International Journal of Biological Macromolecules 47 (2010) 15–20

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Polyethyleneimine–protein interactions and implications on protein stability

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ARTICLE INFO

*Article history:*
Received 10 February 2010
Received in revised form 13 April 2010
Accepted 14 April 2010
Available online 22 April 2010

*Keywords:*
Polycation
Thermodynamic stability
Oxidation

ABSTRACT

Protein stability assessment of seven model proteins in the presence of low molecular weight polyethyleneimine (PEI, MW 2000 Da) was performed. Thermodynamic stability, monitored by circular dichroism (CD) spectroscopy, showed that the polymer did not have a major effect on the melting temperature ($T_m$) of the basic proteins – muscle lactate dehydrogenase (LDH), ribonuclease A, lysozyme and cutinase, while for the acidic ones – human growth hormone, human serum albumin and heart lactate dehydrogenase – there was a shift in $T_m$ towards lower temperatures. The secondary structures of the basic proteins were essentially the same, with none or a slight increase in the CD spectra, in presence of the polymer. In the case of the acidic proteins, the CD spectra were diminished mostly due to phase separation. Assuming a homogeneous distribution of the net charge on the protein surface a quantitative inverse relationship was established between surface charge density of the acidic proteins and the $PEI_{2000}$ concentration required for maximum flocculation. Despite lowering the thermal stability of acidic proteins, $PEI_{2000}$ was seen to protect heart LDH at an increasing oxidative stress.

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## 1. Introduction

Despite the increasing application of protein engineering for improving the stability of proteins, use of soluble chemical compounds as stabilizing excipients in formulations is still largely practised. This is especially true for therapeutic proteins, which are desired to be available in a wild-type form. An understanding of the effect of different stabilizing agents continues therefore to be a topic of great interest for designing protein formulations.

Interaction of polyelectrolytes with proteins is central to several physiological processes as well as to different applications such as protein separations, and immobilization [1–3]. Polyelectrolytes also influence protein stability; examples of both stabilization and inactivation in the presence of positive-/negatively charged polymers are found in the literature [4–6].

Polyethyleneimine (PEI) is a positively charged branched polyelectrolyte that has various biotechnological applications. The polycation is used for precipitating DNA, RNA, and proteins bound to DNA or RNA, as well as acidic proteins [7]. It has found acceptance as a carrier in industrial immobilized biosystems [8]. Moreover, PEI was reported to be a highly efficient vector for delivering oligonucleotides and plasmids both *in vitro* and *in vivo* [9], and since then several efforts have been made to understand and enhance PEI-mediated transfection [10]. It has also been shown to have a stabilizing effect on several enzymes in solution [11–14] as well as in biosensors [15]. In our earlier studies on the effect of PEI on protein stability, we have demonstrated that $PEI_{2000}$ (MW 2000 Da), at a concentration as low as 0.10% (w/v), was highly effective in preventing oxidative damage of porcine muscle lactate dehydrogenase (LDH, EC 1.1.1.27; isoelectric point 8.2) [16]. Light scattering measurements showed the polycation and the enzyme to form a loose complex [17]. PEI helped to maintain the secondary structure of LDH during storage for long periods (at 36 °C) and significantly reduced protein aggregation, however it did not protect the enzyme against thermal denaturation [11,16,18].

The present report is a more extensive investigation of the effect of PEI on protein stability. The study is based on seven model proteins which differ significantly in isoelectric points, molecular masses and stability: heart and muscle lactate dehydrogenase (LDH), recombinant human growth hormone (rhGH), cutinase, human serum albumin (HSA), lysozyme (LYZ) and ribonuclease A (RNAse).

