Domain Organization of the 39-kDa Receptor-associated Protein*

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The 39-kDa receptor-associated protein (RAP) is an endoplasmic reticulum resident protein that binds to the low density lipoprotein receptor-related protein (LRP) as well as certain members of the low density lipoprotein receptor superfamily and antagonizes ligand binding. In order to identify important functional regions of RAP, studies were performed to define the domain organization and domain boundaries of this molecule. Differential scanning calorimetry (DSC) experiments revealed that the process of thermal denaturation of RAP is highly reversible and occurs in a broad temperature range with two well resolved heat absorption peaks. A good fit of the endotherm was obtained with four two-state transitions suggesting these many cooperative domains in the molecule. A number of recombinant fragments of RAP were expressed in bacteria, and their domain composition and stability were characterized by DSC, circular dichroism, and fluorescence spectroscopy. The results confirmed that RAP is composed of four independently folded domains, D1, D2, D3, and D4, that encompass residues 1–92, 93–163, 164–216, and 217–323, respectively. The first and the fourth domains preserve their structure and stability when isolated, whereas the compact structure of the fragment corresponding to D2 seems to be altered when isolated from the parent molecule. Isolated D3 was partially degraded during isolation from bacterial lysates. The isolated D4 was capable of binding with high affinity to LRP whereas neither D1 nor D2 bound. At the same time a fragment containing both D1 and D2 exhibited high affinity binding to LRP. These facts combined with the thermodynamic analysis of the melting process of the fragments containing D1 and D2 indicate that these two domains interact with each other and that the proper folding of the second domain into a native-like active conformation requires presence of the first domain.

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*** The abbreviations used are: RAP, receptor-associated protein; LRP, low density lipoprotein receptor-related protein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; GST, glutathione S-transferase; DSC, differential scanning calorimetry; GdmCl, guanidinium chloride.
RAP requires the delineation of its domain organization. To accomplish this, we performed studies to define the domain structure of RAP and the boundaries of its domains by differential scanning calorimetry (DSC). The results of these studies reveal that human RAP is composed of four independently folded domains. The first NH₂-terminal domain and the second domain appear to interact with each other, whereas the fourth COOH-terminal domain seems to be rather independent within the molecule. The third domain is protolyzed during isolation resulting in destabilization. Binding studies reveal that the first and second domain cooperate to form a high affinity binding site for LRP and that the isolated fourth domain of RAP also binds with high affinity to LRP.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Human RAP cDNA that was cloned into the pGex-2T vector (10) was utilized as a template to amplify by polymerase chain reaction a cDNA encoding the RAP fragments studied. The following oligonucleotides were used as primers: 5'-CCCGTGGATCCTACTCGCGGGAA-3' for fragment 1–82; 5'-CCCGTGATCTGTTACTCGGCAAA-3' and 5'-CATGAATTCTCAGAGTTCGTTGTGCCG-3' for fragment 1–164; 5'-CCCGTGATCTGTTACTCGGCAAA-3' and 5'-CATGAATTCTCAGAGTTCGTTGTGCCG-3' for fragment 1–99; 5'-CCCGTGATCTGTTACTCGGCAAA-3' and 5'-CATGAATTCTCAGAGTTCGTTGTGCCG-3' for fragment 1–92; 5'-CCCGTGATCTGTTACTCGGCAAA-3' and 5'-CATGAATTCTCAGAGTTCGTTGTGCCG-3' for fragment 206–323; 5'-ATACCGGATCCTCAAATGCTATCTGGCC-3' and 5'-GACGGCGAATTCTCAGAGTTCGTTGTGCCG-3' for fragment 216–323. The sense primer was designed with a BamHI cleavage site and the antisense primer with an EcoRI cleavage site. The cleaved pGex-2T vector was used as a template to amplify by polymerase chain reaction, and resultant clones were then sequenced to determine integrity. This experiment was carried out at room temperature for 8 h using a 1/500 ratio of thrombin and its individual fragments by the following equation: 1/[KD] = 1/[Bmax] + [L]/[Bmax], where KD is the dissociation constant. The latter assumes an ordered process in which neighboring domains. The software allows an analysis by either independent or dependent domains. The former is based on the assumption that each domain unfolds independently, regardless of the state of the neighboring domains. The latter assumes an ordered process in which constituent domains unfold sequentially, implying the occurrence of interactions between domains such that the unfolding of any given domain depends on the status of its neighbors. In the absence of domain-domain interactions the two schemes give essentially the same results, whereas the dependent scheme gives a better fit if there is such interaction. Melt temperatures (Tm), calorimetric (ΔHm), and van't Hoff (ΔFm) enthalpies were determined from the DSC curves using the same software. The values of the theoretical heat capacity of the denatured protein and its fragment concentrations were calculated based on the amino acid composition of the protein as described (31, 32). The values of the partial molar heat capacities of amino acid side chains in aqueous solutions that were used for the calculation were taken from Privalov and Makhatadze (31).

