Interaction of the collagen-like tail of asymmetric acetylcholinesterase with heparin depends on triple-helical conformation, sequence and stability

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The collagen-like tail of asymmetric acetylcholinesterase (AChE) contains two heparin-binding domains (HBDs) that interact with heparan sulphate proteoglycans, determining the anchoring of the enzyme at the basal lamina and its specific localization at the neuromuscular junction. Both HBDs are characterized by a cluster of basic residues containing a core with the BBXB consensus sequence (where B represents a basic residue and X a non-basic residue). To study the interaction of such HBDs with heparin we have used synthetic peptides to model the N-terminal and C-terminal sites. CD spectroscopy showed that all peptides are triple-helical at low temperatures, and undergo trimer-to-monomer transitions. Displacement assays of asymmetric AChE bound to heparin were performed using the peptides in both monomeric and triple-helical states. In the monomeric con-

formation, all the peptides were able to displace low levels of AChE depending on the basic charge content. In the triple-helical conformation, peptides containing the consensus sequence showed a large increase in the ability to displace bound AChE. Results suggest that the specific binding of the collagen-like-tail peptides to heparin depends both on the presence of the core sequence and on the triple-helical conformation. Moreover, BBXB-containing peptides that are less stable are more effective in displacing AChE, suggesting that the interaction region needs a significant amount of structural flexibility to better accommodate the ligand.

Key words: cholinergic, ColQ, proteoglycan, triple-helical peptide.

INTRODUCTION

The triple-helical conformation is one of the basic structural motifs found in proteins [1]. This structure has long been recognized as a major component of all collagens and recently a growing number of unrelated molecules have been found to contain triple-helical domains. Examples of such triple-helix-containing proteins include the macrophage scavenger receptor, C1q, pulmonary surfactant apoproteins A and D, mannose-binding protein and the asymmetric form of acetylcholinesterase (AChE) [2,3]. In addition to their structural role in providing rigid rods and proper geometric organization of molecules, triple-helix regions are important for ligand binding [2,4].

AChE (EC 3.1.1.7) is responsible for the rapid hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses, allowing for the precise control of neurotransmission and muscle contraction [5,6]. AChE displays an exceptional polymorphism, exhibiting both globular and asymmetric forms that differ in their macromolecular structure and cellular localization [3]. In particular, asymmetric AChE (A₄, A₈ and A₁₂ forms) consists of one to three catalytic tetramers linked to a collagen-like tail, ColQ, which is coded for by a separate gene [7,8]. The ColQ triple helix is a homotrimer, composed of three identical chains. It contains a central triple-helical domain surrounded by non-collagen-like terminal sequences. The C-terminal region is a globular proline and cysteine-rich domain, while the N-terminal region is a proline-rich domain responsible for the recruitment of the catalytic subunits [9,10]. This general organization as well as the amino acid sequence is highly conserved between the electric fish *Torpedo* and mammalian ColQ [11–14].

The central collagen-like domain in *Torpedo* consists of 180 residues, characterized by Gly-Xaa-Yaa repeats (where Xaa and

Yaa denote two unknown amino acids) [14], and a high proportion of the stabilizing imino acids proline and hydroxyproline (18%), as well as the presence of hydroxylysine [15–17]. The presence of glycine as every third residue and the high imino acid content allow the formation of a triple-helical structure, in which the three helical chains are staggered by one residue with respect to each other, and are supercoiled in a right-handed manner [18,19]. In this conformation, glycine residues are buried in the centre of the superhelix, whereas residues in the Xaa and Yaa positions have substantial exposure to solvent, making them available for interaction with other molecules [2,20].

In asymmetric AChE, the collagen-like subunit ColQ is responsible for the anchoring of the enzyme in the basal lamina as well as for the highly specific localization at the synaptic cleft of the neuromuscular junction [5,15]. Since the whole process of synaptic transmission takes place within 1 ms, it demands a very precise tempo-spatial integration of the components involved [21]. Several molecules have been shown to bind ColQ *in vitro*. These include heparan sulphate proteoglycans (HSPGs) [22,23], laminin, type-I and -V collagens [24], sphingomyelin liposomes [25] and a collagen-like protein of 140 kDa present in *Torpedo* extracellular-matrix extracts [23]. However, at this time, only the binding of HSPGs has been shown to have physiological relevance. The evidence suggests that HSPGs could have a role in the early stages of anchoring as well as in clustering of the enzyme at the neuromuscular junction [22,26,27].

