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Thermofluor-based optimization strategy for the stabilization and crystallization of *Campylobacter jejuni* desulforubrerythrin

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ABSTRACT

Desulforubrerythrin from *Campylobacter jejuni* has recently been biochemical and spectroscopically characterized. It is a member of the rubrerythrin family, and it is composed of three structural domains: the N-terminal desulforedoxin domain with a non-heme iron center, followed by a four-helix bundle domain harboring a binuclear iron center and finally a C-terminal rubredoxin domain.

To date, this is the first example of a protein presenting this kind of structural domain organization, and therefore the determination of its crystal structure may unveil unexpected structural features. Several attempts were made in order to obtain protein crystals, but always without success. As part of our strategy the thermofluor method was used to increase protein stability and its propensity to crystallize. This approach has been recently used to optimize protein buffer formulation, thus yielding more stable and homogenous protein samples. Thermofluor has also been used to identify cofactors/ligands or small molecules that may help stabilize native protein states.

A successful thermofluor approach was used to select a pH buffer condition that allowed the crystallization of *Campylobacter jejuni* desulforubrerythrin, by screening both buffer pH and salt concentration. A buffer formulation was obtained which increased the protein melting temperature by 7 °C relatively to the initial purification buffer. Desulforubrerythrin was seen to be stabilized by lower pH and high salt concentration, and was dialyzed into the new selected buffer, 100 mM MES pH 6.2, 500 mM NaCl. This stability study was complemented with a second thermofluor assay in which different additives were screened. A crystallization screening was carried out and protein crystals were rapidly obtained in one condition. Protein crystal optimization was done using the same additive screening. Interestingly, a correlation between the stability studies and crystallization experiments using the additive screening could be established.

The work presented here shows an elegant example where thermofluor was shown to be a key biophysical method that allowed the identification of an improved buffer formulation and the applicability of this technique to increase the propensity of a protein to crystallize is discussed.

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Introduction

Reactive oxygen species (ROS)[1] such as $O_2^{\cdot-}$, H_2O_2 and ·OH are formed as a normal product of aerobic cellular metabolism; however, in conditions of intracellular oxidative stress, ROS formation can be highly toxic. The most lethal ROS is the hydroxyl radical, that attacks different macromolecules such as proteins, lipids or DNA, and for which there is no known detoxifying biological system [1–3]. The hydroxyl radical is produced by a one electron reduction of hydrogen peroxide, and therefore the generation of this radical is closely related to the hydrogen peroxide concentration. Different proteins, namely catalase or various peroxidases, have been described to be involved in hydrogen peroxide consumption by a two electron reduction reaction resulting in the production of water [4–8]. Over the last few years, rubrerythrins have been described as an alternative system in anaerobic and microaerobic organisms, and have been proposed to have NADH-linked peroxidase activity [9–14].

Rubrerythrins are a family of proteins which contain two domains: a N-terminal erythrin-like domain containing a four helix bundle with a diiron site, and a C-terminal rubredoxin-like domain

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[1] *Abbreviations used:* ROS, reactive oxygen species; DRbr, desulforubrerythrin; Dx, desulforedoxin; DSF, differential scanning fluorimetry; IPTG,isopropyl-1-thio-β-D-galactopyranoside; KPi, potassium phosphate; FHB, four helix-bundle; Rd, rubredoxin.

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containing a $FeCys_4$ center [10,15–17]. These proteins were first isolated from the anaerobic bacterium *Desulfovibrio vulgaris*, and subsequently in other prokaryotes, such as *Clostridium* and *Porphyromonas* species [18–20]; they have also been isolated from archaea, e.g., *Sulfolobus tokodaii* [21], and eukarya, e.g., *Trichomonas vaginalis* [22]. The function of these proteins is, as yet, not fully established, and different activities have been reported for them, such as ferroxidase [23], superoxide dismutase [20], pyrophosphatase [24] and NADH-linked peroxidase activity [9–11], the latter being the most reproducible. Rubrerythrins have been found to complement catalase null strains, and rubrerythrin deletion mutants are known to be more sensitive to oxygen and hydrogen peroxide [18].

Recently, a protein from *Campylobacter jejuni* NCTC 11168 belonging to the rubrerythrin family, named desulforubrerythrin (DRbr) has been isolated and characterized as containing an additional N-terminal domain, desulforedoxin (Dx) comprising a $FeCys_4$ center [25,26]. This protein was proposed to be involved in the H_2O_2 oxidative stress response [25,26]. As of yet, *C. jejuni* desulforubrerythrin is the first example of a protein with this unique domain structural organization, and an interesting target for structure determination, which will further enrich the knowledge of the rubrerythrin superfamily, namely on the different structural domain arrangements and on the kinetic mechanism of hydrogen peroxide reduction.

Protein thermal stability is known to be related with the propensity of proteins to crystallize, and can be measured by heating the protein while monitoring its structural integrity [27]. Increasing temperatures cause proteins to unfold in a cooperative process that occurs within a short temperature range. The midpoint of such thermal transition is called the protein melting temperature (T_m) and corresponds to the temperature where the Gibbs free energy of unfolding is zero and the concentration of native and non-native forms are equal [28].

