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Cyclodextrins as templates for the presentation of protease inhibitors

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Abstract Mono(6-succinylamido-6-deoxy)-β-cyclodextrin was synthesized by classical carbohydrate chemistry and used as a template mono-functionalized with the linear, fully flexible 4C-spacer carboxylate for covalent linkage of the calpain inhibitor leucyl-leucyl-norleucinal. Spectroscopic analyses of the conjugate do not support a self-inclusion of part of the hydrophobic peptide tail, but confirm its intra- or intermolecular interaction with the template moiety that leads to full water solubility. The inhibitory potency of the β-cyclodextrin/peptide aldehyde construct was compared with that of the parent Ac-Leu-Leu-Nle-H against cathepsin B and calpain. Despite the large size of the template the inhibition of cathepsin B was only slightly reduced in full agreement with the X-ray structure of this enzyme which shows full accessibility of the S-subsites. For this enzyme the 4C-spacer is apparently sufficient to guarantee optimal interaction of the peptide tail with the binding cleft. Conversely, for μ-calpain a significantly decreased inhibitory potency was obtained with the conjugate suggesting steric interference of the template in the binding process. These results show that the beneficial properties of the cyclodextrin template can be retained in conjugates with bioactive peptides if attention is paid to optimize in each case the size and nature of the spacer for optimal recognition of the grafted biomolecule.

Key words: Peptide aldehyde; β-Cylodextrin conjugation; Protease inhibitor; Calpain; Cathepsin B

1. Introduction

Cyclodextrins are cyclic oligosaccharides known for their ability to include into their hydrophobic cavity via host-guest complexation a variety of hydrophobic compounds [1]. This property has been extensively exploited to change the physicopharmaceutical properties of lipophilic drugs such as water solubility, bioavailability, improved stability and effectiveness [2], and cyclodextrins at present are widely used as transport-active additives. The covalent attachment of bioactive peptides to cyclodextrins has also been proposed [3,4], although their grafting to the relatively large cyclodextrin carrier might impair recognition by receptor molecules. In fact, covalent linkage of an enkephalin analog to β-cyclodextrin was found to lower significantly the receptor affinity and selectivity, but the clearance rate was reduced [5].

In recent years extensive investigations have been performed by NMR and X-ray analysis on methylated and unmethylated β-cyclodextrin monosubstituted at the C6 of the cyclomaltoheptaose with aromatic chromophores [6], amino acids, related derivatives and dipeptides [7–10]. Self-inclusion of the grafted moieties was found to depend critically upon the spacer [7,8]. The length and flexibility of the spacer are expected to play a similarly decisive role in the mode of presentation of grafted biomolecules to the recognition by receptor molecules.

In the present study we have examined this aspect by using β-cyclodextrin as template in a conjugate with the well established, but poorly water-soluble calpain inhibitor Ac-Leu-Leu-Nle-H [11].

2. Materials and methods

2.1. Materials

All reagents and solvents used in the synthesis were of the highest quality commercially available. The calpain inhibitor Ac-Leu-Leu-Nle-H and human cathepsin B (EC 3.4.22.1) were purchased from Calbiochem (Bad Soden, Taunus), Z-Phe-Arg-NH-Mec and Suc-Leu-Tyr-NH-Mec from Bachem (Heidelberg) and chemicals for buffers from Sigma (München). Human calpain I (μ-calpain) (EC 3.4.22.17) was isolated from human erythrocytes by B. Gerhartz in the laboratory of E.A. Auerswald, München.