Our laboratory has described two heparin-binding consensus sequences in the collagen-like domain of ColQ [28,29], composed of a BBXB motif, where B represents a basic residue and X a non-basic residue [30]. The two consensus sites contain different basic residues, Arg-Lys-Gly-Arg and Lys-Arg-Gly-Lys, and each is surrounded by additional basic residues that may also parti-

Abbreviations used: AChE, acetylcholinesterase; HBD, heparin-binding domain; HSPG, heparan sulphate proteoglycan; GAG, glycosaminoglycan; T_m, melting temperature; ColQ, collagen-like tail of asymmetric AChE; BBXB consensus sequence, B represents a basic residue and X a non-basic residue.

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Figure 1 Scheme of asymmetric AChE

The asymmetric form of AChE, A₁, consists of three tetramers of catalytic subunits associated with ColQ, a collagen-like protein. ColQ contains a central collagen-like domain, an N-terminal domain linked to the catalytic subunits (left), and a C-terminal globular domain (right). The collagen-like region has two HBDs, whose sequences and approximate locations are shown. O, hydroxyproline.

cipate in heparin binding [31] (see Figure 1). The consensus sequences are conserved among species, being found in both *Torpedo* and human [11,12,14], supporting the hypothesis of functional significance.

Given the linear, helical nature of the collagen-like structure, it is interesting to consider the determinants mediating the specificity of heparin recognition by the heparin-binding domains (HBDs) of ColQ. In this study, peptides were designed to model the heparin-binding regions of ColQ. In order to promote a triple-helical conformation in peptides, the most stabilizing tripeptide unit, Gly-Pro-Hyp, was included at the ends of each of the two *Torpedo* ColQ HBD sequences. The results suggest that the adoption of the triple-helical conformation greatly enhances the interaction between ColQ-binding sites and heparin, and that the consensus sequence is required. The interaction of the C-terminal binding site appeared to be stronger than that of the N-terminal binding site, and the lower stability of the former suggests a preference for a flexible binding site.

MATERIALS AND METHODS

Materials

Synthetic peptides containing ColQ sequences were purchased from SynPep Corp. (Dublin, CA, U.S.A.). The (Pro-Hyp-Gly)₁₀ peptide was purchased from Peptides International (Osaka, Japan). Their purity, determined by HPLC, varied from 92 to 97% depending on the peptide. For peptide sequences, see Tables 1 and 2. Heparin–albumin, heparin from bovine lung, heparan sulphate from bovine kidney, chondroitin sulphate from whale and shark cartilage, de-N-sulphated heparin from porcine mucosal heparin, β -galactosidase, alkaline phosphatase, BSA, leupeptin, pepstatin, bacitracin, caproic acid and decamethonium bromide were obtained from Sigma (St. Louis, MO, U.S.A.). Acridine-agarose was kindly provided by Dr Terry Rosenberry (Mayo Clinic, Jacksonville, FL, U.S.A.). Other reagents, when not otherwise specified, were obtained from commercial sources.

CD spectroscopy

Peptides were dried *in vacuo* over P₂O₅ for at least 48 h before weighing. Samples for CD were prepared at a concentration of

Table 1 Sequences of five peptide models of the *Torpedo* collagen-like tail of AChE and their melting temperatures (T_m), mean residue ellipticity at 225 nm (θ_{225}) and basic charge content

The central 15-mer sequences correspond to the natural sequence and the triplets in parentheses are Pro-Hyp-Gly triplets added for stabilization. The natural sequences correspond to the *Torpedo* AChE tail [14]. The basic residues are shown in bold type. The most stable 30-mer, (POG)₁₀, is shown for comparison. O, hydroxyproline.