The protein melting temperature can be determined by different biophysical methods, such as circular dichroism, differential scanning calorimetry and fluorescence spectroscopy. Recently, a new method has been developed by Pantoliano et al. [29] which offers several advantages over those mentioned above [28,30,31]. Differential scanning fluorimetry (DSF) also known as thermofluor or thermal stability assay, has the ability to screen a multiplicity of parallel reaction conditions; it requires only microgram quantities of protein and it uses a common fluoroprobe for all proteins, thus avoiding differences based on the intrinsic properties of each protein. Thermofluor determines the protein melting temperature (T_m), and is based on the interaction between the dye and the hydrophobic regions of the protein, which are exposed upon protein thermal denaturation. The dye (e.g., SYPRO Orange) is required to be solvatochromic with low fluorescence quantum yield in aqueous environment, but highly fluorescent in non polar environments such as the hydrophobic regions exposed when the protein unfolds [32–34]. The assay consists initially, on the non-interaction between the dye and the native folded protein. As the temperature increases the protein becomes thermally denatured, exposing its hydrophobic patches and thus causing a drastic increase in the fluorescence signal due to the strong interaction of the dye with those regions [32,35]. Thermofluor was initially developed as a high-throughput screening method for buffer optimization and ligand-induced stabilization of proteins, but it has since been shown to be quite effective in identifying the best buffer formulation to be used for protein purification, storage [32,33] and crystallization, in screening for ligands and small molecules, and to assess the effect of ionic strength on the protein T_m [27,33,34,36,37].

Herein we show how DSF/thermofluor was successfully applied to the thermal stability study of desulforubrerythrin from *C. jejuni*, allowing the selection of a buffer formulation which was shown to be relevant for the protein crystallization and subsequent structural characterization. Thermofluor stability assays helps identifying promising additive candidates for protein crystal optimization, and this correlation can be an added value to structural projects.

Experimental procedures

Expression and purification of recombinant DRbr

The gene *cj0012c* from *C. jejuni* NCTC11168 was cloned using the pMAL system (New England Biolabs) [38] as previously described [26]. The gene was then transformed into *Escherichia coli* strain BL21DE3 cells, and DRbr overexpression was performed by growing aerobically this strain at 37 °C in M9 minimal medium with the addition of 20 mM glucose, 400 μM $FeSO_4$ and 100 μg/ml ampicilin (final concentrations). Since the protein is predicted as a metalloprotein with three different iron sites, the addition of iron in the growth media revealed to be crucial to obtain the protein with the correct amount of iron incorporated. At the optical density 600 nm of 0.3, it was added 250 μM (final concentration) of isopropyl-1-thio-β-D-galactopyranoside (IPTG), the cells were grown for ca. 16 h, and then were harvested by centrifugation. The cells were finally resuspended in a buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mg/ml lysozyme and 20 μg/ml DNase, and stored at −80 °C.

All purification procedures were performed using a Akta-Prime (GE Healthcare) and were carried out under anaerobic conditions at 4 °C, in a Coy glove box, with an O_2-free atmosphere constituted by a mixture of 95% argon and 5% hydrogen. All buffers were previously degassed and flushed with argon and as a finally step were under vacuum for 15 min.

After defrosting the cells in the resuspension buffer, they were under nitrogen flux for 60 min. Then the cells were broken after passing the cell suspension three times through a French press (35,000 psi). The cell extract was put under nitrogen flux and prepared for ultracentrifugation at 125,000*g* for 1 h at 4 °C, under anaerobic conditions. The soluble fraction obtained from the supernatant was immediately dialyzed overnight (ca. 16 h) against degassed buffer, 20 mM Tris–HCl (pH 7.2), under anaerobic conditions at 4 °C. The soluble fraction was loaded into an anionic exchange Q-Sepharose Fast Flow column (XK 26/10, GE Healthcare) previously equilibrated with 20 mM Tris–HCl (pH 7.2), and eluted at 2 ml/min applying a linear ionic strength gradient from 0 to 1 M NaCl in the same buffer. The DRbr fraction eluted at ca. 0.2 M NaCl, was collected and then dialyzed in anaerobic conditions overnight against 10 mM potassium phosphate (KPi) pH 7.0 buffer. This DRbr fraction was further purified onto a Bio-gel hydroxyapatite type II column (XK 16/40, Bio-Rad), equilibrated with the same buffer. The protein was eluted with a linear gradient from 0 to 1 M KPi at pH 7.0. Fractions containing the protein eluted at ~0.3 M KPi and were concentrated anaerobically in a Diaflo (Amicon) using a YM10 membrane and finally applied to a molecular filtration column, Sephadex 75 (XK 26/60, GE Healthcare), equilibrated with 20 mM Tris–HCl (pH 7.2), 150 mM NaCl. Since the purification was performed under anaerobic conditions the protein was reduced and therefore colorless, so the presence of DRbr was followed throughout the purification procedure by UV/vis spectroscopy under aerobic conditions and by SDS–PAGE [39].

The final protein purification buffer was 20 mM Tris–HCl pH 7.2, 150 mM NaCl and the final protein concentration used in all the thermofluor assays was 0.05 mg/ml.