**Iodination of RAP and RAP Fragments**—RAP and all RAP fragments were iodinated with 125I by IODO-GEN (Amersham Pharmacia Biotech) as described previously (33) with specific activities ranging from 3 to 17 mCi/μg. 

**Solid Phase Binding Assay**—Wells of microtiter plates (Dynatech Immulon 2, Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4 °C with 100 μl of 1 μg/ml LRP. The wells were then blocked with 5% bovine serum albumin in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM CaCl₂ for 1 h at 25 °C. Following washing, the indicated concentrations of RAP, fragment 1–92, fragment 89–164, fragment 1–164, fragment 89–216, or fragment 206–323 were added to the wells and also to control wells coated with just bovine serum albumin. After an overnight incubation at 4 °C, the wells were washed, and 100 μl of 0.1 x NaOH was added. An aliquot was removed and counted. Data were analyzed by nonlinear regression analysis using (Equation 1),

$$B = B_{\text{max}} \frac{[L]}{K_D + [L]}$$  

where B represents the amount of ligand bound, B_{max} is the amount of ligand bound at saturation, [L] is the molar concentration of free ligand, and K_D is the dissociation constant.
**RESULTS**

**Fluorescence-detected Thermal Denaturation of RAP**—Fluorescence spectroscopy and light scattering were used to derive initial information about the conditions for denaturation of RAP in order to identify those under which the protein unfolds reversibly and/or without aggregation. Fig. 1A presents several curves obtained by heating RAP at pH values between 2.9 and 10.5 while monitoring the ratio of fluorescence intensity at 350 nm to that at 320 nm as a measure of the spectral shift that accompanies unfolding. Under such conditions the protein exhibited a sigmoidal denaturation transition whose midpoint ($T_m$) was sensitive to pH. The denaturation process was partially or fully reversible in all cases since the fluorescence parameter returned to a value near the original upon cooling of the heated sample with the highest degree of reversibility occurring between pH 4.3 and 8.7. The protein also exhibited the highest stability in this pH range. At the same time the denaturation was accompanied by partial aggregation of the denatured molecules since simultaneous light scattering measurements indicated an increase in turbidity following denaturation that was not restored upon cooling (an example is shown in Fig. 1B). The turbidity was abolished when RAP was heated at pH 8.7 in the presence of 0.5 M urea (Fig. 1B) with 0.25 M GdmCl producing a similar effect (not shown). Both urea and GdmCl did not change noticeably the stability of the protein at pH 8.7. These conditions were selected for studying the denaturation of RAP with DSC, a technique that gives more information about the structural organization of the molecule.

**DSC-detected Thermal Denaturation of RAP**—The original endotherm of RAP obtained by heating the protein in the calorimeter while monitoring the changes in its heat capacity ($C_p$) is presented in Fig. 2A (solid curve). The endotherm exhibits two well resolved heat absorption peaks that are centered at 43 and 63 °C and reflect denaturation of at least two compact regions with different stability. The denaturation of RAP was highly irreversible since the endotherm was well reproduced when the protein was heated up to 95 °C then cooled and reheated (Fig. 2A, inset). This is in excellent agreement with the fluorescence data even though the concentration of the protein used in the DSC experiment was more than 1 order of magnitude higher. The $C_p$ function of the denatured RAP almost coincides with the theoretically calculated $C_p$ function after 85 °C indicating that the protein was fully denatured at this temperature and that the exothermic processes of aggregation are absent upon denaturation. The high reversibility of the process and the absence of post-denaturation aggregation enabled for accurate determination of the $C_p$ function in the studied temperature region. This allowed us to perform a detailed thermodynamic analysis of the excess heat capacity function ($\Delta C_p(\text{exc})$), i.e. the observed heat absorption on melting of the protein, that provides more definite information on the number of the independently folded domains in RAP. In this analysis one can deconvolute the $\Delta C_p(\text{exc})$ function into simple constituents corresponding to the heat effect of individual two-state transitions that reflect melting of individual domains (see “Experimental Procedures”).