## 2. Materials and methods

### 2.1. Materials

All the chemicals used were of analytical grade and were obtained from standard sources. L-Lactate dehydrogenase (porcine heart; EC 1.1.1.27), albumin (human serum) and ribonuclease A

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doi:10.1016/j.ijbiomac.2010.04.003

(bovine pancreas Type II-A, EC 3.1.27.5) were purchased from Sigma Chemical Co. (St. Louis, MO). L-Lactate dehydrogenase (porcine muscle; EC 1.1.1.27) was purchased from ICN Biomedicals (Aurora, Ohio) and lysozyme (hen egg white; EC 3.2.1.17) was from Fluka BioChemika. Recombinant growth hormone (human) was purchased from the Swedish pharmacy as the product Genotropin® from Pfizer. Recombinant cutinase (*Fusarium* sp., EC 3.1.1.74) purified from culture supernatant of *Saccharomyces cerevisiae* was kindly provided by Professor Maarten Egmond (Unilever Research Laboratories, Vlaardingen, The Netherlands).

Bicinchoninic acid kit for protein determination, D-sorbitol and polyethyleneimine (PEI; average MW 2000 Da; 50% (w/w/) aqueous solution) were procured from Aldrich Chemicals, Belgium.

Stock solutions of 5% (w/v) polyethyleneimine ($PEI_{2000}$) and 4 M sorbitol, respectively, were prepared in water and the pH adjusted to 7.2 using HCl/NaOH solution. Lactate dehydrogenase (LDH) suspensions were extensively dialysed against 5 mM Tris–HCl buffer pH 7.2 and later centrifugued at 13 000 rpm in an Eppendorf centrifuge for 6 min in order to remove any aggregated material.

### 2.2. Circular dichroism

Circular dichroism (CD) scans of protein solutions were performed using a Jasco spectropolarimeter, model J-720, calibrated with (1R)-(-)-10-camphor sulfonic acid. Scan of the protein samples (100 $\mu g\,mL^{-1}$ protein) was performed in the UV range 195–250 nm, at 1 nm increments, 10 nm/min, and a temperature of 25 °C using a sample cell pathlength of 0.1 cm.

The change in secondary structure of the proteins with increasing $PEI_{2000}$ concentrations was followed at 25 °C in a 1 cm pathlength quartz cuvette. The concentrations of proteins were 20 $\mu g\,mL^{-1}$ for human serum albumin and 10 $\mu g\,mL^{-1}$ for heart LDH and human growth hormone, respectively. To avoid changes in pH during titration, both protein and polymer solutions were adjusted to the same pH value. Molar ellipticity $[\theta]$ at 222 nm and α-helical content were calculated according to Morrow et al. [19].

For determination of melting temperature ($T_m$), the ellipticity ($\theta$) of proteins (100 $\mu g\,mL^{-1}$) at 222 nm was followed with increase in temperature from 25 to 95 °C at a scan rate of 60 °C $h^{-1}$. Duplicate samples were scanned, using a 0.1 cm pathlength quartz cuvette.

### 2.3. Measurement of protein concentration

Total protein concentration used for the experiments was determined by the bicinchoninic acid method [20]. Bovine serum albumin was used as the standard protein.

### 2.4. Measurement of LDH activity

The activity of LDH samples (100 $\mu g\,mL^{-1}$) was measured according to the established procedure [21]. To a reaction mixture composed of 100 μL of 0.1 M Tris–HCl buffer (pH 7.5), 21 μL of 30 mM pyruvate and 5 μL of 6.6 mM NADH was added 30 μL of suitably diluted enzyme solution. The initial rate of consumption of NADH was followed at 340 nm using an Ocean Optics USB4000 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme causing the oxidation of 1 μmol of NADH per minute under the specified conditions.

### 2.5. Sequence alignments and electrostatic calculations

Net charge of human growth hormone (PDB code 1HGU), human serum albumin (PDB code 1AO6), porcine heart LDH (PDB code 5LDH) and porcine muscle LDH (PDB code 9LDT) at pH 7.2 was calculated by means of Henderson–Hasselbach equation ($pH = pK_a + \log[A^-]/[AH]$), using their amino acid compositions and the solvent accessible surface by the Voss Volume Voxelator online calculator with all default parameters [22]. Surface charge density of the proteins was calculated as the ratio of net charge of the protein and its solvent accessible surface.

