Aqueous Stability of Human Epidermal Growth Factor 1-48

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Human epidermal growth factor 1-48 (hEGF 1-48, Des(49-53)hEGF) is a single chain polypeptide (48 amino acids; 3 disulfide bonds; 5445 Da) possessing a broad spectrum of biologic activity including the stimulation of cell proliferation and tissue growth. In this study, three primary aqueous degradation products of hEGF 1-48 were isolated using isocratic, reverse phase/ion-pair HPLC. The degradation products were characterized using amino acid sequencing, electrospray ionization mass spectrometry, isoelectric focusing, and degradation kinetics. Results indicate that hEGF 1-48 degrades via oxidation (Met^{21}), deamidation (Asn^{1}), and succinimide formation (Asp^{11}). The relative contribution of each degradation pathway to the overall stability of hEGF 1-48 changes as a function of solution pH and storage condition. Succinimide formation at Asp^{11} is favored at pH < 6 in which aspartic acid is present mostly in its protonated form. Deamidation of Asn^{1} is favored at pH > 6. The relative contribution of Met^{21} oxidation is increased with decreasing temperature, storage as a frozen solution (−20°C), and exposure to fluorescent light.

KEY WORDS: epidermal growth factor; degradation kinetics; reversed-phase/ion-pair HPLC; amino acid sequencing; isoelectric focusing; electrospray ionization mass spectrometry; oxidation; deamidation; succinimide.

INTRODUCTION

Advances in genetic engineering and biotechnology have made the commercial production of peptides/proteins for therapeutic applications an integral part of the pharmaceutical industry (1,2). Proteins are inherently unstable molecules (3), which present unique difficulties in their purification, formulation, storage, and delivery. They possess multiple functional groups in addition to three dimensional structure, which results in degradation via both chemical (modifications involving bond formation or cleavage) and physical (denaturation, aggregation, adsorption, precipitation) pathways.

Polypeptide growth factors, including epidermal growth factor (EGF), have a broad array of pharmacological/biological activities (4–6). These include: stimulation of protein kinase activity; stimulation of RNA, DNA and protein synthesis; stimulation of cell proliferation in a variety of cell types; inhibition of gastric acid secretion; inhibition of Na^+/H^+ exchange; and activation of the synthesis of extracellular molecules. This diverse pharmacology has attracted considerable interest in determining the physiologic roles and potential therapeutic applications of growth factors.

Human epidermal growth factor 1-48 (hEGF 1-48, Des(49-53)hEGF; Figure 1) is a single chain polypeptide (48 amino acids; 3 disulfide bonds; 5445 Da) possessing similar biologic activity to native human epidermal growth factor (7). These studies were undertaken to identify the chemical degradation pathways of hEGF 1-48 and determine the kinetic dependencies of those processes.

MATERIALS AND METHODS

Materials

hEGF 1-48 was obtained from Sibia (San Diego, CA; yeast derived) and PeproTech, Inc. (Rocky Hill, NJ; E. coli derived). All other chemicals were of reagent or analytical grade. All reagents used for amino acid sequence analysis were obtained from Applied Biosystem (ABI, Foster City, CA). PhastSystem, PhastGel/IEF 4-6.5, and the low molecular weight isoelectric focusing calibration kit were obtained from Pharmacia (Piscataway, NJ). Coomassie® Brilliant Blue R-250 was obtained from Pierce (Rockford, Il).

HPLC Methods

Instrumentation. The HPLC system was composed of a Hewlett Packard HP1050 series pump, variable wavelength detector, and autosampler. Samples in the autosampler were maintained at ≈4°C by a constant temperature circulating water bath (Brinkmann, Westbury, NY). A Hewlett Packard Vectra QS/20 equipped with Hewlett Packard Chemstation software was used for data acquisition and integration.

Analytical HPLC Method. The analytical HPLC method was used to confirm purity of the isolated degradation products and to assay samples from kinetic studies. A YMC-Pack Protein-RP (C4) column (4.6 × 250 mm) was used. The mobile phase was comprised of 28–31% (v/v) acetonitrile in water (acn:water), 1.08 g of octanesulfonic acid (sodium salt) per L of acn:water, and 2 mL of phosphoric acid (85%) per L of acn:water. The acn:water ratio was adjusted to achieve a retention time of 16.0 ± 1.0 min. This was necessary since, as like other proteins (8,9), the capacity factor (k′) of hEGF 1-48 is sensitive to small changes in acetonitrile concentration. The injection volume was 25 μL, and the eluent flow rate was 1.0 mL/min. The detector wavelength was 200 nm.

SemiPreparative HPLC Method. The semi-preparative HPLC method was used to isolate the primary degradation products of hEGF 1-48. The analytical method was scaled up to handle higher sample loads. A YMC-Pack Protein-RP (C4) column (10 × 250 mm) was used. The mobile phase was comprised of 28–31% (v/v) acetonitrile in water (acn:water), 1.08 g of octanesulfonic acid (sodium salt) per L of acn:water, and 2 mL of phosphoric acid (85%) per L of acn:water.

