Concerted Motions of the Integrin-binding Loop and the C-terminal Tail of the Non-RGD Disintegrin Obtustatin*

Received for publication, July 2, 2003, and in revised form, August 4, 2003 Published, JBC Papers in Press, August 28, 2003, DOI 10.1074/jbc.M307030200

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Obtustatin is a potent and selective inhibitor of the αβ1 integrin in vitro and of angiogenesis in vivo. It possesses an integrin recognition loop that harbors, in a lateral position, the inhibitory KGKT23 motif. We report an analysis of the dynamics of the backbone and side-chain atoms of obtustatin by homonuclear NMR methods. Angular mobility has been calculated for 90 assigned cross-peaks from 22 off-resonance rotating frame nuclear Overhauser effect spectroscopy spectra recorded at three magnetic fields. Our results suggest that the integrin binding loop and the C-terminal tail display concerted motions, which can be interpreted by hinge effects. Among the integrin-binding motif, threonine 22 and serine 23 exhibit the lowest and the highest side-chain flexibility, respectively. It is noteworthy that the side chain of threonine 22 is not solvent-exposed, although based on synthetic peptides it appears to be the most critical residue for the inhibitory activity of obtustatin on the binding of integrin αβ1 to collagen IV. Instead, the side chain of threonine 22 is oriented toward the loop center and hydrogen-bonded to residues Thr25 and Ser26. This network of interactions explains the restrained mobility of threonine 22 and suggests that its functional importance lies in maintaining the active conformation of the αβ1 inhibitory loop.

Disintegrins represent a group of cysteine-rich peptides released in Crotalidae and Viperidae snake venoms by proteolytic processing of PII metalloproteinases (1). Disintegrins are potent inhibitors of the binding of β1 and β3 integrins to their ligands. Biochemical (2,3) and structural studies (4–7) have disclosed that the inhibitory activity of disintegrins is linked to a tripeptide motif, which mimics the recognition sequence of the integrin ligands. The tripeptide is maintained in the active conformation at the apex of a mobile loop by the appropriate pairing of cysteine residues (8, 9).

Disintegrins are divided into five different groups (10). The first group includes short disintegrins composed of 49–51 residues and four disulfide bonds. The second group is formed by medium sized disintegrins, which contain about 70 amino acids and six disulfide bonds. The third group includes long disintegrins composed of 84 residues cross-linked by seven disulfide bonds. The disintegrin domains of PIII snake venom metalloproteases contain about 100 amino acids with 16 cysteine residues involved in the formation of eight disulfide bonds. These constitute the fourth group of the disintegrin family. Unlike the short, medium, and long disintegrins, which are single-chain molecules, the fifth group is composed of homo- and heterodimers. Dimeric disintegrins contain subunits of about 67 residues with 10 cysteines involved in the formation of four intrachain disulfide bonds and two interchain cysteine linkages. Current biochemical and genetic data support the view that structural diversification among the disintegrin family occurred through disulfide bond engineering (10).

The integrin-binding loop represents a mutational “hot spot.” Unlike the monomeric disintegrins, which most frequently contain the RGD sequence, the dimeric disintegrins exhibit a large sequence diversity in their integrin-binding motifs. The inhibitory activity of disintegrin EC3 from Echis carinatus toward the αβ1 and αβ3 integrins is associated with the MLD sequence of its B-subunit (11); the selective recognition of integrin αβ1 by EMF-10, from the venom of Eristostachus macrohoni, is associated with the MGD(W) motif (12); and the presence of a WGD motif in CC8, a heterodimeric disintegrin from Cerastes cerastes cerastes, enhances its inhibitory effect on α1β1β3, α2β1, and αβ2β3 integrins (13).