Thermofluor assay

Protein melting temperature (T_m) determination was performed by monitoring protein unfolding using the fluoroprobe SYPRO Or-

ange dye (Molecular Probes), which upon binding to the hydrophobic protein regions emits fluorescence that can be measured as a function of temperature. The thermal shift assay was performed on an iCycle iQ5 Real Time PCR Detection System (Bio-Rad), equipped with a charge-coupled device (CCD) camera and a Cy3 filter with excitation and emission wavelengths of 490 and 575 nm, respectively. This equipment can simultaneously detect the fluorescence changes in 96-well plates (low profile plate, Bio-Rad) and thus can be used for parallel thermal stability studies.

In order to optimize the fluorescence signal to noise ratio an assay optimization was performed.

Typical assay volumes are 25 μl, and initially a signal strength optimization is required. Considering that the experimental volume is fixed, two remaining experimental parameters may influence the signal strength – protein and dye concentration. Using a fixed protein concentration of 0.05 mg/ml, increasing dye concentrations were tested (from 1- to 10-fold, diluted from the initial 5000-fold stock in 50 mM HEPES pH 8.0). The 96-well plates were sealed with Optical Quality Sealing Tape (Bio-Rad) and centrifuged at 2500*g* for 2 min immediately before the assay to remove possible air bubbles. For the thermal denaturation the plates were heated from 20 to 90 °C with stepwise increments of 1 °C per minute and a 10 s hold step for every point, followed by the fluorescence reading. The best signal-to-noise ratio was obtained using 0.05 mg/ml protein and 10-fold dye as final assay concentrations, and subsequently these conditions were used for the remaining of this work.

Buffer formulation screening was prepared based on the Solubility kit from Jena Biosciences (100 mM buffer concentration and pH range 3–10) with the addition of increasing NaCl concentrations (0, 150 and 500 mM). The assay was prepared by adding 2.5 μl of protein–dye mixture solution previously prepared in 50 mM HEPES pH 8.0 with 22.5 μl of the different screening buffers. The reference experiment was prepared using the protein purification buffer (20 mM Tris–HCl pH 7.2, 150 mM NaCl).

The additive screening assay was performed by adding aliquots of 2.5 μl of each additive compound (Hampton Research) to 22.5 μl of the protein–dye mixture. In this screen the protein–dye mixture was prepared in the buffer yielding the highest T_m in the previous screening (100 mM MES pH 6.2, 500 mM NaCl). The control experiment was prepared by adding the protein buffer instead of the additive compound.

Protein crystallization

Following the thermofluor analysis, the protein was dialyzed into the new buffer (100 mM MES pH 6.2, 500 mM NaCl) and concentrated up to 25 mg/ml. Crystallization trials were done at the nanoliter (nl) scale with the Classic Screen (Nextal) using a Cartesian Crystallization Robot Dispensing System (Genomics Solutions) and round-bottom Greiner 96-well CrystalQuick™ plates (Greiner Bio-One). Taking into consideration the thermofluor results from the additive screen, two additional crystallization drops per condition screened were prepared with the same protein concentration, but adding for each condition 10 mM cadmium chloride (position *b*) or 10 mM zinc chloride (position *c*). Only one drop ratio 100:100 (nl) was prepared, and drops were equilibrated against 100 μl of reservoir solution. Dark red colored crystals appeared in less than 1 h and only in position *a* for condition A5: 100 mM HEPES pH 7.5, 5% (v/v) isopropanol, 10% (w/v) PEG 4000.

Crystal optimization was performed by the hanging-drop vapor diffusion technique at 20 °C, using the additive screen (Hampton Research) in 48-wells plates (Hampton Research). Subsequently, crystals appeared using the following additives: 100 mM ammonium sulfate (B3), 10 mM sarcosine (D5), 10 mM adenosine-5-triphosphate disodium salt (D10), 10 mM TCEP hydrochloride (D11), 10 mM GSH-GSSG (D12), 3% 1,4-dioxane (G9), 3% methanol (G12).

Results and discussion

DRbr protein is a 25 kDa metalloprotein which belongs to the rubrerythrin family, containing three domains: a N-terminal domain similar to desulforedoxin (Dx), followed by a four helix-bundle (FHB) domain with a diiron site, and finally a C-terminal rubredoxin (Rd) domain [25]. Both desulforedoxin and rubredoxin domains contain a mononuclear Fe-Cys_4 iron center. DRbr is to date the only studied example from the rubrerythrin family containing a N-terminal Dx domain, and no structural information is available. Analyzing all the known crystallographic structures of rubrerythrin family members, it is known that for the cases where the protein is composed of an FHB and an Rd domains, the rubredoxin domain is always close (ca. 13 Å) to the di-iron center on the four-helix bundle domain [16,40,41]. The structural fold of the Dx domain is known from the crystallographic structure of desulforedoxin itself and also from the Dx domain of the 2Fe superoxide reductase (desulfoferrodoxin) [42,43]. Although structural information is known for each individual domain of DRbr, the lack of a complete structural model for DRbr is still limiting our understanding of the structural arrangement of the different domains and their role in the reaction mechanism with hydrogen peroxide.

