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# Biophysical characterisation reveals structural disorder in the nucleolar protein, Dribble ☆

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**Abstract**

Dribble (DBE) is an essential protein in *Drosophila* that belongs to the evolutionarily conserved Krr1p protein family. Proteins in this family are localised in the cell nucleolus and are important for the processing of ribosomal RNAs. However, little is known about their structural and biophysical properties. We have expressed and purified full-length DBE protein from *Escherichia coli*. Consistent with the native role of DBE in RNA processing, recombinant DBE was shown to bind RNA homo-polymers in vitro. By bioinformatics, size-exclusion chromatography, equilibrium sedimentation analysis, controlled proteolysis, and a variety of spectroscopic techniques, we have found that DBE is a monomeric protein in solution containing both α- and β-structures. Moreover, the structure of DBE is expanded and significantly disordered (~45% disordered). Natively disordered proteins are thought to provide a disproportionately large surface area and structural plasticity for nucleic acid binding. We therefore propose that the presence of structural disorder is an important feature of DBE that facilitates the protein to interact with RNAs in the nucleolus.


*Keywords:* Intrinsically disordered protein; Intrinsically unfolded proteins; HRB2; Krr1p; K-homology; Pfg27; RNA binding

*dribble* (*dbe*) is an essential gene in *Drosophila melanogaster* [1]. Mutations in *dbe* are associated with aberrant cleavage of pre-ribosomal RNA precursors and lethality [1]. The gene product, DBE, belongs to a protein family which consists of 22 homologous members to date [2]. Some of the DBE homologues include human HIV-1 Rev-binding protein (HRB2), fission yeast Mis3, and budding yeast Krr1p [2]. While Krr1p is the first member of this evolutionarily conserved protein family to have been identified, DBE has been studied the most among the homologues from multi-cellular eukaryotes [1]. The DBE protein is preferentially localised in a perinucleolar ring structure (in or near the nucleolus) of the cell nucleus [1]. The nucleolus is organised into three compartments, with an innermost fibrillar centre surrounded by dense fibrillar components (DFC) and peripheral granular components (GC) [3]. It is in the DFC and GC where RNA processing such as cleavage, methylation, and pseudouridylation occurs. The expression of *dbe* is ubiquitous in embryonic cells, and DBE appears to be required for the viability of developing cells in different tissues [1]. *dbe* mutations are lethal, and homozygous mutants die at the first instar larval stage of the *Drosophila* life cycle, presumably due to failure in ribosome biogenesis when maternal gene product is exhausted.

DBE contains 345 amino acids and has a theoretical isoelectric point of 9.83. Sequence analysis suggests that DBE and its homologues carry a K-homology (KH) domain (region 136–188 in the DBE sequence; Fig. 1).

☆ *Abbreviations:* DBE, dribble; KH, K-homology; N-/C-terminal, amino/carboxyl terminal; ssRNA, single-stranded ribonucleic acid; UV, ultraviolet.

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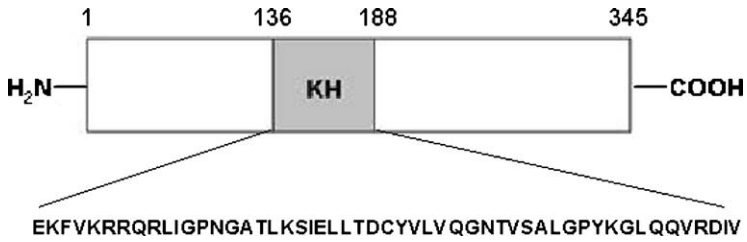


Fig. 1. Sequence analysis by Pfam (http://www.sanger.ac.uk/Software/Pfam) suggests the presence of a K-homology (KH) domain in DBE. The sequence that corresponds to the KH-domain is indicated.

The KH-domain is the most common ssRNA-binding structure after the RNA recognition motif (RRM) [4] and adopts a $\beta\alpha\alpha\beta\beta\alpha$ fold [5]. Three-dimensional structures of the KH-domain from certain proteins have been determined, in isolation or complex with RNA [6–10]. The KH-domain can be found in single or multiple copies in a protein [1,11–14]. The DBE/Krr1p/Mis3/HRB2 and the STAR/GSG/SQG protein families contain only one KH-domain, while examples of multiple-KH-domain proteins include the heterogeneous nuclear ribonucleoprotein K (hnRNP K), ribosomal protein S3, vigilin, and fragile X mental retardation 1 (FMR1). In human hnRNP K, the three KH-domains have been shown to be important for defining the specificity of RNA binding [15]. On top of mediating RNA binding, the KH-domain is involved in protein–protein interactions, such as self-dimerisation [11–14].

