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Research Article

Localization and analysis of nonpolar regions in onconase

E. Y. Kolbanovskaya[a], A. C. Terwisscha van Scheltinga[b], V. G. Mukhortov[a], W. Ardelt[c], J. J. Beintema[b,*] and M. Y. Karpeisky[a,†]

[a]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, Moscow 117984 (Russia)
[b]Departments of Biochemistry and Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen (The Netherlands), Fax + 31 50 3634165, e-mail: beintema@chem.rug.nl
[c]Alfacell Corporation, Bloomfield (New Jersey 07003, USA)

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Abstract. A detailed analysis of the composition and properties of hydrophobic nuclei and microclusters has been carried out for onconase. Two main hydrophobic nuclei in the onconase structure were detected. Their composition and shape were found to be very similar to those of RNase A, in accordance with the predictions made. The nuclei in onconase are more compact, the side-chain atoms of residues included in the nuclei in onconase form more contacts with the environment than in RNase A. The hydrophobic nuclei should be considered as individual structural units along with elements of the secondary structure. Differences in composition and conformation of exposed loops between onconase and RNase A were found. The additional hydrophobic clusters attached to the nuclei in onconase might be involved in the fixation of an appropriate conformation of site(s) for manifestation of the biological activity of onconase. A comparison of amphibian representatives of the RNase A superfamily was also made. The results obtained suggest that the availability of nonpolar residues in established key positions of amino acid sequences determines the characteristic fold of homologous proteins and the structure of the active site cleft.

Key words. Amphibians; hydrophobic nuclei; nonpolar contacts; onconase; ribonuclease A superfamily.

The rapidly increasing number of structures deposited in protein databases allows extraction of useful information for studies on structure, properties, evolution, and other aspects of proteins by members of the scientific community. But it is not always easy to describe features of protein structures in a comprehensible way. While the description of elements of, e.g., secondary structure and residue accessibility is not too problematic, accurately describing hydrophobic contacts in the core regions of proteins is more difficult. This is a handicap in the abovementioned types of studies, because such core regions are the most conserved in protein evolution [1, 2] and are probably also of prime importance in the folding of nascent chains [3, 4]. We have solved this problem in a simple way by describing hydrophobic nuclei in proteins from analysis of deposited coordinates of atoms and producing contact maps and nonpolar-contact networks (Graf schemes), which give a clear and workable representation of the hydrophobic cores of proteins [5, 6].

With this method, we analyzed hydrophobic nuclei in bovine pancreatic ribonuclease (RNase A) [7] and used these results to predict nuclei composition and some other properties of RNase A homologous proteins with

* Corresponding author.
† M. Y. Karpeisky deceased 19 July 2000. The other authors dedicate this paper to the memory of Marat Karpeisky: our dearest teacher, good friend and highly esteemed colleague.

known amino acid sequences [6, 7]. Proteins of the RNase family show remarkable similarity in position and conservation of the nonpolar residues comprising the cores of the two main hydrophobic nuclei found in the RNase A molecule. However, conformation and exposed loops maintained by nonconservative microclusters are less well conserved. One may assume that differences in biological activities of proteins of the RNase family are associated with position, sequence, and conformation of such exposed loops.

Chotia and Lesk [8] have shown that there is an exponential divergence between three-dimensional structure of homologous proteins with increasing sequence difference. While polypeptide folds are generally still superimposable until about 25% sequence difference, they deviate significantly if sequences differ at more than half of the residues. But folding patterns may still retain conserved features, even when sequence similarity cannot longer be recognized. X-ray structures have been published for several members of the RNase A family (angiogenin, EDN, onconase, RNase 4), which differ by more than 50% in amino acid sequence from RNase A and each other. While overall folding patterns of these proteins are still similar and most amino residues contributing to the enzymic active sites have been conserved, there are interesting structural and sequence differences which may be responsible for differences in biological properties.

Members of the RNase A family with the most deviating sequences relative to RNase A originate from amphibian sources (75–80% sequence difference). The best-characterized representative of these is onconase, a protein component of extracts of *Rana pipiens* oocytes and early embryos with high cytostatic and cytotoxic activity against numerous mammalian cell lines in vitro [9], as well as anti-tumor activity in vivo [10, 11]. It also inhibits HIV-1 replication in infected H9 leukemia cells at noncytotoxic concentrations [12]. The amino acid sequence [13] and X-ray structure, refined at 1.7 Å resolution [14], of onconase have been determined. These structural studies showed that onconase belongs to the RNase A family, a finding confirmed by the demonstration of ribonuclease activity against high-molecular-weight ribosomal RNA and certain diribonucleoside monophosphates [15, 16]. The ribonuclease activity is necessary but not sufficient for the anti-tumor effects of onconase [15, 16]. Onconase cytotoxicity has been suggested to be connected with penetration of the protein to the cell cytosol where it degrades cellular RNA [17]. The insensitivity of onconase to the mammalian protein ribonuclease inhibitor (RI) may contribute to its cellular toxicity [15, 16, 18]. The structural basis and mechanism of onconase cytotoxicity are still under investigation.

The distant homology of onconase with RNase A makes onconase very suitable for a comparative analysis of nonpolar regions with earlier studies on RNase A. This analysis confirms the presence of the two hydrophobic nuclei with conserved composition and shape in members of the ribonuclease superfamily [6, 7]. However, one may assume that the biological activity of onconase is associated with the position, sequence, and conformation of exposed loops, which differ in onconase and RNase A. In turn, the conformation of exposed loops is maintained by nonconservative hydrophobic microclusters.

Materials and methods

The method of analysis of nonpolar regions in proteins has been published previously [5, 6].