TLC was carried out on silica gel 60 plates (Merck AG, Darmstadt) and compounds were visualized by fluorescamine, chlorine/*o*-tolidine, H_2SO_4/MeOH; naphthoresorcinol/H_3PO_4. CE was performed on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt) at 25 kV using an underivatized fused silica capillary (67 cm×75 m; length×ID) and 50 mM sodium borate buffer (pH 8.5); HPLC was carried out with Waters equipment (Eschborn, Germany) on Nucleosil 300/C8 (Machery and Nagel, Düren) using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min. NMR spectra were recorded on Bruker AMX500, FAB-MS spectra on Finnigan MAT 900 and MALDI-TOFMS on Bruker Reflex II instruments. CD spectra were measured on a Yobin-Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All data are averages of 10 scans and the spectra were taken at 20°C employing quartz cells of 0.2 cm optical path length. The spectra are reported in terms of ellipticity units per mol of compounds ($[\theta]_M$). The spectra were recorded in water/MeOH (95:5, v/v) and concentrations were determined by weight and peptide content as determined by quantitative amino acid analysis (6 M HCl; 110°C; 48 h); peptide content of Ac-Leu-Leu-Nle-H: 79% and β-cyclodextrin/Leu-Leu-Nle-H: 77%.

2.2. Synthesis of the β-cyclodextrin/L-leucyl-L-leucyl-L-norleucinal conjugate

Boc-Nle-H was obtained by reduction of the related *N*-methyl-*N*-methoxy carboxamide with lithium aluminum hydride [12] and converted to the stereochemically stable semicarbazone Boc-Nle-Sc [13–15]. Upon deprotection of the α-amino group with 25% TFA/DCM the amino acid semicarbazone H-Nle-Sc was extended to the tripeptide Boc-Leu-Leu-Nle-Sc by standard procedures using Boc-Leu-OSu in the acylating steps. Final deprotection again with 25% TFA/DCM led to H-Leu-Leu-Nle-Sc.TFA; homogeneous on TLC ($CHCl_3$/MeOH/H_2O, 80:40:6; $CHCl_3$/MeOH/pyridine/H_2O, 60:15:2:1); FAB-MS: $m/z = 399.4$ ($[M+H]^+$); calcd. for $C_{19}H_{38}N_6O_3$: 398.3; ^{1}H-NMR (d_6-DMSO) was consistent with the structure.

Mono(2,3-di-*O*-acetyl-6-deoxy-6-amino)hexakis(2,3,6-tri-*O*-acetyl)-β-cyclodextrin was synthesized according to known procedures [16,17] and then reacted in pyridine with 1.5 equiv. succinic anhydride. Upon removal of the excess anhydride by washings of the AcOEt solution

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with a 5% $KHCO_3$ solution the succinylated derivative was isolated by flash chromatography (eluent: toluene/EtOH, 2:1) in 85% yield; homogeneous on TLC (toluene/EtOH, 2:1; R_f 0.43); ^{1}H-NMR (d_6-DMSO): δ = 1.97–2.07 (m, 60H), 2.31 (br s, 4H), 3.60–4.50 (m, 28H), 4.64–4.81 (m, 7H), 5.01–5.10 (m, 7H), 5.14–5.26 (m, 7H), 7.72 (br s, 1H); FAB-MS: m/z = 2113.0 (37%, $[M+K]^+$), 2097.0 (40%, $[M+Na]^+$), 2075.0 (100%, $[M+H]^+$), 2057.1 (21%, $[M+H-H_2O]^+$); calcd. for $C_{86}H_{115}O_{57}N$: 2073.61.

Deacetylation of mono(2,3-di-*O*-acetyl-6-deoxy-6-succinylamido)-hexakis(2,3,6-tri-*O*-acetyl)-β-cyclodextrin was performed with KOH in aqueous MeOH for 4 h, then the reaction mixture was diluted with water and treated with Amberlyst 15. Mono(6-deoxy-6-succinylamido)-β-cyclodextrin was isolated in 98% yield from the concentrated aqueous solution by precipitation with acetone; homogeneous on TLC (acetonitrile/H_2O, 7:3; R_f 0.26), HPLC (t_R 6.85 min) and CE (t_M 5.28 min); ^{1}H-NMR (D_2O): δ = 2.50–2.65 (m, 4H), 3.33 (dd, 1H), 3.46 (t, 1H), 3.56–3.74 (m, 12 H), 3.80–4.06 (m, 28 H), 5.02–5.20 (m, 7H); FAB-MS: m/z = 1310.0 (33%, $[M-H+2K]^+$), 1272.2 (100%, $[M+K]^+$), 1234.1 (30%, $[M+H]^+$); calcd. for $C_{46}H_{75}O_{37}N$: 1233.40.

