Chemical and Physical Stability of Chimeric L6, a Mouse–Human Monoclonal Antibody

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Chimeric L6 is a mouse–human monoclonal antibody specific for tumor cell-associated antigens. The factors affecting the physical and chemical stability of chimeric L6 were assessed at elevated temperatures (30–60°C) and by multiple freezing and thawing. Three routes of degradation were observed: chemical degradation to smaller molecular weight species, irreversible aggregation, and formation of a reversible dimer. The specific pathway depended on the stress condition applied and the pH, with maximal overall stability to both thermal stress and multiple freezing/thawing observed at about pH 5.5. Other factors including antibody concentration, buffer concentration, NaCl concentration, and agitation had minimal influence on the stability. Commonly used sugars, polyhydric alcohols, and amino acids effectively prevented freeze/thaw-induced aggregation.

KEY WORDS: chimeric antibody; protein; stability; multiple freezing and thawing; aggregation.

INTRODUCTION

Chimeric L6 is a mouse–human monoclonal IgG_1 antibody specific for the Lewis-Y antigen found on the surface of tumor cells (1). The antibody has been labeled with ^{131}I to target tumor cells for radioimmunotherapy or cancer therapy (2). Factors which affect the stability of the antibody were evaluated to identify a stable composition and determine appropriate storage and handling conditions. Contributing factors to protein degradation often include pH, buffer, temperature, type and concentration of excipients, interfacial denaturation, and multiple freezing and thawing (3,4). To investigate the factors affecting the stability of chimeric L6, thermal stress and multiple freezing and thawing were used to induce chemical and physical degradation under a variety of conditions. The kinetics and mechanisms of degradation of chimeric L6 are discussed.

MATERIALS AND METHODS

Materials

Chimeric L6 at a concentration of 5 mg/mL in 0.05 *M* sodium phosphate buffer and 0.5 *M* NaCl at pH 7.1 was produced by Bristol-Myers Squibb Bio/Chem Division, Syracuse, NY, and stored at 2–8°C. Centricon 30 microconcentrator units were obtained from Amicon. Gel-filtration standards were obtained from Bio-Rad (No. 151-1901). Milli-Q processed water (Millipore) was used for all studies. β-Nicotinamide adenine dinucleotide phosphate reduced form (NADH), L-glutamate dehydrogenase (GDH), and α-ketoglutarate (α-KG) were obtained from Sigma. All other chemicals were analytical grade.

Instrumentation

pH measurements were performed using a Metrohm 632 pH meter. Samples for the pH–rate profile were stored at 30 to 60°C in an appropriate temperature-controlled station. Buffer exchange procedures were carried out using an IEC Model HN-SII centrifuge. UV spectra were recorded with a Shimadzu UV-2101PC scanning spectrophotometer.

Chromatography

The HPLC system consisted of a Shimadzu SPD-6AV UV-vis spectrophotometric detector at 280 nm, dual Shimadzu LC-7A pumps, a SCL-6B system controller, a SIL-6B Auto Injector, and a Perkin Elmer Nelson Analytical Series 900 Interface. A Bio-Sil SEC-250 (30 cm × 7.8-mm) column was used. The mobile phase was 0.2 *M* KH_2PO_4 in 0.9% NaCl at pH 6.8 (adjusted with KOH) at a flow rate of 1.0 mL/min. The elution volume of chimeric L6 (7.5 mL) was in the linear portion of a plot of log (molecular weight) versus elution volume of gel-filtration standards. The molecular weights of chimeric L6 degradation peaks were estimated using the linear portion of this calibration curve. All assays were performed in duplicate on undiluted samples.

Enzyme-Linked Immunosorbent Assay (ELISA)

Immulon II 96-well plates (Dynatech) were coated with antiidiotype antibody 1B (αID 1B) and incubated overnight at 4°C. The wells were then washed and nonspecific sites were blocked with specimen diluent. Chimeric L6 was diluted to 100 ng/mL in specimen diluent, and a standard curve of 5.0 to 100 ng/mL was generated. Stability samples were diluted to an initial concentration of 50 ng/mL in specimen diluent and a series of 1:2 dilutions was made. The reference standard and stability samples were incubated in the wells for 2 hr (each dilution was assayed in quadruplicate). Quality-control samples (40 and 30 ng/mL) were prepared separately from the stock solution and run on each plate. Following this incubation, the solution in each well was removed and each well was then washed. αID 13B-biotin, diluted 1:1000 in conjugate diluent, was then added to each well. The plate was subsequently developed with Vectastain and TMB Chromogen reagent (Vector Labs). The plates were read on a Molecular Devices UV Max Plate Reader at 450 and 630 nm.

Buffer Exchange Procedure

The buffer was exchanged with 4 vol of the desired buffer using Centricon 30 microconcentrator units. The final solution was sterile-filtered through a 0.2-μm low-protein binding polysulfone Acrodisc filter assembly into a sterile Falcon tube. The filter was evaluated for binding and no loss

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of protein concentration was observed by size-exclusion chromatography (SEC).

