Interleukin-1 Receptor (IL-1R) Liquid Formulation Development Using Differential Scanning Calorimetry

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Purpose. To elucidate the solution conditions that confer stability of aqueous IL-1R using differential scanning calorimetry (DSC).

Methods. Optimal pH conditions were determined by monitoring degradation products encountered during accelerated studies (at elevated temperatures) using SDS-PAGE. At the pH optimum, DSC screened for excipients that enhanced thermal stability by shifting the Tm to higher values. Using SEC the relationship between thermal unfolding and stability was investigated by considering if lower Tm's in the presence of preservatives correlated with degradation products at 37°C over time. The degree of aggregation relative to that of a control determined the level of stability achieved.

Results. Circular dichroism (CD) measurements confirmed molecular modeling studies showing IL-1R to be about 39% β-sheet. Two major transitions characterized the DSC data with Tm's observed near 47°C and 66°C. Among 21 excipients screened, NaCl exhibited the greatest stabilizing influences based on shifting the low temperature transition to 53°C. The low temperature transition was later found to comprise two transitions, yielding a total of three melting transitions for IL-1R. High Tm's arising from the presence of preservatives correlated with the order of stability (i.e., 0.065% phenol > 0.1% m-Cresol > 0.9% benzyl alcohol).

Conclusions. The three melting transitions are consistent in origin with the cooperative unfolding of three unique immunoglobulin-like domains of IL-1R. Optimal stability was achieved in 20 mM sodium citrate at pH 6 with sufficient NaCl to attain the tonicity of human serum. A correlation between the predicted ranking of stability and the extent of aggregation was demonstrated using DSC.

KEY WORDS: IL-1R type I; microcalorimetry; protein formulation; stability; aggregation; preservatives; far-uv circular dichroism.

INTRODUCTION

Interleukin-1 receptor type I (IL-1R) is a recombinant human glycoprotein produced in Chinese hamster ovary cells. It consists of 312 amino acids and is believed to play a therapeutic role in inflammation. Finding therapies for inflammatory disorders potentially involves the inhibition of IL-1 cytokines (i.e., IL-1α, IL-1β), which elicit inflammatory immune reactions (1). Such immune responses can be triggered when the IL-1 cytokines bind to membrane-bound IL-1R found on the surface of immune cells (i.e., T and B lymphocytes). IL-1R has been cloned from human T cells (2) and truncated at the transmembrane sequence position to produce a soluble receptor. Soluble IL-1R can act as a specific inhibitor of inflammatory reactions by selectively binding to IL-1 cytokines. The binding portion of the IL-1R molecule comprises three immunoglobulin-like domains (2) and is a member of the immunoglobulin gene superfamily (2,3). Among the three disulfides predicted by the immunoglobulin superfamily sequence homology, there are four additional cysteines that can participate in disulfide linkages (one in each of two domains).

Little is known about the solution conditions that stabilize IL-1R. Therefore, the intent of this study was to determine the conditions that confer stability to this protein in the liquid state with and without preservatives. Determination of the best candidate solutions that offer the greatest chances of achieving optimal shelf-life can be difficult. Solution conditions that need to be considered during stages of formulation development include pH (4), ionic strength, surface interactions (e.g., air, containers), impurities (e.g., residual peroxide (5), metal ions), temperature (6,7) and osmolality. When these conditions are not optimized, protein denaturation is more prone to occur.

In addition to determining which solution conditions give rise to stability, the influence of preservatives on IL-1R is also of interest. Preservatives are commonly used in multidose products. However, the introduction of such compounds may have negative consequences on stability. Many preservative molecules are small, possess a polar moiety and are hydrophobic, which can permit them to penetrate into the protein hydrophobic core (8). Such interactions may facilitate loosening of the conformation and contribute to the overall destabilization of the protein molecule. For this reason, the effect of benzyl alcohol, m-cresol and phenol were studied to evaluate their impact on the stability of IL-1R.

The characterization of protein unfolding has been investigated using several biophysical methods including differential scanning calorimetry (DSC) (6), fluorescence (9), optical rotatory dispersion (8), and circular dichroism (CD) (6,9,10). Using such methods, researchers have found that a loss in compact structure resulting in a nonnative conformational change (i.e., denatured state) has a dramatic impact on aggregation (9,11,12), deamidation (13) and oxidation (14). Many studies using these methods have provided information about the relationship between protein unfolding and degree of stability. High melting temperatures determined by DSC have often been attributed to a favorable level of stability in screening studies (15). With this in mind, DSC was considered as a tool for determining the relationship between the melting transition temperature of IL-1R and stability. In this study, the DSC technique was tested to ascertain its value in ranking the stability of IL-1R in the presence of preservatives.

MATERIALS AND METHODS

Materials

Purified IL-1R was obtained as a bulk drug concentrate in 20 mM tris (pH 7.4) at Immunex Corporation. Protein concentrations were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 1.50 ml/mg cm. All excipients

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used were reagent grade or better and obtained from the sources listed below. Lactose, sucrose, mannitol, NaH_2PO_4, tris, sodium citrate, sodium acetate, glycerol, $CaCl_2$, glycine, and NaCl were obtained from J.T. Baker. Glucose and PVP, poly(vinylpyrrolidone), were obtained from Fluka. Tween 80 was obtained from Mallinckrodt. Lysine, cysteine, ascorbic acid, dextran 40 and alanine were obtained from Sigma Chemicals. L-arginine, phenol and m-cresol were obtained from Aldrich. Benzyl alcohol was purchased from EM Science. Polyethylene glycol (in assorted molecular weights) was obtained from Union Carbide. Pluronic F68 was purchased from BASF Corporation and ethanol (100%) was obtained from Quantum.

