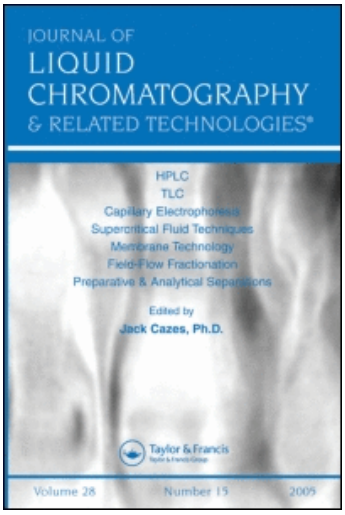

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CAPILLARY ZONE ELECTROPHORESIS OF DEGRADATIVE AND CYCLIC LACTAM DERIVATIVES OF THE GROWTH HORMONE-RELEASING FACTOR PEPTIDE

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ABSTRACT

Capillary zone electrophoresis (CZE) was used to monitor deamidation of the Asn^8 residue in the human growth hormone-releasing factor peptide, GRF(1-44)-NH_2. The deamidation proceeds via cyclic imide formation yielding isomeric Asp^8 and β-Asp^8 containing products. It is demonstrated that GRF peptides differing only by isomerization at a single aspartic acid residue can be separated by CZE at pH 2.5-4.5 as a result of the greater acidity of the β-Asp side-chain carboxylic acid versus that of the normal Asp isomer. The dependence of electrophoretic mobility on the size and charge of GRF peptide fragments was studied by CZE for proteolysis of GRF(1-29)-NH_2 by trypsin and endoproteinase Glu-C. CZE was also used to separate cyclic lactam analogs of GRF that all bear approximately the same net charge and differ only by the ring size or orientation of the lactam bridge.

INTRODUCTION

Human growth hormone-releasing factor (GRF) is a 44-residue peptide amide secreted in the hypothalamus that binds to a receptor in the pituitary gland stimulating the release of growth hormone (1-3). The use of GRF as

a treatment for children with hypothalamic growth hormone deficiency is being studied at several clinical centers (4).

Our research has focused on the design of GRF analogs of high potency and stability (5). Replacement of Gly^{15} by Ala^{15}, for example, enhances the α-helicity and potency of GRF (6). Replacement of Ala^2 by $D\text{-}Ala^2$ confers resistance to cleavage at Ala^2-Asp^3 by the proteolytic enzyme, dipeptidyl aminopeptidase IV, which rapidly deactivates GRF (7). Replacement of Met^{27} by Leu^{27} prevents potential oxidation of Met^{27} to the sulfoxide during the synthesis or formulation of GRF analogs without any loss in intrinsic activity.

As in the case of Met^{27} oxidation, deamidation of Asn residues can result in loss of potency and create other difficulties during the synthesis, formulation, and delivery of the drug. In this paper we report on the use of capillary zone electrophoresis (CZE) to determine the relative stabilities of asparagine residues in GRF towards deamidation. We also report on separations by CZE of proteolytic fragments of GRF(1-29)-NH_2, the minimum sequence with full intrinsic activity and high biological potency. In addition, cyclic GRF analogs (8-10) containing i-(i+4) side-chain to side-chain lactam bridges that stabilize the α-helix (8-10) and confer resistance to proteolysis (9) were also evaluated by CZE. The effects of charge and size of the peptides on their relative electrophoretic mobilities are discussed.

MATERIALS AND METHODS

Peptides

Linear peptides were prepared by standard methods of solid phase peptide synthesis. Cyclic peptides were assembled by the solid phase method and the lactam bridges formed with the peptide anchored to the resin as described earlier (12). All peptides were purified by HPLC, and characterized by amino acid analysis and fast-atom-bombardment (FAB) mass spectrometry.

Proteolytic Digests

Trypsin (TPCK-treated) was obtained from Millipore Corporation (Freehold, NJ). Endoproteinase Glu-C (protease from the V8 strain of *Staphylococcus aureus*) was obtained from Sigma Chemical Company (St. Louis, MO). Proteolytic digests consisted of GRF(1-29)-NH_2 (1.0 mg mL^{-1}) and enzyme (0.05 mg mL^{-1}) in 50 mM NH_4HCO_3, pH 7.8. The digests were allowed to stand at 22°C for 12 hours and were then injected directly into the capillary of the CZE instrument.