Peptide	Peptide sequence	θ_{225} (deg · cm ² · dmol ⁻¹)	T_m (°C)	No. of basic residues
P146	(POG) ROGRKGRGVGRGPRG (POG) ₄	1970	15.5	6
P146A	(POG) ROGAAGAAGVGRGPRG (POG) ₄	2600	18.6	3
P146B	(POG)AOG RGKRG ROGVAGPAG(POG) ₄	2400	11.0	3
P251	(POG) ROGRKRGKTGLKGDIG (POG) ₄	2000	9.8	5
P224	(POG)LOGMLGQ KGEMGPKG (POG) ₄	1700	7.0	2
(POG) ₁₀	(POG)POGPOGPOGPOGPOG(POG) ₄	4000	60.0	0

Table 2 Sequences of two peptide models of the rat collagen-like tail of AChE and their melting temperatures (T_m), mean residue ellipticity at 225 nm (θ_{225}) and ability to displace heparin-bound AChE

The central 12-mer sequences correspond to the natural sequence and the triplets in parentheses are Pro-Hyp-Gly triplets added for stabilization. The natural sequences correspond to the rat AChE tail [11]. The basic residues are shown in bold type. Percentage of AChE displacement was performed with peptides in triple-helical conformation at 5 μ g/ μ l, and is expressed as the mean $\pm$ S.E.M. of two experiments, with each experiment done in triplicate.

Peptide	Peptide sequence	θ_{225} (deg · cm ² · dmol ⁻¹)	T_m (°C)	AChE displacement (%)
P126r	G(POG) ₂ ROGRKGRGVGRG POG(POG) ₄	2690	30.0	15.85 $\pm$ 3.18
P231r	G(POG) ₂ ROGRKRGKGGQKG (POG) ₄	2420	23.9	44.16 $\pm$ 5.14

1 mg/ml, in 10 mM phosphate buffer, pH 7.4, with 150 mM NaCl. The concentration of the samples was confirmed by amino acid analysis. CD spectra were recorded on an Aviv Model 62DS spectropolarimeter with a Hewlett-Packard Peltier thermoelectric

temperature controller. Samples were equilibrated at 0–4 °C for at least 48 h before measuring, and data were recorded in 1-mm-pathlength cells. CD spectra were collected at 0 °C from 260 to 210 nm at 0.5-nm intervals with a 3-s recording time at each point. The results were normalized to mean residue ellipticities (θ). For equilibrium melting transitions, the ellipticity was monitored at 225 nm, and the sample temperature was raised from 0 to 60 °C in increments of 0.3 °C/min. The equilibration time at each point was 2 min and data were collected for 10 s at each point. The data obtained for the equilibrium melting were fitted to a two-state monomer-to-trimer transition model, as described previously [32].

Purification of asymmetric AChE

Asymmetric AChE was purified from *Torpedo* electric organ (Pacific Bio-Marine Laboratory, Venice, CA, U.S.A.) using the protocol of sequential extraction and affinity chromatography described previously by Rosenberry and Richardson [16], with some modifications. Frozen tissue was homogenized in 1 vol. of low-salt buffer (LS) containing 20 mM phosphate buffer, pH 7.0, 50 mM MgCl_2 , 0.5% Triton X-100 and protease inhibitors (100 mM caproic acid, 0.1 mg/ml bacitracin, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ pepstatin) in a Sorvall mixer. The homogenate was filtered to eliminate remaining cartilage and then centrifuged at 9800 g for 1 h at 4 °C. The supernatant was discarded and the pellet was rehomogenized in 0.75 vol. of LS (relative to the initial tissue weight) in a glass-to-glass homogenizer, and centrifuged as above. These steps allowed the elimination of the globular forms of the enzyme. To extract the asymmetric forms, the last pellet was homogenized in 0.36 vol. of high-salt buffer (HS) containing 20 mM phosphate buffer, pH 7.0, 1 M NaCl, 5 mM EDTA and the same protease inhibitors as included in LS. This homogenate was centrifuged at 153000 g for 40 min at 4 °C. The supernatant was retained and the same procedure was repeated with the pellet. Both supernatants obtained from the HS extractions were incubated overnight with 2 ml of acridine-agarose with constant agitation. After the affinity resin was packed in a chromatography column (0.79 $\times$ 20 cm) without flow, the HS extract was passed through at a flow rate of 3 ml/h, the column was washed with 10 vols of HS buffer, and the bound enzyme was eluted with 10 mM decamethonium bromide in HS buffer. AChE activity in the 500- μl fractions collected was determined using the colorimetric method of Ellman et al. [33]. Fractions containing the eluted AChE were pooled and concentrated using Millipore Ultrafree-MC centrifugal filters (30000 Da nominal molecular-mass limit). Final protein concentration was determined by the method of Bradford [34], and the AChE-form composition was verified by sedimentation analysis on 5–20% linear sucrose gradients, as described previously [35]. Sedimentation coefficients for AChE forms were determined by comparison with those for β -galactosidase (16 S) and alkaline phosphatase (6.1 S). The purification procedure was performed in a cold room at 4 °C to ensure that a majority of A_{12} isoform (17 S) was obtained (> 90%), with some amount of A_8 isoform (12.5 S).

