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Thermal-induced denaturation of two model proteins: effect of poloxamer 407 on solution stability

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Summary

In an attempt to enhance the physical stability of two model proteins in solution toward thermal-induced denaturation, we investigated the effect of the nonionic surfactant poloxamer 407 (Pluronic® F-127) with a nonrecombinant (urease) and recombinant-derived (interleukin 2) protein. Our aim was to determine the time-dependent loss in biological activity when urease and recombinant interleukin-2 (rIL-2) were dissolved in pH 7 phosphate buffer (PB) with or without poloxamer 407 (P-407) and stored at elevated temperatures for 96 h. In addition, urease was selected to determine whether denaturation due to the thermal stress was reversible. Resulting percent activity remaining vs time data for each protein were fitted to a mono- (75°C) or biexponential (37 and 50°C) equation and the area-under-the-curve ($AUC|_{0\rightarrow 96}$) calculated. A significantly ($p < 0.05$) greater fraction of the enzymatic activity of urease was irreversibly lost when urease was dissolved in PB only and incubated at 37 and 50°C compared to urease incubated in a PB solution which contained P-407. However, for urease/PB solutions incubated at 75°C, addition of P-407 resulted in an increased rate of enzyme inactivation. Results with rIL-2 suggest that P-407 had no protective effect against thermal-induced denaturation at 50°C. Activity-time profiles described by biexponential equations may suggest an initial rapid unfolding phase followed by a slower unfolding/refolding phase for both proteins evaluated in either solvent system.

Introduction

Protein drugs approved for use in the treatment of a variety of diseases have special formulation and administration requirements. Since these potent protein pharmaceuticals are exposed to a variety of environmental conditions during shipment to worldwide markets, it is important to develop strategies to minimize physical denaturation of these drugs.

It has been reported that the processes that give rise to physical instability of protein pharmaceuticals are denaturation, aggregation, precipitation, and adsorption (Manning et al., 1989). Of these physical processes, denaturation which results in irreversible inactivation of a protein is perhaps the greatest obstacle to the successful formulation of protein pharmaceuticals. In general, irreversible inactivation with subsequent loss

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in biological activity of a protein occurs when a protein has lost all or a fraction of its three-dimensional conformation (tertiary structure). Denaturation of protein pharmaceuticals in solution may arise from extremes in pH, temperature, ionic strength, and solution agitation. Thermal-mediated denaturation would potentially be the predominant mechanism for loss in the biological activity of a protein drug that would be distributed to warm climates. A number of approaches have been suggested to enhance the physical stability of various protein drugs. Strategies that have been employed in the formulation of protein drugs include the use of low ionic strength buffers, avoidance of transition metals, use of carbohydrates, polyalcohols, and certain amino acids, and the incorporation of surfactants (Wang and Hanson, 1988). The latter approach has previously been investigated in our laboratory (Fults and Johnston, 1990; Johnston et al., 1992; Pec et al., 1992; Wang and Johnston, 1993).

Previously, we have shown that the nonrecombinant protein (urease) and the DNA-derived recombinant protein (recombinant interleukin-2; rIL-2), when dissolved in pH 7 PB and subjected to vigorous solution agitation for 96 h at 4°C, were irreversibly inactivated (Wang and Johnston, 1993). However, when similar solutions of both urease and rIL-2 were prepared using P-407 at a concentration of 10 and 0.5% w/w, respectively, and subjected to identical hydrodynamic and temperature conditions, no loss in biological activity was observed over the 96 h test period (Wang and Johnston, 1993). Thus, it was concluded that the nonionic surfactant P-407 might be useful as an excipient in parenteral protein formulations to minimize agitation-induced denaturation with subsequent loss in biological activity when such protein pharmaceuticals were formulated either as an aqueous solution or as a lyophilized powder intended for reconstitution. The purpose of the present study was to investigate whether thermal stress of solutions of two model proteins would result in the irreversible denaturation of the proteins, and whether P-407 might be beneficial as an excipient to minimize/eliminate thermal denaturation of both model proteins in solution.