The main definitions used for analysis are as follows:

1) Hydrophobic family of a residue: a number of amino acid residues having at least two nonpolar groups and/or atoms separated from any analogous groups and/or atoms of the residue under consideration by a certain cutoff distance.
2) Library: a list of hydrophobic families for every residue of a protein under consideration arranged to the sequence of the protein.
3) Nonpolar region of a protein globule: a part of a protein globule comprising amino acid residues selected out of the library such that each has at least one nonpolar neighbor from the library.
4) Hydrophobic nucleus: the most compact part of a nonpolar region of the protein globule comprised of amino acid residues having not less than two nonpolar neighbors at a cutoff distance of 4.5 Å, provided that at least three of them contact each other to form an interacting triangle.
5) Hydrophobic microcluster: a group of amino acid residues, in which every residue has at least one hydrophobic neighbor at a cutoff distance of 4.5 Å; the residues involved do not form interacting triangles.

Results and discussion

RNase A-related proteins have been postulated to have similar folds of the polypeptide chain as RNase A [6, 7]. The validity of this assumption in the case of onconase has been demonstrated by X-ray data [14]. Figure 1 represents the superposition of the polypeptide chains of onconase and bovine ribonuclease. Two out of three α helices and the main part of the antiparallel β strands are quite similar in their length and position in the two structures. A substantial difference between these two

proteins was found in the position and conformation of exposed loops.

Contact maps for onconase. To carry out a detailed analysis of nonpolar regions in onconase, one has to estimate the number of pairs of side-chain nonpolar groups (atoms) that are in contact at a chosen cutoff distance [5, 6]. For convenient representation of noncovalent contacts in onconase, we took advantage of the contact map approach [20–22] that had been successfully used for RNase A [6, 7]. The contact map for all noncovalent contacts in onconase, calculated at a cutoff distance of 4.5 Å, is shown in figure 2. Regular elements of the structure, such as helices (H1, residues 2–10; H2, residues 18–23; H3, residues 41–49), are seen as a thickening along the main diagonal of the map. β strands are represented by contacts arranged in a linear fashion normal to the main diagonal (residues 32–38, 51–59, 63–70, 77–84, 86–91, 94–102). There are some differences in the secondary-structure elements between onconase and RNase A. The second helix in onconase is shorter than that of RNase A and it should be considered as 3_{10}-helix. The regions of β strands 61–63 and 71–75 in RNase A do not exist in onconase (fig. 2). Other helices and antiparallel β sheets coincide rather well for onconase and RNase A (fig. 1). It should be noted that β strands near the C terminus in both proteins are twisted (this region is not strictly normal to the main diagonal on the map; fig. 2). The main distinctions between onconase and RNase A are located in loop regions and in some parts of contact domains (the groups of contacts on the map that do not belong to the secondary-structure elements and determine the spatial orientation of these elements). To minimize differences in the maps, we adjusted the alignment of published

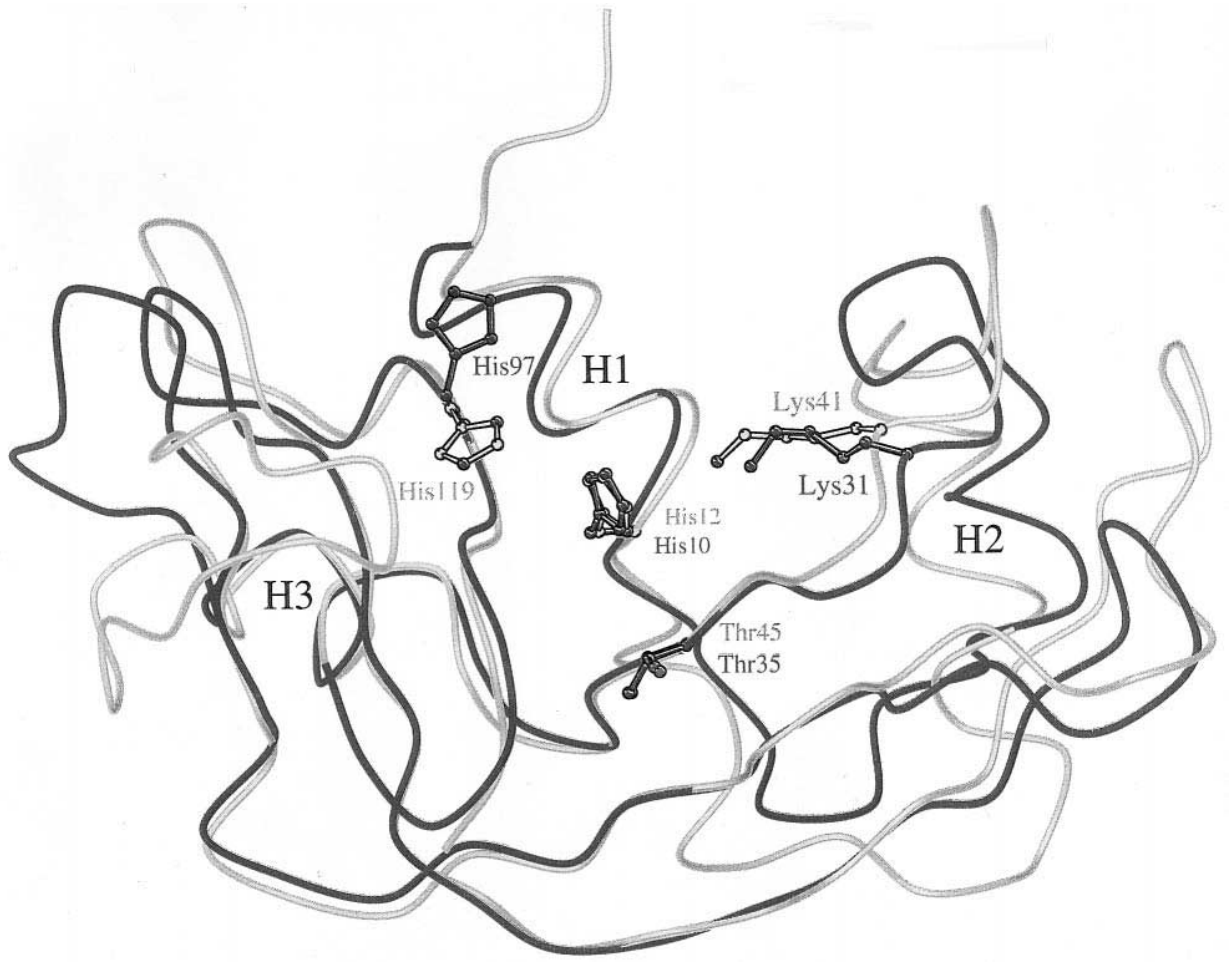

Figure 1. Superposition of polypeptide chain folds of onconase (1onc.pdb [14], black) and RNase A (7rsa.pdb [19], gray) obtained by the alignment of Cα atom coordinates. The main active site residues are indicated. The picture was generated with the O-programme.

