Semisynthesis and segmental isotope labeling of the apoE3 N-terminal domain using expressed protein ligation

Paul S. Hauser,*† Vincent Raussens,§ Taichi Yamamoto,* Gezman E. Abdullahi,**
Paul M. M. Weers,** Brian D. Sykes,†† and Robert O. Ryan,*†

Center for Prevention of Obesity, Diabetes, and Cardiovascular Disease,* Children’s Hospital Oakland Research Institute, Oakland, CA 94609; Department of Nutritional Sciences and Toxicology,† University of California, Berkeley, CA 94720-3104; Center for Structural Biology and Bioinformatics,‡ Laboratory for the Structure and Function of Biological Membranes, Université Libre de Bruxelles, Brussels, Belgium; Department of Chemistry and Biochemistry,§ California State University, Long Beach, CA 90840; and Department of Biochemistry,†† University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Abstract Apolipoprotein E (apoE) is an exchangeable apolipoprotein that functions as a ligand for members of the LDL receptor family, promoting lipoprotein clearance from the circulation. Productive receptor binding requires that apoE adopt an LDL receptor-active conformation through lipid association, and studies have shown that the 22 kDa N-terminal (NT) domain (residues 1–183) of apoE is both necessary and sufficient for receptor interaction. Using intein-mediated expressed protein ligation (EPL), a semisynthetic apoE3 NT has been generated for use in structure-function studies designed to probe the nature of the lipid-associated conformation of the protein. Circular dichroism spectroscopy of EPL-generated apoE3 NT revealed a secondary structure content similar to wild-type apoE3 NT. Likewise, lipid and LDL receptor binding studies revealed that EPL-generated apoE3 NT is functional. Subsequently, EPL was used to construct an apoE3 NT enriched with 15N solely and specifically in residues 112–183. 1H-15N heteronuclear single quantum correlation spectroscopy experiments revealed that the ligation product is correctly folded in solution, adopting a conformation similar to wild-type apoE3-NT. The results indicate that segmental isotope labeling can be used to define the lipid bound conformation of the receptor binding element of apoE as well as molecular details of its interaction with the LDL receptor.

Supplementary key words intein • apolipoprotein E • apolipoprotein

Apolipoprotein E (apoE) is a 299-amino acid protein that is a well-characterized ligand of the LDL receptor. X-ray crystallography of the N-terminal (NT) domain of apoE revealed that, in the absence of lipid, it adopts an elongated four-helix bundle conformation that is stabilized by interhelical hydrophobic interactions (1). While this structure provides detailed information about the organization of apoE NT in a lipid-free state, lipid association is required for adoption of an LDL receptor competent conformation. Thus, it is assumed that apoE NT undergoes a significant conformational change in the presence of lipid to impart LDL receptor binding capability. The LDL receptor recognition sequence has been localized to a region of the protein that encompasses residues 130–172 in the NT domain and includes a sequence element with high positive charge density (2, 3).

An X-ray crystal structure of the extracellular portion of the LDL receptor at endosomal pH has been reported (4). From this structure, a model for LDL receptor function was proposed in which electrostatic interactions between receptor ligand binding repeats and apoE/B-containing lipoproteins facilitate internalization of the receptor/lipid complex at neutral pH (5). Upon receptor internalization and subsequent pH change in the early endosome, the receptor is proposed to release its lipoprotein ligand in response to a pH-induced molecular rearrangement that displaces bound ligand from the receptor. Two im-
portant gaps in our knowledge of this system include a detailed understanding of the receptor-active, lipid-associated conformation of the apoE ligand as well as the molecular details of its binding interaction with the receptor.

In an effort to pursue high-resolution studies of lipid-bound apoE, we employed intein-mediated expressed protein ligation (EPL) (6–8). This approach, which allows for increasingly detailed analysis of structural elements within proteins, involves reconstruction of an intact protein from fragments generated separately. In this study, we used EPL to produce a semisynthetic apoE3-NT comprising the first 183 residues of apoE. The results reveal an efficient and novel approach for elucidation of the conformation of lipid-associated apoE3 NT that potentially provides a means to characterize molecular details of apoE-LDL receptor interactions.

