Two Different Heparin-binding Domains in the Triple-helical Domain of ColQ, the Collagen Tail Subunit of Synaptic Acetylcholinesterase*

Received for publication, February 7, 2003, and in revised form, April 7, 2003
Published, JBC Papers in Press, April 8, 2003, DOI 10.1074/jbc.M301384200

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ColQ, the collagen tail subunit of asymmetric acetylcholinesterase, is responsible for anchoring the enzyme at the vertebrate synaptic basal lamina by interacting with heparan sulfate proteoglycans. To get insights about this function, the interaction of ColQ with heparin was analyzed. For this, heparin affinity chromatography of the complete oligomeric enzyme carrying different mutations in ColQ was performed. Results demonstrate that only the two predicted heparin-binding domains present in the collagen domain of ColQ are responsible for heparin interaction. Despite their similarity in basic charge distribution, each heparin-binding domain had different affinity for heparin. This difference is not solely determined by the number or nature of the basic residues conforming each site, but rather depends critically on local structural features of the triple helix, which can be influenced even by distant regions within ColQ. Thus, ColQ possesses two heparin-binding domains with different properties that may have non-redundant functions. We hypothesize that these binding sites coordinate acetylcholinesterase positioning within the organized architecture of the neuromuscular junction basal lamina.

At vertebrate cholinergic synapses, acetylcholinesterase (AChE)† rapidly hydrolyzes the neurotransmitter acetylcholine, thereby terminating synaptic transmission. This key function does not only require a high catalytic turnover number but also a strategic positioning of the enzyme. This is achieved by the association of AChE catalytic subunits with structural subunits that bring them to the site of action. In the case of asymmetric AChE, the collagen ColQ is responsible for the localization of the enzyme at the vertebrate neuromuscular junction. Inactivation of the ColQ gene in mice or mutations in the human ColQ gene result in the absence of enzyme accumulation at the neuromuscular junction and are the cause of a congenital myasthenic syndrome (type 1c) (1, 2).

As found in other collagens, ColQ contains a central triple-helical domain surrounded by non-collagenous N- and C-terminal domains (Fig. 1A). Each N-terminal domain organizes an AChE tetramer, so the triple-helical structure generates an A12 or asymmetric AChE form with 12 catalytic subunits (3, 4). The collagen domain is characterized by Gly-Xaa-Yaa repeats and a high proportion of the stabilizing proline and hydroxyproline-rich regions. The C-terminal domain is divided into a proline-rich region, important for triple-helix formation (5), and a cysteine-rich region probably involved in the anchorage of AChE at the neuromuscular junction, since mutations in this region prevent the accumulation of AChE in congenital myasthenic syndrome type 1c patients (2).

Heparan sulfate proteoglycans (HSPGs) have been implicated in the anchorage of asymmetric AChE to the synaptic basal lamina by interacting with ColQ through their heparan sulfate (HS) chains. This was proposed after showing that AChE could be specifically solubilized from the tissue with heparinase as well as with HS and heparin (6). Consistently, binding of the enzyme to the cell surface is inhibited after treatment of the myotubes with heparitinase and in cells deficient in HS synthesis (7). Later studies showed that exogously added asymmetric AChE associated specifically with nerve-muscle contact sites in a heparin-sensitive manner (8), suggesting that HSPGs would define its localization at the neuromuscular junction. Perlecan, a basal lamina HSPG, has been proposed as the receptor of collagen-tailed AChE (9), consistent with the recent finding that AChE is not accumulated at the neuromuscular junction in perlecan-null mice (10).

In this context, we have found that the collagen domain of ColQ contains two heparin-binding consensus sequences of the BBXB form (where B represents a basic residue) surrounded by other basic residues and proposed they constitute the sites for HSPG recognition (11). We have characterized the structural properties of those putative heparin-binding domains (HBDs) by using molecular modeling and synthetic peptides (12–14). Here, we analyzed ColQ-heparin interactions by heparin affinity chromatography of the entire A12 AChE, carrying different mutations in ColQ. We first established that recombinant and purified A12 enzymes share the same properties. Then we dissected the participation of the different domains of ColQ as well as of the individual basic residues composing both HBDs to define the determinants of heparin binding. The results obtained provide evidence that expands the role of ColQ-HSPGs interactions to the fine ultrastructural organization of AChE at the synaptic basal lamina. Moreover, they contribute to our

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*This work was supported by Fondo Nacional de Ciencia y Tecnología Grant 2970072 (to P. D.), Fondo de Investigación Avanzada en Areas Prioritarias Grant 2970072 (to P. D.), Fondo de Investigación Avanzada en Areas Prioritarias Grant 3980001, and the CNRS, and the Association Française contre les Myopathies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: AChE, acetylcholinesterase; HBD, heparin-binding domain; HS, heparan sulfate; HSPG, HS proteoglycan; WT, wild-type.
ColQ-Heparin Interactions

