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Biophysical Characterization Reveals Structural Disorder in the Developmental Transcriptional Regulator LBH

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Abstract

Limb-bud and heart (LBH) is a key transcriptional regulator in vertebrates with pivotal roles in embryonic development and human disease. Herein, using a diverse array of biophysical techniques, we report the first structural characterization of LBH pertinent to its biological function. Our data reveal that LBH is structurally disordered with no discernable secondary or tertiary structure and exudes rod-like properties in solution. Consistent with these observations, we also demonstrate that LBH is conformationally flexible and thus may be capable of adapting distinct conformations under specific physiological contexts. We propose that LBH is a member of the intrinsically disordered protein (IDP) family, and that conformational plasticity may play a significant role in modulating LBH-dependent transcriptional processes.

Keywords

vertebrates; LBH; transcriptional regulation; embryonic development; intrinsically disordered proteins

INTRODUCTION

Limb-bud and heart (LBH) is a highly conserved tissue-specific transcriptional regulator in vertebrates with important roles in embryonic development [1–3]. Aberrant gain of function of LBH, which maps to human chromosome 2p21, is associated with partial trisomy 2p syndrome, a rare human autosomal disorder with characteristic cardiovascular, skeletal and postaxial limb defects [2]. Moreover, overexpression of LBH during mouse and chick embryogenesis results in dramatic phenotypes, including cardiovascular anomalies, impaired bone formation and angiogenesis [2,3].

LBH is small nuclear acidic protein of 12.2 kD (105 amino acids) and a theoretical isoelectric point of 4.2 that exhibits no sequence homology to any known protein families [1]. However,

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a conserved nuclear localization signal and a C-terminal glutamic acid-rich putative transcriptional activation domain in the absence of a DNA-binding domain, hinted that LBH may act as a transcription cofactor [1]. This was confirmed by fusion of LBH to the DNA-binding domain of GAL4, which led to transcriptional activation of a reporter gene [1,4]. Both the N-terminal and C-terminal domains of LBH are required for its transcriptional activity [4]. In heart development, LBH predominantly represses the transcriptional activities of key cardiac transcription factors Nkx2.5 and Tbx5 resulting in inhibition of the expression of a common target gene *Nppa* [2]. Furthermore, overexpression of LBH during bone development efficiently represses transcription of the key bone transcription factor Runx2 and the pro-angiogenic factor Vegf [3]. Other *in vitro* studies suggest that LBH may act synergistically with the transcriptional activators AP1 and SRF [4]. Thus, LBH appears to have both transcription co-activator and co-repressor activities. Despite the importance of LBH as a key gene regulator in human development and disease, the structural basis for the function of LBH is not understood.

In an effort to understand structure-function relationships, we overexpressed and purified full length LBH in *E. coli* for biophysical characterization. Employing a diverse array of biophysical techniques, we demonstrate that native LBH is intrinsically disordered with no discernable secondary or tertiary structure exuding rod-like properties in solution. Our structural characterization of LBH provides further insight into the transcription regulator function of LBH in mechanistic terms.