The mono-functionalized β-cyclodextrin was coupled overnight with H-Leu-Leu-Nle-Sc trifluoroacetate in DMF with PyBOP [18] upon neutralization with TEA. The cyclodextrin/peptide conjugate was obtained by direct precipitation from water with acetone in 68% yield; homogeneous on TLC (acetonitrile/H_2O, 7:3; R_f 0.42), HPLC (t_R 13.12 min) and CE (t_M 4.40 min); FAB-MS: m/z = 1614.9 (71%, $[M+H]^+$); calcd. for $C_{65}H_{111}O_{39}N_7$: 1613.69. The semicarbazone derivative was hydrolyzed in MeOH with acetic acid in presence of formaldehyde and the desired β-cyclodextrin/tripeptide aldehyde conjugate was isolated from water by precipitation with acetone in 72% yield; homogeneous on TLC (acetonitrile/H_2O, 7:3; R_f 0.46), HPLC (linear gradient from 100% A to 100% B in 30 min; t_R 13.12 min) and CE (t_M 4.45 min); ^{1}H-NMR (D_2O): δ = 0.88–1.07 (m, 15H), 1.17–1.90 (m, 12H), 2.47–2.80 (m, 4H), 3.07–3.14 (m, 1H), 3.40–4.10 (m, 42H), 4.28–4.42 (m, 2H), 4.97 (d, 1H hydrated aldehyde), 5.05–5.18 (m, 7H), 9.50 (s, 1H, aldehyde); FAB-MS: m/z = 1557.3 (18%, $[M+H]^+$), 1539.3 (100%, $[M+H-H_2O]^+$), MALDI-TOFMS: m/z = 1579.6 $[M+Na]^+$, 1595.6 $[M+K]^+$; calcd. for $C_{64}H_{108}O_{39}N_4$: 1556.65.

2.3. NMR spectroscopy

NMR spectra of the β-cyclodextrin/Leu-Leu-Nle-H conjugate were recorded at 500 MHz in d_6-DMSO (5 mM) and water (10 mM, 10% D_2O/90% H_2O). For 1D and 2D proton spectra the following parameters were used. 1D ^{1}H-NMR (d_6-DMSO): 400 acquisitions, size 16K, sweep width 7575.7 Hz; TOCSY [19,20] (d_6-DMSO): mixing time for MLEV17 40 ms, trim pulse 2.5 ms, size 2K, sweep width 7575.7 Hz in t_1 and t_2, 64 acquisitions, 512 increments [21]; NOESY [22] (d_6-DMSO): mixing time 150 ms, size 2K, sweep width 7575.7 Hz in t_1 and t_2, 64 acquisitions, 512 increments [21]; 1D ^{1}H-NMR (water): with presaturation, 64 acquisitions, sweep width 7575.7 Hz, size 16K and with jump return pulse sequence [23] and same parameter as with presaturation; TOCSY [19,20] (water): with presaturation, mixing time for MLEV17 40 ms, trim pulse 1.5 ms, size 2K, sweep width 7575.7 Hz in t_1 and t_2, 96 acquisitions, 416 increments (TPPI) [24]; NOESY [22] (water): with jump return pulse sequence [23], mixing time 200 ms, size 2K, sweep width 7575.7 Hz in t_1 and t_2, 96 acquisitions, 512 increments (TPPI); ROESY [25] (water): 150 ms spin-lock with water suppression, 96 acquisitions, size 2K, 512 increments (TPPI), spin-lock power 4 kHz, sweep width 7575.7 Hz in t_1 and t_2; prior to transformation of the TOCSY, NOESY and ROESY spectra gaussian window function in t_2 and shifted sine-bell function in t_1 were used.