The protein was purified in three chromatographic steps under anaerobic conditions, as previously described [25]; in solution the protein oligomeric state is a tetramer, as determined in the final purification step by size exclusion chromatography [25]. The purified protein was stored at −80 °C in the buffer 20 mM Tris–HCl pH 7.2, 150 mM NaCl under anaerobic conditions. Although the protein was purified and stored under anaerobic conditions, the crystallization screens were performed at aerobic conditions, this change on the protein environment did not induce any protein degradation or precipitation during the experiment. Several aerobic crystallization screenings were carried out with the "as purified" protein, the results were always without success, no crystals were observed in any condition. Therefore, a thermofluor-based stability study was performed, in order to find a different buffer formulation, where the protein would be more stable in solution, and thus increasing the likelihood of obtaining protein crystals. The data derived from the thermofluor experiments were analyzed taking into consideration two main factors: the midpoint temperature of the protein-unfolding transition (T_m – melting temperature) and the transition slope. The ideal buffer formulation is that where only one transition (folded-unfolded state) is observed with a T_m increase relatively to the initial purification buffer. This increase means the protein is more stable and therefore more energy (heat) is required to unfold it. Since protein unfolding is a cooperative process, the unfolding of a small protein region should induce the immediate unfolding of the remaining protein core; thus an optimal protein stabilization buffer should present a sharp and fast thermal denaturation transition between the folded and unfolded states, detected through high transition slopes, in parallel with a higher T_m.

A preliminary screen was performed in order to choose the best signal-to-noise ratio, where different solutions of dye were diluted into 50 mM HEPES pH 8.0 (1, 2, 5 and 10-fold) and using a protein concentration of 0.05 mg/ml. The different DRbr denaturation curves were analyzed and the first derivative was calculated in order to determine the protein melting temperature for each assay. Based on these assay conditions, a buffer screen was carried out, consisting of 24 different buffers, each with a concentration of 100 mM and a pH range from 3 to 10. Each buffer was

prepared with different salt (NaCl) concentrations: 0, 150 and 500 mM. The T_m values determined from each condition were then compared with the T_m value from the control experiment, with the purification buffer (100 mM Tris–HCl pH 7.2, 150 mM NaCl), and the unfolding temperature shift (ΔT_m) could be calculated. As expected, two of the 24 conditions tested, at pH 3.0 and 3.2, gave no measurable transitions, probably due to their very low pH inducing a protein structure destabilization, which was always observed in the presence and absence of salt. Interestingly, two melting transitions were observed with the following buffers: citric acid pH 4.0, sodium acetate pH 4.5, sodium citrate pH 5.5, sodium/potassium phosphate pH 6.0, bis-tris pH 6.0, ADA pH 6.5, bis-tris propane pH 6.5, both with and without salt present. The buffers PIPPS pH 3.7 and sodium/potassium phosphate pH 5.0 also generated two transitions but only at high salt concentration (500 mM NaCl).

Protein sample homogeneity is potentially the most important factor for successful crystallization, and since the DRbr protein is composed of three different domains, the aim of this work was to identify buffers presenting only a unique melting transition, meaning that the protein is structurally homogenous in that buffer.

A clear unique thermal transition could be detected and measured in buffers with a pH range of 3.7–10 (Fig. 1 A and B). Initial fluorescence at low temperatures was observed in all these buffers, which may be due to the fact that one of the protein domains contains a slightly exposed hydrophobic region, as also proposed for other proteins [32]. The results obtained from these conditions were compared with the control experiment, 100 mM Tris–HCl pH 7.2, 150 mM NaCl, which yields a T_m of 62 °C. Therefore, the best buffers, yielding the highest ΔT_m and a higher slope, were sodium/potassium phosphate pH 5.0, MES pH 6.2, ammonium acetate pH 7.0 and MOPS pH 7.0 (Fig. 1C and D). Considering only the T_m values, the increase in ionic strength (150 mM and 500 mM NaCl) does not seem in general to have any stabilizing effect on the protein. An exception is the buffer condition 100 mM MES pH 6.2, 500 mM NaCl, which clearly shows an increase of the protein-unfolding transition slope (Fig. 1D).

Considering the melting temperature and the protein-unfolding transient slope of DRbr, it can be concluded that it is strongly stabilized by a 100 mM MES pH 6.2 buffer with addition of 500 mM NaCl (Fig. 2). These conditions stabilize DRbr by 7 °C and present a slope increase of 0.9, when compared with the assay using the purification buffer (100 mM Tris–HCl pH 7.2, 150 mM NaCl).

DRbr was initially identified as being degraded in *C. jejuni* in the presence of hydrogen peroxide. Nevertheless, its mRNA levels under those oxidative stress conditions remained constant [26]. Our first attempt at purifying the protein was performed aerobically, but during protein purification a degradation of the major band at 24 kDa (SDS–PAGE) was observed. The generated smaller fragments were sequenced by Edman degradation and confirmed to be degradation products of DRbr, corresponding to a cleavage of the Dx domain. In order to avoid protein degradation, subsequent purifications were performed under anaerobic conditions [25].