**EXPERIMENTAL PROCEDURES**

Materials—All restriction endonucleases were purchased from New England Biolabs (Ozyme, France). Other molecular biology reagents were from Promega Corp. (Madison, WI). Oligonucleotides were synthesized either by Oligo Express or Eurobio (Paris, France). Heparin-agarose was purchased from Pierce. Acridine-agarose was kindly provided by Dr. Terry Rosenberry (Mayo Clinic, Jacksonville, FL). Unless otherwise specified, other reagents were obtained from commercial sources.

Site-directed Mutagenesis—Two different plasmids were constructed using the coding sequences for rat AChE and ColQ1a (15, 16). Both coding sequences were inserted between Torpedo and maxammitals B, scheme of the main deletion mutants and mutants of rat ColQ. From the left, the N-terminal domain (residues 1–186, dark gray), the collagen domain (186–285, light gray) containing the N-terminal HBD (in black) and the C-terminal HBD (in white), and the C-terminal domain (285–450) divided into a proline-rich region (medium gray) and a cysteine-rich region (dark gray).

understanding of the nature of a HBD in the context of the canonical structure of collagen.

**Figure 1. Schematic representation of ColQ.** A, ColQ is a homotrimERIC collagen divided in three major domains. The N-terminal domain is responsible for organizing tetramers of AChE catalytic subunits through the proline-rich attachment domain, PRAD. The central domain forms a collagen triple helix in which the N-terminal and C-terminal HBDs are located. The C-terminal globular domain is composed by a proline-rich region, important for triple-helix formation, and a cysteine-rich region. The sequences of the HBDs are conserved between the major forms of rat ColQ. From the left, the N-terminal domain (residues 1–186, dark gray), the collagen domain (186–285, light gray) containing the N-terminal HBD (in black) and the C-terminal HBD (in white), and the C-terminal domain (285–450) divided into a proline-rich region (medium gray) and a cysteine-rich region (dark gray).

**RESULTS**

**ColQ–Heparin Interactions**—The sequence of ColQ is a homotetramer composed of rat ColQ. From the left, the N-terminal domain (residues 1–186, dark gray), the collagen domain (186–285, light gray) containing the N-terminal HBD (in black) and the C-terminal HBD (in white), and the C-terminal domain (285–450) divided into a proline-rich region (medium gray) and a cysteine-rich region (dark gray).

These results indicated that the affinity chromatography using an acridine-agarose column as a template for site-directed mutagenesis, performed as described by the unpaired Student’s t test. (Eq. 1)

where the parameters $A_1$, $A_2$, $A_3$, and $p$ correspond to the bottom asymptote, the top asymptote, the center, and the variable Hill slope, respectively. Finally, starting from the center parameter value (i.e., $\log(NaCl)$), the NaCl concentration for 50% elution of the bound enzyme was obtained for each curve. This value was used in this study as a measure of the relative affinity of a given $A_2$ AChE mutant for heparin. In the different experiments, statistical analyses were performed using the unpaired Student’s t test.
RESULTS

Production of Recombinant Rat A12 AChE and Its Affinity for Heparin—Xenopus oocytes constitute a very efficient system to produce predominantly A12 AChE forms by injecting defined amounts of both ColQ and AChE mRNAs (16), as shown by the sedimentation profile in a sucrose gradient of oocyte extracts (Fig. 2A).

To evaluate the heparin-binding behavior of recombinant rat asymmetric AChE, its A12 form produced in Xenopus oocytes was isolated from a sucrose gradient, bound to a heparin-agarose column, and eluted from the resin using a linear NaCl gradient. This strategy allows determination of the values of relative affinity for heparin, where a protein with higher affinity is eluted at a higher NaCl concentration (20). A12 AChE purified from Torpedo electric organ was also analyzed as reference.

As shown in Fig. 2, B and C, both Torpedo purified and rat recombinant A12 AChE present the same elution profile, composed of five peaks eluting at the same NaCl concentrations and presenting similar relative abundance. In both cases the majority of the enzyme was eluted in the central peak at a concentration of 0.6 M NaCl. The same elution profile was obtained under different experimental conditions, varying the NaCl concentration increment per fraction, the elution volume, the elution flow rate, and the volume of resin (data not shown). Each peak differed significantly from its neighboring peaks both at the level of NaCl concentration of elution and relative abundance (n = 12, p < 0.0003). This multiplicity might reflect an intrinsic heterogeneity of enzyme species. To examine this possibility, AChE eluted in the first peak was re-loaded onto a second heparin-agarose column, generating the original five peaks in the elution profile (data not shown). This excludes the possibility that the enzyme is composed of chemically different species. Most likely, the observed behavior arises from the multivalent nature of the HBDs in ColQ (12) and the heterogeneity of heparin molecules.