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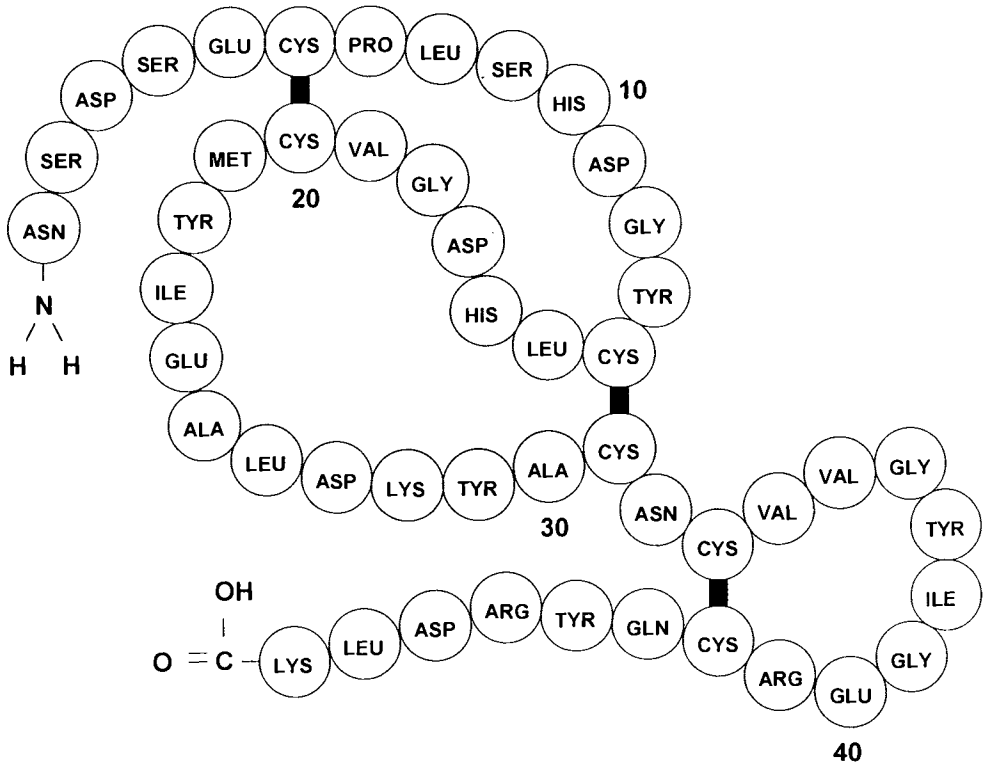

Figure 1. Structure of human epidermal growth factor 1-48 (hEGF 1-48) indicating the amino acid sequence and the location of disulfide linkages.

The acn:water ratio was adjusted as needed to optimize the separation of the degradant being isolated. The injection volume was 300 μL, and the eluent flow rate was 4.0 mL/min. The detector wavelength was 200 nm.

Identification of hEGF 1-48 Degradation Products

Isolation of Primary Degradation Products. Preliminary studies showed that three primary hEGF 1-48 degradation products are resolved by the described HPLC methods. The formation of each degradation product is favored under different storage conditions; One is favored by exposure to fluorescent light (DP1), another is favored in basic solutions (DP2), and the third is favored in acidic solutions (DP3). Therefore, a different incubation condition was chosen to maximize the generation of each degradation product. Solutions of hEGF 1-48 (1 mg/mL) were prepared in sodium phosphate buffer (0.05 M, pH 7.6 & 0.02 M, pH 6.0) and sodium citrate buffer (0.05 M, pH 3.4). The hEGF 1-48 solutions prepared at pH 7.6 & pH 3.4 were incubated in glass volumetric flasks at 30°C (ESPEC Humidity Cabinet Model LHL-112, Osaka, Japan) for 2 weeks and then stored in a refrigerator (≈4°C) prior to degradation product isolation. The solution prepared at pH 6.0 was sparged with oxygen, crimp sealed in a 20 mL glass vial, and then incubated in a fluorescent light cabinet with an intensity of 1000 foot candles (Forma 3890, Marietta, OH; ≈25°C) for 1 month prior to degradation product isolation. Each desired degradation product was isolated from its incubation solution using the semi-preparative HPLC method. Fractions were collected by hand as the degradation product eluted from the column/detector. Collection runs were repeated several times so that fractions could be combined to ensure adequate yield of the degradation product. The collected fractions were dialyzed against water and concentrated using Centricon-3 microconcentrators (Amicon, Beverly, MA). Following isolation, purification, and concentration each isolate was analyzed by HPLC for purity verification and yield estimates (in terms of HPLC peak height response of the degradation products relative to hEGF 1-48 standard). They were then stored in polypropylene cryovials at −70°C (Revco, Asheville, N.C.) until further analyses were performed.

Amino Acid Sequencing. Amino acid sequence analysis was performed with a ABI Model 473A Protein Sequencer System (Applied Biosystems, Foster City, CA), which automates Edman degradation and phenylthiohydantoin-amino acid (PTH-AA) analysis. PTH-AA's are determined using the Model 473A's liquid chromatography (HPLC) system. An ABI-PTH-C18 (2.1 × 220 mm) column was used.

