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Polyol Additives Modulate the In Vitro Stability and Activity of Recombinant Human Phenylalanine Hydroxylase

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Abstract Phenylketonuria (PKU; OMIM 261600), the most common disorder of amino acid metabolism, is caused by a deficient activity of human phenylalanine hydroxylase (hPAH). Although the dietetic treatment has proven to be effective in preventing the psycho-motor impairment, much effort has been made to develop new therapeutic approaches. Enzyme replacement therapy with hPAH could be regarded as a potential form of PKU treatment if the reported in vitro hPAH instability could be overcome. In this study, we investigated the effect of different polyol compounds, e.g. glycerol, mannitol and PEG-6000 on the in vitro stability of purified hPAH produced in a heterologous prokaryotic expression system. The recombinant human enzyme was stored in the presence of the studied stabilizing agents at different temperatures (4 and −20 °C) during a 1-month period. Protein content, degradation products, specific activity, oligomeric profile and conformational characteristics were assessed during storage. The obtained results showed that the use of 50% glycerol or 10% mannitol, at −20 °C, protected the enzyme from loss of its enzymatic activity. The determined ΔG^0 and quenching parameters indicate the occurrence of conformational changes, which may be responsible for the observed increase in catalytic efficiency.

Keywords Additives · Enzyme activity preservation · Human phenylalanine hydroxylase · Phenylketonuria · Protein stabilization

Introduction

Human phenylalanine hydroxylase (hPAH; E.C. 1.14.16.1), a cytosolic enzyme, expressed mainly in the liver and kidney [1, 2], is responsible for the hydroxylation of the essential

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amino acid L-phenylalanine (L-Phe) into L-tyrosine (L-Tyr) in the presence of the cofactor (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) and dioxygen. Besides its key role in the biosynthesis of L-Tyr, the precursor amino acid of several catecholamines (e.g. epinephrine, norepinephrine and dopamine), hPAH is an essential enzyme to maintain L-Phe homeostasis in mammals [3].

Mutations in the *PAH* gene leading to a decrease activity and/or in vivo instability of the hPAH protein have been associated with the autosomal recessive disorder phenylketonuria (PKU; OMIM 261600) and related forms of hyperphenylalaninemia (HPA). PKU and HPA represent the most common inborn error of amino acid metabolism, affecting approximately one in 10,000 Caucasians [3]. The phenotypic outcome of this disorder is highly heterogeneous, ranging from benign HPA (non-PKU HPA) to a severe classic form. When untreated, PKU patients present a severe psycho-motor delay [4] which can be prevented if a dietary restriction in L-Phe is implemented early after birth and maintained for life [5, 6]. Although the dietetic treatment remains the most effective treatment for PKU patients, it presents several limitations, which include: compliance difficult to maintain, especially to adolescents; specific nutritional deficiencies, usually associated with low animal protein content diets [7] and failure to prevent the foetal congenital abnormalities observed in maternal PKU [8]. Administration of the hPAH natural cofactor (BH_4) decrease the levels of circulating L-Phe mainly in non-PKU HPA and mild PKU patients [9, 10].

Enzymatic replacement therapy with recombinant Phe-degrading enzyme, phenylalanine ammonia-lyase (PAL), has been investigated [11, 12]. Nevertheless, PAL administration would not obviate the need for additional Tyr supplementation, while being highly immunogenic [13]. An efficient alternative to PAL therapy would be hPAH administration. The success of such a therapy will always depend on the stability of hPAH enzyme throughout formulation and storage.

Human PAH is a homotetrameric non-heme iron dependent enzyme. Each subunit (452 residues) folds into three distinct domains: (1) an N-terminal regulatory domain (residues 2–117) containing an auto-regulatory sequence and a phosphorylation site (Ser16); (2) a catalytic core domain (residues 118–410) and; (3) a C-terminal oligomerization domain (residues 411–452) containing a dimerization and a tetramerization motifs [14]. In a solution, a dimer-tetramer equilibrium is observed for the recombinant enzyme which is shifted towards the tetrameric form after enzyme incubation with L-Phe, while at physiological conditions the hPAH tetrameric form predominates. The hPAH tetramer is activated 5–6-fold on pre-incubation with L-Phe and demonstrates positive cooperativity for substrate binding [15].

Different approaches have been developed to enhance the in vitro stability of proteins, which include the use of additives in aqueous solutions, chemical modifications (pegylation) and protein engineering (directed mutagenesis) [16]. Additives commonly used as protein stabilizers include polyols, such as sorbitol, glycerol, polyethylene glycol (PEG), and different mono- or disaccharides [17]. Although the most common method for stabilizing liquid protein pharmaceuticals is the use of chemical additives [18], pegylation of recombinant forms of hPAH was, to our knowledge, the only experimental approach studied so far [19]. Taking into account the above-mentioned observations, we aimed to study and compare the efficacy of three polyol additives, namely glycerol, mannitol and PEG-6000, in preserving the biological function of a recombinant form of hPAH, stored at different temperatures, during a 1-month period. The specific enzymatic activity, as well as the oligomeric profile, was studied. In order to determine the influence of the added additive on hPAH structure and thermodynamic stability, urea denaturation curves and fluorescence quenching studies were performed.

Materials and Methods

Materials

The pTrcHis expression system was from Invitrogen (Carlsbad, CA, USA). The substrate L-Phe, HEPES, dithiothreitol (DTT) and mannitol (molecular biology grade) were from Sigma (St. Louis, MO, USA). The (6*R*)-L-*Erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) was purchased from Schircks Laboratories (Switzerland) and catalase from Roche Applied Science (Mannheim, Germany). Glycerol, urea, guanidinium-hydrochloride (Gnd-HCl), PEG-6000 and acrylamide (molecular biology grade) were purchased from Merck Chemicals (Darmstadt, Germany). Otherwise stated, all reagents were of analytical grade.

Expression of Recombinant Wild Type hPAH Protein

For prokaryotic expression of the recombinant enzyme the coding sequence of hPAH [20] (GenBank accession U49897) was used as previously reported [21] to obtain the respective expression construct encoding the hPAH protein (pTrcHis system).

The hPAH was expressed as a fusion protein in *E. coli* (TOP 10) using the 6×His peptide as a fusion partner (6×His-hPAH). Cells were grown at 37 °C, and expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Simultaneously, 0.1 mM ferrous ammonium sulphate [$(NH_4)_2Fe(II)SO_4$] was added to the culture medium. Bacteria were harvested 3 h after induction and disrupted by passage through a Carver Press (Model C, from F.S. Carver Inc, Wabash, IN, USA) at 4,000 psi, after 30 min incubation at 4 °C. The crude extract was centrifuged at 4,000×*g* for 20 min at 4 °C, and the obtained supernatant (soluble fraction) was used to purify the recombinant protein.

