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A glycosylated form of the human cardiac hormone pro B-type natriuretic peptide is an intrinsically unstructured monomeric protein [☆]

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Abstract

The N-terminal fragment of pro B-type natriuretic peptide (NT-proBNP) and proBNP are used as gold standard clinical markers of myocardial dysfunction such as cardiac hypertrophy and left ventricle heart failure. The actual circulating molecular forms of these peptides have been the subject of intense investigation particularly since these analytes are measured in clinical assays. Conflicting data has been reported and no firm consensus on the exact nature of the molecular species exists. Because these clinical assays are immunoassay-based, specific epitopes are detected. It is conceivable then that certain epitopes may be masked and therefore unavailable for antibody binding, thus the importance of determining the nature of the circulating molecular forms of these analytes. This situation is an unavoidable Achilles' heel of immunoassays in general.

A recombinant O-linked glycosylated form of proBNP has been shown to mimic some of the properties of extracted plasma from a heart failure patient. In particular the recombinant and native material co-migrated as diffuse Western-immunostained bands on SDS-PAGE and each band collapsed to an apparent homogeneous band following deglycosylation. Thus, glycosylated-proBNP may be one such circulating form. Here we provide extensive physiochemical characterization for this O-linked protein and compare these results to other described circulating species, non-glycosylated-proBNP and NT-proBNP. It will be shown that glycosylation has no influence on the secondary and quaternary structure of proBNP. In fact, at moderate concentration in benign physiological neutral pH buffer, all three likely circulating species are essentially devoid of major secondary structure, i.e., are intrinsically unstructured proteins (IUPs). Furthermore, all three proteins exist as monomers in solution. These results may have important implications in the design of NT-proBNP/BNP immunoassays.

Keywords

Glycosylated-proBNP; Intrinsically unstructured protein; Circular dichroism; 2-Dimensional nuclear magnetic resonance; Sedimentation equilibrium centrifugation; Clinical immunoassay

B-type natriuretic peptide (BNP)¹ is a cardiac hormone synthesized as a prepro 134-mer protein in atrial and ventricular myocytes. Processing by secretase(s), likely furin, generates a 108-

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mer (proBNP) which is further processed to a 76-mer N-terminal fragment (NT-proBNP) and the active 32-residue hormone (BNP) [1,2]. BNP regulates cardiovascular homeostasis and has been used as drug treatment for congestive heart failure in acutely decompensated patients [3]. Moreover, clinical immunoassays for both BNP and NT-proBNP have been FDA-approved for the diagnosis of left ventricle dysfunction [4-6].

Immunoassays utilize antibodies that react with specific analyte (usually protein) epitope(s). If these epitopes become unreactive due to say proteolytic processing, or masked by interaction with other molecule(s), or are post- or co-translationally modified, then the immunoassay can generate incorrect results. It becomes important then to identify the circulating forms of the analytes. This analysis can include primary sequence, post-translational modifications, and quaternary structure as homo- and hetero-interaction partners. Knowledge of such can aid in the development of immunoassays that accurately detect the circulating species.

Previous characterization of human NT-proBNP whether as a recombinant product or synthetic peptide showed this N-terminal fragment to be a largely unfolded, flexible protein, termed an IUP, which exists as a monomer in solution [7,8]. No comparable data were hitherto available for proBNP and a recently discovered [9] glycosylated form of proBNP. Here we use a combination of CD to assess the global average secondary structure of proBNP and glycosylated-proBNP (HEK-proBNP), 2D-NMR of HEK-proBNP for secondary structure at the α -carbon backbone level, and sedimentation equilibrium AUC for both proteins to assess their respective oligomeric state. Like NT-proBNP, both proBNP and HEK-proBNP are shown to be natively unfolded proteins, IUPs. In addition, all three proteins are observed to be monomers in solution. For proBNP, glycosylation has no major influence on its solution structure.