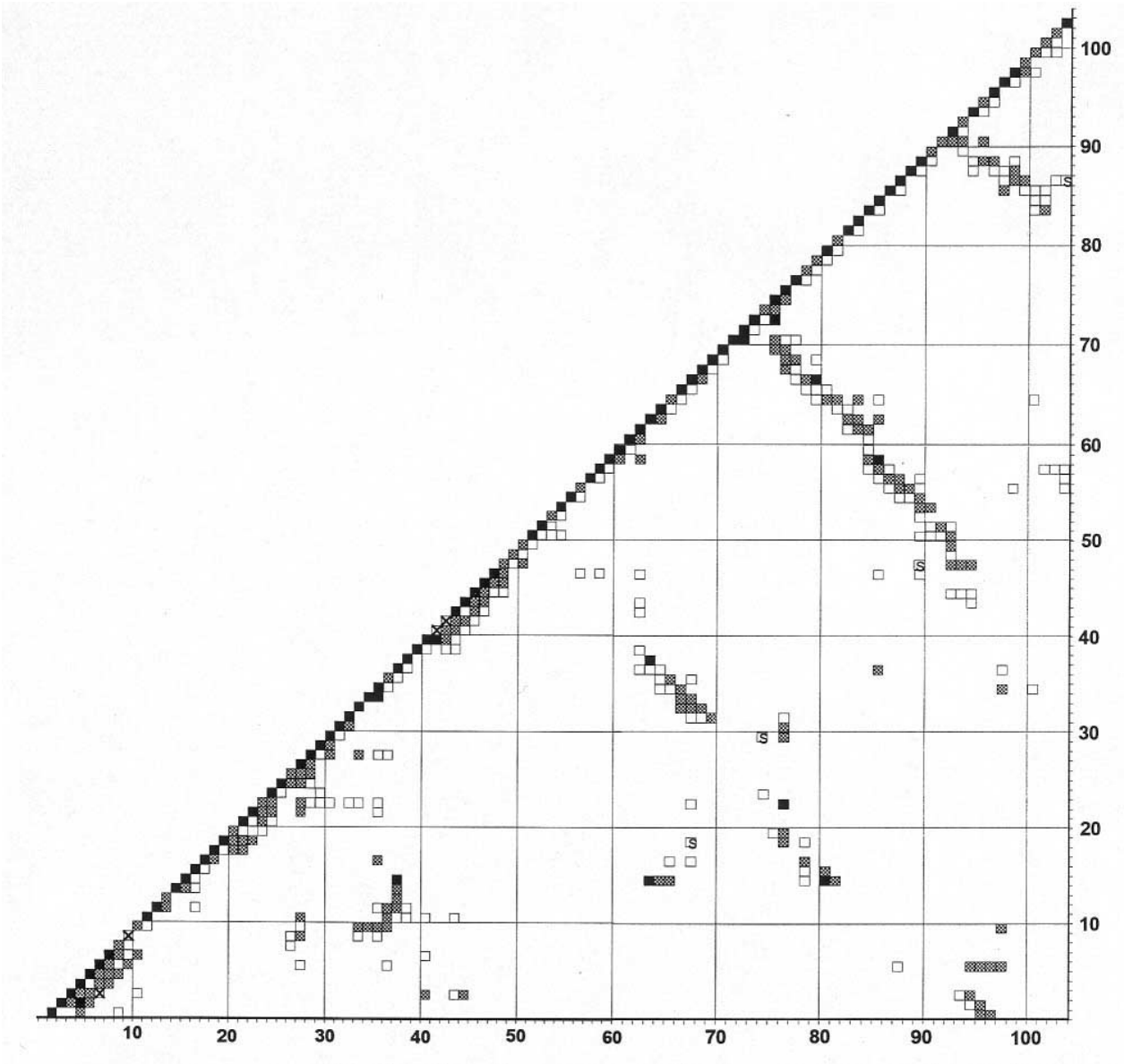

Figure 2. Contact map of all noncovalent contacts in onconase at a cutoff distance of 4.5 Å (1onc.pdb [14]); the amino acid sequence of the protein is plotted on the axes. Number of contacts between residues (i, j) are indicated by squares; white, 0–5; gray, 5–15; black, 15–25; with a cross, >25; the disulfide bridges are marked with the letter S. Three of these bridges are at homologous positions in RNase A. The bridge between residues 87 and 104 only occurs in onconase and other frog RNases.

amino acid sequences [13, 18, 23]. For example, we put Val-17 in onconase at the position of Tyr-25 in RNase A to preserve nonpolar contacts of these residues in the three-dimensional structure. Cys-19 in onconase corresponds to Cys-26 in RNase A and so Asp-18 was found to be an insertion in the alignment of the sequences. Such an adjustment is in agreement with spatial superposition of this region according to X-ray data, but may be in conflict with actual evolutionary descent.

In addition, analysis of contacts between the side-chain nonpolar groups (CH, CH_2, CH_3, and S) of all amino acid residues in onconase on the basis of the constructed contact map at a cutoff distance of 4.5 Å was carried out.

