Protein loop compaction and the origin of the effect of arginine and glutamic acid mixtures on solubility, stability and transient oligomerization of proteins

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Abstract

Addition of a 50 mM mixture of L-arginine and L-glutamic acid (RE) is extensively used to improve protein solubility and stability although the origin of the effect is not well understood. We present Small Angle X-ray Scattering (SAXS) and Nuclear Magnetic Resonance (NMR) results showing that RE induces protein
compaction by collapsing of flexible loops on the protein core. This is suggested to be a general mechanism preventing aggregation and improving stability to proteases and to originate from the polyelectrolyte nature of RE.

Molecular polyelectrolyte mixtures are expected to display long range correlation effects according to dressed interaction site theory. We hypothesize that perturbation of the RE solution by dissolved proteins is proportional to the volume occupied by the protein. As a consequence, loop collapse, minimizing the effective protein volume, is favored in the presence of RE.

**Keywords:** Protein compaction, crowding, molecular polyelectrolytes, loop dynamics, protein stability, co-solutes

**Abbreviations:**
- FKB12: FK506 binding protein
- lmwPTP: Bovine low molecular weight protein tyrosine phosphatase
- MBP: Maltose Binding Protein
- PIC: Polyelectrolyte Induced Compaction
- RE: An equimolar mixture of L-arginine and L-glutamic acid
- \( R_g \): Radius of gyration
- SAXS: Small Angle X-ray scattering
Introduction

Weak protein-protein interactions can be modulated by the presence of co-solutes. Non-specific interactions leading to aggregation of partially unfolded proteins is a physiological relevant process that is minimized by the action of chaperones and possibly by increasing the concentration of small molecular weight co-solutes under stress conditions. Protein aggregation is a common cause of failure of structural biology projects either using NMR or X-ray. The addition of an equimolar mixture of arginine and glutamic acid (RE) was reported by Golovanov et al. (2004) to prevent aggregation and to improve the solubility of a large number of proteins while preserving biologically relevant macromolecular interactions. The same publication reported an increased resistance to degradation by proteases in the presence of 50 mM RE. A large scale study by Vedadi et al (2006) of the influence of added co-solutes on structural proteomics projects showed that RE and n-dodecyl-β-D-maltoside were the two most universal co-solutes in stabilizing a wide variety of different proteins by more than 4°C. Our group have studied the structural effects of RE mixture with bovine low molecular weight protein tyrosine phosphatase (ImwPTP), a protein that forms dimers and higher oligomers in solution. Using $^{129}$Xe-NMR we showed that RE strongly suppresses non-specific interactions of Xe atoms with ImwPTP (Blobel et al 2007). In the same work it was shown that RE increases the population of ImwPTP dimers. Thus, while RE prevents non-specific aggregation of a very diverse set of proteins, it preserves and even increases the affinity of specific
interactions. The origin of these apparently conflicting effects of RE is not well understood.

Here we have studied the effect of RE on three different proteins by Small Angle X-ray Scattering (SAXS) and NMR. Bovine ImwPTP, had been previously studied in our laboratories by a number of different techniques (Åkerud et al. 2002, Bernadó et al. 2003; Blobel et al. 2007; Blobel et al. 2009). Dimerization is a conserved feature of ImwPTP from bacteria to mammals (Blobel et al. 2009b). Dimerization involves loops which are highly mobile in the monomer (Åkerud et al. 2002). Applying the Multivariate Curve Resolution (MCR) approach to SAXS data, as recently described (Blobel et al. 2009), we show that RE causes a selective reduction of the radius of gyration of ImwPTP dimers determined by SAXS. NMR data on the same system map RE induced perturbations to the dimerization loop and the N- and C- terminal extremes of ImwPTP. FK-506 binding protein (FKBP12) is a monomeric, highly stable protein that has a loop, comprising residues Ala81-Thr96, with restricted conformational dynamics. This loop is part of the binding surface for immunosuppressant and peptide substrates (Brath et al. 2006, Brath & Akke 2009). While SAXS data do not detect RE induced changes in FKBP12, NMR chemical shift and relaxation time measurements show that the effect of RE is concentrated in the loop regions of FKBP12. Chymotrypsinogen A contains large loop regions that are cleaved by proteases to generate different forms of chymotrypsin. SAXS results show a clear compaction of chymotrypsinogen and ImwPTP dimer caused by the addition of RE. NMR results identify in ImwPTP and FKBP12 that RE induces perturbations
mainly localized in the loops. RE also induces an increase in the denaturation
temperature of chymotrypsinogen but not of maltose binding protein which does
not have exposed loops.