Materials and methods

Materials

NT-proBNP was a gift from Dade-Behring [7], proBNP was purchased from Hytest, Ltd. (Turku, Finland), and glycosylated-proBNP was obtained through a Materials Transfer Agreement with Scios, Inc. (Fremont, CA). The O-linked glycosylated recombinant protein was grown in HEK293 cells (HEK-proBNP) and purified by affinity chromatography as described for similar material produced in CHO cells [9]. All proteins were exhaustively dialyzed against PBS pH 7.2 containing 0.005% NaN_3 and protein concentration estimated from UV absorbance at 280 nm using calculated extinction coefficients of 0.826 L/g cm for NT-proBNP, and 0.598 L/g cm for both proBNP and HEK-proBNP. Anti-BNP monoclonal antibodies (24C5, epitope 87–98 and 43B12, epitope 102–108) and anti-NT-proBNP monoclonal antibodies (11D1, epitope 31–39 and 15D7, epitope 48–56) were obtained from Hytest, Ltd. Additional materials have been described [7,8].

Purity assessment of proteins

About 12 μg of protein was injected onto a C_{18}RP column (Vydac 218TP54, Hesperia, CA) followed by gradient elution [10]. Chromatographs were monitored at the absorbance of peptide bonds near 214 nm. Following denaturing gel electrophoresis (10–20% SDS–PAGE) of about 2 μg of protein prepared under sample reducing conditions, the gel was stained with GelCode (Pierce Chemical Co., Rockford, IL) or transferred to PVDF- PSQ for Western

¹Abbreviations used: BNP, B-type natriuretic peptide; NT-proBNP, N-terminal pro B-type natriuretic peptide; HEK, human embryonic kidney; HEK-proBNP, glycosylated-proBNP; CHO, Chinese Hamster ovary, RP, reversed-phase, SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; UV, ultraviolet; CD, circular dichroism; IUP, intrinsically unstructured protein; NMR, nuclear magnetic resonance, 2D-NOESY, 2-dimensional nuclear overhauser effect spectroscopy; AUC, analytical ultracentrifugation; and the standard single letter codes are used for the amino acids.

immunostaining [11]. N-terminal sequencing of HEK-proBNP was performed on a Procise 494 (Applied Biosystems, Inc., Foster City, CA) using about 1 nmol of protein absorbed to a ProSorb PVDF cartridge. Two runs of 51 and 60 residues were made. Matrix-assisted laser desorption time-of-flight mass spectrometry was used to estimate the molecular weight of HEK-proBNP.

Circular dichroism

Far-UV CD measurements were performed by Alliance Protein Laboratories (Camarillo, CA) on a JASCO J-715 spectropolarimeter. Sample concentration was about 0.7 mg/ml and spectra were recorded at room temperature in a 0.01 cm path-length quartz cell. A mean residue molecular weight of 113 g/mol was used for NT-proBNP and 110.2 for both forms of proBNP. Spectra are averages of at least 5 scans from 190 to 260 nm and were subtracted from dialyzate buffer background.

Nuclear magnetic resonance

2D homonuclear spectra were recorded on a Varian Inova-600 (Varian Inc., Palo Alto, CA) as described [8]. A working solution of HEK-proBNP was prepared just prior to use by adding 9 volumes of protein at 2.5 mg/ml to 1 volume of D₂O. Spectra were obtained at room temperature and 5 °C with data processed as described [8].

Analytical ultracentrifugation

Analytical sedimentation equilibrium ultracentrifugation was used to determine the quaternary structure for all three proteins. A Beckman XL-A centrifuge (Beckman Instruments, Fullerton, CA) was operated at 4 °C and the absorbance at 280 nm of the protein solution was subtracted from the dialyzate blank as a function of radial position. Rotor speeds of 20,000, 30,000, and 40,000 rpm were selected as appropriate for a given protein at two different concentrations. Data were analyzed using XL-A/XL-I data analysis software version 6.03 based on the Origin data analysis software from Origin Labs Software, Inc. (Northampton, MA). Partial specific volumes were calculated at 4 °C from the known protein sequence using SEDNTERP [12] version 1.08 giving 0.7204 ml/g for NT-proBNP and 0.7193 ml/g for proBNP. The same value as that calculated for proBNP was used for HEK-proBNP since carbohydrate composition was not known. Density was measured (Mettler/Paar DMA 55M) as 1.01025 g/L at 20 °C and calculated to be 1.01204 at 4 °C.

NT-proBNP content by immunoassay

The NT-proBNP content for NT-proBNP, proBNP, and HEK-proBNP was determined by a dual monoclonal antibody sandwich assay on a Dade Dimension RxL Max instrument using products RF523A and RC423A. Samples were assayed in triplicate at 1/1000–1/40,000 dilutions using the supplied sample diluent.

