Permeation of Unfolded Basic Fibroblast Growth Factor (bFGF) Across Rabbit Buccal Mucosa—Does Unfolding of bFGF Enhance Transport?

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Purpose. To investigate whether recombinant human basic fibroblast growth factor (rhbFGF) would permeate freshly-excised rabbit buccal mucosa. In addition, the effect of a permeation enhancer (Na^+ glycocholate) and the possibility of reversibly unfolding the globular protein to a more linear conformation to increase the permeability of the test protein was evaluated.

Methods. The *in vitro* flux of bFGF through freshly-excised rabbit buccal mucosa was determined using side-by-side diffusion systems. Detection of bFGF was performed using gradient elution, reversed-phase high-pressure liquid chromatography (RP-HPLC). Fluorescence spectroscopy and heparin affinity chromatography were used to assess the tertiary structure of bFGF.

Results. Preliminary *in vitro* results have demonstrated that the bFGF flux increased from 1.4 ± 0.13 ng min^{-1} cm^{-2} to 3.2 ± 0.38 ng min^{-1} cm^{-2} with the addition of 15 mM Na^+ glycocholate (NaG) to the donor solution. Subsequent addition of guanidine HCl (GnHCl) to the donor solution (3 M) was not followed by a further increase in the flux of bFGF (2.9 ± 0.26 ng min^{-1} cm^{-2}). However, when the order of addition of the additives was reversed (GnHCl first followed by NaG), the flux of bFGF across rabbit buccal mucosa was increased. Upon addition of GnHCl, there was a significant ($p < .05$) increase in bFGF flux from 1.2 ± 0.15 ng min^{-1} cm^{-2} to 5.0 ± 0.58 ng min^{-1} cm^{-2}. Addition of NaG further increased the flux to 8.5 ± 1.1 ng min^{-1} cm^{-2} which was approximately 3- to 3.5-fold greater than that determined with the protein alone in the absence of any donor phase additives. The percent of parent bFGF remaining following a 3-hr exposure of a bFGF solution to either the mucosal, serosal, or both sides of rabbit buccal mucosa were $54.3 \pm 5.7\%$, $71.8 \pm 6.3\%$, and $36.2 \pm 5.4\%$, respectively with the majority of parent bFGF lost during the first 15 minutes. A model endopeptidase (endoproteinase Arg-C from mouse submaxillary gland) was shown *in vitro* to contribute to the loss in parent bFGF.

Conclusions. The permeation of bFGF across rabbit buccal mucosa may be significantly increased by initially unfolding the protein with GnHCl and then treating the tissue with the permeation enhancer, NaG. Refolding and possible reactivation of bFGF's bioactivity may occur following membrane transport and subsequent dilution into an infinite sink.

KEY WORDS: buccal delivery; basic fibroblast growth factor; protein unfolding; rabbit; transmucosal permeation; denaturation.

INTRODUCTION

Interest continues in the delivery of peptide drugs by non-parenteral routes of administration. Transmucosal drug delivery, especially drug delivery by the nasal, vaginal, rectal, and buccal routes of administration, has assumed a greater importance in the delivery of proteins and peptides. While each alternative, non-parenteral route of drug administration has its own advantages and disadvantages, the buccal route of peptide administration has several distinct benefits. The oral cavity is easily accessible, the buccal mucosa can withstand environmental extremes (changes in pH, temperature, etc.) and the epithelial surface undergoes rapid cellular recovery following local stress (1–2). While the buccal route of administration of peptides has been documented for over ten years, the development of biocompatible and efficient mucoadhesive patches for their administration requires further refinement. Examples of peptides that have been delivered across the buccal mucosa include TRH (2–7), calcitonin (8–9), buserelin (10), oxytocin (5,7,11–12), and octreotide (13). Mucoadhesive buccal patches consisting of a therapeutic peptide homogeneously dispersed in a polymeric matrix have been used for drug delivery across the oral mucosa (7,14–15). However, most mucoadhesive buccal patches have been used to deliver low molecular-weight drug substances rather than large molecular weight proteins and peptides.

Delivery of proteins across the buccal mucosa has met with little success. The buccal route of drug administration is attractive due to avoidance of the 'first-pass' effect, resulting primarily from less acid- and enzyme-induced biochemical degradation of drug substances in the oral mucosa as compared to the gastrointestinal tract, and rapid cellular recovery following irritation of tissue. However, the buccal mucosa represents a significant diffusion barrier. Bioavailabilities of peptide drugs, with and without penetration enhancers, generally range from 1 to 10 percent when administered by this route (16). A number of strategies have been employed to cause significant enhancement in the buccal delivery of peptides. Permeation enhancers have been used to reversibly alter (fluidize) membrane lipids and/or extract membrane lipids (17–18). Protease inhibitors have been evaluated with and without the use of permeation enhancers to increase the mass transport of the peptide or protein by inactivating various enzymes present in buccal mucosa (19–20). Recently, factors such as charge (21–22) and three-dimensional conformation (23) are being modified to potentially increase drug absorption of polypeptides by the buccal route. Tertiary structure of the peptide or protein has been shown to play a significant role in the *in vitro* permeation of various cyclic peptides across a monolayer of Caco-2 cells (23). The present study was undertaken to assess whether intentional unfolding of basic fibroblast growth factor (bFGF) would increase the protein's penetration through freshly-excised rabbit buccal membrane. The hypothesis to be tested was whether the unfolded, presumably more linear conformation of bFGF, would result in increased permeation through rabbit buccal epithelium due to a reduction in molecular dimensions associated with the globular conformation. Additionally, so as to determine whether unfolded bFGF might spontaneously refold to its native state following its transport across buccal mucosa and dilution into an infinite sink (systemic circulation), we investigated whether GnHCl-unfolded bFGF would refold after dialysis.

