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Kinetics of recombinant human brain-derived neurotropic factor unfolding under reversed-phase liquid chromatography conditions

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ABSTRACT

A model describing the pathway of unfolding of recombinant human brain-derived neurotrophic factor (rhBDNF) under reversed-phase high-performance liquid chromatographic conditions is introduced. The unfolding process is divided into two major steps. The first entails a series of events such as the dissociation of the dimer and the effect of the initial contact of the protein with the stationary phase. The second step is the unfolding of the monomer molecule on the hydrophobic surface exhibiting a rate constant comparable with the time scale of chromatography. The kinetics of rhBDNF unfolding is studied with respect to the type and composition of the organic solvents and as function of the temperature of chromatography. The experimental results validate the suggested multistep unfolding model. The activation energy of monomer unfolding in both solvent systems studied is similar, but the unfolding processes are different in 1-propanol and in acetonitrile.

INTRODUCTION

Purified recombinant human brain derived neurotropic factor (rhBDNF) exhibits two peaks under reversed-phase high-performance liquid chromatographic (RPLC) conditions [1]. The two distinct peaks are well separated from each other, and it was observed that the peak I/II ratio decreases as the time rhBDNF spent on the column increases. Reinjection of the individually collected peak I and II showed two peaks under the original elution conditions, indicating the reversibility of the process. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the peaks revealed identical molecular mass to the rhBDNF standard. On-line spectroscopic studies such as light scattering and fluorescence spectroscopy revealed that both peaks are the monomeric form of rhBDNF and that the first peak represents a more folded, while the second a more unfolded conformational form of rhBDNF [1].

The two-peak phenomenon due to conformational changes has been observed previously with commercially available proteins, such as papain, soybean trypsin inhibitor, lysozyme [2–4]. It then was concluded that the peaks are related to a conformational rearrangement of these proteins, due to the combined effects of the adsorption to the stationary phase, low pH, temperature and the time proteins spend in the adsorbed state. The rate of conformational change was measured and the activation energy calculated [5] using such model systems. Protein conformational changes under chromatographic conditions were also measured by *in situ* fluorescence techniques [6,7]. The occurrence of multiple peaks can also be the consequence of dissociation–association equilibrium [8,9], and is not limited to RPLC but can occur under other chromatographic separation modes as well [10,11]. Under hydrophobic interaction chromatography conditions the role of the stationary phase was studied on the conformational changes of α-lactalbumin [12].

rhBDNF exists as a homodimer at physiologi-

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cal conditions [13]; however under reversed-phase conditions only the monomeric forms are present. During RPLC at least two steps should occur. In step one, the dimer rapidly dissociates as the molecule contacts the stationary phase. In step two, the unfolding of the dissociated monomer molecule occurs. The half-life of the second step is comparable to the time scale of chromatography which allows the detection of both the folded and the unfolded conformers in the same chromatogram. An analysis of the kinetics and thermodynamics of the observed conversion of rhBDNF as a function of chromatographic parameters is reported here.

EXPERIMENTAL

Materials

rhBDNF expressed in Chinese hamster ovary cells were purified as described previously [14]. HPLC-grade water and organic solvents were the Burdick & Jackson Labs. (Muskegon, MI, USA) or J.T. Baker (Phillipsburg, NJ, USA). Reagent-grade acids were used without further purification. The reversed-phase columns used in this work were a 30 × 4.6 and a 150 × 4.6 mm Vydac Protein C_4 (Separation Group, Hesperia, CA, USA).

HPLC instrumentation and conditions

The chromatographic system used consisted of an SP 8800 gradient pump, a Spectra 100 variable-wavelength detector (210 nm for these experiments), an SP 8780-SPST autosampler, a ChromJet integrator and a WIN86 data station (Spectra-Physics, Santa Clara, CA, USA). The column temperature was controlled to ±0.2°C by immersing a heat exchanger coil and the column in a thermostated water bath (RTE-110, Neslab Instruments).

An IBM PS/2 computer was used for data acquisition, and a Macintosh IIsi, for calculations and data presentation using Excel and Cricket Graph software.