**Table 1**
Different model proteins used in the study.

| Protein | Source | Molecular mass (kDa) | pI |
|---|---|---|---|
| Lactate dehydrogenase | Porcine muscle | 146 | 8.2 |
| Lactate dehydrogenase | Porcine heart | 146 | 5.6 |
| Albumin | Human serum | 66 | 5.6 |
| Cutinase | Fusarium | 23 | 8 |
| Growth hormone | Human (recombinant) | 22 | 5.3 |
| Lysozyme | Hen egg white | 14.6 | 11 |
| Ribonuclease A | Bovine pancreas | 13.7 | 8.6 |

Protein sequence alignments for heart and muscle LDH (PDB codes 5LDH and 9LDT, respectively) were performed by means of Basic Local Alignment Search Tool (BLASTp 2.2.18) using all default parameters [23]. Electrostatic potential maps were calculated using the online server Protein Continuum Electrostatics [24]. Calculation parameters include the protein internal dielectric of 4, solvent dielectric constant of 80, and ionic strength as 0.1.

## 3. Results and discussion

### 3.1. Polyethyleneimine–protein complexes

Seven proteins, representing acidic and basic proteins of different sizes and complexity of structures, were chosen for this study (Table 1), and the influence of $PEI_{2000}$ on the conformation of these proteins at pH 7.2 was investigated by means of circular dichroism spectroscopy in the far UV range at 25 °C. The ionization of the polymer is ~10% around pH 7 [25]. In accordance with our earlier studies [11], the CD spectra of the basic proteins – muscle LDH, RNAse, lysozyme and cutinase – were either unaffected or were slightly enhanced in presence of 0.10% (w/v) PEI (data not shown). Enhancement of secondary structure of different proteins on interaction with small ligands has been reported earlier [26,27], while in some cases the bound ligands produce an induced CD spectrum [28]. Overlapping of the PEI absorption spectrum at 200–250 nm with the one corresponding to proteins makes it difficult to differentiate between either of these effects in the present study. We have however shown that enhancement in secondary structure is also accompanied by an increase in the activity of enzymes in the presence of PEI [11].

In case of the acidic proteins the ellipticity was diminished and flocculates visible to the naked eye were formed for HSA and LDH immediately after mixing with the polymer. Complexation of oppositely charged macromolecules has been shown to result in formation of coacervates or precipitates that undergo phase separation [29]. Thereby, the diminution of the CD signal was mainly attributed to phase separation of the PEI–protein complexes. CD spectra of HSA in the presence of high concentration of different polyamines showed a reduction of α-helices in favour of β-sheet, attributed to partial unfolding of the protein [30].

The proteins were titrated with increasing concentrations of PEI and ellipticity of the mixture at 222 nm was determined. Fig. 1 shows that at low polymer/protein ratio, ellipticity decreased with an increase in the polymer concentration until a plateau was reached. Ellipticity values in the plateau were in the range −10 to −6 mdeg (versus 3 mdeg for baseline), indicating that loss of secondary structure was partial. Residual α-helices, relative to the protein without additives, were 60, 71 and 86% for heart LDH, HSA and hGH, respectively. This implied the presence of an equilibrium

