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Conformational changes induced by detergents during the refolding of chemically denatured cysteine protease ppEhCP-B9 from *Entamoeba histolytica*

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ABSTRACT

EhCP-B9, a cysteine protease (CP) involved in *Entamoeba histolytica* virulence, is a potential target for disease diagnosis and drug design. After purification from inclusion bodies produced in *Escherichia coli*, the recombinant EhCP-B9 precursor (ppEhCP-B9) can be refolded using detergents as artificial chaperones. However, the conformational changes that occur during ppEhCP-B9 refolding remain unknown. Here, we comprehensively describe conformational changes of ppEhCP-B9 that are induced by various chemical detergents acting as chaperones, including non-ionic, zwitterionic, cationic and anionic surfactants. We monitored the effect of detergent concentration and incubation time on the secondary and tertiary structures of ppEhCP-B9 using fluorescence and circular dichroism (CD) spectroscopy. In the presence of non-ionic and zwitterionic detergents, ppEhCP-B9 adopted a β-enriched structure (ppEhCP-B9$_{\beta 1}$) without proteolytic activity at all detergent concentrations and incubation times evaluated. ppEhCP-B9 also exhibits a β-rich structure in low concentrations of ionic detergents, but at concentrations above the critical micelle concentration (CMC), the protein acquires an $\alpha + \beta$ structure, similar to that of papain but without proteolytic activity (ppEhCP-B9$_{\alpha + \beta 1}$). Interestingly, only within a narrow range of experimental conditions in which SDS concentrations were below the CMC, ppEhCP-B9 refolded into a β-sheet rich structure (ppEhCP-B9$_{\beta 2}$) that slowly transforms into a different type of $\alpha + \beta$ conformation that exhibited proteolytic activity (ppEhCP-B9$_{\alpha + \beta 2}$) suggesting that enzymatic activity is gained as slow transformation occurs.

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1. Introduction

The cysteine protease (CP) EhCP-B9 is highly involved in the virulence of *Entamoeba histolytica*, a protozoan parasite that causes amoebiasis. Together with EhADH112 adhesin, EhCP-B9 forms a 112 kDa complex (EhCPADH112). EhCP-B9 was first identified using monoclonal antibodies and virulence-deficient mutants [1]. *E. histolytica* trophozoites secrete EhCP-B9, which degrades collagen and fibronectin. EhCP-B9 also binds to and lyses red blood cells and degrades haemoglobin, and virulence-deficient mutant poorly expressed EhCP-B9 [2]. Furthermore, overexpression of the *ehcp-b9* gene restores the pathogenic phenotype of a non-pathogenic *E. histolytica* clone, generating amoebic liver abscesses in both gerbil and mouse models [3].

The 446-amino acid (aa) precursor of the EhCP-B9 proteinase (ppEhCP-B9) contains a signal peptide, a propeptide, and a catalytic domain characterised by a catalytic triad comprising Cys, His, and Asn. As a potential target for drug design, the recombinant EhCP-B9 proenzyme has been expressed as an active enzyme in heterologous systems, albeit with very low yields because of strong autoproteolytic activity and instability [2]. To overcome this problem, ppEhCP-B9 was overexpressed in *Escherichia coli* inclusion bodies, which enabled high-yield purification of the denatured enzyme but required a refolding step. In the laboratory, ppEhCP-B9 requires the presence of sodium dodecyl sulphate (SDS) during the refolding step to become an active enzyme, allowing biochemical characterisation of the mature enzyme [4].

EhCP5, another CP from *E. histolytica*, also refolds and activates in the presence of SDS [5]. However, a variety of detergents have not been screened for refolding, and the conformational changes that occur during the refolding of CPs obtained from parasites have not been systematically studied.

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Here, we present the first comprehensive study of the conformational changes that occur during the detergent-assisted refolding of ppEhCP-B9 as monitored using fluorescence and circular dichroism (CD) spectroscopy. These results help explain the role of a variety of detergents in the refolding of ppEhCP-B9, a CP of biotechnological and biomedical importance.

2. Methodology

2.1. Expression and purification of ppEhCP-B9

Recombinant His-tagged ppEhCP-B9 was produced as inclusion bodies in *E. coli* C43 harbouring the pQE80L-*ppEhcp112* plasmid [4]. The transformed bacteria were grown at 37 °C with shaking at 200 rpm in flasks containing 500 mL LB medium supplemented with 50 μg/mL ampicillin. The bacteria were grown to 0.6 OD_{600}, and protein expression was induced with 1 mM IPTG for 18 h at 25 °C. The bacteria were collected by centrifugation at 20,000 ×*g* for 10 min, and the pellet was resuspended in 20 mM Tris–HCl, pH 8.0, and 30 mM NaCl (1 mL buffer per gramme of wet cells) supplemented with 1 μg/mL lysozyme. The pellet was incubated at 4 °C for 1 h and lysed by repeated freezing and thawing in a dry ice bath. The lysate was centrifuged at 20,000 ×*g* for 40 min at 4 °C. The insoluble protein fraction was washed twice with 5 mL of buffer containing 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2% Triton X-100, and 2 M urea. Inclusion bodies were solubilised with 10 mL of buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 8 M urea) for 1 h at 25 °C and centrifuged at 20,000 ×*g* for 1 h to remove any insoluble material; the recombinant protein was purified on a 5 mL pre-packed Ni-Sepharose High-Performance Resin column (GE Healthcare Science, Uppsala, Sweden). The column was washed with 25 mL of deionised water and equilibrated with 15 mL of buffer A. The His-tagged protein was applied to the Ni-Sepharose column, washed with 15 mL of buffer A, and eluted with 10 mL of buffer A supplemented with 500 mM imidazole. Fractions (1 mL) were collected and analysed using SDS-PAGE. Fractions containing purified protein were pooled and dialysed against buffer A without imidazole. The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 78,240 M^{-1} cm^{-1}.

2.2. Refolding and activation of recombinant ppEhCP-B9

Urea-denatured ppEhCP-B9 was refolded as previously reported [4] by gel filtration in the presence of SDS; however, auto-activation and processing were observed. To properly observe the effect of detergents on the formation of secondary and tertiary structures, urea was removed by gel filtration followed by the addition of detergents as described in the following section.