Electrophoresis

All separations were performed on a Spectra PHORESIS 1000 instrument (Spectra Physics, San Jose, CA) equipped with a fast-scanning variable-wavelength UV-vis detector. Fused-silica capillaries with polyimide outer-coatings were coiled in cartridges thermostated by circulating air. Sample injection was by electromigration at 15 kV for 1-2 s and separation was performed at a constant 25 kV, 25°C. A 70 cm × 75 μm i.d. capillary (375 V cm^{-1}) was used for separations of the degradative derivatives of GRF (deamidated peptides and proteolytic fragments) and a 95 cm × 50 μm i.d. capillary (263 V cm^{-1}) was used for separations of the cyclic lactam derivatives. Capillaries were washed with running buffer (3-5 min) immediately prior to injection and treated with 0.1 M NaOH (3-5 min) followed by water (3-5 min) between runs. Phosphate running buffers were prepared by mixing equimolar solutions of Na_2HPO_4 and H_3PO_4.

RESULTS AND DISCUSSION

Degradation of Asparagine Residues in GRF Peptides

Deamidation of asparagine residues is an important nonenzymatic degradation pathway for many peptides and proteins (13-15). In neutral and alkaline solutions the Asn side-chain can acylate the peptide-bond nitrogen

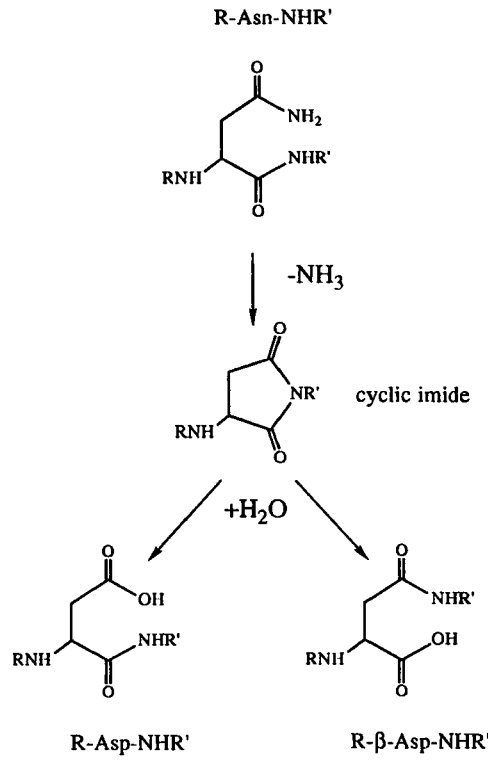

Figure 1. Deamidation of asparagine residues via cyclic imide formation.

of the adjacent amino acid residue with the loss of ammonia, forming a cyclic imide (Figure 1). The imide intermediate is usually rapidly hydrolyzed to give an isomeric mixture of normal and β-linked aspartic acid residues in place of the original asparagine residue.

Deamidation of Asn residues is general-base catalyzed (16) and hence rates of deamidation depend on the types of buffers present and increase with increasing pH and increasing buffer concentration. Rates are also strongly influenced by sequence (17) and conformation (18) and vary widely among proteins and peptides. For example, the Asn^{8} residue in the parent human

hormone, GRF(1-44)-NH_2 (H-YADAIFTNSY[10]RKVLGQLSAR[20]KLLQDIM-SRQ[30]QGESNQERGA[40]RARL-NH_2), has a half-life of five days at pH 7.4, 37°C in 0.25 M Na_2HPO_4/H_3PO_4 buffer whereas Asn[35] shows no detectable deamidation after two weeks under these conditions (19).

With the exception of human and pig GRF all other known mammalian sequences contain an Asn[28] (3). One objective of this work to study the relative stability of this residue with respect to deamidation. Friedman and coworkers (20) recently found by HPLC that [Ser[8], Leu[27]]-GRF(1-32)-NH_2 had an approximately ten-fold longer half-life in aqueous solution (pH 7.4, 37°C) than [Leu[27]]-GRF(1-32)-NH_2 and an approximately two-fold longer half-life compared to [Ser[8], Leu[27], Asn[28]]-GRF(1-32)-NH_2. Although the degradation products of the Asn[28]-containing analog were not isolated and identified (18), these results do suggest the likely deamidation of Asn[28], albeit at a considerably slower rate than Asn[8].