Electrospray Ionization Mass Spectrometry. Isolates were analyzed on a Finnigan TSQ 70 mass spectrometer (Finnigan MAT, Inc., San Jose, CA) fitted with a Vestec electrospray (ES) interface (Vestec Corporation, Houston, TX). The instrument was tuned and calibrated to a resolution of 300 using a 17 M bovine insulin solution (Sigma Chemical Co., St. Louis, MO) in the direct infusion solvent. Samples were acquired in the profile mode scanning from m/z 500 to 20000 in 10 sec. The m/z value of each individual peak was determined by accumulation of data from 3 to 10 scans. Each peak was smoothed and the top half manually centroided. The molecular weight estimates represent the mean of the values calculated from each peak in the ES series. The av-

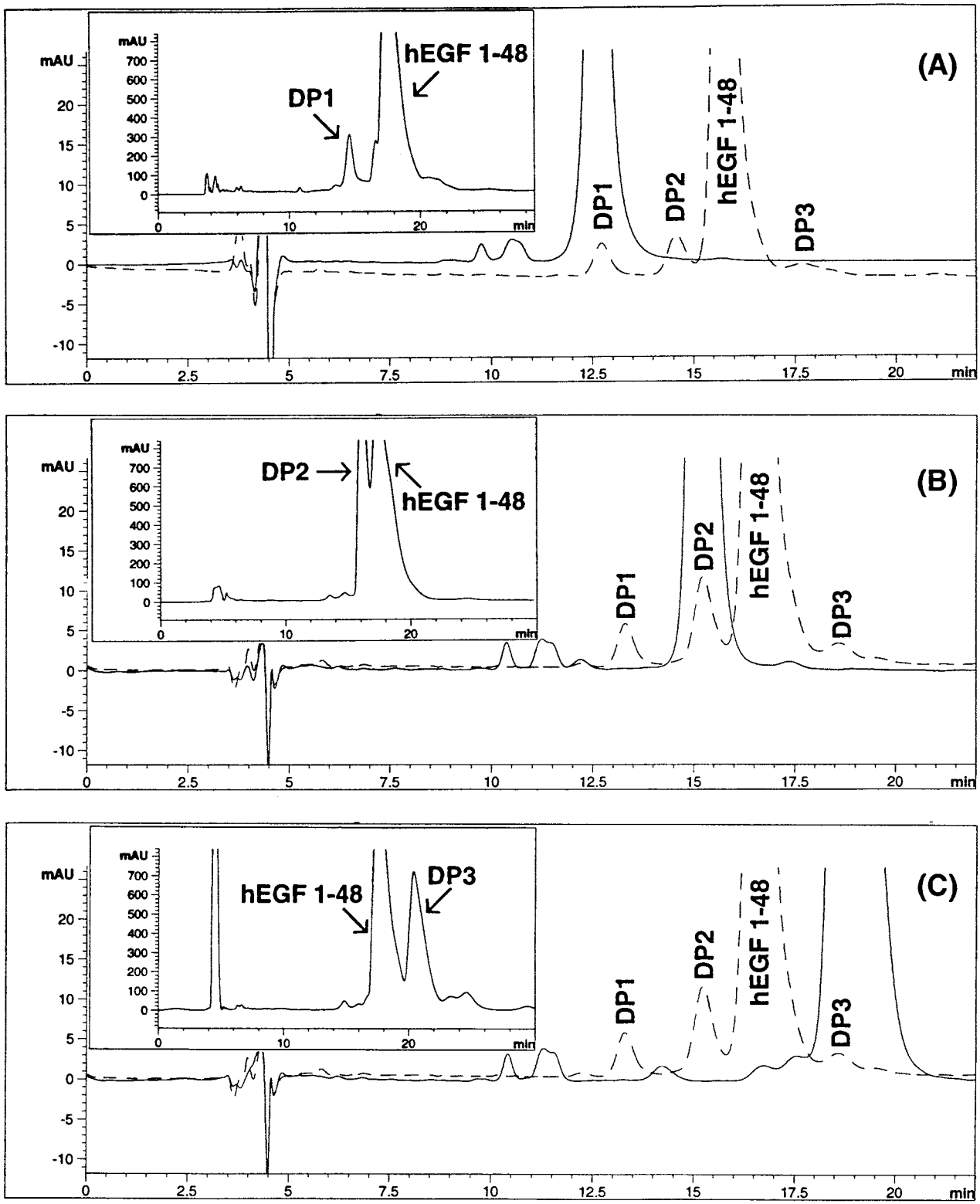

Figure 2. Analytical HPLC chromatograms indicating the purity of isolated degradation products (solid lines) compared to partially degraded hEGF 1-48 standards (initial conc. ≈50 μg/mL) showing the resolution of the three primary degradation products, DP1, DP2, and DP3 (broken lines). The unidentified peaks in the chromatograms of each isolate represent minor contamination with hEGF 1-48, the other degradation products and/or extractables from the microconcentrators. The insets show representative semi-preparative HPLC chromatograms obtained during purification of each degradation product. Incubation conditions for generation of each degradation product are noted in Materials and Methods. Key: A) DP1 Isolate; B) DP2 Isolate; C) DP3 Isolate.

erage of duplicate analyses is reported. The estimated uncertainty is ±1 dalton.

Isoelectric Focusing. The isolates were further concentrated approximately 10-fold using a SpeedVac® evaporator/concentrator system (Savant Instrument, Farmingdale, NY) to enhance detection of the bands. Isoelectric points were determined using the PhastSystem (Pharmacia, Piscataway, NJ), generally according to the procedure described by the manufacturer. The gel medium was composed of polyacrylamide, containing Pharmalyte® carrier ampholytes. Isoelectric points of the degradation products were determined by comparing their migration distances relative to the marker proteins in the calibration kit.