Materials and Methods

Materials

Urease (from jack beans, type IV, lot 55F-9357) having an activity of 63 000 U/g and Na_2HPO_4 were purchased from Sigma (St. Louis, MO). rIL-2 formulated with human serum albumin (HSA) and mannitol was generously provided as a gift by Hoffmann-La Roche, Inc. (Nutley, NJ). Poloxamer 407, N.F. XVII grade, was a gift from the BASF Corp. (Parsippany, NJ) and was used as received. KH_2PO_4, sodium hydroxide, and EDTA tetrasodium salt were obtained from Fisher (Fair Lawn, NJ) and used as received.

Human peripheral blood lymphocytes (PBLs) were obtained from whole blood using techniques in our laboratory (Johnston et al., 1992). Growth medium employed in the bioassay of rIL-2 was Iscove's Modified Eagle Medium (IMEM) (Gibco Laboratories) with 10% v/v fetal calf serum (FCS). Cell density calculations were performed using a hemocytometer and trypan blue (Gibco Laboratories) to stain viable lymphocytes.

Bioassay of rIL-2 was determined using flat-bottomed, 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). Harvested lymphocytes were pulsed with [^{3}H]thymidine (1 μCi of [^{3}H]thymidine; specific activity, 1 μCi/μmol) (Amersham, Arlington Heights, IL). DNA fragments labelled with [^{3}H]thymidine were harvested on filter paper (Whitaker M.A. Bioproducts) using a Brandel cell harvester (model no. M-125, Gaitherburg, MD). Dried filter papers were combined with 3 ml of Aquasol® scintillation fluid (Research Products Inc., Rockford, IL) and counted in a liquid scintillation counter (Beckman, model no. LS 5801, Irvine, CA) at 2 sigma error.

The biological activity of urease was determined as described previously (Fults and Johnston, 1990) using a urea nitrogen assay kit purchased from Sigma (St. Louis, MO).

Instruments

A Perkin-Elmer lambda 2 UV/Vis spectrophotometer (Norwalk, CT) was used to determine the biological activity of urease.

Methods

Protein bioactivity determinations. The activity of urease was determined as described previously (Fults and Johnston, 1990). In brief, the urea nitrogen kit measured the amount of ammonia generated as a result of urea hydrolysis. The assay kit was based on the reaction of ammonia with alkaline hypochlorite and phenol which was catalyzed by sodium nitroprusside to produce indophenol. The indophenol was then determined spectrophotometrically at 570 nm and was proportional to the amount of ammonia that was liberated.

The biological activity of rIL-2 was assessed as described previously (Johnston et al., 1992) using standard techniques in our laboratory. Briefly, freshly harvested PBLs were evaluated for their proliferative response when stimulated with rIL-2 by determining the amount of [^{3}H]thymidine incorporated.

Protein physical stability studies during thermal cycling. To assess the percent loss in the biological activity of urease in aqueous solutions incubated at various temperatures, a known concentration of substrate (urea; 30 mg/ml) was allowed to react with samples of the thermally stressed test solutions containing urease to convert most of the urea to ammonia as previously described (Wang and Johnston, 1992). In brief, a concentration of enzyme (protein) was selected based on previous work (Wang and Johnston, 1992) that converts a known amount of urea to NH_3 such that the absorbance readings remain in the linear portion of the calibration curve (Wang and Johnston, 1992).

The urease solutions incubated at 37, 50 and 75°C were freshly prepared in triplicate by adding urease powder to 30 ml of phosphate buffer (pH 7.0) to yield a urease concentration of 0.33 mg/ml (= 20.8 IU activity/ml). The pH 7.0 phosphate buffer was prepared using double deionized water containing 1 mM EDTA to prevent the possibility of metal-ion catalyzed hydrolysis of essential sulfhydryl groups on urease (Tirrell and Middleman, 1978). The three 30 ml solutions of urease were then individually divided into two equal volumes. All six vials were then placed in a water bath maintained at 37 ± 0.1°C. At 0, 2, 4, 8, 12, 24, 48, 72, and 96 h, three 0.1 ml samples were removed from each of the six vials. The three urease solution samples obtained from each of three vials comprising the first set of three vials were then analyzed for enzymatic activity immediately following removal from the 37°C water bath. Samples obtained from the second set of three vials were analyzed for urease activity after the solutions were allowed to cool passively to room temperature. Similar sets of urease solutions were prepared and studied at 50 and 75°C. Samples (0.1 ml) which were retrieved from solutions incubated at 37, 50 and 75°C were allowed to remain undisturbed at room temperature for 1 h prior to determination of urease activity.