Circular Dichroism

The far-UV CD spectrum was collected using a J-600 spectropolarimeter (Jasco). Prior to examination of the sample, the instrument was calibrated with ammonium d-10-camphorsulfonic acid. The protein was evaluated in 10 mM NaH_2PO_4 buffer (pH 7.4) at a concentration of 0.291 mg/ml. A cuvette with 0.1 mm path length contained the degassed sample. Buffer evaluated under identical conditions was subtracted from the protein scan. Scans were carried out at 1 nm step resolution from 240 to 195 nm at a speed of 10 nm/min. The sensitivity was 20 mdeg with a time constant of 2 sec. Nine scans were accumulated to improve the signal to noise ratio. The secondary structure estimates were determined by deconvoluting the spectrum into five structure elements using convex constraint analysis (CCA) as described (16,17).

Differential Scanning Calorimetry Studies

The bulk drug concentrate (7 mg/ml) was dialyzed against the corresponding formulation to be evaluated for at least 20 hr at 4°C. After removal from the dialysis tubing, the solution was diluted further with buffer to attain a final protein concentration of approximately 5 mg/ml. Initial studies involved the addition of excipients to a control (20 mM sodium citrate, pH 6) and the examination of their effect on the IL-1R Tm. The excipients tested and amounts used in these studies are listed in Table I.

Calorimetric measurements were carried out using a MicroCal MC-2 DSC. The scan rate in each case was 68°C/hr. The thermograms were background corrected and rescans employed to evaluate the reversibility of the unfolding transitions. The extent of reversibility was measured by dividing area recovered (enthalpy) on the second upscan by the area of the first upscan. In this study 90°C was set as the upper temperature limit, which was beyond the completion of the highest Tm of IL-1R. The Tm values for transitions on the second scans were identical to those on the first. All data manipulation was performed using the Origin software provided with the instrument.

Stability Studies

Dialyzed formulations were diluted to a concentration of 1 mg/ml and 1 ml volumes placed within 2 ml type I glass vials under aseptic conditions. The vials were then subsequently stoppered, capped and crimped. One vial per formulation condition per temperature was prepared and set up with timepoints

Table I. List of Excipients Evaluated by Microcalorimetry (DSC)

	Excipient	Conc. (g/ml) in buffer	Mole Ratio [Ms/Mp]*	Tm (°C)
	Control	0.00	—	48.1
	Ascorbic Acid	0.05	2037	36.7
Sugars	Mannitol	0.0517	2037	46.7
Sugars	Lactose	0.0972	2037	49.7
Sugars	Sucrose	0.0972	2037	49.7
Sugars	Glucose	0.0512	2037	49.6
Polymers	PVP (MW,10000)	0.01	7	48.9
Polymers	PEG (MW, 300)	0.0003	7	49.4
Polymers	PEG (MW, 1000)	0.001	7	49.1
Polymers	PEG (MW, 3350)	0.00335	7	48.7
Polymers	Dextran 40	0.0392	7	48
Polyols	Glycerol	0.01	779	48.7
Polyols	Ethanol	0.0051	779	48.6
Polyols	Ethanol	0.05	7617	43.8
Salts	NaCl	0.00584	717	53.1
Salts	$CaCl_2$	0.0111	717	41.1
Amino Acids	Glycine	0.01	955	46.2
Amino Acids	L-Lysine	0.01947	955	48.3
Amino Acids	L-Cysteine	0.01614	955	51.3
Amino Acids	L-Alanine	0.01187	955	46.2
Amino Acids	L-Arginine	0.0232	955	49.2
Surfactants	Pluronic F68	0.0001	4	46.6
Surfactants	Tween 80	0.001	5	45.8
	Glucose/NaCl	.0512/.00584	2037/717	52.2

* Ms = moles of excipient, Mp = moles of protein

of initial, day 1, day 7 and day 60. Samples were incubated at 2° to 8°C (control), 30°C, 37°C and 50°C. Stability studies were carried out first on seven formulations to determine the optimum pH within the range of pH 3.0 to 9.0. The buffering systems chosen included sodium citrate (pH 3.0), sodium acetate (pH 4.0 and 5.0), sodium citrate (pH 6.0), NaH_2PO_4 (pH 7.0) and tris (pH 8.0 and 9.0). All formulations (unless otherwise specified) contained 50 mM buffer, 100 mM NaCl and protein. The preservatives, 0.65% phenol, 0.1% m-cresol and 0.9% benzyl alcohol, were placed on stability in a 20 mM sodium citrate (pH 6) formulation containing 100 mM NaCl. Differential scanning calorimetry studies were carried out on these preservative formulations as described above.

Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS-PAGE)

Nonreduced SDS-PAGE silver stained gels were prepared to qualitatively assess stability (i.e., breakdown, aggregation). A 1 μg protein load per well was used on a Novex 4–20% Tris-glycine gradient gel. Gels were run at 35 mA for 50 min, subsequently stained using 1 ml of 19.4% silver nitrate per 100 ml silver staining solution, and then developed.

Size Exclusion Chromatography (SEC)

A Biosil 250 column was used with a Waters 625 HPLC system to quantitatively evaluate aggregation and breakdown products. A flow rate of 1 ml/min in PBS (pH 6.8) elution buffer was used at ambient temperature. Detection of eluting components was measured at 220 nm (Waters 991 photodiode array detector). Aggregate and native (nondegraded protein) compositions were determined by the percent areas of high molecular weight species (eluting between 5 and 7.25 min) and the main peak (eluting from 7.25 to 9 min), respectively. Integration was carried out from the elution baseline.

RESULTS AND DISCUSSION

IL-1R Structure

An understanding of the structure of IL-1R was needed to assist in the interpretation of the DSC results. Quantitative assessment of the secondary structure of IL-1R by far-UV CD suggested that this molecule was 39% β-sheet (Figure 1), which agrees with the predicted molecular model of IL-1R (Figure 2). The model was generated using FOLDER, a distance geometry-based homology modeling program (18). The primary sequence of IL-1R suggested three immunoglobulin-like domains in tandem followed by a putative transmembrane region. Each of the three immunoglobulin-like domains showed significant sequence homology to the immunoglobulin domains of 3D structures. Although sequence homology searches revealed significant homology between IL-1R and members of the immunoglobulin superfamily, it was not possible to select an unambiguous template from the large number of immunoglobulin domains with known 3D structures. In the model, a 3D structure of a variable light chain domain from the protein data bank structure with code 3FAB was used as a template for all three domains of IL-1R. Sequence alignment between the 3FAB-VL domain and IL-1R domains was optimized visually to bury hydrophobic amino acids. Three of the seven strands that compose the immunoglobulin-like domain could be unambiguously aligned because of the two conserved cysteines in strands B and F and a Trp residue in strand C. Sequence alignment for the other four strands, A, D, E, and G, was less obvious and was optimized to satisfy chain closure constraints and the hydrophobic interior. The structural constraint from the two additional disulfide crosslinks and conserved glycosylation sites further restricted the number of possible sequence alignments.

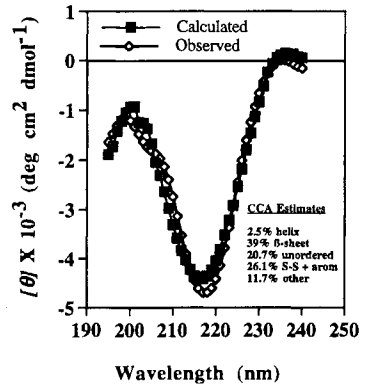

Fig. 1. Comparison of experimentally observed far-UV CD spectrum of IL-1R to a calculated fit determined from Convex Constraint Analysis. Secondary structure elements are listed.

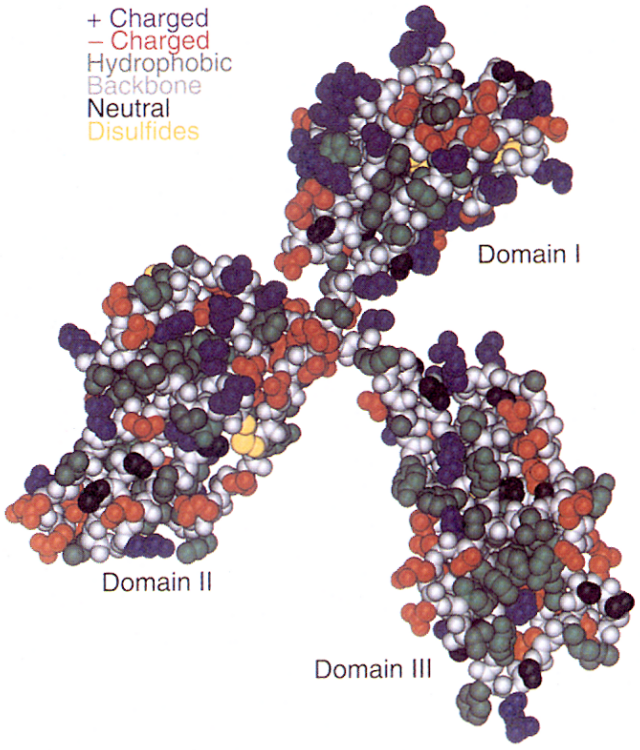

Fig. 2. A molecular model of IL-1R showing regions of charge (color coded). Domain I contains the N-terminus and domain III possesses the C-terminus.

The three domains of the IL-1R molecule are unique in terms of the nature of the immunoglobulin folds and support the CD β-sheet assessment. Polypeptide molecular weights for the domains are 12.5 kD (domain I), 13.4 kD (domain II) and 14.4 kD (domain III) based on amino acid sequence. Another distinguishing characteristic in structure was that domains I and II where found to have one N-linked glycosylation site whereas domain III was found to possess four. Glycosylation has been reported to have a favorable impact on protein stability in some cases (19,20).