Chemical denaturants or chaotropes have long been utilized in biochemistry to intentionally unfold proteins for subse-

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quent analysis. Examples of chaotropes include urea, sodium dodecyl sulfate (SDS), and guanidine hydrochloride (GnHCl). Many GnHCl-denatured enzymes have been shown to refold to the native structure or state with complete recovery of their enzymatic activity in the presence of select pharmaceutical additives, e.g. detergents (24–27). Even without the assistance of detergents, several enzymes have been shown to spontaneously and completely refold to the native state and regain total enzymatic activity when incubated with GnHCl (28–29). The purpose of the present study was to determine whether GnHCl-denatured bFGF would permeate rabbit buccal mucosa *in vitro* to a greater extent than the native (folded) form. In addition, the rate and extent of bFGF permeation through rabbit buccal mucosa was investigated *in vitro* with and without the addition of the permeation enhancer, sodium glycocholate (NaG). Lastly, we sought to determine whether a representative endopeptidase from the oral cavity might contribute to the biochemical degradation of bFGF.

MATERIALS AND METHODS

Recombinant human bFGF was generously supplied by Scios, Inc. (Mountain View, CA) and used as received. The molecular weight of bFGF (146 a.a.'s) is 17.1 kDa with an isoelectric point (pI) of approximately 9.1. The protein's secondary structure is comprised mainly of beta turns. Guanidine hydrochloride, sodium glycocholate, and endoproteinase Arg-C from mouse submaxillary gland (470 Units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue Isolation

Male, New Zealand albino rabbits were obtained from Mertles Rabbitry (Thompson Station, TN) and weighed an average of 2.5 ± 0.13 kg. Rabbits were sacrificed with an overdose of pentobarbital sodium (90 mg/kg) and the cheek removed and placed immediately into iced normal saline. Using the method of Robinson *et al.* (2) with a fine-point tweezers, the connective tissue on the serosal side of the excised buccal membrane was removed. The research adhered to the Principles of Laboratory Care (NIH publication #85-23, revised 1985).

Assessment of Protein Unfolding

To ensure that bFGF was unfolded following incubation with GnHCl, we conducted an experiment that monitored the ratio of the emission intensities at 350 nm and 307 nm ($F_{350/307}$) using fluorescence spectroscopy (30). In brief, a solution of bFGF (10 μg/mL in pH 7.2 phosphate buffered saline) was incubated with GnHCl over a concentration range of 0.0 to 3.2 M and the $F_{350/307}$ ratio determined at each GnHCl concentration. The excitation wavelength was set at 277 nm and the emission spectra from 290–360 nm obtained using a Perkin Elmer LS 50B fluorimeter (Beaconsfield, Buckinghamshire, UK). The slit width used for excitation and emission of the bFGF solution was 5 nm. The scan speed was 99 nm/min. Background intensity due to phosphate buffer was subtracted from the intensity values obtained following a scan of either bFGF or the protein and GnHCl. The native state associated with bFGF consistently resulted in an $F_{350/307}$ ratio value of 0.25 ± 0.02.

Diffusion Studies

Isolated buccal tissue was immediately placed in a side-by-side diffusion cell as described previously (14). The area of the orifice between the donor and receptor compartments was 0.71 cm^2 and both compartments had an individual volume of 3 mL. Both the donor and receptor solutions were continuously stirred at 150 rpm throughout the 180 min experiment and maintained at 22.0 ± 0.1 °C using a PolySciences model 1157 circulating water bath (Niles, IL).

Effect of Donor Phase Additives on bFGF Diffusion

These experiments were conducted to determine the effect of GnHCl and NaG on the permeation of bFGF through excised rabbit buccal mucosa. NaG was used as a prototype penetration enhancer to increase bFGF paracellular flux across buccal mucosa. Three different types of experiments were conducted. The protocol is briefly summarized below. After placing the buccal tissue in a model DC-100B side-by-side diffusion cell (Somerville, NJ), the donor phase which consisted of 7 μl of a bFGF stock solution (4.3×10^3 μg/mL in pH 5.5 citrate buffer containing 1 mM EDTA and 9% sucrose) added to 2.993×10^3 μl of pH 7.2 phosphate buffered saline (PBS) was placed in the donor cell to yield a final bFGF concentration of 10 μg/mL. PBS without bFGF was added to the receptor compartment. In the first experiment, the donor phase which contained bFGF was added at time zero and the receptor phase sampled (50 μl) with replacement of fresh buffer at 0, 1, 2, 5, 10, 20, 40, and 60 min. Following collection of the 60 min sample, NaG was added directly to the donor phase to a final concentration of 15 mM and the receptor phase sampled at 80, 100, and 120 min. Lastly, following collection of the 120 min sample, GnHCl was added to the donor compartment to a final concentration of 3 M and the receptor phase sampled at 140, 160, and 180 min. This experiment was performed in triplicate.