Solution Stability

pH–Rate Profile

The bulk solution of chimeric L6 was diluted to 1 mg/mL in phosphate buffered saline (0.018 *M* phosphate buffer/0.2 *M* NaCl) at pH 7.2 and aliquots were adjusted to the desired pH using 1 *N* HCl or NaOH. The solutions (1-mL aliquots) were then transferred to 3-mL Type I flint glass vials, sealed with PTFE-coated combination seals/stoppers, and stored upright in a 60°C oven. Samples were removed at appropriate time intervals and stored at 2–8°C. SEC was used to quantitate remaining antibody and degradation peaks.

Effect of Protein Concentration, Temperature, Buffer, and NaCl

Various solutions of chimeric L6 (Table I) were prepared by exchanging the original buffer with the desired buffer system by the procedure described previously. Aliquots (0.5 mL) of the test solutions in 3-mL flint glass vials were stored at 50, 40, 30, and 2–8°C. At appropriate time intervals samples were removed and frozen at −70°C until analysis by SEC. A sample stored at −70°C was used as the control.

Effect of Agitation

A 2-mL sample of chimeric L6 in a 15-mL sterile Falcon conical polypropylene tube was agitated on an Eberbach shaker at 200 rpm for 24 hr at room temperature. Samples were removed periodically, assayed directly by SEC, and compared to a control solution.

Measurement of Ammonium Ion Concentration

The concentration of ammonium ion in stressed samples was measured spectrophotometrically with a coupled-enzyme assay (5). The ammonium ion liberated as a result of deamidation of side chains of L-asparagine and/or L-glutamine is incorporated into α-KG, resulting in a Schiff's base which is enzymatically reduced by GDH. This is accompanied by oxidation of β-nicotinamide adenine dinucleotide, reduced form (NADH), to NAD^+, resulting in a decrease in absorbance at 340 nm. Excess concentrations of α-KG, GDH, and NADH were used so that the only limiting reagent would be ammonium ion.

A cuvette containing 100 μL of 10 m*M* α-KG, 100 μL of 0.256 m*M* NADH, 15 μL of GDH (58 U), and 735 μL of 0.05 *M* phosphate buffer with 0.5 *M* sodium chloride, pH 7.2, was allowed to equilibrate in the cell compartment of the spectrophotometer at 340 nm at ambient temperature. After establishment of a stable baseline, the measurement of ammonium ion was initiated by adding 50 μL of a sample of partially degraded chimeric L6. An initial drop in absorbance due to dilution was observed, followed by a change in absorbance due to consumption of ammonium. The amount of ammonium present was calculated using a molar absorptivity (ε) of 6200 M^{-1} cm^{-1} for NADH. After a plateau was reached, indicating consumption of the liberated ammonium by GDH, 2 μL of ammonium chloride (0.1 *M*) was added to ensure that the assay was working properly.

Multiple Freezing and Thawing

Effect of pH

Portions of chimeric L6, 1 mg/mL in 0.018 *M* phosphate buffer and 0.2 *M* NaCl, pH 7.2, were adjusted to pH 4 to 8 using 0.1 *N* HCl or NaOH. Aliquots of each solution (1 mL) in 3-mL Type I flint glass vials were frozen at −20°C for approximately 2 hr and then thawed at ambient temperature for 30–45 min. Samples were returned to the freezer and the procedure was repeated. Control samples were stored at −70°C. The percent of the monomer remaining was measured directly using SEC.

Effect of Protein, Buffer, and Salt Concentrations

Solutions of chimeric L6 with varying buffer, protein, and salt concentrations which were prepared for thermal degradation were also subjected to multiple freezing and thawing.

Table I. Solution Stability of Chimeric L6

Buffer	Effect	[B] (*M*)[a]	[NaCl] (*M*)	pH	[L6] (mg/mL)[b]	$k_{obs} \times 10^3$ wk^{-1} 30°C	40°C	50°C	E_a[c] (kcal/mol)
Phosphate	Control	0.05	0.5	7.1	5.02	16.8	44.0	181	23.0
Phosphate	[B]	0.01	0.5	7.1	4.74	15.6	34.0	168	23.0
Phosphate	[L6]	0.05	0.5	7.2	1.00	11.4	38.0	204	28.0
Phosphate	[L6]	0.05	0.5	7.2	9.51	17.3	39.8	172	22.3
Phosphate	[NaCl]	0.05	0.1	7.2	4.96	17.3	36.0	155	21.2
Citrate	Control	0.05	0.5	5.5	5.08	2.69	6.61	30.4	23.5
Citrate	[B]	0.01	0.5	5.5	5.24	0.41	5.69	22.5	39.0
Citrate	[L6]	0.05	0.5	5.5	0.89	2.03	4.31	37.0	28.1
Citrate	[L6]	0.05	0.5	5.5	10.6	1.93	6.10	34.3	27.9
Citrate	[NaCl]	0.05	0.1	5.5	5.23	2.20	10.6	58.6	31.9

[a] Buffer concentration.
[b] Chimeric L6 concentration.
[c] Activation energy.