MATERIALS AND METHODS

Preparation of apoE(1-111)

Taking advantage of the lone cysteine at position 112 in human apoE3 NT together with the requirement that the desired EPL reaction includes a substrate polypeptide possessing an NT cysteine, apoE(1-111) was cloned and expressed as a Saccharomyces cerevisiae-derived vacuolar ATPase intein domain and chitin binding domain fusion protein using the pYBI vector (New England Biolabs) as previously reported (9). To facilitate optimal intein-mediated fusion protein cleavage (10), valine 111 was mutated to alanine using the QuikChange method (Stratagene) according to the manufacturer’s instructions. Expression and purification procedures for apoE(1-111) followed standardized protocols previously established for generating intein-mediated thioester-adducted proteins (11). Briefly, saturated overnight cultures of ER2566 Escherichia coli cells harboring the vector encoding the apoE(1-111) fusion protein were inoculated into 2xYT media containing 50 µg/ml ampicillin, grown to OD 600 = 0.6, and induced with 1 mM isopropyl thiogalactopyranoside. After 6 h at 30°C the cells were pelleted by centrifugation (8,000 ×g, 15 min), solubilized with buffer A (20 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 8.0) containing 1% Triton X-100, and stored at −20°C. Dissolved cell pellets were combined, passed through a microfluidizer, sonicated, and centrifuged at 12,000 g for 20 min. Isolated clarified cell extract was passed over a chitin bead column preequilibrated with buffer A containing 1% Triton X-100. The column was washed with 10 column volumes of detergent-free buffer A and fusion protein cleavage induced by addition of 2-mercaptoethanesulfonic acid (MESNA) to a final concentration of 60 mM. Flow was arrested for 16–24 h at 22°C and eluted with two bed volumes of buffer A containing 5 mM MESNA. The sample was dialyzed against deionized water, lyophilized, and stored at −20°C. ApoE(1-111)-MESNA was further purified by semipreparative C8 reversed-phase HPLC on a Perkin-Elmer Series 200 chromatography system.

Preparation of apoE(112-183)

Human apoE(112-183) polypeptide was prepared from engineered apoE(1-183) starting material by cyanogen bromide (CNBr)-induced cleavage. Recombinant apoE3(1-183) containing engineered mutations at positions 111 (Val→Met) and 125 (Met→Ala) was expressed and purified as described elsewhere (12). Briefly, saturated overnight cultures were inoculated into M9 minimal media supplemented with 13.3 mM glucose, 0.1 mM CaCl2, 2 mM MgSO4, and 50 µg/ml ampicillin. At OD 600 = 0.6, the culture was induced with 2 mM isopropyl thiogalactopyranoside. After 6 h at 30°C, bacteria were pelleted by centrifugation at 8,000 g for 15 min and the culture supernatant collected, concentrated by ultrafiltration, and subjected to heparin affinity chromatography and semipreparative C8 reversed-phase HPLC. Where indicated, apoE3 NT was expressed in M9 medium supplemented with 15NH4Cl (99% purity; Cambridge Isotope Laboratories) as the sole nitrogen source. All subsequent processing and ligation steps were identical to those described above for the unlabeled protein. Isolated apoE(1-183) was then subjected CNBr digestion (13). ApoE(1-183) was dissolved in 80% formic acid (5 mg/ml). CNBr was added to achieve a CNBr/methionine ratio >100 and the sample incubated in an oxygen-free environment for 24 h in the dark. Reactions were quenched by the addition of a 10-fold excess of deionized water and lyophilized to dryness. This procedure was repeated three times to remove residual formic acid. Complete digestion resulted in enrichment in apoE(112-183) containing the desired NT cysteine. ApoE(112-183) was further purified by semipreparative C8 reversed-phase HPLC on a Perkin-Elmer Series 200 chromatography system.