Although the accuracy of the deconvolution analysis depends mainly on the accuracy of the experimentally determined $C_p$ function, in the case of RAP the main challenge is the proper selection of the base line for accurate determination of $\Delta C_p(\text{exc})$. Despite the well defined beginning and ending of the process, the occurrence of the denaturation in a broad temperature range (25–85 °C) results in difficulties in the accurate determination of this parameter ($\Delta C_p(\text{exc})$). When the base line was drawn arbitrarily as presented in Fig. 2A (dashed lines, a and b), the total enthalpies of the process were found to be slightly different, 152 and 170 kcal/mol. Of significance, the deconvolution analysis resulted in three two-state transitions in the first case and four transitions in the second case (Fig. 2B) reflecting the melting of that many domains, respectively, in the protein. To select between these two schemes an additional study with individual RAP fragments was performed.

**DSC-detected Thermal Denaturation of the 1–216 and 216–323 Fragments of RAP**—The deconvolution of the RAP endotherm into three two-state transitions (see above) is in agreement with the three-domainal composition of RAP proposed on the basis of triplicate internal sequence similarity with residues 18–112, 113–218, and 219–323 forming three individual domains (22). To test this model we expressed two fragments as follows: 1–216 that was expected to comprise two domains, and 216–323 corresponding to the third predicted domain. Note that we selected slightly different boundaries between predicted domains 2 and 3 (at 216–217) to correlate with the boundaries between exons encoding corresponding regions (Fig. 3A). The fragments were expressed in a bacterial system as fusion proteins (see “Experimental Procedures”). Digestion of GST-RAP1–216 generated fragment 1–216 in high yield. In
In contrast, the GST-RAP<sub>216–323</sub> fusion protein was not readily cleaved by thrombin (see "Experimental Procedures"), most likely due to the presence of acidic residues near the cleavage site. To avoid this complication we expressed a longer version of this fragment starting at residue 206, and we obtained high yields of the final 206–323 fragment. This fragment exhibited a CD spectrum typical for α-helical proteins (Fig. 4A, inset). Comparison of its melting properties with that of the 216–323 fragments by fluorescence spectroscopy revealed that both fragments exhibit similar sigmoidal transitions in the same range.
temperature range (Fig. 4B) indicating that they both form cooperative structure with similar stability. The CD-detected melting curve of the 206–323 fragment (Fig. 4A) also revealed a cooperative unfolding transition with a similar $T_m$ as that measured by fluorescence. When melted in the calorimeter the 206–323 RAP fragment exhibited a symmetrical heat absorption peak in the same temperature range that was readily fitted with a two-state transition with a denaturation enthalpy ($\Delta H$) of 45 kcal/mol and a $T_m$ of 43.1 °C (Fig. 5 and Table I).

These results indicate that the 216–323 region of RAP forms a stable cooperative domain and suggest that the extra 10 residues in 206–323 fragment most probably are not a part of this domain.

Denaturation of the 1–216 fragment occurred at higher temperature with an enthalpy of denaturation of 119 kcal/mol (Fig. 5 and Table I). The combined enthalpy of the two fragments, 1–216 and 206–323, was very close to that of the whole RAP ($\Delta H = 170$ kcal/mol) indicating that all of the compact structure is present in the fragments. The deconvolution analysis of the endotherm of the 1–216 fragment revealed three two-state transitions in contrast to the expected two suggesting that this fragment of RAP contains three cooperative domains. Thus results using these two fragments of RAP suggest that RAP may consist of four independently folded domains, three of which are formed by a region containing residues 1–216 and the remaining fourth one by a region containing residues 217–323. They are in agreement with the alternative four-domainal model of RAP (Fig. 2, lower curve, and Fig. 5, upper curve).

Further Study of the Domain Structure of the 1–216 RAP Region—To explore further the domain structure of the 1–216 region of RAP, several smaller fragments were expressed (Fig. 3C). The selection of the size of these fragments was based on the following assumptions. First, based on the deconvolution results (see above), we roughly divided the 1–216 region into three portions 1–72, 72–144, and 145–216. Then, taking into account that the border of the COOH-terminal domain (216–323) coincides with the intron position, we selected the nearest intron position at 82–83 and 163–164 as possible boundaries between domains. Finally, we analyzed the probability of secondary structure formation by analysis of the RAP sequence to