Effects of Excipients

Solid excipients (Table V) were weighed, added directly to 10 mL of chimeric L6, and mixed with a vortex mixer until completely dissolved. For L-arginine and L-lysine, 500 mg of the amino acid was slurried with 0.25 mL of concentrated HCl and 425 μL of sterile water for injection (SWFI) and then added to 9 mL of chimeric L6. All of the solutions were sterile-filtered through a 0.22-μm Acrodisc filter, dispensed in 1-mL portions into 3-mL Type I flint glass vials, and subjected to freeze/thaw cycling.

RESULTS AND DISCUSSION

Solution Stability at 60°C

pH–Rate Profile

The stability of chimeric L6 was examined from pH 4.5 to pH 8.6 at 60°C using SEC. Under these conditions four major degradation peaks were observed. Two lower molecular weight species (D_1 and D_2) at relative retention times of 1.05 and 1.24, respectively, were formed primarily at pH values above 5. Higher molecular weight species, A_1 and A_2, were formed at pH values below 5 and had relative retention times of 0.84 and 0.68 (void volume), respectively. Comparison of the retention volumes to the calibration curve suggests that A_1 was a dimer and A_2 had a molecular weight of at least 2×10^6 daltons. Representative SEC chromatograms of freeze/thaw and thermally degraded chimeric L6 are illustrated in Fig. 1.

Loss of chimeric L6 from aqueous solution followed pseudo-first-order kinetics at pH values of 4.5 and above. The pseudo-first-order rate constants (k_{obs}) at pH 4.5–7.2 were calculated from the slopes of plots of ln(percentage remaining) versus time. The rate constants were not corrected for possible buffer or ionic strength effects. Due to limited data at pH 8 and 8.6 the rate constants were estimated from single values of percentage remaining after 24 hr, assuming first-order kinetics. Solutions of chimeric L6 at pH 4.0 degraded rapidly and did not follow first-order kinetics, so rate constants were not calculated.

The pH dependence of the degradation of chimeric L6 at 60°C is illustrated in Fig. 2. The overall pH–rate profile shows that the antibody is most stable in the pH range of 5.5 to 6.0 at 60°C. Above pH 5.5, D_1 and D_2 were the major degradation peaks. The shape of the curve in this pH range suggests a base-catalyzed degradation pathway, possibly deamidation of L-asparagine or L-glutamine residues, peptide bond cleavage, or disulfide bond cleavage. The extent of aggregation as measured by formation of A_1 and A_2 was not significant under these conditions. Analysis by ion-exchange chromatography indicated that degraded samples containing peaks D_1 and D_2 exhibited additional peaks with shorter retention times than the parent. This is consistent with formation of species with less net surface positive charge, which could have resulted from any of the aforementioned processes.

Below pH 5.5 the degradation of chimeric L6 exhibited acid catalysis. Although D_1 and D_2 formed at all pH values, aggregation to A_2 (large molecular weight aggregate) appeared to be the main pathway responsible for acid-catalyzed degradation. Visible aggregates were not observed in any of the samples.

The line in Fig. 2 represents the theoretical curve for pH-dependent degradation of chimeric L6 based on the following equation:

$$k_{obs} = \frac{k_{H^+}[H^+]^2}{K_a + [H^+]} + \frac{k_o[H^+]}{K_a + [H^+]} + \frac{k_{OH}K_w}{K_a + [H^+]} \quad (1)$$

where k_{obs} is the observed rate constant, k_{H^+} is the rate constant for acid-catalyzed degradation, $[H^+]$ is the hydrogen ion concentration, K_a is the effective ionization constant, k_o is the rate constant for the uncatalyzed reaction,

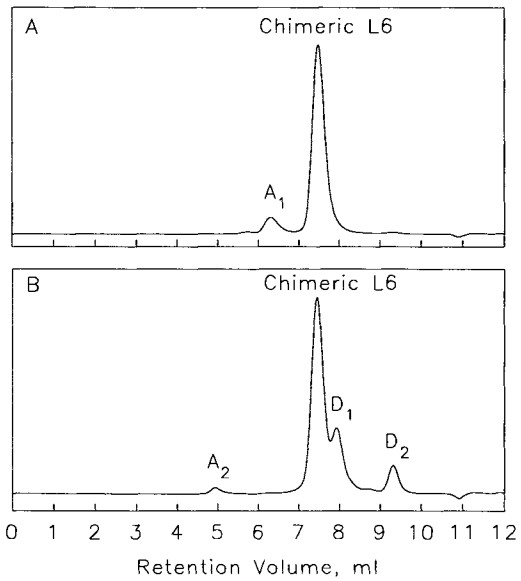

Fig. 1. Typical SEC of (A) chimeric L6 subjected to 35 freeze/thaw cycles over 14 days and (B) chimeric L6 at pH 7.2 stored at 60°C for 4 days.