Kinetic Studies

Solutions of hEGF 1-48 (50 μg/mL) were prepared in sodium citrate (0.05 M, pH 3.8–5.3) and sodium phosphate (0.10 M, pH 6.0–7.6; 0.02 M, pH 6.0) buffers containing 0.01% (w/v) polysorbate 80. The polysorbate 80 was included to prevent surface adsorption of hEGF 1-48. Aliquots of these solutions were stored under controlled conditions in glass vials or ampoules. Constant temperature storage chambers, circulating water baths, and a fluorescent light cabinet (1000 foot candles) were used. Samples were withdrawn at various time points, and assayed by HPLC. The pH of each sample was verified at the end of each kinetic run; no changes in pH were found. The kinetics of degradation were determined as a function of pH, temperature, and fluorescent light exposure. The apparant first-order rate constants, k_{obs}, were estimated by log-linear least-squares fits of the % hEGF 1-48 remaining vs. time. Estimates of the initial formation rate constants for each primary degradation product, k_i, were made by least-squares fits of the degradation product concentration (% total peak area) vs. time. Degradation product concentrations at the first three to four available assay points were used. Initial rate fits resulting in correlation coefficients (r) > 0.8 were used for comparisons. The k_i estimates are reported in units (time^{-1}) associated with apparent first-order processes, which is consistent with the method of data analysis. However, it is recognized that the reaction mechanisms may be of some different order. The data analysis provided reasonable k_i estimates to identify overall trends and relative differences between degradation pathways as a function of pH and storage condition.

RESULTS AND DISCUSSION

Analytical and semi-preparative HPLC chromatograms which characterize the isolation and purification of the three primary hEGF 1-48 degradation products, DP1, DP2, and DP3, are shown in Figure 2. Approximate concentrations of the DP1, DP2, and DP3 isolates were 50, 60, and 70 μg/mL respectively. Results of amino acid sequencing, electrospray ionization mass spectrometry, and isoelectric focusing analyses for each degradation product are summarized in Table I.

Identification of DP1

The analytical data suggests that the lone methionine residue (Met^{21}) in hEGF 1-48 has been oxidized to the sulfoxide form (hEGF-ox). The key finding in this identification was the mass spectrometry result, which showed a gain of 15.4 daltons. This is consistent with the expected mass difference (+16 Da) following Met-sulfoxide formation. Although the side chains of His and Tyr present in hEGF 1-48 are also potential oxidation sites, oxidation of Met^{21} seems more likely due to the susceptibility of Met residues to oxidation by even atmospheric oxygen (1). No difference was found between hEGF 1-48 and DP1 by amino acid sequencing or isoelectric focusing analyses. These results were also expected following Met-sulfoxide formation. Met-sulfoxide is easily reduced to methionine by treatment with reducing agents, such as dithiothreotal, especially under acidic conditions (10). Since the Edman degradation is carried out under acidic conditions in the presence of dithiothreotal, methionine oxidation to a sulfoxide would not be detected by sequencing analysis. Also, the isoelectric point would not change following methionine oxidation since no ionizable groups are affected. Other variants of epidermal growth factor and insulin-like growth factor were found to be susceptible to methionine oxidation (11–13), which further supports our identification. The proposed reaction mechanism for the degradation of hEGF 1-48 via methionine oxidation is shown in Figure 3A.

Identification of DP2

The results of amino acid sequencing, mass spectrometry, and isoelectric focusing analyses of DP2 suggests the N-terminal asparagine (Asn^1) of hEGF 1-48 has undergone deamidation to form an iso-aspartic acid residue (hEGF-da). DP2 was resistant to Edman degradation at Asn^1. This indi-

Table I. Summary of Amino Acid Sequencing, Electrospray Ionization Mass Spectrometry, and Isoelectric Focusing Analyses of Isolated Degradation Products

Degradation product	Amino acid sequencing	Mass spectrometry[a]	Isoelectric point[b]
DP1	Consistent with hEGF 1-48	+15.4 Da	4.55
DP2	Sequencing blocked. N-terminal resistant to Edman degradation	+1.4 Da	4.40
DP3	1st ten residues consistent with hEGF 1-48. Residue 11 resistant to Edman degradation.	−17.9 Da	4.94

[a] Mass difference from hEGF 1-48.
[b] Isoelectric point of hEGF 1-48 was determined to be 4.55.

cated a chemical modification which blocked the sequencing reaction occurred at this residue. In Edman degradation, phenylisothiocyanate (PITC) couples with the terminal α-amino group of the protein to form a phenylthiocarbamyl (PTC) conjugate. The N-terminal amino acid is then selectively cleaved as a heterocyclic derivative (an anilothiazolinone) and converted to a more stable isomer (a phenylthiohydantoin; PTH-AA) prior to identification. A structural residue which cannot form the heterocyclic derivative, such as an iso-aspartic acid residue (iso-Asp), will be resistant to Edman degradation (14–16). Mass spectrometry of DP2 showed a gain of 1.4 daltons. This is consistent with the expected mass difference (+1 Da) following Asn deamidation. The isoelectric point of DP2 (pI 4.40) was less than that of hEGF 1-48 (pI 4.55). This is the expected result following Asn deamidation (forming an iso-Asp residue), since an ionizable carboxyl group is formed. Our identification is also supported by the findings of DiAugustine et al. (15), in which