2.4. Determination of kinetic constants

Continuous fluorometric inhibition assays were performed and evaluated as described in detail elsewhere [26,27]. Inhibition of cathepsin B (27 pM) was assayed at 12 and 30°C with the substrate Z-Phe-Arg-NH-Mec (10 μM) in 1.2 ml of 0.3 mM sodium acetate buffer (pH 5.5) containing 2 mM EDTA, 0.015% Brij-35, 1 mM dithiothreitol (added freshly), 1% DMSO (from added substrate solution). The inhibitors Ac-Leu-Leu-Nle-H (dissolved in DMSO) and β-cyclodextrin/Leu-Leu-Nle-H (dissolved in water) were added in 1–10 μl aliquots resulting in final concentrations of 1–125 nM in the assay. Inhibition of calpain (10–15 nM) was measured at 12°C with the substrate Suc-Leu-Tyr-NH-Mec (250 μM) in a total volume of 1.2 ml of 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM dithiothreitol (added freshly), 200 μM $CaCl_2$, 0.015% (by vol.) Brij-35 and 1% DMSO (from the substrate solution). After 10–15 min, when calpain was fully activated and the rate of substrate release constant, inhibitor solutions (1–10 μl) were added resulting in final concentrations of 0.1–0.3 μM for Ac-Leu-Leu-Nle-H and of 0.4–10 μM for β-cyclodextrin/Leu-Leu-Nle-H.

The rate constants k_{on}, k_{off} (mechanism A), k_5, k_6 (mechanism B), and the dissociation constant of the initial complex, K_i (mechanism B), were obtained from the presteady-state phase of the progress curves as described by Morrison [28]. The equilibrium dissociation constant for mechanism A was calculated from the rate constants ($K_i = k_{off}/k_{on}$) or measured in steady-state inhibition experiments when the presteady-state phase was too fast to be resolved. For mechanism B the overall inhibition constant K_i^* was calculated from the equation: $K_i* = K_i \cdot k_6/(k_5 + k_6)$ [28].

3. Results and discussion

To allow for flexible display of the tripeptide aldehyde Leu-Leu-Nle-H in the conjugate, the mono(6-succinylamido-6-deoxy)-β-cyclodextrin containing a 4C-spacer was synthesized by classical methods of carbohydrate chemistry as outlined in Fig. 1. The intermediate mono(6-azido-6-deoxy)-β-cyclodextrin was peracetylated prior to the phosphine-mediated reduction of the azido group to the amino function and subsequent acylation with succinic anhydride in order to avoid formation of succinic acid esters. Deacetylation generated the β-cyclodextrin derivative mono-functionalized at the linear, fully flexible 4C-spacer as carboxylic acid. The tripeptide aldehyde H-Leu-Leu-Nle-H was prepared as the semicarbazone according to known procedures [12–15] and was then linked to the cyclodextrin derivative via the PyBOP procedure [18]. Formation of intra- and/or intermolecular ester bonds with the free hydroxyl functions was not observed to occur under these conditions. Finally, weak acid hydrolysis of the semicarbazone in the presence of excesses of formaldehyde led to the desired conjugate in good overall yield as a homogeneous and well characterized compound.