MATERIALS and METHODS

Cloning, protein expression and purification

The murine *Lbh* open reading frame (Image Clone 6813866; ATCC) was cloned via BamHI and SmaI sites into pGEX2T vector (Promega) using PCR technology. The pGEX2T-LBH expression vector was transformed into *E. coli* BL21 StarTM (DE3) cells (Invitrogen). A single colony of transformed cells was grown in 50 mL PG minimal medium (50 mM Na₂HPO₄, 50 mM KH₂PO₄, 5 mM Na₂SO₄) supplemented with 100 µg/mL carbenicillin, 2 mM MgSO₄, 56 mM NH₄Cl, 0.6% glucose, and 0.2× of a trace metal mixture [5] at 37°C overnight to make a starter culture. Upon inoculation of 15 mL of starter culture in 500 mL of PG medium plus supplements, the culture was allowed to grow at 37°C to an optical cell density of OD₆₀₀ = 0.8. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was grown for an additional 4 h at 37°C before harvesting. For ¹⁵N-isotopic labeling of LBH, NH₄Cl in the culture medium was replaced with an identical amount of ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Inc.). Cells (~3 g/500 mL of culture) were harvested by centrifugation, washed once in 50 mM phosphate buffer, pH 7.3, 500 mM NaCl (4 mL/g of cells), and lysed in GST-binding buffer (50 mM phosphate buffer, pH 7.3, 500 mM NaCl, 1 mM dithiothreitol; 10 mL/g of cells) using an EmulsiFlex-C3 homogenizer (Avestin, Inc.). After centrifugation for 30 min at 35,000g, the soluble fraction containing the GST-LBH fusion protein was loaded at a flow rate of 1 mL/min onto tandem 5 mL GStrap FF affinity columns (equilibrated at 4°C in GST-binding buffer) connected to an AKTAbasic 100 Explorer FPLC system (GE Healthcare). The columns were washed with 50 mL of GST-binding buffer containing 0.01% Triton X-100, and subsequently with detergent free GST-binding buffer until the absorbance at 280 nm returned to baseline. GST-LBH protein was eluted with 50 mM Tris-HCl, pH 8.0 containing 500 mM NaCl, 2 mM dithiothreitol, and 10 mM glutathione. Fractions containing the GST-LBH protein were combined and exchanged back into GST-binding buffer using a HiPrep 26/10 desalting column (GE Healthcare). The GST affinity tag was cleaved off with 10 units of Thrombin (GE Healthcare) per mg of GST fusion protein and incubation at 4 °C for 16 h. The cleavage reaction was loaded (1 mL/min) onto the tandem GStrap FF affinity columns, which retained cleaved GST and any uncleaved GST-LBH. The cleaved LBH that

was not retained was collected, concentrated to ~1 mL using a 3K ultrafiltration concentrator (Millipore) and loaded onto a Superdex™ 75 16/60 size-exclusion column (GE Healthcare) equilibrated at 4 °C in 50 mM phosphate buffer, pH 6.5, and 250 mM NaCl. LBH was separated from all other protein components by isocratic elution with this buffer at a flow rate of 1 mL/min. The purity of recombinant LBH was determined by analytical SDS-PAGE followed by Coomassie staining of the gels.

SEC analysis

Size-exclusion chromatography (SEC) on purified LBH was performed using a Hiload Superdex 200 column interfaced to a GE Akta FPLC system equipped with a UV detector within a chromatography refrigerator at 4°C. The protein samples were loaded onto the column at a flow rate of 1ml/min and the data were automatically acquired using the UNICORN software. The protein was prepared in 50 mM Tris, 200 mM NaCl, 5 mM β-mercaptoethanol and 1 mM EDTA at pH 8.0 and the starting concentrations injected onto the column were between 40–50 μM. In order to determine the observed molecular mass (M_{obs}) of LBH, a calibration curve was generated by plotting the logarithm of M_{obs} of a set of globular protein markers as a function of their elution volume (V_e) and fit to linear regression according to the following relationship:

$$\text{Log}[M_{obs}] = aV_e + b \quad (1)$$

where a is the slope and b is the y-intercept of the $\text{Log}[M_{obs}]$ versus V_e plot. The M_{obs} of LBH was then calculated using Eq (1) from the knowledge of its V_e and the constants a and b obtained from the calibration curve.

ALS experiments

Analytical laser scattering (ALS) experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static laser light scattering detector coupled in-line with a Wyatt Optilab rEX refractive index detector and interfaced to a Hiload Superdex 200 column under the control of a GE Akta FPLC system within a chromatography refrigerator at 4°C. LBH samples were loaded onto the column at a flow rate of 1ml/min and the data were automatically acquired using the ASTRA software. The protein was prepared in 50 mM Tris, 200 mM NaCl, 5 mM β-mercaptoethanol and 1 mM EDTA at pH 8.0 and the starting concentrations injected onto the column were between 40–50 μM. The angular- and concentration-dependence of Rayleigh scattering intensity of recombinant LBH was fit to the built-in Zimm equation [6,7]:

$$(Kc/R_\theta) = ((1/M) + 2A_2c) [1 + ((16\pi^2 r^2 / 3\lambda^2) \sin^2(\theta/2))] \quad (2)$$

where R_θ is the excess Raleigh ratio due to protein in the solution as a function of protein concentration c (mg/ml) and the scattering angle θ (42°, 90° and 138°), M is the molecular mass of protein, A_2 is the second virial coefficient, λ is the wavelength of laser light in solution (658 nm), r is the radius of gyration of protein, and K is given by the following relationship:

$$K = [4\pi^2 n^2 (dn/dc)^2] / N_A \lambda^4 \quad (3)$$

where n is the refractive index of the solvent, dn/dc is the refractive index increment of the protein in solution, N_A is the Avogadro's number ($6.022 \times 10^{23} \text{ mol}^{-1}$) and λ is the wavelength of laser light in solution (658 nm). Weighted average values of r and M for the recombinant LBH were respectively calculated from the slope and the y-intercept of linear fits of a range

of (Kc/R_θ) versus $\sin^2(\theta/2)$ plots for a total of 480 data slices in the limit of low protein concentrations along the elution profile at three scattering angles as described previously [6, 7].

CD studies

Circular dichroism (CD) measurements were conducted on a Bio-Logic MOS450 spectrometer equipped with a CD accessory and thermostatically controlled with a water bath. All data were acquired on a 10 μ M LBH in 50 mM Sodium phosphate at pH 8.0 and processed using the Biokine software. CD spectra were collected at 25°C using a quartz cuvette with a 2-mm pathlength in the wavelength range 180–300 nm and normalized against a reference spectrum to remove the buffer contribution. The reference spectrum of the buffer was obtained in a similar manner. Data were recorded with a slit bandwidth of 2 nm at a scan rate of 3 nm/min. The data set represents an average of 4 scans acquired at 1 nm intervals.

SSF measurements

Steady-state fluorescence (SSF) spectra were collected on a Jasco FP-6500 spectrofluorometer using a 5-mm pathlength cuvette at 25°C. Emission spectra were acquired from 300 to 500 nm using an excitation wavelength of 295 nm and a 3-nm bandwidth for both excitation and emission. Data were collected on a 10 μ M LBH in 50 mM phosphate, 0.1 mM 2-mercaptoethanol containing either 50 or 250 mM NaCl at pH 6.5. Fluorescence spectra of the buffer solution were recorded and subtracted from the protein spectra.

NMR spectroscopy

For nuclear magnetic resonance (NMR) analysis, 0.5 mM ^{15}N -isotopic labeled LBH was prepared in 50 mM phosphate, pH 6.5, 250 mM NaCl, and 5% D_2O . Two-dimensional ^1H – ^{15}N HSQC spectra were collected at 25 °C with a Bruker DMX500 NMR spectrometer (500 MHz for protons) equipped with pulsed-field gradients, four frequency channels, and a triple resonance cryoprobe with an actively shielded z -gradient. For the ^1H – ^{15}N HSQC experiment, the data were recorded by using a pulse sequence in which the HSQC detection scheme was optimized to avoid water saturation [8] and by using the States-TPPI method [9] in the indirect dimension, with a relaxation delay of 1 s. The data were obtained with spectral widths of 1520 and 7000 Hz in f_1 (^{15}N) and f_2 (^1H), respectively, and with 256 and 1024 complex points, respectively in the t_1 and t_2 dimensions. A total of 8 transients were acquired for each hypercomplex t_1 point with ^1H and ^{15}N carriers positioned at 4.71 and 120 ppm, respectively. The program NMRPipe [10] was used to process the data. Proton chemical shifts are given with respect to the HDO signal taken to be 4.71 ppm relative to external TSP (0.0 ppm) at 25 °C. The ^{15}N chemical shifts were indirectly referenced.

Structural order and disorder prediction

The relative order/disorder of the amino acid composition of LBH was predicted by the neural network program PONDR® VL-XT [11,12], which is available from Molecular Kinetics, Inc. at <http://www.pondr.com>.