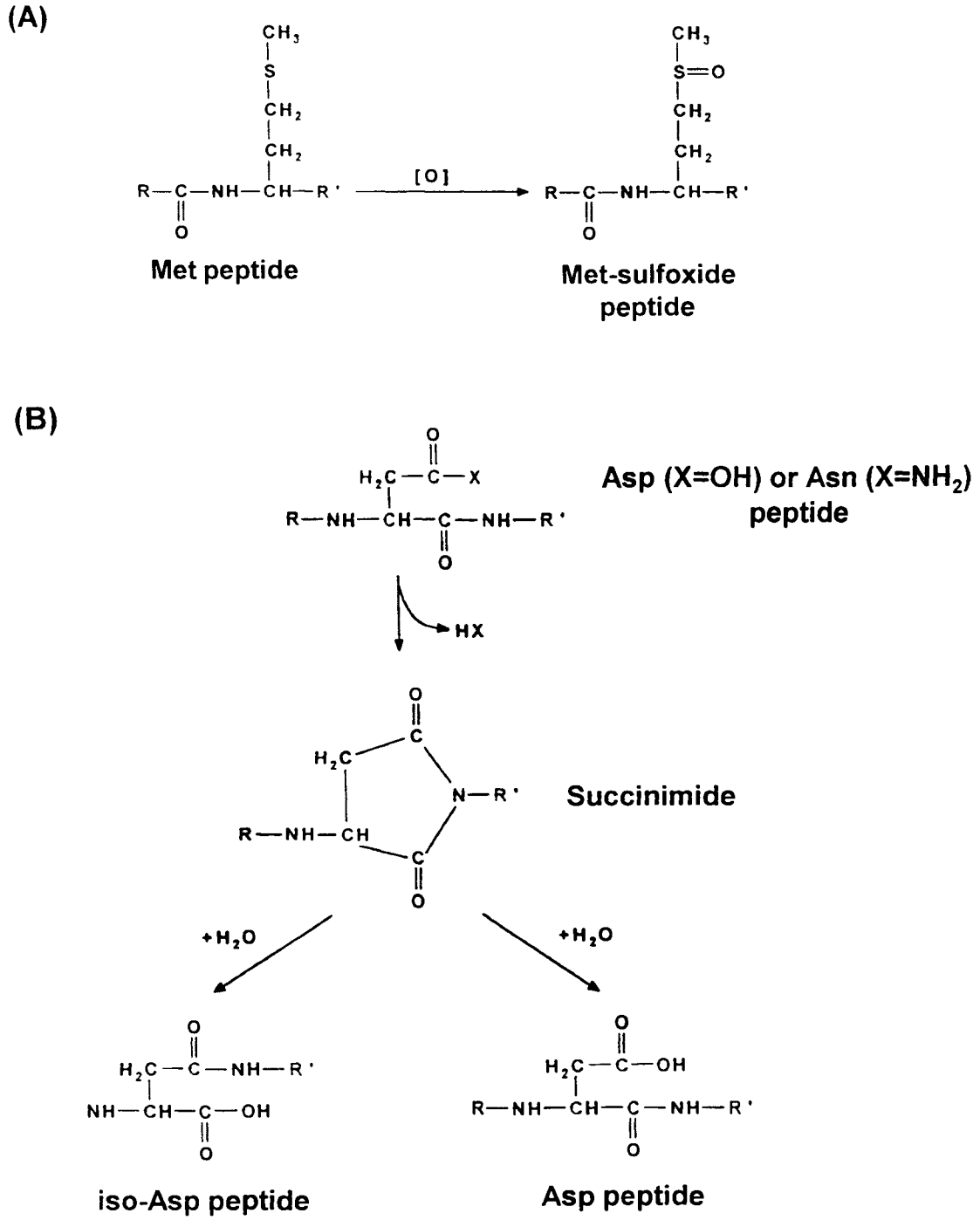

Figure 3. Reaction mechanisms for: A) Oxidation of Met to Met-sulfoxide, B) Deamidation at Asn, or succinimide formation/isomerization at Asp.

evidence for an iso-Asp variant of mouse epidermal growth factor resulting from the deamidation at the N-terminal asparagine was reported. The generally accepted reaction mechanism for Asn deamidation via a succinimide intermediate (1) is shown in Figure 3B.

Identification of DP3

The results of amino acid sequencing, mass spectrometry, and isoelectric focusing analyses of DP3 suggests the conversion of Asp^{11} in hEGF 1-48 to a stable Asp-succinimide group (hEGF-suc). Edman degradation was blocked at Asp^{11}, indicating a chemical modification occurred at this residue. Although aspartyl groups can undergo isomerization which would result in resistance to Edman degradation (as with DP2), the mass spectrometry result showed a loss of 17.9 daltons. This is not consistent with an isomerization reaction since no change in mass would be expected. However, it is consistent with the loss of 1 H_2O molecule (-18 Da), associated with succinimide formation. A stable succinimide group would also be resistant to Edman degradation (17). Isoelectric focusing provided additional evidence for this identification since the isoelectric point of DP3 (pI 4.94) was greater than that of hEGF 1-48 (pI 4.55).