A conserved characteristic among disintegrins is the presence of an acidic residue in the C-terminal part of the integrin-binding motif. The crystal structure of the extracellular segment of integrin αβ3 in complex with an RGD ligand (14) shows that the peptide fits into a crevice between the αβ3 propeller and the β3 A-domain. The arginine side chain is held in place by interactions with αβ3 carboxylates 218 and 150, whereas the glycine residue makes several hydrophobic interactions with αβ3 and the aspartate of the ligand interacts primarily with residues in the β3 A-domain. Thus, the conserved aspartate residue appears to be responsible for the binding of disintegrins to the integrin receptors that share a β-subunit, whereas the two other residues of the integrin-binding motif (RG, MG, WG, ML, VG) dictate the integrin specificity.

Obtustatin, a short disintegrin isolated from the venom of Vipera lebetina obtusa (15) exhibits unique structural and functional features. With only 41 residues, obtustatin represents the smallest disintegrin reported to date. It contains an integrin recognition loop that is two residues shorter than that of the other disintegrins and whose amino acid sequence (20WKTSLTSHY28) strongly departs from the primary structure of the loops...
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\[ \theta = \arctan(2/\sqrt{\Omega}) \]  

(4) Unmodified NOESY and ROESY spectra can be associated with values of the \( \theta \) angle equal to 0 and 90°, respectively. Therefore, the angle at which the NOE and ROE effect contributions are equal, and thus the spectral signal is cancelled provides a measure of the ratio \( R \),

\[ 0 = \sigma_{\text{NOE}} - \tan^2 \theta \]  

(5) where \( \sigma(\theta) \) is a scaling factor that does not affect the zero-crossing angle (\( \theta \)) value (19). \( R \) can be determined measuring the value of \( \theta \), where NOE and ROE contributions cancel, and its value relates to angular internal motions.

\[ R = \frac{\sigma_{\text{NOE}}}{\sigma_{\text{ROE}}} = \tan^2 \theta \]  

(6) Since the spectral density \( J(\theta) \) depends only on the angular component of internal motions (Equation 3), low and high values of \( \theta \) are inversely proportional to the degree of angular flexibility of a given vector.

Variations on \( \theta \) values are largest for motions in the time scale of 100–300 ps; therefore, these motions are the main contributors to the internal dynamics detected here. In this time scale, \( \theta \) seems to have a parallel behavior to the maximum of the \( S^2 \) Lipari-Szabo order parameter for that range of \( \tau_c \) values (100–300 ps). Thus, \( \theta \) provides an estimation of the relative angular flexibility for a given NOE vector in a similar fashion to that extracted from relative differences in \( S^2 \) in traditional heteronuclear relaxation studies for the \( ^{1}H-^{1}H \) or \( ^{13}C-^{1}H \) backbone.

**NMR Data Acquisition and Analysis—**All two-dimensional spectra were recorded on Bruker AVANCE spectrometers working at proton frequencies of 500, 600, and 700 MHz. Triple resonance cryogenic NMR probes were used for the measurements at 500 and 600 MHz, and a conventional triple resonance probe was used for the 700-MHz experiments. All spectra were processed with XWIN-NMR version 3.1 and analyzed with SPARKY (20) software packages on a Pentium 4 work station running the Linux operating system. An independent set of off-resonance ROESY spectra was recorded on each spectrometer at 25 °C. The off-resonance pulse sequence for off-resonance experiments was obtained from Ref. 18 and adapted to our spectrometers. In general, experimental parameters were as similar as possible to those reported previously for BPTI (18). Relaxation delay was 3.0 s including presaturation of water resonance. All ROESY experiments had a mixing time of 100 ms, spectral width of 10 ppm, and transmitter offset at the water position equivalent to 4.69 ppm at 25 °C. For each experiment, 512 \( t_1 \) and 2048 \( t_2 \) complex data points were acquired. The number of scans was 24 for all experiments. A regular on-resonance ROESY was recorded at each spectrometer with the same parameters as for the off-resonance ROESY experiments for detection of positive cross-peaks indicating chemical exchange processes. Values for \( \gamma_2 \) were 7180, 9259, and 10,000 Hz for the 500-, 600-, and 700-MHz experiments, respectively, corresponding to ~50% of the used spectral width, as recommended in the literature. Table I shows values of frequency offsets and equivalent \( \delta \) at the transmitter frequency (middle of the spectrum) used in all off-resonance ROESY experiments for evaluation the optimum range of offset frequencies (19). ROESY cross-peaks were assigned using obtustatin resonances from the BioMagResBank (BMRB entry 5410). Cross-peaks were fitted to Lorentzian curve peak shapes and integrated using SPARKY internal commands. ROESY cross-peaks showing severe overlapping were disregarded for further analysis. Graphical representation, second-order polynomial fitting, and zero-crossing identification for cross-peak intensity versus \( \delta \) dependence were carried out using homemade gnuplot macros and Unix shell scripts. In general, \( \delta \) value at the zero-crossing point did not significantly depart from values obtained by analysis of peak volumes or data heights.