To assess the steady-state flux of bFGF in the presence of NaG only, a second experiment was conducted with stirring conditions, temperature, and sample collection times similar to the initial experiment described above. However, in this experiment, no additional chemical substances were added to the donor phase at 120 min (Fig. 2). The third and final experiment was similar to the first experiment described above except that the order of addition of NaG and GnHCl were reversed (Fig. 2). Reversal in the order of addition of NaG and GnHCl to the donor compartment was investigated because it was possible that the rate and extent of bFGF permeation might be unchanged during hour three relative to hour two in the first experiment due to a smaller molecular radius associated with unfolded bFGF. In the unfolded state, bFGF would presumably assume a more linear conformation with less restricted motion and be capable of freely passing through an extended pore structure of buccal membrane resulting from pretreatment with NaG during hour two (Fig. 2).

Degradation of bFGF *In Vitro* by Tissues Enzymes

This experiment was conducted to determine the percent of intact parent bFGF which remained when a bFGF solution was placed in contact with either the serosal, mucosal, or both the serosal and mucosal sides of freshly-excised rabbit buccal

membrane. Buccal tissue harvested as described previously was placed in the side-by-side diffusion cell with the mucosal side facing the donor compartment and the serosal side facing the receptor compartment. Basic FGF was added to 6 mL of pH 7.2 phosphate buffered saline (PBS) to yield a final concentration of 72 μg/mL. The bFGF/PBS solution was then equally divided and 3 mL placed in each compartment. The surface area of the membrane exposed to each bathing solution was 0.71 cm^2. Samples (25 μl) were obtained through the sampling port of each compartment at 0, 1, 2, 4, 8, 15, 30, 60, 120, and 180 min with replacement of fresh PBS. Both the donor and receptor solutions were continuously stirred throughout the 180 min experiment and maintained at 22.0 ± 0.1 °C using a thermostatted circulating water bath. All samples were assayed for bFGF using gradient elution, reversed-phase high pressure liquid chromatography (RP-HPLC) described later. For experiments which assessed the rate and extent of loss of parent bFGF when both sides of the excised tissue were simultaneously exposed to the bFGF/PBS solution, the tissue was cut so that the area of the circular tissue specimen was 0.71 cm^2 to correspond to the surface area exposed to the bFGF solution when the mucosal and serosal sides of the membrane were evaluated individually.

Buccal Membrane Pretreatment Study

This study was conducted to determine whether preincubation of freshly-excised buccal tissue in a 3 M solution of GnHCl would inactivate tissue-bound enzymes responsible for biochemical degradation of bFGF. Presumably, pretreatment of the tissue in a solution of 3 M GnHCl would denature enzymes contained in the tissue and potentially result in detection of a larger fraction of parent bFGF following simultaneous exposure of both sides of the buccal membrane to a bFGF/PBS solution. This study was necessary to exclude the possibility of enhanced transport of bFGF in the diffusion studies employing various donor phase additives in which GnHCl was added to intentionally disrupt the tertiary structure of bFGF. If addition of GnHCl to the donor phase in those experiments denatured bFGF, as well as tissue-bound enzymes responsible for bFGF's degradation, any degree of enhanced transport could then be attributed to either increased transport of a more linear conformation of bFGF and/or a reduction in the degradation of bFGF by inactive (denatured) enzymes present in the buccal tissue.

In the GnHCl-pretreatment study, freshly-excised rabbit buccal membrane (total area of mucosal and serosal sides = 1.42 cm^2) was incubated for 30 minutes in an iced 3 M solution of GnHCl. Following the 30-min incubation period, the tissue underwent 5 washes with pH 7.2 PBS. The tissue was then placed in 3 mL of a bFGF/PBS solution (72 μg/mL) maintained at 22 °C and the bathing solution sampled (50 μl) with replacement of fresh PBS at 0, 1, 2, 4, 8, 15, 30, 60, 120, and 180 min. Samples were assayed for parent bFGF by gradient-elution RP-HPLC as described later in the report.

Endopeptidase-mediated bFGF Degradation *In Vitro*

The following experiment was conducted to determine whether a representative endopeptidase would chemically degrade bFGF *in vitro*. The model endopeptidase used was obtained from mouse submaxillary gland. The concentration of the endopeptidase selected was based on the assay for the activity of the enzyme. Activity of the endopeptidase can be determined spectrophotometrically by conversion of Nα-p-Tosyl-L-Arginine methyl ester to Nα-p-Tosyl-L-Arginine at 25 °C and pH 8.0. The absorbance of the solution is monitored at 247 nm continuously for five minutes (31). One unit of the endopeptidase activity will hydrolyze 1.0 μmol of Nα-p-Tosyl-L-Arginine methyl ester per minute at pH 8.0 and 25 °C. The suggested concentration for determination of the enzyme's activity is 1–3 U/mL (Sigma; Assay No. P-5171, 1994).