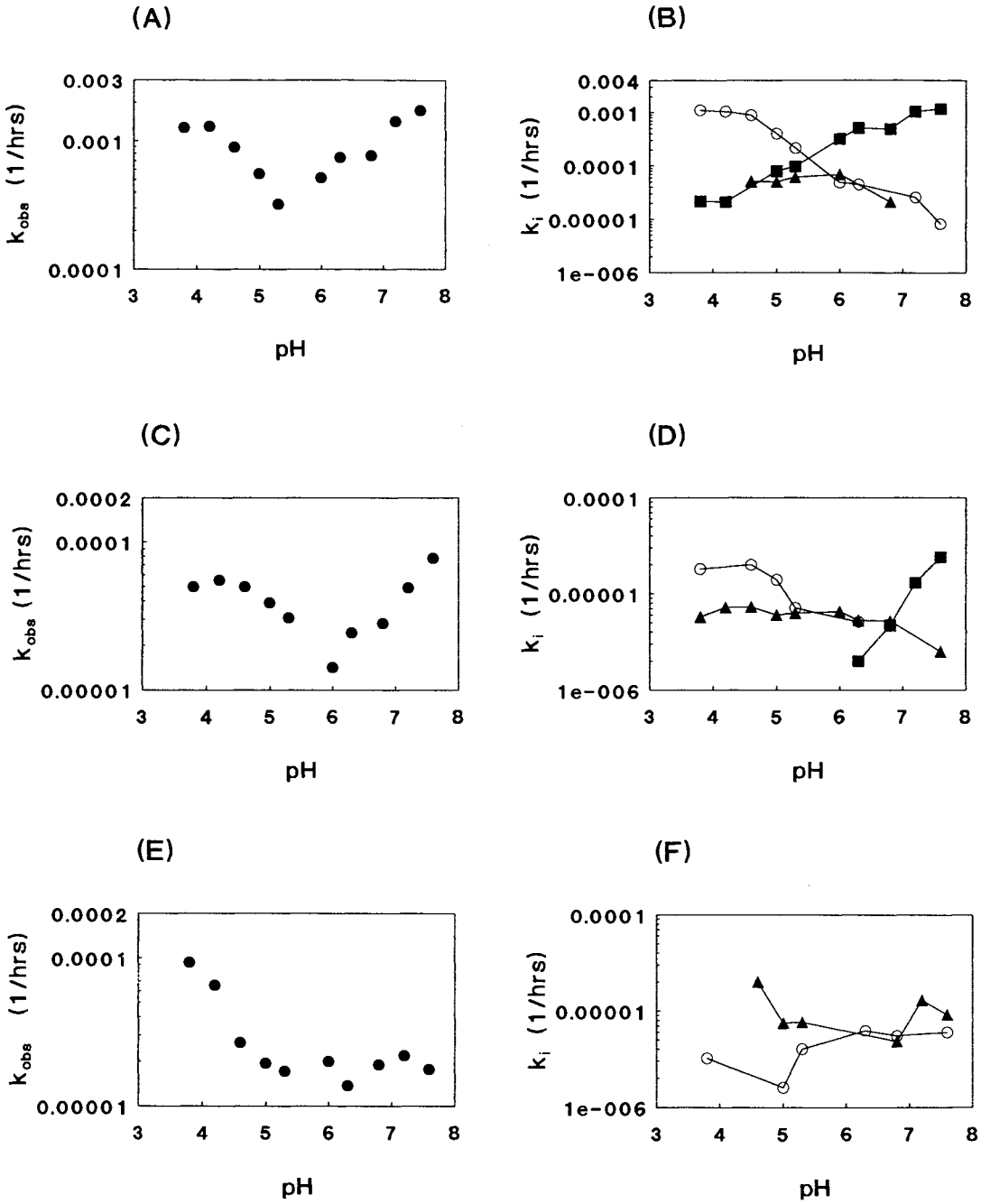

Figure 4. pH-rate profiles indicating the degradation of hEGF 1-48 (●) and the formation of of hEGF-ox (▲), hEGF-da (■), and hEGF-suc (○) at 30°C (A & B), 4°C (C & D), and −20°C (E & F).

Table II. Activation Energy (E_a) Estimates for Each Degradation Pathway Made from the Slopes of the Arrhenius Plots (ln k_i vs. 1/T)

Degradation pathway	E_a (kcal/mol)	S.E.[a]	A[b]	S.E.
Oxidation (Met^{21})	10.48	1.38	10.34	0.20
Deamidation (Asn^{1})	23.64	1.00	33.85	0.14
Succinimide formation (Asp^{11})	19.44	1.10	25.75	0.16

[a] Standard error of the estimate.
[b] Preexponential constant.

This is the expected result following conversion of an Asp residue to a stable Asp-succinimide, since a carboxyl group is lost. If Asp was converted to an iso-Asp no difference in the isoelectric point would be expected.

Interestingly, George-Nascimento et al. (16) previously reported that Asp^{11} is nonenzymatically converted to isoaspartate in human epidermal growth factor. Their conclusion was reached on the basis of reactivity of a purified derivative of human epidermal growth factor with L-isoaspartyl-D-aspartyl methyltransferase, an enzyme that methylates the side-chain carboxyl group of L-isoaspartyl but not normal L-apartyl residues. However, their results showed that the purified derivative was methylated in very low yields until it was oxidized and denatured with performic acid, or digested with thermolysin. On this basis, they hypothesized that the initial poor methylation resulted from steric hinderance due to secondary structure. Another possibility more consistent with our results is that a stable Asp-succinimide (not a substrate for the methyltransferase) was converted to the iso-Asp residue during the performic acid and thermolysin treatments. It is generally accepted that aspartyl isomerizations proceed through a succinimide intermediate (Figure 3B; 1,17,18). The first reported characterization of a stable succinimide from a protein solution was reported by Violand et al. (17), in which they suggest that one reason succinimide forms of proteins are not isolated and characterized on a routine basis may be due to the conditions (such as pH or temperature) these modified proteins are subjected to during analyses. Our results support that contention.

pH-Rate Profiles

The pH-rate profiles representing the degradation rates of hEGF 1-48 (k_{obs}) and the formation rates (k_i) of hEGF-ox, hEGF-da, and hEGF-suc are shown in Figure 4. Differences in the pH dependencies were found, when the profiles obtained at 30°C, 4°C, and −20°C were compared.