**Hydrodynamic Calculations—**For comparison with experimental data and for initial estimation of global correlation time \( \tau_m \), HydroNMR version 5.5a software (21) was employed with the obtustatin NMR structure retrieved from the Protein Data Bank (PDB code 1MPZ). The disulfide bond network of obtustatin, along with the r.m.s. deviation value of 1.1 Å for all residues among the 20 NMR conformers (0.6 Å for the 29 best-defined residues) (17), provide the level of overall structure globularity and rigidity required for this type of analysis. Temperature and solvent viscosity for calculations were set to 25 °C and 0.010 poise, respectively, as recommended in the software manual. Theoretical rigid limit values of \( \theta \) for each set of off-resonance ROESY experiments were estimated using the calculated value of \( \tau_m \).

\[ \theta = \arctan(2/\sqrt{\Omega}) \]  

(4) 

of all known disintegrins. The KTS sequence within this novel integrin-binding loop endows obtustatin with selective inhibitory activity of the in vitro adhesion of integrin \( \alpha_\beta_1 \) to collagen IV (15). Obtustatin is also a potent inhibitor of angiogenesis in vivo in the chicken chorioallantoic membrane assay, and it impairs the development of Lewis lung tumor in the mouse (16) by about 50%. Hence, obtustatin represents a potential lead molecule for the design of inhibitors of angiogenesis in cancer therapy.

An in depth understanding of the structure-function correlation of obtustatin requires a detailed analysis of its structure and dynamics. We have recently determined the solution structure of obtustatin (17) by NMR spectroscopy. The obtustatin structure revealed that the KTS motif is oriented toward a side of the active loop instead of being located at the apex of a \( \beta \)-hairpin as in the known RGD-containing disintegrin structures. Moreover, doubling of the NMR signals in the aromatic region of residues Trp20, His27, and Tyr28, which are located at the base of the integrin-binding loop, indicated that the side chains of these residues exist in alternate conformations and undergo a slow exchange process in the millisecond time scale. The aim of this study was to analyze the dynamics of the backbone and side chain atoms of obtustatin in detail. The major conclusions from our results are that the integrin binding loop and the C-terminal tail of obtustatin display concerted motions in the 100–300-ps time scale and that the active site residue threonine 22 is important for maintaining the conformation of the active loop. In the loop size, composition, and flexibility, along with the distinct orientation of the KTS tripeptide, may underlie the structural basis of obtustatin’s unique selectivity and specificity for integrin \( \alpha_\beta_1 \).

**MATERIALS AND METHODS**

**Sample Preparation—**Obtustatin was purified from the venom of *V. lebetina obtusa* (Latexon, France) as described (15). Protein purity was assessed by SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (using an Applied Biosystems DE-Pro spectrometer and \( \alpha \)-hydroxyacinamic acid saturated in 0.1% trifluoroacetic acid in 50% acetonitrile as matrix). The isoaverage-averaged molecular mass (\( M + 2 \)\(^{12}C \)) of native obtustatin was 4324.2 Da, which accurately matches the calculated mass from its amino acid sequence (SwissProt accession number P83469). Obtustatin was dissolved at a final protein concentration of ~2.5 mM in a mixture of 10% D\(_2\)O and 90% H\(_2\)O in a 5 mm Wilman NMR tube, and the pH of the solution was adjusted to 3.0 with HCl.