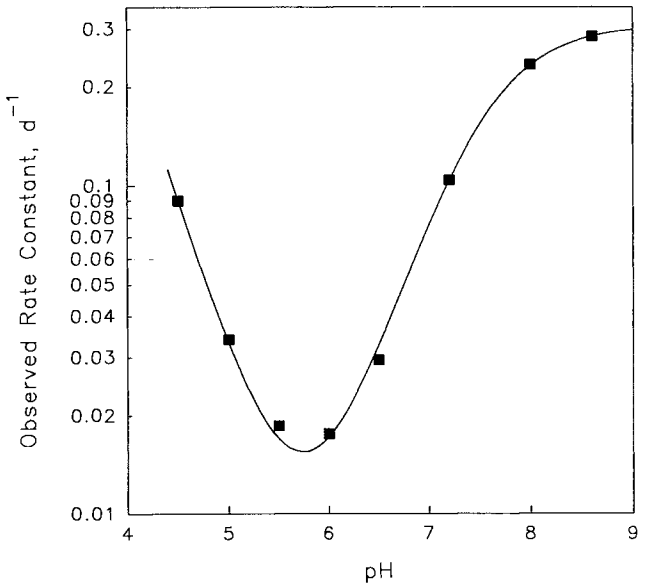

Fig. 2. Solution stability of chimeric L6 at 60°C. The symbols represent averages of three determinations and the solid line is the theoretical curve calculated using Eq. (1).

k_{OH} is the base-catalyzed rate constant, and K_w is the ionization constant for water. The calculated parameters are summarized in Table II. The equation predicts the behavior reasonably well at pH 4.5 and above, but under more acidic conditions (pH <4.5), where irreversible aggregation is the predominant degradation pathway and pseudo-first-order behavior is not followed, the model is not applicable.

Treatment of the kinetic data in the same manner as a conventional small molecule yielded an effective pK_a of 7.5 at 60°C. It is defined as an effective pK_a since it most likely reflects multiple ionizable groups involved in degradation. The effective pK_a is distinct from the p*I* of the protein (9.5–10.5 at 25°C) determined by isoelectric focusing (IEF) (6). The p*I* represents ionization of all ionizing groups on the protein, in contrast to the pK_a, which represents the specific ionizing groups in the kinetically important regions of the protein.

Effect of Temperature, Protein, Buffer, and Salt Concentrations

The stability of chimeric L6 was investigated under varying conditions of temperature, buffer type, and antibody, buffer, and NaCl concentration. The data in Table I suggest that the stability of chimeric L6 at pH 7.1–7.2 at all temperatures (30–50°C) was not significantly influenced by phosphate buffer concentration (0.01 to 0.05 *M*), antibody concentration in the range of 1–10 mg/mL, or NaCl concentration (0.1 to 0.5 *M*). In citrate buffer at pH 5.5, the overall stability was greater than at higher pH values but no correlation between stability and the solution composition was observed (Table I). Distinctions or comparisons among the formulation parameters at pH 5.5 are difficult due to the greater stability compared to pH 7.2. Even after 12 months at 30°C, <10% loss was observed by SEC. Therefore, trends among the various citrate-buffered solutions are difficult to observe.

Arguments for and against thermal stress as an accurate estimate of lower temperature stability of proteins have been presented (7–9). The overwhelming conclusion that can be made from the examples put forward is that the applicability of Arrhenius behavior to proteins depends on the specific protein in question. Due to the possibility of multiple chemical and physical degradation pathways, the observed kinetic behavior at any given temperature will be determined by the predominant degradation processes and the temperature dependence of each process. In the case of chimeric L6, Arrhenius behavior was apparent at pH 7.2 in the temperature range of 30–50°C, with an apparent activation energy of 23 kcal/mol. At pH 5.5 the degradation was too slow to accurately determine activation energies, but the results follow the expected trend. To relate the results from high-temperature studies to normal storage conditions, the stability of chimeric L6 was monitored at 2–8°C, the recommended storage temperature. As indicated in Table I, minimal loss of monomeric L6 by SEC (<2%) was observed at 2–8°C after 18 months. Extrapolation of the high-temperature SEC data to 2–8°C results in estimates for t_{90} of 6 and 75 years at pH 7 and 5.5, respectively, which are consistent with the available data. As with conventional pharmaceuticals, the use of high-temperature stability testing is advocated in appropriate situations for predicting trends and initial screening of formulation variables.