Protein Purification

Purification of the expressed recombinant protein was essentially performed as described elsewhere [21] using a nickel chelating resin (Ni-NTA resin; Qiagen, Venlo, The Netherlands) packed in a 9 cm polypropylene conical column (Poly-Prep columns; BioRad, Hercules, CA, USA), at 4 °C. The column was washed with 50 mM imidazole buffer (50 mM phosphate buffer, 300 mM NaCl, pH 7.8; 10% glycerol; 50 mM imidazole), and the recombinant proteins were eluted with 150 mM imidazole buffer. After purification, the 6×His-hPAH protein was dialysed (Spectra/Por®4; MWCO: 12–14 kDa) O.N. at 4 °C against a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA).

When necessary, the leader fusion peptide 6×His was excised from the recombinant fusion protein by cleavage with enterokinase (EK; Invitrogen). In this assay, 50 μg of purified enzyme were incubated with 1 U of EK in 200 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$ (ratio EK/fusion protein (*w*/*w*) of about 1:30) for 5 h at 4 °C.

Aliquots of the purified recombinant protein were stored at 4 and −20 °C, for 1 month, in the presence of several additives: 50% (*v*/*v*) glycerol, 10% (*v*/*v*) mannitol and 10% (*v*/*v*) PEG-6000. All additive solutions were filter-sterilized.

Electrophoresis and Protein Quantification

In order to assess the purity grade and integrity of the isolated recombinant protein, SDS-PAGE (10% gel) followed by Coomassie Brilliant Blue R250 staining was used. Protein

purity was assessed by densitometric scanning, followed by analysis using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA; version 4.5). The protein concentrations were determined by the method of Bradford [22] using bovine serum albumin as a standard.

Assay of hPAH Activity

The biological activity of hPAH was measured by quantifying the amount of Tyr produced by 1 mg of protein during a 1-min period (nmol Tyr $min^{-1} mg^{-1}$), as previously described [21], either in the activated form (pre-incubation with L-Phe) or in the non-activated form. The reaction mixture, performed in a 250 μL of final volume contained 1 mM L-Phe, 0.1 M Na-HEPES, pH 7.5, 0.2 M NaCl, 1,600 U mL^{-1} catalase, 10–15 μg of purified recombinant hPAH, 5 mM DTT, 100 μM $(NH_4)_2Fe(II)SO_4$ and 75 μM BH_4. The obtained Tyr was quantified indirectly by the nitrosonaphtol method [23] using fluorimetric detection (λ_{exc} 460 nm; λ_{em} 570 nm).

Size Exclusion Chromatography

The oligomeric profile of the recombinant protein, during storage, was estimated using size exclusion chromatography (SEC). Samples (40 μg of hPAH) were run, at 4 °C, on a Bio-Sil SEC250 300 mm×7.8 mm column (BioRad) connected to a Shimadzu HPLC system (Shimadzu Europe, Duisburg, Germany). Data were collected on a registrator/integrator model C-R6A (Shimadzu). The mobile phase (20 mM Na-HEPES and 0.2 M NaCl pH 7.0) was pumped at a flow rate of 0.5 mL min^{-1}. Identification of the peaks corresponding to aggregated, tetrameric and dimeric forms was performed by comparison with a calibration curve obtained by running thyroglobin (670,000 Da), bovine γ-globin (158,000 Da), chicken ovalbumin (44,000 Da) and equine myoglobin (17,000 Da) as standard proteins (BioRad).

Fluorescence Measurements

Intrinsic protein fluorescence is mainly due to the presence of the naturally fluorescent aminoacid Tryptophan (Trp) and to a lesser extent to Tyr. Since Trp emission maximum is strongly dependent of the polarity of its environment, fluorescence spectroscopy can provide valuable information on protein's conformational changes. Therefore, emission spectrum of hPAH was determined to monitor protein denaturation throughout storage. All data were obtained in a Hitachi F-2000 spectrofluorimeter (Hitachi High-Technologies, Germany) at constant temperature (25 °C), using λ_{exc} of 295 nm (slit 6 nm), λ_{em} from 300 and 460 nm (slit 4 nm) and a scan speed of 240 nm s^{-1}. Samples contained approximately 40 μg mL^{-1} of recombinant protein in order to maintain $A_{295}<0.02$ (minimizing inner filter effect). All spectra were corrected for blank emission and the inner filter effect (where appropriate).

Equilibrium Denaturation Assays

Analysis of urea or Gdn-HCl denaturation curves is a powerful tool to estimate protein conformational stability (ΔG^0). To achieve equilibrium denaturation, samples containing 40 μg mL^{-1} of protein, 0.1 M Na-HEPES (pH 7.5), 200 mM NaCl, 1.25 mM EDTA and 0–7.5 M Urea or 4 M Gdn-HCl were kept protected from light, for 18 h at 25 °C [24]. After incubation, fluorescence intensity was measured at λ_{em} of 337, 345 and 355 nm using a λ_{exc} of 295 nm.

Unfolding curves were analyzed using the three-state mechanism. For the unfolding transition, $N \leftrightarrows I \leftrightarrows D$ (where N is the native state, I is an intermediate state and D the denatured state), each step may be assumed to follow a two-state mechanism. The fraction of the intermediate state, f_{I}, in the reaction $N \leftrightarrows I$ can be obtained from Eq. 1:

$$\mathrm{f}_I = \left(b_{\mathrm{n}}^0 + a_{\mathrm{n}}[C] - y\right)/\left(b_{\mathrm{n}}^0 + a_{\mathrm{n}}[C] - b_{\mathrm{i}}^0 - a_{\mathrm{i}}[C]\right) \tag{1}$$

and the fraction of denaturation, $I \leftrightarrows D$, was calculated from Eq. 2:

$$\mathrm{f}_D = \left(b_{\mathrm{i}}^0 + a_{\mathrm{i}}[C] - y\right)/\left(b_{\mathrm{i}}^0 + a_{\mathrm{i}}[C] - b_{\mathrm{d}}^0 - a_{\mathrm{d}}[C]\right) \tag{2}$$

Where y is the fluorescence intensity measurement at a given urea concentration, $[C]$; b_{n}^0, b_{i}^0 and b_{d}^0 are the fluorescence intensity values for the N, I and D at 0 M urea, respectively; and a_{n}, a_{i} and a_{d} are the slopes of the pre-transition and post-transition baselines, respectively. The Gibbs energy change upon denaturation, ΔG_{NI} and ΔG_{ID}, were calculated by the linear extrapolation model [25], assuming a two-state transition for each step, according to the following equations:

$$\Delta G_{\mathrm{NI}} = -RT\ln K_{\mathrm{NI}} = -RT\ln(f_{\mathrm{I}}/(1-f_{\mathrm{I}})) \tag{3}$$

$$\Delta G_{\mathrm{ID}} = -RT\ln K_{\mathrm{ID}} = -RT\ln(f_{\mathrm{D}}/(1-f_{\mathrm{D}})) \tag{4}$$

$$\Delta G_{\mathrm{NI}} = \Delta G_{\mathrm{NI}}^0 - m_{\mathrm{NI}}[C] \tag{5}$$

$$\Delta G_{\mathrm{ID}} = \Delta G_{\mathrm{ID}}^0 - m_{\mathrm{ID}}[\mathrm{C}] \tag{6}$$

Where, for each step, K_{NI} K_{ID} are the equilibrium constants; ΔG_{NI}^0 ΔG_{ID}^0 are the Gibbs energy change upon denaturation in the absence of urea (in water) and m_{NI} and m_{ID} are the slope of the linear correlation between ΔG_{NI} or ΔG_{ID} and $[C]$, respectively.