Buffer exchange was performed by gel filtration using a PD10 Desalting Column (GE Healthcare Science, Buckinghamshire, England) pre-equilibrated with buffer B (10 mM Tris–HCl, pH 8.0, and 50 mM NaCl). Subsequently, 2.5 mL samples of 42 μM denatured ppEhCP-B9 were applied to the column and eluted with 3.5 mL buffer B. All experiments were performed in buffer B, and the protein was diluted to a final concentration of 3 μM in each refolding assay. Under these conditions, ppEhCP-B9 was soluble and stable at 4 °C for up to one month. To determine the effect of detergents on the secondary and tertiary structures of ppEhCP-B9, β-lauryl maltoside (β-LM), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), cetyltrimethylammonium bromide (CTAB), or SDS were added to final millimolar concentrations equivalent to 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 times the experimentally determined critical micelle concentration (CMC), as determined by fluorimetric assay (Chattopadhyay and London [6]). The correspondence between CMC and millimolar concentrations is indicated in Table 1. The protein was either analysed immediately or incubated at 25 °C for 24, 48, or 72 h prior to analysis. To obtain enzymatically active ppEhCP-B9, dithiothreitol (DTT) was added at a final concentration of 10 mM.

Table 1
Detergents used in ppEhCP-B9 refolding.[a]

Detergent	Concentration (mM)[b]	Experimental CMC[c] (mM)
β-LM	0.017, 0.04, 0.08, 0.17, 0.33, 0.67, 1.67	0.167
CHAPS	0.45, 1.13, 2.25, 4.5, 9.0, 18.0, 45.0	4.5
CTAB	0.017, 0.04, 0.08, 0.17, 0.33, 0.67, 1.67	0.167
SDS	0.2, 0.5, 1.0, 2.0, 4.0, 8.0, 20.0	2.0

[a] All assays in this study were conducted at 25 °C in 10 mM Tris HCl, pH 8.0, 50 mM NaCl.
[b] Detergent concentrations (mM) represent 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 fold of CMC.
[c] Experimental CMC was determined using a fluorimetric method as described in [6].

Additionally, ppEhCP-B9 was refolded by dialysis in the presence of 0.5 or 2.0 times the CMC (concentrations at which major conformational changes occur) of each detergent for 24 h, and far UV-CD and fluorescence spectra were obtained at each detergent concentration.

2.3. Fluorescence emission measurements

Steady-state measurements were performed using a Fluoromax-3 spectrofluorometer (Horiba, Japan) with a temperature-controlled cell holder in 1 cm pathlength quartz cuvettes. Emission spectra were recorded between 300 and 400 nm with a fixed excitation wavelength of 290 nm. The experiments were performed at 25 °C. All spectra represent the average of three scans and were corrected by subtraction of the corresponding blank spectrum.

2.4. CD measurements

Far-UV CD experiments were performed on a Jasco-815 spectropolarimeter (Jasco Inc., USA) equipped with a Peltier-type cell holder that permits temperature control. Spectra were recorded between 200 and 250 nm using a 1 mm pathlength quartz cuvette. The Dichroweb programme (http://dichroweb.cryst.bbk.ac.uk/) was used for spectral deconvolution and secondary structure prediction analysis [7–11].

2.5. Substrate gel electrophoresis assay and fluorimetric activity assay

Proteinase activity was determined by substrate-gel electrophoresis using 10% gelatine as a substrate as previously reported [2,5]. Briefly, 50 μL samples were mixed with equal volumes of 2× Laemmli buffer and incubated for 20 min at 37 °C. Then, 15 μL of each sample was loaded into a substrate gel. Following electrophoresis, the gel was incubated for 1 h in 2.5% (v/v) Triton X-100 and then for 24 h in 100 mM sodium acetate, pH 4.5 containing 1% (v/v) Triton X-100 and 20 μM DTT at 37 °C prior to staining with Coomassie Brilliant Blue. Clear bands were indicative of proteolytic activity.

3. Results

Conformational changes of denatured-urea-free ppEhCP-B9 induced by non-ionic (β-LM), zwitterionic (CHAPS), and ionic (SDS, CTAB) detergents as chaperones were examined. We found that the detergents caused major conformational changes at concentrations between 0.5 and 2.0 times the CMC. We then analysed conformational changes of ppEhCP-B9 that occurred after refolding by dialysis in the presence of two detergent concentrations (0.5 and 2.0 CMC) as shown in Sections 3.1, 3.2, and 3.3. The behaviour of ppEhCP-B9 differed

depending on the detergent, the detergent concentration, and the duration of incubation.

3.1. Effect of non-ionic and zwitterionic detergents on the conformation of ppEhCP-B9

ppEhCP-B9 was readily soluble and did not aggregate in the absence of detergents or when β-LM (non-ionic) or CHAPS (zwitterionic) was added. The CD spectra of ppEhCP-B9 in the absence or in the presence of β-LM or CHAPS (Figs. 1 and 2, panels A and C) were symmetric and exhibited minima between 210 and 220 nm. The spectra were slightly broader in the presence of CHAPS than in the presence of β-LM. The CD spectra had the same shape and intensity from 0.1 to 10.0 times the CMC for both detergents. In addition, the CD spectra remained unaltered during the course of the experiment (72 h) (data not shown). Therefore, at all concentrations tested, these detergents exhibited minimal or no effect on the secondary structure of ppEhCP-B9. Under the conditions used in our study, the estimated α-helical and β-sheet contents were approximately 16 and 38%, respectively, in β-LM.

In the presence of 8 M urea, ppEhCP-B9 exhibits a red-shifted fluorescence-emission spectral centre of mass (SCM) of 359 ± 1 nm. In the absence of urea and detergent, ppEhCP-B9 exhibits an SCM of 351 ± 1 nm (Fig. 1B). We observed a slight blue shift of SCM (348 ± 1 nm) and increased fluorescence intensity upon the addition of β-LM, which were independent of detergent concentration.