It is evident that many eukaryotic proteins are either totally or partially disordered in their native states, and about 30% of all eukaryotic proteins are estimated to be somewhat disordered [16–18]. Dunker et al. [19] have reviewed on intrinsically disordered proteins and maintain an online database for this group of proteins (http://www.disprot.org). Disordered proteins include nuclear proteins, such as the nucleosomal-binding protein 1, non-histone chromosomal protein HMG-T, and various ribosomal proteins. The function of disordered proteins varies from RNA binding, protein scaffolding, signal transduction, and cell cycle control to transcriptional and translational regulations. The intrinsic disorder in proteins is thought to provide structural plasticity and a disproportionately large surface area for interactions with proteins or nucleic acids [17].

As an initial step to understand the structure and function of the Krr1p family of proteins, we overexpressed and purified the full-length *Drosophila* DBE protein for biophysical and biochemical characterisation. Supporting the role of DBE in ribosomal RNA processing, we demonstrated the ability of the recombinant DBE protein to bind RNA in vitro. Moreover, using controlled proteolysis, size-exclusion chromatography, analytical ultracentrifugation, and a variety of spectroscopic methods, we investigated the degree of structural disorder, solvent exposure, secondary structure, and oligomeric status of this essential *Drosophila* protein.

## Results

### *DBE is a RNA-binding protein in vitro*

We overexpressed and purified the recombinant full-length DBE protein from *Escherichia coli* to study its biochemical and biophysical properties experimentally. Approximately 10 mg of homogeneous protein was obtained per litre of culture (data not shown).

Since DBE is involved in ribosomal RNA processing [1], we first tested the recombinant DBE protein for its ability to bind RNA. At 4 °C, purified DBE was capable of binding to the RNA homo-polymer resin, poly-G (Fig. 2). We show that DBE also interacted with poly-U, poly-A, and poly-C homo-polymers at similar affinity (data not shown). When different concentrations of heparin (a RNA analogue) were added to the reaction mixture, the binding of DBE to RNA homo-polymer resin was either reduced or abolished (Fig. 2, lanes 5–8). In contrast, no binding was observed between chicken egg white lysozyme and any of the RNA homo-polymer resins even in the absence

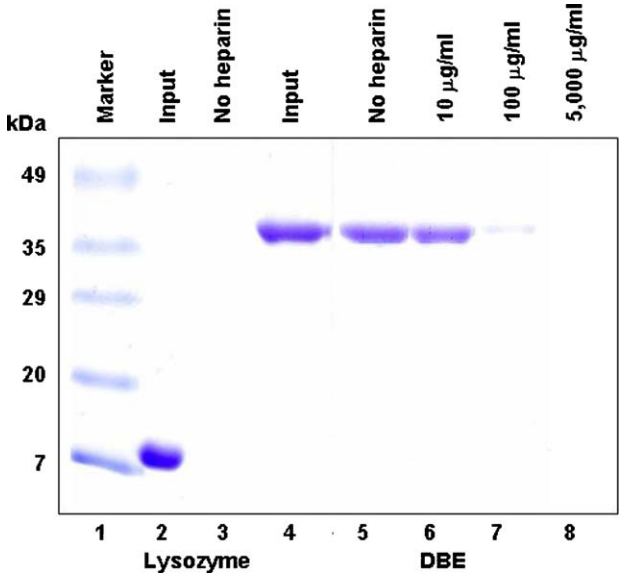


Fig. 2. RNA binding of DBE in the absence and presence of heparin. DBE was allowed to bind to the RNA homo-polymer poly-G at 4 °C in 10 mM Tris–HCl, 100 mM NaCl, 2.5 mM $MgCl_2$, and 0.5% Triton X-100, pH 7.5, with various concentrations of heparin. Chicken hen egg white lysozyme was used as a negative control.

of heparin (Fig. 2, lane 3; data not shown). Since lysozyme has a theoretical isoelectric point higher than that of DBE (11.3 and 9.8, respectively), the result indicates that the binding of DBE to RNA is not merely due to non-specific electrostatic interactions. Similar results were obtained when the experiment was repeated at room temperature (data not shown) at which the circular dichroism (CD) and fluorescence spectroscopic measurements were made (see below).

*DBE is proteolytically sensitive*

Bioinformatics predicts that DBE is 46.38% disordered in structure (Fig. 3). When the DBE sequence is analysed using different disorder prediction algorithms, the C-terminal region is invariably found to be disordered (Fig. 3). One feature of natively disordered proteins is that they are extremely proteolytic sensitive [17]. Therefore, we subjected DBE to trypsin digestion under native conditions. Fig. 4A shows the SDS–PAGE profile of DBE protein collected from the digestion mixture at designated time intervals. We found that the protein was highly susceptible to proteolytic digestion (Fig. 4A). Rapid and progressive fragmentation of the DBE protein was clearly observed, and no intact protein remained after 3 h of proteolytic digestion (Fig. 4A). Although some fragments of a low molecular weight were accumulated (Fig. 4A, lanes 7–11, as indicated by the arrows) which may suggest the presence of some compact structures, a large proportion of the protein structure must be exposed to solvent for the observed proteolysis to occur. Thus, the structure of DBE is significantly disordered.