To assess whether P-407 would minimize/eliminate denaturation with subsequent loss in the biological activity of urease when a urease solution was thermally stressed, an additional set of three 30 ml solutions of urease were prepared as above but in a PB containing P-407 at a concentration of 1% w/w. The individual urease solutions were again equally divided into two sets of three vials which were incubated and sampled as described above with determination of enzymatic activity either immediately following removal of the samples from the vials in the water bath or subsequent to the samples being allowed to cool to room temperature. The urease solutions were sampled at the same time points as used for the urease solutions that contained no P-407. For each temperature studied, the mean percent enzymatic activity of urease remaining at each time point was calculated relative to the mean value of the activity determined at time $t = 0$ h and plotted as the percent activity remaining vs time.

To determine the effect of thermal stress on the biological activity of rIL-2, a set of three 10 ml rIL-2/pH 7.0 PB solutions that contained 3×10^4 U of rIL-2 (= 1.88 μg rIL-2) were prepared under sterile conditions. The rIL-2 solutions were incubated in a water bath at 50°C for 96 h. Triplicate samples were removed from individual vials at 0, 2, 4, 8, 12, 24, 48, 72, and 96 h to assess the biological activity of rIL-2. All samples were immediately frozen at −70°C until the time of bioassay.

The effect of P-407 on the biological activity of rIL-2 solutions when subjected to thermal stress was evaluated by a similar procedure to that for the urease solutions. An additional set of three rIL-2/pH 7.0 PB solutions were prepared as described above. However, rIL-2 solutions included in this set contained P-407 at a concentration of 0.5% w/w. All rIL-2 solutions were sampled at the same time points as the rIL-2 solutions containing no P-407. Samples collected at each time point from test solutions with or without P-407 were plated in triplicate and the mean value of the [^{3}H]thymidine incorporated at a given time point compared to the [^{3}H]thymidine uptake by lymphocytes at time $t = 0$ h. Results were expressed as the percent rIL-2 activity remaining vs time.

Data analysis. Using nonlinear least-squares regression analysis, a biexponential (37 and 50°C) or monoexponential (75°C) equation was determined for each set of percent activity remaining vs time data obtained for urease solutions. Table 1 lists the mean values for the rate constant(s) and area-under-the-curve (AUC_{0-96}) as well as the value of the square of the multiple correlation coefficient (R^2) for each data set analyzed by nonlinear least-squares regression. The rate constants (k_1 and k_2) and corresponding AUC_{0-96} values for a group of three urease solutions were then compared to appropriate groups for statistical significance using Student's t-test to determine whether the enzyme was irreversibly denatured and whether the addition of P-407 would minimize/eliminate thermally induced denaturation (Table 2). For activity-time data described by a biexponential equation, k_1 and k_2 represent the rate constants associated with the initial and terminal exponential phases, respectively.

As with urease solutions, a biexponential equation was determined for each of the mean

TABLE 1

Rate constants and areas-under-the-curve ($AUC|_{0\to96}$) for the fitted activity-time profiles