DRbr is composed of three domains, and its theoretical isoelectric point (pI) is around 5.5 for the full-length protein. The pI of each individual domain is 6.1 for Dx, 5.1 for FHB and 7.9 for Rd. The fact that the Dx domain of the protein was cleaved off during aerobic purification may suggest that this domain is more exposed to the solvent and to degradation. Therefore, a buffer pH change from 7.2 to 6.2, which is closer to the pI of the individual Dx domain, may neutralize its charge, thus decreasing the charge repulsion with the other protein domains (Rd and FHB) and consequently stabilize the folded protein conformation. Concomitantly, the increase in salt concentration to 500 mM would help

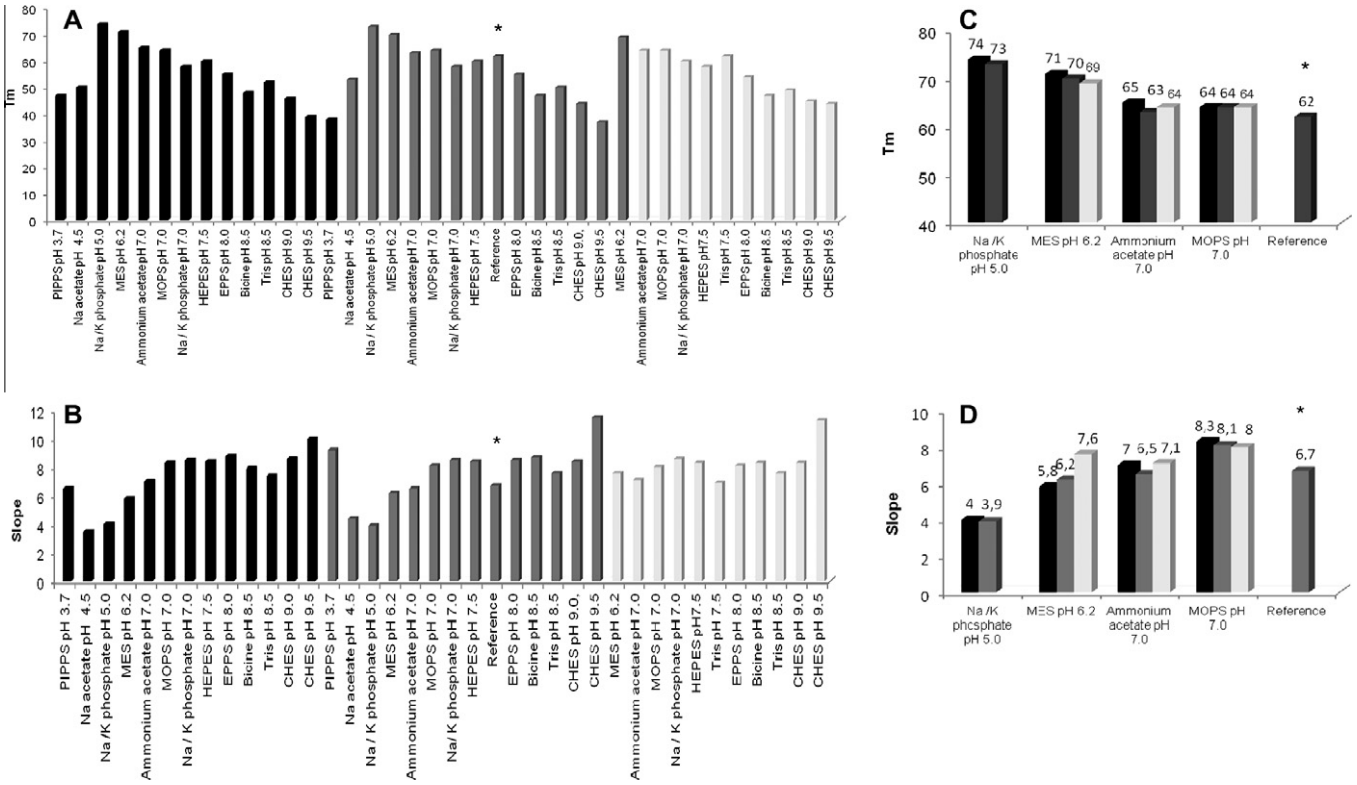

Fig. 1. Midpoint temperatures of the protein-unfolding transition (T_m) (A and C) and its slope (B and D) for *Campylobacter jejuni* DRbr. (A and B) in the presence of the buffers that gave a clear thermal transition, and (C and D) in the presence of the four best buffers: sodium/potassium phosphate pH 5.0, MES pH 6.2, ammonium acetate pH 7.0, MOPS pH 7.0. In this study the concentration of salt (NaCl) was zero (black), 150 mM (gray) and 500 mM (light-gray). The control experiment represented as reference (∗) was prepared with the final purification buffer, 20 mM Tris-HCl pH 7.2, 150 mM NaCl.