**Fig. 4.** Fluorescence- and CD-detected thermal denaturation of COOH- and NH$_2$-terminal recombinant fragments of RAP. A shows changes in ellipticity at 222 nm of the COOH-terminal 206–323 fragment; the CD spectra of this fragment at 25 ($\alpha$) and 95 °C ($b$) are presented in the inset. The experiment was performed in 10 mM phosphate buffer, pH 8.7. B and C illustrate fluorescence-detected melting curves of different variants of the COOH- and NH$_2$-terminal domain, respectively. Numbers indicate boundaries of the individual fragments. The experiments were performed in 20 mM Gly buffer, pH 8.7, with 0.25 M GdmCl.
check if the selected boundaries coincide with the disruption in the regular structure (Table II). The accuracy of this prediction was confirmed by comparing the predicted results with the three-dimensional structure of the NH₂-terminal fragment (18–112) determined by NMR (23). The comparison gave excellent correspondence of the α-helices and random coil distribution in this region and also forced us to shift the COOH terminus of the first putative domain from the intron position at 82–83 to the end of the theoretically predicted and NMR-determined third α-helix (73–88). The other putative boundaries at 164–165 and 216–217 were found to be in regions predicted to exist as random coils, 160–167 and 208–221, that are excellent candidates for the interdomainal connectors.

When melted in the calorimeter, recombinant 1–99 fragment exhibited a symmetrical endotherm that was readily fitted by a two-state transition with ΔH = 56 kcal/mol (Fig. 5 and Table I) indicating that this fragment forms an independently folded compact domain. The shorter recombinant version, 1–92, exhibited a comparable endotherm with similar enthalpy (Table I) indicating that the extra 7 residues do not contribute to the stability of this domain; in fact the 1–99 fragment was even destabilized by about 2 °C (see Table I and Fig. 4C) suggesting that these extra residues may perturb the structure. Overall, these findings are in a good agreement with the NMR data (23) demonstrating that the COOH-terminal 20 residues of the 18–112 fragment used in these experiments are not in a well

![Figure 5. Original differential scanning calorimetry curves of RAP and its recombinant fragments and their deconvolution analysis. All melting curves were obtained in 20 mM Gly, pH 8.7, 0.25 M GdmCl. Original DSC curves are presented by thick solid lines; broken lines indicate the manner in which the excess heat capacity function for each curve was determined; thin solid lines represent the component two-state transitions and the best fits obtained by deconvolution. Domain compositions of RAP and its fragments are presented schematically at the right; the domains, D1, D2, D3, and D4, are numbered from NH₂ to COOH terminus.](image-url)

**TABLE I**

| Protein/fragments | Transition 1 | Transition 2 | Transition 3 | Transition 4 | Total enthalpy |
|-------------------|--------------|--------------|--------------|--------------|---------------|
|                   | T_m (°C)    | ΔH (kcal/mol)| T_m (°C)    | ΔH (kcal/mol)| T_m (°C)    | ΔH (kcal/mol)| T_m (°C)    | ΔH (kcal/mol)| T_m (°C)    | ΔH (kcal/mol)| T_m (°C)    | ΔH (kcal/mol) |
| RAP-(1–323)       | 41.0         | 47           | 51.2         | 39           | 60.4         | 41           | 65.9         | 43           | 170          |
| 206–323 fragment  | 43.1         | 45           | 54.1         | 42           | 61.6         | 36           | 66.1         | 41           | 119          |
| 1–216 fragment    | 60.8         | 39           | 60.6         | 41           | 63.8         | 41           | 82           |
| 1–164 fragment    | 65.8         | 56           | 67.9         | 59           | 59           |
| 1–175 fragment    | 60.6         | 41           | 63.8         | 41           | 82           |
| 1–199 fragment    | 54.0         | 41           | "           | "           | 41           |
| 82–323 fragment   | 41.1         | 45           | 53.6         | 37           | 82           |

Assignment*: D4, D3, D2, D1

*: Not observed (see text).
*: D1, D2, D3, and D4, individual domains numbered from NH₂ to COOH terminus as in Fig. 5.
defined conformation. The other shorter version, 1–82, did not exhibit a noticeable transition in the calorimeter (not shown). When this fragment was heated and its fluorescent properties monitored, a broad transition with a \( T_m \) much lower than that of 1–92 or 1–99 fragments was detected (Fig. 4C) indicating that the missing residues from position 83–92 of RAP are important for stabilization of this domain. Thus amino acids 1–92 of RAP represents the minimal sequence to form a stable compact NH2-terminal domain.