**Off-resonance ROESY—**Internal motions were analyzed using the off-resonance ROESY method described by Schleucher and Wijmenga (18). Briefly, cross-peak build-up rates in NOESY1 and ROESY spectra can be described as follows,

\[ \sigma_{\text{NOE}} = \frac{1}{20} \left( \frac{\hbar_{\text{NOE}}}{4\pi} \right)^2 J(r) (2J(2\omega_0) - J(0)) \]  

(1)

\[ \sigma_{\text{ROE}} = \frac{1}{20} \left( \frac{\hbar_{\text{ROE}}}{4\pi} \right)^2 J(r) (2J(0) + 3J(\omega_0)) \]  

(2)

where \( J(r) \) is a distance-dependent term that depends on the average internuclear distance and on distance fluctuations (radial component of internal motions). \( J(\omega) \) is the spectral density, which depends only on the angular component of internal motions (19). Thus, the radial component can be eliminated when the ratio of NOE and ROE rates is considered (18).

\[ R = \frac{\sigma_{\text{NOE}}}{\sigma_{\text{ROE}}} = \frac{6J(2\omega_0) - J(0)}{2J(0) + 3J(\omega_0)} \]  

(3)

In a ROESY experiment, the angle \( \theta \) at which a spin \( i \) with a chemical shift \( \gamma_2 \) and an offset \( \Delta \) is defined as follows.

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1 The abbreviations used are: NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; ROE, rotating frame nuclear Overhauser effect spectroscopy; r.m.s., root mean square.
RESULTS AND DISCUSSION

NMR relaxation methods for studying protein dynamics rely on the determination of internal motions of the vector formed by a particular bond, typically N–H or C–H (22–24). A major drawback of this approach is that the samples need to be isotope-labeled. In addition, only very local motions, which may indirectly indicate the existence of nonlocal flexibility, are determined. Recently, new methods for the detection of internal motions by homonuclear NMR have been developed (18, 25–27). These exploit the fact that the NOESY and ROESY cross-peak intensities depend on both the internuclear distances and internal motion (28, 29). Moreover, intraresidue and sequential NOESY Hβ-HN cross-peak intensities are directly related to the dihedral angles φ and ψ, respectively. Thus, the backbone flexibility information is more accurate and can be directly extracted from the experimental data. A method to separate and analyze the contribution of the internal motions to the off-resonance ROESY spectra has been reported (18). The ratio between NOE and ROE signal intensities is related to internal motions. Off-resonance ROESY experiments measure a weighted average of NOE and ROE. The NOE to ROE ratios are obtained from a series of off-resonance ROESY spectra, and a parameter θ0, describing internal angular motions in a time scale of 100–300 ps, is calculated. We have applied this approach to obtustatin in order to characterize its internal motions. Our aim is, in particular, to validate the proposed hinge mechanism of the integrin recognition loop (17) and to determine the dynamics of the KTS integrin-binding motif and the C-terminal tail.

NMR Experimental Data—Of the 37 nonproline amino acids of obtustatin, NMR off-resonance ROESY data have been analyzed for 33 intraresidue and 35 sequential resolved Hβ-HN cross-peaks from spectra recorded at 500, 600, and 700 MHz (see Fig. 2). The remaining intraresidue and sequential Hβ-HN cross-peaks were not taken into consideration due to spectral overlap, resonance broadening, or the absence of the corresponding NOE interaction. All analyzed cross-peaks corresponded to negative intensities (opposite to diagonal sign) in a regular ROESY spectrum, indicating the absence of chemical exchange in the millisecond time scale. In addition, nine intraresidue or sequential cross-peaks involving at least one side-chain proton of the αβ integrin recognition motif residues Lys21, Thr22, and Ser23 and five other side-chain cross-peaks into the hinge residues Trp20, His27, and Tyr28, were analyzed. For establishing relative differences of θ0 values of well and ill defined residues in the NMR solution structure of obtustatin (17), six Hβ-HN intraresidue cross-peaks involving methylene or methyl atoms in the well defined amino acids Thr23, Arg8, Gln9, Leu12, Ala15, and Thr18 were included in the study. Cysteine Hβ atoms were excluded from the selection because of possible underestimation of the dispersion of atomic coordi-