Optimal pH

Accelerated stability studies were performed on seven solutions ranging in pH from 3.0 to 9.0. A comparison of these formulations run as nonreduced gels after 7 days at 30° and 50°C is shown in Figure 3. In lane 1 at both temperatures is a standard that was used as a control. The standard exhibits a major band that migrates on the gel at about 59 kD. This molecular weight is higher than the polypeptide molecular weight of 35.9 kD and can be attributed to glycosylation that contributes to the hydrodynamic volume of the protein. Interestingly, at low pH conditions (below pH 6) at 30°C there was a large amount of aggregation (as shown in lanes 2 through 4). Above pH 6 at 30°C there was considerably more breakdown (as shown by the low molecular weight species detected on the gel). In fact, the greatest breakdown was observed at pH 8 under these conditions. At 50°C there was a greater propensity toward aggregation at pH conditions ranging from 6 to 8. However, in both cases evaluated, pH 6 was shown to possess minimal aggregation and breakdown. Therefore, pH 6 was chosen as the pH optimum with which to carry out further formulation development studies.

Sodium citrate was used as the formulation buffer since it offered sufficient buffer capacity at pH 6. A 20 mM sodium citrate buffer concentration was selected for subsequent studies to permit room for the addition of excipients while remaining isotonic with blood (discussed further below). The DSC result in Figure 4A shows the baseline-corrected and concentration-normalized thermogram for IL-1R under these solution conditions. There were two distinct unfolding transitions near 48°C and 65°C. The transition at 48°C was chosen to screen a series of excipients because it was more predominant, less obscured and occurred at a lower temperature, suggesting it was more thermally labile than the transition at 65.5°C. As discussed below, the transition envelope at 48°C comprised two transitions that exhibited the same shifting movement consistent with stability. Therefore, the strategy was to screen for excipients that would raise the Tm of the low temperature endotherm, indicating a positive contribution to conformational stability of the molecule (15).

Excipient Screening Using DSC

The excipients studied included a broad array of compound types (i.e., sugars, polymers, polyols, salts, surfactants and amino acids) (Table I). Without the addition of excipient to the formulation, the lower Tm occurred near 48°C, so it was possible to characterize the impact of a given excipient as potentially stabilizing or destabilizing based upon whether the Tm shifted above or below this temperature, respectively. Destabilizing excipients therefore included ascorbic acid, mannitol, ethanol, calcium chloride, glycine, L-alanine, Pluronic F68 and Tween 80. It is noteworthy that ethanol at a concentration of 0.05 g/ml was destabilizing in contrast to the same excipient with about a 10-fold lower concentration, which was slightly stabilizing. Such behavior has been reported previously in the case of alcohol, indicating that this excipient can stabilize proteins at low concentration levels (21). This observation sug-

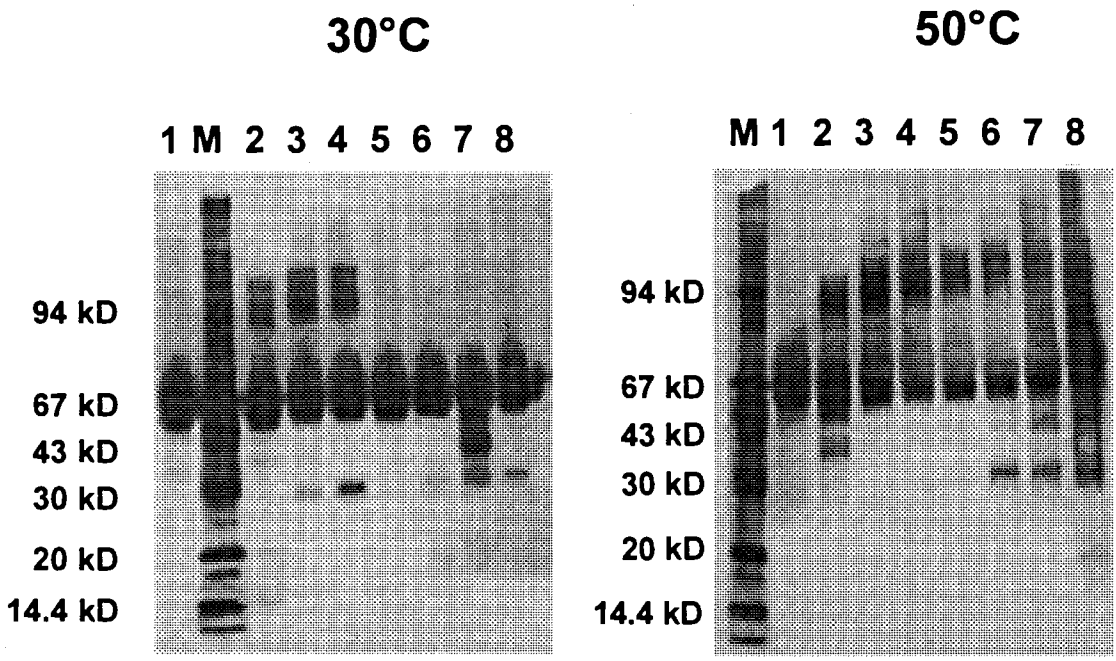

Fig. 3. Nonreduced SDS-PAGE gels showing product changes after 7 days accelerated stability as a function of pH. Lane assignments are standard material (lane 1), pH 3 (lane 2), pH 4 (lane 3), pH 5 (lane 4), pH 6 (lane 5), pH 7 (lane 6), pH 8 (lane 7), and pH 9 (lane 8). The lane designated "M" exhibits the migration of the molecular weight markers.