To facilitate the analysis of the elution profiles of AChE from heparin-agarose and the comparison between different A12 AChE mutants, the elution profiles were expressed as cumulative areas versus log[NaCl]. The NaCl concentrations at which 50% of the bound enzyme was eluted were derived from these curves (see "Experimental Procedures"), and relate directly to their affinity for heparin. When expressed in this form, both Torpedo and recombinant rat enzymes presented the same elution curve, with 50% of the enzyme eluting at 0.6 M NaCl (Fig. 2D, Table I). These results show that the recombinant rat A12 AChE behaves exactly as the purified A12 enzyme from Torpedo electric organ, so that Xenopus oocytes constitute a good expression system for A12 AChE.

The Heparin-binding Activity Is Localized in the Collagen Domain of ColQ—By analyzing the ColQ sequence, it is possible to identify two heparin-binding consensus sequences of the BBXB type in the collagen domain of ColQ. Other clusters of basic residues also exist within the N- and C-terminal non-collagenous domains of ColQ, and it has been suggested that the C-terminal domain could participate in binding to HSPGs.
**TABLE I**

Names, description, and relative affinity of the different A_{12} AChE variants

The nomenclature of the mutants was designed to show the state of the N-terminal and the C-terminal HBDs, separated by a slash. When the site is intact it is labeled with a plus sign (e.g. N\(^+/\)) and when it is inactivated it is labeled with a minus sign (e.g. N\(^-\)). Single and double substitutions are specified. For instance, in N\(^+/\)H11001 the N-terminal HBD is intact, and in the C-terminal HBD lysine 229 was replaced by an alanine. For more complex mutations a conceptual notation is used. For example, in C\(\rightarrow\)N\(^+/\) the C-terminal HBD is inactivated, and in the N-terminal HBD the basic residues from the N-terminal BBXB motif were replaced by the basic residues from the C-terminal motif, as if the C-terminal site was “moved” to the N-terminal position.

Basic residues are shown in bold, and mutated residues are highlighted in black. The concentration of NaCl required for elution of 50% of the enzyme bound to heparin-agarose columns was obtained from the fitting equation (see “Experimental Procedures”) and is expressed in M ± S.D., \(n\) varied from 2 to 4; the precise values are given in the figure legends.

| \(A_{12}\) enzyme name | Description | [NaCl] of elution |
|------------------------|-------------|------------------|
| Torpedo \(A_{12}\)     | Asymmetric AChE purified from *Torpedo* electric organ | 0.604 ± 0.012 |
| Recombinant \(A_{12}\) | Asymmetric AChE from rat expressed in *Xenopus* oocytes | 0.606 ± 0.008 |
| ΔN                    | Deletion of residues 106 to 210 of ColQ | 0.506 ± 0.007 |
| ΔC                    | Deletion of residues 140 to 244 of ColQ | 0.470 ± 0.008 |
| ΔCt                   | Deletion of residues 362 to 450 of ColQ | 0.626 ± 0.006 |
| ΔCol                  | Deletion of residues 105 to 276 of ColQ | Not retained |
|QN                     | Deletion of residues 187 to 450 of ColQ | Not retained |