Results and discussion

Purity of NT-proBNP, proBNP, and HEK-proBNP

A C₁₈RP chromatogram monitored at 214 nm for all three protein preparations is displayed in Fig. 1. Both NT-proBNP and proBNP elute as major single peak species with essentially identical retention times. In contrast, HEK-proBNP elutes as a broad unresolved peak with a shorter retention time. This result is expected for glycosylation in general (shorter retention time) and heterogeneity of both the number of O-linked sites and structure at each site (broad peak). The inset in Fig. 1 shows the corresponding SDS–PAGE analysis of these proteins. The order of electrophoretic migration mirrors the molecular weights of the proteins, namely that NT-proBNP migrates faster than proBNP, which in turn migrates faster than HEK-proBNP.

Again, the broad unresolved band for HEK-proBNP is due to glycosylation heterogeneity. This observation was also noted for proBNP produced in CHO cells [9].

There are 14 serine and 5 threonine residues as potential sites of O-linked carbohydrate attachment in the 108 residues of proBNP and no potential N-linked sites. Previously, all of the carbohydrate moieties were localized to the 76-residue NT-proBNP fragment with S₃₇, S₄₄, T₄₈, S₅₃, and T₇₀ as complete attachment sites and T₃₆ and T₅₈ as partial attachment sites [9]. The carbohydrate sites in HEK-proBNP were inferred from “blank cycle” Edman sequencing [13]. We used intact protein loaded at approximately 1 nmol and performed two sequencing runs. In the first, 51 residues were identified (Fig. 2). Blank cycles were found at S₃₇, S₄₄, and T₄₈. The second run gave 60 residues (Fig. 2). This time 4 blank cycles were observed. The first three were identical to those from run 1 confirming that result. In addition, T₅₈ was also identified from a blank sequencing cycle. We were unable to assess T₇₀ by our procedure. Thus, three of the five complete carbohydrate sites (S₃₇, S₄₄, and T₄₈) for CHO proBNP were found for HEK-proBNP including an additional complete site (T₅₈) previously assigned to be partially glycosylated [9].

Further confirmation on the glycosylation site assignments in HEK-proBNP were obtained from Western blot of NT-proBNP and HEK-proBNP with antibodies that reacted with epitopes in the BNP portion of the molecule (43B12 and 24C5) or the NT-proBNP fragment (11D1 and 15D7). Fig. 3 shows that HEK-proBNP, but not NT-proBNP, reacted with 43B12 and 24C5 as expected. Like wise, 11D1 and to a lesser extent 15D7 immunostained NT-proBNP but not HEK-proBNP. The epitopes for these two antibodies contains a known carbohydrate site, S₃₇ and T₄₈, respectively. It seems reasonable to conclude that these carbohydrate modifications disrupt antibody immunoreactivity.

Secondary structure

Circular dichroism—Fig. 4 is a composite spectrum of the far-UV CD for all three proteins. As with NT-proBNP before [7,8], there is no α -helix minimum or β -sheet maximum at their respective corresponding wavelengths. Hence, proBNP and its glycosylated counterpart are largely devoid of secondary structure suggesting that neither the extension of the 76-residue NT-proBNP to the 108-residue proBNP nor the addition of the carbohydrates to proBNP have significant influence on the average solution structure of this protein. Indeed, the minimum molar ellipticity for each suggests appreciable amounts of a random coil flexible structure: NT-proBNP at 199 nm, $-18,500 \text{ deg cm}^2/\text{dmol}$; proBNP at 200 nm, $-15,000 \text{ deg cm}^2/\text{dmol}$; and HEK-proBNP at 199 nm, $-14,000 \text{ deg cm}^2/\text{dmol}$ [14]. All three proteins can be classified then as IUPs.

Nuclear magnetic resonance—A more detailed picture of the α -carbon backbone structure of HEK-proBNP was obtained from 2D homonuclear NMR at room temperature. This technique has been previously used to probe the backbone region of NT-proBNP [8]. Fig. 5 illustrates the 2D-NOESY for the amide region of HEK-proBNP. The observed amide resonances only span less than 1 ppm, from 7.8 to 8.7 ppm, indicating the protein is not in a folded conformation. In most cases, a folded protein will have amide resonance extended downfield to 10.5 ppm. O-linked sugar resonances are commonly located between 3 and 5.2 ppm, which here are overlapped with the α and β regions of the protein. Even though there is severe resonance overlap, useful structural information can still be obtained. The lack of significant off-diagonal NH(*i*) – NH(*i* + 1) (Fig. 5a) and less H α (*i*) – NH(*i* + 1) (Fig. 5b) NOE cross-peaks for a large portion of the sequence demonstrates very little solution structure at the backbone level and confirms the IUP nature of HEK-proBNP.