Under step-dialysis conditions in the presence of β-LM, the far UV-CD spectra of ppEhCP-B9 in 8 M urea (Fig. 1C) were characteristic of denatured proteins, presenting an ellipticity signal near zero between 250 and 225 nm. The CD spectra recorded at 0.5 and 2.0 CMC (β-LM) and 0 M urea were comparable to previously obtained spectra in which the urea was removed by gel filtration before the addition of detergent. In this case, the estimated α-helical and β-sheet contents were approximately 14 and 38%, respectively. In the intrinsic emission fluorescence spectra, an expected blue shift of approximately 11 nm was observed after the removal of urea by dialysis in the presence of detergents, and a blue shift of approximately 8 nm was observed after the removal of urea by gel filtration in the absence of detergents. Interestingly, regardless of the method used, all samples in 0 M urea exhibited the same SCM (348 ± 1 nm), consistent with the CD results. Moreover, on decreasing the urea concentration from 8 to 0 M, the fluorescence intensity decreased by approximately 65 and 52% at 0.5 and 2.0 CMC of β-LM, respectively (Fig. 1D).

In the presence of CHAPS (using the gel filtration method), ppEhCP-B9 exhibited estimated α-helical and β-sheet contents of approximately 10 and 38%, respectively. The Fluorescence SCM was blue shifted from 349 ± 1 nm at 0.1 CMC to 347 ± 1 nm at 10.0 CMC with a concomitant 1.3-fold increase in emission intensity (Fig. 2, panel B).

After removing urea using dialysis, the CD spectrum of ppEhCP-B9 in 0.5 CMC CHAPS exhibited a slight negative peak near 211 nm, indicating that removal of urea altered the helical content of the protein (Fig. 2C). However, major changes were observed in the fluorescence spectra (Fig. 2D), showing that the tertiary structure was modified by urea elimination. In this case, the fluorescence emission intensity decreased 1.8-fold at 0.5 CMC and 1.2-fold at 2.0 CMC. The total blue shift observed upon dialysis was 14 nm (from 363 ± 1 nm in 8 M urea to 349 ± 1 nm in 0 M urea), whereas upon gel filtration, a blue shift of 10 nm was observed. Notably, the SCM obtained in the absence of urea using both methods was the same.

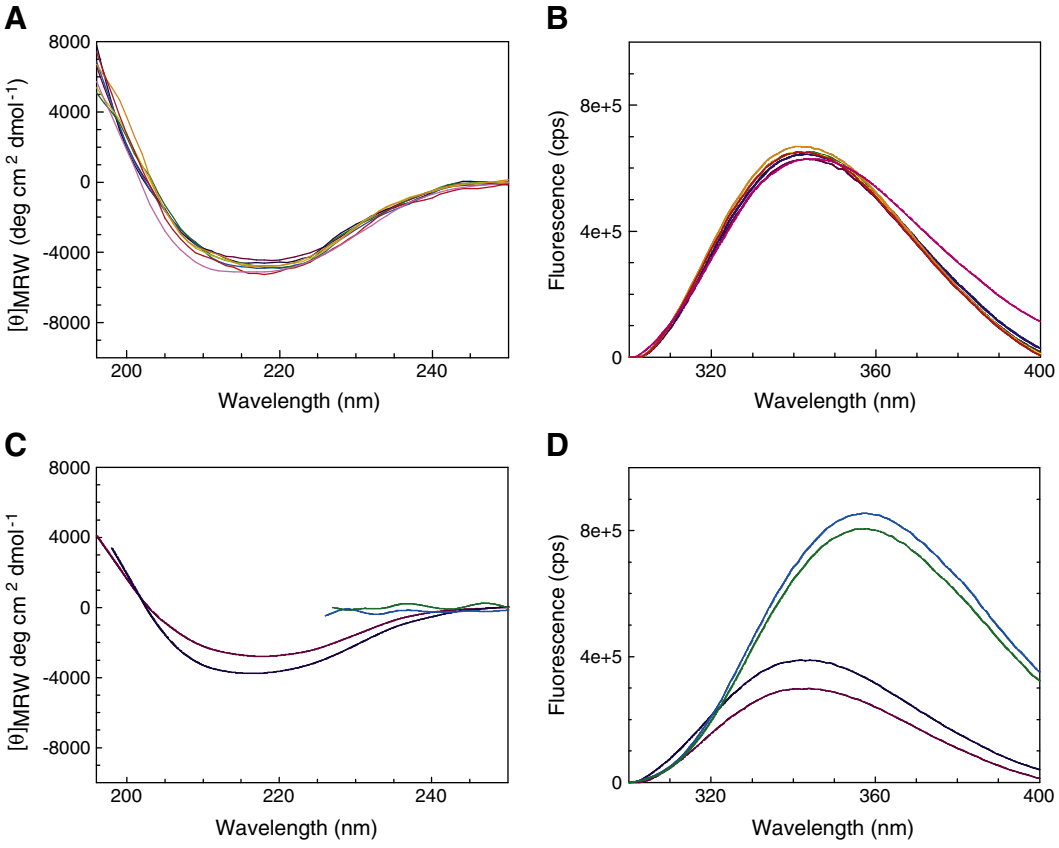

Fig. 1. *Far-UV CD (A, C) and fluorescence emission (B, D) spectra of ppEhCP-B9 in the presence of β-LM detergent.* Panels A and B: Urea was removed from purified ppEhCP-B9 by gel filtration, and the enzyme was diluted 1:10 in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl in the absence (magenta) or presence of detergent at 0.1 (purple), 0.25 (dark blue), 0.5 (blue), 1.0 (green), 2.0 (yellow), 4.0 (orange), or 10.0 (red) times the CMC. Panels C and D: Denatured ppEhCP-B9 in 8 M urea and 0.5 (green) or 2.0 (cyan) times the CMC of β-LM was step dialysed to 0 M urea in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl containing 0.5 (purple) or 2.0 (blue) times the CMC of β-LM. The enzyme concentration used was 3 μM.