| N-terminal HBD (114-128) | C-terminal HBD (216-236) |
|--------------------------|--------------------------|
| WT (N\(^+/\)C\(^-\))    | GDLGPRGKGRGKPP GRKQGKDGS | 0.606 ± 0.008 |
| N\(^+/\)C\(^-\)        | GDLGPRGDRGKPP GRKQGKDGS | 0.174 ± 0.006 |
| N\(^+/\)C\(^-\)        | GDLGPRGDRGKPP GRKQGKDGS | Not retained |
| N\(^+/\)C\(^+\)        | GDLGPRGDRGKPP GRKQGKDGS | 0.540 ± 0.001 |
| N\(^-\)C\(^+\)        | GDLGPRGDRGKPP GRKQGKDGS | 0.412 ± 0.007 |
| C\(\rightarrow\)N\(^+/\) | GDLGPRGKGFKPP GRKQGKDGS | 0.401 ± 0.002 |
| N\(^+/\)N\(\rightarrow\)C | GDLGPRGDRGKPP GRKQGKDGS | 0.539 ± 0.025 |
| R118\(\)A/C\(^-\)     | GDLGPRGKGFKPP GRKQGKDGS | 0.335 ± 0.013 |
| R121\(\)A/C\(^-\)     | GDLGPRGKGFKPP GRKQGKDGS | 0.357 ± 0.003 |
| K122\(\)A/C\(^-\)     | GDLGPRGKGFKPP GRKQGKDGS | 0.255 ± 0.004 |
| K122\(\)P/C\(^-\)     | GDLGPRGKGFKPP GRKQGKDGS | 0.363 ± 0.014 |
| R124\(\)A/C\(^-\)     | GDLGPRGKGFKPP GRKQGKDGS | 0.297 ± 0.012 |
| N\(^+/\)R21\(\)8A     | GDLGPRGDRGKPP GRKQGKDGS | 0.475 ± 0.005 |
| N\(^+/\)R21\(\)8P     | GDLGPRGDRGKPP GRKQGKDGS | 0.593 ± 0.019 |
| N\(^+/\)R22\(\)3A     | GDLGPRGDRGKPP GRKQGKDGS | 0.522 ± 0.004 |
| N\(^+/\)K22\(\)6A     | GDLGPRGDRGKPP GRKQGKDGS | 0.346 ± 0.006 |
| N\(^+/\)R22\(\)7A     | GDLGPRGDRGKPP GRKQGKDGS | 0.355 ± 0.004 |
| N\(^+/\)R22\(\)7P     | GDLGPRGDRGKPP GRKQGKDGS | 0.485 ± 0.007 |
| N\(^+/\)K22\(\)9A     | GDLGPRGDRGKPP GRKQGKDGS | 0.404 ± 0.013 |
| N\(^+/\)K23\(\)3A     | GDLGPRGDRGKPP GRKQGKDGS | 0.632 ± 0.011 |
| N\(^+/\)R21\(\)8A-K23\(\)3A | GDLGPRGDRGKPP GRKQGKDGS | 0.508 ± 0.004 |
To define the contribution of different domains to heparin binding, AChE oligomers containing ColQ with different deletions were expressed and analyzed. In ΔCt, the last 88 residues of ColQ were deleted, corresponding to the cysteine-rich region of the C-terminal domain (Fig. 1B). The remainder of the C-terminal domain was not removed because it is required for triple-helix folding. ΔCol contains a deletion of 171 amino acids in the central region of the collagen domain of ColQ that eliminates the two HBDs, but 7 proline-rich Gly-Xaa-Yaa triplets were left to ensure the formation of a stable triple helix. Finally, QN contained only the N-terminal domain of ColQ, allowing the organization of AChE tetramers to occur independently of the collagen domain. Fig. 3A shows the sedimentation profiles of the different ColQ deletion mutants co-expressed with AChE in *Xenopus* oocytes. As expected, ΔCt and ΔCol were able to produce asymmetric forms of AChE, whereas QN only produced a tetramer of catalytic subunits (G4 form). AChE expressed with ΔCt presented a sedimentation profile similar to wild type (WT), whereas asymmetric forms produced with ΔCol presented higher sedimentation coefficients, consistent with a decreased asymmetry of the molecules due to a shortened collagen tail.

The A12 ΔCol and ΔCt forms as well as the QN G4 form were isolated from the sucrose gradients and run on heparin-agarose columns. As shown in Fig. 3B, QN and ΔCol completely lost their ability to bind heparin, whereas ΔCt presented an elution profile similar to WT (Fig. 3C). The NaCl concentration for elution of 50% of the bound ΔCt enzyme (n = 2) did not differ from the WT enzyme (n = 3) (p > 0.1).

**Fig. 3.** The heparin-binding activity of AChE is localized in the collagen domain of ColQ. A, sedimentation profiles of AChE co-expressed with different ColQ constructs. ΔCt lacks the distal region of the C-terminal domain, and ΔCol lacks the central collagen domain. Both were able to produce A12 forms (16 S and 21 S, respectively). QN presents the N-terminal domain of ColQ and was able to produce only G4 AChE (10 S). The AChE forms used for heparin-binding assays were isolated from the peaks shaded in gray and are represented schematically. B, retention of the different AChE variants (from A) in heparin-agarose columns. The values correspond to the mean ± S.D. C, elution profile of the WT A12 AChE and of the ΔCt mutant, expressed as cumulative areas. The NaCl concentration for elution of 50% of the bound ΔCt enzyme (n = 2) did not differ from the WT enzyme (n = 3) (p > 0.1).
under physiological conditions. Indeed, an equilibrium binding assay performed at 0.15 M NaCl using heparin-coated plates showed that only 16% of the N-terminal enzyme was able to bind heparin (Fig. 4A). On the other hand, the fact that N-terminal still presented some heparin-binding capacity suggested that the basic residues surrounding the BBXB motif may also participate in this interaction. To assess this possibility, almost all basic residues constituting both putative HBDSs were substituted by alanines (N-C, Table I). In this case, only 14% of the N-terminal mutant enzyme was retained by heparin-agarose, with only 5% able to bind heparin under equilibrium conditions (Fig. 4A). These results demonstrate that the N- and C-terminal HBDSs, comprising BBXB motifs surrounded by basic residues and localized in the collagen domain of ColQ, are responsible for the interaction of A12 AChE with heparin.