RESULTS and DISCUSSION

Recombinant LBH can be purified to apparent homogeneity

In order to facilitate biophysical studies aimed at elucidating the mechanisms by which LBH modulates transcriptional processes, we overexpressed and purified mammalian LBH protein from *Escherichia coli*. The protocol we developed (see Materials and Methods) reproducibly yielded 20–30 mg of purified full length LBH from 1 L of culture, which was of $\geq 95\%$ homogeneity as judged by SDS-PAGE (Fig. 1B, lane 5). It should be noted that the recombinant

LBH additionally contains N-terminal Gly-Ser residues as a result of using a pGEX-2T expression vector (Fig. 1A) and has a theoretical molecular mass of 12.3 kD.

Hydrodynamic analysis reveals that LBH lacks a globular fold

In an effort to gain an insight into the globular fold of LBH, we conducted a detailed size-exclusion chromatography (SEC) analysis (Fig. 2A). Interestingly, our data reveal that LBH elutes with an apparent molecular mass of about 33 kD using a set of globular proteins as reference. This value is approximately three-fold greater than its theoretical molecular mass of 12.3 kD and could suggest that LBH exists as a higher-order oligomer in solution. However, since determination of molecular mass of proteins from such analysis is highly dependent upon their globular shape, an alternative scenario could be that LBH lacks an intrinsic globular fold allowing it to behave as a structurally disordered polymer. To distinguish between these two possibilities, we carried out analytical laser scattering (ALS) measurements (Fig. 2B). Fit of these data to Zimm equation yielded values of 11.8 ± 0.5 kD and 234 ± 6 Å for the molecular mass and radius of gyration for the recombinant LBH, respectively. Thus, the molecular mass determined for LBH from ALS analysis on the basis of first principles of hydrodynamics with no assumptions is in an excellent agreement with a theoretical value of 12.3 kD and suggests that LBH exists as a monomer in solution. Additionally, a value of 234 Å for the radius of gyration of LBH strongly suggests that LBH lacks a globular fold and most likely behaves as a rod-like macromolecule. Given that structural disorder is a hallmark of eukaryotic transcriptional regulators [13,14], the possibility that LBH may belong to what have come to be known as intrinsically disordered proteins (IDPs) [15] deserved further investigation.

Spectroscopic studies indicate that LBH lacks secondary and tertiary structural features

To further investigate the extent to which LBH may be structurally disordered, we carried out steady-state fluorescence (SSF) and circular dichroism (CD) measurements (Figs. 3A and 3B). Consistent with the above observations, SSF and CD data further indicate that recombinant LBH is largely unfolded. In Figure 3A, fluorescence spectrum of LBH shows that the single tryptophan residue (Trp-80) within the protein emits with λ_{max} around 350 nm. Given that this is the λ_{max} observed for free tryptophan residues in solution, it can be argued that the tryptophan residue within LBH is completely accessible to the aqueous solvent and that LBH is likely to contain a non-globular fold. That this is the case is further corroborated by CD measurements. As shown in Fig. 3B, CD spectrum of LBH in the far-UV region (180–240 nm) is largely characterized by a negative band centered around 200 nm, which is reminiscent of a structurally disordered protein with little or no secondary structure. Additionally, the CD spectrum of LBH in the near-UV region (240–300 nm) is completely devoid of a band around 280 nm, which would be expected for a non-globular protein with one or more tryptophan residues. Taken together, our SSF and CD data argue strongly that LBH lacks any discernable secondary or tertiary structure.