In this *in vitro* experiment, the percent of intact parent bFGF which remained following incubation with endopeptidase (3.0 U/mL) with or without GnHCl (3 M) in the solution medium (72 μg bFGF/mL in pH 7.2 PBS) was determined. The experiments with and without GnHCl were conducted in triplicate and at a constant temperature of 22 ± 0.1 °C. If the endopeptidase cleaved the parent bFGF molecule into smaller molecular weight fragments in the absence of GnHCl, then presumably a disappearance profile would be expected. However, since previous pilot studies (data not shown) have demonstrated a reduction in the enzymatic activity of the endopeptidase when incubated with 3 M GnHCl and negligible loss of parent bFGF when the protein is incubated with GnHCl alone, the only explanation for detection of less parent bFGF in a bFGF/endopeptidase solution containing GnHCl would be enhanced biochemical degradation of bFGF due to the exposure of previously inaccessible reactive residues in the unfolded state.

bFGF Refolding Following Dialysis

To assess the potential of bFGF to refold to the native state when diluted into an infinite sink, presumably similar to the situation anticipated following membrane transport and dilution in the blood, an experiment was conducted which employed two analytical techniques to monitor tertiary structure. Specifically, 3 mL of a bFGF solution (72 μg/mL in pH 7.2 PBS) was incubated in the presence of 3 M GnHCl for fifty minutes. At various time points during the incubation and 25 min following dialysis against a pH 7.2 PBS solution using Slide-A-Lyzer® cassettes (Pierce, Rockford, IL), the $F_{350/307}$ ratio and the percent of bFGF bound to a heparin affinity column were determined. The solution volume removed at each time point was 40 μl; 20 μl for analysis using fluorimetry and 20 μl for heparin affinity chromatography. The method to determine the percent of bFGF which remained in the native state using heparin affinity chromatography is briefly described below (30). The heparin affinity column allowed only the native conformation of bFGF to bind to the stationary phase. A commercially-available concentrating solution for use with the Slide-A-Lyzer® technique was not necessary since the internal volume of the cassette did not increase over the course of the 25-min dialysis period due to the dilute concentration of bFGF used. Similarly, removal of three, 40 μl samples throughout the experiment represented only 4% of the total bFGF solution volume (or amount) and, for the purpose of these experiments, was assumed to be negligible.

To determine the percent of bFGF which remained in the native three-dimensional conformation, a Toso Haas heparin

TSK-gel column (7.5 mm i.d. × 7.5 cm length) was used (Supelco, Bellefonte, PA). Experiments were conducted using a Beckman System Gold™ (Fullerton, CA) equipped with a model 166 UV detector, 128 solvent module, and Gold Nouveau™ software processed by a model 350-P90 IBM personal computer. The injector used was a model 7725i Rheodyne injector (Cotati, CA) with a sample loop volume of 20 µl. The column was equilibrated with 100 mM potassium phosphate buffer which contained 1 mM Na_2EDTA and maintained at pH 6.5 (mobile phase A). Mobile phase B contained 3 M NaCl in the same buffer system. The flow rate was maintained at 1 mL/min. Mobile phase B was gradually increased from 0 to 80% of the total flow rate from 1 through 14 min. Composition of mobile phase B was decreased from 80 to 0% from 14 through 16 min. The detection wavelength used for all analyses was 215 nm. Peak areas associated with bFGF samples collected at 30 and 50 min during the incubation and 25 min following the dialysis procedure were compared to the mean value of the peak area obtained at time $t = 0$ min and expressed as the percent of bFGF in the native state/conformation.

Quantitation of bFGF by RP-HPLC

Basic FGF was assayed using gradient elution RP-HPLC according to the method of Sluzky *et al.* (30). In brief, a Vydac C4 column (4.6 mm × 15 cm), equilibrated with 0.1% trifluroacetic acid (TFA)/water, was eluted using an acetonitrile (ACN) gradient created by 0.08% TFA/ACN (2 min at 11.5%/min, 5 min at 1%/min, 20 min at 0.5%/min). The flow rate was 1.2 mL/min and detection was at 215 nm. All analyses were conducted using the chromatographic system described above for the heparin affinity chromatography.

RESULTS

bFGF Unfolding Using Fluorescence Spectroscopy

As can be noted in Figure 1, the $F_{350/307}$ ratio increased from 0.25 ± 0.02 to approximately 1.6 ± 0.07 as the concentration of GnHCl was increased from 0 to 2.8 M. The concentration-dependent unfolding curve was sigmoidal in appearance. The GnHCl concentration which represented approximately one-half the plateau $F_{350/307}$ ratio was about 1.7 M. A further increase in the $F_{350/307}$ ratio was not observed at GnHCl concentrations greater than 3.3 M (data not shown).

Diffusion of bFGF Through Buccal Mucosa

The flux of bFGF through rabbit buccal membrane is tabulated for each 60-min interval for each of the three types of diffusion experiments shown in Figure 2. As can be noted in Figure 2, addition of NaG to the donor phase significantly increased ($p < .05$) the flux of bFGF through rabbit buccal epithelium from a mean value of 1.4 ± 0.13 ng min^{-1} cm^{-2} to 3.2 ± 0.38 ng min^{-1} cm^{-2}. However, addition of GnHCl at 120 min did not result in an additional increase in bFGF permeation (Table I). For comparative purposes, the flux of bFGF through rabbit buccal membrane following addition of NaG at 60 min is listed in Table 1 (2.5 ± 0.27 ng min^{-1} cm^{-2}).