Table II. Kinetic Parameters Calculated using Eq. (1) for Degradation of Chimeric L6 at 60°C

Parameter	Result
k_{H^+} (M^{-1} day^{-1})	2668
k_{OH} (M^{-1} day^{-1})	9.24×10^5
k_0 (day^{-1})	5.78×10^{-3}
pK_a	7.5

Effect of Agitation

Proteins are often subject to aggregation and precipitation due to denaturation at interfacial surfaces, including the air–liquid interface (10). Continual shaking creates a large effective surface area and can lead to significant denaturation of surface active proteins. The susceptibility of a protein to this type of surface phenomenon has implications in the production process, solution manipulation, and shipping.

Chimeric L6 was shaken at 200 rpm at ambient temperature for 24 hr to assess its potential for denaturation and aggregation due to shipping and handling. The results are summarized in Table III. Essentially no changes in monomer concentration, aggregates, or degradation peaks were evident by SEC. This indicates that chimeric L6 is not denatured at the air–liquid interface and should withstand normal handling conditions.

Measurement of Ammonium Ion Concentration

Deamidation of proteins upon storage at elevated temperatures leads to liberation of ammonium ion (3,5), which can be quantitated to determine the extent of degradation by this process. Selected samples of chimeric L6 which had been subjected to thermal degradation were assayed for free ammonium ion using a coupled-enzyme assay as shown in Fig. 3 (5). A 10 mg/mL solution of chimeric L6 in 0.05 *M* sodium phosphate buffer and 0.5 *M* NaCl at pH 7.2 was stored for 3 weeks at 50°C. The amount of monomeric L6 remaining as determined by SEC was 59%, and the degradation peaks corresponded to lower molecular weight fragments, including D_1 and D_2. The amount of ammonium de-

Table III. Effect of Shaking (200 rpm for 24 hr) on Stability of Chimeric L6 in pH 7.1 Phosphate Buffer at Ambient Temperature

Time (hr)	Peak area percentage[a]			
	A_1	L6	D_1	D_2
0	0.29	93.95	3.06	1.57
0.5	0.29	94.05	3.07	1.54
1	0.14	94.36	2.98	1.58
2	0.20	94.12	3.19	1.56
4	0.32	94.07	3.12	1.60
6	0.14	94.21	3.07	1.63
24	0.18	94.05	3.39	1.69

[a] A_1, dimer; D_1, degradation peak at a relative retention time of 1.05; D_2, degradation peak at a relative retention time of 1.24.

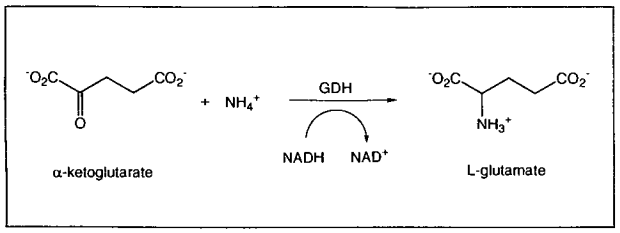

Fig. 3. Scheme illustrating the reaction of ammonium ion in the coupled enzyme assay.

tected in the sample was 0.19 m*M*. Assuming a molecular weight of 150,000 for the antibody, mole fractions of 0.025 and 0.030 for asparagine and glutamine residues (1), respectively, and an average amino acid molecular weight of 133 daltons, the amount of ammonium formed corresponded to deamidation of approximately 4% of the total number of Asn and Gln residues in the protein.

The SEC assay is not capable of distinguishing small changes, such as deamidation, in the antibody molecule. However, deamidation of a single amino acid residue in the IgG molecule (which would approximate 4% of the total residues) would not likely result in the degree of fragmentation observed by SEC. This would suggest that other degradation pathways, such as base-catalyzed hydrolysis of the peptide backbone of the antibody or β-elimination of cysteine or other residues, may also contribute to the overall loss of chimeric L6.

The liberation of ammonium ion was also assessed in a succinate-buffered solution of chimeric L6 at pH 5.5. After storage of a 10 mg/mL solution in 0.05 *M* succinate buffer containing 0.5 *M* NaCl at pH 5.5 at 50°C for 2 weeks, the amount of free ammonium measured was 0.024 m*M*, corresponding to approximately 0.6% deamidation. Therefore this degradation pathway was not considered significant at lower pH. This result is consistent with results reported previously (3), where deamidation is more favorable under neutral and alkaline conditions.

Further support for the lack of significant deamidation is the stereochemical environment of the side chain amide bonds. Examination of the primary sequence of chimeric L6 revealed that most glutamine and asparagine residues had bulky neighboring groups including tyrosine, threonine, tryptophan, and phenylalanine (1). The sterically hindered environments dictated by the secondary and tertiary structures of this IgG molecule would be expected to reduce its susceptibility to deamidation (3,11). Furthermore, it has been demonstrated that deamidation may involve formation of a succinimide intermediate, which, depending on the bonds cleaved during hydrolysis, can also lead to racemization or transpeptidation (3,12). The formation of this intermediate is influenced by the folding of the polypeptide chain about the deamidation site. In native proteins the conformation near Asp and Asn residues generally restricts the approach of the peptide bond to the side-chain carbonyl carbon, thereby limiting the reactivity in the native state, and therefore deamidation is often accelerated in the denatured state (12). This has been confirmed for bovine pancreatic ribonuclease (13) and bovine and human growth hormones (14).

