Longer peptides may well adopt stable secondary structure which is not compatible with an immunoglobulin combining site created in response to native conformation. If small antigenic peptides are conformationally flexible, however, they may be capable of being induced into native conformation by antibody binding despite the entropic forces to be overcome.

Materials and Methods

Theoretical Conformational Analysis. Conformational energies were calculated by using ECEPP[2] (empirical conformational energy program for peptides) (Zimmerman et al., 1977) which uses the empirical potential energy functions and energy parameters derived by Momany et al. (1975). The total conformational energy of the molecule is calculated as the sum of the electrostatic, nonbonded, and torsional interaction energies between all atoms; hydrogen atoms are considered explicitly. The hydrogen bond energy is included in the nonbonded energy component. In order to obtain the precise location of the minimum energy conformation of a peptide in conformational space, ECEPP was used in conjunction with a function-minimizing subroutine (Powell, 1964). The energy was minimized with respect to all dihedral angles of each residue; minimization was terminated when the conformational energy changed by less than 0.001 kcal/mol between successive calculations.

Hydrogen bonds were located in low-energy minima by an algorithm that analyzes all possible hydrogen bonds by calculating all H–A distances where H is a polar hydrogen atom and A is a proton acceptor (an oxygen or nitrogen atom). If the H–A distance is less than or equal to 2.3 Å, the two atoms are considered to be in a hydrogen bond (Zimmerman et al., 1977).

Peptide Synthesis. Peptides were synthesized on a polyamide resin (Chemical Dynamics, South Plainfield, NJ) with a cleavage-resistant sarcosylethylenediamine spacer arm using N^{α}-FMOC-amino acid derivatives, *t*-Boc and *t*-Bu side chain protecting groups, and standard coupling procedures (Arshady et al., 1979, 1981; Atherton et al., 1978, 1981). The FMOC group was cleaved by 20% piperidine between couplings, and a sample of the peptide resin was removed at each coupling step in the synthesis for subsequent deprotection and radioimmunoassay. The side chain protecting groups were cleaved by 2 M HBr in acetic acid/TFA (1:1) with the fully deprotected peptide remaining attached by its carboxy terminus to the resin. The peptide sequences representing the amino terminus of cyt *c* were N-acetylated before side-chain deprotection using 10% acetic anhydride and 10% diisopropylethylamine in dimethylformamide. Two free synthetic peptides, which represent regions 41–49 and 1–9 in rat cyt *c*, were custom-synthesized by Peninsula Labs, San Fransisco, CA, and used to screen anti-rat cyt *c* antisera for reactivity to the antigenic sites which are the subjects of this study.

The composition of all the synthetic peptides used in this study, whether synthesized by Peninsula or in our laboratory, was confirmed by amino acid analysis.

Immunization Procedures. Cytochrome *c* is only weakly immunogenic in monomeric form; thus, it was used either polymerized with 0.1% glutaraldehyde by the method of Reichlin et al. (1970) or conjugated to the immunogenic protein carrier BGG by using glutaraldehyde (Reichlin, 1980). Standard procedures were used for generating immune responses in rabbits by intradermal injection into positions along the back and in the hind footpads. A primary immunization of 100 μg of polymerized protein or protein conjugate in complete Freund's adjuvant was followed in 2–3 weeks by a secondary immunization in incomplete Freund's adjuvant. Subsequent immunizations, to keep serum antibody titers elevated, took place every 4 weeks and used 100 μg of immunogen in incomplete Freund's adjuvant. The polyclonal antisera were affinity purified on rat cyt *c*–Sepharose columns to isolate only those antibodies that bind to the native protein antigen.

RIA of Rabbit Antibodies on Microtiter Plates. Microtiter plates were coated with antigen (10^{-7} M) incubated for 2–6 h and then blocked for several hours with 10% horse serum in phosphate-buffered saline. The antigens were either crystalline monomeric cytochromes *c* or peptides conjugated to a polylysine carrier to ensure that the peptides bind to the microtiter plates. For the conjugation, 10 mg of carrier poly(L-lysine) (M_r 260 000) in 1 mL of 5 mM potassium phosphate solution, pH 7.0, was added to 4 mg of peptide in 0.1 mL of dimethylformamide. CDI (40 mg) was added and the reaction allowed to proceed at room temperature for 2 h with stirring. Reactants and products were separated by dialysis.