The molecular charge (q) versus pH profiles in Figure 2a were calculated for the model peptides, [Leu[27], X[28]]-GRF(22-32)-OH (X = Asn, Asp, β-Asp), using the computer program and pK_a data of Skoog and Wichman (21). A literature value (21) for the average pK_a of Asp side-chains of 3.9 was used in the calculations. The pK_a of the β-Asp side-chain was estimated to be 2.1 which is an average pK_a for the α-carboxylic acid groups of C-terminal Asn residues. This value was taken as a rough initial estimate owing to the structural similarity between β-Asp residues and C-terminal Asn residues. Experimental CZE results for these model peptides conform fairly closely with the predicted q versus pH properties (Figure 2b).

Deamidation of the Asn[8] residue of GRF(1-44)-NH_2 in dilute alkaline buffer solution was monitored by CZE (Figure 3). The six Arg and two Lys residues in GRF(1-44)-NH_2 make it a highly basic peptide carrying a high charge in the pH range of interest. Unfortunately, interactions with the wall of the fused-silica capillary result in peak broadening, tailing, and hence rather inefficient separation. The ratio of β-Asp to Asp products of 4:1 is

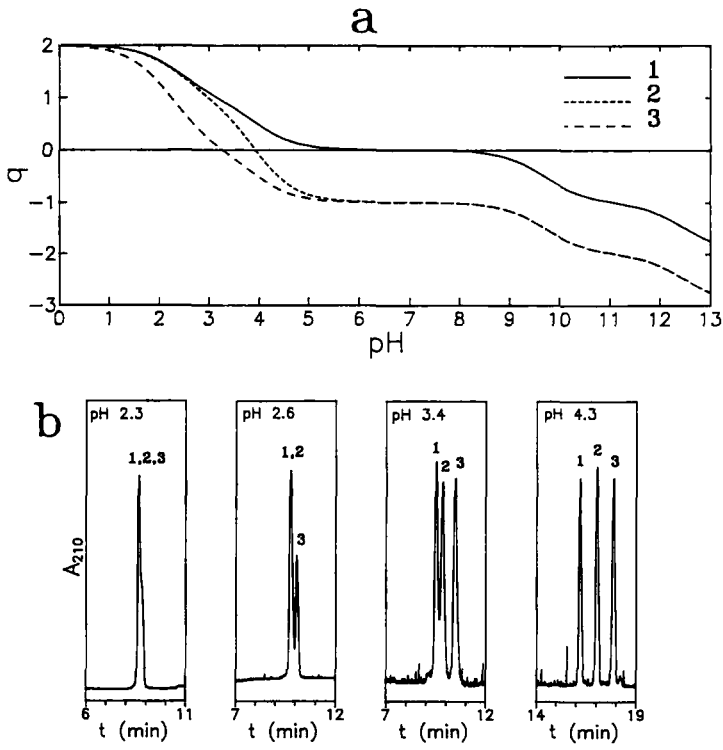

Figure 2. (a) Calculated charge versus pH profiles and (b) electropherograms at varying pH for the synthetic model peptides, [Leu[27], Asn[28]]-GRF(22-32)-OH, 1, [Leu[27], Asp[28]]-GRF(22-32)-OH, 2, and [Leu[27], β-Asp[28]]-GRF(22-32)-OH, 3. The pH of the 25 mM Na_2HPO_4/H_3PO_4 running buffer is shown in the upper left corner of each panel.

characteristic of deamidation via cyclic imide (14). No deamidation of Asn[28] was detected by CZE for [Leu[27], Asn[28]]-GRF(22-32)-OH (25 mM sodium borate, pH 9.2) after standing for one week at 22°C. Thus Asn[28] deamidates many-times slower than Asn[8] under these conditions, if at all. The side-chains of Arg[29] and/or Leu[27] may sterically hinder cyclic imide formation at Asn[28]. On the other hand, the residues adjacent to Asn[8] (Thr[7] and Ser[9]) should present less steric hindrance to cyclic imide formation at Asn[8].