### Table I

| Offset (Hz) | 500 MHz | 600 MHz | 700 MHz |
|------------|---------|---------|---------|
| 21,000     | 21.0    | 22.00   | 25.5    |
| 18,000     | 24.6    | 20,000  | 28.0    |
| 16,000     | 27.7    | 18,200  | 31.0    |
| 15,000     | 29.6    | 16,500  | 34.0    |
| 13,000     | 34.0    | 15,000  | 37.0    |
| 11,000     | 39.9    | 14,200  | 39.1    |
| 10,000     | 43.1    | 13,100  | 42.0    |
|            |         | 12,000  | 45.0    |
|            |         | 11,100  | 48.2    |

θ0 is the value of θ for the frequency at the middle of the spectrum.

![Fig. 1. Alanine 15 intraresidue (solid line) and sequential (dashed line) fitted curves for NOE Hβ-HN cross-peaks intensity with respect to θ values at 500- (top), 600- (middle), and 700-MHz (bottom) proton frequencies. Experimental points have been plotted as crosses.](https://www.jbc.org/}

Fig. 1.
nates due to disulfide bond restraints. H_{δ}H_{δ} cross-peaks for Cys36, which had been unambiguously assigned in NOESY spectra at 100 ms (17), were taken into consideration. However, the H_{δ}H_{δ} resonances of Cys36 were excluded due to overlapping NOE cross-peaks.

Internal Motions and θ Values—The intensity of all analyzed cross-peaks was fitted to a second order polynomial function of the θ value at the three different magnetic fields. Fig. 1 illustrates the polynomial fitting for intraresidue and sequential H_{δ}H_{δ} NOE cross-peaks of alanine 15. θ values were calculated for each magnetic field and normalized with respect to the corresponding theoretical rigid limit. Although θ values for intra-H_{δ}H_{δ} NOE cross-peaks were expected to be larger than the equivalent sequential θ values, because the accessible energy regions in the Ramachandran plot are wider for ψ than for φ dihedral angle, both situations, intraresidue larger than sequential and sequential larger than intraresidue, were observed. This indicates that the internal motions are not preferentially associated with any component of the dihedral angle. In Fig. 2, the values of θ at the three magnetic fields for all measured backbone ROESY cross-peaks have been plotted against the amino acid sequence. The reliability of any θ value has been scored by the r.m.s. deviation of the corresponding polynomial fitting. θ values of all of the backbone cross-peaks extracted from the spectra at 500 and 600 MHz showed an r.m.s. deviation below 10%. The r.m.s. deviations of equivalent cross-peaks in the 700-MHz spectra were slightly larger because the signal to noise ratios in the 500- and 600-MHz spectra were better than those at 700 MHz. This is due to the sensitivity enhancement effect of the cryoprobe. These results are also in line with previous off-resonance ROESY experiments, which indicate best results for small molecules at low magnetic fields (18, 25). In general, side-chain cross-peaks showed larger r.m.s. deviation values than the intraresidue cross-peaks. The likely reason for this is the additional contributions to the ROE/NOE intensities due to other motions, such as the contribution of the methyl and methylene rotation. Furthermore, spin diffusion or chemical exchange may contribute to the measured cross-peak intensity and degrade the accuracy of the θ measurements.