Rabbit antisera were affinity purified by affinity chromatography on rat cyt *c* covalently coupled to cyanogen bromide activated Sepharose 4B (Jemmerson & Margoliash, 1980). The antibody preparations were incubated on antigen-coated plates at various dilutions for 2–4 h; to measure nonspecific binding, plates coated with BSA or carrier polylysine alone were used. The plates were washed with phosphate-buffered saline, and an ^{125}I-labeled, affinity-purified goat anti-rabbit antibody was added. After incubation overnight, the plates were washed, dried, and counted (Pierce & Klinman, 1977).

RIA of Peptide–Polyamide Conjugates. In protic solvents, polyamide resins swell to about 10 times their dry size (Stahl et al., 1980). The large pore size of the beaded polymer in aqueous solutions thus allows access to immunoglobulin

[2] The computer program ECEPP and its description and geometric and energy parameters are available on magnetic tape (QCPE 286) from the Quantum Chemistry Program Exchange, Chemistry Department, Indiana University, Bloomington, IN 47401.

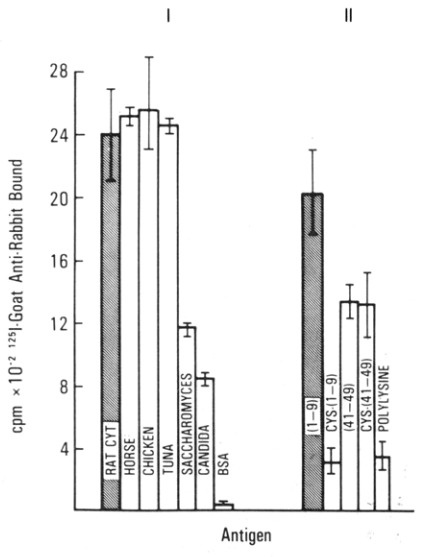

FIGURE 1: RIA of affinity-purified rabbit antiserum to polymerized rat cyt *c* on two groups of antigens. Values shown were from assays where antibody was limiting. (I) Eukaryotic cytochromes *c* and a BSA control; (II) synthetic peptide sequences from rat cytochrome *c* coupled to polylysine carrier and a polylysine control.

molecules during radioimmunoassay. Antibody binding was assessed by binding 200 μL of specific rabbit antibody to 1 mg of the peptide sequences bound to polymer and quantitating the amount of bound immunoglobulin with the addition of 30 000 cpm of an ^{125}I-labeled goat anti-rabbit immunoglobulin-constant region according to the procedure of Smith et al. (1977).

One-milligram samples of dry peptide–polyamide sample were swollen and washed 3 times with 0.1 M borate buffer, pH 8.5, containing Tween 20 (0.2%) and NaCl (0.5 M). Affinity-purified rabbit antibodies from anti-cyt *c* antisera were diluted with this buffer containing 0.5% bovine serum albumin to a protein concentration of 15 μg/mL. At this concentration, peptide antigen on the resin was in excess. Nonspecific binding to the peptide sequences was measured with preimmune rabbit immunoglobulin at 15 μg/mL prepared from the sera of naive rabbits by ammonium sulfate fractionation.

RESULTS

Production of Specific Antisera. The isolation of a population of antibodies from rabbits immunized with polymerized cytochrome *c* which are specific for the evolutionarily conserved antigenic site within the first four residues on mammalian cyt *c* has been described in a recent publication from this laboratory (Jemmerson et al., 1985).

In order to produce antisera with reactivity to the other surface region of interest, i.e., the β-bend region within residues 41–49 in cytochrome *c*, a different immunization procedure was used. Rabbits were primed with a rat cyt *c*/BGG conjugate in complete Freund's adjuvant and then boosted with polymerized cyt *c*. The affinity-purified antisera from these rabbits were screened, by using the solid phase RIA described above, on a variety of cytochromes and on two peptides with the same sequence as two regions from rat cyt *c*, from residues 41–49 and from *N*-acetyl residue 1 to residue 9, with the results shown in Figure 1. It can be seen that this polyclonal antisera contains populations of antibodies directed to both the amino-terminal region of cytochrome *c* and the region around residues 44 and 45.