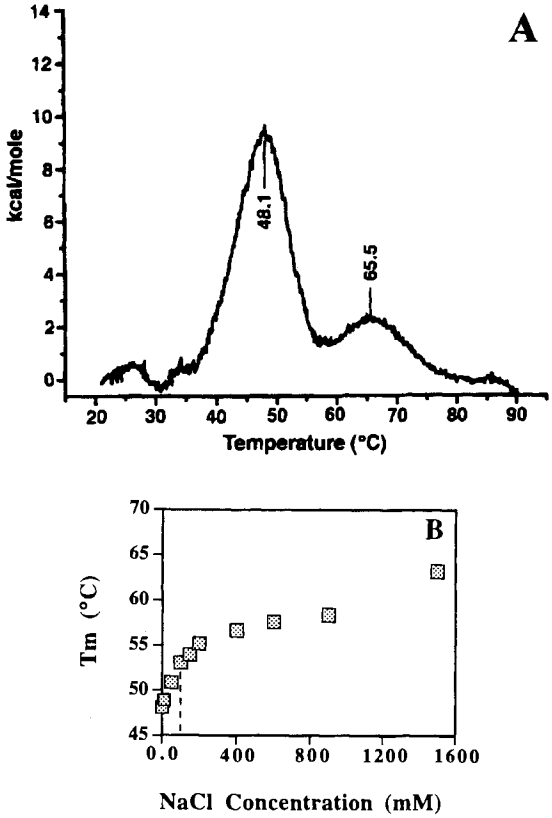

Fig. 4. Concentration-normalized DSC thermogram of IL-1R in the control solution consisting of **(A)** 20 mM sodium citrate (pH 6) without the addition of excipients and **(B)** a plot showing the Tm behavior as a consequence of NaCl addition. In (B), the 100 mM NaCl concentration is marked by the dashed vertical line.

gested that the excipient concentration needed to be considered in assessing candidates that could afford a significant impact on stability. To further address this issue and ascertain the potency of an excipient, a mole ratio parameter was included, as shown in Table I. Here the moles of excipient (Ms) were evaluated as a ratio to the number of moles of protein (Mp).

Slightly stabilizing influences were observed among the polymers (at relatively low concentrations) with the exception of dextran, which had no significant impact on Tm. Although further investigation of the polymers' effects on Tm was not carried out, evidence in the literature suggests that the presence of PEG at higher concentrations would have a destabilizing impact on Tm (22). Among the PEG molecular weights tested, the order of acquired stabilization was 300 MW > 1000 MW > 3350 MW. These results suggested that low molecular weight PEG was more favorable than high molecular weight PEG in contributing to the stabilization of IL-1R at low concentration levels. Perhaps this arose as a consequence of the low molecular weight polymer being least hydrophobic (22). Poly (vinylpyrrolidone) (PVP) exhibited a small stabilizing impact on IL-1R that may be concentration dependent. For example, PVP has been reported to stabilize human IgM at low concentrations (below 2%) (23).

Although cysteine exerted a stabilizing influence as determined by an increase in Tm, the transition was broad and upon a second scan exhibited no reversibility. We ascribe this to the ability of cysteine to act as a reducing agent at elevated temperatures and note that significant aggregation was observed when removing the sample from the DSC. Therefore, the position of the Tm in this case was somewhat misleading.

Sodium chloride alone afforded the most positive influences by shifting the Tm up to 53.1°C at a mole ratio of 717:1. Therefore, a combination of glucose and NaCl was investigated to see if together they could contribute further to stability. This combination resulted in a lower Tm than that of NaCl alone at the same mole ratio. Such a loss in thermal stability was hypothesized to involve dilution of NaCl interactions with charged sites on the protein when glucose was added. Thus, further studies of excipient combinations were discontinued and more focus was placed on the concentration dependence of NaCl.

The stabilizing effects of NaCl on IL-1R suggested direct interactions between salt ions and charged groups of the protein (Figure 2). If stabilization was attributed to these interactions alone, addition of salt would eventually lead to charged site saturation, at which point no further increase in Tm would occur. However, as shown in Figure 4B a continued increase in Tm was observed that did not exhibit a maximum value within the range of NaCl concentrations tested. In fact, the Tm appeared to increase at a concentration of 1500 mM, a point beyond which charged sites would be completely saturated.

Salt ions can also affect water structure, which in turn has been implicated to affect protein conformational stability (24). The above data suggest that both direct interactions of the salt with the protein (i.e., ion-dipole, ion-ion interactions) and a change in water structure play a stabilizing role in the native state of IL-1R. This might be conceptualized in terms of strengthening intrachain hydrophobic interactions of the compact native structure.

Examination of Preservatives

To study the impact of preservatives in pharmaceutically meaningful solutions using DSC, it was desirable to make the solutions isotonic (i.e., 300 mOsmol/kg). Therefore, 100 mM NaCl in a 20 mM sodium citrate buffer solution was chosen to carry out the preservative investigation since it gave rise to an osmolality of approximately 260 mOsmol/kg (contributions of the protein being negligible). Under these formulation conditions, added preservative would keep the osmolality within the range of 300 ± 50 mOsmol/kg.