Fig. 4B shows the proteolytic digestion of DBE that was pre-incubated with heparin. Under such conditions, no proteolytic digestion of the DBE protein was observed after incubation with trypsin for up to 30 min (Fig. 4B, lane 6). In contrast, extensive proteolysis occurred in the absence of heparin as expected (Fig. 4B, lanes 2 and 3). This suggests ligand binding may lead to an increase in the order of the protein or block access of the enzyme to the proteolytically sensitive region.

*The major secondary structure of DBE is random coil*

We next investigated the secondary structure content of DBE. Fig. 5A shows the far-UV CD spectra of DBE acquired at various temperatures. Secondary structure content was estimated from the deconvolution of the native spectrum using DICHROWEB [20]. DBE is estimated to contain 34% helices, 7% strands, and 14% turns. Most of


Fig. 3. Disorder regions in DBE predicted by PONDR-VLXT (http://www.pondr.com), DisEMBL (http://dis.embl.de), and GlotPlot (http://globplot.embl.de). Amino acid residues in these disorder regions are indicated by a line underneath the protein sequence. Secondary structure predicted by NNPREDICT (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html) is also shown, where 'H' and 'E' indicate helix and strand, respectively. PONDR-VLXT also predicts that DBE is overall 46.38% disordered.

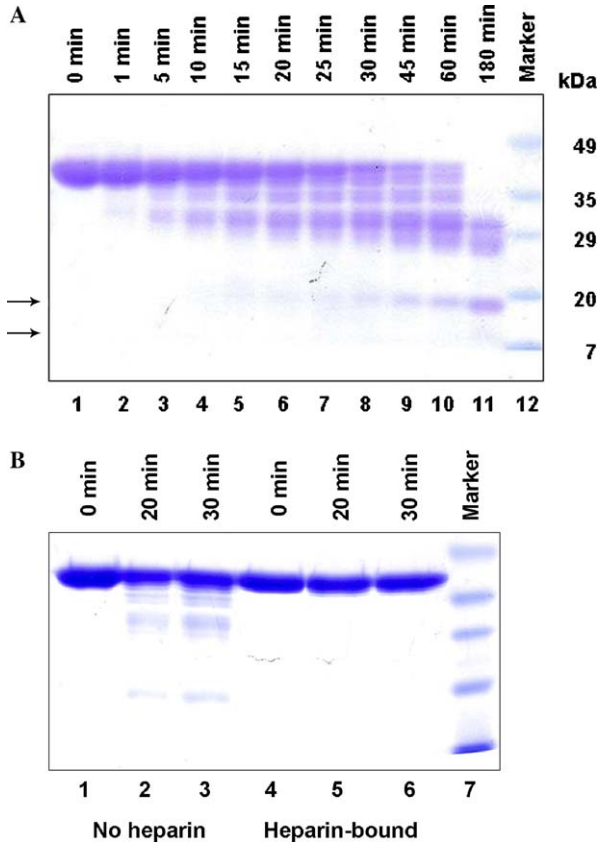


Fig. 4. (A) Controlled proteolysis of DBE by trypsin at room temperature. Aliquots were removed from the reaction mixture at designated time intervals when the reaction was quenched using SDS-containing buffer. Accumulated fragments are indicated by an arrow. (B) Trypsin proteolysis after binding to heparin. DBE was incubated with or without heparin followed by trypsin digestion for 0, 20, and 30 min.

these structures are persistent even at a higher temperature of 55 °C. Notably, there is also a considerable amount of random coil (45%), which is in good agreement with the PONDR-VLXT prediction (Fig. 3). In other words, our CD data suggest that DBE possesses some regular secondary structure but also contains significantly disordered regions.