Delineation of the Antigenic Region around Residue 44. Sequential peptide synthesis coupled with RIA with the reactive antisera described above was used to further delineate the residues within this region that are essential for the binding of antibodies to this region in cyt *c*. Since solid phase peptide synthesis begins at the carboxy terminus and proceeds toward the amino terminus, it is possible to delineate only the amino terminus by sequential sampling, and multiple syntheses are necessary to determine the residues at the carboxy terminus that are integral to the antigenic site. In order to decide at which carboxy-terminal residue, beyond residue 49, to initiate synthesis, we inspected the X-ray crystal structure of tuna cyt *c* (Takano & Dickerson, 1980), the coordinates for which were obtained from the Protein Data Bank, Brookhaven National Laboratory, by using interactive computer graphics. Residues 41–49 occur at a β bend, centered at residues 44 and 45, on the exterior of the molecule. The side chains of residues beyond 47 were buried in the interior of the molecule. Synthesis was started, therefore, at residue 47, in the solid phase, on the polyamide resin and continued through residue 41. The antigenicity of increasingly longer peptide sequences from residue 47 to residue 41 is shown in Figure 2. It can be seen that sequences with a carboxy terminus at residue 47 are antigenic but not until glutamine at residue 42 is added.

Multiple syntheses to determine the carboxy terminus of this determinant were also performed with the results shown in Figure 2b, which shows that although there is weak antigenicity in the short peptides that contain Gly-Gln this is greatly enhanced by the addition of Phe-46. Subsequent synthesis and RIA of Gln-Ala-Ala-Gly-Phe established this as the shortest peptide with antigenicity in this region of cytochrome *c*. Thus, the antigenic determinant in rat cyt *c* around residue 44 requires the residues at positions 42 and 46 for efficient binding to an immunoglobulin binding site raised in response to the whole molecule. Whether this requirement is due to enhanced conformational stability conveyed on the isolated peptide sequence by the presence of these residues or due to their direct involvement in the immunoglobulin binding site was investigated by conformational energy calculations as described below.

Residues Important in the Antigenicity of CH_3CO(1–4). The determinant in the first four residues of the amino terminus of cyt *c* described by Jemmerson et al. (1985) is evolutionarily conserved throughout the vertebrates. Mammals have the sequence *N*-Ac-Gly-Asp-Val-Glu, but substitution of Ile for Val occurs in avian species and Ala for Glu occurs in tuna. The antisera to polymerized cyt *c* described in that study show reduced binding to chicken and pigeon cyt *c* but equivalent binding between the mammals and tuna cytochromes. The binding of this antisera was measured during the sequential synthesis of two sequences which represent residues 1–4 of mammalian cyt *c* and tuna cyt *c*. Results, shown in Figure 3a,b, indicate that acetylated glycine as residue 1 is essential to binding. Although some binding can occur with alanine in all of the other three positions, valine in position 3 greatly improves the affinity. The conformational space of this determinant will be described in the following section.

Conformational Energy Calculations. (A) Conformational Stability of Native Structure in Peptides in Region 41–47. The antigenicity of peptide sequences in the β-bend region of cyt *c* between residues 41 and 47 can be seen from Figure 2a,b to require glutamine (residue 42) and phenylalanine (residue 46). Shorter peptides between these two points do not bind to antibody raised to whole cyt *c*. Type II β bends are stabilized largely by local interactions between the central two residues, in this case, Ala-44–Gly-45, and an $i \rightarrow i + 3$ hydrogen bond (Nemethy & Scheraga, 1980). The sequence