Physical Stability

The physical stability of chimeric L6 was assessed by subjecting it to multiple freezing and thawing, a stress condition which has been applied in the assessment of protein stability (4). The effect of freezing at −20°C and subsequent thawing of chimeric L6 solutions was evaluated as a function of pH. The results in Fig. 4 indicate a significant pH dependence of aggregation after 35 cycles of freezing and thawing. The major degradation peak observed by SEC was A_1, with an approximate molecular weight corresponding to a dimer. Maximal amounts of A_1 formed at pH 6.5 and minimal amounts at pH below 5.5 or greater than 8.0. The aggregation was reversible since incubating a sample containing 13% A_1 at 37°C for a few hours resulted in nearly complete dissociation to the parent antibody (<0.3% A_1 remaining). This suggests that the dimer formed as a result of noncovalent interactions and is supported by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which showed bands similar to those of the monomeric antibody and no higher molecular weight bands under nonreducing conditions (data not shown).

The pH of maximal aggregate formation (pH 6–7; Fig. 4) did not correspond to the p*I* (9.5 to 10.5). Theoretically, aggregation of the native antibody would be expected to be maximal at the p*I* of the protein where the net surface charge is zero and hydrophobic interactions would be greatest. However, multiple freezing and thawing involves phase changes, adding another dimension to the complexity of aggregate formation. Freezing can induce physical changes in the solutions, including partial unfolding of the protein, precipitation of certain salts, pH changes, concentration of various solution components, and potential alterations of interactions between the protein and water (15). Any physical changes in the solutions could potentially destabilize the protein and cause aggregation.

The aggregation behavior of chimeric L6 is in agreement

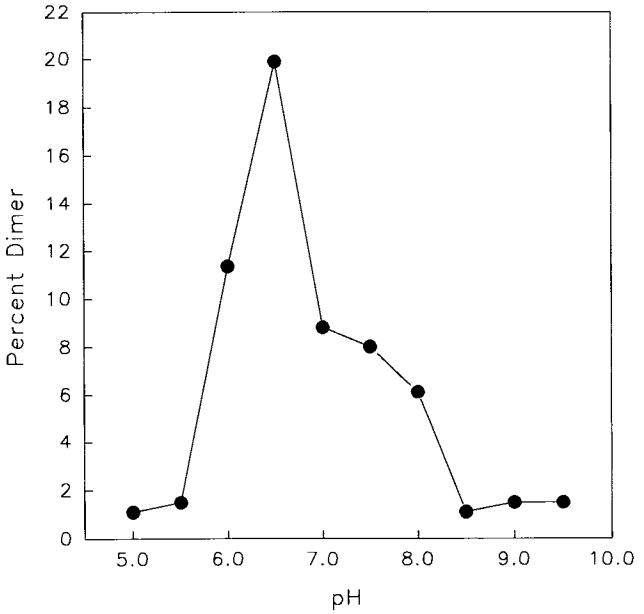

Fig. 4. Aggregation of chimeric L6 as a function of pH after multiple freezing and thawing (35 cycles).

with the work of Soltis and Hasz, who demonstrated that nonthermal IgG aggregates, formed in isolated IgG preparations, were almost entirely dissociated at low pH levels, in contrast to heat-induced aggregates which were not dissociated under the same conditions (16). In addition, Hansson has presented evidence to suggest that the bonds responsible for thermal IgG aggregation are of a different nature than those which form as a result of multiple freezing and thawing (17).

Effects of Phosphate, NaCl, and Antibody Concentrations

To study the variables which affect the freeze/thaw-induced dimerization of chimeric L6, solutions of varying concentrations of buffer, salt, and antibody were evaluated at pH 7.2. At this pH a significant amount of dimer formation was observed (Fig. 4). Table IV summarizes the results. Aggregation was greater at a buffer concentration of 0.05 *M* compared to 0.01 *M* (11.8 vs 2.5%, respectively). The effect is not likely a function of ionic strength differences since NaCl (0.5 *M*) was the main determinant of ionic strength. The behavior of sodium phosphate upon freezing may contribute to the differences observed. Changes in pH upon freezing can be significant (up to 3 pH units) due to precipitation of Na_2HPO_4 (15) and the effects would be expected to be concentration dependent. Thus, pH changes, phase changes, crystallization of water, and freeze-concentration of the protein could potentially influence the protein conformation and aggregation.