Quenching Assays

Fluorescence quenching provides a means of probing the accessibility of Trp residues to small molecules (quenchers) thus giving information about protein's conformation. Fluorescence quenching experiments were performed by the step-wise addition of quenching solutions (5 M KI containing 10 mM $Na_2S_2O_3$ or 5 M acrylamide) to a solution of the studied protein at 40 $\mu g.mL^{-1}$ [26]. The fluorescence quenching data were analyzed according to the Stern–Volmer and the modified Stern–Volmer equations [27].

Analysis of Results

Sigma Plot (Jandel Scientific Corp.) was used for fitting the denaturation three-state model to the observed data. The kinetic parameters were calculated by non-linear regression analysis of the experimental data using the SigmaPlot SPSS Enzyme Kinetics module 1.1 software.

Results

In order to investigate the effect of storage on the biological function of the hPAH protein, the experiments were carried out using aliquots of the enzyme, which were never submitted to freeze-thawing cycles, being always discarded after use. After thawing, and before analysis, the protein solutions were centrifuged and the obtained supernatant was filtered to remove any insoluble aggregates. Whenever possible, a control assay, consisting of recombinant protein stored in buffered solution without additives (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) was introduced in the study. Results were expressed as the average of three independent assays.

Protein Quantification and Enzymatic Activity

The effect of 10% mannitol, 50% glycerol and 10% PEG-6000 on the hPAH protein content during storage at different temperatures is shown in Fig. 1. A significant reduction of the protein concentration was observed in all the tested solutions stored at 4 °C (Fig. 1a). After 48 h in the presence of 10% glycerol or 10% PEG-6000, a significant decrease in hPAH content was found, 57±2% and 46±10% respectively. However, in the presence of 10% mannitol only after 15 days of storage, a pronounced diminishing of soluble protein (46±6%) was detected.

At −20 °C (Fig. 1b) in the absence of additives (buffered solution), the protein was recovered after thawing in a precipitate form making impossible to detect any soluble hPAH. Therefore, no control solution was introduced in these experimental conditions. As expected, the addition of the tested stabilizers to the hPAH solution stored at −20 °C prevented protein loss throughout time. After 1 month, it was possible to recover 90±6% and 42±4% of protein using 10% mannitol and 50% glycerol, respectively. However, in the presence of 10% PEG-6000, only 6±3% of hPAH remained in a soluble form after that period.

When aliquots of the studied solutions (4 and −20 °C storage) were examined using SDS-PAGE (Fig. 2), it was found that a decrease in hPAH content, as well as the appearance of low molecular weight molecules occurred only at 4 °C in the presence 10% mannitol. This electrophoretic pattern remained unchanged upon addition of a protease inhibitor cocktail (results not shown).

The protein concentration in the stored solutions correlated with the determined specific enzymatic activity (Fig. 3). The observed decrease in protein content occurred simultaneously with the reduction of enzymatic activity, except for the hPAH stored in 10% mannitol, at 4 °C, after 8 days, in which the recovered enzyme (104±7%) presented only 52±12% of residual activity (Fig. 3a). The storage of recombinant protein, at −20 °C in solutions containing 10% mannitol or 50% glycerol resulted in the preservation of enzymatic activity (Fig. 3b). After 1 month, the hPAH showed 83±2% and 70±10% relative activities when stored in 50% glycerol or 10% mannitol, respectively. Nevertheless, in the presence of PEG-6000, the low amounts of recovered protein showed no enzymatic activity.

Size Exclusion Chromatography

The oligomeric state of the recombinant proteins, stored at −20 °C, either in 10% mannitol or 50% glycerol, was assessed, using size exclusion chromatography. As shown in Fig. 4a, the produced hPAH was expectedly resolved into three main components [28]: aggregated

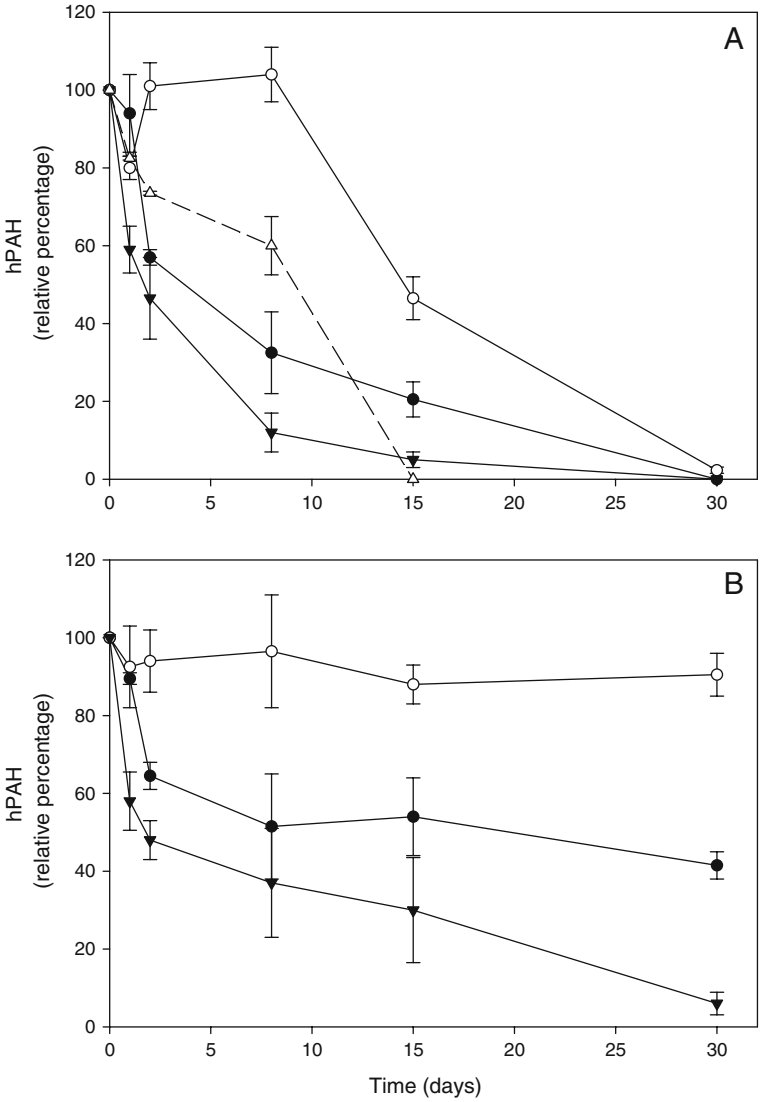

Fig. 1 Quantification of hPAH protein stored at 4 °C (**a**) and −20 °C (**b**), during 30 days, in buffered solution (*empty triangle*), 10% mannitol (*empty circle*), 50% glycerol (*filled circle*) and 10% PEG-6000 (*filled inverted triangle*). Data are shown as mean±SD of three independent assays, each performed in triplicate