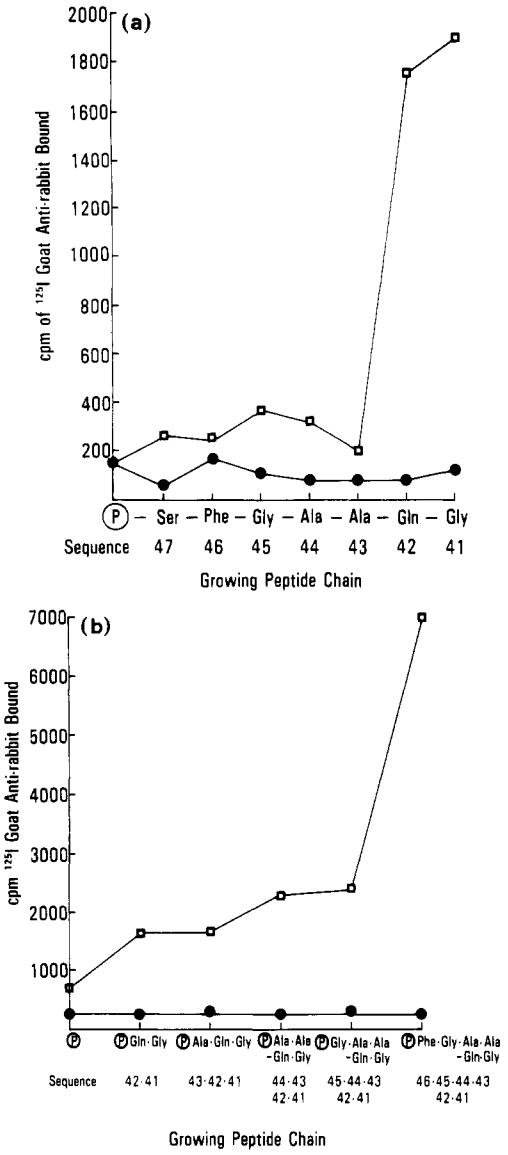

FIGURE 2: (□) Binding of rabbit anti-rat cytochrome *c* antibodies to polyamide resin bound synthetic peptides from region 41–47 of rat cytochrome *c*. (●) Non specific binding to these sequences by preimmune rat antibodies. (a) The peptides become increasingly longer from the carboxy terminus toward the amino terminus. (b) The peptides become increasingly longer toward the carboxy terminus.

Ala-Gly has a high β bend forming probability (Zimmerman & Scheraga, 1978a); thus, short peptide structures containing this sequence might be expected to retain some native structure. The necessity of residues 42 and 46, therefore, to antibody binding may not be because they are an integral part of the antigenic domain but because they stabilize secondary structure that is essential to the presentation of the antigenic site containing the only sequence difference between rat and rabbit cyt *c* in this region at position 44. To test this possibility, energy minimizations were performed on peptide sequences around residue 44 starting with residues 43–46 (Ala-Ala-Gly-Phe) and increasing the length of the peptide sequentially to residues 41–47. The conformation this region adopts in the crystal structure of native cyt *c* was used as the starting conformation.

The crystal structure of tuna cyt *c* provided two geometries for the oxidized structure and one for the reduced structure (Takano & Dickerson, 1980).[3] These were used for the

[3] The coordinates for the geometry of tuna cyt *c* were obtained from the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

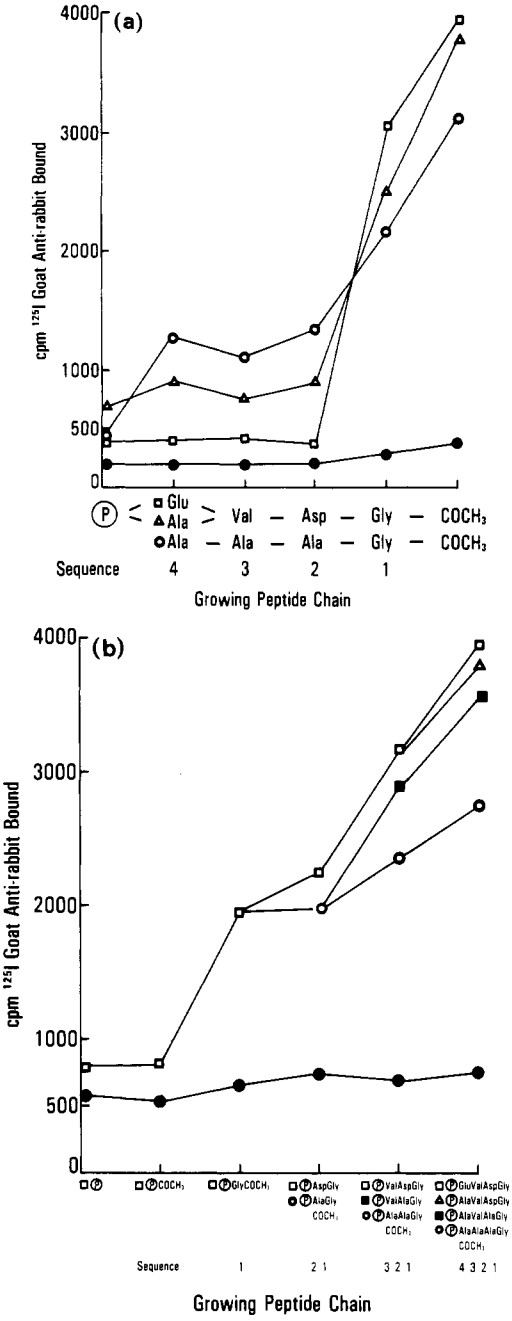

FIGURE 3: Binding of rabbit anti-rat cytochrome *c* antibodies to polyamide resin bound synthetic peptides from region 1–4 of rat (□) and tuna (△) cyt *c* and two nonnaturally occurring analogues (○, ■). Nonspecific binding to these sequences by preimmune rat antibodies was very similar and is shown only for the rat sequence (●). (a) The peptides become increasingly longer from the carboxy terminus toward the amino terminus. (b) The peptides become increasingly longer toward the amino terminus.