At 30°C (Figures 4A & 4B), the pH of maximum stability is ≈5.3. The major pathway contributing to hEGF 1-48 degradation at pH > 6 is deamidation, which increases with increasing pH. This finding is consistent with previous reports describing the pH dependencies of deamidation via a succinimide intermediate (1,19,20). The apparent inflection in the pH-rate profile at pH 6.5 is consistent with a previous report by Capasso et al. (21), in which the kinetics of succinimide ring formation in the deamidation process of an asparaginyl peptide were studied. A similar inflection was found in the pH-rate profile for the asparaginyl peptide at pH 6.5–7.0; this was attributed to a change in the rate determining step. At pH <5 the major pathway contributing to hEGF 1-48 degradation is succinimide formation, which increases with decreasing pH to an apparent constant rate at pH's of approximately 4.6 or less. Stephenson and Clarke (22) reported greater than 10,000-fold increases in the half-lives of aspartyl peptide degradation (via succinimide formation) when compared to aspartyl β-methyl ester peptides at pH 7.4. This suggests the protonated form of Asp^{11} (acidic pH) would preferentially undergo succinimide formation, since the hydroxyl group from the protonated aspartyl residue is as good (or better) a leaving group than the OR-group from the ester (23); the pK_a range for the side chain carboxyl of aspartyl groups in proteins ranges from 3.0 to 4.7 (Average = 3.8). On this basis, the curvature in the pH-ratio profile in the acidic region can be related to the fraction of Asp^{11} present in its protonated form. Methionine oxidation is a relatively minor degradation pathway at 30°C. Its pH dependence is less clear, although the oxidation rate appears to decrease slightly with increasing pH. This is consistent with previous reports that an acidic medium is favorable for the selective conversion of methionine residues into sulfoxides (24,25), and the oxidation rate remains constant in the pH interval from 1 to 5 (26).

Although the overall characteristics of the pH-rate profile at 4°C (Figures 4C & 4D) are similar to those at 30°C, the pH of maximum stability shifts to a slightly higher pH (≈6.0). This suggests the temperature dependencies differ for each degradation pathway. For instance, it is apparent (comparing Figures 4B & 4D) that methionine oxidation is a more significant pathway, relative to deamidation and succinimide formation, at 4°C than at 30°C.

The changes in physico-chemical characteristics which accompany freezing and thawing of drug solutions, including pH shifts and the concentration of solutes or drugs, can have significant impact on the stability profiles (27). For instance, the characteristics of pH and temperature dependencies on drug stability determined in the non-frozen state may not be extended to frozen solutions. This was apparent in the pH-rate profiles obtained for hEGF 1-48 samples stored as frozen solutions at −20°C (Figures 4E & 4F). At −20°C, the degradation rate of hEGF 1-48 is relatively constant at pH ≥5; this can be attributed to the elimination of the deamidation degradation pathway, which is the predominant degradation pathway at pH > 6 for non-frozen hEGF 1-48 solutions. Differences in the pH dependencies of succinimide formation rates were also apparent when comparing frozen and non-frozen solutions. Significant succinimide formation was found at pH > 6; this was not the case for non-frozen solutions. These changes in deamidation and succinimide formation behavior subsequent to freezing are consistent with a pH shift to a more acidic pH (based on the pH dependencies of these pathways in non-frozen solutions at 4°C and 30°C), and may be related to the well reported observation that sodium phosphate-buffered solutions can undergo marked pH drops (more than 3 pH units) upon freezing (28). In addition to differences in the pH dependencies of the deamidation and succinimide formation pathways between frozen and non-frozen solutions, the relative contributions of each degradation pathway to the overall stability of hEGF

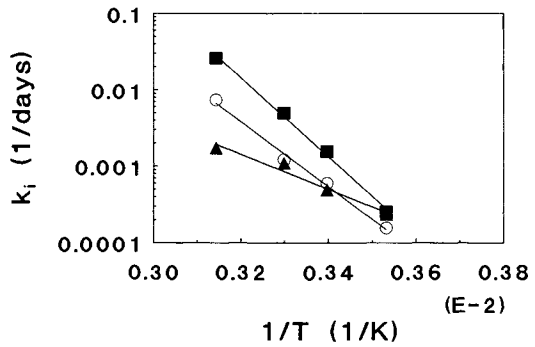

Figure 5. Arrhenius plots of k_i for the formation of hEGF-ox (▲), hEGF-da (■), hEGF-suc (○) at pH 6.0 (0.02 M sodium phosphate/0.01% polysorbate 80).

1-48 was affected. Interestingly, at −20°C the major degradation pathway is methionine oxidation. Accelerated oxidation of sulfhydryls during freezing and in the frozen state may be related to increased oxygen concentration in ice (29); the oxygen concentration in a "partially" frozen solution at −3°C was reported to be 1150-fold greater than at 0°C (30).