Together, these results are consistent with a model explaining the RE-
induced promiscuous solubility and stability enhancement by the compaction of
loops and other poorly structured regions onto the surface of the folded protein
core. This compaction effect would efficiently reduce aggregation involving
unstructured regions and would also explain the RE-enhanced stability to
proteases, which preferentially hydrolyze locally unfolded regions. We propose
that RE, and other polyelectrolytes, induce protein compaction to minimize the
perturbation by the protein of long-range electrostatic interactions between
molecular polyelectrolyte co-solutes.

MATERIALS AND METHODS

RE buffer preparation

A stock solution of 1 M RE was prepared by dissolving a mixture of pure L-
arginine (Sigma) and L-glutamic acid (Sigma) in water. The solubility of the
mixture is higher than that of L-glutamic acid alone. The stock solution (pH 6.5)
was used to prepare samples in different buffers.

Sample preparation.
Chymotrypsinogen A ($M_r = 20.4$ kDa) was bought from Sigma. Recombinant human FKBP12 ($M_r = 11.8$ kDa) and lmwPTP ($M_r = 18.1$ kDa) were expressed and purified as explained elsewhere (Standaert et al. 1990; Wo et al. 1992). From the same batches of chymotrypsinogen A and FKBP12, pairs of samples, one with the maximum RE concentration and the other without RE, were prepared in 25 mM phosphate (pH = 6.5) and 1mM TCEP•HCl. Samples with intermediate RE concentrations were obtained by mixing of the two standard samples. The lmwPTP buffer consisted of 200 mM potassium phosphate, 3 mM sodium azide and 10 mM TCEP•HCl at a pH = 6.0. In all cases, total protein concentrations were determined by UV absorption.

Small Angle X-ray scattering.

Synchrotron radiation X-ray scattering data was collected following standard procedures on the X33 beamline at the European Molecular Biology Laboratory (EMBL) on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) (Roessle et al. 2007). Scattering curves were recorded on a MAR345 image plate detector. Chymotrypsinogen A was measured at a concentration of 0.36 mM (7.3 mg/ml) and FKBP12 at 0.76 mM (9 mg/ml) at a temperature of 20°C. Both proteins were measured in the presence of 0, 50, 100 and 200 mM RE. lmwPTP was measured in zero and 50 mM RE at ten different protein concentrations (1.00, 0.80, 0.60, 0.55, 0.48, 0.41, 0.34, 0.25, 0.17 and 0.056 mM; 18 to 1.01 mg/ml) at 37°C. lmwPTP samples were prepared in both non-RE and RE buffer by successive dilution of a 1 mM lmwPTP sample with the corresponding buffer. Measurements up to 0.60 mM lmwPTP in non-RE buffer
were already reported in an earlier publication of our group (Blobel et al. 2009). Scattering curves of the buffer were collected before and after each acquisition of a protein sample to avoid systematic error. The scattering curves covered a momentum transfer range of $0.0956 < s < 5.0455 \text{ nm}^{-1}$. The scattering due to buffer was subtracted by averaging the buffer measurements enclosing the actual protein measurement. All data manipulations were performed with the program PRIMUS (Konarev et al. 2003).

The forward scattering $I(0)$ and the radius of gyration $R_g$ were evaluated with the Guinier approximation assuming that, at very small angles ($s < 1.3/R_g$), the intensity is represented as $I(s) = I(0) \exp(-sR_g^2/3)$ (Guinier 1939). The actual concentration of the lowest lmwPTP concentration sample (0.056 mM) was determined from its $I(0)$ value by extrapolation of the $I(0)$ obtained at 0.17 mM lmwPTP.

**Multivariate Curve Resolution Alternating Least Squares (MCR-ALS) analysis of the SAXS curves.**

The SAXS curves of 0.17, 0.25, 0.34 and 0.41 mM lmwPTP ranging from $s = 0.185 – 1.666 \text{ nm}^{-1}$, consisting of 659 points, were jointly analyzed by MCR-ALS as described elsewhere (Blobel et al. 2009). The maximum $s$-value was chosen to ensure that only positive intensities for all scattering curves were present up to 1.666 nm$^{-1}$. The large divergences in the intensities up to 0.0185 Å$^{-1}$ of the lowest used concentration curve (0.17 mM) restricted the analysis to lower angle measures. The error due to the concentration matrix, resulting from its deviation
from the dissociation constant, was scaled in respect to the previous publication
to make up 5% of the total error, giving a scaling factor $\varepsilon = 500$.