backbone dihedral angles and for appropriate side chain angles. Where sequence changes between rat and tuna occur, and where side chain dihedral angles were not specified by the crystal structure, values compatible with the backbone structure were selected from the low-energy minima of blocked single amino acid residues (Zimmerman et al., 1977). The H bonding distance, d_h, of the β bend between the carbonyl oxygen of residue 43 and the amide hydrogen of residue 46 was used as an index for retention of native structure after minimization. Values of this parameter and divergence from d_h for the native molecule are given in Table I.

It can be seen from this table that native backbone structure may be retained by all of the peptide sequences on isolation

Table I: β-Bend H-Bonding Distances for Peptide Sequences between Residues 41 and 47 of Rat Cytochrome *c*

peptide sequence	antigenicity	d_{hm}[b] reduced	d_{hm}[b] oxidized 1	d_{hm}[b] oxidized 2	$d_{hm} - d_{hn}$[a] reduced	$d_{hm} - d_{hn}$[a] oxidized 1	$d_{hm} - d_{hn}$[a] oxidized 2
43–46	–	2.20	2.42	1.98	0.09	0.10	0.19
43–47	–	2.64	2.47	2.04	0.42	0.05	0.13
41–45	–	2.04	2.10	1.98	0.27	0.45	0.19
42–46	+	3.15	2.36	2.81	0.84	0.16	0.64
41–46	+	3.06	2.62	2.25	0.75	0.10	0.08
42–47	+	2.56	3.13	2.07	0.25	0.61	0.10
41–47	+	3.20	2.37	2.05	0.59	0.58	0.12

[a] d_{hm} = H-bonding distance for minimized peptides. [b] d_{hn} = H-bonding distance for native conformation = 2.31 Å for reduced crystal structure and 2.52 and 2.17 Å for the two oxidized crystal structures.

Table II: Low-Energy Conformations of CH_3-CO-Gly-Asp-Val-Ala-CO-CH_3

conformation[a] for the sequence Gly	Asp	Val	Ala	*E* (kcal/mol)
C*	A	A	D	−13.8
C*	A	A	A	−13.6
D	E	A	C	−13.4
A*	G	A	C	−12.7
C*	A	A	C	−12.6
C*	C	A	C	−12.4
C*	A	C	C	−11.0
C*	A	C	A	−10.9

[a] Backbone conformation in conformational letter code.[4]

from the whole molecule although there are changes in orientation of side chains. Computer drawings of some of these peptides are shown in Figure 4 to illustrate these differences. It can be seen that the shorter nonantigenic peptides move into bends with a stronger hydrogen bonding interaction than is found in the native structure.

(B) Conformational Space of CH_3-CO-Gly-Asp-Val-Ala-CO-CH_3. The results of the immunological studies described above indicate that acetylated glycine (residue 1) is essential to binding and that there is improved binding as the peptide chain is lengthened toward glutamic acid or alanine (residue 4). It seems unlikely that such a short peptide would have a unique conformation that binds to antibody. To explore the conformational space of this peptide, 234 conformations were chosen as starting points for energy minimization from the low-energy conformations of the dipeptides Gly-Asp and Val-Ala (Zimmerman & Scheraga, 1978a,b). These represented combinations of all the different backbone conformations within 2 kcal/mol of the global minimum of the dipeptides. In addition to this, three native backbone conformations (from the crystal structure of oxidized and reduced tuna cyt *c*) were also minimized. Conformations within 3 kcal/mol of the global minimum are shown in Table II; the backbone conformation of each amino acid residue is described by a conformational letter code (Zimmerman et al., 1977) in order to focus on the general features of the conformation rather than exact values of dihedral angles.[4]

None of the conformations in Table II are close to the native conformations of the amino terminus of cyt *c*, that is, FCAA, DCAA for oxidized cyt *c*, and FFAA for reduced cyt *c*. All of the native conformations are also not stable when isolated from the whole protein since after minimization FCAA and DCAA converged to CCAA with only DCAA remaining in the same region of conformational space. After minimization, all 237 starting points gave conformations with a fairly continuous spectrum of energy values between −5.5 and −13.8 kcal/mol, indicating that this peptide does not have a unique low-energy minimum in its conformational space.