and high oligomeric forms (26%), a major fraction corresponding to tetramers (60%) and a minor component of dimers (14%). No monomer fraction was detected. In the presence of 5 mM L-Phe the dimer⇆tetramer equilibrium was shifted towards the tetrameric form, being identified 80% tetramers and 5% dimers (Fig. 4b). After protein purification, no changes were observed in the described oligomeric profile for hPAH in buffered solutions, in 10% mannitol or 50% glycerol (results not shown). Since PEG-6000 revealed a negligible effect in preventing the loss of hPAH, this compound was not included in the study. However, when aliquots of the protein solution in 10% PEG-6000, stored at 4 or −20 °C, were

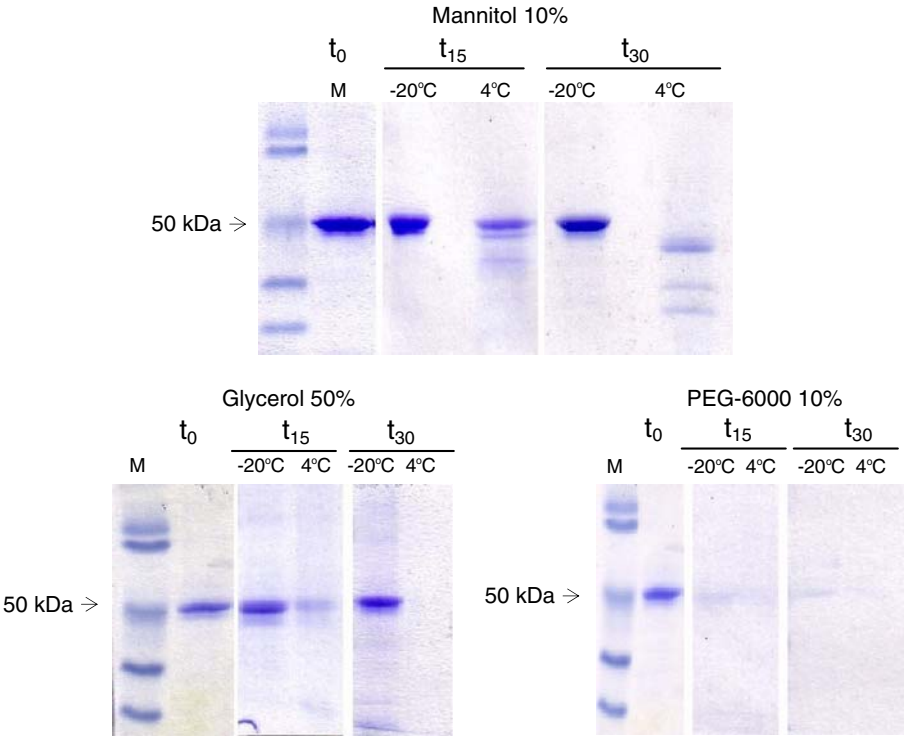

Fig. 2 SDS-PAGE analysis of hPAH protein after 15 days (t_{15}) and 30 days of storage (t_{30}), at 4 °C and −20 °C, in the presence of 10% mannitol, 50% glycerol and 10% PEG-6000. *M* molecular weight marker

analyzed, a main peak of aggregated forms was detected (results not shown). After 8 days at 4 °C in buffered solution the hPAH was recovered mainly as aggregated forms (≈82%), whereas after 15 days at the above temperature in 10% mannitol or 50% glycerol, the recombinant enzyme was detected mainly as a dimer (≈70%).

The relative percentage of the obtained forms after 30 days at −20 °C is presented on Table 1. Both 10% mannitol and 50% glycerol resulted in similar oligomeric patterns. When comparing with the profile obtained after protein purification (buffered solution), the relative percentage of aggregated forms found after 1 month was lower (9% in 10% mannitol and 2% in 50% glycerol), the tetramers content was similar (44% in 10% mannitol and 60% in 50% glycerol), but the relative amount of dimeric forms was higher (48% in 10% mannitol and 38% in 50% glycerol). As shown in Table 1, after 30 days at −20 °C, in 10% manitol or 50% glycerol, the dimeric forms maintained their capacity to assemble into tetrameric forms (dimer⇆tetramer equilibrium) after incubation with the substrate (5 mM L-Phe).

Catalytic Properties

Steady-state kinetic studies were performed on the purified recombinant protein. As seen in Fig. 5, the enzyme preparations revealed a rate concentration curve for L-Phe similar to that previously reported [29], including a positive cooperativity of L-Phe binding and a marked substrate inhibition, which was observed at concentrations of L-Phe≥1 mM. Since after a