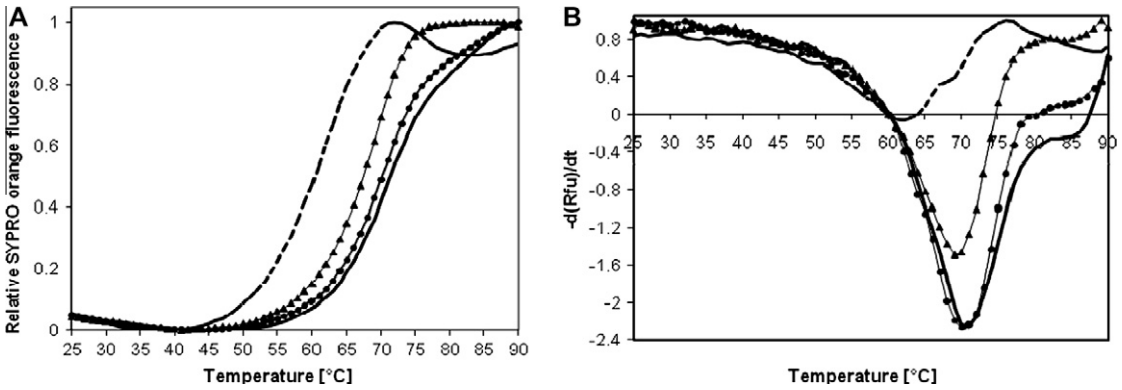

Fig. 2. Curves obtained from the fluorescence data, comparing the best buffer (100 mM MES pH 6.2) in combination with salt concentration, zero (solid line),150 mM (closed circles) and 500 mM (closed triangles) with the control experiment, 100 mM Tris–HCl pH 7.2, 150 mM NaCl (dotted line). (A) Normallized variation in SYPRO Orange fluorescence with temperature; (B) first derivative (d(Rfu)/dT) of the data represented in (A).

maintain the protein in solution, by decreasing the ionic interactions. Following the definition of the best buffer and pH, pure DRbr protein was dialyzed into 100 mM MES pH 6.2, 500 mM NaCl.

In addition, an additive screening (Hampton Research) was carried out in order to identify possible small molecules, e.g., salts, metal ions, cofactors, detergents or reducing agents that might help crystallize the target protein. Although this additive screen has been mainly used for crystallization optimization, it comprises a list of small molecules that can affect protein solubility, stability and crystallization, and therefore it was used to improve protein stability using thermofluor-assay.

The results obtained from the thermofluor assay using the additive screen for DRbr showed that the majority of the conditions (ca. 62%) from a total of 95 conditions tested resulted in a clear unique thermal transition (Fig. 3A and B), for instance the conditions with multivalent ions (e.g., Cd, Ca, Co, Mg, Zn), salt (e.g., KCl, LiCl, NaCl) and organic solvents (e.g., 2-propanol, methanol, acetone). The remaining conditions can be divided into two main categories: two or more fluorescence transitions (ca. 25%), probably due to the thermal transitions of the different DRbr protein domains, in conditions such as carbohydrates (glucose and sucrose), reducing agent (GSH-GSSG), chelating agent (EDTA), sodium thiocyanate, potassium sodium tartrate or sodium citrate; and no measurable transition (13%), such as compounds of the class of detergents (e.g., *n*-octyl-β-D-glucoside, *n*-dodecyl-β-D-maltoside).

The results from the best additives, i.e., those with only a single transition, were compared with the control assay which had a T_m of 69 °C and slope of 6.3 (Fig. 3C and D). The selected additives were divalent metal ions (cadmium, calcium, cobalt, magnesium, zinc and nickel), a linker (glycyl-glycyl-glycine), one polyamine (hexamine cobalt(III) chloride), two chaotrope agents (urea and trimethylamine HCl), a reducing agent (TCEP), one carbohydrate (myo-inositol), one polyol (glycerol), one volatile organic solvent (acetone) and one non-detergent (NDSB-195). Taking into consideration the increase in T_m, the best four additives selected were cadmium, cobalt, zinc and TCEP (Fig. 3C and D).

Combining the results obtained from the two thermofluor screens (buffer and additives), the protein initially purified in 20 mM Tris–HCl pH 7.2, 150 mM NaCl, was dialyzed into the buffer 100 mM MES pH 6.2, 500 mM NaCl. The protein was aerobically concentrated up to 25 mg/ml in a vivaspin 500 (Sartorius stedim) and a crystallization screen was performed using the Classic Screen (Nextal). In addition, the protein was also incubated with cadmium and zinc ions, the best two additives out of the final four mentioned above and which yielded the highest T_m (Fig. 4). Therefore, three different conditions were used for the crystallization screen: position *a*, DRbr dialyzed into the buffer 100 mM MES pH 6.2, 500 mM NaCl; position *b*, DRbr with 10 mM cadmium chloride; position *c*, DRbr with 10 mM zinc chloride. Within less than one hour, small dark red colored needle-shaped crystals were observed in position *a* (DRbr in the optimized buffer and without additives) for crystallization condition A5 (100 mM HEPES pH 7.5, 10% (v/v) isopropanol and 20% (w/v) PEG 4000, Fig. 5). It is interesting to note that the predicted protein pI is 5.5 and the crystallization condition had a pH of 7.5. The difference between pH and pI in this case is +2, which is consistent with the highest likelihood for crystallizing acidic proteins (0–2.5 pH units above their pI) [44].

In order to optimize DRbr protein crystals, a second crystallization screen was performed using the additive screen (Hampton Research). At this stage, protein crystals were observed with different additives: ammonium sulfate, sarcosine, 1,4-dioxane, methanol and TCEP hydrochloride, the latter being the one that gave the most promising crystals. The additives yielding crystals can be analyzed in correlation with the results obtained from the thermofluor assay using the same additive screen (Table 1). Although for three additives the T_m is 68 °C (with a ΔT_m of only ca. 1 °C) and therefore this difference is not significant. In the case of 1,4-dioxane the T_m is lower but shows a higher slope when compared with the control assay. The most promising additive to improve the quality of protein crystals is the reducing agent TCEP which gave an increase of both T_m (+6 °C) and transition slope (+0.4) (Fig. 3). This comparison suggests that the positive hits (higher T_m and slope) from the thermofluor additive screen assay can be selected and further used on the crystallization screening to improve crystal quality. The pre-selection of additives using the thermofluor approach has an advantage concerning the amount of protein required, since thermofluor uses much lesser protein than the required for setting up 96 different crystallization additive conditions.