The influence of [NaCl] on chimeric L6 aggregation is less clear. In 0.05 *M* phosphate buffer at pH 7.1 the aggregate increased to 11.8 and 16% in 0.5 and 0.1 *M* NaCl, respectively, after 38 freeze/thaw cycles (Table IV). The salt type and concentration could potentially alter the aggregation properties of proteins in a variety of ways. Examples include effects of the amount of tightly bound nonfreezing water (18), solute heterogeneity upon thawing (19), and crystallinity or amorphous character of the frozen state (20). Interferon-γ aggregation is enhanced with the addition of sodium chloride, possibly due to a masking effect of the chloride ions on the positive charge of the protein (3). Similar behavior has also been observed in β-lactamase (21), α-lactalbumin (22), and ribonuclease T_1, which is stabilized by preferential binding of anions and cations to specific sites on the protein (23). Other contributing effects of salts include lyotropic effects, alterations in the water structure, and a balance between strengthening and weakening forces of anions and cations (24).

Under conditions at which chimeric L6 aggregation is relatively high (0.05 *M* sodium phosphate, 0.5 *M* NaCl at pH 7.1), the antibody concentration in the range of 1–10 mg/mL did not have a significant effect on aggregation (Table IV). The lack of a significant concentration effect suggests that other mitigating factors more profoundly influence the hydrophobicity of the solution and potential conformational changes upon freezing or thawing that lead to aggregation. Furthermore, the insensitivity of dimerization to the protein concentration indicates that a classical bimolecular collision mechanism cannot entirely explain the aggregation of chimeric L6.

Effect of Excipients

The results in Table V indicate that most common sugars including lactose, sucrose, glucose, and mannitol effectively prevented freeze/thaw-induced aggregation (2–4 vs 15% dimer). The mechanism is nonspecific, as other polyhydric alcohols such as glycerin and dextran 40 achieved similar stabilization. Surfactants such as Pluronic F68 and polysorbate 80 (Tween 80) did not appreciably prevent the aggregation, suggesting that it may not be a surface phenomenon. Solutions of chimeric L6 in the presence of 5% glycerin were also chemically stable and were resistant to aggregation induced by freezing and thawing. Glycerin is an acceptable excipient and has been used in many marketed products.

All amino acids that were examined, including glycine, L-arginine, L-lysine, and L-alanine, reduced aggregation effectively. Amino acids including glycine and alanine have been used routinely for stabilization of monoclonal antibodies and other biological compounds (25). However, L-cysteine was not acceptable due to formation of a precipitate.

Dextran 40, human serum albumin (HSA), nicotinamide, and sodium taurocholate were also found to prevent aggregation effectively (Table V). Ethanol was incompatible with chimeric L6, as it caused precipitation of the protein.

The primary variable affecting the physical stability of chimeric L6 toward freezing and thawing was apparently the sodium phosphate buffer and the resultant pH changes upon

Table IV. Effect of Multiple Freezing and Thawing on Stability of Chimeric L6 in Sodium Phosphate Buffer

					Peak area percentage			
					Initial		38 cycles	
Effect	[B] (*M*)[a]	[NaCl] (*M*)	pH	[L6] (mg/mL)[b]	A_1[c]	L6	A_1[c]	L6
Control	0.05	0.5	7.1	5.02	1.5	95.9	11.8	85.3
[B]	0.01	0.5	7.1	4.74	1.8	95.9	2.5	96.8
[NaCl]	0.05	0.1	7.2	4.96	1.9	95.7	16.0	80.2
[L6]	0.05	0.5	7.2	1.00	1.4	95.5	8.3	89.9
[L6]	0.05	0.5	7.2	9.51	1.4	95.7	9.7	88.3

[a] Buffer concentration.
[b] Chimeric L6 concentration.
[c] Dimer.

Table V. Effect of Excipients on Freeze/Thaw-Induced Aggregation of Chimeric L6 in 0.05 *M* Sodium Phosphate Buffer/0.5 *M* NaCl, pH 7.2

Excipient	Percentage[a]	[L6] (mg/mL)	22 freeze/thaw cycles: A_1 peak area percentage[b]	22 freeze/thaw cycles: Percentage L6 remaining
Control	—	5.2	15	82.1
Lactose	5	5.1	2.3	96.6
Sucrose	5	5.2	2.1	100
Glucose	5	5.3	2.0	97.9
Mannitol	5	5.2	3.9	96.9
PEG 300	5	4.8	5.3	104
Glycerin	5	4.6	1.9	99.4
Ethanol	5	4.8	5.6	94.4
Pluronic F68	1	3.5	9.7	91.0
Polysorbate 80	1	4.0	15	85.0
Glycine	5	4.9	1.9	98.1
L-Arginine	5	4.9	1.4	96.1
L-Lysine	5	4.4	2.1	98.1
L-Alanine	5	5.3	2.4	97.4
L-Cysteine	0.1	5.1	2.6	93.0
Dextran 40	5	5.0	2.7	96.2
Human serum albumin	1.25	4.9	1.3	98.8
Nicotinamide	5	4.8	2.1	95.3
Sodium taurocholate	2	4.7	4.9	97.6

[a] w/v for solids and v/v for liquids.
[b] A_1, dimer.

freezing. Incorporation of certain excipients such as sugars could possibly alter the precipitation behavior of Na_2HPO_4 (i.e., inhibit the crystallization) and consequently minimize aggregate formation.