The mobile phase systems were evaluated: (I) Mobile phase A was 10 m*M* H_3PO_4 (pH 2.2) and mobile phase B was 1-propanol–water (45:55, v/v) with an overall H_3PO_4 concentration of 10 m*M*. (II) Mobile phase A was 0.1% trifluoroacetic acid (TFA) (pH 2.2) and mobile phase B was acetonitrile with 0.1% TFA.

In all experiments 10-min linear gradients were used with a 9%/min gradient rate and a 1 ml/min flow-rate. The starting and finishing B concentration varied and will be described in the text. rhBDNF samples were prepared in water (1 mg/ml) and the injection volume was 10 μl.

The experimental design for the kinetics evaluation is described in detail in a previous work [5]. The on-column incubation time for rhBDNF is defined as the time from the injection until the start of the gradient program. The total protein residence time is the sum of the incubation time and the elution time. All kinetics experiments in this work were performed using 0, 5, 10, 15 and 20 min on-column incubation times. During the incubation period the column was washed with the starting mobile phase with 1 ml/min flow-rate.

The model and kinetics of unfolding

A model which is proposed to describe the array of events a native rhBDNF (D_N) molecule goes through during reversed-phase chromatography is displayed in Fig. 1.

The first series of events described by the model is the adsorption of the rhBDNF homodimer (D_S) to the surface, following its dissociation into identical folded monomers [$(M_F)_S$] due to the synergistic effects of the initial reversed phase conditions. In a previous denaturation model [5] which described the on-column denaturation of monomeric proteins under similar reversed-phase conditions, a fast unfolding step was suggested prior to the observed slow unfolding step. It was stated that the fast unfolding step should be related to the initial contact of the protein with the hydrophobic stationary phase [5]. In the case of rhBDNF a similar step was

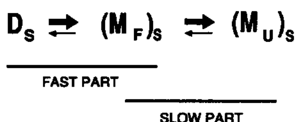

Fig. 1. A model for rhBDNF unfolding under reversed-phase conditions. D_S = Folded dimer on the surface; $(M_F)_S$ = folded monomer; $(M_U)_S$ = unfolded monomer.

assumed and included in the proposed model. During the adsorption and unfolding process a number of intermediates can be expected, some having half-life comparable to the time scale of chromatography. However, these intermediates were not detected by the current method. The next event is the unfolding of the adsorbed monomers, which are noted as $(M_U)_S$. For practical reasons the unfolding process can be divided into two major steps, one with a fast and a second with a much slower kinetics. The fast step includes the adsorption and dissociation of rhBDNF on the surface and the generation of folded monomers by the initial contact with the stationary phase. This step cannot be studied with the current chromatographic method. The slow step is the unfolding of the monomeric conformer(s) during the on-column incubation period. This second step exhibits a half-life which is comparable to the time scale of chromatography and thus it is the focus of the present study.

The fact that two different but interrelated conformations of rhBDNF are present on the chromatogram, in conjunction with their residence time dependent distribution, provides an opportunity to measure the kinetics of the unfolding. Using the change in the area of peak I as the function of the incubation time, the rate constant of unfolding on the stationary phase surface can be calculated [5]. Three assumptions have been made to simplify the mathematical expression of the above described kinetic model:

(a) The effect of the elution on the rhBDNF unfolding is independent of the incubation time and constant as long as the elution conditions are kept unchanged,

(b) The overall rate constant (k) of unfolding is expressed as

$$k = k_{F \rightarrow U} + k_{F \leftarrow U}$$

However, since both conformers are in the adsorbed state during on-column incubation the reverse reaction is infinitely slow and it can be assumed that

$$k = k_{F \rightarrow U}$$

(c) The rate of unfolding follows pseudo first-order kinetics, which can be written as

$$A = C\,e^{-kt} \quad \text{or} \quad \ln A = \ln C - kt$$

where C = a constant which includes the effect of the dissociation of the dimer, the fast unfolding step, the delay time and the effect of gradient elution on the unfolding of rhBDNF, k = rate constant of the slow unfolding step, t = incubation time and A = the amount of rhBDNF eluted from the column (peak area). The slope of the ln A *vs.* t plot will give k, the rate constant of unfolding on the bounded phase surface. In the following experiments we used the conversion instead of peak area for the rate constant calculation. Conversion was calculated as $a = A_I/(A_I + A_{II})$, where A_I and A_{II} are the area of peak I and peak II, respectively.