In order to evaluate the absolute degree of flexibility and to normalize the data obtained at different magnetic fields, a value for the θ at the theoretical limit (ωr, ≫ 1) was calculated from the graphical representation of S2 with respect to ωr. The global correlation time, τm of 2.8 ns obtained from a hydrodynamic calculation was used for the evaluation of the θ at the rigid limit. The estimated values for the theoretical rigid limits of θ at 500, 600, and 700 MHz were 31.6, 32.7, and 33.4°, respectively. However, many residues showed θ values larger than this rigid limit. Such a behavior has been related to the deviations of the true θ values due to the finite duration of gradients and adiabatic ramps during the mixing time (18). The absence of secondary structure in obtustatin (17) may also lead to overestimation of τm in the hydrodynamic calculation, affecting thereby the values of the theoretical rigid limits of θ. Nevertheless, this nonideal behavior did not preclude the use of the θ rigid limit at each magnetic field for normalization and comparison of the absolute θ values. On the other hand, differences in normalized θ values show better agreement with respect to differences in theoretical S2 order parameters calculated in model situations (data not shown). Hence, the individual values of θ at the different magnetic fields were normalized with respect to the corresponding rigid limit values, and a

| NOE cross-peak | Normalized mean θ |
|----------------|--------------------|
| Thrα H_{δ}H_{δ} (w) | 0.85 ± 0.05 |
| Argα H_{δ}H_{δ} (w) | 0.83 ± 0.04 |
| Glnα H_{δ}H_{δ} (w) | 0.75 ± 0.03 |
| Alaα H_{δ}H_{δ} (w) | 0.74 ± 0.07 |
| Thrα H_{δ}H_{δ} (w) | 0.71 ± 0.04 |
| Thrα H_{δ}H_{δ} (r) | 0.73 ± 0.08 |
| Lysα H_{δ}H_{δ} (r) | 0.81 ± 0.05 |
| Lysα H_{δ}H_{δ} (r) | 0.80 ± 0.06 |
| Thrα H_{δ}H_{δ} (r) | 0.82 ± 0.04 |
| Thrα H_{δ}H_{δ} (r) | 0.55 ± 0.12 |
| Thrα H_{δ}H_{δ} (r) | 0.69 ± 0.08 |
| Lysα H_{δ}H_{δ} (r) | 0.50 ± 0.11 |
| Serα H_{δ}H_{δ} (r) | 0.86 ± 0.08 |
| Leuα H_{δ}H_{δ} (r) | 0.71 ± 0.09 |
| Trpα H_{δ}H_{δ} (h) | 0.74 ± 0.09 |
| Hisα H_{δ}H_{δ} (h) | 0.83 ± 0.11 |
| Tyrα H_{δ}H_{δ} (h) | 0.81 ± 0.09 |
| Tyrα H_{δ}H_{δ} (h) | 0.62 ± 0.12 |
| Thrα H_{δ}H_{δ} (l) | 0.74 ± 0.09 |
| Serβ H_{δ}H_{δ} (l) | 0.78 ± 0.08 |
Mean-normalized value of $\theta^0$ was determined. Values of $\theta^0$ for ROE cross-peaks for side chains of selected well defined residues in the NMR conformers, residues at the hinge region, and residues at the recognition site are shown in Table II. Although the highest flexibility was expected to be associated with residues Lys21–Ser26 and Leu38–Gly41, which exhibit the largest r.m.s. deviation in the NMR solution conformers of obtustatin (17) (Fig. 3, bottom panel), the lowest values of mean-normalized backbone $\theta^0$ were associated with residues Cys19, Trp20, His27, Tyr28, Leu38, and Gly41 (Fig. 3, upper panel). We interpret this result on the basis of a hinge motion at the basis of the loop. Thus, bundle superpositions using residues with the lowest r.m.s. deviation or highest dihedral order parameter values may produce this distortion in proteins which exhibit relative mobility between different structural regions.