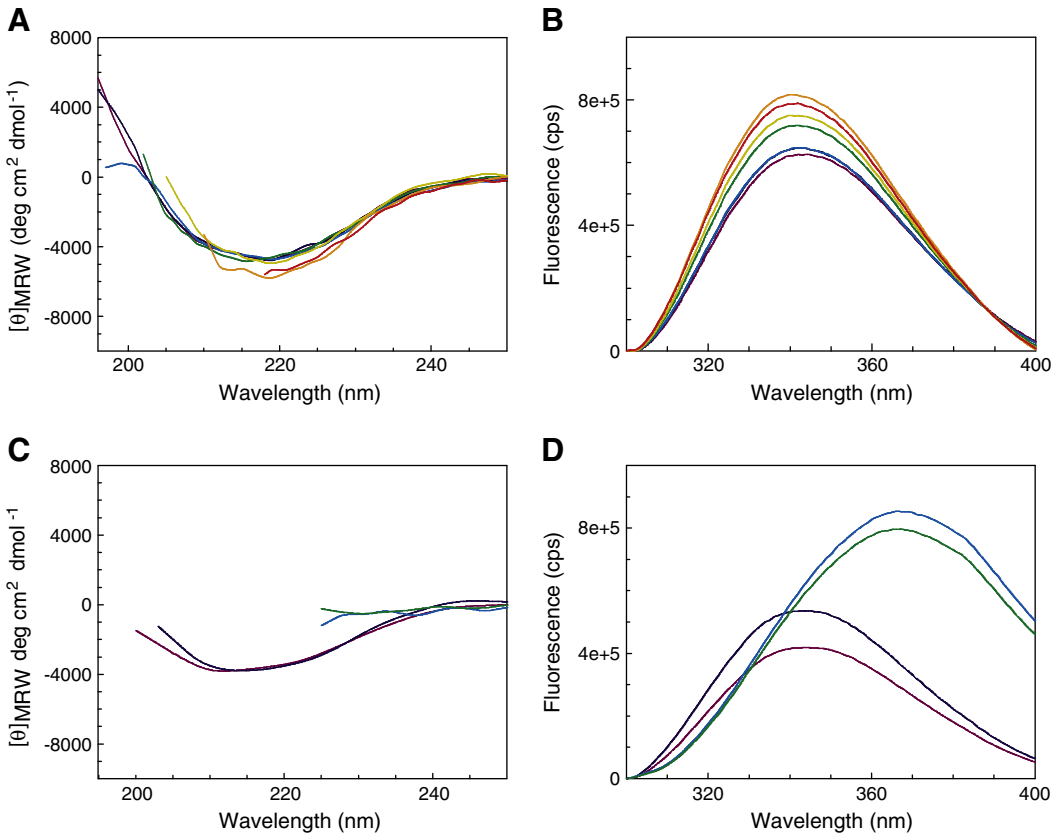

Fig. 2. *Far-UV CD (A, C) and fluorescence emission (B, D) spectra of ppEhCP-B9 in the presence of CHAPS detergent.* Panels A and B: Urea was removed from purified ppEhCP-B9 by gel filtration, and the enzyme was diluted 1:10 in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl in the presence of detergent at 0.1 (purple), 0.25 (dark blue), 0.5 (blue), 1.0 (green), 2.0 (yellow), 4.0 (orange), or 10.0 (red) times the CMC. Panels C and D: Denatured ppEhCP-B9 in 8 M urea and 0.5 (green) or 2.0 (cyan) times the CMC of CHAPS was step dialysed to 0 M urea in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl containing 0.5 (purple) or 2.0 (blue) times the CMC of CHAPS. The enzyme concentration used was 3 μM.

3.2. Effect of the cationic detergent CTAB on the secondary and tertiary structure of ppEhCP-B9

Far-UV CD spectra of ppEhCP-B9 in the presence of the cationic surfactant CTAB are shown in Fig. 3, panel A. At 0.1 CMC CTAB, the spectra resemble those of enriched β-strand proteins, exhibiting approximately 5% α-helical and 42% β-sheet contents. We denoted this structure together with those obtained in non-ionic and zwitterionic detergents as ppEhCP-B9$_{\beta 1}$. At 0.25 and 0.5 of the CMC, precipitation occurred, and no secondary structure could be assigned. Increasing the CTAB concentration relative to the CMC resulted in a small shoulder in the spectrum centred at 208 nm, indicating a possible transition (Fig. 3A) from a conformation enriched in β-sheet structure towards a structure with increased helical content, denoted here as ppEhCP-B9$_{T}$. At CTAB concentrations above the CMC, the CD spectra of ppEhCP-B9 resemble that of papain. We denoted this conformation as ppEhCP-B9$_{\alpha+\beta 1}$ and it exhibited approximately 15% α-helical and 33% β-strand contents. The transition between these structures is visualised in the inset of Fig. 3A, depicting major spectral changes between 0.5 and 2.0 CMC. Intrinsic-emission fluorescence spectra of ppEhCP-B9 at various concentrations of CTAB are shown in Fig. 3B. The intensity of the emission signal increased 1.5-fold as the CTAB concentration was increased, with a maximum emission at 4.0 and 10.0 times the CMC of CTAB. In addition, a red shift was observed from 346 ± 1 nm to 351 ± 1 nm when the surfactant concentration was increased from 0.1 to 10.0 CMC, suggesting that aromatic residues become less quenched as secondary structure is acquired.

During dialysis in the presence of 0.5 CMC CTAB, ppEhCP-B9 precipitated, and no secondary structure was present (based on the CD spectra, Fig. 3C). Therefore, dialysis was performed at 0.1 CMC; however, aggregation was observed, similar to that observed at 0.5 CMC (data not shown). When urea was removed by dialysis at 2.0 CMC CTAB, ppEhCP-B9 acquired a conformation that resembles the conformation of ppEhCP-B9$_{\alpha+\beta 1}$ with slight differences (approximately 12% and 32% α-helical and β-strand contents, respectively). The fluorescence spectra obtained at 0.5 CMC were noisy in 0 M urea due to precipitation. In this case, the emission intensity decreased 6-fold when denaturant was removed, and the spectral line was considerably broadened. The SCM changed from 357 ± 1 nm to 348 ± 1 nm (Fig. 3D). A slight reduction in the fluorescence intensity related to 8 M urea was observed at 0 M urea when dialysis was performed at 2.0 CMC of CTAB, and the SCM shifted from 357 ± 1 nm to 351 ± 1 nm. This final SCM is red shifted from the SCM observed for β-LM and CHAPS. Again, the final SCM was the same (351 ± 1 nm) for both methods.