Binding to heparin and displacement experiments

Binding and the displacement experiments were performed in MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) coated with heparin coupled to albumin. The plates were incubated with 200 μl of 16 $\mu\text{g}/\text{ml}$ heparin–albumin in coating buffer (15 mM Na_2CO_3 /35 mM NaHCO_3 , pH 9.6) overnight at 4 °C with constant agitation. To determine non-specific binding, the plates were coated with BSA under the same conditions. Plates were

washed once with 250 μl of washing buffer (PBS, pH 7.4/0.05% Tween-20) for 10 min at room temperature and incubated with 100 μl of blocking buffer (PBS, pH 7.4/0.05% Tween-20/0.5% BSA) for 2 h at 37 °C. Finally, the plates were washed four times with 250 μl of washing buffer as stated above.

For binding experiments, coated plates were incubated with 50 μl of A_{12} AChE at concentrations ranging from 5 pM to 100 nM in incubation buffer (20 mM phosphate buffer, pH 7.4/150 mM NaCl) for 3 h at 37 °C or for 18 h at 4 °C with constant agitation. The supernatant was recovered, the plates were washed twice with 100 μl of incubation buffer for 5 min and AChE was measured in the supernatant and washes (both representing the unbound enzyme) as well as directly in the plates (bound enzyme). The K_d values were obtained by fitting the curve to a one-site binding hyperbola using the software PRISM (GraphPad Software, San Diego, CA, U.S.A.). Both protocols gave consistent K_d values.

For displacement experiments, the heparin–albumin-coated plates were incubated with 50 μl of 20 nM A_{12} AChE in incubation buffer for 18 h at 4 °C with constant agitation. AChE activity was measured in the recovered supernatant to estimate the amount of bound AChE and to verify that the binding was homogenous in all the plates. Plates were then washed twice and incubated with 50 μl of different concentrations of glycosaminoglycans (GAGs) or peptides in incubation buffer. For GAGs, plates were incubated for 3 h at 37 °C with concentrations ranging from 0.1 $\mu\text{g}/\text{ml}$ to 2 mg/ml. For experiments determining displacement by the monomeric conformation, peptides were first equilibrated for 2 h at 37 °C, and plates were then incubated for 3 h at 37 °C. For experiments determining displacement by peptides in triple-helical conformation, peptides were equilibrated for at least 1 week at 2–4 °C to promote triple-helix formation, and the plates were incubated for 18 h at 4 °C. In both cases, incubation was performed in 50 μl of incubation buffer with peptides in concentrations ranging from 0.1 to 5 $\mu\text{g}/\mu\text{l}$. This is equivalent to a range of 35–1765 μM for monomers and 12–590 μM for trimers. To compare both experiments it is necessary to express the concentration as the weight concentration in order to have the same number of polypeptide chains (or the same number of basic residues). In all the displacement experiments, AChE activity was measured in the supernatant as well as directly in the plates, using the colorimetric method of Ellman et al. [33]. Statistical analyses were performed using the unpaired Student's *t* test.

RESULTS

Model peptide design

A set of 30-mer peptides was designed to model two HBDs of the collagen-like tail of asymmetric AChE and to evaluate their interaction with heparin (Table 1). Sequences of 15 residues incorporating each consensus sequence and surrounding basic residues were synthesized with a C-terminal cap of (Pro-Hyp-Gly) $_4$ to provide stability and a nucleation site, and with one additional Pro-Hyp-Gly triplet at the N-terminus for stability (see [36] and [37] for a discussion of design features). The C-terminus was blocked by amidation to avoid charge repulsion, which decreases stability [32]. The amino acid sequences used in the peptides were designed using the assumption that all the proline residues in the Yaa position are hydroxyproline, based on the amino acid analysis of *Torpedo* ColQ [15–17]. Each peptide is designated by its N-terminal residue in the *Torpedo* collagen-like tail sequence [14], P146 corresponding to the N-terminal HBD, and P251 to the C-terminal one (see Table 1).