The Two HBDSs in the Collagen Domain of ColQ Have Different Affinities for Heparin—To analyze the behavior of each HBDS separately, two ColQ mutants were generated by interrupting a single BBXB motif and leaving the other intact. Thus, N-C allowed the analysis of the C-terminal HBDS and N-C of the N-terminal HBDS (Fig. 1B). In both cases, the modified BBXB sequence was substituted by DPXB (Table I). Both mutants were fully retained in heparin-agarose as the WT enzyme but eluted from the column at different NaCl concentrations (Fig. 5A). Whereas the N-C enzyme eluted at 0.54 M NaCl, the value for N-C was 0.41 M, implying that the C-terminal HBDS has a significantly higher affinity for heparin than the N-terminal HBDS. Interestingly, both N-C and N-C mutants presented lower affinities for heparin than the WT enzyme (0.61 M), suggesting that WT affinity results from the simultaneous or cooperative participation of both HBDSs.

The Difference between the Two HBDSs Does Not Simply Result from the Different Number or Nature of Basic Residues—Although ColQ HBDSs share a minimum GRPBBXB sequence, the two sites differ both in the number and nature of basic residues. The C-terminal HBDS presents two additional basic residues that are not found in the N-terminal HBDS. To evaluate whether these extra basic residues could explain the higher heparin affinity of the C-terminal HBDS, these residues were replaced by alanines (N-C, Table I). Fig. 5B shows that the resultant mutant had a slightly lower affinity for heparin than N-C, eluting at a concentration of 0.51 M NaCl. This suggests that the extra basic residues at the C-terminal HBDS participate in heparin interactions even if their contribution is small. However, N-C presented a heparin affinity significantly higher than that of the N-terminal HBDS (N-C), showing that the basic charge difference

**Fig. 4.** Only the two HBDSs present in ColQ are responsible for the interaction of AChE with heparin. A, binding of different mutants of A12 AChE to heparin-agarose columns in 0.1 M NaCl (dark gray bars) or to heparin-coated plates in equilibrium conditions in 0.15 M NaCl (light gray bars). In N-C, only the two central basic residues of both HBDSs were mutated, whereas in N-C almost all basic residues were substituted with alanines. Values are the mean ± S.D., n = 3. B, the A12 AChE mutants retained in the heparin-agarose column were eluted using a linear gradient of NaCl, and the elution profiles were expressed as cumulative areas. The elution values determined from the curves differed significantly between WT and N-C (p < 0.0000005, n = 3).

**Fig. 5.** The two HBDSs have different affinity for heparin. A, cumulative areas of the elution profiles from heparin-agarose columns of the WT A12 AChE, of N-C, which only presents a C-terminal intact HBDS, and of N-C, which contains only the N-terminal HBDS. The elution values of both mutants were significantly lower than that of WT (p < 0.0005 for N-C and p < 0.0001 for N-C), and different between them (p < 0.0001, n = 3). B, elution profiles as cumulative areas of A12 AChE having only one of the two HBDSs, either intact or altered. In N-C and N-C, one of the HBDSs has been inactivated, whereas the other remains intact. N-C presents only the C-terminal domain intact, in which the BBXB motif has been replaced by the N-terminal sequence. Inversely, C-N-C presents only the N-terminal HBDS with the BBXB sequence from the C-terminal HBDS. Finally, in N-C, the N-terminal HBDS has been inactivated, and in the C-terminal HBDS the indicated basic residues were replaced by alanines, leaving the central GRPBBXB sequence intact. See Table I as a guide. N-C elution value was significantly lower than that of N-C (p < 0.0005, n = 3), whereas N-C (n = 3) and N-N-C (n = 4) had equal elution values (p > 0.9) as did N-C and C-N-C (p > 0.9, n = 3).
between the two HBDs does not underlie their different affinities for heparin.