The order of addition of NaG and GnHCl to the donor phase produced a vastly different outcome in bFGF flux through rabbit buccal membrane. As can be noted in Table I and Figure 2, addition of GnHCl to the donor phase at 60 min significantly ($p < .05$) increased the flux from 1.2 ± 0.15 ng min^{-1} cm^{-2} to 5.0 ± 0.58 ng min^{-1} cm^{-2}. Addition of NaG at 120 min produced a greater than 7-fold increase in the flux of bFGF through the buccal membrane to 8.5 ± 1.1 ng min^{-1} cm^{-2}.

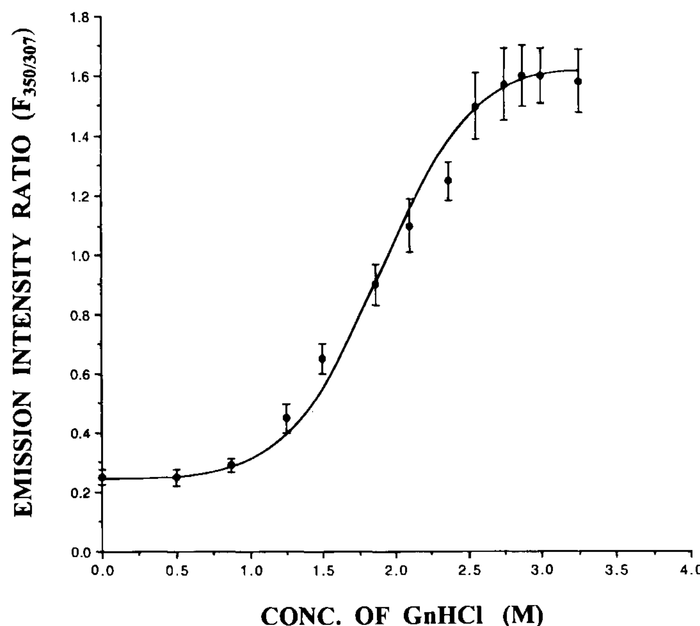

Fig. 1. The effect of GnHCl on bFGF unfolding as assessed using fluorescence spectroscopy. All data points represent the mean values ± standard deviation of duplicate determinations. Curve through symbols does not represent a mathematical fit of the data.

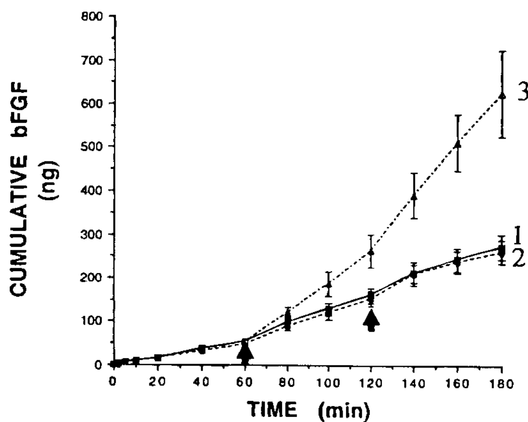

Fig. 2. The effect of donor phase additives on the permeation of bFGF through rabbit buccal mucosa. Curve 1 (—■—): Addition of bFGF (10 µg/mL) at 0 min, NaG (15 mM) at 60 min, and GnHCl (3 M) at 120 min. Curve 2 (---●---): Addition of bFGF (10 µg/mL) at 0 min, NaG (15 mM) at 60 min. Curve 3 (-·-·▲-·-·): Addition of bFGF (10 µg/mL) at 0 min, GnHCl (3 M) at 60 min, and NaG (15 mM) at 120 min. All data points represent the mean values ± standard deviation of triplicate determinations. Lines through mean values are included to visualize the trend and do not represent a mathematical fit of the data.

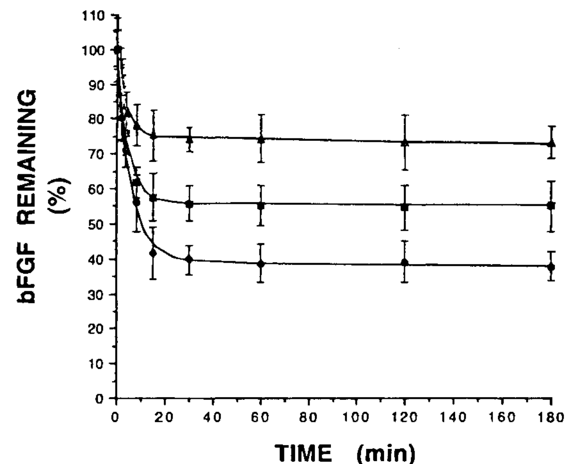

Fig. 3. Residual bFGF following exposure of a pH 7.2 bFGF/PBS solution (72 µg bFGF/mL) to either the serosal (▲), mucosal (■), or both sides (●) of rabbit buccal mucosa *in vitro*. All data points represent the mean values ± standard deviation of triplicate determinations. Lines through the mean values represent a standard biexponential fit of the observed data.