RESULTS AND DISCUSSION

The effect of on-column incubation time, unfolding kinetics

In a series of experiments the conversion of rhBDNF was determined as a function of incubation time. The injected sample was incubated on the RPLC stationary phase at a constant flow rate and solvent composition for a period of time, followed by gradient elution. In the first series of experiments the water–1-propanol–phosphoric acid system was used with linear gradient elution, where the B solvent concentration increased from 5 to 95% in 10 min. Fig. 2 displays a series of chromatograms of rhBDNF after 0, 5, 10, 15 and 20 min on-column incubation at 22.5°C. The disappearance of peak I with the corresponding increase of peak II as a function of incubation time is clearly illustrated.

Plots of the logarithm of peak I conversion (ln a) as a function of the incubation time were constructed at the various temperatures and the typical kinetic curves are shown in Fig. 3. Regression analysis provides the slopes which show good linearity. The reliability of the data was checked by a reproducibility study in which each incubation point was repeated with three different rhBDNF samples in duplicate. No carry over was observed between the runs and the total peak areas (peak I + II) were constant within *ca.* 5%, indicating good protein recovery. The linearity of the kinetic curves confirms the as-

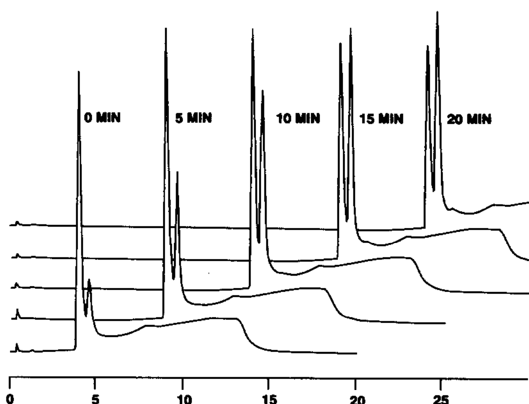

Fig. 2. Chromatographic behavior of rhBDNF as a function of on-column incubation time at a mobile phase composition of 1-propanol–water (2.25:97.75, v/v) in which the total H_3PO_4 concentration is 10 mM. Chromatographic conditions: Column = 30 × 4.6 mm Vydac Protein C_4; mobile phases: A = 10 mM H_3PO_4(pH 2.2); B = 1-propanol–water (45:55, v/v) in which the total H_3PO_4 concentration is 10 mM; gradient: 5–95% B solvent in 10 min; flow-rate 1 ml/min; sample: 10 μl of a 1 mg/ml rhBDNF sample; detection at 210 nm; column temperature: 22.5°C.

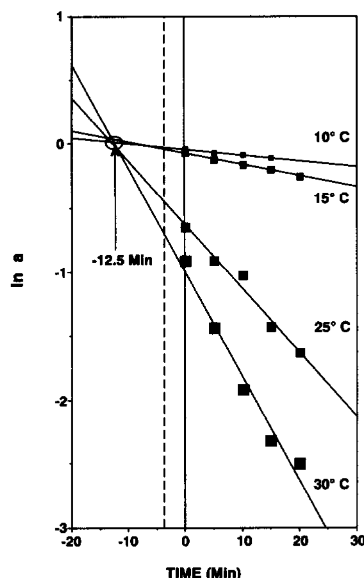

Fig. 3. First order kinetic plots of the unfolding of rhBDNF on a C_4 phase at various column temperatures as a function of on-column incubation time. ln a = logarithm of peak I conversion. Incubation solvent: 10 mM H_3PO_4 and 2.25% 1-propanol (pH 2.2) in water.

sumed pseudo first order unfolding mechanism. Table I lists the average rate constants and their relative standard deviations obtained from the reproducibility experiments at various selected temperatures. R.S.D. values between 2.3 and 9.4% are acceptable in this type of measurement where a multistep calculation has been carried out on the original experimental data.