3.3. Effect of the anionic detergent SDS on the secondary and tertiary structures of ppEhCP-B9

Far-UV CD spectra of ppEhCP-B9 immediately after the addition of SDS at various concentrations are shown in Fig. 4, panel A. The helical content of ppEhCP-B9 increases with increasing SDS concentration. In particular, at concentrations below the CMC, the CD spectra are symmetric and exhibit minima between 210 and 220 nm. Although this spectra are similar to spectra obtained in the presence of non-ionic and zwitterionic detergents (ppEhCP-B9$_{\beta 1}$), this conformation denoted as ppEhCP-B9$_{\beta 2}$ is able to slowly transform (48–72 h) into a native-like conformation (ppEhCP-B9$_{\alpha+\beta 2}$) able to acquire proteolytic activity characterised by a slight increase in helical content.

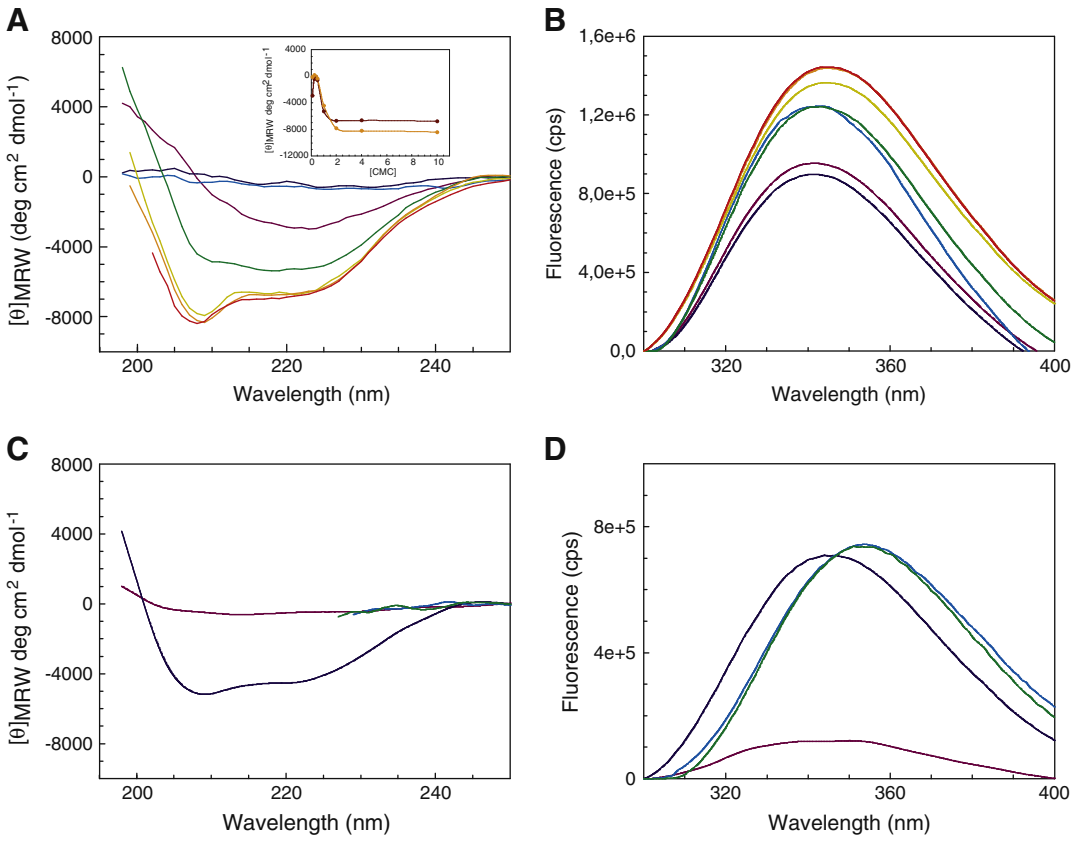

Fig. 3. *Far-UV CD (A, C) and fluorescence emission (B, D) spectra of ppEhCP-B9 in the presence of CTAB detergent.* Panels A and B: Urea was removed from purified ppEhCP-B9 by gel filtration (Panel A and B), and the enzyme was diluted 1:10 in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl in the presence of detergent at 0.1 (purple), 0.25 (dark blue), 0.5 (blue), 1.0 (green), 2.0 (yellow), 4.0 (orange), or 10.0 (red) times the CMC. Panels C and D: Denatured ppEhCP-B9 in 8 M urea and 0.5 (green) or 2.0 (cyan) times the CMC of CTAB was step dialysed to 0 M urea in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl containing 0.5 (purple) or 2.0 (blue) times the CMC of CTAB. The enzyme concentration used was 3 μM.

When the CMC of SDS was reached, we observed the conformation previously denoted as ppEhCP-B9$_{\alpha+\beta1}$. Major changes occurred between 0.1 and 2.0 CMC, and no further changes in the secondary structure were observed at concentrations greater than 2.0 CMC (Fig. 4A, inset), where the secondary structure was approximately 25% α-helix and 50% β-sheet. This conformation was not able to gain proteolytic activity. Intrinsic fluorescence emission spectra of ppEhCP-B9 at various SDS concentrations are presented in Fig. 4B. In contrast to the results obtained in the presence of CTAB, the intensity of the fluorescence decreased by 66% when the SDS concentration was increased. The lowest fluorescence signal was observed at 2.0 CMC SDS, and higher concentrations of SDS did not cause any additional change in the fluorescence emission signal. At all SDS concentrations, the SCM remained the same: 350 ± 1 nm. For the dialysis refolding at 0.5 and 2.0 times the SDS-CMC, the range in which the largest conformational changes occur, the CD spectra resembled the previously described ppEhCP-B9$_{\alpha+\beta1}$ conformation, exhibiting minima at 208 and 222 nm (Fig. 4C). For the fluorescence spectra (Fig. 4D), the intensity was 1.6-fold lower at both CMCs assayed. In the absence of urea, the SCM was 350 ± 1 nm, the same value as that observed when detergent was added. Notably, the fluorescence intensity at 0.5 CMC when urea was removed by dialysis was 60% lower than that obtained when urea was removed by gel filtration.