Table 1
Kinetic constants for the inhibition of cathepsin B and μ-calpain by the β-cyclodextrin/Leu-Leu-Nle-H conjugate and Ac-Leu-Leu-Nle-H

		Cathepsin B			μ-Calpain			
		k_{on} ($\times 10^{-5}$) (M^{-1} s^{-1})	k_{off} ($\times 10^4$) (s^{-1})	K_i (nM)	K_i (nM)	k_5 ($\times 10^3$) (s^{-1})	k_6 ($\times 10^4$) (s^{-1})	K_i^* (nM)
β-Cyclodextrin/	30°C			8.5 ± 0.54 (9)				
Leu-Leu-Nle-H	12°C	0.40 ± 0.0064	0.8 ± 0.10	2.0 (8)	3470 ± 449	3.7 ± 0.77	1.0 ± 0.16	91 (6)
Ac-Leu-Leu-Nle-H	30°C			2.2 ± 0.055 (15)				
	12°C	2.80 ± 0.064	2.8 ± 0.12	1.0 (11)	122 ± 26	2.8 ± 0.28	0.77 ± 0.16	3.2 (7)

See Section 2.4 for experimental conditions and definition of constants. The best estimates obtained by nonlinear regression analysis are given together with their standad errors and the number of experiments (in parentheses).

1D and 2D ^{1}H-NMR spectra were recorded in DMSO and water in the attempt to define the mode of display of the peptide moiety on the cyclodextrin template. TOCSY and NOESY spectra (data not shown) allowed us to assign all resonances of the tripeptide aldehyde including the spacer residue. Moreover, with the 1D and TOCSY spectra in H_2O/D_2O quantification of the hydrated (87%) and non-hydrated aldehyde (13%) was achieved. With the TOCSY and NOESY spectra, however, only a partial assignment of the carbohydrate moiety could be achieved in both solvents. The carbohydrate signals were found to be more dispersed in water than in DMSO. While the anomeric protons of the seven glucopyranose units overlap in water ($\delta = 5.18$–5.05 ppm), the residual protons of the modified unit are partly well separated. Mono-attachment of the peptide moiety to the symmetric cyclodextrin molecule via the succinyl spacer leads to an asymmetric cyclodextrin derivative with concomitant partial resolution of the resonances in the 1D ^{1}H-NMR spectrum. This dispersion may, therefore, not necessarily reflect self-inclusion of the peptide as suggested previously in mono-derivatized cyclodextrins [29,30]. The NOESY spectra in both solvents and the ROESY spectrum in water show sequential NH-CHα, NH-side-chain and NH-spacer NOEs (ROEs) within the peptide chain and the spacer residue. The amide proton of the derivatized glucopyranose unit exhibits several NH-carbohydrate proton NOEs, whereas no NOE could be detected from the peptide backbone amides as well as from the CHα protons to the carbohydrate moiety. The absence of these NOEs in water and DMSO would exclude self-inclusion of part of the peptide chain. However, the weak NOEs observed between the methyl groups of the leucine and/or norleucine side chains to the carbohydrate portion are supportive of an interaction of the peptide with the cyclodextrin surface in a manner similar to the capped structural model [7]. Apparently, this type of interaction is sufficient to convert the poorly water-soluble Ac-Leu-Leu-Nle-H into a fully water-soluble cyclodextrin conjugate.

A comparison of the CD spectrum computed from the spectra of mono(6-deoxy-6-succinylamido)-β-cyclodextrin and Ac-Leu-Leu-Nle-H with that of the β-cyclodextrin/Leu-Leu-Nle-H conjugate shows that the dichroic contributions of the two component parts are not additive (Fig. 2). A noticeable red shift of the negative ππ* maximum with a strong increase in intensity is observed that would be consistent with a less hydrophilic environment of the peptide backbone [31] and thus with the capped structural model in which the peptide portion is interacting with the top of the cylindrical cyclodextrin.

Regarding the inhibitory potency of the tripeptide aldehyde when covalently linked to β-cyclodextrin, it showed the same reversible slow-binding inhibition of cathepsin B and μ-calpain as Ac-Leu-Leu-Nle-H. Under the experimental conditions used the inhibition of cathepsin B by both tripeptide aldehydes follows a simple single-step mechanism A [28], whereas the inhibition of calpain by both inhibitors was described best by the mechanism B [28] where a rapidly formed initial complex (K_i) is slowly converted to a tightened complex (K_i^*). The molecular basis of this two-step mechanism is not known.