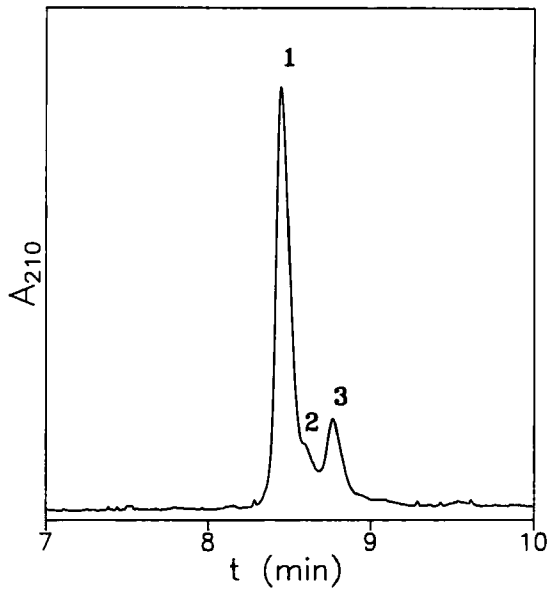

Figure 3. CZE of GRF(1-44)-NH_2, 1, after standing in 25 mM sodium borate, pH 9.2, at 22°C for 54 hours, showing the deamidation products [Asp^8]-GRF(1-44)-NH_2, 2, and [β-Asp^8]-GRF(1-44)-NH_2, 3. The running buffer was 25 mM Na_2HPO_4/H_3PO_4, pH 3.7.

Proteolytic Fragments of GRF(1-29)-NH_2

The CZE map of the endoproteinase Glu-C digest of GRF(1-29)-NH_2 is shown in Figure 4a. The three peaks were identified by comparison of the absorbance at 210 nm versus 280 nm and by CZE of an admixture of the digest with a synthetic GRF(1-3)-OH standard. The aromatic rings in GRF(1-29)-NH_2 that absorb at 280 nm belong to Tyr^1, Phe^6, and Tyr^{10}. The GRF(4-25)-OH fragment, 3, has a longer t_m than might be predicted solely on the basis of q (Table 1) which is probably due, in part, to the much larger size of this peptide compared to GRF(25-29)-NH_2, 1, or GRF(1-3)-OH, 2. Coulombic interactions with the silica wall of the capillary may also slow the mobility of GRF(4-25)-OH. The two smaller C-terminal and N-terminal fragments do elute in order of decreasing q.

TABLE 1

Proteolytic Fragments of GRF(1-29)-NH_2

# [a]	Fragment	q [b]	n [c]	t_m [d]
1	(26-29)-NH_2	2.0	4	8.3
2	(1-3)-OH	-0.7	3	10.4
3	(4-25)-OH	3.3	22	11.5
4	(12-20)-OH	2.0	9	7.8
5	(9-11)-OH	1.0	3	8.0
6	(13-20)-OH	1.0	8	8.9
7	(21-29)-OH	1.3	9	9.6
8	(22-29)-OH	0.3	8	10.3
9	(1-11)-OH	0.3	11	11.2
10	(1-8)-OH	-0.7	8	17.0

[a] labeled peak in Figure 4.
[b] calculated charge at pH 4.3.
[c] number of amino acid residues.
[d] migration time.