Analytical methods

Protein purity and/or ligation reaction progress was monitored by SDS-PAGE using either a 10–20% acrylamide gradient tricine gel or a fixed 18% acrylamide slab gel. Gels were stained with Amido Black 10B. MALDI-TOF mass spectrometry was performed on a Bruker Daltonics autoflex LRF as described previously (9). Nondenaturing gradient gel electrophoresis was performed on 4–20% acrylamide gradient Tris-glycine slab gels (Invitrogen). Samples were electrophoresed for 18–20 h at 20 mA and stained with GelCode Blue (Pierce Chemical Co.).

EPL

Ligation reactions employed purified apoE(1-111)-MESNA and apoE(112-183) protein fragments. Typical ligation reactions were carried out in 150–250 µL incubations containing purified (1–1.5 mM) apoE(1-111) and 2–3 mM apoE(112-183) in an approximate 2:1 molar ratio to drive the reaction to near completion (9). Protein fragments were dissolved in 20 mM NaH2PO4, 150 mM NaCl, 2 mM urea supplemented with 5% (w/v) MESNA, and 10 mM Tris(2-carboxyethyl)phosphine (final pH 8.5). Samples were incubated at 37°C for 96 h with gentle stirring. Ligation reaction progress was assessed by SDS-PAGE and quantified by densitometry of the stained gel using ImageJ software for Macintosh (14, 15).

Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectroscopy analysis was performed on a Jasco 810 spectropolarimeter. Scans were recorded between 185 and 260 nm in 20 mM sodium phosphate and 20 mM DTT, pH 7.4, at a protein concentration of 0.5 mg/ml in a 0.2 mm path length cuvette. Secondary structure content was calculated as previously described (16). For guanidine HCl-induced denaturation studies, apoE NT samples (0.2 mg/ml) were incubated overnight at a given denaturant concentration to attain equilibrium, and ellipticity was measured at 222 nm in a 1 mm path length cuvette.

LDL receptor binding assay

For LDL receptor binding studies, 2.5 µg of a soluble human LDL receptor (sLDLR; NT residues 1–699) (17) in 20 mM Tris (pH 7.2), 2 mM CaCl2, and 90 mM NaCl was incubated with 1.5 µg Trp-null apoE3 NT labeled on Cys112 with the fluoros-
cent probe N-(iodoacetyl)-N-(5-sulfo-1-naphthyl) ethylenediamine (AEDANS) and complexed with dimyristoylphosphatidylcholine (DMPC) to form reconstituted HDL (rHDL) particles as above (18). Interaction between AEDANS–Trp null apoE3 NT•DMPC and sLDLR was detected by fluorescence resonance energy transfer between excited Trp residues in the sLDLR and the AEDANS moiety covalently attached to Trp null apoE3 NT•DMPC (17). Unlabeled wild-type (WT) apoE3 and EPL apoE3 NT were complexed with DMPC (5:1 lipid to protein weight ratio) as described by Weers, Narayanaswami, and Ryan (18) and employed as competitor ligands in receptor binding assays. In this assay, the ability of the unlabeled competitor ligands to bind sLDLR is measured as a function of changes in AEDANS fluorescence intensity (excitation 280 nm; emission 470 nm) after 10 min of incubation at 25°C (final sample volume 300 µl). Fluorescence measurements were made on a Perkin-Elmer LS50b luminescence spectrometer.

NMR spectroscopy

NMR experiments were performed on 0.3–0.5 mM 15N-labeled apoE3(1-183) or segmental (residues 112–183) 15N-labeled apoE3 NT in 500 µl sodium phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, 20 mM DTT, and 100 µM EDTA, pH 7.3–7.4), containing 10% D2O, 0.5 mM NaN3, and 0.25 mM 2,2-dimethyl-2-silapentane-5-sulfonate as internal chemical shift references. 1H–15N heteronuclear single quantum correlation (HSQC) spectra were obtained at 30°C using the gNhqc pulse sequence from the Vnmrj 2.1D software applied to a Varian Inova 600 MHz NMR spectrometer with the following parameters: 256 and 1024 complex points for 15N and 1H dimensions, respectively. The spectral widths were 2432 Hz in 15N and 8398 Hz in 1H dimension. The carrier positions were at 118 and 4.75 ppm in 15N and 1H dimension, respectively. Data were processed using NMRpipe (19) and analyzed using NMRview (20). Spectral assignment of Gly residues was achieved by comparison of the chemical shifts of Gly resonances in uniformly 15N-labeled apoE3 NT (21).