**NMR measurements.**

$^{15}$N HSQC spectra at different RE concentrations were measured using 500 MHz
Varian or Bruker spectrometers. HSQC spectra of 1.0 mM FKBP12 were
recorded at 20°C in 25 mM phosphate and 1mM TCEP•HCl at a pH = 6.5 in the
presence of 0, 50, 100, 200, 300 or 400 mM RE. HSQC spectra of 0.1 mM
ImwPTP were measured at 37°C in 100 mM potassium phosphate, 1 mM sodium
azide and 1 mM TCEP•HCl at a pH = 6.0 with 0, 50, 100, 200, 400 or 600 mM
RE. $^{15}$N relaxation measurements of 0.94 and 1.4 mM FKBP12 were
accumulated on a 600 MHz Varian spectrometer. Spectra were processed with
NMRPipe (Delaglio et al. 1995). Further data processing was done using in
house written programs running in Matlab®.

$<R_2/R_1>$ in the presence of 50 mM RE were scaled using the core residues not
affected by chemical exchange to correct for the different viscosity of the two
solvents. The scaling factor (1.077) would correspond to a ratio of viscosities of
1.038, in good agreement with the measured value of 1.041 (Blobel et al. 2007).

**Denaturation measurements.**

Denaturation experiments were performed in 96-well plates on a FluoDia T70
fluorimeter by following the increase of fluorescence of SYPRO Orange (Sigma
Aldrich) in the presence of 10 µM protein in 25 mM phosphate buffer containing
150 mM NaCl at pH 6.5 for chymotrypsinogen A and pH 7.5 for maltose binding
protein. The melting temperature was calculated by fitting a two state model to the unfolding curve using an in-house program written in Matlab\textsuperscript{©}.

**RESULTS**

*lmwPTP dimer compaction and stabilization*

The dimerization and further oligomerization of lmwPTP was studied in the presence of 50 mM RE and in RE-free buffer by measuring SAXS curves at ten different lmwPTP concentrations. Figure 1 shows the apparent radius of gyration ($R_g$) and maximum dimensions ($D_{max}$) of the particles present in solution as a function of the total protein concentration.

**Insert Figure 1 here**

The measured values are intermediate between those expected on the basis of the pure monomer and pure dimer X-ray structures (Zhang et al. 1994, Tabernero et al. 1999), consistent with an oligomerization equilibrium described with earlier NMR measurements (Åkerud et al. 2002, Bernado et al. 2003, Blobel et al. 2007). We have previously demonstrated that the presence of RE increased the stability and therefore the population of lmwPTP dimer in the equilibrium mixture (Blobel et al. 2007). In apparent contrast with these observations, samples in RE buffer consistently show smaller apparent $R_g$
values. The difference is greater at intermediate concentrations where the proportion of dimer is the highest. The apparent $D_{max}$ values increases with the total protein concentration up to a plateau value at 0.17 and 0.34 mM in the presence and in the absence of RE, respectively. In RE-free buffer the plateau value of $D_{max}$ value agrees with the dimensions of the dimer observed in crystals. In contrast, $D_{max}$ is approximately 5 Å smaller in the presence than in the absence of RE. At higher concentrations $D_{max}$ become larger than expected for a dimer indicating the presence of higher oligomers.

Pure SAXS curves were extracted for each of the two main components in the mixture by simultaneous analysis of all curves in the concentration range from 0.17 to 0.41 mM using the MCR-ALS algorithm (Blobel et al. 2009). At higher concentrations at least one more component contributes significantly to the experimental curves preventing further analysis. Figure 2 shows the extracted pure curves for the monomer and dimer of ImwPTP in the presence of RE when fitted to the crystallographic models of both species with CRYSOL (Svergun et al. 1995).

The extracted curve for the monomer is in good agreement with the one expected from the crystal structure ($\chi^2 = 2.4; R_g \text{ crystal} = 16.45 \text{ Å}, R_g \text{ exp} = 15.91 \text{ Å}$) but the curve for the dimer shows a lower agreement ($\chi^2 = 3.4$). The $R_g$ value obtained from the extracted curve is 21.1 Å which is smaller than the one
predicted from the crystal structure ($R_g$ crystal = 23.3 Å). As a reference, the $R_g$
values of the individual species extracted from the data measured in the absence
of RE were 16.4 Å and 23.9 Å for the monomer and dimer respectively (Blobel et
al. 2009). Thus, both $R_g$ values extracted from the pure curves and the evolution
of $D_{max}$ values suggest that RE is causing a compaction of the lmwPTP dimer.
The compaction is also manifested in the poor agreement of the crystallographic
structure of the dimer to the MCR-ALS derived SAXS curve ($\chi^2 = 3.4$, see above)
that indicates that RE perturbs its structure. The MCR-ALS method provides
estimates of the dimer dissociation constant of 0.79 ± 0.06 mM in the presence of
RE and 1.62 ± 0.12 mM in RE free buffer. These results confirm the stabilization
of lmwPTP dimers by RE observed with different techniques (Blobel et al. 2007).
The MCR-ALS analysis of the concentration dependent SAXS dataset has the
capacity to deconvolute the thermodynamic changes and the compaction effects
exerted by RE.