Integrin-binding Loop Flexibility and Proposed Hinge Motions—Obtustatin shows several flexible regions along the whole amino acid sequence. Fig. 4 depicts the information inferred from the $\theta^0$ values for intrar residue and sequential $H_\alpha$-HN NOEs mapped on the mean NMR structure using the “sausage” representation (30). In general, as expected, increased flexibility is associated with solvent-exposed residues calculated by PROCHECK (31) like Thr22, Gly23, Arg24, Cys25, Trp26, Lys27, and Ser28 (Fig. 3). Residues Cys6, Cys7, Cys10, Ala15, Gly16, and Cys19, which are part of the core of the protein cross-linked by disulfide bonds, display values of mean-normalized $\theta^0$ close to or above 1 (Fig. 3), which correspond to the rigid limit. With the exception of the integrin recognition loop residues, the data show good agreement between regions with high NMR structural dispersion and increased flexibility. The integrin-binding loop shows larger dispersion in the NMR structures than that expected from the flexibility estimations. This further supports the hypothesis of an overall movement of this loop (17). Superposition of the ensemble of solution structures of obtustatin (PDB code 1MPZ) shows that the local motions of the integrin binding loop are mainly lateral within a range of $-35^\circ$ and with maximal displacement of about 5 Å. In
This has been shown that the C-terminal tail of another short disintegrin, echistatin, is involved in modulating the binding affinity of the disintegrin toward integrin $\alpha_\beta_2$ (33). Thus, an echistatin C-terminal peptide inhibited echistatin-integrin $\alpha_\beta_2$ binding, activated the integrin to bind immobilized ligands, and increased fibrinogen binding to peptide-treated platelets (34). The NMR solution structures of the RGD-containing disintegrins echistatin (5), kistrin (4), flavoridin (6), and albolabrin (7) showed that, similar to obtustatin, their C-terminal portions are in close spatial proximity to the RGD loop. Senn and Klaus (6) have reported that the C-terminal residues of flavoridin (Cys$^{64}$-Trp$^{67}$) are connected to the RGD loop. The authors suggested that the C terminus of the disintegrin could act as a secondary integrin-binding determinant.

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From this evidence, we interpret that the C-terminal tail and the integrin-binding loop have been proven to be a determinant for conferring integrin specificity to cyclic RGD peptides with a known conformation (3) and to the short sized, obtustatin-related disintegrins echistatin and eristatin (9). Thus, the critical importance of Thr$^{22}$ may be linked with a structuring mechanism of the integrin-binding loop for proper recognition of the $\alpha_\beta_2$ integrin rather than with a direct role in receptor binding.

**Conclusion**—The two major findings from our homonuclear NMR study of the backbone and/or side chain dynamics of obtustatin are that threonine 22 is important for maintaining the conformation of the active loop and that the integrin-binding loop and the C-terminal tail display a concerted movement, which we propose is due to a hinge effect articulated at residues Trp$^{25}$, His$^{27}$, and Tyr$^{28}$. Fast recognition and fitting processes are typically brought about by mobile segments in protein structures (37, 38). Thus, the conformation and dynamics of the integrin-binding loop, along with the flexibility and internal motions of the side chains of active site residues 21KTS$^{23}$ of obtustatin are probably important for fast recognition of integrin $\alpha_\beta_2$. On the other hand, the composition and architecture of the integrin recognition loop, maintained in its active conformation by a network of hydrogen bonds between Thr$^{22}$ and other loop residues, may determine the selective inhibition of the $\alpha_\beta_2$ integrin receptor by obtustatin. In this sense, if the binding mechanism of obtustatin is similar to that observed in the crystal structure of the extracellular segment of integrin $\alpha_\beta_2$ in complex with a RGD ligand, the residues Lys$^{21}$ and Ser$^{23}$ represent the functional homologues of the arginine and aspartic acid residues, respectively. We are currently investigating the hypothesis that Lys$^{21}$ confers $\alpha_\beta_2$ recognition specificity and Ser$^{23}$ is a major determinant of the binding affinity for the $\beta_2$ subunit.

**Acknowledgment**—We thank the Servicio Central de Soporte a la Investigación Experimental of the University of Valencia for providing access to the NMR facility and high performance computing facilities.

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*Acknowledgment*—We thank the Servicio Central de Soporte a la Investigación Experimental of the University of Valencia for providing access to the NMR facility and high performance computing facilities.
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J. Biol. Chem. 2003, 278:45570-45576.
doi: 10.1074/jbc.M307030200 originally published online August 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307030200

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