The curves in Fig. 3 intersect with zero conversion at −7 for the low temperature and −12 min for the higher temperature data. The total residence time for a protein in these experiments is the sum of the incubation and the elution times. At zero incubation time the residence time is equal to the elution time, which is about 3.7 min. Since in Fig. 3 the time axis is the experimental incubation time, assuming a one step model the theoretical intersection point should be at −3.7 min with zero conversion. The difference between the theoretical (−3.7 min) and the extrapolated intersection points indicates the presence of other step(s) prior to the measured monomer unfolding. The fact that the low-temperature intersection point differs from the intersection point at higher temperatures might indicate that the unfolding mechanism is different in the two temperature ranges. These experimental data validate the above described model which suggests more than one process during the chromatography of rhBDNF on a hydrophobic stationary phase.

The effect of other mobile phase conditions

The second mobile phase system of this evaluation was based on water–acetonitrile–TFA mixtures. The chromatographic conditions remained the same except that we changed the A solvent to water and the B solvent to 100% acetonitrile, with both solvents containing 0.1% TFA. The general trend (*i.e.*, the first peak disappears as the incubation time increases) was similar to what was observed with the 1-propanol–phosphoric acid-based mobile phase. The elution times for the two peaks were comparable, but the peaks were much sharper due to the organic gradient. The measured kinetics of unfolding in this solvent system also indicated a pseudo first order mechanism. The calculated rate constants are listed in Table I. The rate constants are of the same order of magnitude

TABLE I

RATE CONSTANTS FOR UNFOLDING OF rhBDNF UNDER RPLC CONDITIONS

Temperature (°C)	*n*-Propanol		Acetonitrile, Rate constant ($\times 10^{-4}$ s^{-1})
	Rate constant ($\times 10^{-4}$ s^{-1}), average	R.S.D. (%)	
10.0	0.7		0.2
15.0	1.6	6.4	
20.0	4.2	9.4	1.7
22.5	4.7	7.1	
25.0	6.1	3.0	
27.5	12.8	7.9	
30.0	13.8	4.2	5.2
32.5	21.2	6.9	
35.0	22.3	2.3	
40.0			15.9

though their values are significantly lower in comparison to the previous solvent system.

Activation energy of rhBDNF unfolding

Since the rate constant of unfolding was measured at different temperatures, the activation energy of the process can be calculated from the ln k *vs.* $1/T$ curve according to the Arrhenius equation [15]. In Fig. 4, the logarithm of rate constants at different temperatures (ln k) for both mobile phase systems were plotted against $1/T$, and the slopes of the curves were determined by linear regression. The calculated activation energies in acetonitrile–TFA and in 1-propanol–phosphoric acid-based mobile phases were 26.2 and 24.6 kcal/K mol (1 cal = 4.14 J), respectively. Since the reproducibility of the activation energy determination is about 5% the activation energies are not significantly different in the two solvent systems. In general, the calculated activation energies for rhBDNF are in agreement with values obtained for other proteins (*ca.* 20 kcal/K mol) [5].

The effect of temperature on the elution of rhBDNF

Fig. 5 shows the change of the elution times for both peaks in both solvents. In the 1-propanol based mobile phase the elution times for both peaks I and II decrease as a function of temperature, as it is predicted based on the theory of HPLC. However, in the acetonitrile based system the behavior is anomalous. The elution time for peak I increases with temperature, which in protein RPLC is usually the consequence of a conformational change occurring during chromatography. The developing new conformational state has more hydrophobic

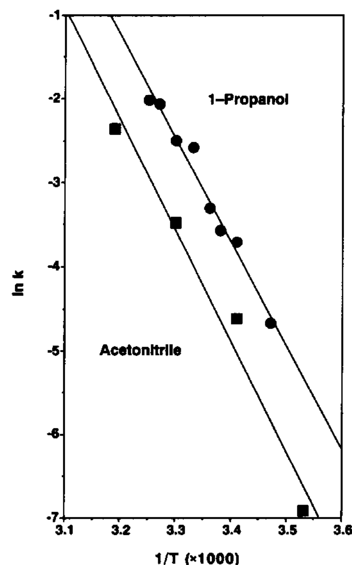

Fig. 4. The Arrhenius plots of the rate constants of rhBDNF unfolding in different mobile phase systems, 1-propanol–phosphoric acid and acetonitrile–TFA; ln k = logarithm of the rate constant; $1/T$ = inverse absolute temperature (1/K).