ImwPTP NMR studies map the sites perturbed by RE

In order to further characterize the structural effects caused by the addition of RE
at a residue level, we measured changes in the NMR chemical shifts of 100 μM
ImwPTP as a function of RE concentration in the range of 0-600 mM. At this
protein concentration the population of dimer is negligible. Chemical shift
differences between 0 and 50 mM RE along the protein sequence are shown in
Figure 3. The solvent accessibility of each residue and the location of secondary
structure elements are also shown. The most perturbed regions correspond to loops. The large loops ranging from residues 46 to 55 and from residues 120 to 135 that form the dimerization interface of ImwPTP show large perturbations. The most perturbed residues include charged, polar uncharged as well as hydrophobic groups. Although the perturbed residues in general are solvent exposed there are other residues with higher solvent accessibility that are unaffected.

**Insert Figure 3 here**

**FKBP12 NMR studies also map RE perturbations to loops**

In order to check if the effect of RE on loop residues is a general feature, we studied the effect of different RE concentrations on the $^1$H chemical shift and $^{15}$N relaxation rates of FKBP12.

Figure 4 shows the $^1$H chemical shift changes of FKBP12 induced by the addition of 50 mM of RE, the solvent accessibility of the different residues and the location of secondary structure elements. The mobility of different regions of FKBP12 in the presence and absence of 50 mM RE was characterized by $^{15}$N relaxation. The residue specific $R_2/R_1$ values, corrected for the viscosity differences between the two buffers, are shown in Figure 5.

**Insert Figure 4 here**
RE induced chemical shift perturbations of FKBP12 are mainly associated to loops and do not correlate with solvent accessibility or residue type. Relaxation data confirm that the residues that are more perturbed by the addition of RE are located in loops and probably reflect chemical exchange effects. The largest $R_2/R_1$ differences are observed for Gly86 and are clearly outside the experimental error. Smaller differences are observed for residues Gly83, His87, Ile91 and Thr96 in the same loop. While the differences are subtle, the result was confirmed at two different FKBP12 concentrations. At the higher concentration Arg42, located in a second mobile region, shows a significant decrease of $R_2/R_1$ values in the presence of 50 mM RE. The fact that this residue is only affected at high concentrations suggests that the effect may result from minor aggregation being suppressed by the addition of RE.

**Chymotrypsinogen A loops are collapsed on the folded core**

SAXS data reveal the compaction of the ImwPTP dimer but do not detect any changes in the ImwPTP monomer. This suggests that the effect of RE on the conformational space sampled by small loops is too modest to cause a detectable change by SAXS. To directly characterize the effect of RE on a monomeric protein using SAXS we selected chymotrypsinogen A that contains
large disordered loops not observed in the crystal structure of the protein. As a control we measured SAXS data of FKBP12 under the same conditions.

SAXS curves of chymotrypsinogen A and FKBP12 were measured under equivalent conditions in the presence of 0, 50, 100 and 200 mM RE. The $R_g$ values extracted from the curves are plotted vs. RE concentration in Figure 6. As a reference, the $R_g$ expected from the published crystal structures (1chg Freer et al. 1979, 1d7h Burkhard et al. 2000) are indicated.

The $R_g$ of chymotrypsinogen A in the absence of RE is 22.3 Å and decreases to 17.8 Å in 200 mM RE. Most of the compaction takes place below 50 mM RE. The presence of RE does no affect de $R_g$ value of FKBP12 and this value is in excellent agreement with the one predicted from its crystal structure confirming that the RE buffer does not perturb the SAXS measurements.

The $R_g$ value calculated from the crystallographic structure of chymotrypsinogen A is 17.3 Å. Since the mobile loops are not present in the structure, this value reflects the dimensions of the folded core. The compact form of chymotrypsinogen A in the presence of 200 mM has a $R_g$ value of only 17.8 Å, just slightly larger than that of the protein core observed in the crystal. Comparison of the complete SAXS curve with the ordered part of chymotrypsinogen A using CRYSOL shows a not very good agreement in the absence of RE that dramatically improves in the presence of 200 mM RE (Figure 7). These results suggest that the mobile loops of chymotrypsinogen A collapse onto the surface of the folded core in the presence of RE.