RESULTS

Semisynthesis rationale

The goal of the present semisynthesis strategy was to generate an apoE3 NT protein specifically isotope labeled in the receptor recognition segment of the protein (i.e., residues 130–172). The overall design strategy is outlined in Fig. 1. Since EPL requires an NT cysteine nucleophile to initiate protein ligation (11, 22), the ε3 isofrom of apoE NT, which contains a lone cysteine at position 112, is well suited for EPL. To begin, we independently generated a C-terminal thiol-stabilized apoE(1-111) fragment and an apoE(112-183) fragment. This choice eliminated the need for mutagenic introduction of a potentially disruptive cysteine elsewhere in the protein and employed an apoE fragment (i.e., residues 112–183) that encompasses the entire LDL receptor recognition sequence. To generate the apoE(1-111) fragment, thiol-induced, intein-mediated cleavage of a protein chimera was performed (9). On the other hand, CNBr cleavage of a modified intact apoE3 NT generated the apoE(112-183) fragment. Ligation of these fragments to reconstruct intact apoE3 NT, with a peptide bond between residues 111 and 112 and without extraneous tags or tails, involved incubating the fragments in the presence of excess thiol to promote the rate-limiting thioesterification reaction (23).

EPL

ApoE(1-111)-MESNA (~13 kDa) was produced in high yield (Fig. 2), and the isolated fragment was highly soluble and stable in a range of aqueous buffers at high concentration. The purity of the ~8 kDa apoE(112-183) ligation substrate fragment is also shown. Upon incubation of these fragments, a time-dependent appearance of a product band with electrophoretic mobility identical to that of control apoE3 NT was observed. When excess apoE(112-183) was present, ligation efficiency increased to ~70–80% under these conditions. Similar yields have been reported for ligations that employ two similarly sized fragments >8 kDa (24). Ligation progress was monitored by SDS-PAGE, and the reaction was quenched by dialysis. Following ligation, the sample was processed to remove unreacted substrate fragments. A combination of heparin affinity chromatography and reversed-phase HPLC yielded a purified semisynthetic apoE3 NT (Fig. 2B).

Characterization of semisynthetic apoE3 NT

Mass spectrometry analysis of the ligation product (21,278 Da) and WT recombinant apoE3 NT (21,197 Da) agree well with the calculated masses of these proteins based on their amino acid composition (21,270 Da and 21,192 Da, respectively). To examine whether the ligation product folds in solution to adopt a conformation similar to native apoE3 NT, far-UV CD spectroscopy was performed (Fig. 3). Control and ligated apoE3 NT spectra were overlapping with dual minima at 208 and 222, corresponding to an α-helix content of 64%, consistent with earlier reports on apoE3 NT (12, 25, 26). Guanidine HCl-induced denaturation studies (Fig. 4) revealed that control apoE3 NT and ligation product were stable in solution, yielding native to unfolded transition midpoints in the range of 2–2.5 M guanidine HCl. Based on this, we conclude that, following ligation, EPL generated apoE3 NT folds in solution to adopt a stable conformation characteristic of apoE3 NT.