DBE thermally denatures in two steps as monitored by CD ellipticity at 222 nm, which is a measure of α-helical content (Fig. 5B). In the first step, the protein melted with a slight increase in ellipticity indicating little loss in helical structure. The transition was reversible and had a mid-point at ~35 °C, which may correspond to the reversible unfolding of a low-stability structure. The second step, however, involved a major loss in secondary structure and concomitant aggregation. This step was irreversible and began only at a high temperature of ~90 °C. Notably, in between the two transitions, i.e., from 40 to 90 °C, a slight increase as opposed to a decrease in helical content was observed. As temperature could promote secondary structure formation by enhancing hydrophobic interactions, the observed increase in structure is indicative of that DBE is an intrinsically unstructured protein. This experiment was repeated at the same and lower protein

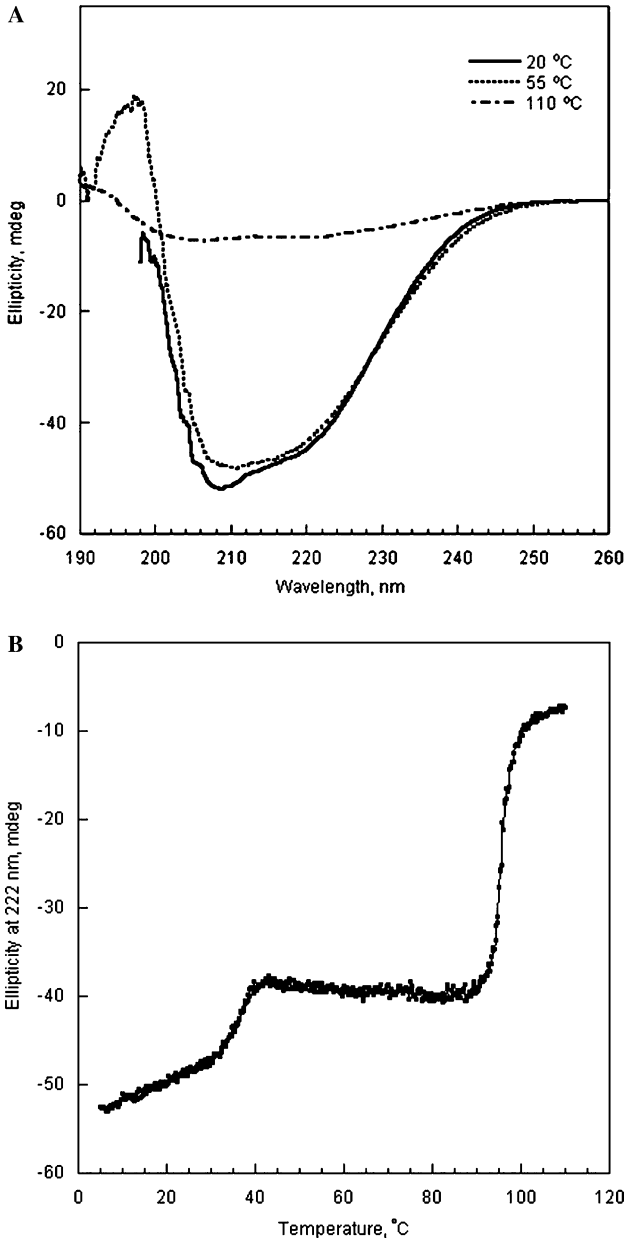


Fig. 5. (A) The CD spectra of DBE at 20, 55, and 110 °C. The native spectrum (20 °C) was subjected to secondary structure analysis using DICHROWEB [20]. (B) Thermal denaturation of DBE as monitored by CD spectroscopy at 222 nm.

concentrations of DBE, and the same results were obtained (data not shown).

### *Solvent exposure of hydrophobic residues in DBE*

Fluorescence spectroscopy was used to study the solvent exposure of DBE. Under all pH conditions studied, the emission maximum of DBE was close to 350 nm, indicating extensive exposure of the tryptophan fluorophores (Fig. 6A). Even so, a gradual red-shift in the emission maximum toward 350 nm was observed as the pH decreased from 7.0 to 2.0. This suggests that the tryptophan residues became increasingly exposed to the solvent. Consistent