## Insert Figure 7 here
The additional interactions between the loops and the protein core are expected to increase the proteins thermodynamic stability. Indeed, chymotrypsinogen A melting temperature increases by nearly 4K in the presence of 200 mM RE (Table 1). As a comparison, we measured also the stability of maltose binding protein (MBP), a highly soluble periplasmic protein that does not have large disordered loops on its surface. RE had no effect on the stability of MBP, although the stabilization by sodium sulphate and the destabilization by sodium thiocyanate, two classical Hoffmeister type cosolutes (Tadeo et al. 2007), and also the stabilization by its ligand, maltose, could be clearly observed.

Insert Table 1 here

DISCUSSION

Reported effects of 50 mM RE on the solution properties of proteins include: a) Increase in protein solubility (Golovanov et al. 2004); b) Increase in the second virial coefficient (Valente et al. 2005); c) Decrease of non-specific interactions of Xe atoms with proteins (Blobel et al. 2007); d) Preservation of specific intermolecular interactions (Golovanov et al. 2004); e) Increase of the stability of oligomers (Blobel et al. 2007); f) Increase in protein thermal stability (Vedadi et al. 2006); g) Increase in the resistance to proteolytic degradation (Golovanov et al. 2004).

Some of the observed effects are clearly related and suggest that RE causes a reduction in the exposure of hydrophobic regions of the protein that are
responsible for non-specific protein-protein interactions measured by the second
virial coefficient and favouring aggregation, and also for the reduced interaction
with a non-specific hydrophobic probe like Xe atoms. On the other hand, the
preservation of specific interactions with other macromolecules suggests that RE
is not affecting equally all exposed regions of the protein. The observed
enhancement of the stability of the dimers in the case of ImwPTP indicates an
effect of RE in oligomerization processes. Moreover, the increase in thermal
stability observed with many, but not all, of the proteins whose solubility is
enhanced by the addition of RE indicates also suggests an effect on the
unfolding equilibria. The entropic cost of rigidifying loops upon compaction could
reduce or compensate the energetically positive intramolecular interactions
explaining the negligible thermal stabilization of some proteins in RE containing
buffers.

Finally, the surprising observation of the increased stability to proteases of
proteins that are not significantly thermally stabilized by RE remains unexplained.
It has been speculated that it has a completely different origin from other RE
effects, and is related to competitive inhibition of contaminating proteases by the
added amino acids (Golovanov et al. 2004).

The effects of RE on three unrelated proteins, all of which display disordered
loops on their surface (Figure 8), have been measured by NMR and SAXS. While
NMR provides information at an atomic scale, SAXS provides a low resolution
view of large amplitude changes induced by the co-solutes. NMR chemical shifts
and relaxation data on FKBP12 and ImwPTP presented in the results section and
elsewhere (Blobel et al 2007) show that the residues most perturbed by RE are located in loop regions. Conversely, exposed residues in structured regions are typically not perturbed by RE suggesting that the effect of RE is associated with changes in the conformation of flexible regions, rather than accessible to cosolutes per se.

SAXS measurements provide complementary information with respect to NMR. In the published crystallographic structure of chymotripsinogen A 23 of the 245 residues have atoms that could not be located in the electron density. The portion of the protein that is observed in the crystal structure roughly corresponds to the folded protein core. The $R_g$ calculated from this structure is much smaller than the experimentally observed one in the absence of RE in which the larger volume occupied by the disordered loops is also detected. However, the SAXS curve of chymotrypsinogen A in 200 mM RE is in good agreement with the one calculated using only the folded core. The solution $R_g$ value is only slightly larger than the one calculated for the ordered core strongly suggesting that the disordered loops have collapsed onto the protein core in 200 mM RE.

Despite the effects of RE on the chemical shifts of FKBP12 and monomeric ImwPTP, RE does not have any significant effect on the measured $R_g$ values for these two proteins, suggesting that the volume sampled by the loops of these two proteins in the absence of RE is smaller than in the case of chymotrypsinogen A. However, a significant compaction was observed for the ImwPTP dimer. It is likely that compaction of the dimer interface, which is formed by the interaction of symmetry-related loops from both protomers, causes changes in the dimer
dimensions that are large enough to be observed by SAXS. Thus, the compaction of ImwPTP dimer and that of chymotrypsinogen A can be seen as two examples of minimization of the effective protein volume in the presence of RE.