The other fragment, 1–164, including the NH2-terminal domain and the putative second domain, exhibited an endotherm that was readily fitted with two two-state transitions indicating the melting of two domains (Fig. 5 and Table I). The dependent scheme (see “Experimental Procedures”) gave a better fit suggesting that these domains interact with each other. It should be mentioned that the longer version of this fragment, 1–175, exhibited similar DSC-detected curve that was fitted dependent scheme (see “Experimental Procedures”) gave a better fit suggesting that these domains interact with each other. It should be mentioned that the longer version of this fragment, 1–175, exhibited similar DSC-detected curve that was fitted

**Table II**

| Predicted structure | Determined for 18–112 fragment by NMR | α-Helical content (%) |
|---------------------|--------------------------------------|-----------------------|
| 1–18                | C                                    | 1–17 portion is missing in the fragment 18–22 (well ordered conformation) 81% 18–88 region 74%* |
| 19–36               | H                                    | 23–34 (first helix, H1) |
| 37–39               | C                                    | 36–37 (a sharp bend) |
| 40–65               | H                                    | 39–65 (second helix, H2) |
| 66–72               | C                                    | 66–71 (type IV and type I turns) |
| 73–88               | H                                    | 73–88 (third helix, H3) |
| 89–92               | (type IV turn)                       |
| 89–115              | C                                    | 93–112 (no well defined conformation) |
| 116–124             | H                                    | |
| 125–133             | C                                    | |
| 134–159             | H                                    | 89–164 fragment |
| 160–167             | C                                    | |
| 171–180             | C                                    | |
| 183–196             | H                                    | |
| 197–200             | C                                    | 171–180 C |
| 201–207             | H                                    | |
| 208–221             | C                                    | |
| 222–274             | H                                    | 197–200 C |
| 275–282             | C                                    | 222–274 H |
| 283–293             | H                                    | 275–282 C |
| 294–301             | C                                    | 283–293 H |
| 304–310             | H                                    | 294–301 C |
| 311–313             | C                                    | 304–310 H |
| 314–323             | H                                    | 311–313 C |

* The GGBSM method (see “Experimental Procedures”) gave 59.2% of α-helical, 37.4% coil, and 3.3% extended conformation for the whole RAP; only regions in α-helical (H) and coil (C) conformations are presented. This prediction is consistent with the CD analysis that yielded 60% α-helix, 6% β-sheet, 16% turns, and 18% other.

**Preparation and Characterization of Other RAP Fragments—**To clarify further the relationship between the first and the second domains in RAP we expressed an 82–323 fragment lacking the first domain as well as some shorter fragments (Fig. 3D). When melted in the calorimeter the 82–323 fragment exhibited an asymmetrical endotherm that was fitted with two two-state transitions whose enthalpies and \( T_m \)s are similar to that of the first two transitions in RAP (Fig. 6 and Table I). Since in RAP the first and the second transitions correspond to the melting of domains D4 and D3, respectively, the transitions observed in the 82–323 fragment most probably reflect melting of these domains. A shorter fragment containing residues 89–216 that includes domains D2 and D3 exhibited a symmetrical heat absorption peak that was fitted with a single two-state transition. The enthalpy and \( T_m \) of this transition were similar to that of the second transition in RAP, suggesting that it reflects melting of D3. No observable transition corresponding to the third transition in RAP was observed by DSC in both fragments suggesting that their regions corresponding to the third transition in RAP were not involved in the formation of the compact structure. Thus the results obtained with smaller fragments that represent more simple systems in terms of domain composition allow us to conclude unambiguously that RAP consists of four domains with approximate boundaries at 92–93, 163–164, and 216–217 and that deconvolution of the endotherm of RAP into 4 two-state transitions (Fig. 2B, lower curve) closely describes the denaturation process of this complex molecule.

**Assignment of the Individual Transitions in RAP—**Comparison of the enthalpies and melting temperatures of the individual transitions in RAP and its various fragments (Fig. 5 and Table I) allows one to assign these transitions to the melting of certain domains. For example, the first transition in RAP undoubtedly reflects the melting of the COOH-terminal domain D4 (217–323) since its \( T_m \) and \( \Delta H \) are similar to that in the corresponding fragment 206–323. This domain is the less stable in the protein and seems not to interact with the other domains since its separation does not influence its stability. Similarly, the fourth transition in RAP, the third transition in the 1–216 fragment, and the second transition in the 1–164 fragment all have their \( T_m \) and \( \Delta H \) similar to that in the 1–92 fragment and reflect the melting of the first NH2-terminal domain D1 (1–92). This domain is the most stable, and its stability is also preserved upon separation from the other domains. At the same time, as mentioned above, it may interact with the neighboring second domain D2 (93–164). Most likely, the latter melts in the third transition of RAP, in the second transition of 1–216 fragment, and in the first transition of 1–164 fragment whose enthalpies and \( T_m \)s are similar. This leaves the second transition in RAP and the first transition in 1–216 fragment to reflect the melting of the third domain D3 (164–216).
to the second domain (93–164) do not form a meltable structure. To test these suggestions, we expressed the 89–164 and 159–216 fragments that correspond to the second and third domain, respectively, and characterized their stability and folding status by different methods.