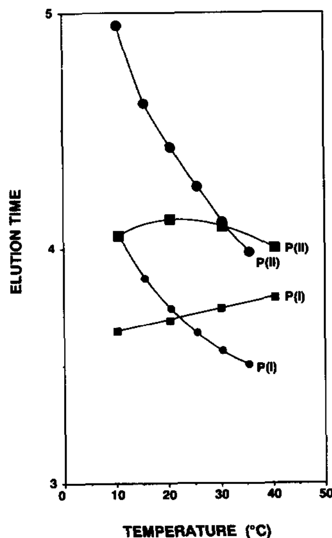

Fig. 5. The elution times as a function of column temperature in different mobile phase systems. The elution was performed with identical gradient conditions (5–95% B solvent in 10 min). ● = 1-Propanol–phosphoric acid–water; ■ = acetonitrile–TFA–water. P(I) and P(II) correspond to the folded and unfolded conformers, respectively. The elution time is in minutes.

contact points which ultimately lead to longer retention. Since acetonitrile is a known helix inducer [16] it seems that during the separation of rhBDNF a helical or helix-like conformation is generated which is reflected in an increase in elution time. Similar anomalous behavior has been observed with helix-forming peptides [17] and recombinant human growth hormone [18]. The behavior of peak II is somewhat different. The retention curve goes through a maximum, after which the retention decreases again. It can be hypothesized that even in the more unfolded state an intermediate helix-like structure exists which will then be destroyed by the harsher mobile phase conditions. Under conditions below the temperature corresponding to maximum retention the helix-inducing forces dominate, while above the maximum, at higher temperatures the unfolding is favored.

The difference in chromatographic behavior in the two studied solvent systems can be explained by the existence of different unfolding pathways. It is well known that the activation energy represents the energy difference between two conformational states, but does not provide information regarding the pathway as the molecule changes from one conformation to another.

The effect of organic solvent concentration

We have shown that the appearance of an rhBDNF chromatogram can be manipulated by changing the temperature, incubation time and the type of the organic solvent. Significant differences in peak shape, elution time and peak ratios were obtained as a function of those parameters. Next we explored the effect of organic solvent concentration on the unfolding of rhBDNF, by measuring the rate constants and following changes in the peak ratio. The incubation was performed at various B concentrations for various incubation times, prior to elution with a constant 9%/min gradient rate, and the temperature was kept at 20°C. The calculated rate constants were plotted as a function of organic solvent concentration for both solvent systems. The plots displayed in Fig. 6 show that in kinetic terms, based on the rate constants, 1-propanol–phosphoric acid is more effective on the rhBDNF unfolding than acetonitrile–TFA.

The rate constant of unfolding provides conditions where the unfolding can be carried out more efficiently in a kinetic term. Plotting the

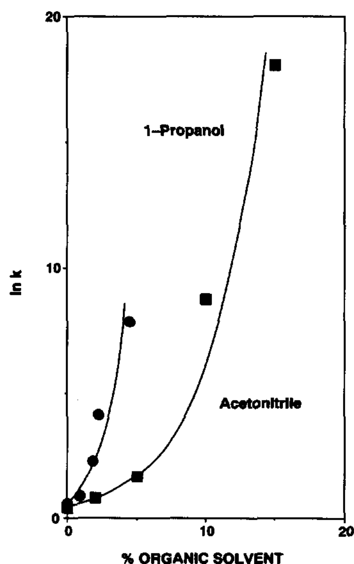

Fig. 6. The effect of incubation solvent concentration on the rate of rhBDNF unfolding. Temperature = 20°C; ln k = logarithm of the rate constant.