Thermodynamic parameters were determined by fitting the DSC data shown in Figure 5 to a non-two-state model (attempts to apply a two-state function did not adequately account for the unfolding process). The calorimetric enthalpy (ΔH_{cal}) of the melting transitions was taken as the areas of the protein endotherms for each unfolding transition. The van't Hoff enthalpies (ΔH_v) were determined by the equation $\Delta H_v = 4R\,Tm^2/\Delta T_{1/2}$, where R is the gas constant and $\Delta T_{1/2}$ is the sharpness of the unfolding endotherm (expressed as the temperature width at half-height of the heat absorption peak) (25). When fitting the unfolding endotherms to two transitions, a poor fit was obtained. A better fit was achieved when the predominant transition was deconvoluted into two rather than one unfolded transition as suggested by a $\Delta H_{cal}/\Delta H_v$ ratio of approximately 2 (26). Therefore, three unfolding transition temperatures accounted for the two endotherms of the DSC data. Identification of each unfolding

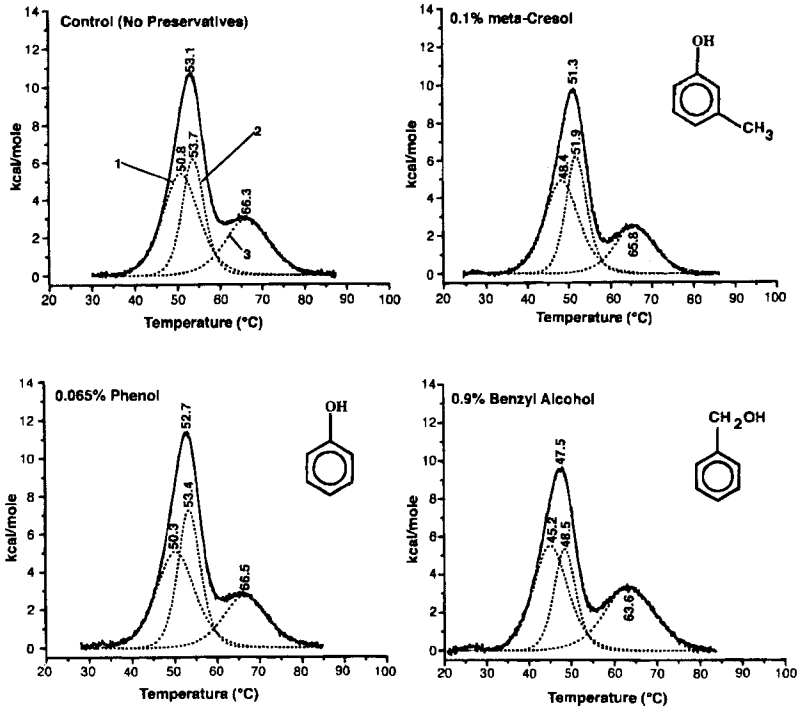

Fig. 5. Deconvoluted melting transitions of IL-1R in the presence of three preservatives. The control solution consists of 20 mM sodium citrate (pH 6) and 100 mM NaCl. Preservative amounts added to the control solution in each case are designated in the figure.

transition was arbitrarily numbered from 1(lowest temperature) to 3 (highest temperature), with corresponding Tm's numbered in the same way (i.e., Tm1, Tm2, Tm3). Hence, in the case of the control, Tm1 = 50.8°C, Tm2 = 53.7°C and Tm3 = 66.3°C. Since three unique immunoglobulin-type domains make up the IL-1R molecule (Figure 2), the discovery of three unfolding domains seemed appropriate. However, investigation into the possibility that these transitions might also arise from different glycosylated variants was assessed with two isolated glycoforms of IL-1R. The DSC results (data not shown) exhibited the same transition profile in each case, providing some evidence that glycoform variants were not responsible for the unfolding transitions associated with the molecule. Although it is tempting to assign the three Tm's to the three domains of this molecule, further experimental studies are needed to elucidate these assignments. We can only conclude that three identifiable melting transitions originate

Table II. Thermodynamic and Preservative Potency Parameters

	ΔH_{cal} (kcal/mol)	ΔH_v (kcal/mol)	$\Delta H_{cal}/\Delta H_v$	Reversibility (%)	P_f*
Control					
Tm1	55.5	73.0	0.76	87.8	
Tm2	47.4	123.5	0.38	86.2	
Tm3	34.8	68.5	0.51	93.2	
0.9% Benzyl Alcohol					
Tm1	59.8	74.7	0.80	51.8	0.009
Tm2	36.3	121.8	0.30	54.4	0.009
Tm3	51.5	58.5	0.88	59.0	0.004
0.1% m-Cresol					
Tm1	55.4	75.4	0.73	70.9	0.04
Tm2	43.1	124.9	0.35	69.4	0.03
Tm3	31.9	74.4	0.43	100.5	0.008
0.065% Phenol					
Tm1	60.4	69.8	0.86	78.3	0.01
Tm2	51.6	119.4	0.43	79.4	0.006
Tm3	37.0	67.4	0.55	98.5	–0.004

*P_f is the normalized preservative force on protein unfolding.

from the unfolding behavior of three unique domains in the IL-1R molecule.