Two variations of P146 were also synthesized. In P146A, the

Figure 2 Conformation and stability of model peptides

(A) CD spectra of peptides P146 (○), P251 (□), P146A (△), P146B (▽) and P224 (◇) at 2 °C. The results were normalized to mean residue ellipticities. (B) Equilibrium thermal transition curves monitored at 225 nm for the same peptides, with increments of 0.3 °C/min, from 0 to 60 °C. The data were fitted to a two-state trimer-to-monomer transition model. All spectra were recorded at 2 °C in 10 mM phosphate buffer, pH 7.4, with 150 mM NaCl.

basic residues of the BBXB consensus sequence were substituted by alanines, while in P146B the three basic residues in the region surrounding the consensus sequence were replaced by alanines, leaving the BBXB sequence intact. An additional peptide, P224, was also synthesized as a control, corresponding to a ColQ sequence that contains two basic residues but with no consensus sequence. The 30-mer consisting only of the most stabilizing tripeptide unit, (Pro-Hyp-Gly)₁₀, served as an additional standard.

Conformation and stability of AChE model peptides

The CD spectrum of all ColQ model peptides showed typical triple-helical features at low temperatures, including a maximum near 225 nm (Figure 2A). This 225-nm maximum decreases with increasing temperature, giving a sharp thermal transition (Figure 2B), which has been shown previously to correspond to the trimer-to-monomer transition [38].

The conformation and stability of peptides modelling the two HBDs were compared (Figure 2 and Table 1). The prototype triple-helical 30-mer model peptide, (POG)₁₀ (corresponding

to Pro-Hyp-Gly), has a mean residue ellipticity near 4000 deg·cm²·dmol⁻¹ at the 225-nm maximum, and a melting temperature (*T*_m) of 60 °C. The magnitudes of the 225-nm maximum for P146 (1970 deg·cm²·dmol⁻¹) and P251 (2000 deg·cm²·dmol⁻¹) suggest they both have significant triple-helix content, but less than that determined for (POG)₁₀. The melting temperature of P146, corresponding to the N-terminal HBD, was 15.5 °C, while P251, corresponding to the C-terminal HBD, was less stable (*T*_m = 9.8 °C). The increased stability of P146 compared with P251 is likely to be a consequence of the higher imino acid content in its 15-residue ColQ sequence (2 hydroxyprolines and 1 proline compared with 1 hydroxyproline). In addition, arginine residues in the Yaa position have been shown to be very stabilizing [36], and P146 has two arginines residues in the Yaa position compared with one for P251.

The effect of the basic residues on the conformation and stability of P146 was examined. The replacement of the consensus basic sequence Gly-Arg-Lys-Gly-Arg with Gly-Ala-Ala-Gly-Ala in P146A led to an increased ellipticity at 225 nm (2600 deg·cm²·dmol⁻¹) and an increase in thermal stability (from *T*_m = 15.5 °C for P146 to *T*_m = 18.6 °C for P146A). These results suggest that the high density of basic charges in the consensus region in P146 results in a small destabilization, and perhaps a small loss of triple-helix content, which is reversed when these BBXB residues are replaced by alanines. Replacement of the surrounding basic residues by alanines, while retaining the consensus sequence, led to a small increase in the magnitude of the 225-nm peak (2400 deg·cm²·dmol⁻¹) and a decrease in thermal stability (*T*_m = 11 °C) for P146B. It is likely that this decreased stability is a result of the loss of two stabilizing arginine residues in the Yaa position [36]. The control peptide P224 also formed a triple helix, with mean residue ellipticity at 225 nm of 1700 deg·cm²·dmol⁻¹ and *T*_m of 7.0 °C.

The equilibrium thermal transitions indicated that at low temperature, e.g. 4 °C, all the peptides are more than 99% triple-helical, whereas at temperatures higher than the *T*_m (e.g. 37 °C), all peptides remain monomeric and are not associated in a triple-helical form (Figure 2B). Using different temperatures, it is possible to evaluate the activity of the peptides in both monomer and triple-helical forms.