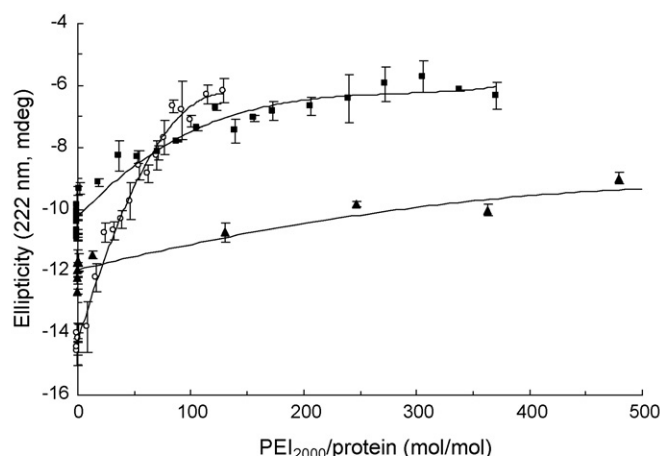


**Fig. 1.** Ellipticity at 222 nm obtained from CD spectra of human growth hormone (▲, 10 μg/mL), human serum albumin (■, 20 μg/mL) and heart LDH (○, 10 μg/mL) during titration with increasing amounts of $PEI_{2000}$.

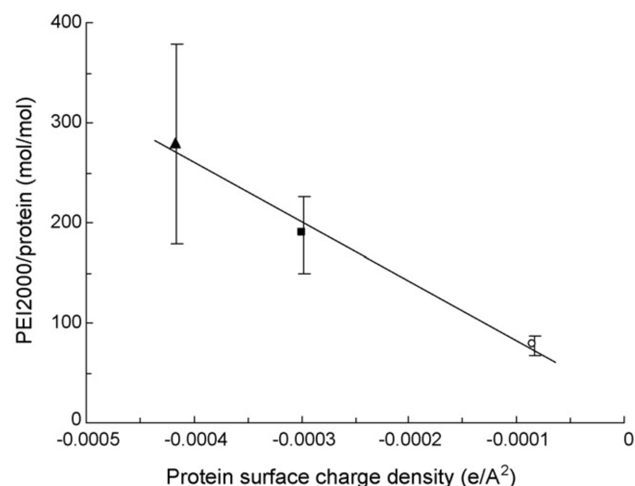


**Fig. 2.** Relationship between $PEI_{2000}$/protein ratio (mol/mol) for maximum flocculation and protein surface charge density (e/Å$^2$) of human growth hormone (▲), human serum albumin (■) and heart LDH (○).

mixture between soluble protein–PEI complex with uncomplexed protein and PEI.

From the data in Fig. 1, the stoichiometric polymer/protein ratio at the point of maximum loss of ellipticity (i.e. when maximum quantity of the protein has been precipitated as insoluble complex) was determined by the intersection of two straight lines – one fitted to the data obtained with low polymer concentration while the other, parallel to the abscissa, calculated using the data forming the plateau at high polymer concentration. Unlike coacervation where intrapolymer complexes are formed by one polymer chain binding several proteins [31], a large number of PEI molecules were complexed per mole of protein. As shown in Table 2, the number of polymer molecules needed for precipitation of the proteins was increasing in the order heart LDH > HSA > hGH, and did not seem to be correlated with the total or solvent exposed numbers of acidic residues. For example, human serum albumin with larger number of acidic residues than heart LDH required more PEI for precipitation. A quantitative inverse relationship was however found between the amount of polymer needed for precipitation and the surface charge density of the proteins (Fig. 2). Even though the calculation of the proteins' surface charge density was done assuming a homogeneous distribution of the charges, it is most likely an important feature determining the degree of flocculation, and is in agreement with previous reports relating binding constant of high molecular weight polymers to protein surface charge density [32].

### 3.2. Heart versus muscle LDH

The differential in interaction of PEI with porcine heart and muscle LDH, respectively, is of interest since the proteins are similar in their catalytic function and molecular weight and shape, but differ significantly in their catalytic parameters, isoelectric points and thermodynamic stability. The heart LDH is found to be more stable protein of the two with a denaturation temperature of 71 °C compared to 61 °C for muscle LDH (see Table 3). This observation is consistent with studies of LDH from chicken and beef which show higher thermal stability for heart than muscle enzyme [33].