LDL receptor binding activity

Control WT apoE3 NT and semisynthetic apoE3 NT were complexed with DMPC to generate rHDL (18). Native PAGE analysis revealed comigration of WT and ligated apoE3 NT rHDL (Fig. 5). To examine the relative ability of semisynthetic apoE3 NT rHDL to serve as an LDL receptor ligand, a fluorescence resonance energy transfer-based competition binding assay (17) was performed. As shown in Fig. 6, both WT apoE3 NT•DMPC and EPL apoE3 NT•DMPC induced a concentration-dependent decrease in AEDANS fluorescence intensity (excitation 280 nm) that is characteristic of binding to sLDLR. Thus, WT apoE3 NT and EPL apoE3 NT rHDL are able to compete with AEDANS Trp-null apoE3 NT•DMPC for binding to sLDLR.
NMR studies of segmental isotope-labeled apoE3 NT

A goal of apoE3 NT semisynthesis is the generation of a segmental isotope-labeled protein for heteronuclear multidimensional NMR spectroscopy. Previous studies in our laboratory have shown that a 58-residue peptide fragment of apoE3 NT corresponding to residues 126–183 fails to adopt secondary structure in buffer yet forms an extended α-helix in the presence of 50% (v/v) trifluoro ethanol cosolvent or the micelle forming lipid dodecylphosphocholine (27, 28). In the present approach, we sought to extend this work by examining the structure of this region of the protein within the context of an intact NT domain. By culturing E. coli in minimal media containing 15NH4Cl as the sole nitrogen source, efficient labeling was achieved (12). 15N-labeled WT apoE3 NT was then used as starting material for production of labeled apoE(112–183). Subsequent ligation with MESNA-apoE(1–111) resulted in generation of a semisynthetic apoE3 NT that harbors 15N isotope only in residues 112–183. For comparison, two-dimensional 1H–15N HSQC spectra were obtained for uniformly 15N-labeled and segmental 15N-labeled apoE3 NT at pH 7.4 (Fig. 7). In this HSQC experiment, the observed cross-peaks correlate the chemical shift of amide protons with the amide nitrogen for each of the 15N-labeled amino acids in the polypeptide chain. From this data, we can see that segmental isotope-labeled semisynthetic apoE3 NT resonances coincide with a subset of resonances manifest by uniformly 15N-labeled apoE3 NT, thereby affording significant simplification of the NMR spectrum for EPL-generated apoE3 NT. The HSQC resonances for segmental isotope-labeled apoE3 NT were well dispersed with minimal spectral crowding outside the central region of the spectrum. In contrast, uniformly 15N-labeled apoE3 NT contained a much higher resonance density arising from overlapping cross-peaks, consistent with a greater number of labeled residues present in this sample. Based on peak intensity, clustering, and their position in the spectrum, it is likely that peaks around 1H ppm 8.0 and 15N ppm 125–132 ppm are the result of contaminating denatured protein. Further examination of the HSQC signature using complementary NMR experimentation may clarify the nature of these aberrant peaks.

Examination of overlaid HSQC spectra in the 1H ppm 7.6–9.2 and 15N ppm 104–111 region highlights differences between the segmental and uniformly 15N-labeled proteins (Fig. 8). Assignment of glycine resonances, which predominate this spectral region, reveals that three of the 10 Gly cross-peaks present in uniformly 15N-labeled apoE3 NT are missing from the spectrum of segmental isotope-labeled apoE3 NT. This result is anticipated because the apoE3 NT sequence contains three Gly in the segment from 1–111 and seven in the segment from 112–183. Figure 8 also depicts representative examples of Gly resonances that are virtually coincident in the two samples (e.g., Gly173 and Gly182) and resonances that are slightly shifted between the two samples (e.g., Gly113 and Gly127). The high degree of resonance overlap between segmental and uniformly 15N-labeled apoE3 NT confirms the expected relatedness of the two proteins and documents the reduced complexity afforded by segmental isotope labeling. A similar simplification is observed in the region corresponding to the Gln side chain amino group spectral pairs (1H ppm 6.0–8.0 to 15N ppm 108–113) due to a reduction in the number of Gln from 18 in uniformly 15N-labeled samples to five in segmentally 15N-labeled apoE3 NT. Whereas spectral crowding makes determination of the 18 spectral pairs difficult, the observation of doublet pairs for the five glutamines in segmentally labeled apoE3 NT is evident.