Contacts between nonpolar groups. Following the proposed definitions, the nonpolar environment for every onconase residue has been identified (data not shown). An analysis of the composition and structure of the

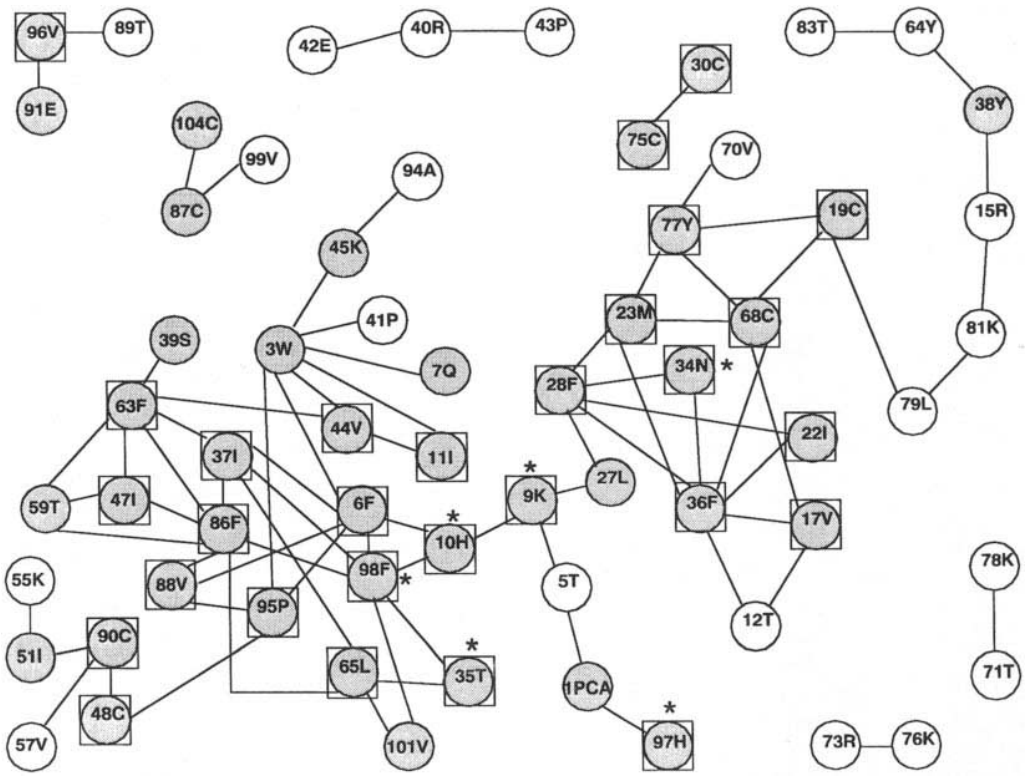

Figure 3. Graf scheme of the nonpolar-contact network in onconase at a cutoff distance of 4.5 Å. Squares, conservative and invariant residues in the RNase A superfamily. Residues marked with an asterisk belong to the active site of the enzyme. Residues marked with gray circles are invariant or conservative in the sequences of frog proteins (fig. 7).

nonpolar contact network of each residue in a family resulted in the construction of a scheme of nonpolar contacts among residues. These data were used to reveal nonpolar regions and to identify two nuclei and seven microclusters (fig. 3). Their composition is summarized in table 1.

The hydrophobic nuclei in onconase (as well as in RNase A) include not merely the classical nonpolar amino acid residues but also polar and charged ones (for example Thr-59, His-10, Asn-34, Lys-9). Lys-9 was assigned (as Gln-11 in RNase A) to the first nucleus and Leu-27 (as Leu-35 in RNase A) to the second. Four cysteine residues (comprising disulfide bridges 19–68 and 48–90) are involved in nonpolar interactions with amino acid residues of hydrophobic nuclei. The spatial arrangement of the hydrophobic nuclei in onconase is shown in figure 4. The most compact part of the hydrophobic nucleus $\gamma 1$ comprises the residues of the first α helix, interacting with residues of the third α helix, and their mutual orientation is stabilized due to nonpolar contacts with Phe-63, Leu-65, Phe-86, Val-88, and Phe-98 of the principal β strands. The hydrophobic nucleus $\gamma 2$ comprises the residues of the second α helix and the

Table 1. Hydrophobic nuclei (γ) and microclusters (mc) of onconase (1onc.pdb [14]).

$\gamma 1$	1<E; 3W; 5T; 6F; 9K*; 10H; 11I; 35T; 37I; 44V; 45K; 47I; 48C; 51I; 59T; 63F; 65L; 86F; 88V; 90C; 95P; 98F; 101V (*7Q; 39S; 41P; 55K; 57V; 94A; 97H*)
$\gamma 2$	12T; 15R; 17V; 19C; 22I; 23M; 27L*; 28F; 34N; 36F; 38Y; 64Y; 68C; 77Y; 79L; 81K (*70V; 83T*)
mc1	87C; 99V; 104C
mc2	40R; 42E; 43P
mc3	58L; 85K
mc4	89T; 91E; 96V
mc5	30C; 75C
mc6	71T; 78K
mc7	73R; 76K

Minimal number of contacts between residues = 2; minimal number of nonpolar neighbors = 2. Residues associated with nucleus due to contacts with one nonpolar neighbor are italicized.
* Residues that comprise a bridge between $\gamma 1$ and $\gamma 2$.