Further optimization trials of DRbr crystals are under way, using the original crystallization condition with the reducing agent TCEP (Fig. 5 B), which gave the best results in the thermofluor screen (higher T_m and slope) and also the one which gave the most promising crystals. The aim is to obtain crystals of the best possible quality, suitable for X-ray diffraction measurements.

Concluding remarks

A thermofluor approach was used as an optimization screen for the DRbr protein stability and solubility, and this information was subsequently used for optimization crystallization trials. The buffer formulation which greatly stabilized DRbr was 100 mM MES pH 6.2, 500 mM NaCl, where the protein exhibited a +7 °C increase

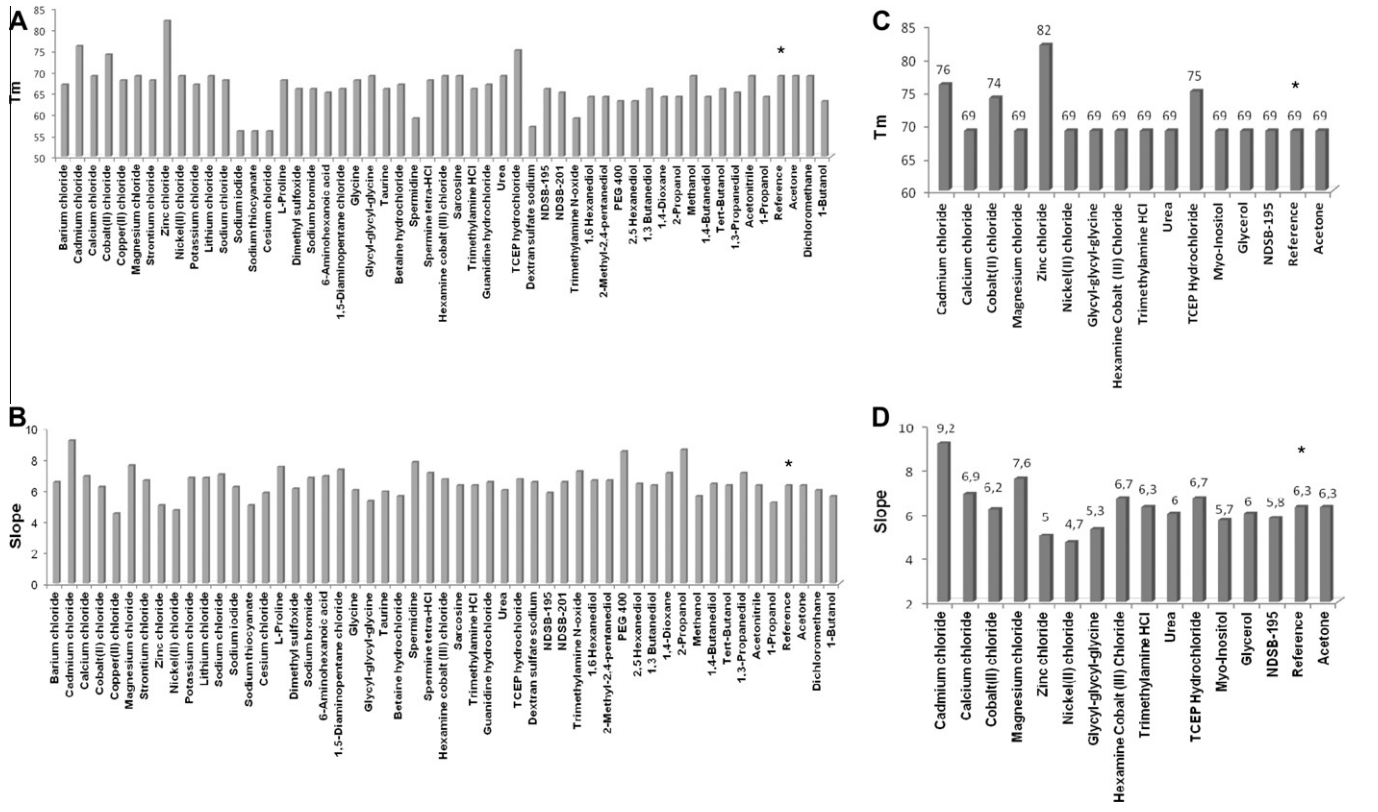

Fig. 3. Midpoint temperatures of the protein-unfolding transition (T_m) (A and C) and its slope (B and D) for *Campylobacter jejuni* DRbr. (A and B) in the presence of the additives that gave a clear thermal transition, and (C and D) in the presence of the best additives selected. The control experiment is represented as reference (∗) and the protein buffer was 100 mM MES pH 6.2, 500 mM NaCl.