The thermodynamic stability of chymotrypsinogen A is increased in the presence of RE. In principle this could be caused by stabilization of the folded form or destabilization of the unfolded state. To check if RE can destabilize unfolded states we measured the melting temperature of MBP in different conditions. MBP is larger than chymotrypsinogen A, but it does not have any large disordered loops. If RE had a general destabilizing effect on unfolded proteins, denaturation temperature of MBP would be expected to increase in the presence of RE. The results in Table 1 show that RE has no effect on the stability of MBP in contrast to classical osmolites like Na$_2$SO$_4$ or NaSCN which show a clear stabilization and destabilization, respectively. The stabilization resulting from maltose binding could also be clearly observed for MBP. These results are consistent with the hypothesis that chymotrypsinogen A stabilization occurs in the folded state and is a consequence of the additional interactions between the core and the collapsed loops.

A unified explanation for RE effects on proteins

The results presented in this work show a common theme in the three systems studied: the addition of RE selectively affects disordered loops on the protein
surface leading to compaction, probably by forcing their collapse onto the stably folded protein core. This observation suggests a unified and general explanation for all the effects caused by RE on many different proteins. Disordered regions protruding from the protein surface of an otherwise well folded protein are often mediating non-specific interactions and protein aggregation and are also especially susceptible to proteolytic degradation. By collapsing these regions onto the protein core, non-specific interactions and proteolytic degradation are reduced.

The additional intramolecular interactions between the collapsed loops and the core may stabilize the folded state and contribute to the increased thermal stability in some cases. The enhanced stability would be a consequence of the collapse of the loops and not the other way around (i.e. the lower exposure of disordered regions may be the primary effect and not a consequence of a higher thermal stability of the protein core). Thus, in some cases it may be possible that the collapse of small loops contributes to reduce aggregation and increase protein solubility but has no measurable influence in the thermal stability of the protein core, as has been observed by Golovanov et al. (2004).

Protein compaction, as observed in the presence of RE has been previously observed with much larger concentration of sugars (Kim et al. 2003), polyols (Kaushik and Bhat 1998) and trimethylamine N-oxide (TMAO, Stanley et al. 2008). Reduction of protein aggregation by these co-solutes has been associated to intramolecular burying of hydrophobic patches.
The origin of the RE effect: the Polyelectrolyte Induced Compaction model

Which is the mechanism by which RE causes the collapse of disordered loops on the protein surface? Arginine is one of the most common additives used to prevent aggregation during refolding of denatured proteins (De Bernardez Clark, 2001) and has been shown to prevent heat-induced aggregation (Shiraki et al, 2002). Either arginine or glutamic acid separately reduce aggregation induced by partial thermal unfolding at 50 mM concentration although higher concentrations of arginine have larger effects (Shiraki et al. 2002). Arginine prevents aggregation during refolding of lysozyme by dilution from 8M urea but glutamic acid induces aggregation under these conditions. Protection from heat-induced aggregation by arginine and glutamic acid shows no correlation with the isoelectric point of the protein (Shiraki et al. 2002). Very high concentrations of arginine (0.5-2 M) decrease the thermal stability of proteins (Arakawa and Tsumoto, 2003). The aggregation suppressing effect of arginine has been related to its capacity to bind to both hydrophobic and hydrophilic amino acid side chains offsetting its preferential exclusion from protein surfaces suggested by surface tension measurements (Arakawa et al. 2007). An alternative explanation based on the formation of arginine clusters has been suggested by Das et al. (2007).

The use of glutamic acid as co-solute is limited by its low solubility. Sodium glutamate at high concentration stabilizes both lysozyme and BSA (Arakawa and Timasheff 1984). The solubility of glutamic acid can be increased to much higher concentrations in the presence of equimolar amounts of arginine. The increase in
protein solubility in 50 mM RE equimolar mixture is not observed in the presence of the same concentration of the individual amino acids (Golovanov et al 2004). RE also has a larger effect than arginine on the second virial coefficient of lysozyme (Valente et al 2005).

In contrast to other co-solutes, the RE mixture increases protein solubility at low concentrations. The non-additive effects of the components of the RE mixture and the differences from classical co-solutes like Na$_2$SO$_4$ and NaSCN on the melting temperature of MBP suggests that RE acts, at least in some cases, by a different mechanism. It has been speculated that charged amino acids interact and mask oppositely charged groups on the protein surface while the hydrophobic parts of the side chains of arginine and glutamic acid cover adjacent hydrophobic groups preventing aggregation (Golovanov et al 2004). However, preferential interaction would result in protein destabilization as the denatured state usually has more accessible interaction sites. In the presence of 50 mM RE a significant number of proteins are stabilized by more than 4°C (Vedadi et al 2006) or not affected.