[4] Conformations are classified in terms of the region of the φ, ψ map in which they occur. The boundaries of the regions which appear in Table II are as follows:

A	$-110 \leq \phi < -40$	$-90 \leq \psi < -10$
A*	$40 < \phi \leq 110$	$10 < \psi \leq 90$
C	$-110 \leq \phi < -40$	$50 \leq \psi < 130$
C*	$40 < \phi \leq 110$	$-130 < \psi \leq -50$
D	$-180 \leq \phi < -110$	$20 \leq \psi < 110$
E	$-180 \leq \phi < -110$	$110 \leq \psi < 180$
G	$-180 \leq \phi < -110$	$-90 \leq \psi < -40$

Discussion

It has been established by Jemmerson & Margoliash (1979b) that rabbit cyt *c* when polymerized by gluteraldehyde is an autoimmunogen and that 80% of the antigenicity of the antibody raised against the self-protein in rabbits can be accounted for by its binding to a CNBr cleavage product representing the first 65 residues of the molecule. From cross-reactivity studies, these authors concluded that the antigenicity of this region of the molecule involved residues at positions 44 and 62. We immunized rabbits with polymerized rat cyt *c* which differs from the rabbit molecule at only one position, 44, in the 1–65 region. The resulting antisera, affinity purified on cyt *c*-Sepharose, bound not only rat cyt *c* but also a wide range of mammalian cytochromes (Jemmerson et al., 1985). It did not, however, bind a 10-residue synthetic peptide, representing region 41–49 in the native molecule, when presented to the antibody coupled to polylysine or to BSA with either a water-soluble carbodiimide or glutaraldehyde (unpublished results); in fact, most of the reactivity of this antisera was to the evolutionarily conserved sequence in mammals, *N*-Ac-Gly-Asp-Val-Glu (Jemmerson et al., 1985). Since the first 10 residues at the amino terminus are common to all mammalian cytochromes *c*, this constitutes B-cell recognition of a "self" sequence.

The results presented in Figure 3 demonstrate which residues in this tetrapeptide are important for binding. The theoretical conformational analysis presented here shows that the peptide adopts a wide statistical ensemble of conformational states. Since the peptide binds to antibody raised in response to the restricted conformational state that pertains to the whole cytochrome, and this binding appears to involve at least three of the four residues in the peptide, this may be evidence that this peptide is induced into native conformation by antibody binding. The predominant conformation of one of the residues important in this interaction, valine at position 3, in all of the conformational states of the peptide with energies within 2 kcal/mol of global minimum is that of this residue in the native conformation. This factor may lower the energy required to induce conformation in the entire peptide.

It is conceivable, however, that this amino acid sequence in native cyt *c* also has conformational flexibility since it is at the amino terminus of the folded peptide chain. If this were the case, polyclonal antisera raised to the native molecule may