The 159–216 fragment was partially degraded during isolation from bacterial lysates. Mass spectroscopic analysis revealed several fragments resulting from cleavage at Arg-205, Arg-203, Arg-195, and Lys-193. When the mixture of these shorter fragments was analyzed by CD, a spectrum typical for a random coil was observed in the far UV region (not shown) suggesting that the truncated domain is destabilized and unfolded. In contrast, fragment 89–164 (D2) was isolated intact. When it was heated in the calorimeter, no heat absorption peak was observed, in agreement with the above suggestion (Fig. 6A, lower curve). However, at low temperature the heat capacity of the fragment was significantly lower than the theoretical heat capacity of the unfolded fragment suggesting compact packing of its hydrophobic residues (compare curves 1 and 3 in Fig. 6B). The heat capacity increased in a sigmoidal manner upon heating and at high temperature reached the value calculated for the unfolded fragment. When the fragment was heated to 90 °C, cooled, and heated again to 130 °C, the sigmoidal change of the heat capacity was highly reproducible (compare thin and bold curves 1 in Fig. 6B). When the fragment was then heated again to 130 °C, cooled, and the process repeated several times, its heat capacity function was noted to approach that calculated for the completely unfolded fragment in a broad temperature range (compare curves 2 and 3 in Fig. 6B). Comparison of the temperature-induced heat capacity changes of the native fragment (curves 1) with that of the unfolded fragment (curves 2 and 3) suggests that the hydrophobic core of the fragment becomes exposed to the solvent in a sigmoidal manner. This process is accompanied by a very low change of the enthalpy (compare the 3rd transition in RAP in Fig. 6A with the heat capacity change of the native fragment in Fig. 6B). This suggests that fragment 89–164 contains some compact structure that unfolds cooperatively upon heating, but the structural changes occur with a much lower enthalpy in isolated D2 than D2 in the parent RAP or fragments that containing D1 along with D2. To characterize further compact structure of the isolated D2, we performed CD experiments. CD measurements revealed that fragment 89–164 has a spectrum in the far UV region typical for α-helical proteins (Fig. 7A, inset) with an α-helical content of 25%. Heating of the fragment while monitoring ellipticity at 222 nm produced a sigmoidal transition indicative of cooperative unfolding for the secondary structure (Fig. 7A). Furthermore, the fragment exhibited a near UV CD spectrum with a negative and positive maxima at 268 and 291 nm, respectively, reflecting an asymmetry in the environment of its aromatic groups, Tyr and Trp, i.e. the presence of the tertiary structure (Fig. 7B, inset). The
intensity of these maxima decreased in a sigmoidal manner upon heating (Fig. 7B) suggesting cooperative unfolding of the tertiary structure. In agreement, the fluorescence spectrum of the fragment had a maximum at 341 nm indicating hydrophobic environment of its Trp residue(s). Upon heating (Fig. 7C) or titrating with GdmCl or urea (data not shown) the maximum was shifted toward a longer wavelength in a sigmoidal manner indicating cooperative unfolding of the fragment with the exposure of its Trp residue(s) to the solvent.

The above results indicate that the 89–164 fragment corresponding to the second domain in RAP forms a cooperative domain that contains secondary and tertiary structure and exhibits a typical sigmoidal transition upon denaturation. However, its structure appears to be different from that in the parent RAP molecule and in all fragments containing the first NH2-terminal domain since when isolated its denaturation occurs with a very low enthalpy (see above and Fig. 6). In the absence of D1 (i.e. in the 89–216 and 82–323 fragments), D2 also seems to denature with a low enthalpy. The fact that the 89–216 fragment has a higher α-helical content than one could expect if its D2 region would be in a random coil conformation (compare the value of 47% determined by CD measurement