Among the three unfolding thermal transitions of IL-1R, Tm3 achieved the highest levels of reversibility (Table II). In fact, the reversibility measured for this transition in all preservative studies (with the exception of benzyl alcohol) was greater than 93%, suggesting that the estimated enthalpies in these cases were thermodynamically meaningful. The average enthalpy of Tm3 for the cases in which the reversibility exceeded 93% (i.e., control, m-cresol, phenol) was 34.6 ± 2.6 kcal/mol. Additionally, the $\Delta H_{cal}/\Delta H_v$ ratio in every case was less than 1, indicating that intermolecular cooperation and molecular association play a role in thermal unfolding (27). It is worth mentioning that Tm2 in every case had the lowest $\Delta H_{cal}/\Delta H_v$ ratio, suggesting that it was most actively involved in intermolecular association processes of unfolding. Tm3 in all cases was observed to be least affected by the presence of the preservatives. Although Tm1 and Tm2 appeared to be affected more than Tm3 by the preservatives in each case, Tm1 in general appeared to be affected the greatest. This behavior was consistent with the predicted order of thermal stability implied by the melting transitions, which was expected to be Tm3 > Tm2 > Tm1. The Tm1 transition therefore described a domain that was most vulnerable to unfold in contrast to Tm3, which could be considered to represent the most stable domain.

The observed impact of the three preservatives on the unfolding of IL-1R relative to the control are shown in Figure 5. In the cases presented, there was a consistent destabilizing influence afforded by the presence of the preservative as shown by the shift of the predominant transition envelope toward low Tm (comprising Tm1 and Tm2). Furthermore, the behavior of Tm1 and Tm2 followed that of the predominant transition; thus, the major transition in the raw data indicated stability in a given solution environment. This observation supports the use of the predominant transition in the excipient screening studies to measure an excipient's influence on conformational stability. Using the deconvoluted data, estimates were made concerning thermodynamic parameters obtained from the calorimetric measurement (including reversibility) and are displayed in Table II.

To account for the differences in preservative concentration and to compare potency among the preservatives' ability to destabilize IL-1R, a new term was created. The change in unfolding transition for a given component can be described by $\Delta Tmi = Tmi_c - Tmi_p$, where "i" denotes the component transition to be evaluated (i.e., 1,2,3), the subscript *p* refers to the presence of preservative, and subscript *c* indicates that i^{th} component of the control. Dividing the change in unfolding transition by the mole ratio (Ms/Mp, previously defined) one obtains a new term, $P_f = \Delta Tmi/(Ms/Mp)$, where P_f is the estimated normalized preservative force on protein unfolding. Table II includes the P_f values for the preservatives taken from the data presented in Figure 5. A negative P_f value indicates a stabilizing force. In the case of phenol, even though a slightly stabilizing force was observed in Tm3, we acknowledge that this positive change resulted from a 0.2°C increase in Tm3 relative to the control (Table III) and we therefore suggest that this result is insignificant since it falls within the precision of the DSC measurement (± 0.2°C). In contrast to the small effects observed with phenol, m-cresol had the greatest destabilizing impact among the preservatives tested (as exhibited by the high P_f values), perhaps due in part to its enhanced hydrophobic properties associated with the methylated aromatic ring.

Verification of DSC Predictions

Using the Tm's presented in Figure 5, it seemed clear from the DSC data that formulation stability should presumably follow control > 0.065% phenol > 0.1% m-Cresol > 0.9% benzyl alcohol. To assess product stability over time, a quantitative parameter was needed that could be related to the DSC data in order to substantiate the results predicted. Size exclusion chromatography was chosen for this purpose. This technique could be used to quantify the amount of aggregation and breakdown the product experienced over time. The SEC data for a 60-day time point incubated at the physiological temperature of 37°C (for the identical solutions tested by DSC) are shown in Figure 6. Aggregation was the primary form of instability observed from these data. There was little evidence of breakdown under the solution conditions investigated; however, aggregation did seem to follow the behavior predicted by the DSC data. That is, the solution containing benzyl alcohol clearly exhibited the greatest amount of aggregation followed by m-cresol and finally the phenol-containing formulation. Clearly, a correlation was observed as listed in Table III that as one moves from phenol to benzyl alcohol (down the table), a decrease in Tm in each case correlates well with an increase in aggregation and a decrease in remaining native protein. The reliance of the DSC to predict the impact of each preservative condition on aggregation was therefore demonstrated.

Table III. DSC Melting Temperatures and SEC *In Vitro* Stability Data at 37°C

	DSC			SEC			
				7 day		60 day	
	Tm1 (°C)	Tm2 (°C)	Tm3 (°C)	Agg (%)	Native (%)	Agg (%)	Native (%)
Control	50.8	53.7	66.3	0.66	98.93	1.50	97.54
Phenol	50.3	53.4	66.5	1.02	98.62	3.07	96.02
m-Cresol	48.4	51.9	65.8	1.37	98.25	5.10	93.92
Benzyl Alcohol	45.2	48.5	63.6	2.93	96.92	16.46	83.09