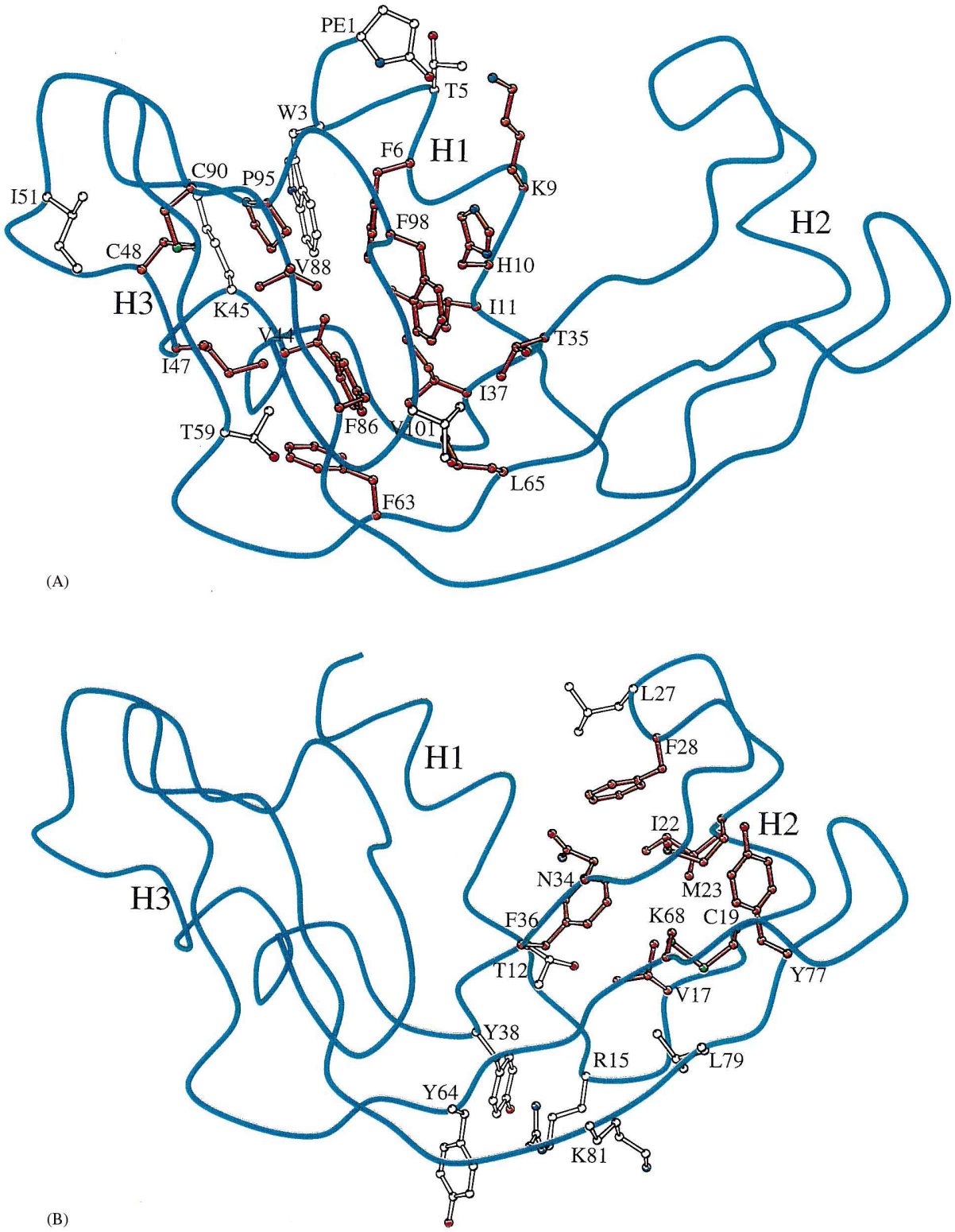

Figure 4. Mutual orientation of γ nuclei and secondary-structure elements in the onconase $\gamma 1$ nucleus (*A*) and $\gamma 2$ nucleus (*B*). The side-chains of the conservative or invariant nucleus residues within the superfamily of RNase A are shown in red; the side-chains of the nonconservative ones in white. H1, H2, and H3 mark the corresponding helix regions in the onconase structure.

Table 2. Quantitative characteristics of hydrophobic nuclei of RNase A and onconase (Onc).

	$\gamma 1$ RNase A	$\gamma 1$ Onc	$\gamma 2$ RNase A	$\gamma 3$ RNase A	$\gamma 2$ Onc	
N (c.p.)	98	131	16	56	85	number of contacting atom pairs (CH, CH_2, CH_3, and S)
N (t)	81	77	23	45	53	total number of nonpolar contacting atoms
N (c/at)	2.42	3.4	1.39	2.49	3.21	average number of contacts per contacting atom
N (c/res)	8.52	11.3	4.57	9.33	0.63	average number of contacts per residue
N (n)	2.6	3.2	2.0	2.5	3.0	average number of neighbors per residue
T/nuc	0.13	0.5	0.14	0.25	0.69	average number of triangles per residue
(A.r.)/R	0.23	0.3	0.71	0.50	0.13	average number of associated residues per residue
$\langle B \rangle$	12.0	11.9	16.5	11.6	12.6	average value of temperature factor for side-chain (CH, CH_2, CH_3, and S) atoms

orientation of this helix is stabilized by nonpolar contacts with the residues of β strands: Phe-36 and Tyr-77.

The hydrophobic nuclei in onconase differ in shape and composition, and in the numbers of intranuclear contacts and associated residues. The quantitative characteristics of these nuclei are listed in table 2, with the corresponding characteristics of nuclei in RNase A for comparison. The nuclei in onconase are more compact than in RNase A. The side-chain atoms of residues comprising the nuclei in onconase form more contacts with the environment and therefore the average number of neighbors per residue and the average number of triangles per residue exceed the corresponding values for RNase A. The temperature factor values were used to characterize the atomic mobility in hydrophobic nuclei (table 2). The average values of the factor $\langle B \rangle$ for side-chain atoms of residues of the two main nuclei in onconase and RNase A are very close and can be compared with the average $\langle B \rangle$ values of atoms in α helices or β sheets. Thus the nuclei are relatively rigid parts of the protein globule and might be considered similar structural elements as those of the secondary structure.

Comparison of hydrophobic nuclei in onconase and RNase A. According to our predictions, two main hydrophobic nuclei of RNase A should be strictly conserved in onconase [6, 7]. Indeed, superposition of Graf schemes of the nonpolar-contact network in onconase and RNase A supports this assumption (fig. 5). The amino acid compositions and spatial arrangements of the nuclei ($\gamma 1$ and $\gamma 3$ in RNase A, and $\gamma 1$ and $\gamma 2$ in onconase) are very similar. The spatial superpositions of side-chains of amino acid residues, comprising homologous nuclei $\gamma 1$–$\gamma 1$ and $\gamma 2$–$\gamma 3$ in onconase and RNase A are represented in figure 6. This concurrence of the main hydrophobic nuclei confirms their importance for the maintenance of the structure and the particular function of the homologous proteins.