Temperature Dependence and the Effects of Fluorescent Light Exposure

The sensitivities of the hEGF 1-48 degradation pathways (pH 6.0) to temperature were evaluated using Arrhenius relationships. Activation energy (E_a) estimates for each degradation pathway (Table II) were made from the slopes of the Arrhenius plots (ln k_i vs. 1/T; Figure 5) based on the relationship

$$\ln k = \ln a - \frac{E_A}{RT}$$

where k is the reaction-rate constant, R = 1.987 cal/K-mole, T is the absolute temperature, and A is the preexponential constant. The results substantiate the interpretation of the pH-rate profiles. For instance, the largest E_a (23.64 kcal/mol) was found for the deamidation pathway. Therefore, Asn^1 deamidation is reduced to a greater extent with decreasing temperature than the other pathways. Since deamidation was favored in basic solution, the shift in the observed pH of maximum stability to a higher pH at lower temperatures is expected. Likewise, the smallest E_a (10.48 kcal/mol) was found for the oxidation pathway. Therefore, oxidation at Met^{21} is least sensitive to changes in temperature. This explains the increased relative contribution of methionine oxidation to hEGF 1-48 degradation at lower temperatures.

Table III. Shelf-Life Estimates (T90; $0.105/k_{obs}$) for hEGF 1-48 at pH 6.0 (0.02 M Sodium Phosphate/0.01% Polysorbate 80)

Temperature (°C)	Observed[a] shelf-life (days)	Predicted[b] shelf-life (days)
45	1.0	1.0
30	5.9	6.1
10	91.2	90.4
8	****	120.9
2	****	296.7

[a] Shelf-life estimated directly from the apparent first-order rate constant determined from the log-linear least squares fits for loss of hEGF 1-48 vs. time.

[b] Shelf-life estimated from the apparent first-order rate constant predicted from the Arrhenius relationship (ln k_{obs} vs. 1/T; E_a = 22.99 kcal/mol, A = 34.10).

Similarly, the temperature dependence of the overall degradation process at pH 6.0 was characterized using Arrhenius plots (ln k_{obs} vs. 1/T; Figure 6). The resultant shelf-life estimates (T90) are shown in Table III. Good agreement is found between the observed and predicted shelf-life estimates (T90) in the 10 to 45°C temperature range. The expected shelf-life varies from approximately 4 to 10 months over the accepted refrigerated temperature range of 2–8°C.

The hEGF 1-48 degradation rate increases with increasing exposure to fluorescent light; The observed degradation rate of solutions packaged in clear ampoules ($k_{obs} \times 10^3$ = 1.23 ± 0.09 hr^{-1}) is > amber ampoules ($k_{obs} \times 10^3$ = 0.757 ± 0.062 hr^{-1}), which is > than foil-covered ampoules ($k_{obs} \times 10^3$ = 0.410 ± 0.059 hr^{-1}). As expected (10,12), the increase in hEGF 1-48 degradation rate under these conditions is primarily due to an increase in methionine oxidation (Figure 7).

CONCLUSIONS

hEGF 1-48 was shown to degrade in aqueous solution by methionine oxidation, asparagine deamidation, and aspartyl succinimide formation. The degradation kinetics were characterized in terms of pH, temperature, and exposure to flu-

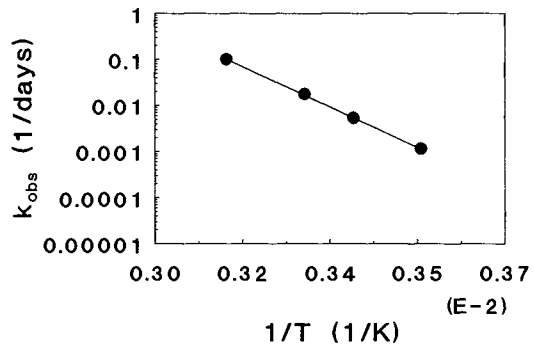

Figure 6. Arrhenius plot of k_{obs} for the degradation of hEGF 1-48 at pH 6.0 (0.02 M sodium phosphate/0.01% polysorbate 80).

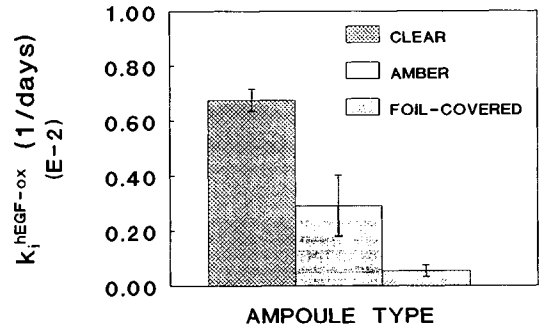

Figure 7. Formation of hEGF-ox as a function of fluorescent light exposure at pH 6.0 (0.02 M sodium phosphate/0.01% polysorbate 80). Error bars represent the standard error of the estimate.

orescent light. Since the production, isolation, processing, and formulation of hEGF 1-48 and other recombinant proteins are often carried out under aqueous conditions, the aqueous stability can have a large impact on many aspects of the development process for protein-based pharmaceuticals. An understanding of the degradation process in aqueous solution is necessary so that rational steps can be taken to eliminate or reduce the reactions which could potentially lead to a loss of biological activity.

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