DISCUSSION

The objective of this study was to employ EPL methods to generate a functional apoE3 NT for structural analysis of lipid-bound apoE by NMR spectroscopy. This approach
fragment encompassing residues 112–183. In the final ligation product, two amino acids substitutions were introduced to facilitate the design. Val111 was converted to Ala to promote intein-mediated cleavage of the precursor chimeric, and Met125 was converted to Ala to prevent unwanted CNBr cleavage at that site.

Characterization studies of the ligation product provided evidence that semisynthetic apoE3 NT folded in solution to adopt an amphipathic α-helix bundle that possesses intrinsic lipid binding activity and is able to function as an LDL receptor ligand. The yield of ligation product was up to 70%, suggesting this method can be used to generate large quantities of specifically labeled protein. The versatility of the EPL approach relates to the ability to introduce labels or modifications in specific regions or sites within a protein while the other portion remains unaffected. For example, by introducing any number of modifications, including unnatural amino acids (30, 31), biophysical probes (32), posttranslational modifications (33), or isotope labels (34, 35), into one fragment, EPL can be used to study structural and functional aspects of proteins that might otherwise be difficult to address. Indeed, EPL applications for the study of posttranslational modifications of proteins have proved particularly useful for their ability to create large quantities of proteins with modifications that would otherwise be difficult to achieve by traditional recombinant or protein purification methods (22). By modifying one or both fragments prior to ligation, EPL can produce proteins that contain unique characteristics that allow for the study of protein function as it pertains to alternative conformational state transitions (24, 36), posttranslational affects (37), and protein-protein interactions (24, 32). Given that the only requirements of EPL are the presence of an active cysteine at position 1 of the C-terminal fragment and a leaving group moiety on the NT fragment (11), modifications made elsewhere in the fragments will be imparted to the ligated protein product that allow for discriminating functional studies to be performed.

complements a recent study by Weisgraber and colleagues who reported an X-ray structure of apoE4 bound to dipalmitylophosphatidylcholine at 10 Å resolution (29). While the apoE4 lipid complexes reported in this study were LDL receptor competent, the limited resolution did not afford discernable secondary structure or provide direct evidence of the detailed determinants of the receptor active conformation. The design adopted in this study involved joining apoE fragments together via a peptide bond to create an intact NT domain that is similar to WT apoE3 NT. Despite subtle differences, the LDL receptor binding curves for WT apoE3 NT•DMPC and EPL apoE3 NT•DMPC were consistent with binding activity. Whereas one fragment (apoE residues 1–111) was conveniently generated following intein-mediated cleavage of a precursor protein chimera, CNBr cleavage of a modified apoE3 NT yielded a

Fig. 3. Far-UV CD spectra of apoE NT constructs. Far-UV CD spectra of WT apoE3 NT (open circles) and EPL apoE3 NT (closed circles). Spectra were recorded in 50 mM sodium phosphate, pH 7.4.

Fig. 4. Effect of guanidine-HCl on the secondary structure content of apoE3 NT. Guanidine-HCl induced denaturation of WT apoE3 NT (open circles) and EPL apoE3 NT (closed circles). Protein unfolding was monitored by the function of changes in molar ellipticity at 222 nm upon incubation with given concentrations of guanidine HCl.

Fig. 5. Native PAGE analysis of apoE3 NT rHDL. Samples were applied to a 4–20% acrylamide gradient slab gel and electrophoresed for 18–20 h at 20 mA under nondenaturing conditions. Lane 1, native PAGE standards; lane 2, WT apoE3 NT•DMPC; lane 3, EPL apoE3 NT•DMPC.
In this study, our goal was to generate a segmental isotope-labeled apoE3 NT. The ability to introduce stable isotopes specifically into the receptor binding region of this protein affords new opportunities for detailed structural analysis. Because apoE3 NT is biologically active in a lipid-associated state, characterization of its structural properties in this environment is desired. While such studies are feasible by NMR spectroscopy, the spectra of helix-rich apolipoproteins undergo significant spectral compression upon complexation with lipid (38). While previous NMR spectroscopy studies generated a resonance assignment of lipid-free apoE3 NT (21), spectral complication associated with lipid complexation requires an alternative approach for ascertaining details of the LDL receptor active conformation. In order to decrease spectral overlap, we adopted a strategy to specifically label the region of interest. A similar approach has been employed to investigate apoE interdomain conformational adaptation using a stable isotope-enriched C-terminal domain (39). This differs from the goal of this study, which aims to investigate conformational adaptation of the receptor binding region of the NT domain upon association with lipid to confer LDL receptor binding activity.