The inhibition constants (K_i) of Ac-Leu-Leu-Nle-H and its β-cyclodextrin conjugate determined in the present study (Table 1) are considerably lower than those previously published

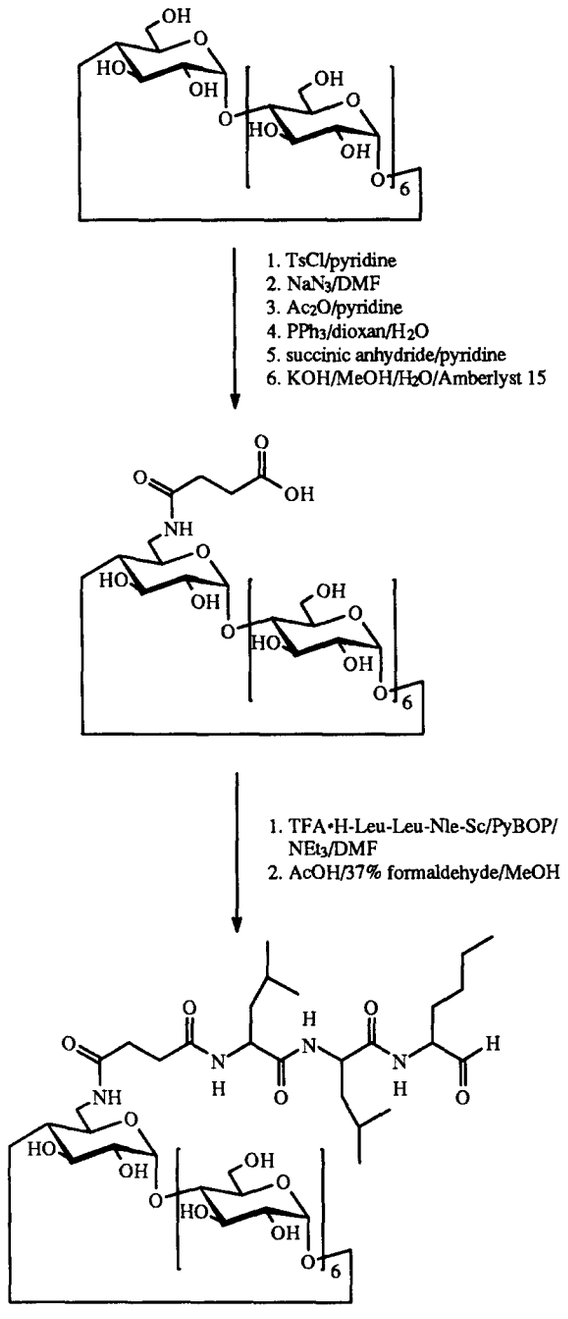

Fig. 1. Synthesis of the β-cyclodextrin/Leu-Leu-Nle-H conjugate.

for the calpain inhibitor (150 nM for cathepsin B and 190 nM for calpain I [11]). These differences might be explained by the fact that the former values were obtained in stopped assays rather than by detailed kinetic analysis. In control experiments β-cyclodextrin (up to 100 μM) did not affect the activity of cathepsin B and calpain. The related kinetic data confirm that potential complexation of the substrate and/or of the released fluorophore by the cyclodextrin does not disturb the measurements, although such complexation is known to affect both the intensity and location of the fluorescence emission maxima of sequestered fluorophores [32]. Moreover, inhibition of both enzymes by Ac-Leu-Leu-Nle-H was not affected when β-cyclodextrin (up to 10 μM) was added to the