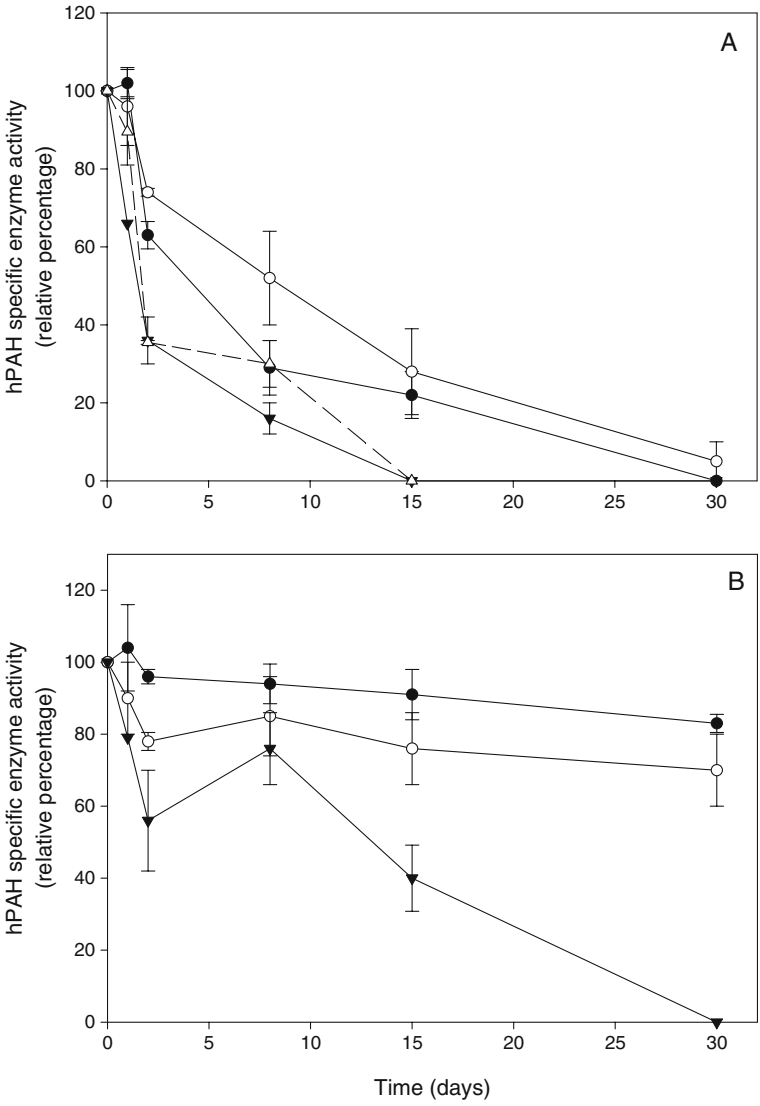

Fig. 3 Specific enzymatic activity of hPAH protein stored at 4 °C (**a**) and −20 °C (**b**), during 30 days, in buffered solution (*empty triangle*), 10% mannitol (*empty circle*), 50% glycerol (*filled circle*) and 30% PEG-6000 (*filled inverted triangle*). Data are shown as mean±SD of three independent assays, each performed in triplicate at standard conditions

1-month storage (t_{30}), in 10% mannitol or 50% glycerol, changes in the V_{max} values and in the affinity for L-Phe were observed (Table 2), the enzymatic assays were also performed in protein solutions containing 10% mannitol and 50% glycerol, after purification (t_0). At this time point, the recombinant hPAH stored in 10% mannitol revealed lower cooperativity and V_{max}, a lesser extend of substrate inhibition, and a higher $S_{0.5}$ (lower affinity), resulting in a catalytic efficiency ($K_{cat}/S_{0.5}$) of only 0.1. The $K_{cat}/S_{0.5}$ of the enzyme preparation in 50% glycerol was about 2-fold higher than that found for the enzyme dissolved in buffered solution. Higher catalytic efficiencies were also observed for the enzymes preserved during

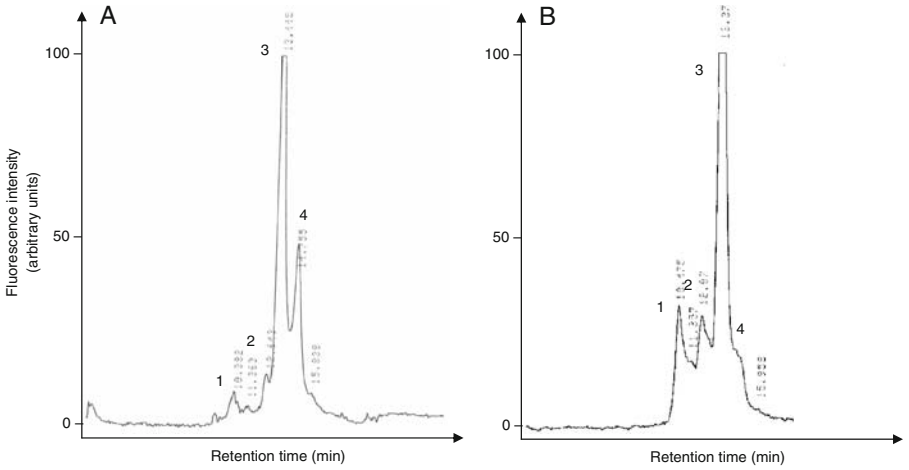

Fig. 4 Analytical size-exclusion chromatography of purified recombinant hPAH protein, in the non-activated (**a**) and after activation with 5 mM L-Phe (**b**), on a Bio-Sil SEC250 HPLC column equilibrated in 20 mM Na-HEPES and 0.2 M NaCl (pH 7.0, 0.5 mL min^{-1}); *1* aggregated forms, *2* octameric/hexameric forms, *3* tetrameric forms and *4* dimeric forms

1 month either in 10% mannitol (2.9-increase) or 50% glycerol (2-fold). The increased efficiency was mainly a result of the reduced $S_{0.5}$.

Unfolding Monitored by Fluorescence Spectroscopy

The hPAH contains three conserved Trp residues (hPAH residue 120, 187 and 326) and presents a fluorescence emission maximum at 335 nm (excitation at 295 nm). Upon incubation with high urea concentration a 10 nm red shift in the maximum value of fluorescence to about 350 nm is observed [24], as expected for solvent exposed Trp residues. Therefore, fluorescence spectroscopy was selected for monitoring denaturation that could occur along time storage. However, throughout the study it was not possible to observe any change in the emission maximum of the −20 °C stored enzyme either when

Table 1 Relative percentage of oligomeric forms of recombinant hPAH in the non-activated (−L-Phe) and activated states (+L-Phe), after 30 days of storage, at −20 °C, in the presence of 50% glycerol and 10% mannitol.

	Pre-incubation	Relative percentage		
	(5 mM L-Phe)	Aggregates	Tetramers	Dimers
Buffered solution[a]	−	18	56	16
	+	16	75	5
Glycerol 50%	−	2	60	38
	+	6	70	25
Mannitol 10%	−	9	44	48
	+	3	77	20

[a] Recombinant hPAH after purification. Results represent the average of three independent experiments

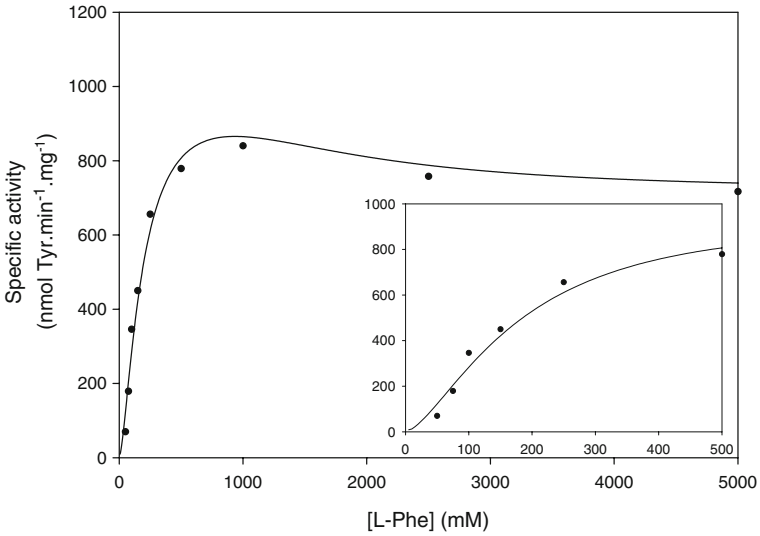

Fig. 5 The effect of substrate (L-Phe) concentration on the catalytic activity of the purified hPAH. The enzyme activity was assayed at standard conditions (0–3 mM L-Phe, 75 μM BH_4 and 25 °C). The *inset* represents the data obtained in the concentration range 0–500 μM L-Phe demonstrating the positive cooperativity

preserved in 10% mannitol or in 50% glycerol (results not shown). These results suggest that no major structural changes occurred.