Protein sequence alignments (BLASTp) of heart and muscle LDH subunits display high sequence homology with 75% identity (Table 4). Both enzymes contain equal number (140) of acidic groups (glutamic and aspartic acid residues), but differ in the number of solvent exposed acidic groups, 72 for heart LDH versus 56 for muscle LDH. On the other hand, muscle LDH has higher number of total basic groups (lysine and arginine), i.e. 148 versus 128 for heart LDH, but the difference is smaller on the surface (72 versus 68). The surface charge density of muscle LDH at pH 7.2 was determined to be $+6.16 \times 10^{-4}$ e/Å$^2$ in contrast to $-0.84 \times 10^{-4}$ e/Å$^2$ for heart LDH. The electrostatic surface representations of heart and muscle LDH clearly demonstrated that heart LDH is richer in negative electrostatic potential patches (Fig. 3).

In an earlier study, Joshi et al. correlated the number of positive residues of growth hormones of different origins with the degree of interaction with polyanions [6]. Interaction of human α-synuclein with polyethyleneimine was attributed to its C-terminal region that is very rich in acidic residues [34]. Electrostatic interactions of PEI with negative patches on the surface of the protein would seem to be the main reason for the differences between the complexes of the homologous enzymes with PEI, since the polymer in principle interacts with the native protein. As shown in our earlier study, PEI exhibits binding even to muscle LDH with a net positive charge [17], but the overall binding strength may be diminished by repulsive interactions hence not resulting in flocculation of the complex.

**Table 2**
Polymer/protein ratio obtained for acidic proteins from CD titration curves with $PEI_{2000}$.

| Protein | mol PEI/mol protein | Total acidic residues | Solvent exposed acidic residues | Protein surface charge (e/Å$^2$) |
|---|---|---|---|---|
| rhGH | 278 | 24 | 11 | $-4.16 \times 10^{-4}$ |
| HSA | 188 | 100 | 56 | $-2.98 \times 10^{-4}$ |
| Heart LDH | 77 | 140 | 72 | $-0.84 \times 10^{-4}$ |

**Table 3**
Effect of PEI (0.10%, w/v) and sorbitol (2 M) separately and in combination on melting temperatures of proteins at pH 7.2.

| Protein | $T_m$ (°C) | | | |
|---|---|---|---|---|
| | No additive | PEI | Sorbitol | PEI + sorbitol |
| Basic proteins | | | | |
| Lysozyme | 77 | 79 | ND | ND |
| Ribonuclease A | 62 | 62 | 69 | 71 |
| Muscle lactate dehydrogenase | 61 | 60 | 69 | 71 |
| Cutinase | 60 | 60 | 66 | 66 |
| Acidic proteins | | | | |
| Heart lactate dehydrogenase | 71 | 48 | 75 | 60 |
| Albumin | 81 | 72 | >95 | 86 |
| Growth hormone | 82 | 76 | 91 | 86 |

Error in $T_m$ is approximately ±0.7 °C.
ND, not determined.