Quaternary structure

The oligomeric nature of NT-proBNP and proBNP has produced conflicting results. An earlier study stated that each may be a trimer or tetramer based on a predictive assessment of coiled-coil domains in the NT-proBNP fragment and elution positions of each on size-exclusion chromatography run under benign neutral pH conditions [15]. A detailed physicochemical study using CD, size-exclusion chromatography, and sedimentation equilibrium AUC [7] demonstrated that NT-proBNP at moderate concentration in neutral pH physiologically relevant buffer conditions was actually a monomer. No data using AUC had been reported previously for proBNP and HEK-proBNP.

Fig. 6 is a composite of three AUC runs for NT-proBNP, proBNP, and HEK-proBNP, respectively. The absorbance at 280 nm was plotted versus radial position at the speeds and protein concentrations indicated in the figure legend. Data were fitted to a single, ideal species model and the corresponding residuals of these fits are displayed above each plot. Two other manipulations of the data were performed: as the primary data were cast into plots of apparent molecular weight versus concentration and the natural logarithm of the absorbance versus the square of the radial position (data not shown). For the former, all plots were either horizontal (flat) or very slightly positive indicating a homogeneous, non-interacting species. In the latter case, all plots were essentially linear implying again, a single non-interacting species.

NT-proBNP gave a molecular weight of 8214 (Fig. 6a) in excellent agreement with a previously reported value of 8351 [7]. The calculated molecular weight for unmodified NT-proBNP is 8458 so both results indicate that NT-proBNP is a monomer. Fig. 6b displays the data and corresponding curve fit for proBNP. An experimental molecular weight of 12,019 was determined and is to be compared to a calculated value for unmodified proBNP of 12,037. Thus, proBNP is also a monomer. This result supplants an earlier conclusion that human proBNP is a trimer or tetramer [14]. The analysis of HEK-proBNP is not as straightforward because without the carbohydrate composition it is not possible to accurately calculate the partial specific volume. Nonetheless, using the same calculated value of 0.7193 as for unmodified proBNP generates a molecular weight of approximately 21,000 or about 9000 more. In point of fact, the partial specific value for HEK-proBNP will actually be less than proBNP so the experimentally determined molecular weight will be slightly less than ~21,000. Mass spectrometry gave a rather broad peak [16] centered at ~17,000 (data not shown). A more detailed analysis as before [9] would be required to obtain more accurate data but clearly glycosylation has no influence on the oligomeric state of proBNP.

Immunoassay of NT-proBNP content of all three proteins

Stock protein solutions for all three samples were converted to mg/ml NT-proBNP and were 0.69, 0.48, and 0.49 mg/ml for NT-proBNP, proBNP, and HEK-proBNP, respectively. Samples were diluted 1/1000–1/40,000 with sample diluent contained in the assay kit so on-scale readings were obtained. Triplicate sample analysis gave 0.32, 0.26, and 0.009 mg/ml of NT-proBNP for the above three analytes, respectively. Recovery was therefore about 50% for the two non-glycosylated samples. This may be due to the precise nature of the calibrator(s) which is(are) not known. In contrast, the recovery for HEK-proBNP was <2% suggesting that some of the O-linked carbohydrate sites may interfere with antibody binding. Alternately, perhaps some constituent in the HEK-proBNP solution per se may be severely interfering with the assay. To test this possibility an equal volume mixture of diluted NT-proBNP and HEK-proBNP was prepared and compared to the non-mixed diluted value for each. The expected value for the mixture was found, thereby ruling out an interfering substance in the HEK-proBNP sample.

Conclusion

Several circulating versions of BNP or its processed fragments have been described [17]. These include BNP, proBNP, and NT-proBNP [16], plus a recently isolated glycosylated form of proBNP [9]. The first three are detected by immunoassays that use antibodies to specific epitopes, therefore knowledge of the molecular structure of each analyte can be important factors in designing clinical immunoassays. We are unaware of any reported assay to detect glycosylated NT-proBNP/proBNP.