Degradation of bFGF by Tissue-Bound Enzymes

As shown in Figure 3, the percent of parent bFGF remaining when the serosal side of freshly-excised rabbit buccal membrane was exposed to a solution of bFGF was 76 ± 7.1 at 15 min and reached a final value of 71.8 ± 6.3 percent at 180 min. In contrast, exposure of a solution of bFGF to the mucosal side of the buccal membrane resulted in a decrease in the percent bFGF detected to 57 ± 6.8 at 15 min with a final value of 54.3 ± 5.7 percent at 180 min. Simultaneous exposure of both sides of the membrane to a bFGF solution resulted in 41.8 ± 8.1 percent of the bFGF being detected at 15 min and 36.2 ± 5.7 percent of the parent bFGF present at 180 min. For all three profiles, the percent bFGF remaining versus time resulted in a biexponential loss profile with the faster rate of bFGF degradation occurring for about the first 15 min following buccal membrane exposure to bFGF.

Membrane Pretreatment

Pretreatment of the buccal membrane with 3 M GnHCl did not significantly increase the percent of parent bFGF detected following exposure of a bFGF solution to buccal membrane when compared to control (non-treated) buccal tissue. As can be noted in both Figures 3 and 4, simultaneous exposure of both the serosal and mucosal sides of a control (non-treated) buccal membrane to the bFGF solution resulted in approximately 60 percent loss in the amount of parent bFGF detected at 15 min and at times thereafter. In contrast, buccal membrane pretreated with GnHCl resulted in 56.2 ± 4.9 percent of the bFGF being detected at 15 min and a final value of 46.3 ± 3.7 percent which remained at 180 min. Thus, approximately 5 percent more of the parent bFGF remained at 180 min when the bFGF solution was incubated with pretreated tissue compared to the percent of bFGF detected following exposure to control tissue. As with the disappearance profiles in Figure 3, the data yielded a biexponential relationship with most of the intact, parent molecule lost during the first 15 min.

Degradation of bFGF by a Representative Endopeptidase

It can be noted in Figure 5 that at the concentration of the endopeptidase selected (3 U/mL), the effect of the enzyme on

Table 1. Effect of Donor Phase Additives on the Flux of bFGF Through Rabbit Buccal Membrane

	bFGF FLUX (ng/min/cm^2)		
TIME INTERVAL	0–60 min	60–120 min	120–180 min
——Curve 1	1.4 ± 0.13	[a]3.2 ± 0.38	[a]2.9 ± 0.26
——Curve 2	1.1 ± 0.14	[a]2.5 ± 0.27	[a]2.5 ± 0.24
——Curve 3	1.2 ± 0.15	[a]5.0 ± 0.58	[a,b]8.5 ± 1.1

[a] Indicates significant ($p < .05$) increase compared to flux observed for the 0–60 min time interval.
[b] Indicates significant ($p < .05$) increase compared to corresponding values for Curves 1 and 2.

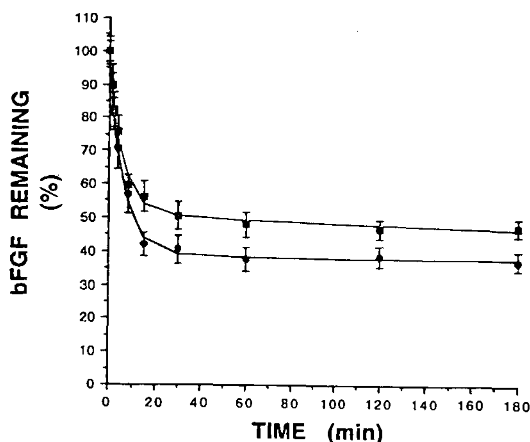

Fig. 4. Residual bFGF following exposure of a pH 7.2 bFGF/PBS solution (72 μg bFGF/mL) to both sides of either control (●) rabbit buccal membrane or membrane previously incubated in 3 M GnHCl for 30 min (■). All data points represent the mean values ± standard deviation of triplicate determinations. Lines through the mean values represent a standard biexponential fit of the observed data.

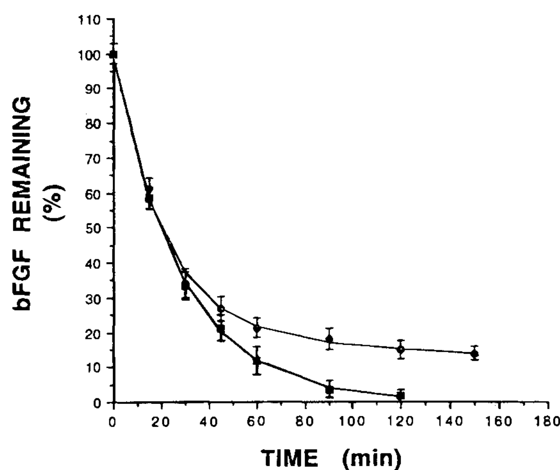

Fig. 5. Effect of mouse submaxillary endopeptidase (3 U/mL) on the percent of parent bFGF detected *in vitro* for a pH 7.2 bFGF (72 μg/mL)/PBS solution without (■) or with (●) 3 M GnHCl. All data points represent the mean values ± standard deviation of triplicate determinations. Lines through the mean values simply connect the data points and do not represent a mathematical fit of the data.