3.4. Refolding and activation of ppEhCP-B9

The activity acquisition of ppEhCP-B9 was monitored under all detergent conditions using zymograms obtained at 0, 24, 48, and 72 h. In presence of cationic, zwitterionic, or non-ionic detergents, no evidence of proteolytic activity was apparent even after 72 h of incubation (data not shown). In contrast, a proteolytic band appeared at 47 kDa in the presence of SDS at concentrations below 0.5 CMC (Fig. 5A). Under our experimental conditions, the activity gain was a slow process. A 47 kDa band exhibited activity in the substrate gels after 48 h of incubation in the presence of SDS (Fig. 5B). This proteolytic activity was clearly observed after 72 h of incubation. Therefore, we measured the CD and fluorescence spectra of ppEhCP-B9 obtained at various incubation times at 0.25 CMC (up to 72 h) under refolding/activation conditions (Fig. 5, panels C and D). As described previously, the CD spectra recorded immediately following the addition of SDS were symmetric (ppEhCP-B9$_{\beta2}$) but after 24 and 72 h of incubation, the CD spectra exhibited minima at 222 nm and a shoulder at approximately 208 nm, this change corresponded to estimated α-helical and β-sheet contents of 9 and 37% respectively. For fluorescence emission, we observed no change in the SCM (355 ± 1 nm) at 0, 24, and 72 h of incubation. However, at 72 h, the fluorescence intensity emission signal was 40% lower. In contrast, neither the CD spectra nor the fluorescence emission intensity of ppEhCP-B9 were dependent on β-LM, CHAPS, or CTAB incubation time.

4. Discussion

CPs in *E. histolytica* are good candidates for diagnostics and drug design targets. CPs from this parasite are classified in three main clades: A, B and C. The CPs of clade B differ from the CPs of clade A in the length of their pro-regions and catalytic domains. EhCP-B sequences contain hydrophobic regions that might constitute transmembrane helices for GPI-attachment moieties near or at the C-terminus. In contrast, no EhCP-A sequences possess these hydrophobic regions [12].

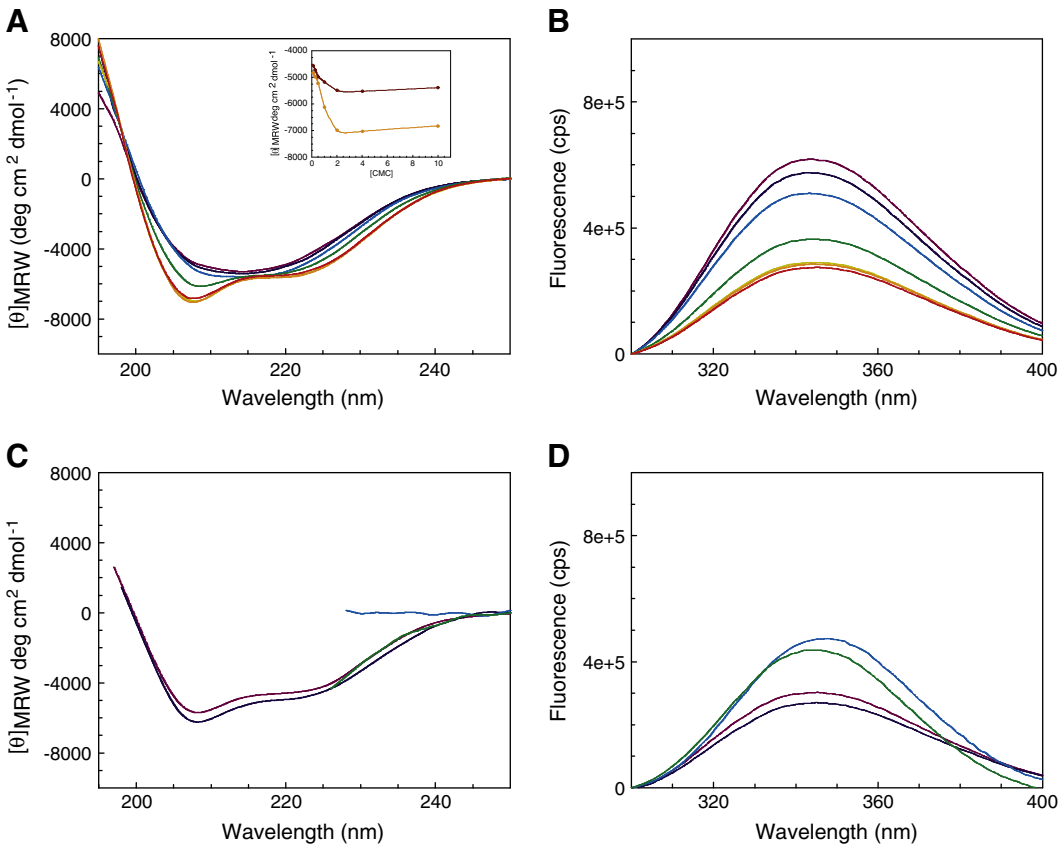

Fig. 4. *Far-UV CD (A, C) and fluorescence emission (B, D) spectra of ppEhCP-B9 in the presence of SDS detergent.* Panels A and B: Urea was removed from purified ppEhCP-B9 by gel filtration (Panel A and B), and the enzyme was diluted 1:10 in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl in the absence (magenta) or presence of detergent at 0.1 (purple), 0.25 (dark blue), 0.5 (blue), 1.0 (green), 2.0 (yellow), 4.0 (orange), or 10.0 (red) times the CMC. Panels C and D: Denatured ppEhCP-B9 in 8 M urea and 0.5 (green) or 2.0 (cyan) times the CMC of SDS was step dialysed to 0 M urea in 10 mM Tris HCl, pH 8.0, and 50 mM NaCl containing 0.5 (purple) or 2.0 (blue) times the CMC of SDS. The enzyme concentration used was 3 μM.

EhCP-B9, a clade B CP involved in the virulence of *E. histolytica*, occurs as a complex with an adhesin associated with the parasite membrane. It is possible that surfactants might improve the refolding of this protein. Therefore, four chemical detergents, including a non-ionic, a zwitterionic, a cationic, and an anionic detergent, were tested as chaperones. Screening strategies using various surfactants have been used to study the refolding of lysozyme, rhodanese, cytochrome c, citrate synthase, and carbonic anhydrase [13–17]. For example, SDS and CTAB improve the refolding yield of carbonic anhydrase, whereas the non-ionic detergent POE(10)L is ineffective. Moreover, POE(10)L acts as a successful artificial chaperone for the refolding of citrate synthase [15,17].