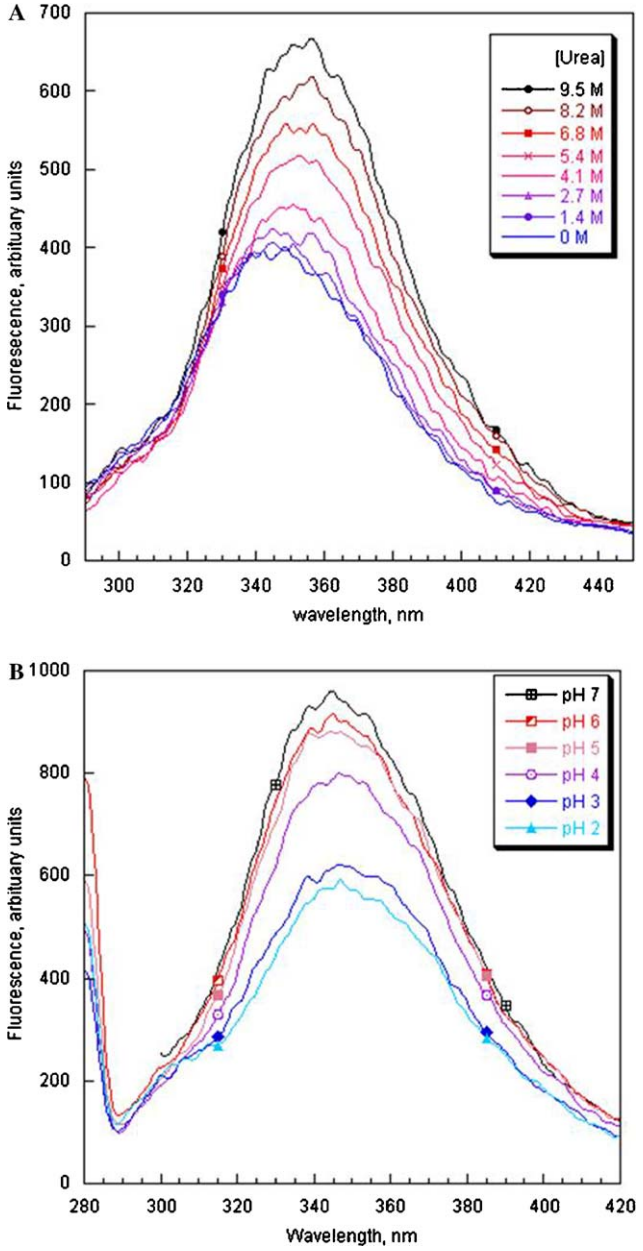


Fig. 6. Emission spectra of DBE in various concentrations of urea (A), and at various pHs (B). The excitation wavelength is 280 nm for all the spectra shown.

with this, tyrosine fluorescence (a small emission peak at 303 nm) also became more observable with decreasing pH.

The emission spectra of DBE in various concentrations of the chemical denaturant, urea, are shown in Fig. 6B. A red-shift in the emission maximum toward 350 nm was again observed as urea concentration increased. There was also an enhancement in the emission intensity, which suggests there was less fluorescence quenching and that the protein became progressively denatured. Excitation at 255 nm also gave similar results (data not shown).

Our emission data indicate that there is a change in structure upon denaturation of DBE, which is further supported by changes in excitation spectra (data not shown) and absorption spectra (data not shown). However, in the native state, the hydrophobic residues of DBE are more exposed than usual in fully folded proteins, which is, again, consistent with the structural disorder feature of the protein.

Table 1a
Stokes radius of DBE determined in three analytical gel filtration chromatography experiments

| Column used | Stokes radius (Å) |
|---|---|
| Superdex 75 | 33.4 |
| | 33.3 |
| Superdex 200 | 32.5 |
| | Average = 33.1 ± 0.3 |

Table 1b
Theoretical Stokes radius calculated for different conformational states of DBE

| Hypothetical conformational states of DBE | Theoretical Stokes radius calculated according to [28] (Å) |
|---|---|
| Native globular | 27.4 |
| Molten globule | 30.5 |
| Native pre-molten globule | 41.2 |
| Native disordered | 52.1 |
| Unfolded | 59.6 |

### *Hydrodynamic properties of DBE*

To investigate the hydrodynamic dimensions of DBE, size-exclusion chromatography was used. Table 1a shows the Stokes radius of DBE measured in three independent experiments. An average value of 33.1 Å was obtained. Such value is smaller than the theoretical Stokes radius calculated [21] for globular DBE (27.4 Å) but close to that of a molten globule (30.5 Å). Natively disordered proteins usually structurally resemble a pre-molten globule state, which is a species that is more unfolded than the molten globule [28] (Table 1b). We, therefore, propose that DBE is partially collapsed and possesses extended, disordered structure. Since we did not observe any species having a large hydrodynamic radius, our data suggest that DBE exists as a monomer in solution.

To further confirm its monomeric status, DBE was subjected to sedimentation equilibrium studies. Fig. 7 shows a representative data set. The data were fitted to a single ideal species and the mean $M(1-\bar{V}\rho)$ determined was $10{,}725 \pm 350$, which gives a calculated relative molecular mass of $41{,}831 \pm 1400$. This is close to the monomer relative molecular mass of 39,840 calculated from the primary sequence of DBE.