the degradation of bFGF was significant. When the enzyme was incubated with bFGF in the absence of GnHCl, the percent of bFGF which was detected at 120 min was approximately 2.4 ± 0.7. However, when 3 M GnHCl was present in the bFGF/endopeptidase solution, the percent of bFGF which remained at 60 min was approximately 21.1 ± 2.8 percent with 12.3 ± 1.6 percent remaining after 180 min. The effect of GnHCl on the enzymatic activity of the endopeptidase was first observed after approximately 30 min (Fig. 5). Thereafter, the enzymatic activity of the endopeptidase was diminished and approximately 10 percent more of the substrate (parent bFGF) remained intact at 180 min (Fig. 5) when GnHCl was included in the incubation solution.

bFGF Refolding Following Dialysis

As shown in Figure 6, both fluorescence spectroscopy ($F_{350/307}$ ratios) and heparin affinity chromatography were uti-

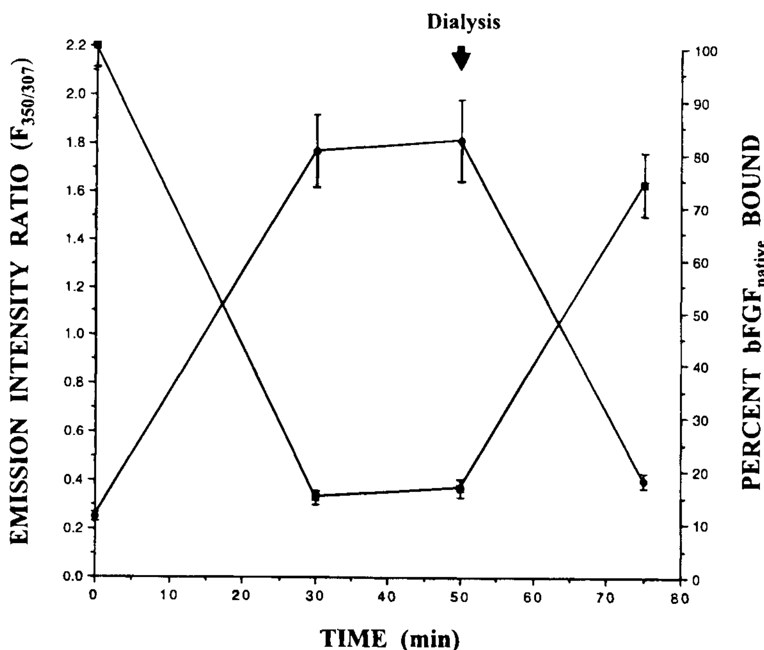

Fig. 6. The effect of addition and subsequent removal (by dialysis) of GnHCl on the unfolding/refolding of bFGF using fluorescence spectroscopy (—●—) and heparin affinity chromatography (—■—). Arrow indicates the time at which dialysis was performed. All data points represent the mean values ± standard deviation of duplicate determinations.

lized to demonstrate refolding of the bFGF molecule. Approximately 16 ± 1.6 percent of the bFGF remained in the native conformation 30 min following exposure of the protein to 3 M GnHCl. The corresponding $F_{350/307}$ ratio was 1.78 ± 0.15 at the 30 min sampling time point. At 50 min, analysis of bFGF solution samples demonstrated that the mean values of the $F_{350/307}$ ratio and the percent native bFGF bound were not significantly different than values determined at 30 min. Following dialysis, results using heparin affinity chromatography demonstrated that approximately 74 ± 6.1 percent of the bFGF existed in the native state or native three-dimensional conformation, whereas data from fluorescence measurements indicated an $F_{350/307}$ ratio in close agreement to the value normally observed for the native state (0.25 ± 0.02).

DISCUSSION

The present study has demonstrated that transport of bFGF through rabbit buccal membrane *in vitro* may be significantly increased by initially unfolding the protein with GnHCl and subsequently using the permeation enhancer NaG. GnHCl has long been used in biochemistry to intentionally denature (unfold) proteins for subsequent analysis. This was demonstrated with bFGF in Figure 1 using fluorimetry to monitor the extent of unfolding in the presence of GnHCl. However, it has long been recognized that GnHCl may assist in the refolding process of many enzymes (24–29). Many of the enzymes refolded by addition of GnHCl resume the conformation of the native state and hence regain complete enzymatic activity (24–29).

In the present study, GnHCl was used to purposely denature bFGF to an unfolded, presumably more linear conformation, to enhance protein permeation through rabbit buccal mucosa. Our initial diffusion study in which NaG was added at 60 min followed by GnHCl at 120 min demonstrated no further increase in bFGF flux upon addition of GnHCl. The results suggested that either 1) the molecular dimension of unfolded bFGF was already small enough to easily pass through the membrane which had previously been exposed to NaG during the 60–120 min time interval, or 2) that enhanced transport of unfolded bFGF was offset by an equivalent loss in parent bFGF due to increased susceptibility of reactive sites within the bFGF molecule in the unfolded state. While it is possible that GnHCl added to the donor phase at 120 min inactivated tissue-bound proteases responsible for bFGF's degradation, our data would suggest the opposite (Fig. 4). That is, inactivation of proteolytic enzymes contained in buccal tissue by exposure to GnHCl does not account exclusively for the enhanced permeation of bFGF noted in Figure 2 and Table I. In addition, results of these initial diffusion studies would also suggest that GnHCl alone does not sufficiently alter the structure of excised rabbit buccal membrane to facilitate enhanced permeation of bFGF (120–180 min interval of curve 1 in Fig. 2). It should also be noted that the molecular dimensions (diameter) of the compact, globular structure of bFGF in the native state as determined by X-ray diffraction studies is approximately 4 nm (32). However, the dimensions of the denatured (unfolded) state are difficult to determine due to the numerous possible "random" conformations.