Unfolding of the hPAH protein in urea was monitored by fluorescence emission at 345 nm (λ_{exc} 295 nm) as a function of the denaturant concentration. The urea equilibrium unfolding curves for the hPAH protein in buffered solution as well as in 10% mannitol or 50% glycerol showed the typical three-state form [24]. The thermodynamic parameters for denaturation at pH 7.5 and 25 °C were calculated using Eqs. 1–6, as shown in Table 3. When compared with the reference protein (buffered solution), the hPAH in 10% mannitol or 50% glycerol showed lower values for the Gibbs energy changes between the native and denatured states in water, ΔG^0, as well as for the *m* values for both the transition states. The transition midpoints (C_m) of the protein stored in the presence of 10% mannitol was

Table 2 Kinetic properties of the hPAH enzyme preserved in 50% glycerol and 10% mannitol, following purification (t_0) and after 30 days of storage (t_{30}), at −20 °C.

	V_{max} (nmol Tyr $min^{-1}mg^{-1}$)	$S_{0.5}$ L-Phe (μM)	*h*	$k_{cat}/S_{0.5}$ ($\mu M^{-1}min^{-1}$)
Buffered solution[a]	1,000	186	1.8	0.29
Glycerol 50% (t_0)	1,100	109	1.5	0.59
Mannitol 10% (t_0)	642	355	1.4	0.10
Glycerol 50% (t_{30})	714	77	1.6	0.52
Mannitol 10% (t_{30})	670	45	1.5	0.83

Results represent the average of three independent experiments

$k_{cat}/S_{0.5}$ represents the catalytic efficiency and was determined assuming a monomeric M_R of 50,000 Da.

[a] Recombinant hPAH after purification. All the assays were performed at standard conditions (75 μM BH_4, 25 °C)

Table 3 Thermodynamic parameters for the equilibrium unfolding of the recombinant hPAH protein after 30 days storage, at −20 °C, in 10% mannitol or 50% glycerol.

	First transition state				Second transition state			
	C_m (M)	ΔG^0_{NI} (kJ mol^{-1})	m_{NI} (kJ L mol^{-2})	$\Delta\Delta G^0_{NI}$	C_m (M)	ΔG^0_{ID} (kJ mol^{-1})	m_{ID} (kJ L mol^{-2})	$\Delta\Delta G^0_{ID}$
Buffered solution[a]	1.5	20	9.0	–	5.0	69	13.0	–
Glycerol 50%	1.6	17	10.0	−3.0	5.8	45	7.8	−24
Mannitol 10%	2.2	10	4.6	−10.0	5.9	58	9.8	−11

Urea unfolding of hPAH was monitored by fluorescence intensity as described in "Material and Methods". The thermodynamic parameters (ΔG^0, C_m, and m), for both states, were calculated using Eqs. 5 and 6. Results represent the average of three independent experiments

[a] Recombinant hPAH after purification

increased more significantly by 1.4 fold (first transition state) as compared with those of the protein stored in buffered solution.

Quenching Assays

To get an insight into the average exposure of the Trp residues during storage, fluorescence quenching studies, using KI and acrylamide as quenchers, were performed. A downward curvature for the Stern–Volmer plots was observed for the enzyme preserved in buffered solution as well as in 10% mannitol or 50% glycerol, after 1 month at −20 °C. The determined K_{sv} (Table 3) clearly increased upon storage. A 3.4 fold and a 12-fold increase were observed in the presence of 50% glycerol and 10% mannitol, respectively. Likewise, the fraction accessible for quenching (f_a) increased from 0.52 to 0.88 (50% glycerol) and 0.75 (10% mannitol). These results indicate that after 1 month in the presence of additives the enzyme presents an increased exposure of Trp residues to the ionic quencher.

When acrylamide was used as a quencher, the Stern–Volmer plots showed an upward deviation. As stated by Knapskogg [26], the observed deviation probably reflects a conformational change of protein occurring at high acrylamide concentrations. The determined K_{sv} (obtained by extrapolation from the initial plots of the quenching curves) were increased in the presence of additives (Table 4). A 1.3 fold and a 1.9 fold increase were obtained in the presence of 50% glycerol and 10% mannitol, respectively.

Table 4 KI and acrylamide quenching constants of the hPAH protein after 30 days storage, at −20 °C, in 10% mannitol and 50% glycerol.

	KI		Acrylamide
	K_{SV} (M^{-1})	f_a	K_{SV} (M^{-1})
Buffered solution[a]	1.7	0.52	4.5
50% glycerol	5.9	0.88	5.8
10% mannitol	12.0	0.75	8.6

K_{sv} and f_a were determined as described by Lehrer (27) according to the modified Stern–Volmer equation. Results represent the average of three independent experiments

[a] Recombinant hPAH after purification

Discussion

Human phenylalanine hydroxylase has been classified as an unstable protein, which rapidly loses its enzymatic activity in post-mortem tissues or in vitro, after purification [30]. It presents a short half-life, which has been estimated to be 9.4 h in hepatoma cells and 2 days in the liver [31]. Although hPAH has been extensively characterized regarding to its enzymatic characteristics and thermodynamic properties [24], no studies have been performed in order to understand and overcome the observed in vitro loss of function. Among the most used methods for the long-term storage of proteins, freeze-thawing and freeze-drying are the most common [32]. However, the freezing and drying processes can cause protein denaturation and consequently methods to preserve the macromolecule biological activity during both phases have been sought. Of the several freezing-induced stresses, those that are routinely recognized include cold temperature, exposure to concentrated solutes due to crystallization of water and crystallization of solutes as well as pH changes when buffer salts are involved. Resistance to all of these stresses can be increased by the addition of additives including sugars, polymers, polyols, methylamines and certain salts and amino acids, which usually are chosen on an empirical basis since the protective effect is variable and depends on the protein characteristics.