## Discussion

We have purified and biophysically characterised the full-length recombinant DBE protein expressed in *E. coli*, which is a representative protein of the Krr1p nucleolar protein family. This family of proteins is important for the processing of ribosomal RNAs and crucial for the via-

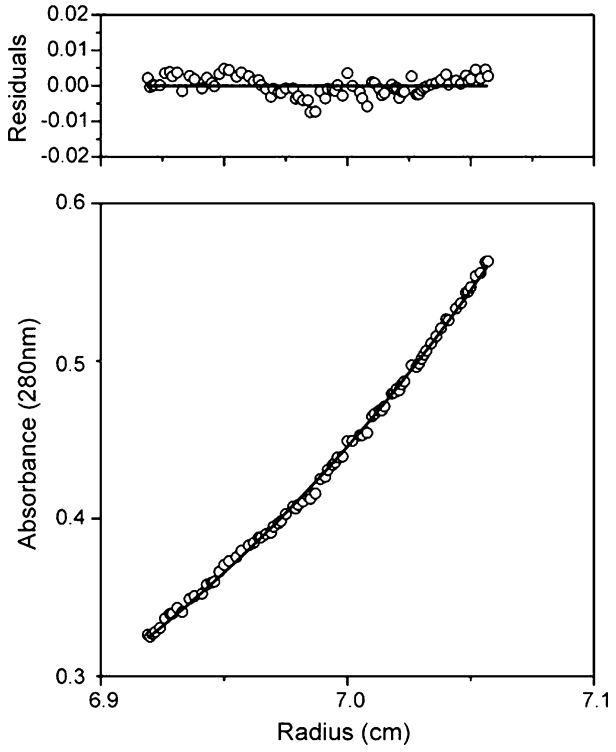


Fig. 7. Sedimentation equilibrium analysis of DBE. A representative set of data collected at an $A_{280}$ of 0.4 and a speed of 11,000 rpm is shown (open circles) fitted to a single ideal species model (solid line). The residuals for the fit are shown above and are randomly distributed around zero. The molecular mass determined was 41,831 ± 1400, indicating the protein is a monomer.

bility of the organisms [1,22,23]. We have noted the following features of DBE which are relevant to its role as a RNA-processing protein in the nucleolus.

*Structural disorder of DBE*

Five lines of evidence suggest DBE is a significantly disordered protein. First, DBE contains a high proportion of disorder-promoting residues such as glutamate, serine, and lysine; and bioinformatics tools predict a high degree of disorder in its structure (Fig. 3). Second, the protein is extremely sensitive to proteolysis (Fig. 4), which is a shared feature of intrinsically disordered proteins [17]. The third line of evidence comes from far-UV CD spectroscopy (Fig. 5). Deconvolution of the native spectrum of DBE shows that there is up to 45% random coil in the protein, consistent with the bioinformatics predictions. Fourth, as shown by fluorescence spectroscopy, the native DBE protein has an emission peak close to that of free tryptophans (350 nm) (Fig. 6), indicating a significant portion of the tryptophan residues are exposed to solvent at physiological pH. Fifth, the Stokes radius of DBE as measured by size-exclusion chromatography is in between those calculated for the globular and fully unfolded conformations (Table 1a). Altogether, we therefore propose that DBE possesses structural disorder. Overall, DBE is as compact as a molten globule. Notably, a significant degree of disorder is also predicted for other homologues of DBE, including Krr1p, Mis3, and HRB2 (data not shown), which further suggests that this is a general feature of this protein family.

Gunasekaran et al. [24] proposed that structural disorder in proteins effectively reduces the size of proteins and in turn, reduces cellular crowding and/or cell size since disordered proteins often have a disproportionately large intermolecular interfaces. For a condensed region like the cell nucleolus where DBE and its homologues reside, such an advantage appears to be important. It is thus not surprising to note that many nucleolar proteins are natively disordered. For example, nucleolin and fibrillarin, which are ribosomal RNA-processing enzymes and localised in the dense fibrillar component of the nucleolus, are, respectively, 56% and 44% disordered as predicted by PONDR-VLXT [16]. In addition, disordered structure also provides structural plasticity for protein and/or nucleic acid binding [17] (see The RNA binding properties of DBE), so the disorder observed in DBE could be functionally important, both for its subcellular localization and ligand binding.

*DBE is monomeric*

We have shown DBE is monomeric by both size-exclusion chromatography (Table 1a) and sedimentation equilibrium analysis (Fig. 7). Since the expression level of DBE inside the cell is relatively low [1], the experimental data collected at micro-molar level imply that cellular DBE exists as monomers although it does not rule out the possibility that the protein can oligomerise upon binding to other cellular factors. For instance, the STAR/GSG/SQG proteins, which represent another family of single-KH-domain proteins [1], form homo-dimers [11–14]. Further, one STAR/GSG/SQG protein, SAM68, has also been demonstrated to self-dimerise only in the presence of RNA [12].