In earlier studies, we characterized a 58-residue peptide fragment of apoE corresponding to residues 126–183. Since this peptide was not present in the context of the intact NT domain, it failed to adopt a native conformation in solution (13). Whereas structural data were obtained in the presence of a helix-inducing cosolvent (27) or a lipid mimetic detergent (28), it is uncertain if the...

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**Fig. 6.** LDL receptor binding activity of apoE3 NT rHDL. Receptor binding activity was assessed by monitoring changes in AEDANS fluorescence intensity upon introduction of WT apoE3 NT•DMPC (solid line) and EPL apoE3 NT•DMPC (dashed line) to a preincubated solution of sLDLR (2 µg) and AEDANS-labeled Trp-null apoE3 NT•DMPC (1.5 µg protein). Samples were excited at 280 nm and emission monitored at 470 nm.

**Fig. 7.** NMR spectroscopy of apoE3 NT. Two-dimensional $^1$H-$^15$N HSQC spectra of uniformly $^15$N-labeled apoE3 NT (black) and segmentally $^15$N-labeled apoE3 NT (red) taken at 600 MHz (pH 7.3–7.4) are depicted. Dashed region outlines the expanded region shown in Fig. 8.
absence of the remainder of the domain influences the properties of the peptide. Thus, in order to examine the structure of this segment of the protein within the context of the intact NT domain, the present EPL approach was pursued. Whereas such methods are not required for smaller apolipoproteins (e.g., apoC-I, apoC-II, or apoC-III), intact apoE NT in the presence of lipid is sufficiently large that spectral crowding and overlap will hamper resonance assignment and high-resolution structure determination (40, 41).

It is evident from the characterization studies presented that the EPL-generated apoE3 NT adopts a native conformation. Indeed, the segmental $^{15}$N-labeled apoE3 NT gave rise to an HSQC spectrum that largely coincides with the corresponding spectrum of uniformly $^{15}$N-labeled apoE3 NT. Overall, >50% of the residues comprising the 112–183 $^{15}$N-labeled fragment directly overlay with corresponding residues in uniformly $^{15}$N-labeled apoE3 NT, strongly indicating that the two proteins adopt similar conformations and display similar chemical environments for key residues. Many other nonoverlapping residues are closely positioned to corresponding residues in the overlaid HSQC, confirming their chemical relatedness. Despite close monitoring of the protein conditions between samples, small changes in protein conformation due to slight differences in pH and temperature can cause deviations in the resulting HSQC spectra, which may explain the observed small shifts in some peaks. Additionally, slight deviations observed in three of the segmentally labeled glycines (Gly113, Gly120, and Gly127; see Fig. 8) may be ascribed to their proximity to site-specific mutations introduced at positions 111 and 125 to facilitate EPL. While this may explain the observed chemical shift deviation for these residues, it also confirms that the two amino acid substitutions present in EPL-generated apoE3 NT do not induce major changes to the overall chemical environment and/or structural architecture of the protein.

Taken together, the results obtained indicate EPL has been successfully applied to construction of an intact, functional apoE3 NT. Furthermore, it is apparent that segmental isotope labeling offers a feasible approach to simplify NMR spectra of apoE3 NT for study of its lipid-bound, receptor active conformation. Currently, efforts are underway to evaluate segmental isotope-labeled apoE3 NT in complex with lipid.

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