The second difference between the two HBDs lies in the identity of basic residues that constitute each BBXB motif: RKGR for the N-terminal and KRGK for the C-terminal domain, with both sequences conserved among species. To assess whether this could explain the different affinities for heparin, we designed two new ColQ mutants in which the BBXB motifs were exchanged between the sites. Having eliminated the C-terminal motif, the remaining N-terminal BBXB sequence was replaced by that of the C terminus and vice versa (Fig. 1B, Table I). As shown in Fig. 5B, the C→N/C- mutant, which presents only the C-terminal BBXB motif located at the N-terminal position, eluted from the heparin-agarose column at the same NaCl concentration as N'/C-. Similarly, N'→N/C-, which presents the N-terminal consensus sequence at the C-terminal position, behaved exactly as did N'/C+. These results indicate that the different heparin affinities of the two HBDs are not explained by the identities of the basic residues that constitute each BBXB motif.

**Contribution of Individual Basic Residues to Heparin Interactions**—To evaluate individual residue contributions within the two binding sites, point mutations were carried out in each HBD to eliminate basic charges while trying to alter the local conformation as little as possible. Arginine or lysine residues located in the Xaa position as well as lysines in the Yaa position of Gly-Xaa-Yaa triplets were substituted by alanines, whereas proline was used to replace arginines in the Yaa position. Previous studies show arginine and proline to be equally stabilizing, whereas the stability of arginine in the Xaa position as well as lysines in both positions were equivalent to alanine (22).

As shown in Fig. 6A, all N-terminal HBD mutants displayed decreased heparin affinities, suggesting the involvement of all residues from this HBD in heparin binding. According to the relative affinity loss presented by each mutant, the importance of each basic residue was ranked as Lys-122 > Arg-218 > Arg-124 > Arg-118 = Arg-121. 

In the C-terminal HBD, all mutations generated a significant change in heparin affinity (Fig. 6B). The mutation of any basic residue from the common GRPGBGB sequence led to a decrease in heparin affinity. Residues were ranked in the following order of importance: Lys-226 > Lys-229 > Arg-227 > Arg-228. Unexpectedly, the mutation of the most distal surrounding basic residues led to an increase in heparin affinity.

**Effect of Local Stability of the Triple Helix on Heparin Affinity**—Cumulative areas of the elution profiles from heparin-agarose columns were expressed as cumulative areas. A, Lys-122, from the N-terminal HBD, was substituted either by alanine or by proline. In those mutants, the N-terminal HBD is inactivated. N'/C-, which presents an intact N-terminal HBD, is shown for comparison. The elution value of K122A/C was significantly higher than that of K122A/C (p < 0.005, n = 3). B, single-point mutations of the C-terminal HBD. In all of them, the N-terminal HBD is inactivated. The elution value for all the mutants differed significantly from N'/C+ (p < 0.001–0.000001, n = 2 for N'/R223A and N'/K235A, n = 3 for N'/C- and N'/R218P and n = 4 for the rest).
Contribution of the Distant Environment to Heparin Binding—Because local conformation seemed to be important in modulating heparin affinity, the possibility that regions distant from the HBDs could influence ColQ-heparin interactions was investigated. For this, ColQ deletions were designed to subject each HBD and its local environment to the same distant environment. The local environment was defined as the five triplets flanking each BBXB motif, so that each broader “mega”-HBD comprised 12 triplets. A single mega-HBD was then conserved, and the entire sequence (35 triplets) between the N-terminal limits of these domains was deleted (Figs. 1B and 8A). Thus, ΔN contained the C-terminal HBD and vice versa for ΔC, both surrounded by the most C-terminal and N-terminal regions of ColQ. Both constructs were able to produce truncated asymmetric AChE forms in *Xenopus* oocytes, exhibiting identical sedimentation profiles in sucrose gradients characterized by increased S values (Fig. 8D). As shown in Fig. 8C, ΔN eluted from the heparin column at 0.5 M NaCl, a value lower than that of N/C/C/ –, whereas the ΔC mutant eluted at 0.47 M NaCl, higher than N/C/C/ (see Fig. 9 for a summary of relative heparin affinity of all the mutants). This indicates that although the C-terminal HBD still exhibits a higher affinity for heparin than the N-terminal HBD, these affinities converge when these sites are located in the same structural context, suggesting that even distant sequences in ColQ are able to affect the heparin affinity of each HBD.

**DISCUSSION**

ColQ plays a fundamental role in anchoring asymmetric AChE at the synaptic basal lamina. The collagen nature of
ColQ confers on it the capacity to interact with multiple ligands, which may allow precise regulation of its localization. Several lines of evidence have suggested that ColQ-HSPG interactions are responsible for retention of AChE in the basal lamina and for its specific localization at the neuromuscular junction. In agreement with this idea, the collagen domain of ColQ contains two basic clusters that we have proposed to mediate ColQ-HSPG interactions by binding to HS chains. We have previously characterized those putative HBDs by molecular modeling and by the use of synthetic peptides. In this work, by analyzing the entire recombinant asymmetric AChE, we show that (i) the heparin-binding capacity of ColQ resides exclusively in two HBDs, (ii) the two HBDs exhibit different properties, and (iii) heparin affinity is determined by the combined contribution of sequence and triple-helix conformation. Besides its relevance to AChE localization, this study contributes to the general understanding of collagen-ligand interactions.