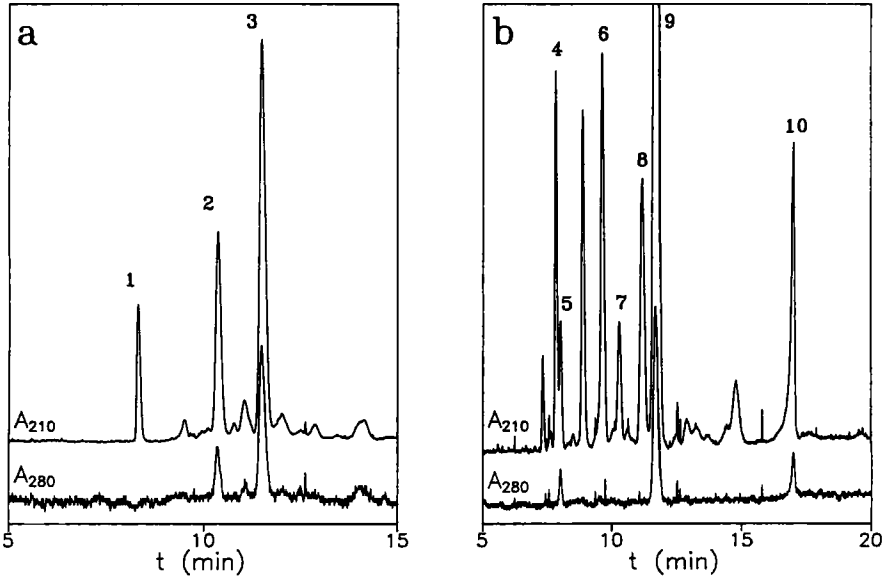

Figure 4. CZE of proteolytic fragments of GRF(1-29)-NH_2 after digestion with (a) endoproteinase Glu-C and (b) trypsin. See Table 1 for identification of labeled peaks. The running buffer was 25 mM Na_2HPO_4/H_3PO_4, pH 4.2.

TABLE 2

Cyclic Lactam Analogs of
[D-Ala^2,Ala^{15}]-GRF(1-29)-NH_2

#[a]	Lactam Bridge[b]	n_r[c]	t_m[d]
1	Lys^{21}-Asp^{25} [e]	20	22.2
2	Lys^{21}-Glu^{25}	21	22.4
3	Dpr^{21}-Glu^{25}	18	22.7
4	Orn^{21}-Asp^{25}	19	22.8
5	Lys^{8}-Asp^{12}	20	25.9
6	Asp^{8}-Lys^{12}	20	25.9
7	D-Asp^{8}-Lys^{12}	20	26.3
8	D-Asp^{8}-D-Lys^{12}	20	26.6
9	Asp^{8}-D-Lys^{12}	20	26.9

[a] labeled peak in Figure 5.
[b] Dpr = 2,3-diaminopropionic acid, Orn = ornithine.
[c] number of atoms in cyclic lactam ring.
[d] migration time.
[e] This peptide contained L-Ala^2 instead of D-Ala^2.

The CZE of a tryptic digest of GRF(1-29)-NH_2 is shown in Figure 4b. All the predicted tryptic fragments (Table 1) were identified by CZE of admixtures of the digest with synthetic standards or previously isolated and identified fragments (22). The nonenzymatic cleavage at Asn^8-Ser^9 that generates GRF(1-8)-OH , **10**, and GRF(9-11)-OH , **5**, is thought to result from formation of a C-terminal cyclic imide (22). The tryptic fragments of GRF(1-29)-NH_2 are all close to the same length (n) and elute roughly in order of decreasing q. The migration times of fragments **4**-**10** correlate reasonably well (r = 0.965) to the semiempirical model of Grossman and coworkers (23): $1/t_m = (0.071 \pm 0.009)[\ln(q+1)/n^{0.43}] + (0.090 \pm 0.007)$.