We detected at least five main conformational states of ppEhCP-B9. One state exhibited high β-sheet content (ppEhCP-B9$_{\beta 1}$), another state resembled papain CD spectra (ppEhCP-B9$_{\alpha+\beta 1}$), a third state represented a transitional state between the first two species (ppEhCP-B9$_T$) neither of these conformations were able to gain proteolytic activity. A β-enriched fourth state (ppEhCP-B9$_{\beta 2}$) does not exhibit immediate proteolytic activity however is prone to activation, at long incubation times, by slowly gaining helical content and being transformed into ppEhCP-B9$_{\alpha+\beta 2}$ structure. These conformations are described in detail in Sections 4.1 and 4.2.

4.1. ppEhCP-B9 exists in a β-sheet-rich conformation

The non-active conformation was detected in the absence of detergent, at all concentrations of β-LM and CHAPS and at CTAB concentrations of 0.1 CMC. This conformation is characterised by symmetrical CD spectra exhibiting minima near 215 nm, which may correspond to approximately 16% α-helical and 38% β-sheet contents.

Although ppEhCP-B9 exhibits similar characteristics between these detergents at the secondary structure level, it may have different tertiary contacts as determined based on differences in the fluorescence emission spectra. Independent of detergent concentration, β-LM and CHAPS did not significantly alter the fluorescence emission spectra of ppEhCP-B9, with a 3 nm blue shift compared to the spectrum obtained without detergent. This suggests that β-LM and CHAPS did not affect the tertiary structure of ppEhCP-B9. These observations were confirmed by the results of the dialysis refolding experiment. CTAB and SDS, even at low concentrations, had effects opposite to those of non-ionic detergents, and the SCM of ppEhCP-B9 was shifted to higher wavelengths by approximately 2 nm (relative to non ionic detergents). This shift may be related to increased tryptophan exposure because both detergents are well-known denaturants. In the presence of CTAB, the fluorescence emission intensity of ppEhCP-B9 increased as the concentration of CTAB added to the protein solution was increased. This increase might be explained by quenching because ppEhCP-B9 possesses seven tryptophans, five of which are very close to each other in a three-dimensional model built using cathepsin L as the scaffold. Therefore, the relaxation of electrons may be channelled by non-radiative events, such as energy transfer to neighbouring atoms. As the detergent concentration is increased, ppEhCP-B9 acquires a loosely packed structure, suggesting less tryptophan quenching with a corresponding gain in fluorescence emission. In contrast, the intensity of the ppEhCP-B9 fluorescence spectrum decreased and was red shifted in the presence of SDS. The conformation acquired in the presence of SDS might enhance the solvent-exposure of tryptophans in ppEhCP-B9. Subsequently, the intensity is reduced because energy is lost in a non-radiating manner. This conformation exhibits increases in secondary

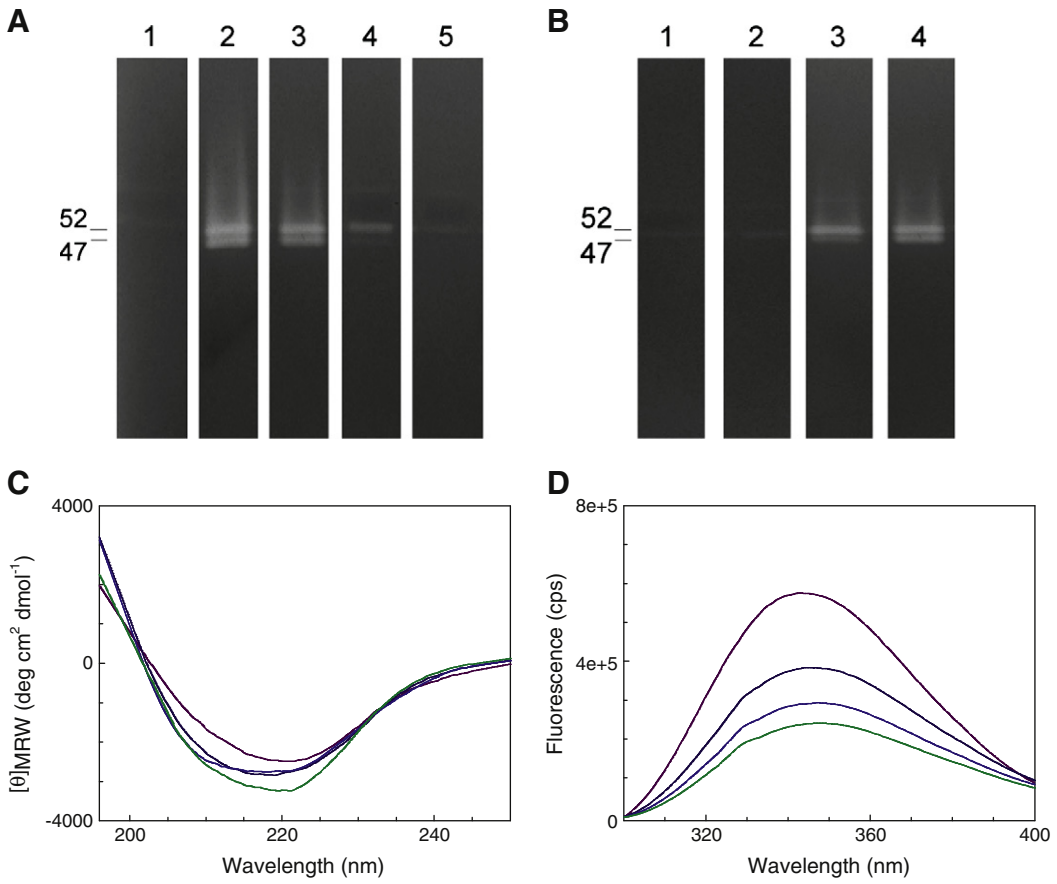

Fig. 5. *SDS-assisted activation of ppEhCP-B9.* (A) A zymogram of purified and refolded protein in the absence (lane 1) or presence of 0.1 (lane 2), 0.25 (lane 3), 0.5 (lane 4), and 2.0 (lane 5) times the CMC of SDS. The samples were incubated for 72 h at 25 °C under refolding conditions. (B) Zymogram of purified and refolded protein in 0.25 times CMC of SDS at 0 h (lane 1), 24 h (lane 2), 48 h (lane 3) and 72 h (lane 4). (C) CD spectra and (D) fluorescence emission spectra of ppEhCP-B9 incubated in 0.25 CMC SDS for 0 h (purple), 24 h (dark blue), 48 h (blue) and 72 h (green). The protein concentration used was 3 μM in all cases.

structure and proteolytic activity as a function of incubation time (up to 72 h).