We would like to suggest an alternative model, the Polyelectrolyte Induced Compaction (PIC), that could explain the loop compaction effect caused by RE. The model is based on the following premises:

a) Arginine and glutamic acid are both molecular polyelectrolytes. Each of them has at neutral pH three charged interaction sites, even though the global charge is +1 for Arg and -1 for Glu. The molecular polyelectrolyte nature of arginine
could be further enhanced by the formation of clusters as suggested by mass

b) The interaction between sites of different molecules will introduce a spatial
correlation between them. The asymptotic behaviour of the correlation function
will be either monotonic or oscillatory depending on the concentration of the
electrolyte. Dressed interaction site theory (DIST) predicts a shifting to lower ionic
concentrations, of the point at which the leading asymptotic term turns from a
strictly monotonic decay to a damped oscillatory behaviour in the case of
molecular electrolytes (Gonzalez-Moruelos et al 2005). Model calculations for a
20 Å rigid rod with four equally charged sites and spherical, single site, counter
ions predict that the onset of the transition would take place at a concentration of
25 \( \mu \)M. At this point the effective decay length would be 262 Å. This distance is
the effective decay length of the effective potential between oppositely charged
species and it has the same order of magnitude as the dimensions of a large
macromolecule. While equivalent calculations for a real arginine-glutamic acid
system are not feasible at this point we assume that, due to their molecular
polyelectrolyte nature, the interaction length will be significantly longer than
expected for simple ions.

c) In the absence of protein, RE solutions will reach a minimum energy state
which will include the effect of the long range correlation between arginine and
 glutamate.

d) The presence of the protein is expected to perturb the electrolyte solution and
increase the free energy of the system. If the protein can adopt different
conformations, those that minimize the occupied volume will also minimize the perturbation and will be favoured, leading to compaction.
e) PIC is reminiscent of other phenomena favouring protein states that occupy the lowest possible volume, such as steric macromolecular crowding or surface tension effects opposing the formation of the solvent cavity needed to accommodate the protein. However, it has a different origin and dependency on structural and dynamic factors. In particular, according to our hypothesis, PIC is associated to a mixture of interacting co-solutes and originates from the dynamic correlation between different chemical species. Thus it may not be quantified by surface tension measurements and is expected to depend on the translational diffusion rates of the electrolytes and the perturbing protein.
f) The correlation between molecular electrolytes affects their relative motions taking place at a velocity related to their translational diffusion which is large in the case of small molecules, such as arginine and glutamic acid. The perturbation induced by a protein in the solution will be different depending on its mobility. For a denatured or unfolded protein, local diffusion is fast and the perturbation will be minimal. Thus, in contrast to macromolecular steric crowding, small polyelectrolytes are not expected to stabilize proteins by destabilizing the denatured state. On the other hand, loops protruding from the surface of a large protein and exploring a considerable volume will cause a large perturbation on the electrolyte system since their translational motion is restricted to that of the complete protein. Thus, RE favours the reduction of protein volume by favouring intramolecular interactions of loops with the folded core. The burying of exposed
sites would be finally responsible for the observed reduction of non-specific interactions and concomitant increase in solubility and stability to proteases. Although the PIC model explains qualitatively the observed behaviour of RE in different systems, it is still speculative and it is difficult to derive quantitative predictions that could be tested. In addition, it is likely that other mechanisms may be simultaneously contributing to the observed effects. However, the PIC model provides a unified explanation for the different effects of RE observed and can be generalized to other systems, leading to qualitative predictions, some of which have been verified.

If the polyelectrolyte nature of RE is responsible for the observed effects, other polyelectrolyte systems should show similar behaviour. We have tested over 50 different mixtures of organic polyelectrolytes and observed stabilization of chymotrypsinogen A by more than 4°C in 46 mixtures and in half of the cases the stabilization was higher than 10°C at co-solute concentrations below 600 mM. These results will be reported in detail elsewhere (Sornosa et al. in preparation).

A large number of small biological molecules such as amino-acids or polyamines can also be considered as polyelectrolytes and may exert similar effects to proteins in cellular environments. The PIC model could also explain the chaperone activity of intrinsically disordered proteins ERD10 and ERD14 produced by plants under stress conditions (Kovacs et al 2008). Their chaperone activity is associated to the presence of charged repetitive regions that can be described as polyelectrolytes.
We suggest that the PIC model provides a new conceptual framework to understand the modulation of protein solution properties by multiple charged molecules. This framework can direct the search for new stabilizing additives for biotechnological applications and help to understand biologically relevant transient interactions involving intrinsically disordered chaperones.