Groups	Parameters			
	k_1	k_2	$AUC|_{0\to96}$	R^2
A1	0.121 ± 0.2258 [a,b]	0.003 ± 0.0010	4563 ± 176	0.9884
A2	1.346 ± 1.0860	0.002 ± 0.0014	4131 ± 77	0.9845
A3	0.059 ± 0.0208	$3.84 \times 10^{-4} \pm 1.211 \times 10^{-4}$	6453 ± 41	0.9996
A4	0.136 ± 0.0224	$3.11 \times 10^{-4} \pm 3.424 \times 10^{-4}$	5174 ± 510	0.9895
B1	0.135 ± 0.0235	0.006 ± 0.0057	3699 ± 543	0.9884
B2	0.273 ± 0.2915	0.007 ± 0.0038	4868 ± 542	0.9982
B3	0.139 ± 0.0365	0.006 ± 0.0025	5712 ± 712	0.9968
B4	0.040 ± 0.0421	0.009 ± 0.0015	6722 ± 396	0.9972
C1	0.539 ± 0.0248	N.A.	186 ± 8.5	1.0000
C2	0.546 ± 0.0435	N.A.	184 ± 14.5	1.0000
C3	1.125 ± 0.2350	N.A.	91 ± 17.1	1.0000
C4	0.720 ± 0.0154	N.A.	139 ± 3.0	1.0000
D1	0.900 ± 1.3481	0.010 ± 0.0060	3395 ± 579	0.9877
D2	0.060 ± 0.0130	0.022 ± 0.0199	2668 ± 595	0.9762

A1, urease in PB incubated and assayed at 37°C; A2, urease in PB incubated at 37°C and assayed at room temperature; A3, urease in P-407 (1% w/w) incubated and assayed at 37°C; A4, urease in P-407 (1% w/w) incubated at 37°C and assayed at room temperature; B1–B4 and C1–C4 correspond to same groups as A1–A4 but evaluated at 50 and 75°C, respectively. D1, rIL-2 in PB incubated at 50°C; D2, rIL-2 in P-407 (0.5% w/w) incubated at 50°C. N.A., not applicable; R^2, square of the multiple correlation coefficient.

[a] Mean value.

[b] Standard deviation of the mean.

TABLE 2

Statistical comparison of fitted parameters for groups listed in Table 1

Groups	Parameters		
	k_1	k_2	$AUC\|_{0\to 96}$
A1 vs A2			
A3 vs A4			
A1 vs A3			* [a]
A2 vs A4			* [a]
B1 vs B2			
B3 vs B4		* [a]	
B1 vs B3			* [a]
B2 vs B4			* [a]
C1 vs C2		N.A.	
C3 vs C4		N.A.	
C1 vs C3	* [a]	N.A.	* [a]
C2 vs C4	* [a]	N.A.	* [a]
D1 vs D2			

A1, urease in PB incubated and assayed at 37°C; A2, urease in PB incubated at 37°C and assayed at room temperature; A3, urease in P-407 (1% w/w) incubated and assayed at 37°C; A4, urease in P-407 (1% w/w) incubated at 37°C and assayed at room temperature; B1–B4 and C1–C4 correspond to same groups as A1–A4 but evaluated at 50 and 75°C, respectively. D1, rIL-2 in PB incubated at 50°C; D2, rIL-2 in P-407 (0.5% w/w) incubated at 50°C. N.A., not applicable.

[a] Significant difference ($p < 0.05$) using Student's t-test.

activity remaining vs time profiles for the two solutions containing rIL-2. The mean values for the rate constants (k_1 and k_2) and $AUC|_{0\to 96}$ are listed in Table 1 and compared for statistical significance using Student's t-test analogous to data treatment for urease solutions (Table 2). This study only examined the potential protective effect of P-407 on the solution stability of rIL-2 when subjected to thermal stress. The renaturation of the thermally stressed rIL-2 at 50°C and room temperature was not investigated.

Results

Urease stability in thermally stressed solutions

Statistical results using Student's t-test to compare rate constants (k_1 and k_2) and AUC_{0-96} values between different groups for statistical significance are shown in Table 2. Figs 1–3 show the best-fit curves for the four different groups of urease solutions incubated at 37, 50 and 75°C, respectively. Urease in PB incubated at 37 and 50°C and assayed at elevated temperature when compared to samples assayed at room temperature showed no significant differences in k_1, k_2, and AUC_{0-96}. However, urease in PB which con-