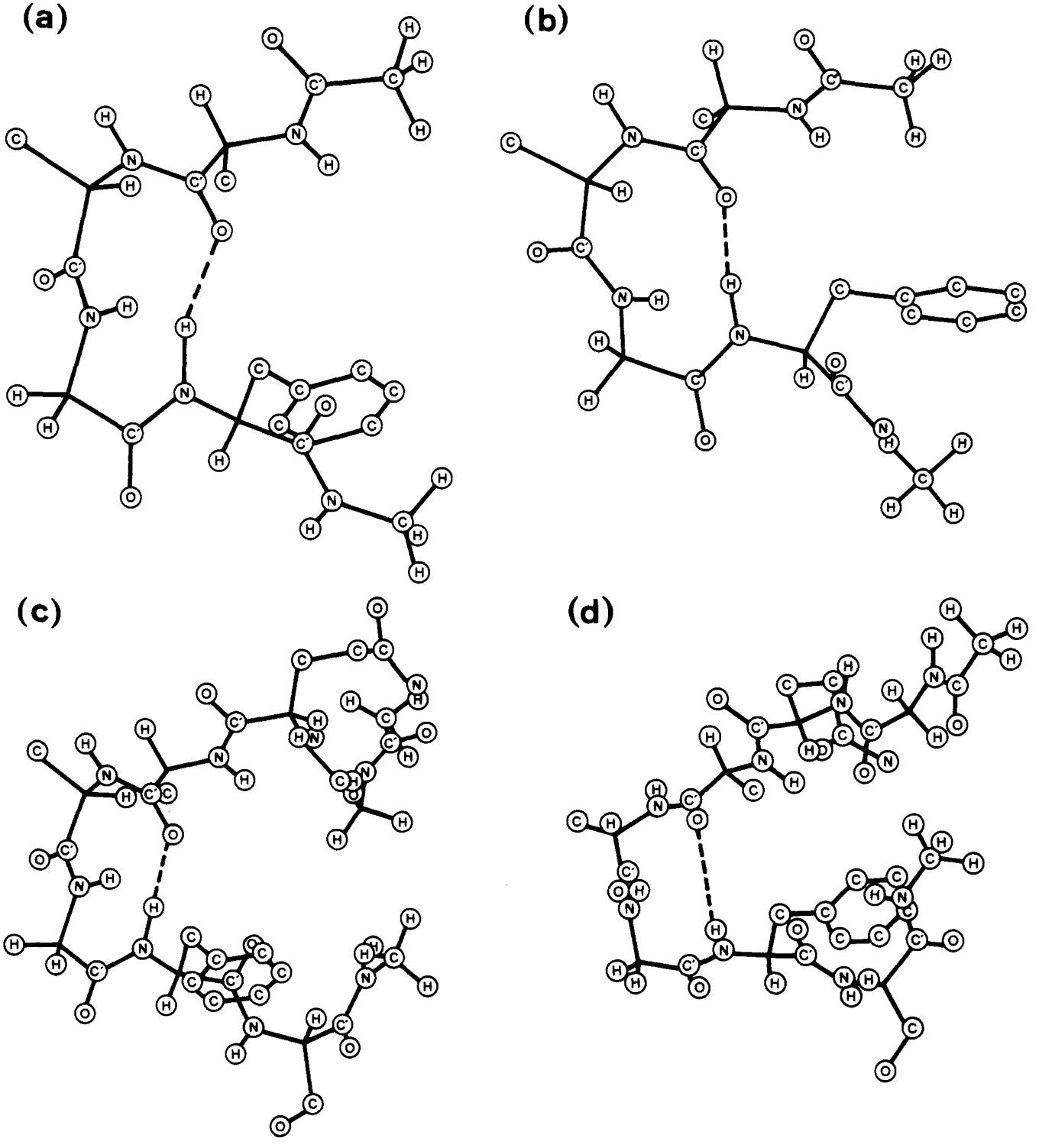

FIGURE 4: Computer drawings of peptide sequences from tuna cyt *c*. The sequences have *N*-acetyl and *N'*-methyl amide blocking groups. Side-chain hydrogen atoms beyond the C^{α} atom are omitted to simplify the representation. C = carbon; C' = carbonyl carbon; N = nitrogen; O = oxygen; H = hydrogen. Dotted lines represent hydrogen bonds. (a) 43–46 (Ala-Ala-Gly-Phe) in the conformation it adopts in the crystal structure of oxidized cyt *c*.[3] (b) 43–46 after energy minimization using the structure shown in (a) as the starting conformation. (c) 41–47 (Gly-Gln-Ala-Ala-Gly-Phe-Ser) in the conformation it adopts in the crystal structure of reduced cyt *c*.[3] (d) 41–47 after energy minimization using the structure shown in (c) as the starting conformation.

contain a population of antibody binding sites all of which bind the same sequence but in different conformations. However, if there is a great deal of flexibility in this region of cyt *c*, it is not reflected in the thermal fluctuations of the atomic positions in crystal structures (Northrop et al., 1980a, 1981). Molecular dynamics calculations also do not indicate that the amino terminus is a particularly flexible region in this molecule (Northrop et al., 1980a,b). Further studies to establish this point are required.

The response to rat cyt *c* coupled to BGG is clearly different than the response to polymerized rat cyt *c*. Although a large component of the polyclonal antisera is directed to the acetylated amino terminus, there is a population of the antibody that binds to peptide 41–49 (see Figure 1). This binding is not blocked by the inclusion of a cysteine at the N-terminus as is the case with the binding to the amino-terminal peptides which indicates that the binding site is within the 41–49 sequence. There also exists a population of antibodies that bind

to the yeast cytochromes (see antigen group I); since there are only two sequence differences between these cytochromes and rat cyt *c* in the region 41–49, this population may well be identical with the antibody that binds to peptide 41–49.

Using sequential peptide synthesis, we have clearly demonstrated that the shortest peptide within region 41–49 with antigenicity to this antisera is Gln-Ala-Ala-Gly-Phe (see Figure 2). Although we have not yet determined whether this peptide adopts a unique stable conformation or whether it is structureless, like the amino-terminal peptide, we have determined that the addition of residues beyond 41 and 45 in the sequence does not appear to stabilize longer peptides in native β-bend conformation. Thus, the antigenicity conferred by Phe-46 and Gln-42 is most likely due to the direct interaction of the side chains of these residues with the antibody binding site.