NMR analysis shows that LBH may exist in multiple conformations

In order to further assess the relative degree of structural disorder in LBH, we also measured its two-dimensional ^1H - ^{15}N HSQC NMR spectrum (Fig. 3C). In this analysis, the ^1H and ^{15}N chemical shifts (δ) of directly bonded ^1H - ^{15}N pairs (i.e., backbone and side chain amide groups) are correlated. As shown in Fig. 3C, the majority of backbone amide proton resonances exhibited poor chemical shift dispersion (~ 7.9 – 8.7 ppm), indicating substantial regions of structural disorder. In addition, many resonances were broadened, indicating interconversion between multiple conformations occurring within the "intermediate" NMR timescale. Since the amide nitrogen present in the side chains of both Gln and Asn contains two bonded protons ($-\text{NH}_2$), this group yields a pair of resonances with the same ^{15}N chemical shift but different ^1H chemical shifts. Thus, pairs of resonances are observed in the upfield

regions (Fig. 3C, $^{15}\text{N}\delta \sim 113$ ppm and $^1\text{H}\delta \sim 6.9$ ppm and ~ 7.7 ppm), as expected for the $-\text{NH}_2$ group in the side chains of the 3 Asn and 4 Gln residues (Fig. 4A, N24, N94, N100, Q43, Q88, Q92, and Q105). Interestingly, a pair of resonances was observed in the downfield regions (Fig. 3C, $^{15}\text{N}\delta = 129$ ppm and $^1\text{H}\delta = 10.1$ ppm) for the N ϵ H of the aromatic indole side chain of the single tryptophan (Trp-80), indicating slow exchange between two different conformations. Taken together, our NMR analysis suggests that the LBH may not only be structurally disordered but that it may also exhibit conformational flexibility allowing it to exist in multiple conformations.

In silico analysis confirms that LBH is largely disordered with little propensity for globular fold

There is growing evidence that IDPs exhibit notable structural features at the primary sequence level [11,12,15,16]. Indeed, bioinformatics analysis of the primary amino acid composition of LBH revealed an enrichment of disorder-promoting residues (Fig. 4A). These include nearly 35% of charged residues (Arg/Lys/Glu/Asp), 10% of polar residues (Ser/Thr) and another 15% of helix breakers (Pro/Gly). On the other side, LBH is severely devoid of hydrophobic residues (Met/Leu/Ile/Val) that dominate in globular proteins. It should be noted that such tendency of LBH for intrinsic disorder is highly conserved among all vertebrate LBH orthologues (Fig. 4A). The LBH amino acid sequence was further subjected to computer analysis using the PONDR® VL-XT software, which utilizes a set of neural network predictors to calculate the probability that amino acid residues exist in either structurally ordered or disordered peptide regions (Fig. 4B) [11,12]. As displayed in Fig. 4B, 70 of 105 LBH residues (approximately 66.7%) are predicted to be disordered and are located primarily in two peptide regions spanning residues 15–38 and 60–105. Interestingly, the predicted nuclear localization signal (NLS, residues 56–63) of LBH overlaps with the most ordered region spanning residues 39–59, whereas the Glu-rich putative transcriptional activation domain (residues 67–104) resides in the longest disordered region.

CONCLUSION

We have purified and biophysically characterized the mammalian LBH protein expressed in *E. coli*, which is a representative of the vertebrate LBH transcription cofactor family. Our data reveal that native LBH is a monomeric protein with a high degree of structural intrinsic disorder that exhibits multiple traits of IDPs. IDPs fulfill key molecular recognition functions in transcriptional regulation, DNA repair and cell signaling [13–15,17] and are abundantly represented among proteins associated with human disease [18,19]. This is noteworthy as abnormal LBH gain-of-function is implicated in human congenital heart disease, as well as in breast cancer [2,20]. Taken together, our data suggest that conformational plasticity may play a significant role in LBH transcriptional regulator function, as it allows LBH to form multi-partner interactions with different transcription factor classes. Finally, like other IDPs, LBH may undergo a ‘disorder-to-order’ transition upon binding to its target molecules, and, depending on the target-induced conformational changes, may acquire different functional activities.

Abbreviations used

ALS	Analytical laser scattering
CD	Circular dichroism
GST	Glutathione S-transferase
HSQC	Heteronuclear single quantum coherence

IDP	Intrinsically disordered protein
IPTG	Isopropyl- β -D-thiogalactopyranoside
LBH	Limb-bud and heart protein
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
SEC	Size-exclusion chromatography
SSF	Steady-state fluorescence

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Figure 1.