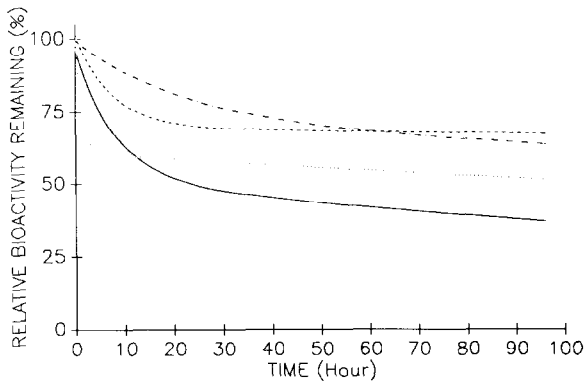

Fig. 1. Percent relative enzymatic activity of urease remaining vs time. (———) Urease in phosphate buffer (PB) incubated and assayed at 37°C, (· · · · · ·) urease in PB incubated at 37°C and assayed at room temperature, (– · – · –) urease in PB with 1% w/w poloxamer 407 (P-407) incubated and assayed at 37°C, and (------) urease in PB with 1% w/w P-407 incubated at 37°C and assayed at room temperature.

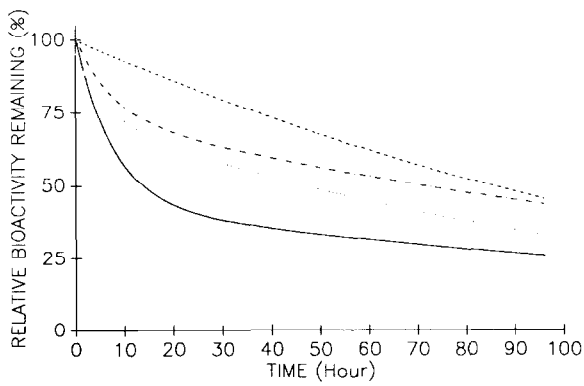

Fig. 2. Percent relative enzymatic activity of urease remaining vs time. (———) Urease in PB incubated and assayed at 50°C, (· · · · · ·) urease in PB incubated at 50°C and assayed at room temperature, (– · – · –) urease in PB with 1% w/w P-407 incubated and assayed at 50°C, and (------) urease in PB with 1% w/w P-407 incubated at 50°C and assayed at room temperature.

tained P-407 and which had been incubated and assayed at 50°C showed a significant difference in the value of k_2 when compared to the corresponding samples assayed at room temperature. Urease in PB which had either been incubated and assayed at 75°C or incubated at 75°C and assayed at room temperature, when compared to corresponding urease/PB solutions which contained P-407, showed a significant difference in the value of k_1. Urease solutions prepared with PB when compared to corresponding solutions in PB which contained P-407 showed significant differences in AUC_{0-96} values at all temperatures studied. Thus, it would appear that a certain fraction of enzymatic activity was irreversibly lost when urease was incubated at each temperature. The fraction of enzymatic activity irreversibly lost for urease solutions incubated at 37, 50, and 75°C decreased in the following order: $\text{activity}_{75°C} > \text{activity}_{50°C} > \text{activity}_{37°C}$.

rIL-2 stability in thermally stressed solutions

Fig. 4 represents the fitted curves for two groups of rIL-2 solutions incubated at 50°C. As shown in Fig. 4 and Table 2, no significant difference was found for the rate constants (k_1 and k_2)

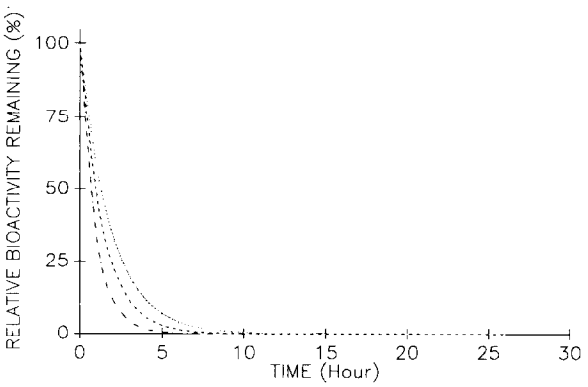

Fig. 3. Percent relative enzymatic activity of urease remaining vs time. (———) Urease in PB incubated and assayed at 75°C, (· · · · · ·) urease in PB incubated at 75°C and assayed at room temperature, (– · – · –) urease in PB with 1% w/w P-407 incubated and assayed at 75°C, and (------) urease in PB with 1% w/w P-407 incubated at 75°C and assayed at room temperature.