The demonstration in this study that native conformation is not essential for antigenic peptides to bind to antibodies raised against the whole protein indicates that the association energy between antigen and antibody is sufficient in some cases to induce conformation. This supports the concept derived from previous studies that anti-protein (Celado & Strom, 1972; Lubeck & Gerhard, 1982; Parham, 1984) and anti-peptide (Niman et al., 1983) antibodies may invoke conformational changes in cross-reactive protein antigens.

Added in Proof

The apparently low mobility of the amino terminus of cyt *c* discussed above has been further examined (Tainor et al., 1985) and is attributed to crystal contact.

Acknowledgments

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Effects of the Replacement of a Double Bond by a Cyclopropane Ring in Phosphatidylethanolamines: A ^2H NMR Study of Phase Transitions and Molecular Organization†

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ABSTRACT: The thermotropic behavior and molecular properties of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-dihydrosterculoyl-*sn*-glycero-3-phosphoethanolamine (PDSPE) have been investigated by ^2H NMR spectroscopy using samples selectively labeled at the 5′-, 9′-, 10′-, and 16′-positions of the *sn*-2 chains. Comparison with the corresponding phosphocholine analogues (POPC and PDSPC), obtained as intermediate synthetic products, was used to monitor the role of the polar head group. Replacement of the choline moiety by ethanolamine increased the gel to liquid-crystal transition temperature by 10–32 °C and led to a significantly higher ordering of the fatty acyl chains in the liquid-crystalline bilayer state. The lateral compression effect, due to the smaller area per polar head group in PE, results in a bilayer to hexagonal phase transition at elevated temperatures. The effects on both PC and PE due to replacement of the olefinic group by a cyclopropane unit are similar. A decrease in the temperature of the gel to liquid-crystal phase transition, T_c, is observed upon introduction of a cyclopropane ring; it goes from 26 °C in POPE to ≈10 °C in PDSPE. In addition, a very significant broadening of the transition profile is observed. These observations are consistent with the poor packing ability of mixed saturated and cyclopropane-containing chains due to the bulky substituent effect. The temperature of the bilayer–hexagonal phase transition of PE samples was decreased by 15–20 °C on replacement of oleoyl chains by dihydrosterculoyl chains at the *sn*-2 position. These effects of dihydrosterculic acid have been tentatively correlated with the observation that most of the olefinic chains are replaced by the cyclopropane analogue when certain microorganisms enter the stationary phase of their growth cycle or respond to differing environmental conditions. According to what is observed in biologically relevant model compounds, it may be assumed that this transformation will prevent the lipid molecules from entering the nonviable gel state as well as allow the membrane to contain small proportions of lipid molecules in nonbilayer structures. It also confers a lesser temperature sensitivity of the acyl chain order parameter.

Fatty acids containing a cyclopropane unit are found in a large variety of microorganisms and protozoa where they may occur as permanent constituents of the lipids, such as in *Lactobacillus plantarum* and *Salmonella typhimurium* (Christie, 1969), or, more frequently, appear at a given stage of the growth cycle. For example, in *Escherichia coli*, most double bonds of the fatty acid chains are converted into the cyclopropane-containing analogue when the bacteria enter the stationary phase (Goldfine, 1972). The biological reasons for this energy-consuming transformation are not understood, and very little research has been devoted to this class of fatty acids. The first attempt to define the role of these structures on membrane organization was performed by Silvius & McElhaney (1979) using model phosphatidylcholines (PC) containing two identical fatty acid chains (either olefinic or containing a cyclopropane ring). The cyclopropane moiety was found to raise the gel to liquid-crystal transition temperature (T_c) by some 15 °C for cis systems. However, since naturally occurring phospholipids generally contain mixed acyl chains, one saturated (located mainly at the *sn*-1 position) and one unsaturated, we have investigated model systems with this type of structure. A ^2H NMR investigation of phosphocholine (PC) bearing a cyclopropane-containing fatty acyl chain (Dufourc et al., 1983) established that the effects of replacing an olefinic group with a cyclopropane ring in a mixed-chain system are significantly different from what had been previously observed on PC containing a homogeneous acyl chain composition (Silvius & McElhaney, 1979).

In the present paper, we shall now consider phosphatidylethanolamines (PE) since they represent a ubiquitous class of

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