**Fig. 7. Heat-induced unfolding of 89–164 fragment detected by circular dichroism (A and B) and fluorescence (C).** A, changes in ellipticity at 222 nm of the 89–164 fragment; the CD spectra of this fragment in the far UV region at 5 and 95 °C are presented in the inset. B, changes in ellipticity at 268 and 291 nm of the 89–164 fragment; the CD spectra of this fragment in the near UV region at 5, 25, 50, and 95 °C are presented in the inset. C, solid curve and thin broken curve represent changes of the fluorescence parameter of the fragment upon its heating and cooling, respectively. The experiments were performed in 10 mM sodium phosphate buffer, pH 8.7 (A), and in 20 mM Gly buffer, pH 8.7 (B and C).
with the predicted value of 41% in Table II) is in agreement with this speculation. Thus, the presence of the first domain is required for the second domain to be folded properly into a native-like conformation suggesting that these two domains interact in the parent molecule. To test the functional importance of these interactions, we investigated the binding properties of the individual domains and combinations thereof to LRP.

**Binding of RAP Domains to LRP**—To measure the binding of LRP with RAP, a solid phase assay was employed. In this assay LRP was first coated to microtiter wells, and then increasing concentrations of $^{125}$I-labeled RAP or its fragments, 1–92, 89–216, 1–164, and 206–323, were added. Following incubation and washing, the amount of bound radioactivity was determined. As a control, LRP was omitted during the coating step, and the binding of iodinated RAP and RAP fragments to bovine serum albumin-coated wells was measured. In all cases the amount of radioactivity bound to these wells was less than 1% of the binding to the LRP-coated wells. The results of this experiment are shown in Fig. 8, and the apparent $K_D$ values derived from non-linear regression analysis of the data are summarized in Table III. RAP bound to LRP with an apparent $K_D$ of 3.1 nM, a value in excellent agreement with that previously published (10). Fragment 206–323 also bound to LRP with a high affinity, but fragment 1–92 and 89–164 failed to bind to immobilized LRP. Interestingly, fragment 1–164 bound to LRP with high affinity, although the amount bound appears lower than that observed for RAP or fragment 206–323. Thus, the fourth domain of RAP binds LRP, whereas the first and second domains of RAP are both required to form a second high affinity site on RAP that is recognized by LRP.

**DISCUSSION**

There are several commonly used approaches for the establishment of the domain structure of complex multimodular proteins whose three-dimensional structures are not known. They are based either on the results of limited proteolysis, on the analysis of the exon/intron map, or on internal amino acid sequence homology. The latter was used to predict the existence of three domains in RAP on the basis of the triplicate internal sequence similarity (20, 22). Although this approach usually gives accurate predictions, there are a number of examples indicating that some homologous sequences, often called modules, can form more than one independently folded domain. For example, the serine proteinase module in several proteins was found to consist of two domains (34–37) and the fibrinogen-like module to consist of three domains (38, 39). Thus the structure predicted by any of the above approaches requires experimental verification to check if the predicted domains satisfy folding criterion, i.e. if they are properly folded into a compact structure that unfolds cooperatively in a two-step manner. DSC is the most appropriate method for such testing since it allows one to evaluate the number of cooperative domains in a protein and its fragments by monitoring their unfolding process (40, 41). In this investigation we have utilized DSC measurements to establish that RAP consists of four independently folded cooperative domains.

The unfolding of RAP occurs in a broad temperature range creating difficulties in the accurate determination of the excess heat capacity of the process and its subsequent thermodynamic analysis. To simplify the analysis we turned to recombinant RAP fragments that represent a more simplified system. We initiated our studies with two fragments, 1–216 and 206–323. The first fragment (1–216) was predicted to be composed of two domains, whereas the second fragment (206–323) was predicted to be composed of a single domain (22). In agreement with the prediction, DSC measurements revealed that the 206–323 fragment denatured as a cooperative unit characterized by a single two-state transition. This fragment was found to be stable as a separate domain and was unfolded only upon heating as detected by three independent methods, spectrofluorimetry, CD, and DSC. This is in contrast to the previous report based on NMR data that a 219–323 fragment from RAP is largely unfolded when isolated (22). Although the extra 3 residues present in our 216–323 fragment may account for its proper folding, the alternative explanation of this discrepancy may result from a comparatively low thermal stability of the fourth domain. Indeed, this domain begins to unfold at about 30 °C in neutral buffer and thus could be unstable under conditions employed in the NMR study. Whichever explanation is correct, the data obtained in our study has provided us with the background for the selection of the appropriate conditions for the determination of the three-dimensional structure of the 216–323 fragment by NMR, a study that is currently in progress. Our studies also demonstrated that fragment 1–216 of RAP contains three domains in contrast to two predicted by others (22). To confirm that this is not an artifact of the deconvolution analysis but an internal property of the system, we expressed two shorter versions of this fragment, 1–164 and 1–92, and demonstrated that they contain 2 domains and 1 domain, respectively. Thus, the data are consistent with the presence of four domains in RAP.