The main differences between the two proteins were found in the composition and the conformation of exposed loops. The second small and less compact hydrophobic nucleus $\gamma 2$ in RNase A (see table 2), maintaining the structure of the exposed loop comprising the B2 site of the active center (the binding site of the leaving group), does not exist in the onconase and in many other members of the RNase superfamily [24]. Other regions of difference are also located near the surface of the protein globule. As mentioned above, the structural basis and mechanism of onconase cytotoxicity are still unknown. The additional nonpolar contacts in onconase might maintain the appropriate conformation of the site(s) responsible for its biological activity and should be the objects of special interest for further investigations. Of particular interest could be the region around the long hydrophobic microcluster (Lys-81–Arg-15–Tyr-38–Tyr-64) attached to the second hydrophobic nucleus and the region of the first nucleus around residue Trp-3, which forms many nonpolar contacts in onconase but is lacking in RNase A. It would also be interesting to investigate the functional role of Thr-59, which belongs to nucleus $\gamma 1$ (fig. 5).

The active site region of proteins is created from amino acid residues which belong to different parts of the polypeptide chain and it forms numerous noncovalent contacts with the environment [7]. Some of these contacts are nonpolar and are related to hydrophobic nuclei residues. One might assume that nonpolar contacts between the residues of hydrophobic nuclei and some residues of the active site region provide necessary conditions for stabilizing the enzyme-substrate complex in a catalytically competent conformation. The active site residues in onconase as well as RNase A form a network of nonpolar contacts with residues of the hydrophobic nuclei (fig. 3).

The main catalytic and binding residues of the active sites in both enzymes from a similar network of nonpolar contacts. An unexpected difference found in the present work is the existence of nonpolar contacts between catalytic His-97 and Pyr-1 in onconase. Previously, a hydrogen bond was established between Pyr-1

N and Val-96 O [14]. This directs the $O^{\varepsilon 1}$ atom of Pyr-1 into the active site [14]. The noncovalent contacts might fix the position of the His-97 imidazole ring in the active site of onconase. To appreciate the possible significance of this difference for catalysis, one has to keep in mind that His-97 should be involved directly in transition state stabilization in the course of the transesterification reaction (like His-119 in RNase A).

The results of these analyses indicate a direct connection between the structure of the nonpolar regions and the active site region in homologous proteins.

Comparison of frog ribonucleases. The pyrimidine-specific RNase A superfamily constitutes a group of homologous proteins with well-characterized and sequenced members isolated from many mammalian, avian, reptilian, and amphibian sources [24]. Distantly related members of the RNase A superfamily differ at more than 50% of their amino acid positions. In amphibians, five members of the RNase A superfamily have been identified [13, 25–28]. Three other amphibian members of the RNase A family [25–29]—an RNase from bullfrog (*R. catesbeiana*) liver and two sialic acid-binding lectins from bullfrog and Japanese frog (*R. japonica*) oocytes—differ at about 50% of their amino acids from onconase and might be expected to have a more similar three-dimensional structure than other investigated members of the RNase A family. This has indeed been demonstrated for the sialic acid-binding