**Table 4**
Protein sequence alignments of heart (query) and muscle (subject) LDH using all default BLASTp 2.2.18 parameters.

```
Query ID lcl|7928                              Subject ID: 7929
Description : 5LDH:A|PDBID|CHAIN|SEQUENCE      Description: 9LDT:A|PDBID|CHAIN|SEQUENCE
Query Length: 333                              Subject Length: 331

>lcl|7929 9LDT:A|PDBID|CHAIN|SEQUENCE
Length=331
Score = 526 bits (1356), Expect = 2e-154, Method: Compositional matrix adjust.
Identities = 250/331 (75%), Positives = 292/331 (88%), Gaps = 1/331 (0%)

Query  1    ATLKEKLIAPVAQQETTIPDNKITVVGVGQVGMACAISILGKSLTDELALVDVLEDKLKG  60
Sbjct  1    ATLKDQLIHNLLKEEH-VPHNKITVVGVGAVGMACAISILMKELADEIALVDVMEDKLKG  59

Query  61   EMMDLQHGSLFLQTPKIVANKDYSVTANSKIVVVTAGVRQQEGESRLNLVQRNVNVFKFI  120
Sbjct  60   EMMDLQHGSLFLRTPKIVSGKDYNVTANSRLVVITAGARQQEGESRLNLVQRNVNIFKFI  119

Query  121  IPQIVKYSPNCIIIVVSNPVDILTYVAWKLSGLPKHRVIGSGCNLDSARFRYLMGEKLGV  180
Sbjct  120  IPNIVKYSPNCKLLVVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGV  179

Query  181  HPSSCHGWILGEHGDSSVAVWSGVNVAGVVLQQLNPEMGTDNDSENWKEVHKMVVESAYE  240
Sbjct  180  HPLSCHGWILGEHGDSSVPVWSGVNVAGVSLKNLHPELGTDADKEHWKAVHKEVVDSAYE  239

Query  241  VIKLKGYTNWAIGLSVADLIESMLKNLSRIHPVSTMVQGMYGIENEVFLSLPCVLNARGL  300
Sbjct  240  VIKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGIKENVFLSVPCILGQNGI  299

Query  301  TSVINQKLKDDEVAQLKNSADTLWGIQKDLK  331
Sbjct  300  SDVVKVTLTPEEEAHLKKSADTLWGIQKELQ  330
```

Negatively charged residues glutamic and aspartic acid are highlighted in red, and positively charged residues histidine, lysine and asparagine are highlighted in blue.

### 3.3. Thermal stability of PEI–protein complexes

CD thermal scans at 222 nm (characteristic of α-helical structure) were performed in order to investigate thermal stability of the model proteins in presence of 0.10% (w/v) PEI. Table 3 shows the midpoint of the transition ($T_m$) in the presence of PEI alone and in combination with sorbitol, calculated by using a sigmoidal model with slight modification [35].

PEI did not appear to have a notable effect on the $T_m$ value of the basic proteins – lysozyme, RNAse, muscle LDH and cutinase. This effect seems to be similar to that reported for polyethylene glycol (the non-ionic polymer with a similar backbone as PEI), which also does not increase the thermodynamic stability of proteins; the low molecular weight PEGs can in fact have a destabilizing influence through interaction with non-polar regions of the protein molecules [36–38]. Not surprisingly, the thermal stability of