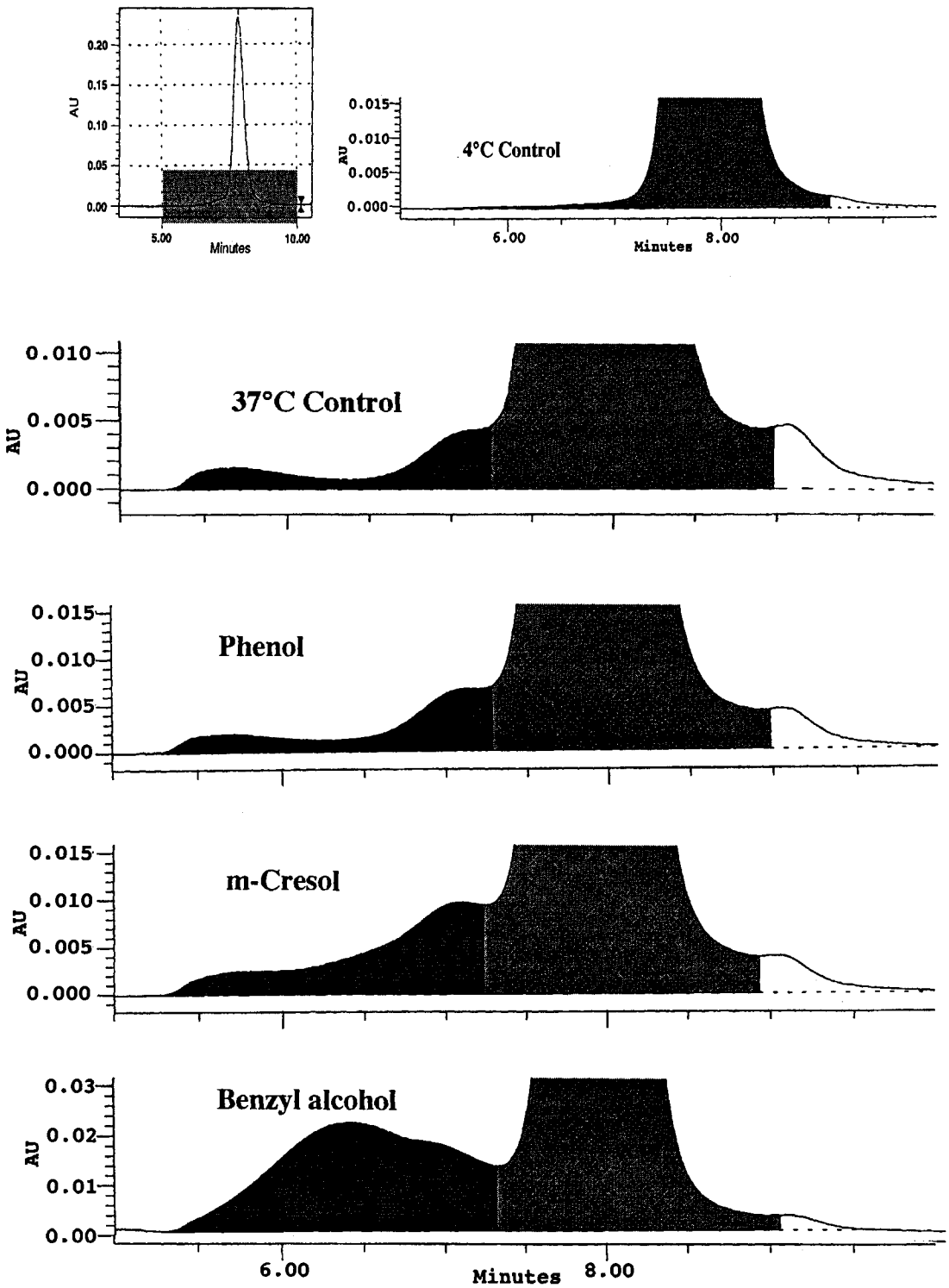

Fig. 6. Size exclusion chromatography of IL-1R after incubation for 60 days at 37°C. The dark shaded region is the portion of the trace evaluated as percent aggregation, and the light shaded region is the integrated main peak considered as percent native protein. Note that the 37°C control contains no preservative and in the upper right is the preheated control stored at 4°C.

CONCLUSIONS

The preceding results and discussion suggest that formulating at pH 6 in sodium citrate buffer with sufficient NaCl to remain isotonic with serum contributes to optimal stability of IL-1R. Aggregation was found to be the predominant pathway of instability and breakdown was minimal at the accelerated conditions tested. Differential scanning calorimetry was used successfully to assess the nature of the excipients on overall stability. This technique can be applied to the optimization of solution conditions when preservatives are required. Among all the excipients tested, NaCl had the greatest impact on thermal stability, perhaps due in part to its effectiveness to interact favorably with charged sites on the protein surface and minimize electrostatic protein-protein interactions. The DSC data was able to correctly predict the rank and order of stability in the presence of the preservatives tested (i.e., 0.065% phenol > 0.1% m-Cresol > 0.9% benzyl alcohol). An explanation for the correlation between the DSC and SEC presumably involves reversible unfolding followed by a rate-limiting irreversible step that leads to the formation of aggregate. The DSC results also suggest that intermolecular cooperation and molecular association play a role in the thermal unfolding process of IL-1R in the presence and absence of preservatives. Finally, the discovery of three distinct Tm's is consistent with the interpretation that IL-1R unfolds as three unique domains.

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