Purification of recombinant LBH from *E. coli*. (A) Coding region of the recombinant pGEX-2T-LBH expression vector depicting the thrombin cleavage site and the BamHI restriction enzyme recognition sequences (italics) used to directionally clone the LBH coding region into pGEX-2T. (B) SDS-PAGE analysis with Coomassie staining. M: Molecular weight standard; lane 1: total soluble lysate; lane 2: proteins from the soluble lysate that were not retained on the GST Sepharose High Trap HP affinity column; lane 3: proteins that were retained and subsequently eluted from the GSTrap HP affinity column; lane 4: protein products after thrombin digestion; and lane 5: purified LBH resolved by Superdex™ 75 size-exclusion chromatography.

Figure 2.

Hydrodynamic studies of LBH. (A) SEC analysis of LBH. In the upper panel, elution profile of LBH (red) is superimposed upon the elution profiles of globular protein markers (black). Note that the elution of LBH and protein markers were recorded at 280 nm (A_{280}) using a UV monitor. In the lower panel, a plot of the logarithm of the observed molecular mass (M_{obs}) of known protein markers versus their elution volume (V_e) is shown. The data points in the order of increasing V_e and decreasing $\text{log}[M_{\text{obs}}]$ correspond to 128-kD dimeric albumin, 64-kD monomeric albumin, 48-kD ovalbumin, 20-kD chymotrypsinogen; and 16-kD ribonuclease, respectively. The solid line through the data points represents a linear fit to Eq (1). (B) ALS analysis of LBH. In the upper panel, the differential refractive index (Δn) of LBH (black) is superimposed upon the extent of its scattering intensity, expressed in terms of the excess Rayleigh ratio (R_θ), at three scattering angles of 42° (red), 90° (green) and 138° (blue) as a function of elution volume (V_e). Note that Δn is a dimensionless parameter expressed here in absolute terms. In the lower panel, a representative plot of $[Kc/R_\theta]$ versus $\sin^2(\theta/2)$ is shown

at a protein concentration corresponding to the maximum values of R_{θ} at the three scattering angles. The solid line through the data points represents a linear fit to Eq (2).

Figure 3.

Spectroscopic characterization of LBH. (A) Tryptophan fluorescence spectrum of LBH. (B) CD spectrum of LBH. $[\theta]$ represents the molar ellipticity defined by the equation: $[\theta] = (10^5 \Delta A / cl)$, where ΔA is the difference between the absorbance of left circularly-polarized and right circularly-polarized light in mdeg (the observed ellipticity), c is the protein concentration in μM and l is the cuvette pathlength in cm. (C) Two-dimensional ^1H - ^{15}N HSQC NMR spectrum of uniform ^{15}N -isotopic labeled LBH. The final concentration of LBH was 0.5 mM in 50 mM phosphate buffer, pH 6.5, containing 250 mM NaCl and 5% D_2O . Spectra were collected at 25°C with a Bruker DMX500 NMR spectrometer (500 MHz for protons) equipped with a triple resonance cryoprobe.

Figure 4.

In silico analysis of LBH. (A) Sequence alignment of LBH proteins from mouse (NCBI ID: NP_084275.3), rat (NP_001123352.1), human (NP_112177.2), orangutan (NP_001125165.1), bovine (NP_001092622.1), dog (XP_853968.1), chicken (NP_001026209.1), finch (XP_002198437.1), *Xenopus laevis* (NP_001081507.1), salmon (ACI34372.1) and zebrafish (NP_956814.1) showing a high degree of conservation (58–99%) of disorder-promoting amino acid residues in LBH proteins across vertebrate species. Dark shading represents identity at a given amino acid residue, whereas light shading represents amino acid residue similarity. Conserved protein motifs predicted by the primary amino acid sequence [1] are shown on the top of the alignment. NLS = nuclear localization signal. (B) PONDR® VL-XT analysis. Amino

acids with PONDR scores ≥ 0.5 are classified as being in 'disordered' peptide regions and those with scores < 0.5 are classified as being in 'ordered' peptide regions.