*The RNA-binding properties of DBE*

We showed that DBE binds RNA homo-polymers in vitro (Fig. 2) and that the binding is inhibited by the RNA analogue heparin in a concentration-dependent manner (Fig. 2). KH-domains are known to bind RNA in other proteins, so this may also be the case [4]. One feature of the RNA-binding ability of KH-domain proteins is that the binding is mediated by multiple-KH-domains [15,25]. Since DBE is a single-KH-domain protein, the protein may require dimerisation prior to RNA binding [12]. We cannot however exclude the possibility that DBE and other members of the DBE/Krr1p/Mis3/HRB2 family bind RNA via other structured regions. For instance, a putative N-terminal Pfg27 domain was predicted for DBE and its homologues using SUPERFAMILY (with an $E$ value of $1.1 \times 10^{-17}$ for DBE; see Supplementary material). This Pfg27 domain is an α-helical RNA-binding domain first identified from *Plasmodium falciparum* [26].

Another possibility is that DBE binds RNA via its disordered structures, which can be inferred indirectly from

the protection of DBE from trypsin digestion upon binding to the RNA analogue, heparin (Fig. 4B). It is worth noting that many natively disordered proteins are involved in RNA binding [17,18]. One advantage of having disordered structure for RNA binding is that it possesses a large surface for molecular interactions when compared to a globular protein of identical size [17]. This is particularly advantageous in a condensed region like the nucleolus, as suggested above (Structural disorder of DBE).

In summary, the full-length *Drosophila* DBE protein was overexpressed and purified from *E. coli*. DBE has been characterised to be a monomeric protein containing both α- and β-structures. Moreover, the structure of DBE is significantly disordered. Such structural disorder is relevant to its RNA-binding ability as a nucleolar protein. Our RNA-binding data further support a role of DBE in ribosomal RNA processing.

## Materials and methods

*Plasmid.* The *dbe* open-reading frame was cloned, via *Nco*I and *Not*I sites, into a modified form of the pET-3a vector (Novagen) which carries additional cloning sites and an upstream ribosome-binding site.

*Protein expression and purification.* The pET-3a-*dbe* plasmid was used to transform *E. coli C41*(*DE3*) cells for overexpression [27]. The transformed cells were then grown in Luria Broth medium containing 100 μg/ml ampicillin and 2% glucose at 37 °C to make a starter culture. Upon inoculation, the culture was allowed to grow at 37 °C for 4 h and protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactoside (IPTG). The culture was then grown overnight at 25 °C before harvesting. The protein was extracted in 50 mM sodium phosphate buffer (pH 7.4) with 5 mM PMSF using sonication. After centrifugation, the soluble fraction, supplemented with 0.1% (*v*/*v*) β-mercaptoethanol, was filtered and loaded onto a 5-ml HiTrap SP HP column (Amersham Biosciences) connected to an AKTA Explorer (Amersham Biosciences). A linear NaCl gradient was used (buffer A = 50 mM sodium phosphate, pH 7.4; buffer B = 50 mM sodium phosphate, 1 M sodium chloride, pH 7.4) with a flow rate of 5 ml/min to elute proteins. The DBE protein was eluted at ~60 mM NaCl, as confirmed by SDS–PAGE. The DBE-containing fraction from the HiTrap SP HP column was then concentrated and loaded onto a HiLoad Superdex 200 prep grade column (Amersham Biosciences) for the final step of purification. The elution buffer (50 mM sodium phosphate, 200 mM sodium chloride, pH 7.4) was run at a flow rate of 2.5 ml/min, and the DBE protein was eluted at ca. 180 ml. The isolated protein appeared as a homogeneous band on SDS–PAGE, and the identity of DBE was confirmed by mass spectrometry (Applied Biosystems 4700 Proteomics Analyzer; data not shown).

*UV–visible absorption spectroscopy.* Absorbance at the UV–visible range was monitored using a Beckman DU7500 photo-diode array spectrophotometer. The denatured and native protein samples at 35 μM were prepared, respectively, in 50 mM sodium phosphate (pH 7.4) in the presence or absence of 9 M urea. The samples were equilibrated at room temperature for 3 h prior to the measurements.

*Fluorescence spectrometry.* A Perkin-Elmer LS-50B luminescence spectrophotometer was used for fluorescence measurement. The cell holder was thermostated with a circulating water bath. An excitation wavelength of 280 nm was used (excitation at 255 nm gave similar results). A bandpass of 4 nm and 6 nm was used for the excitation and emission wavelengths, respectively. Urea in various concentrations was prepared using 50 mM sodium phosphate (pH 7.4), and the pH buffers were prepared with 25 mM sodium acetate. Both urea and pH buffers were filtered and then mixed with the protein samples (1.1 μM). Afterwards, the sample mixtures were equilibrated at room temperature for at least 3 h.