4.2. $\alpha + \beta$ conformation of ppEhCP-B9

ppEhCP-B9 can adopt a conformation characterised by a CD spectrum exhibiting two minima, of which the signal at 208 nm is more intense than that at 222 nm, resembling those of papain and cathepsins ($\alpha + \beta$ proteins). In this case, ppEhCP-B9 is estimated to have approximately 15–25% α-helical and 33–50% β-strand contents. This conformation was observed in the presence of CTAB and SDS at concentrations higher than the CMC, suggesting that ppEhCP-B9 interacts with micelles of both detergents.

In the presence of SDS, two types of behaviour were observed for ppEhCP-B9. At SDS concentrations equal to or greater than the CMC, an $\alpha + \beta$ secondary structure was rapidly obtained; however, this conformation (ppEhCP-B9$_{\alpha+\beta1}$) did not exhibit proteolytic activity, regardless of the incubation time. The $\alpha + \beta1$ conformation was also achieved by dialysis at 0.5 or 2.0 CMC. In contrast, when SDS was used below 0.5 CMC, the ppEhCP-B9 acquired a different $\alpha + \beta$ secondary structure after 24 h incubation with the detergent. The ppEhCP-B9$_{\alpha+\beta2}$ structure showed minima at 222 nm and a shoulder at approximately 208 nm. These changes in CD spectra were concomitant with an increase in activity after 48 h or 72 h of interaction with SDS under our experimental conditions.

These results are consistent with previous reports describing the folding reaction in the presence of detergents. In most cases at surfactant concentrations below the CMC, conformations enriched in β-sheet content are common, whereas α-helical structure is promoted above the CMC [18–20]. It is commonly observed that detergent-protein complexes exhibit spectra characteristic of α-helix enrichment, even when the native protein exhibits low helical content [17].

CTAB and SDS caused a red shift in fluorescence emission spectra but induced opposite effects on the intensity of fluorescence signal. While CTAB leaded to an increase in emission, SDS originated a decreased in the spectroscopic observable. This finding indicates that the detergent-ppEhCP-B9 complexes are similar in each case regarding their secondary structure but differs in their tertiary interactions. This difference might occur because the negatively charged head of SDS primarily interacts with positive side chains that are exposed to the solvent, whereas the positive moiety in CTAB interacts primarily with negative charges on the protein-exposed surface.

4.3. Activation of ppEhCP-B9

Because ppEhCP-B9 occurs in a complex with adhesin, a long-chain detergent (such as β-LM) might mimic the cell membrane and facilitate protein refolding and activation; however, this was not observed. Instead, SDS was the only surfactant that was effective at promoting the refolding and activation of ppEhCP-B9 within a defined concentration range. Moreover, it is remarkable that the enzymatic activation of CP EhCP-A5 (a member of clade A) was three-fold more effective with SDS than with Triton X-100 or octylglycoside [5]. Furthermore, Kumari and Jagannadham [21] demonstrated that with 2 mM SDS, the CP heynein adopts a SDS-induced molten globule structure with a considerable gain in α-helix content, albeit exhibiting a lack of tertiary structure and a loss of activity. Therefore, it would be interesting to determine whether SDS can be used for the

refolding of CPs from *E. histolytica*, despite the differences in structure between clades.

Low concentrations of SDS might lead to a native structure, although this process is slow, as evidenced by the long incubation times required to obtain active enzyme. The typical refolding incubation times for other CPs are 16, 24, or even 72 h [22–26]. Activation with low levels of ionic SDS and tetradecyltrimethylammonium bromide (TTAB) has been reported for *Thermomyces lanuginosus* lipase (TlL), whereas at higher concentrations of detergent, inhibition occurred [27]. *E. coli* β-galactosidase was also activated at SDS concentrations between 35 and 70 mM; however, an interaction between SDS and β-galactosidase induces changes in the tertiary structure, disrupting the tetrameric structure while conserving activity [28].

Our results suggest that the unfolded polypeptide ppEhCP-B9$_U$ can follow at least three possible refolding routes, depending on the experimental conditions. One route leads to a stable, β-rich conformation (ppEhCP-B9$_{\beta1}$), which cannot acquire either a native-like secondary structure or proteolytic activity, as observed in the presence of any concentration of non-ionic β-LM or zwitterionic CHAPS. Another route leads ppEhCP-B9 to rapidly adopt a papain-like secondary structure ppEhCP-B9$_{\alpha+\beta1}$, which occurs at concentrations above 0.5 CMC SDS and above the CMC of CTAB. At these detergent concentrations, the conformation of ppEhCP-B9$_{\alpha+\beta1}$ is very stable but cannot be activated, most likely due to a large kinetic barrier. The third route, the only route leading to a gain of activity, involves an on-pathway (ppEhCP-B9$_{\beta2}$), β-sheet-enriched intermediate that slowly (due to a high kinetic barrier) adopts a different type of α + β secondary structure ppEhCP-B9$_{\alpha+\beta2}$ that occurs at SDS concentrations below 0.5 CMC.

5. Conclusions

The effects of four detergents on the conformation of urea-denatured ppEhCP-B9 were studied using CD and fluorescence spectroscopy; of these detergents, only SDS was able to refold the protein to an active conformation. These results suggest that ppEhCP-B9 may adopt a native structure and undergo further activation in the absence of urea in a narrow range of SDS concentrations (below 0.5 CMC) and extended incubation times up to 72 h. This study sheds light on the role of SDS in the refolding of ppEhCP-B9, a CP from *E. histolytica*, which is of biomedical and biotechnological interest.

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