Heparin-Binding Capacity of ColQ Resides Exclusively in the Two HBDs Present in Its Collagen Domain—In addition to the HBDs in the collagen domain of ColQ, its C-terminal domain has also been implicated in asymmetric AChE localization at the neuromuscular junction. In congenital myasthenic syndrome type Ic patients, several mutations in the ColQ gene impair folding of ColQ, avoiding enzyme secretion. In some cases, there are point mutations in the C-terminal domain of ColQ in which asymmetric AChE forms are produced but are not accumulated at the neuromuscular junction (2). Because the interaction of AChE with HSPGs has been proposed to represent a crucial step for AChE retention and localization, it has opened the question of whether these mutations in the C-terminal domain of ColQ modify its interaction with HSPGs. We assessed this possibility and unambiguously established that only the collagen domain of ColQ interacts with heparin. AChE oligomers containing the collagen domain with or without a C-terminal domain presented a full binding to heparin, whereas oligomers lacking the collagen domain lost all binding capacity.

Heparin Interaction Depends upon Basic Residues, but Special Rules Apply in Collagens—Heparin binding has been extensively studied in globular proteins. In most cases, basic residues interact via electrostatic interactions with sulfate or carboxylate groups in the heparin molecule (23). However, in certain proteins such as thrombospondin, heparin binding involves tryptophan and serine residues (24). For ColQ, we showed that the interaction is primarily electrostatic, since the elimination of basic charges completely abolishes heparin binding. This is consistent with chemical modification studies, in which maleylation of the ε-NH₂ groups of lysine residues eliminated the tendency of asymmetric AChE to form glycosaminoglycan-dependent aggregates (25), and with calorimetric measurements that indicate that heparin binding is predominantly electrostatic.²

For heparin-binding proteins where basic residues are involved, different consensus sequences have been described according to the structural context in which they are expressed. Whereas the BBXB sequence is found preferentially in β-strands, the most frequent sequence in α-helices is BBXBXB, generating in both cases a segregation of the basic residues on one face of the structure (26). Although these motifs are absent in several heparin-binding collagens (27), one such consensus sequence was present in ColQ and helped us to initially define the putative HBDs (11). Sequences that interact with heparin have been identified in collagens type I and type V/XI. In type I collagen α1(I)₂α2(I)₂, the residues that have been implicated in heparin binding are concentrated only in two triplets (i.e. KGHRG in α1(I)₁ and KGIRGH in α2(I)₂) (28), whereas in collagens α1(V)₃ and α1(XI)₁α2(XI)₂α1(II) basic residues are spread throughout the sequence (i.e. KXGPRGXRGRGVPRGXR present in α1(V), α1(XI)₁, and α2(XI) chains) (29). In ColQ, our results showed that the most important residues for heparin interaction are concentrated in the sequence GRPGBBGB, differing from those found in collagens. They all have in common the presence of basic residues, but no consensus sequence for triple-helical proteins seems to emerge.

The nature of basic residues is an important factor for heparin binding. Arginine binds tighter to heparin than lysine, since it has a higher potential for forming hydrogen bonds, and the guanidinium group may form an intrinsically stronger electrostatic interaction with a sulfate anion than the ammonium group (30). This has been clearly shown using non-collagenous heparin-binding peptides containing different proportions of arginine and lysine residues (30, 31). However, in each HBD we found the most important residue to be a lysine. This could be a particularity of collagen, where arginines in Yaa position play an important role in the stability of the triple-helical structure (22). Molecular modeling studies show that arginines can form hydrogen bonds with the backbone of the same or the neighboring polypeptide chains (32). This was frequently observed in the model of ColQ, where arginine side chains were packed against the triple helix (12), reducing their availability to interact with other molecules. This seems to be the case of two arginines in Yaa position located C-terminal to the GRPGBBGB motif in the N-terminal HBD of Torpedo that are not conserved in mammals, where these positions are occupied by prolines (Fig. 1A). Consistent with this, a decrease in stability was observed when these arginines were replaced by alanines in triple-helical peptides modeling the Torpedo N-terminal HBD (13). Thus, depending on the contacts they establish, arginines could alternatively participate directly in heparin interactions or play a structural role, which in turn can affect the accessibility of the neighboring residues to the ligand. In collagens, interaction with heparin involves basic residues coming from different polypeptide chains (12), and the contribution of basic residues varies depending on the collagen chain composition. Thus, it seems very difficult to define a general consensus sequence for heparin binding in triple-helical collagens.