The results presented in Figure 4 demonstrate that pretreatment of buccal tissue with GnHCl does not significantly increase the amount of bFGF that was detected following exposure of the protein to freshly-excised rabbit buccal membrane. Therefore, the activity of tissue-bound enzymes that could potentially be responsible for the degradation of bFGF were not significantly affected by tissue pretreatment with GnHCl. The results would support the premise that the increase in bFGF flux observed during the 60–120 min interval in curve 3 of Figure 2 was due to a reduction in overall molecular dimension following exposure to GnHCl rather than inactivation of tissue-bound enzymes responsible for degradation of bFGF. Data contained in curve 1 of Figure 2 also suggested that addition of GnHCl at 120 min did not alter the tissue structure such that there was a significant increase in bFGF flux during the 120–180 min period (Table I). Apparently, the order of addition of the permeation enhancer and chaotrope was extremely important in increasing the flux of bFGF across rabbit buccal epithelium. Once bFGF has been unfolded, the addition of the permeation enhancer augments transport of the more linear, high energy unfolded form of bFGF across the buccal mucosa.

The enzymes in rabbit buccal tissue responsible for the chemical degradation of bFGF play a significant role in the protein's transport. Our data demonstrate that the mucosal side of the buccal epithelium resulted in less parent bFGF being detected at 180 min than when a bFGF solution of identical concentration was exposed to the serosal side of the membrane. Previous work in our laboratory (14) has shown just the opposite trend for the degradation of oxytocin by rabbit buccal membrane. Lastly, the extent of degradation of bFGF by tissue-bound proteases was not additive. That is, approximately 28 and 46 percent of the original amount of bFGF was lost when a bFGF/PBS solution was exposed to the serosal and mucosal sides of freshly-excised rabbit buccal membrane, respectively. However, when the entire buccal membrane (both sides; area = 1.42 cm^2) was exposed to a bFGF/PBS solution of identical concentration, a final value of approximately 64 percent of the parent molecule was lost at 180 min rather than 74 percent.

The effect of endopeptidase obtained from mouse submaxillary gland on the degradation of bFGF supports the data shown in Figures 2 and 3. However, endopeptidase is potentially one of many enzymes contained in buccal tissue that could be responsible for the degradation of bFGF. Buccal tissue is known to contain many amino- and carboxypeptidases which may also participate in the degradation process for proteins. The potential for both an increase in the rate and extent of bFGF degradation by the endopeptidase in the presence of GnHCl was investigated to determine whether unfolding of bFGF exposed highly-reactive sites of the protein to the action of the endopeptidase. Since our data failed to demonstrate that endopeptidase-mediated loss of parent bFGF occurred at an accelerated rate and to a greater extent in the presence of GnHCl even though a pilot study had demonstrated loss of enzymatic activity by GnHCl, it would suggest that, as expected, the chaotrope begins to denature and inactivate the endopeptidase's capacity to degrade bFGF. Since the activity of the endopeptidase is reduced in the presence of GnHCl and GnHCl alone does not cause a significant loss of parent bFGF (unpublished observations), a percent bFGF remaining versus time profile which lies below the corresponding profile when

GnHCl was absent can only be explained by enhanced degradation of bFGF when the protein is in the unfolded state (Fig. 5). Such a phenomenon was observed with the dissociation of insulin oligomers to mono-mers (33) and α-chymotrypsin degradation (33).

The above finding is important for the transmucosal delivery of proteins since it would be undesirable to induce or accelerate degradation of the unfolded protein during membrane transport. Further experimentation is required to determine whether unfolded bFGF would be more susceptible to chemical degradation in the presence of other proteases. These data with a model endopeptidase would also suggest that the enhanced flux of bFGF across rabbit buccal membrane observed after addition of GnHCl to the donor phase (60–120 min interval for curve 3 in Figure 2; Table 1) was due to enhanced transport of unfolded bFGF and does not represent an equilibrium between enhanced transport of unfolded bFGF and increased degradation caused by exposure of reactive sites.

In conclusion, we have demonstrated that enhanced permeation of bFGF across rabbit buccal mucosa was achieved by first unfolding the protein by exposure to GnHCl and then treating the membrane with NaG. Since enhanced transport was demonstrated for the denatured (presumably more linear) conformation of bFGF, the question arises as to whether the protein refolds to the native state following transport across the buccal epithelium. Dilution of bFGF into an infinite sink, for example the systemic circulation, following its transport across the buccal mucosa should theoretically cause the protein to refold, especially in the absence of GnHCl. Using fluorescence spectroscopy and heparin affinity chromatography, it was demonstrated (Fig. 6) that 74% of the bFGF molecules assumed the native conformation following removal of GnHCl by a 25-min dialysis procedure. Such refolding is important for transmucosal protein drug delivery since pharmacologic response is associated with the native (non-denatured) state. Therefore, based on the results of this preliminary study, use of GnHCl or other chaotropes, along with permeation enhancers, may represent a strategy to improve the transmucosal delivery of proteins.

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