In order to identify polyol additives presenting an hPAH-stabilizing effect, a short-term storage study was performed using three different polyols (glycerol, mannitol and PEG-6000) as well as different storage temperatures (4 and −20 °C). Our data demonstrated that depending on temperature it is possible to preserve the biological activity of hPAH using two of the investigated additives, i.e. glycerol and mannitol. When stored at 4 °C, a significant loss of hPAH enzyme was observed (Fig. 1a), irrespective of the presence of additives. Taking into consideration that after thawing of the stored protein solution, the samples were centrifuged, the decrease in the recombinant enzyme content suggests the presence of aggregated insoluble forms, which are thereby removed before analysis. Interestingly, in the presence of 10% mannitol, fragments of low molecular weight were detected in the soluble hPAH recovered after 15 and 30 days, despite the addition of a protease inhibitor cocktail. These results suggest that chemical hydrolysis of the polypeptide chain is favoured under these experimental conditions. Since human PAH presents several amino acid motifs, namely Asn-Gly, Asn-Ser, Gln-Gly and Asp-Pro, localized in fairly flexible and hydrophilic regions, which could be a target for chemical instability [33], a detailed characterization of the obtained products is being undertaken.

At −20 °C glycerol and mannitol were able to protect the protein. After 1 month, the 10% mannitol enzyme solution lost 30% of residual activity, while in 50% glycerol only a 17% of residual activity was lost. As expected in the absence of those additives, it was impossible to recover any soluble protein, since it aggregated.

From the tested additives, PEG exerted no effect over the maintenance of hPAH content and activity. Although a commonly used protein stabilizing agent, a 10% concentration of PEG did not lead to any improvement on the preservation of hPAH biological activity, neither at 4 °C nor at −20 °C. This difference in stabilization might be due to the fact that PEG is essentially a non-polar polyol and can bind to the hydrophobic sites of proteins thus promoting unfolding [34]. Therefore this could explain the finding of a main peak of aggregated forms in hPAH solutions of 10% PEG-6000.

It is well known that, in solution, hPAH exists in a delicate balance between dimers and tetramers, and that mutations in the aminoacid sequence usually results in production of unstable proteins which are purified mainly as inactive soluble aggregates [35]. Therefore, the hPAH oligomeric profile was monitored throughout the experiment. Our results suggest

that the presence of polyol additives prevented hPAH aggregation and preserved the necessary oligomeric assembly. The probable explanation for the obtained lower enzymatic activity when the protein was stored in buffered solution, at 4 °C, is that the protein exists mainly in its dimeric form, which it is known to present lower enzymatic activity [36]. Nevertheless, monomeric forms of hPAH were never detected. This observation could indicate: (1) that the dimers are very stable molecules and consequently very difficult to dissociate; or (2) that the monomers are very unstable forms, which in solution will never be observed. It must be emphasized that although mannitol contributed to the maintenance of 90% of protein (after 30 days of storage) the recovered enzyme activity was only 70% of the initial value since the percentage of dimeric forms were higher than the percentage of tetramers.

To study in more detail the stabilizing effect of the additives, their role in the conformational stability of hPAH was examined using urea-induced denaturation assays. This allowed evaluating the urea-induced denaturation free energy change (ΔG^0), which refers to the thermodynamic stability of the overall protein conformation. Unexpectedly, for both the transition states the buffered protein solution was more stable than the protein stored in mixed solvent ($\Delta\Delta G^0$ negative values). This negative effect was more pronounced for the second transition state where in buffered solution the enzyme is approximately 10 and 20 kcal/mol more stable than when stored in 10% mannitol or 50% glycerol, respectively. Data in Table 3, besides presenting a difference in the stabilization free energy of the protein in different solutions, also show that the denaturation midpoints (C_m) were slightly higher for both transition states and that the denaturation m value, in general, was lower. The m value is the dependence of the unfolding free energy of denaturant concentration in the transition region of the denaturation curve (the function of the slope of the transition). For many proteins, it has been shown to correlate with the amount of protein surface area exposed to solvent upon unfolding. Therefore, the decreased m values indicate a change in the structure of the intermediate and the denatured state (lower exposure of aminoacid residues) probably reflecting the existence of a residual amount of secondary structure. It must be noted that during the urea-induced denaturation assays, the increase of fluorescence intensity did not reach a plateau even at high urea concentrations, thus confirming the existence of residual structures from the native/intermediate enzyme at this high urea concentrations.

To further investigate any protein conformational change occurring upon storage in the presence of additives, Trp fluorescence quenching assays were conducted, thereby following any change in this aminoacid environment. The obtained values for K_{SV} and f_a, were compatible with the presence of a lower compactness of the hPAH structure, when stored at −20 °C in the presence of the studied additives. Since in hPAH, internal protein dynamics involving the tertiary and quaternary structures of the enzyme seem to play an important role in the regulation of its biochemical properties, we looked for the kinetic characteristics of the stored enzyme. The enzymatic properties of hPAH were in agreement with the obtained quenching data. The observed increment in the catalytic efficiency, positive cooperativity of substrate binding, substrate inhibition and affinity for L-Phe, are probably due to an increase in the active site accessibility to the substrate resulting from the conformational changes induced by glycerol or mannitol. Moreover, the observed effect seems to be similar to the one induced by L-Phe binding to the protein. This effect has been reported to cause large conformational changes in the catalytic domain [26, 37–39], which in turn are believed to trigger large-scale structural changes throughout the entire protomer, including a displacement of the autoregulatory sequence, which partly covers the active site in the resting state, resulting in a catalytically high activity state of hPAH.

In summary, two of the studied polyols, i.e. glycerol and mannitol, were able to preserve the enzymatic activity of the recombinant human hPAH protein stored at −20 °C during 1 month. Glycerol contributed to the presence of the characteristic oligomeric profile (mainly tetrameric forms) and was able to stabilize a form of hPAH presenting a positive cooperativity to L-Phe. Two possible mechanisms for protein stabilization by additives have been described: the preferential hydration model proposed by Timasheff [40], and the approach proposed by Bolen et al., by which the stabilizing effect is attributed mainly to a large contribution from interactions with exposed backbone groups in a partially unfolded state [41]. The fact that for the recombinant hPAH the thermodynamic property did not show a dominant contribution toward stability in the presence of polyol additives indicates that the retention of enzyme activity is mainly due to conformational changes. One explanation could be that in the presence of 10% mannitol or 50% glycerol the catalytic site in hPAH is folded in such a way that it is well suited for substrate binding. This mechanism has already been reported for the observed stability of ferric horse myoglobin in the presence of glycerol [42]. Moreover, modifying the microenvironment of the enzyme prevented aggregation, which is one of the main causative factors described for the inactivation of hPAH.