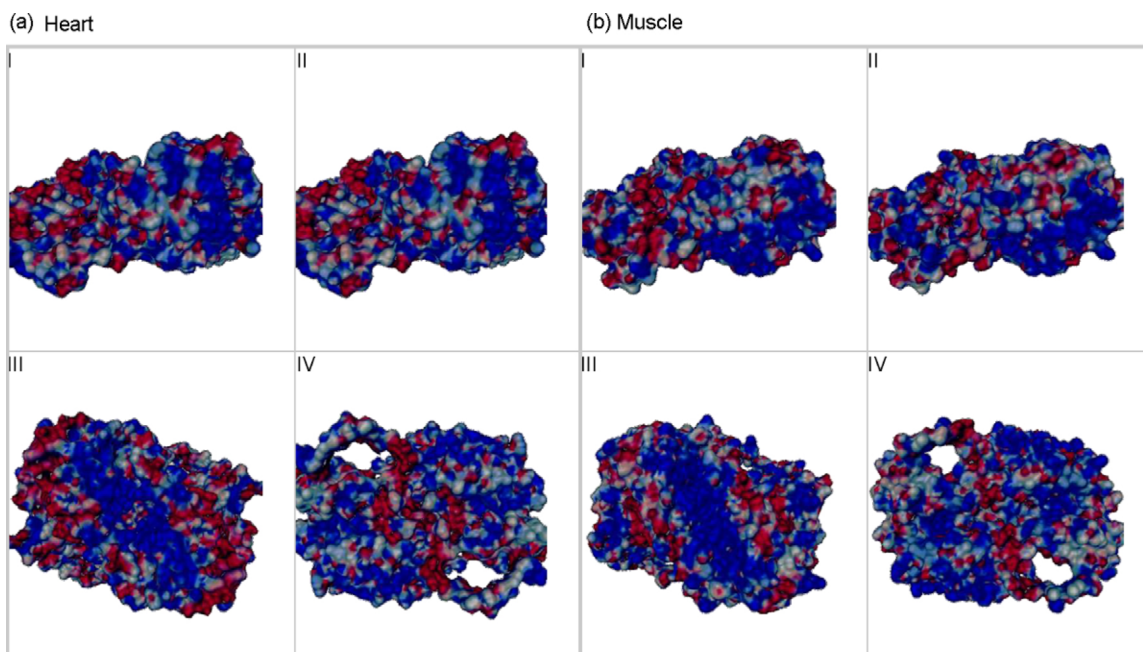


**Fig. 3.** Calculated electrostatic potential maps of (a) heart LDH and (b) muscle LDH with positive potentials (blue) and negative potentials (red). The color ranges in kcal mol$^{-1}$ e$^{-1}$ – negative charge from −2 to 0. The different views are indicated as I and II: side views, III: bottom view, IV: top view-active site surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the acidic proteins was significantly reduced, as indicated by a shift in $T_m$ values towards lower temperatures. Decrease in $T_m$ for heart LDH in the presence of PEI was as high as 23 °C while human growth hormone showed a least decrease of 6 °C. Consistent with these results, Joshi et al. found that bovine and porcine growth hormones (pIs 8.3 and 6.8, respectively) bind the polyanion heparin with a reduction of their thermal stability [6], while hGH (pI 5.3) exhibited lower interaction and $T_m$ remained unchanged. The ellipticity of HSA was shown to be significantly decreased in the presence of a cationic surfactant cetyltrimethylammonium chloride [39]. The above information supports the hypothesis that electrostatic interactions between oppositely charged polymer/proteins pairs affect the existing protein–solvent and intramolecular protein interactions that maintain the conformation and thereby influence negatively the thermodynamic stability. The additional involvement of other interactions, such as H-bonding with protein C=O and C–N groups observed with different polyamines [30], and hydrophobic interactions suggested in case of low molecular weight PEG [38], cannot however be ruled out.

Polyhydric alcohols like sorbitol raise the $T_m$ of various proteins [38,40,41]. The addition of 2 M sorbitol increased the $T_m$ for all the proteins used in this study as well as that of the PEI–protein complexes (Table 3). The augmented water organization and the preferential exclusion effect explain the phenomenon of stabilization by polyols [40]. As a result, sorbitol partially or totally restored the reduced $T_m$ values of the acidic proteins caused by interaction with PEI. In accordance with these observations, the studies on bovine serum albumin (pI 5.6) performed by Kumar et al. showed that polyols restored the negative effect on the thermodynamic stability caused by polyethylene glycol, which seemed to be correlated with an increased apparent solubility of the proteins [38].

The increasing PEI concentration from 0 to 0.10% (w/v) in presence of 2 M sorbitol, showed a tendency to increase the $T_m$ value of muscle LDH (slope: 3.5 °C/mM $PEI_{2000}$), while in the absence of sorbitol, the $T_m$ was reduced (slope: −1.8 °C/mM $PEI_{2000}$). This indicated that more than one event contributes to the thermal denaturation of LDH. Increase in temperature promotes both protein unfolding and oxidation processes [42]; moreover the unfolded protein is more prone to oxidation. In the presence of sorbitol the protein remains preferentially in a folded state but some degree of oxidation may not be prevented. The effect of PEI is a combination of its protective effect against oxidation and inducing loss in conformation due to the electrostatic interactions as described above. Of course, in the case of heart LDH the electrostatic interaction driving protein unfolding prevail over the antioxidant effect of PEI, but the protective effect should be evident under conditions where oxidative stress increases, e.g. in the presence of metal ions (see below).