Binding and displacement assays

An ELISA-like assay system was established to determine the ability of peptides to compete with the binding of ColQ. Whole asymmetric AChE is used in these assays to take advantage of the very sensitive colorimetric assay for AChE activity [33]. Experiments were carried out at two different temperatures so that the peptides could be studied in both their monomeric and trimeric forms. Conditions were established at two temperatures, 4 °C for 18 h and 37 °C for 3 h (see the Materials and methods section), such that comparable results were obtained. In both cases, the binding of asymmetric AChE to heparin was concentration-dependent and saturable. Equivalent values of *K*_d were obtained for assays at both temperatures (Figure 3A), with *K*_d = 5.11 ± 5.65 nM at 4 °C and *K*_d = 11.86 ± 6.27 nM at 37 °C. This binding can be completely displaced by heparin, but not by chondroitin sulphate or de-N-sulphated heparin, showing that AChE is effectively bound to heparin in a specific manner (Figure 3B). Heparin is normally used as a model of the physiologically relevant heparan sulphate [39], which differs from heparin in the lower degree of sulphation. At higher concentrations, heparan sulphate is also able to displace the bound enzyme (Figure 3B).

Figure 3 Binding of asymmetric AChE to heparin-coated plates

(A) Binding assays were done both at 37 °C for 3 h and at 4 °C for 18 h, using A₁₂ AChE concentrations ranging from 5 pM to 50 nM. The curve shown here is one representative experiment for the 37 °C experimental condition. The non-specific binding was determined in albumin-coated plates and was subtracted from the heparin-binding values obtained. For the 37 °C assay, the K_d obtained was 11.86 ± 6.27 nM, and for the 4 °C assay the K_d was 5.11 ± 5.65 nM (means $\pm$ S.D. of five independent experiments for each condition, each one performed in triplicate). The r^2 values of the fitting in each condition were 0.9954 ± 0.0036 and 0.9893 ± 0.0077 respectively. (B) Displacement of heparin-bound asymmetric AChE by heparin (●), heparan sulphate (■), chondroitin sulphate (▲) and de-N-sulphated heparin (▼). Heparin-coated wells were preincubated with 20 nM asymmetric AChE, and then GAGs were added at concentrations ranging from 0.1 μg/ml to 2 mg/ml. Incubations were carried out for 3 h at 37 °C. Means $\pm$ S.D. of four experiments are shown. In both (A) and (B), AChE activity was measured in the supernatant as well as directly in the plates.

Displacement of AChE by monomeric collagen-like peptides

To evaluate the ability of the different peptides in monomeric form to release asymmetric AChE bound to immobilized heparin, displacement experiments were carried out at 37 °C, with peptides previously equilibrated at this temperature to ensure their monomer state. As shown in Figure 4(A), all peptides were able to release a small amount (< 20%) of AChE in a concentration-dependent manner. At low peptide concentrations (1.8 μg/μl), the order of ability of the peptides to release the bound enzyme was P146 > P251 > P146A > P146B > P224. At the highest concentration tested (5 μg/μl), P146, P251 and P146A were able to release 18.5% of the bound AChE, whereas P146B and the control peptide P224 led to the release of 9 and 3% respectively. The order of displacement at low concentration correlates with the number of basic residues present in these monomeric peptides

Figure 4 Displacement curves of heparin-bound asymmetric AChE by peptides in monomeric and triple-helical conformation

Heparin-coated wells were preincubated with 20 nM asymmetric AChE, and then peptides were added. For peptides in monomeric conformation (A), peptides were pre-equilibrated for 3 h at 37 °C and then added at concentrations ranging from 0.1 to 5 μg/μl (35–1765 μM). Incubations were carried out for 3 h at 37 °C. For peptides in triple-helical conformation (B), peptides were pre-equilibrated for at least 1 week at 2–4 °C and added at concentrations ranging from 0.1 to 5 μg/μl (12–590 μM). Incubations were carried out for 18 h at 4 °C. Means $\pm$ S.E.M. of four independent experiments are shown, with each experiment done in triplicate. AChE activity was measured in the supernatant as well as directly in the plates. In (A) and (B), peptides P146 (●), P251 (■), P146A (▲), P146B (▼) and P224 (◆) are shown. (POG)₁₀ (●) is also shown in trimeric conformation in (B). Note the different scaling of the panels. (C) Comparison of the ability of *Torpedo* and rat peptides in monomeric (white bars) and triple-helical (shaded bars) conformations to displace heparin-bound AChE, at 1.8 μg/μl (635 μM for monomers and 212 μM for trimers). Peptides presenting a significant difference between conformations were P146 ($P < 0.005$), P251 ($P < 0.0005$), P146B ($P < 0.00001$) and P231r ($P < 0.02$). In the triple-helical conformation, P126r was significantly different from P231r ($P < 0.02$), and P146 was significantly different from P251 ($P < 0.005$), P146B ($P < 0.001$) and P146A ($P < 0.0005$).