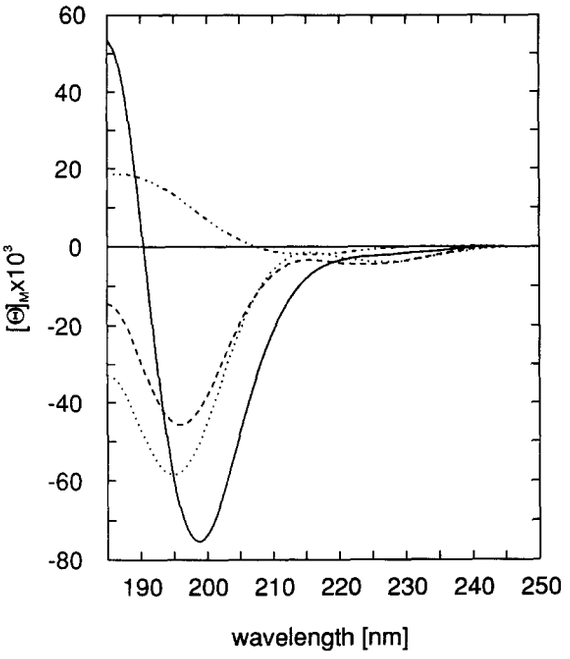

Fig. 2. CD spectra of Ac-Leu-Leu-Nle-H (· · · ·), mono(6-succinylamido-6-deoxy)-β-cyclodextrin (· · · · · · · ·) and of the β-cyclodextrin/Leu-Leu-Nle-H conjugate (——) in water/MeOH (95:5); for comparison the spectrum computed from the dichroic contributions of the two component parts (- - - - -).

assays. These results confirm that the observed inhibition is completely due to the peptide aldehyde moiety of the β-cyclodextrin conjugate.

As indicated by the dissociation constants (K_i), the affinity of the tripeptide aldehyde for cathepsin B is only 2–4-fold reduced upon its conjugation to the β-cyclodextrin template (see Table 1). The slightly weaker inhibition of cathepsin B by the β-cyclodextrin conjugate compared to the free tripeptide aldehyde is mainly due to its 7-fold lower association rate constant compared to the 3.5-fold lower k_{off} rate. The lower k_{on} rate could derive from some steric hindrance in the binding process that results from the bulky cyclodextrin template. Conversely, the lower k_{off} rate would indicate a contribution, although weak, of the cyclodextrin moiety in stabilizing the enzyme/inhibitor complex. An inspection of the X-ray structure of human cathepsin B [33] reveals as peculiar feature of this enzyme the presence of an occluding loop with two histidines which limits C-terminally the size of the substrate, whereas the S-subsites are easily accessible and similar to the binding cleft in papain. In the X-ray structure of the papain/stefin B complex [34] the P_1 and P_2 side chains interact tightly with the related enzyme binding subsites, but the P_3 side chain of methionine was already found to be only in a loose contact with the enzyme surface. Since a substrate-like binding is expected for the tripeptide aldehyde, the 4C-spacer used in the cyclodextrin-conjugate is apparently fully sufficient for optimal binding of the inhibitor portion.

The calpain activity is known to be rather quickly destroyed by autolysis of the Ca^{2+}-activated enzyme. Therefore, pre-steady-state inhibition kinetics with calpain could be measured only by lowering the temperature to 12°C where the rate of substrate release was found to be nearly constant over a period of 2 h. Under these conditions the covalently attached β-cyclodextrin template effected a 28-fold higher dissociation constant of the initial enzyme-inhibitor complex (K_i) and of the tightened complex (K_i^*), respectively, whereas it did not affect significantly the rate constants of the slow conversion between the two complexes (k_5, k_6). Unfortunately, the X-ray structure of calpain is not yet available; thus a rational explanation of the experimental data is not possible, although the strongly reduced inhibition potency of the tripeptide aldehyde in the conjugate would primarily suggest sterically hindered access to the binding cleft.

The results presented in this study show that the highly promising properties of cyclodextrin/bioactive peptide conjugates can be exploited with minimal loss of bioactivity if attention is paid to optimize in each case the spacer in order to allow for recognition of the grafted biomolecule.

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