### 3.4. PEI protects heart LDH against oxidation

Both muscle and heart LDH undergo inactivation in the presence of $Cu^{2+}$ as a result of metal catalysed oxidation. The heart LDH retained only 10% residual activity after 2 h incubation with 100 μM $Cu^{2+}$ (Fig. 4). In our earlier report, we have shown that polyethyleneimine protected several proteins against metal catalysed oxidation [16]; protection was achieved against both copper- and iron-based oxidations in contrast to the chelating agents conventionally used, which are more selective in their protective effect [18].

In contrast to muscle LDH whose activity was enhanced with 0.10% (w/v) $PEI_{2000}$ [11], the initial heart LDH activity was reduced by 10–15%, with a low initial rate of inactivation during the first 2 h at 25 °C (Fig. 4). Fig. 4 clearly shows that heart LDH samples containing $PEI_{2000}$ and $PEI_{2000}$ + $Cu^{2+}$ have a similar inactivation profile, demonstrating the protection provided by the polymer. Despite the

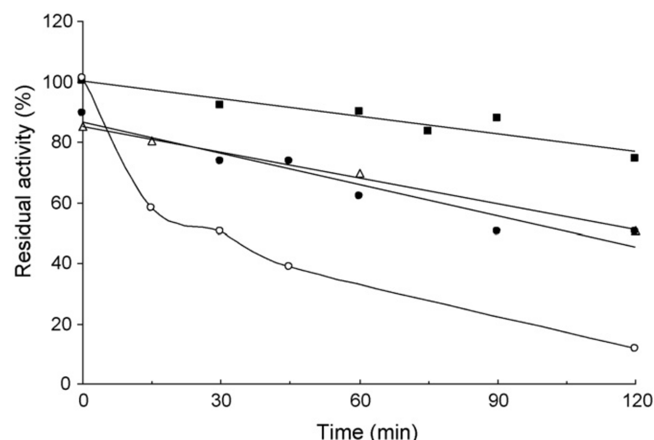


**Fig. 4.** Effect of $PEI_{2000}$ on the activity of heart LDH. The different symbols denote: (■) LDH, (○) LDH + 100 μM $CuSO_4$, (△) LDH + 0.10% (w/v) $PEI_{2000}$, and (●) LDH + 0.10% (w/v) $PEI_{2000}$ + 100 μM $CuSO_4$. One hundred percent of activity corresponded to 16.5 U/mg.

adverse effect of PEI on the activity of heart LDH, the stability of the enzyme on exposure to oxidizing conditions was maintained. In the presence of $Cu^{2+}$ ions, the heart LDH lost 90% of the initial activity after 2 h, while such an activity loss was reached after 3 days of incubation when the solution was supplemented with 0.10% (w/v) $PEI_{2000}$.

## 4. Conclusion

When studying the stability of proteins as a result of amino acid changes or additives, the resistance to the different denaturing forces has to be taken into consideration. PEI was demonstrated to interact with proteins in different ways. On one hand, it impairs the thermodynamic stability, leading to a loss of secondary structure and decrease in $T_m$ of acidic proteins, while on the other it protects against metal catalysed oxidation. This protection was previously attributed to its chelating as well as radical scavenging properties. The results from the present study further emphasize the importance of considering both combined as well as counteracting effects of excipients added to protein formulations. There is a delicate balance of intra- and intermolecular forces exerted by stabilizing additives which will have varying results on different proteins. As a consequence, even when general patterns of stabilization can be defined, protein-additive combinations should be studied for each particular protein, also in the case of highly related ones.

## Acknowledgements

We thank Stina Lindman, Department of Biophysical Chemistry, Lund University for her helpful advice on CD measurements. This work was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), PICTO 9/30725 ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), SIDA (Swedish International Development Cooperation Agency), and FCEyN-UNLPam (Facultad de Ciencias Exactas y Naturales – UNLPam).

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