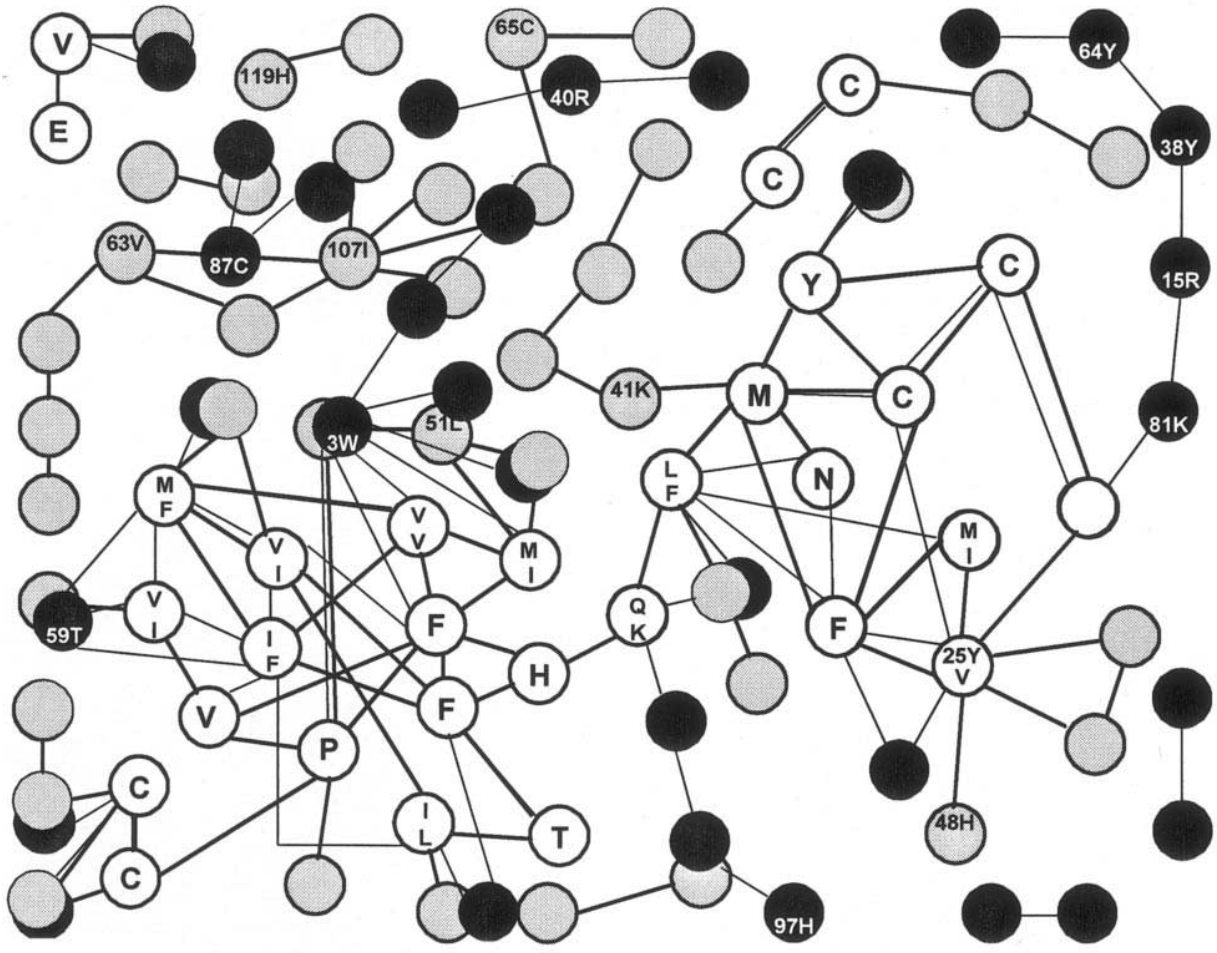

Figure 5. Superposition of Graf schemes of the nonpolar-contract network in onconase and RNase A at a cutoff distance of 4.5 Å. White circles, residues of the nonpolar-contact network that are conservative or invariant in onconase and RNase A; gray circles, residues of the nonpolar-contact network that exist only in RNase A; black circles, residues of the nonpolar-contact network that exist only in onconase (see fig. 3 for other residue numbers). The letters at the top of circles mark the residues of RNase A; letters at the bottom of the circles mark the residues of onconase. Thin lines, nonpolar contacts in onconase; thick lines, nonpolar contacts in RNase A.

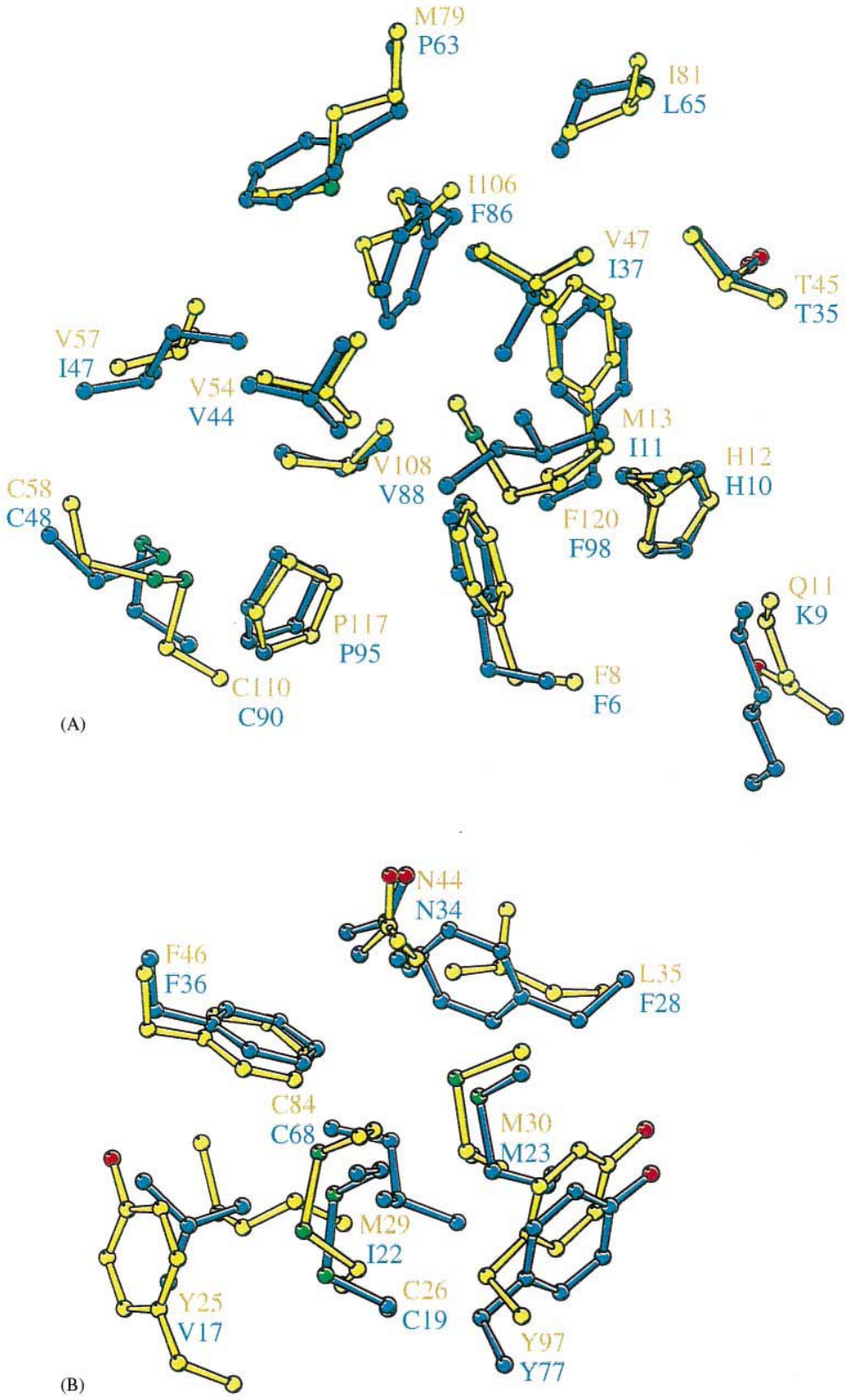

Figure 6*A*, *B*. Spatial superposition of side-chains of amino acid residues comprising two main hydrophobic nuclei in onconase and RNase A. Blue denotes onconase (1onc.pdb [14]), yellow denotes ribonuclease A (7rsa.pdb [19]). (*A*) Nuclei $\gamma 1-\gamma 1$ in onconase and RNase A, respectively. (*B*) Nuclei $\gamma 2-\gamma 3$ in onconase and RNase A, respectively.