In this report, we provide a detailed physical description of NT-proBNP, proBNP, and HEK-proBNP in benign neutral pH physiologically relevant buffer at moderate protein concentrations. First, from CD analysis for all three proteins and 2D-NMR for HEK-proBNP, all proteins were found to possess little secondary structure and show appreciable amounts of random coil. Like NT-proBNP then [8], proBNP and HEK-proBNP are IUPs. Next, their respective oligomeric states were assessed from AUC experiments. These results demonstrate that, again like NT-proBNP [8], proBNP, and HEK-proBNP are monomers in solution. For proBNP, this observation contradicts an earlier study [15] stating that proBNP is either a trimer or tetramer. Our data lend further support to the conclusion of [9] that the “high-molecular-weight component” discussed previously [15,17] is unlikely to be an oligomer of NT-proBNP or proBNP. Interestingly, the four identified O-linked carbohydrate sites in HEK-proBNP have no obvious influence on either the secondary or quaternary structure of proBNP and presumably NT-proBNP as well. Lastly, using a commercial immunoassay for human NT-proBNP analysis we obtained unexpected results. Both NT-proBNP and proBNP gave ~50% recovery from the known protein concentration in contrast to <2% for HEK-proBNP. This could be a consequence of the antibody epitopes containing one or more of the known O-linked carbohydrate sites² and points to the importance of determining the molecular structure of these clinical analytes. If specific pathological conditions can be correlated with an increased glycosylated-proBNP fraction then development of a G-NT-proBNP assay takes on added importance.

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²Perhaps this assay would be better named NG-NT-proBNP to indicate the lack of reactivity to glycosylated NT-proBNP.

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Fig. 1. C₁₈RP-HPLC and (inset) SDS-PAGE for NT-proBNP, proBNP, and HEK-proBNP. About 12 μ g of each sample was injected on a C₁₈ column and elution affected with a trifluoroacetic acid/acetonitrile gradient. Overlay of three separate runs all monitored at 214 nm with sample name next to corresponding peak. Inset shows 10–20% SDS-PAGE with sample name above lanes. Approximately 2 μ g was loaded into each lane.

HPLG**S**PG**S**A**S**DLE**T****S**GLQEQRNHLQGKL**S**ELQVE 34

Q**T****S**LEPLQE**S**PRP**T**GVWK**S**REVA**T**EGIRGHRKMV 68

LYTLRAPRSPKMVQGS~~G~~CFGRKMDRISSSSGLGC 102

KVLRRH

Fig. 2.

Primary sequence of proBNP and HEK-proBNP sequencing results. N-terminal sequencing run 1 was to W₅₁ and run 2 to G₆₀. Bold, italicized, underlined S or T denotes identified residue during run 1 or 2. Boxed S or T represents a “blank cycle” during run 1 or 2. Italicized S or T signifies potential O-linked sites not identified in this report.

Fig. 3.

Western blot of NT-proBNP and HEK-proBNP. PVDF membranes were probed with monoclonal antibody 43B12, 24C5, 11D1, or 15D7. Sample names appear above lanes and epitope sequence is listed below antibody. Approximately 0.2 μ g was loaded into each lane.

Fig. 4. Far-UV CD for NT-proBNP, proBNP, and HEK-proBNP. Sample concentrations were 0.69, 0.7, and 0.68 mg/ml, respectively. Spectra were recorded from 190 to 260 nm at room temperature in a 0.01 cm path-length quartz cell. Labeled arrow identifies sample spectrum.

Fig. 5.
2D-NOESY NMR for HEK-proBNP at 25 °C. (a) NH-NH region. (b) NH-αH region. Sample prepared in 10% D₂O.

Fig. 6. Sedimentation equilibrium analytical ultracentrifugation runs for NT-proBNP, proBNP, and HEK-proBNP. Absorbance at 280 nm versus radial position (in cm) with corresponding residuals from fitting to a single, ideal species model. (a) NT-proBNP at 0.27 mg/ml and 40 K rpm. (b) proBNP at 0.15 mg/ml and 30 K rpm. (c) HEK-proBNP at 0.15 mg/ml and 30 K rpm.