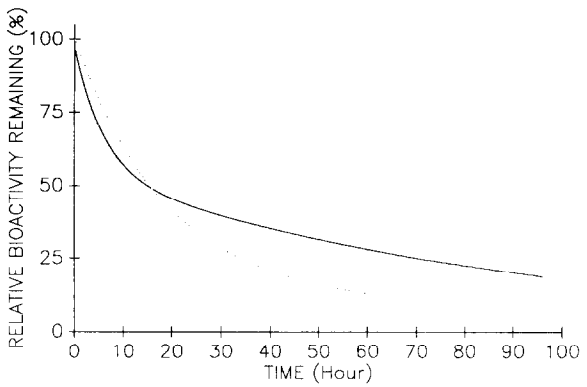

Fig. 4. Percent relative biological activity of rIL-2 remaining vs time. (———) rIL-2 in PB incubated at 50°C, and (· · · · · ·) rIL-2 in PB with 0.5% w/w P-407 incubated at 50°C.

and the $AUC|_{0\to 96}$ values for the percent biological activity remaining vs time profiles for rIL-2 solutions with (0.5% w/w) or without P-407 that were incubated at 50°C. At 96 h, the percent of biological activity remaining was approx. 10 and 25% of initial activity for rIL-2 incubated in phosphate buffer with or without P-407, respectively. This would suggest that P-407 had no protective effect against thermally induced denaturation of rIL-2 at 50°C.

Discussion

The present investigation has demonstrated that the biological activities of a nonrecombinant protein and a DNA-derived recombinant protein were adversely affected when a protein solution was thermally stressed beyond physiological temperatures. This has important implications for protein pharmaceuticals in solution that are subjected to changes in temperature during formulation and/or shipping. Since physical instability of protein drugs may arise from denaturation, aggregation, precipitation, and adsorption, strategies must continue to be developed that will minimize/eliminate this undesired process. Irreversible loss in the biological activity of a protein drug renders the product of no therapeutic value. As discussed previously, reversible denaturation

is defined as unfolding of a protein caused by an increase in temperature which can be reversed by subsequent lowering of the temperature. Recovery of biological activity upon lowering the temperature is considered reversible denaturation (Manning et al., 1989). Irreversible denaturation is any unfolding process which does not allow the native structure to be regained simply by lowering the temperature. Although a protein which is irreversibly denatured may still be returned to its native state by addition of denaturant followed by dialysis, the process is still defined as irreversible (Manning et al., 1989).

Previously, we have shown that agitation-induced denaturation with subsequent loss in biological activity of urease and rIL-2 in solution could be prevented by incorporation of P-407 (Wang and Johnston, 1993). Thus, it was necessary to determine whether P-407 might have a protective effect for protein solutions that were thermally stressed. As shown in Figs 1 and 2, a significantly ($p < 0.05$) smaller fraction of the total enzymatic activity was irreversibly lost when urease was dissolved in phosphate buffer which contained P-407. However, for urease solutions evaluated at 75°C, addition of P-407 resulted in an increased rate of urease inactivation (Fig. 3). Thus, it can be concluded that for urease in solution and heated to temperatures of 37 and 50°C, a protective effect from thermal-induced irreversible denaturation was observed when P-407 was present when compared to thermally stressed urease solutions which contained no P-407. However, a certain fraction of the enzymatic activity of urease in aqueous solutions which were incubated at 37 and 50°C and which contained either no P-407 or P-407 at a concentration of 1% w/w was permanently lost and the denaturation of urease at these temperatures would be classified as irreversible, since full enzymatic activity was not regained following a return of the solutions to room temperature (Manning et al., 1989).