To our knowledge, this is the first study on the effect of different polyol compounds, such as glycerol, mannitol and PEG-6000, on the in vitro stability of purified hPAH produced in a heterologous prokaryotic expression system and allowed screening for polyol compounds to be used in a long-term storage study (12 months) which is presently running in our laboratory. Additionally, preliminary data also point out for an effective stabilization by polyols upon hPAH lyophilisation [43]. However, the changes taking place in the protein and the most likely conformation acquired in polyol solutions are still unknown. Using Far-UV Circular Dichroism and Dynamic Light Scattering, studies are being undertaken to identify changes occurring both at secondary and tertiary structural levels, respectively. Furthermore, the possibility that the presence of additives would retard certain chemical degradations, usually occurring during protein storage, cannot be excluded. One must also take into consideration that it remains unclear whether the strategies found to stabilize two-state proteins will work well for proteins with equilibrium intermediates. Stabilizing these proteins requires specifically stabilizing the native state regarding to the intermediates, as these are expected to lack activity. Nevertheless, the knowledge of the physicochemical processes that cause irreversible changes in the structure and biological activity of hPAH could greatly aid in the rational development of formulations designed to enhance the stability of the protein product and to maintain its efficacy during production, purification, storage and ultimate application of the protein medicinal product.

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References

1. Lichter-Konecki, U., Hipke, C. M., & Konecki, D. S. (1999). *Molecular Genetics and Metabolism, 67*, 308–316.
2. Moller, N., Meek, S., Bigelow, M., Andrews, J., & Nair, K. S. (2000). *Proceedings of National Academy of Sciences USA, 97*, 1242–1246.
3. Scriver, C. R. (2007). *Human Mutation, 28*, 831–845.
4. Surtees, R., & Blau, N. (2000). *European Journal of Pediatrics, 159*(Suppl 2), S109–S113.
5. (1993). *Arch Dis Child*, *68*, 426–427.

6. (2001). *Pediatrics*, *108*, 972–982.
7. de Freitas, O., Izumi, C., Lara, M. G., & Greene, L. J. (1999). *Nutrition Reviews, 57*, 65–70.
8. Widaman, K. F., & Azen, C. (2003). *Pediatrics, 112*, 1537–1543.
9. Kure, S., Hou, D. C., Ohura, T., Iwamoto, H., Suzuki, S., Sugiyama, N., et al. (1999). *Journal of Pediatrics, 135*, 375–378.
10. Blau, N., & Erlandsen, H. (2004). *Molecular Genetics and Metabolism, 82*, 101–111.
11. Sarkissian, C. N., Shao, Z., Blain, F., Peevers, R., Su, H., Heft, R., et al. (1999). *Proceedings of National Academy of Sciences USA, 96*, 2339–2344.
12. Gamez, A., Sarkissian, C. N., Wang, L., Kim, W., Straub, M., Patch, M. G., et al. (2005). *Molecular Therapeutic, 11*, 986–989.
13. Sarkissian, C. N., & Gamez, A. (2005). *Molecular Genetics and Metabolism, 86*(Suppl 1), S22–S26.
14. Flatmark, T., & Stevens, R. C. (1999). *Chemical Reviews, 99*, 2137–2160.
15. Thorolfsson, M., Ibarra-Molero, B., Fojan, P., Petersen, S. B., Sanchez-Ruiz, J. M., & Martinez, A. (2002). *Biochemistry, 41*, 7573–7585.
16. Frokjaer, S., & Otzen, D. E. (2005). *Nature Reviews Drug Discovery, 4*, 298–306.
17. Crommelin, D. J., Storm, G., Verrijk, R., de Leede, L., Jiskoot, W., & Hennink, W. E. (2003). *International Journal of Pharmaceutics, 266*, 3–16.
18. Wang, W. (1999). *International Journal of Pharmaceutics, 185*, 129–188.
19. Gamez, A., Wang, L., Straub, M., Patch, M. G., & Stevens, R. C. (2004). *Molecular Therapeutic, 9*, 124–129.
20. Kwok, S. C., Ledley, F. D., DiLella, A. G., Robson, K. J., & Woo, S. L. (1985). *Biochemistry, 24*, 556–561.
21. Leandro, P., Rivera, I., Lechner, M. C., de Almeida, I. T., & Konecki, D. (2000). *Molecular Genetics and Metabolism, 69*, 204–212.
22. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
23. Kaufman, S. (1987). *Methods in Enzymology, 142*, 3–17.
24. Kleppe, R., Uhlemann, K., Knappskog, P. M., & Haavik, J. (1999). *Journal of Biological Chemistry, 274*, 33251–33258.
25. Pace, C. N. (1986). *Methods in Enzymology, 131*, 266–280.
26. Knappskog, P. M., & Haavik, J. (1995). *Biochemistry, 34*, 11790–11799.
27. Lehrer, S. S. (1971). *Biochemistry, 10*, 3254–3263.
28. Knappskog, P. M., Flatmark, T., Aarden, J. M., Haavik, J., & Martinez, A. (1996). *European Journal of Biochemistry, 242*, 813–821.
29. Kaufman, S. (1993). *Advances in Enzymology and Related Areas of Molecular Biology, 67*, 77–264.
30. Woo, S. L., Gillam, S. S., & Woolf, L. I. (1974). *Biochemical Journal, 139*, 741–749.
31. Chang, N., Kaufman, S., & Milstien, S. (1979). *Journal of Biological Chemistry, 254*, 2665–2668.
32. Wang, W. (2000). *International journal of pharmaceutics, 203*, 1–60.
33. Powell, M. F. (1996). In R. Pearlman & Y. J. Wang (Eds.), *Formulation, characterization and stability of protein drugs* (pp. 1–140). New York: Plenum Press.
34. Shulgin, I. L., & Ruckenstein, E. (2006). *Biophysical Chemistry, 120*, 188–198.
35. Pey, A. L., Desviat, L. R., Gamez, A., Ugarte, M., & Perez, B. (2003). *Human Mutation, 21*, 370–378.
36. Bjorgo, E., de Carvalho, R. M., & Flatmark, T. (2001). *European Journal of Biochemistry, 268*, 997–1005.
37. Shiman, R., Gray, D. W., & Pater, A. (1979). *Journal of Biological Chemistry, 254*, 11300–11306.
38. Andersen, O. A., Flatmark, T., & Hough, E. (2002). *Journal of Molecular Biology, 320*, 1095–1108.
39. Stokka, A. J., Carvalho, R. N., Barroso, J. F., & Flatmark, T. (2004). *Journal of Biological Chemistry, 279*, 26571–26580.
40. Timasheff, S. N. (1998). *Advances in Protein Chemistry, 51*, 355–432.
41. Bolen, D. W., & Baskakov, I. V. (2001). *Journal of Molecular Biology, 310*, 955–963.
42. Barteri, M., Gaudiano, M. C., & Santucci, R. (1996). *Biochimica et Biophysica Acta, 1295*, 51–58.
43. Lino, P. R., Almeida, A. J., de Tavares, A. I., & Leandro, P. (2009). *FEBS Journal, 276*, 323.