Figure 5 Inverse correlation between thermal stability and ability to displace heparin-bound AChE of triple-helical peptides

The percentage of displacement of heparin-bound asymmetric AChE by BBXB-containing peptides in triple-helical conformation at $5 \mu\text{g}/\mu\text{l}$ was plotted against their thermal stabilities, and a linear fitting was performed, considering the S.E.M. as weight. The r^2 of the linear regression was 0.9953.

(Table 1), suggesting that the effect observed could be non-specific and dependent on the amount of positive charge.

Displacement of AChE by triple-helical peptides

Since the folding rates were quite slow ($t_{1/2} \approx 15\text{--}75$ min), peptides were equilibrated for 1 week at $2\text{--}4^\circ\text{C}$ to ensure triple-helix formation. The ability of these triple-helical peptides to displace bound AChE was determined at 4°C . Peptides P146, P146B and P251, the three peptides containing a BBXB motif, showed a dramatic increase in their ability to displace AChE compared with their monomeric forms (Figures 4B and 4C). At $5 \mu\text{g}/\mu\text{l}$ peptide, almost all of the bound AChE was displaced for P251 and P146B, while about 60% was displaced for P146. The BBXB motif in the context of the triple-helical conformation was a far more potent competitor for AChE binding of heparin than seen in the monomer form. Peptides P146A and P224, the two peptides that lack a BBXB consensus sequence, show the same ability to displace AChE as seen in the monomeric state (Figure 4C), and no displacement was observed for $(\text{POG})_{10}$ (Figure 4B). The order of the ability of peptides in their triple-helical conformations to displace bound AChE was $\text{P251} \geq \text{P146B} > \text{P146} \geq \text{P146A} > \text{P224} > (\text{POG})_{10}$ (Figure 4B).

These results suggest that in the trimeric state binding is no longer proportional to the basic charge content, but rather depends on the presence of the BBXB motif. Notably, P146B has a greater ability to displace bound AChE than P146, even though it has replaced three of the six basic residues with alanines. The studies indicate that P251, the peptide that models the C-terminal HBD, is a more effective competitor than P146, the peptide which models the N-terminal one, even though P146 forms a more stable triple helix than P251 (Table 1, Figures 4B and 4C).

To further explore a possible relationship between the displacement ability and stability of the triple-helical peptides, two additional peptides were considered. P126r and P231r follow the same basic design as the previously analysed peptides, but contain twelve residues from the N-terminal and C-terminal HBDs from the rat ColQ sequence, respectively, and an additional N-terminal Pro-Hyp-Gly triplet for higher stability

(Table 2). Both form stable triple helices, with T_m values of 30 and 23.9°C , respectively, and show a lower ability to displace bound AChE than the *Torpedo* BBXB-containing peptides, as expected by their higher stability. A plot of the displacement versus the T_m values of the five BBXB-containing peptides (Figure 5) shows that there is an inverse correlation between heparin binding and stability.

DISCUSSION

Peptides have been useful for studying the structure, dynamics and folding of triple helices, as well as their interaction with other molecules [2,40]. In this work, peptides of the form $(\text{Gly-Xaa-Yaa})_n$ were designed to model the two heparin-binding sites in the triple-helical tail of asymmetric AChE, and the design strategy for incorporating ColQ sequences into stable triple-helical peptides was successful. These peptides were used to study the interaction of these domains with heparin, a GAG that is used widely as a model of heparan sulphate, the active component of HSPG [22]. By varying the assay temperature, it was possible to evaluate the effect of conformation on the activity of the peptides. Both monomer and triple-helical forms of the ColQ model peptides that contained the BBXB consensus sequence were shown to displace AChE from heparin, but the degree of interaction was greatly enhanced by the presence of the triple-helical conformation.

Monomer forms of all peptides studied, including those containing BBXB sequences of the two heparin-binding sites, were found to show non-specific interactions with heparin based largely on the amount of basic charge present in the peptide. The maximum displacement of AChE observed was 18.5% for the most highly charged peptide P146. The observation of a similar amount of elution by protamine, a highly basic nuclear protein, supports the non-specific nature of the interaction [28]. Earlier studies done on peptides that were unable to associate in trimers showed similar abilities for the N- and C-terminal HBD sequences to elute asymmetric AChE from a heparin-agarose column [29]. This is consistent with the results reported here for the high-temperature conditions used to promote the monomer form of peptides P146 and P251.