| RAP or RAP fragment | Apparent $K_D$ ($\text{nM}$) |
|---------------------|-----------------------------|
| RAP-(1–323)         | $3.1 \pm 0.4$               |
| 1–92                | ND$^a$                      |
| 89–164              | ND$^a$                      |
| 1–164               | $9.0 \pm 2.9$               |
| 89–216              | ND$^a$                      |
| 206–323             | $6.8 \pm 1.3$               |

$^a$ No binding detected.
this work, we expressed fragments with alternative boundaries and compared their stability with that of the corresponding domains in the whole molecule. This approach is illustrated by our analysis of the first domain of RAP. Among the three variants expressed, both 1–92 and its longer version, 1–99, exhibited two-state transition in the same temperature range as in the parent protein. On the other hand, the shorter version, 1–82, was dramatically destabilized clearly indicating that the missing 83–92 residues are important for its stability. Indeed, the three-dimensional structure of the first domain shows that residues 83–88 are involved in the formation of the third α-helix (23). A similar situation was observed with the fourth domain, 216–323, where fragments 216–232 and 206–233 have the same stability as the corresponding domain in the parent molecule (this work), whereas the 219–232 fragment may be substantially destabilized (23). The above results indicate unambiguously that the boundaries between D1 and D2 and D3 and D4 are at 92–93 and 216–217, respectively. The boundary between D2 and D3 at 164–165 proposed in this paper is less well defined since we were unable to prepare variants of D2 and D3 domains and test their boundary in the same manner. The 164–216 fragment (D3) was partially degraded upon expression and purification, and isolated 89–164 fragment (D2) seems to adopt the conformation that is different from that in the parent molecule. These complicate direct comparison of their stability with that in the parent molecule. Meanwhile, the fact that the 1–164 and 1–175 fragments which include D1 and D2 exhibited similar stability (Table I) suggests that the boundary of D2 does not extend beyond residue 164. The 216–323 fragment, encompassing domain 4, has the same stability as that found in the intact molecule, suggesting that it does not interact with the neighboring domains. Domain 4 can bind directly to LRP with high affinity and therefore represents a functionally important region of RAP. The low thermal stability of this RAP domain is of interest and may be physiologically important. This portion of RAP is likely to be more flexible and, if so, may more readily interact with LRP and other members of the LDL receptor superfamily. Although the isolated first domain, the most thermally stable in the parent molecule, melts at the same temperature as D1 in RAP, our data provide evidence for its interaction with D2. The deconvolution analysis of the DSC profile of 1–164 fragment (including D1 and D2) suggests some interaction between them. Additional evidence for an interaction between D1 and D2 was obtained when D2 was expressed as an isolated fragment. Several lines of evidence obtained by CD, spectrofluorimetry, and DSC indicate that it has a high content of secondary structure, a hydrophobic core, and some tertiary structure that undergo disruption in a cooperative manner, and thus it forms a compact independently folded domain. At the same time its enthalpy of denaturation was much lower than that of D2 in the parent molecule. In fact we were unable to determine it reliably due to a very low endothermic effect in the region of its denaturation (Fig. 6B). This suggests that an interaction between D1 and D2 influences the folding stability of the second domain. Interestingly, in solid-phase binding assays, neither D1 nor D2 fragment bound to LRP, whereas 1–164 fragment including these domains exhibited strong binding. Thus, it appears that the isolated second domain has a significantly altered structure from that present in D1D2 fragment or in RAP and that the presence of the first domain is important for its proper folding into a native-like active conformation.

In summary, our results indicate that human RAP consists of four independently folded domains corresponding to residues 1–92, 93–163, 164–216, and 217–323. The first second domain interact to form a high affinity binding site for LRP, and the fourth domain also contains a high affinity binding site for LRP indicating that RAP contains multiple, independent binding sites for LRP. Defining the domain organization of RAP and the folding status of its isolated domains will now allow for structural studies of individual domains and for a more comprehensive understanding of the functional properties of RAP.