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Table 1. Co-solute induced changes in melting temperature

<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\Delta T$ * K&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsinogen A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50 mM RE</td>
<td>+1.4</td>
</tr>
<tr>
<td>Chymotrypsinogen A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200 mM RE</td>
<td>+3.9</td>
</tr>
<tr>
<td>MBP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50 mM RE</td>
<td>+0.5</td>
</tr>
<tr>
<td>MBP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200 mM RE</td>
<td>+0.7</td>
</tr>
<tr>
<td>MBP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200 mM Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>+3.1</td>
</tr>
<tr>
<td>MBP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200 mM NaSCN</td>
<td>-7.8</td>
</tr>
<tr>
<td>MBP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 mM maltose</td>
<td>+6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 25 mM phosphate, 150 mM NaCl plus the indicated cosolute. Protein concentration was 10 $\mu$M. The estimated uncertainty in $\Delta T$ is 1K.

<sup>b</sup> pH 6.5

<sup>c</sup> pH 7.5
**FIGURE CAPTIONS**

Figure 1 Apparent radius of gyration ($R_g$) and maximum dimension ($D_{max}$) observed as a function of lmwPTP concentration in the absence (filled dots) and presence (empty circles) of 50 mM RE. The theoretical values for the monomer (dashed dotted line) and dimer (dashed line) are indicated.

Figure 2 MCR-ALS extracted pure SAXS curves for lmwPTP monomer (red) and dimer (green) and their fit to the corresponding crystal structures (dashed lines) by CRYSOL (Svergun et al. 1995). An equivalent analysis to a SAXS dataset measured in the absence of RE yield SAXS curves of both species that were in excellent agreement with crystallographic structures (see Figure 5A in Blobel et al. 2009). The minimization error as a function of the assumed dimer dissociation constant is shown in the inset.

Figure 3 $^1$H$\alpha$ chemical shift changes induced by 50 mM RE in lmwPTP (absolute values). Dashed line is the average $^1$H$\alpha$ chemical shift change measured for buried residues in the protein and therefore are not affected by RE. The localization of secondary structure elements is indicated. Filled rectangles represent $\alpha$-helices and empty ones $\beta$-sheets strands. Relative solvent accessibilities ($rel\ S_{exp}$) are indicated. The residues most affected by RE are labelled and their accessibility is shown by filled bars.
Figure 4. $^1$H chemical shift changes induced by 50 mM RE in FKBP12 (absolute values). Dashed line is the average $^1$H chemical shift change measured for buried residues in the protein and therefore are not affected by RE. The localization of secondary structure elements is indicated. Filled rectangles represent $\alpha$-helices and empty ones $\beta$-sheets strands. Relative solvent accessibilities ($\text{rel } S_{\text{exp}}$) are indicated. The residues most affected by RE are labelled and their accessibility is shown by filled bars.

Figure 5. $R_2/R_1$ data for 0.94 mM (A) and 1.4 mM (B) FKBP12 in RE-free buffer (filled circles) and in the same buffer plus 50 mM RE (open circles). The RE data has been corrected for viscosity. Residues showing the largest differences are indicated by downward arrows. $\alpha$-helices and $\beta$-sheet strands are represented by filled and open rectangles, respectively.

Figure 6. SAXS derived radius of gyration ($R_g$) of chymotrypsinogen A (dashed line) and FKBP12 (continuous line) at different RE concentrations. The experimentally obtained $R_g$s are shown as circles. $R_g$ values calculated from the crystal structures are shown by horizontal lines.

Figure 7. a) SAXS scattering curves of chymotrypsinogen A recorded in the presence of different concentrations of RE (0 blue, 50 mM yellow, 100 mM magenta, and 200 mM turquoise) and CRYSOL fits to the crystallographic structure 1chg. Experimental data are shown by circles and the fit by lines. The y-
axis showing the scattering intensity $I(s)$ is presented in log scale. Curves are

displaced in the $y$-axis for proper visualization. b) Point-by point error $(I(s)_{\text{exp}} - I(s)_{\text{calc}})/(\sigma(s)_{\text{exp}})$, of the fitting of each curve.

Figure 8  Crystallographic structures of  a) lmwPTP monomer, b) lmwPTP dimer, c) FKBP12, and  d) chymotrypsinogen A with surface loops highlighted. Disordered loops not observed in the crystal structure of chymotrypsinogen A are indicated by dashed lines.