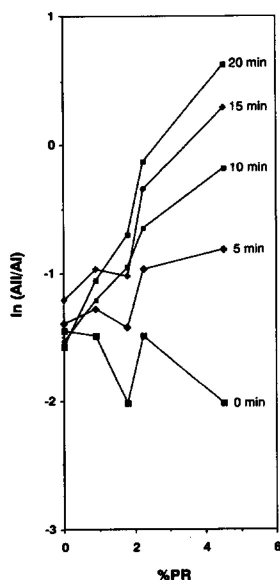

Fig. 7. The logarithm of peak area ratio as a function of the incubating solvent concentration at different incubation times. Mobile phase: 1-propanol–phosphoric acid. All experiments were performed at 20°C. PR = 1-Propanol.

ratio of the area of the unfolded AII to the area of the folded AI is a different way to follow the unfolding process. Fig. 7 displays the logarithm of peak ratios for rhBDNF as a function of 1-propanol concentration, at different incubation times. Similar curves were obtained for acetonitrile. The first interesting observation is that on the curves in both solvent systems a local maximum and minimum can be seen. This observation is similar to what has been observed in solution denaturation studies by organic solvents. At low concentration organic solvents can facilitate the denaturation by a favorable access to the protein molecules, while at intermediate concentrations they can prevent denaturation by shielding the proteins. At higher organic solvent concentration the disruption of the protein structure became dominant.

The second observation is that in 1-propanol, at zero incubation time, the AII/AI ratio decreases as the organic solvent concentration increases. This means that 1-propanol apparently protects rhBDNF against unfolding. The phenomenon has been observed before and was explained by the decreased hydrophobicity of the stationary phase due to the adsorption of 1-propanol. The first interaction of the protein molecule with the surface is cushioned by this adsorbed hydroxyl group rich layer. However on this time scale the effect of 1-propanol on the unfolding is not strong enough to generate more unfolded molecules and significantly alter the peak ratio. In the case of acetonitrile, at zero incubation the peak ratio does not change with increasing concentration. The adsorbed acetonitrile does not form a protective layer, but the unfolding is not fast enough to shift the peak ratio. At longer incubations the disruptive effect of the organic solvents is more dominant, with the peak ratios increasing as more unfolded molecules are generated.

CONCLUSIONS

Based on the above reported systematic study on the effects of a variety of reversed-phase elution conditions on the kinetics of unfolding of rhBDNF a number of conclusions can be drawn:

(a) Increasing column temperature and incubation time enhances the peak I to peak II conversion and the rate of unfolding increases with higher organic solvent concentrations,

(b) The activation energy of rhBDNF unfolding is similar in both of the studied solvent systems, and they are also in the same order of magnitude as has been reported for other proteins,

(c) 1-Propanol as opposed to acetonitrile provides some protection against rhBDNF unfolding by shielding the hydrophobic stationary phase,

(d) The unfolding mechanism is apparently different in the two solvent systems, *e.g.* acetonitrile generates a more hydrophobic conformer, as can be observed in the anomalous increase in retention with increasing column temperature.

Reversed-phase elution conditions synergetically combine the effects of low pH, organic solvents, temperature and hydrophobic surface on protein conformation. The denaturing conditions occasionally lead to odd peak shapes or multiple peaks during the chromatography of a single pure protein sample. However, the phenomena are not always apparent because the half-life of the protein unfolding is either much

shorter or much longer than the duration of the chromatographic process. Only in cases when the time-scale of the two events, conformational change and chromatography, are comparable can the phenomena reproducibly be observed [19–21].

The relevance of the above reported systematic study is apparent both from an analytical and preparative point of view. In protein analysis the unfolded form is usually favored because the larger complementary surface allows the resolution of minor variants, such as degradation and oxidation products and/or other production microheterogeneities. Knowing the effects of various chromatographic parameters, proteins can be directed into the unfolded state, consequently increasing the probability of detecting microheterogeneities buried in the folded state. It has to be mentioned that the above studies were performed in the traditionally used low-pH elution systems. It is known that most reversed-phase protein analysis should be performed at low pH were the least interaction with the silica-based stationary phase is expected and consequently the best resolution can be observed. In protein purification, when the unfolding during RPLC might be irreversible, appropriate conditions can be selected where the folded conformation is favored.

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