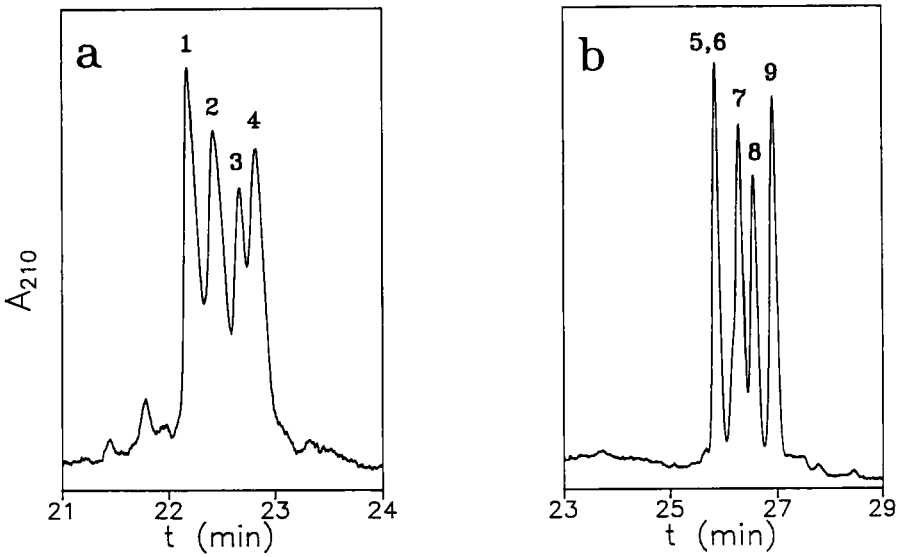

Figure 5. CZE separation of cyclic lactam analogs of [D-Ala^2, Ala^{15}]-GRF(1-29)-NH_2. See Table 2 for identification of labeled peaks. The running buffer was 125 mM Na_2HPO_4/H_3PO_4, pH 2.2.

Cyclic Lactam Derivatives

CZE separations of two mixtures of cyclic lactam derivatives of [D-Ala^2, Ala^{15}]-GRF(1-29)-NH_2 (Table 2) are shown in Figure 5. The first series of peptide homologs, **1-4**, have side-chain to side-chain lactam bridges between residues 21 and 25 with ring sizes (n_r) varying from 18 to 21 atoms (Figure 5a). The second series consists of four diastereomers, **6-9**, containing all combinations of D and L configurations at the Asp^8 and Lys^{12} bridge-head residues of the cyclic lactam and an isostere, **5**, with the bridge-head residues reversed, i.e. (Lys^8-Asp^{12})-bridged (Figure 5b).

In the absence of any differences in charge among these various cyclic peptides, migration through the capillary should ideally depend on how the conformations of the peptides affect their diffusion rates. There does not appear to be any simple relationship between t_m and n_r. We speculate that

the rather highly constrained lactam bridges of **3** (n_r = 18) and **4** (n_r = 19) may disrupt the α-helical conformation of the peptide thereby increasing its hydrodynamic radius and retarding diffusion. These observations are in general agreement with those of Felix and coworkers who recently reported (10) that both the α-helicity, as measured by circular dichroism, and *in vitro* bioactivity of *cyclo*^{21,25}-[Ala^{15}]-GRF(1-29)-NH_2 analogs (n_r = 18-21) decreased sharply for $n_r \leq 19$.

The co-migration of the isosteric peptides, **5** and **6**, seems quite reasonable considering that this pair differ only by a reversal of the positions of the Lys and Asp bridge-head residues which has been reported (8) not to result in any significant changes in conformation or biological activity. It is interesting to note that the diastereomers, **6-9**, have different electrophoretic mobilities in CZE. Circular dichroism studies (9) have shown that these analogs all assume similar levels of α-helical conformation. More subtle conformational differences, which are outside the scope of circular dichroism, may account for these differences in electrophoretic mobility.

CONCLUSIONS

Separation of the asparagine-containing GRF peptides and their Asp and β-Asp deamidation products demonstrates that the variation of pH may be used to obtain optimal separation by CZE of peptides that differ only by a single residue. This type of analysis should, in principle, be applicable to similar "point mutations" in larger peptides and proteins (24-27). However, the troublesome solute-wall interactions that often occur in bare silica capillaries (28) is a practical obstacle exemplified here by the much greater peak broadening and tailing observed for the highly basic GRF(1-44)-NH_2 parent (Figure 3) as compared to the smaller less basic [Leu^{27}, X^{28}]-GRF(22-32)-OH model peptides (Figure 2b).

CZE is well suited for proteolytic mapping of proteins (26,29), a valuable method for primary structure determination and use as a "finger-

print" to ascertain the identity and purity of a synthetic peptide or protein. One advantage of CZE over HPLC for the analysis of proteolytic digests is that elution times can be related to known physicochemical properties of the solutes in a relatively straightforward fashion (23). The rudimentary model (21) used to calculate molecular charge at a given pH employs average pK_a values and contains no provisions for local environmental effects on ionizable groups (30).

The separations of the cyclic lactam GRF derivatives demonstrates the capability of CZE to discriminate between subtle structural differences in relatively large biomolecules. Although no simple relationship between the structures of these cyclic peptides and their CZE migration times was established, such electropherograms may contain useful information about the hydrodynamic properties and conformations of molecules.

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