Binding Activity by ELISA

The previous discussion centered primarily on the use of SEC as an indicator of protein stability. However, the SEC method is unable to detect small changes in a large molecule. An antiidiotype binding ELISA was used to quantitate the binding activity of selected stressed stability samples of chimeric L6 (Table VI). The antiidiotype 1B antibody (αID 1B) binds to the murine F_{ab} portion of chimeric L6 and is therefore a potential indicator of chemical and/or conformational changes in the antigen binding region of the protein. The results for samples subjected to heat stress and multiple freeze/thaw cycles are summarized in Table VI.

Comparison of the percentage antibody remaining by SEC and ELISA indicates a large degree of variability in the ELISA assay for samples stored at elevated temperature (50°C). Although the two methods are both indicators of protein concentration, they actually measure different species. SEC readily separates protein species with large differences in molecular weight and therefore measures gross changes in the protein structure, such as aggregation or fragmentation. On the other hand, the ELISA measures binding of an antiidiotype antibody to the murine F_{ab} region of the protein. Alterations in this portion of the molecule or in portions that directly affect either the conformation or the binding of chimeric L6 to the antibody may cause changes in concentration determined by the ELISA method. Lower molecular weight fragments or aggregates may also have binding activity and falsely elevate the concentration remaining using this assay.

Despite the differences between the SEC and the ELISA assays, the results from both methods indicate that chimeric L6 is more stable at pH 5.5 than at pH 7.1. The high-temperature data were used as an indicator of conditions favoring good stability. Stability under more realistic

Table VI. Percentage of Chimeric L6 Remaining as Measured by SEC and Antiidiotype Binding ELISA

Conditions	pH 7.1[a] SEC	pH 7.1[a] ELISA	pH 5.5[b] SEC	pH 5.5[b] ELISA
Control	100	100 (12.3)[c]	100	100 (3.7)
2–8°C/1 mo	n.d.[d]	n.d.	101	91.4 (10.0)
2–8°C/2 mo	96.2	89.5 (15.0)	n.d.	n.d.
50°C/1 day	95.5	75.6 (10.6)	n.d.	n.d.
50°C/3 days	87.7	126 (4.0)	n.d.	n.d.
50°C/1 wk	78.6	77.9 (5.7)	93.6	86.4 (18.9)
50°C/2 wk	65.9	72.1 (4.5)	93.5	105 (9.5)
50°C/3 wk	59.8	66.3 (6.8)	n.d.	n.d.
50°C/4 wk	n.d.	n.d.	78.0	119 (4.6)
50°C/2 mo	n.d.	n.d.	76.7	115 (7.6)
67 freeze/thaw cycles	92.7	107 (6.1)	95.0	111 (2.4)

[a] 9.5 mg/mL chimeric L6 in 0.05 *M* sodium phosphate buffer/0.5 *M* NaCl, pH 7.1.
[b] 10.6 mg/mL chimeric L6 in 0.05 *M* sodium citrate buffer/0.5 *M* NaCl, pH 5.5.
[c] Coefficients of variation for ELISA assay, performed in quadruplicate.
[d] Not determined.

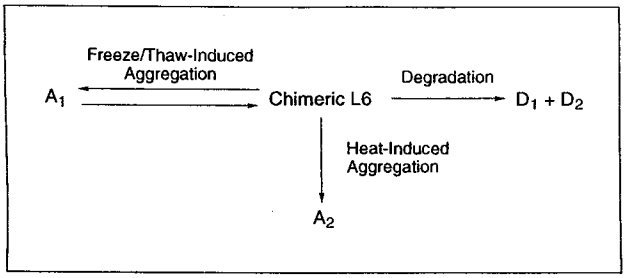

Fig. 5. A proposed scheme for the overall disappearance of chimeric L6 from aqueous solution.

storage conditions (2–8°C) confirmed the observations made at 50°C, and there was also good correlation between the SEC and the ELISA methods.

Summary of Degradation Scheme

A proposed scheme for the overall disappearance of chimeric L6 from aqueous solution is illustrated in Fig. 5. There are at least three processes which contribute to loss of chimeric L6: chemical degradation, heat-induced aggregation, and aggregation induced by multiple freezing and thawing. The major factor influencing the rate and route of degradation is pH. Comparison of the stability profiles after thermal stress and multiple freezing and thawing suggests that optimal stability can be achieved at pH 5.5. At higher pH values thermal degradation and freeze/thaw-induced aggregation are greater, and irreversible aggregation is predominant under thermal stress at pH values below 5.5.

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