*Circular dichroism spectroscopy.* A Jasco J800 CD spectrometer was used for far-ultraviolet CD measurements. The protein concentration used was at 10 or 20 μM and the buffer was 50 mM sodium phosphate (pH 7.4). A pathlength of 0.1 cm was used. Spectral deconvolution for secondary structure content estimation was made with CONTIN using DICHROWEB [20].

*Thermal denaturation.* Thermal denaturation was performed using a Jasco J800 CD spectrometer coupled to a Peltier temperature control unit. The loss of structure was monitored by CD at 222 nm. For the data shown, 20 μM DBE in 50 mM sodium phosphate (pH 7.4) and a pathlength of 0.1 cm were used. The scanning rate was 50 mdeg/h.

*Size-exclusion chromatography.* Purified DBE (35 μM) was subjected to size-exclusion chromatography using Amersham Superdex 75 and Superdex 200 columns connected to an AKTA Explorer Protein Purification System (Amersham Biosciences). A sample loop of 500 μl and a flow rate of 0.5 ml/min were used. The elution buffer used was 50 mM sodium phosphate and 200 mM sodium chloride (pH 7.4). The sample and calibration standards (Amersham Gel Filtration LMW Standard Proteins Kit) were filtered, loaded in sequential order, and their elution profiles collected for Stokes radius determination. The Stokes radius was calculated according to the following empirical equation [28]:

$$\log R_s = 0.357\ \log M_w - 0.204, \quad (1)$$

where $R_s$ is the Stokes radius and $M_w$ is the molecular weight.

*Analytical ultracentrifugation.* Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge as described previously [29]. Samples were prepared in 50 mM sodium phosphate and 200 mM sodium chloride (pH 7.4), and data were acquired as an average of 25 absorbance measurements at a wavelength of 280 nm and a radial spacing of 0.001 cm. Sedimentation equilibrium experiments were performed at 4 °C and rotor speeds of 11,000 and 15,000 rpm, with protein loading concentrations corresponding to a measured $A_{280}$ of 0.4–0.8. Data were analysed using the Beckman analysis software running under Microcal Origin v3.78 in terms of a single ideal solute to obtain the buoyant molecular mass, $M(1-\bar{V}\rho)$, as previously described [29]. Residuals were calculated by subtracting the best fit of the model from the experimental data. In all cases, a random distribution of the residuals around zero was noted as a function of the radius. The molecular mass was calculated using the solvent density of 1.01531 g/ml and partial specific volume $\bar{V}$ based on the amino acid composition of 0.7324 ml/g, both calculated using the software SEDNTERP (http://www.rasmb.bbri.org).

*Controlled proteolysis.* Five hundred micrograms of DBE (45 μM) was incubated at room temperature with 200 ng of trypsin. Twenty microlitres of the sample was removed from the reaction mixture at different time intervals and SDS-containing buffer was added immediately to quench the reaction followed by heating. The samples collected were subjected to SDS–PAGE analysis. In another trypsin digestion experiment, 500 μg of DBE was incubated with 0 or 100 μg heparin for one hour prior to addition of trypsin. Digestion was allowed to proceed for 0, 15 or 30 min before it was quenched as described above.

*RNA-binding assay.* The ability of DBE to bind RNA was assayed with RNA homo-polymers (poly-A, poly-U, poly-C, and poly-G) linked to agarose or Sepharose (Sigma, catalogue numbers P2769, P1908, P8563, and P9827, respectively). The procedure was adapted from [11]. Briefly, 40 μl of the DBE protein at 10 μM was individually mixed with each of the RNA homo-polymer resins (in excess) at 4 °C. Five hundred microlitres of the binding/washing buffer (10 mM Tris–HCl, 100 mM NaCl, 2.5 mM $MgCl_2$, and 0.5% Triton X-100, pH 7.5) was then added and the mixture was incubated with rocking for at least 30 min at 4 °C. The binding/washing buffer used was supplemented with 0, 10, 100 or 5,000 μg/ml heparin. After incubation, the mixtures were washed five times with the corresponding binding/washing buffer. Any protein bound to the resin was then released with the addition of SDS-containing buffer and then detected by SDS–PAGE. Chicken egg white lysosyme (Sigma) at 10 mg/ml was used as a negative control. For the assays on pH- and urea-denatured proteins, the binding/washing buffers used were 25 mM sodium acetate, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5%

Triton X-100, pH 2.0, and 9 M urea in 10 mM Tris–HCl, 100 mM NaCl, 2.5 mM $MgCl_2$, and 0.5% Triton X-100, pH 7.5, respectively. The samples to be denatured were equilibrated in the corresponding binding/washing buffers for three hours before being added to the RNA resin.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.02.153.

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