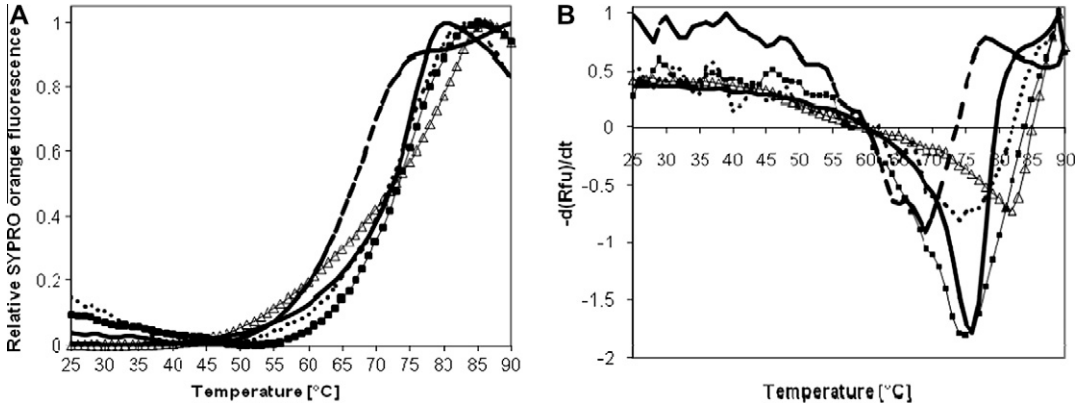

Fig. 4. Curves obtained from the fluorescence data for the control experiment, 100 mM MES pH 6.2, 500 mM NaCl (dashed line) with T_m of 69 °C (reference), and in the same buffer but with addition of the following additives: cadmium (solid line) with T_m of 76 °C, cobalt (dotted line) with T_m of 74 °C, zinc (triangles) with T_m of 82 °C and TCEP (squares) with T_m of 75 °C. (A) Normallized variation in SYPRO Orange fluorescence with temperature; (B) First derivative (d(Rfu)/dT) of the data represented in (A).

in T_m in comparison with the initial storage and purification buffer (20 mM Tris–HCl pH7.2, 150 mM NaCl). Afterwards, DRbr was dialyzed into the selected buffer and used in crystallization screens, where protein crystals appeared in less than one hour. A comparison between the thermofluor assay and the crystallization screen using the Hampton additive screening kit shows a relationship between the formation of protein crystals and an optimized buffer formulation with higher T_m and transition slope values. One of the major advantages of the thermofluor method is the low amount of protein required. Therefore this technique can be used as a pre-selection of the most promising additive conditions that would induce protein stabilization and therefore improve crystal quality.

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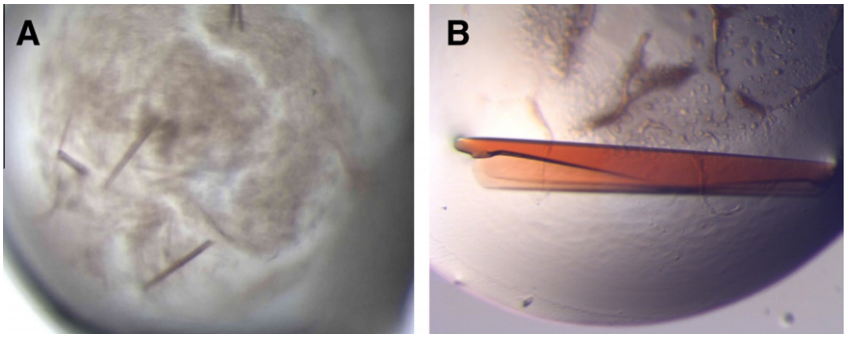

Fig. 5. *Campylobacter jejuni* DRbr crystals. (A) Crystals obtained from the robot plate (drop size 100 nl) using the Classic Lite screen (Nextal). The protein concentration used was 25 mg/ml in the buffer 100 mM MES pH 6.2, 500 mM NaCl. The crystallization condition (A5) was 100 mM HEPES pH 7.5, 5% (v/v) isopropanol, 10% (w/v) PEG 4000. (B) Optimized crystals using vapor diffusion hanging drop technique, the crystallization condition was using 1 μl of DRbr at concentration of 25 mg/ml plus 0.8 μl of the mixture 100 mM HEPES pH 7.5, 14% (w/v) PEG 8000; 10% (v/v) glycerol and 0.2 μl of 100 mM TCEP.

Table 1
Additives that led to the formation of *Campylobacter jejuni* DRbr protein crystals.[a]

	T_m[b]	Slope[b]
100 mM ammonium sulfate (B3)	68	6.1
10 mM sarcosine (D5)	68	6.3
10 mM triphosphate disodium salt (D10)	Two transitions	–
10 mM TCEP hydrochloride (D11)	75	6.7
10 mM GSH-GSSG (D12)	More than 2 transitions	–
3% 1,4-dioxane (G9)	64	7.1
3% methanol (G12)	68	5.6

[a] DRbr crystals were optimized using the initial crystallization condition 100 mM HEPES pH 7.5, 5% (v/v) isopropanol, 10% (w/v) PEG 4000 with different compounds from the additive screen (Hampton Research).
[b] T_m and the transition slope for each condition from the thermofluor assay results.

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