The large increase in interaction that is observed when the AChE peptides are in a triple-helical form must be due to the geometric distribution of basic residues that offers a maximal potential for interaction with heparin. Molecular modelling of the collagen-like domain of ColQ shows that each HBD forms a broad basic belt around the triple helix where four heparin molecules can be accommodated simultaneously [31]. Each heparin can interact with three to five basic residues at the same time, and those basic residues come from different chains. This generates a geometric arrangement of basic residues that will not be found in monomers.

Prior studies have reported that ligand-binding activity by collagen and collagen-like peptides is dependent on the presence of the triple-helical conformation. Examples include the binding of a monoclonal antibody to type-III collagen [41], cell adhesion to two regions of type-IV collagen [42,43], and the binding of acetylated or oxidized low-density lipoprotein to the macrophage scavenger receptor collagen-like domain [44]. All of these collagen-like binding sites consist entirely of Gly-Xaa-Yaa sequences, and show a conformational requirement. Other collagen-like binding sites have been reported which are independent of conformation. These include a cell-binding site to a non-Gly-Xaa-Yaa region of type-IV collagen inserted into the triple helix [45], and type-II collagen epitopes for antibodies found in rheumatoid arthritis that react with monomeric peptides

and may have been generated against partially unfolded collagen [46,47].

In addition to a conformational requirement, the studies reported here demonstrate that heparin binding is dependent on the Xaa and Yaa residues in the (Gly-Xaa-Yaa)_n triple-helix sequence. The peptides were designed to distinguish between basic residues in the consensus sequence and those basic residues in the region surrounding the consensus sequence. All peptides containing BBXB sequences showed a large increase in interaction with heparin when adopting the triple-helix conformation. Peptides that contain basic residues, but lack the BBXB sequence, show no difference in the displacement of heparin-bound AChE between monomer and trimer forms. These results indicate that the interaction with heparin requires the consensus motif BBXB in the context of a collagen triple helix, where basic residues from different chains of the trimer may constitute the binding site for one heparin. It is interesting to note that HBDs described in other collagen triple helices may contain variations of this consensus sequence, such as the BXBB sequence found at two sites in type-I collagen [48,49].

The two triple-helical HBDs in ColQ have similarities in sequence as well as conformation, yet peptide P251, which models the C-terminal HBD, is more effective than peptide P146, which models the N-terminal domain, in displacing AChE from heparin. This is also true for P126r and P231r that model the N- and C-terminal HBDs of the rat asymmetric AChE. The stronger interaction of the C-terminal binding domain compared with the N-terminal one was also observed in a study of recombinant rat asymmetric AChE with specific mutations that altered one of the two heparin-binding sites (P. Deprez, E. Krejci, J. Massoulié and N. C. Inestrosa, unpublished work). The agreement of results on intact recombinant molecules with those on peptides supports the use of triple-helical peptides as tools for characterizing collagen-ligand interactions. The greater ability of the C-terminal heparin-binding site to interact with heparin is not related simply to net positive charge, since it has one less basic residue than the N-terminal HBD. The lack of a direct relationship with charge is even more notable when considering the strong displacement capacity of P146B, equal to that of P251 and greater than that of P146, yet it has three less basic charges than these other peptides.

In contrast to the lack of correspondence with basic charge content, the ability of these peptides to interact with heparin presents a strong inverse correlation with thermal stability of the triple helix. The order of ability of the five peptides with BBXB sequences to displace heparin-bound AChE is P251 ≥ P146B > P146 > P231r > P126r, whereas the reverse order shows the stability of the peptides. The observed relative capacity of peptides to displace heparin suggests that increased structural flexibility may promote interactions, through a greater ability to accommodate heparin. A requirement for triple-helix flexibility has been proposed to occur in regions involved in the collagenase cleavage site [50], and in a monoclonal antibody binding site [37], and could be a general feature of triple-helix recognition.

Thus studies on model peptides indicate a requirement for both conformation and the consensus sequence in concert, and shows how surrounding sequences that modulate flexibility may influence binding. Studies are in progress to determine the basis of local triple-helix stability in terms of the identity of the residues in the Xaa and Yaa positions to better understand the relation between sequences of collagen-like HBDs, local destabilization and interaction with heparin.

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