The biexponential fit of the data may suggest an initial rapid unfolding phase followed by a slower unfolding/refolding phase. The unfolding of proteins is typically reversible, whereas irreversible denaturation of proteins induced by an elevation in temperature is characterized by subsequent aggregation or refolding of protein structures into new structures different from the native conformations. These new structures, which are distinctly different from the native conformation, form kinetically or thermodynamically stable structures which are biologically inactive. However, in our studies, which evaluated the sensitivity of urease to thermal-mediated denaturation, aggregation is highly unlikely since the urease solutions used were very dilute (Klibanov, 1983).

The mechanism by which P-407 protected urease from thermal-induced denaturation at 37 and 50°C was potentially due to its chemical structure. The poloxamers are a family of polyols having regular and predictable physicochemical properties. Polyols have been demonstrated to stabilize protein solutions (Wang and Hanson, 1988). The stabilizing effects of polyhydric alcohols on the heat-caused reversible denaturation of lysozyme and ribonuclease have been reported (Gerlsma, 1968; Gerlsma and Stuur, 1972). The authors suggested that the stabilizing effects of polyols result from the strengthening of intrahydrophobic bonds, reducing the interaction of the protein with water and the penetration of water into the hydrophobic interior, and from a lowering of the dielectric constant of the medium. Stabilization may also result from binding of the nonionic polymer to the enzyme forming steric exclusion of the substrate (urea) at the active site(s) (Wang and Hanson, 1988), however, we have no direct experimental evidence to support this premise. The mechanism by which polyols increase the solution stability of proteins is still poorly understood, but the effects of polyols on water and protein intramolecular interactions play a major role. A final possibility for an increase in the residual enzymatic activity of urease incubated at 37 and 50°C in solutions which contained P-407 may potentially be due to micellar solubilization of urease since the concentration of P-407 selected (1% w/w) exceeds the critical micelle concentration (CMC) of P-407 in aqueous solutions (Rassing and Attwood, 1983).

Inclusion of P-407 afforded no increase in the fraction of rIL-2 that was biologically active when an rIL-2 solution was incubated at 50°C for 96 h. Although bioassay was not performed at 50°C, it

can be concluded from the data that rIL-2 underwent irreversible denaturation when incubated at 50°C in either phosphate buffer solution with or without P-407. This conclusion is based on the fact that if renaturation of rIL-2 had occurred upon a reduction in temperature, the shape of the activity-time profiles shown in Fig. 4 should have been reasonably linear with activity values of approx. 100%. A loss in the biological activity of rIL-2 incubated at 50°C confirms our earlier findings which demonstrated an approx. 15% loss in the biological activity of rIL-2 when incubated for 72 h in a phosphate buffer solution which contained P-407 (Johnston et al., 1992). Presumably, there is a critical temperature between 37 and 50°C at which the rIL-2 is irreversibly denatured. It should be noted, however, that our present and previous (Wang and Johnston, 1993) results concerning the solution stability of rIL-2 subjected to various hydrodynamic and temperature conditions in the presence of P-407 cannot necessarily be extended to predictions of solution stability for the entire class of recombinant-derived protein pharmaceuticals.

Urease is an example of a protein (enzyme) with no approved therapeutic indication for animals or humans. However, with the purification and scale-up of larger quantities of enzymes being used for enzyme deficiency diseases, the findings presented here are of significance. While this model nonrecombinant protein could potentially be denatured in vivo by enzymes (protease), trace amounts of metal ions that serve as catalysts, etc., we have demonstrated in vitro that a solution of the enzyme was not denatured by P-407 to any significant extent at or below 37°C and that agitation-induced denaturation of the type and magnitude imposed in previous work may be prevented when P-407 is added to a urease solution (Wang and Johnston, 1993). In addition, we have previously demonstrated that the enzymatic activity of urease was maintained when formulated with P-407 (30% w/w) and injected intraperitoneally to rats (Pec et al., 1992). Hence, it would follow that newly discovered enzymes for treating enzyme deficiency diseases might potentially be protected from agitation- and thermal-induced (< 37°C) irreversible denaturation with subsequent loss in the biological activity by inclusion of P-407 in an aqueous solution of the enzyme or as a component in a lyophilized preparation of the protein which is intended for reconstitution.

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