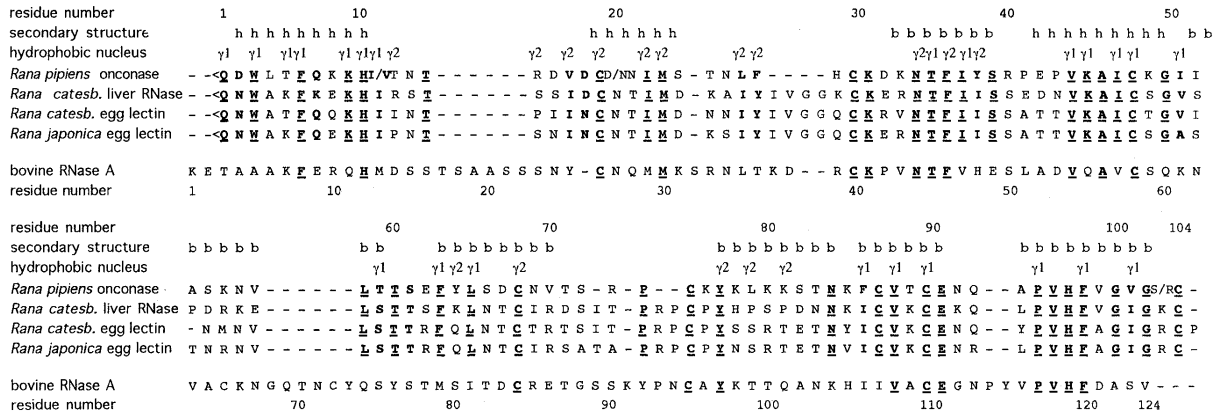

Figure 7. Alignment of amino acid sequences of frog members of the RNase A superfamily [13, 25–27] (numbering according to the onconase sequence) and bovine RNase. A natural variant of onconase differs at positions 11, 20, and 103. Secondary-structure elements (h, helix; b, strand) and hydrophibic nuclei are also included. Bold and underlined, conserved residues; bold only, conservative replacements; < Q, pyroglutamyl residue.

lectin from bullfrog oocytes [29]. As expected, the two lectins exhibit RNase activity, but also show lectin activity towards sialic acid-rich glycoproteins. This lectin activity is essential for cytostatic effects of these two proteins. Onconase and the bullfrog liver RNase have no lectin activity [29]. These similarities and differences in structure and properties between frog RNases justify an additional analysis of hydrophobic nuclei in these proteins on the basis of the onconase structure.

Amino acid sequences of frog proteins are listed in figure 7. The total number of conservative and invariant residues in the sequences is 56, 33 (59%) of which belong to the hydrophobic nuclei. These residues are marked in figures 3 and 7. We have also included in figure 7 a natural variant of onconase. These substitutions of the residues in this variant do not alter the composition of the hydrophobic nuclei (Val-11 is a conservative substitution in the $\gamma 1$ nucleus and two other residues are located in exposed regions of the protein). The structure and composition of the nuclei are clearly strictly conserved. The region of the first nucleus around residue Trp-3, which forms many nonpolar contacts in onconase, is also conserved in frog proteins but not in RNase A and might be responsible for maintaining the appropriate conformation of the site connected with their cytotoxicity. As discussed above, the regions of difference in the network of nonpolar contacts should be located near the surface of a protein globule. The long hydrophobic microcluster (Lys-81–Arg-15–Tyr-38–Tyr-64) attached to the residue Leu-79 in the second hydrophobic nucleus maintains the structure of the exposed region in onconase (fig. 4B). This region is not conserved in the frog protein sequences (fig. 7). The hydrophobic microcluster (Lys-81–Arg-15–Tyr-38–Tyr-64) might be important for manifestation of the biological activity of onconase. It should also be pointed out that Thr-59, which belongs to nucleus $\gamma 1$ (fig. 4A) in onconase, is substituted to Ser in other frog proteins. The functional role of the Thr-59 residue is unknown, and though the chemical nature of Ser and Thr is conservative, Ser should not be able to support the same nonpolar contacts as Thr (lack of a $-CH_3$ group). So there might be some changes in the structure of nucleus $\gamma 1$ near that residue. Another striking feature is the great reduction in size of the loop between the α helix H2 and β strand 32–38 in onconase compared to all other members of the superfamily, including the three other frog RNases.

Conclusions. Detailed analysis of nonpolar regions in onconase confirmed the observations previously made for RNase A and some other proteins [6, 7]. In each protein, a number of separate regions are formed by nonpolar groups. The hydrophobic nuclei are characterized by a dense packing of residues, a large number of mutual contacts and, consequently, by high stability. The conservation of their composition and spatial position in homologous proteins reveals that the hydrophobic nuclei play an important functional role in maintaining the general fold of these proteins and the appropriate conformation of the active site.

Amino acid residues of a nonpolar chemical nature occupy key positions in the spatial organization of the hydrophobic nuclei. Such residues as Phe, Leu, Val, met, Ile, and Trp form the central part of the nuclei

with the most 'populated' hydrophobic families. Comparison of hydrophobic nuclei in onconase and RNase A revealed that the network of nonpolar contacts formed by these central residues is conservative even where there is considerable divergence of amino acid sequence of representatives of the RNase A superfamily. The conformational folding of disulfide-intact RNase A was recently shown to proceed through the formation of an intermediate that is stabilized predominantly by hydrophobic interactions (so-called hydrophobic collapse) [4]. Furthermore, the experimental data suggest that the rate-limiting transition state on the refolding pathway results from the formation of an ordered structure within this intermediate [4]. The two main hydrophobic nuclei found in molecules of RNase A-like proteins should be the best candidates for such ordered structures and might be considered structural elements similar to α helices and β sheets. The main conclusion to be drawn is that the availability of nonpolar residues in established key positions of amino acid sequences should determine the characteristic folding of homologous proteins and the structure of the active site cleft.

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