Triple-helix Structural Properties as Modulators of Heparin Affinity—Previous studies using synthetic peptides have shown a negative correlation between affinity and triple-helical stability (13). Here, ColQ mutants in which a same residue was substituted by alanine or proline, generating an identical array of basic residues but likely to be subjected to different local stability, showed different affinities for heparin. However, contrary to the peptides, we found that the presence of a proline, a residue that stabilizes triple-helical conformation, generated a mutant enzyme with more affinity for heparin than the presence of an alanine. A possible explanation for this discrepancy is that the 30-mer peptides and the full-length ColQ present different levels of local stability. In fact, in order to force the peptides to adopt a triple-helical conformation in solution, it was necessary to surround the HBD sequences with Gly-Pro-Hyp triplets, the most stabilizing sequences in collagen (21). On the other hand, the HBDs in ColQ are surrounded by triplets with low proline content, and as shown by molecular modeling, this can lead to a local untwisting of the triple helix facilitated by the repulsion between chains created by the high basic charge content (12). We propose that affinity for heparin is optimal in an intermediate range of local stability. In highly

² E. Doss-Pepe, P. Deprez, N.C. Inestrosa, A. Kirkpatrick, J. A. M. Ramshaw, and B. Brodsky, unpublished data.
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stable regions the triple helix is too rigid to be able to accommodate the ligand, whereas in very low stability regions the three polypeptide chains could be locally untwisted so that the geometrical arrangement of basic residues necessary for heparin binding would not be generated.

Local conformation, and hence heparin affinity, seems also to be influenced by the distant sequences, as seen in the JN and ΔC mutants. When surrounded by the same distant environment, the two HBDs showed closer affinities for heparin than when located in their original individual environment. A component of the distant environment would be the structural features of the surrounding triple-helical sequences per se, and these may in turn be influenced by the constraints imposed by the other domains in ColQ, as by the presence of 12 catalytic subunits. The effect of the distant environment may also explain the fact that the wild type enzyme with both HBDs exhibits a greater affinity for heparin than each HBD independently. We have previously shown that triple-helical content and stability increase upon heparin binding to a HBD (14). Thus, such a perturbation induced by heparin binding to one HBD could propagate along the triple helix, altering the conformation of the other HBD and increasing its affinity for heparin. Considering that collagen thermal stability is so close to body temperature, it is possible to speculate that its conformation will be sensitive to subtle changes in the milieu, allowing for a fine-tuned regulation of the interaction with other molecules.

Two Different HBDs: More Than One Function?—The presence of two HBDs with different properties that are conserved between Torpedo and mammal opens new perspectives regarding their function in AChE localization and, more generally, in basal lamina organization.

A possible interpretation for the differences observed between the two HBDs is that they reflect differences in specificity. This specificity can operate at different levels. One possibility is that each HBD has a preference for specific microdomains in the HS molecule, determined by specific sulfation patterns (33). The fact that distal basic residues in the C-terminal HBD seem to interfere with heparin binding supports this idea. This HBD, with an ample distribution of basic charges, is most likely a binding site for HS, whereas the GRPGBBGB motif with more concentrated basic charges would be preferred by heparin-like (i.e., highly sulfated) domains in HS. These specific sulfation microdomains could be located in different regions of a single HS chain, in different HS chains of a single proteoglycan, or even in different proteoglycans. Another possibility is that each HBD presents a specific preference for different glycosaminoglycans. In this context, a possible candidate is chondroitin sulfate. It has been suggested previously that chondroitin sulfate proteoglycans interact with asymmetric AChE (34, 35), and we have observed previously a direct interaction between chondroitin sulfate and the peptides modeling the N- and C-terminal HBDs, whose characteristics also differed between the two sites (14).

We propose that the glycosaminoglycan microdomains recognized by each HBD would be located in different layers of the basal lamina. It is interesting to note that the length of ColQ triple helix, measured both in electron micrographs (3, 25) as in the structural model (12), is around 50 nm, which corresponds to the distance between pre- and postsynaptic membranes (36). Therefore, the distance between the two HBDs (~25 nm) represents an important fraction of the space between these membranes, so that their binding to different layers of the extracellular matrix would serve to orient the enzyme for proper function.

Despite its “simple” and linear structure, the collagen triple helix provides a very complex scenario in terms of ligand interactions, in particular heparin interactions. The importance of the local conformation of the triple helix as a key modulator of the interaction with heparin, and potentially with HSPGs, opens new perspectives on the dynamic of the basal lamina.

Acknowledgments—We thank Drs. Barbara Brodsky and Jean Massoulié for helpful discussions and together with Stefan Deuber for critical reading of the manuscript.

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