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Activity-dependent neurotrophic factor, ADNF, determines the structure characteristics of Colivelin, a fusion protein of ADNF9 and Humanin analog

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Abstract: A 24-amino acid long peptide, Humanin, protects neurons from Alzheimer's disease (AD)-related cell toxicities at sub-nM-uM concentrations. Activity-dependent neurotrophic factor (ADNF) is a glia-derived neurotrophic peptide, which protects neurons from tetrodoxin treatment and AD-related and amyotrophic lateral sclerosis-related insults at fM concentrations. An attempt was made to further improve the activity of Humanin by fusing this peptide to ADNF9, a 9-amino acid long core peptide of the ADNF. This fusion resulted in a novel molecule, termed Colivelin, with the neuroprotective activity at fM range, which is $\sim 10^3$–10^7 fold higher than the activity of Humanin and Humanin analogs and follows the activity profile of fM-active ADNF9. We have characterized the structural properties of Colivelin and compared with those of ADNF9 and Humanin in water and phosphate-buffered saline (PBS). The secondary structure of Colivelin was similar to that of ADNF9, but not that of Humanin, and hence was not the average of the contributions of the two peptides fused. Colivelin was stable and monomeric in PBS, consistent with the monomeric property of ADNF9, while Humanin showed strong tendency to self-associate. Thus, it is evident that the structural properties of Colivelin resemble those of ADNF9, rather than those of Humanin. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: humanin; ADNF; colivelin; fusion peptide; sedimentation analysis; circular dichroism

INTRODUCTION

A 24-amino acid long peptide, Humanin (HN), and various analog peptides have been shown to protect neurons dose-dependently at sub-nM-μM range from a variety of neurotoxic insults, including highly toxic Aβ peptides [1–8]. Among various analogs developed, AGA(C8R)-HNG17 (PAGASRLLLLTGEIDLP) showed an activity at 10 pM, which is about 10^5-fold more active than the parent HN molecule (MAPRGFSCLLLLTSEIDLPVKRRA) active at ~1 μM [9]. Activity-dependent neurotrophic factor (ADNF) is a glia-derived neurotrophic peptide, which also protects neurons from tetrodoxin treatment and AD- and amyotrophic lateral sclerosis-related insults at fM concentrations [10–12]. ADNF9 (SALLRSIPA) is a 9-amino acid long core peptide of ADNF and shows a unique activity profile; it is active at fM concentration, but loses the activity at ~10 nM for unknown reasons [10–12]. With the hope that a fusion of these peptides may enhance the activity of HN, a 26-amino acid long peptide termed Colivelin was created by fusing the ADNF9 to the *N*-terminus of AGA-(C8R)HNG17; i.e. it has a sequence of SALLRSIPAPAGASRLLLLTGEIDLP [9]. This resulted in a novel peptide with a neuroprotective activity of 100 fM range, similar to ADNF9, but with no activity loss at higher concentration [9]. It thus appears that Colivelin acquired only the highly active part of ADNF9. However, the mechanism of such an enhanced activity of Colivelin relative to HN is totally unknown. Previously, we have shown that HN has a tendency to self-associate in phosphate-buffered saline (PBS) [13], while ADNF9 forms a stable monomer [14]. It is thus essential to understand the properties of Colivelin in relation to the structural characteristics of HN and ADNF9. This report studies the secondary structure and self-association of Colivelin in PBS as well as in water using circular dichroism (CD) and analytical ultracentrifugation.

MATERIALS AND METHODS

Materials

All the peptides used, i.e. Colivelin, ADNF9, and AGA-(C8R) HNG17, were purchased from Peptide Institute and obtained as powder.

CD Analysis

As a stock solution, 5 mg of Colivelin was dissolved in 1 ml of water and used as a stock solution. We have

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previously observed that direct dissolution of ADNF9 and HN or its Ser14Gly analog (HNG) into PBS resulted in the formation of particulates and poor solubility [13,14]. This stock peptide solution in water was further diluted with water or 2X PBS to generate peptide solutions at different concentrations in water or PBS. The pH of the peptides in water ranged from 3.3 to 3.8 and that in PBS ranged from 6.5 to 7.0 depending on the peptide concentration. The diluted samples were incubated at room temperature for at least 30 min. CD measurements were carried out on a Jasco J-715 spectropolarimeter at room temperature using 0.05 cm cell. Alternatively, the temperature was controlled using a Peltier cell holder and a PTC-348WI temperature controller. For temperature-controlled measurements, 0.1 cm cell was used. The mean residue ellipticity was calculated from the solvent-subtracted spectrum using the peptide concentration, the path-length of the cell, and the mean residue weight of 102.

Sedimentation Analysis

The sedimentation equilibrium experiments were performed at 20 °C on a Beckman analytical ultracentrifuge XL-I (Beckman-Coulter) using a 4-hole An60Ti rotor, standard double-sector centerpieces, and quartz windows. In each cell, 120 µl of sample solution was loaded and the PBS was used as a reference solution. Concentration profiles were monitored by absorbance at 230 nm. The peptide samples were prepared as described for the CD analysis, i.e. by first dissolving the peptide in water and diluting it into PBS. The peptide concentrations of loaded samples were 0.1, 0.15, and 0.25 mg/ml and the rotor speeds were set at 40 000, 47 000, and 55 000 rpm. Scans were recorded every 2 h and the equilibrium of the system was judged by the unchanged last three scans before data acquisition. All scans were globally fitted to a single species model to determine the weight average molecular weight by nonlin software implemented in the Beckman-Coulter software package. The partial specific volume was theoretically calculated from the amino acid composition of the peptide using Sednterp program [15] as 0.773. The sedimentation velocity experiments were performed at 20 °C using standard double-sector centerpieces and quartz windows. The peptide sample at 0.5 mg/ml in PBS was prepared similarly to that used for sedimentation equilibrium. The rotor speed was set at 60 000 rpm. The sedimentation velocity data were analyzed by a program SEDFIT [16].

RESULTS AND DISCUSSION

The secondary structure of HN was previously characterized by CD as a disordered structure at low peptide concentration, which undergoes self-association and formation of β-sheet structure with increasing peptide concentration [13]. On the contrary, ADNF9 showed a disordered structure independent of the peptide concentration [14]. It would be interesting to see whether the fusion of these two peptides generates a peptide resembling HN or ADNF9. For this purpose, a fusion peptide Colivelin, was chemically synthesized and has previously been characterized for biological activities [9]. Here the structural properties of the fused peptide were characterized by the same technique used before, i.e. CD. The far UV CD spectra of Colivelin were obtained as a function of peptide concentration. Figure 1(A) shows the spectra in water. The spectra at 0.1 (black solid line) and 0.5 (gray solid line) mg/ml are nearly identical and show a monotone decrease as the wavelength was decreased. It thus appears that the structure of Colvelin is mostly disordered. A drastic change was observed when Colvelin concentration was increased to 0.75 mg/ml (black dot line). The spectra were qualitatively similar at 0.75–2 mg/ml (gray dot line at 1 mg/ml and black dash line at 2 mg/ml). The spectra at these high peptide concentrations show a minimum at ~215 nm, indicating the presence of β-sheet structure [13]. It appears that the β-sheet structure slightly increases with the peptide concentration, as the negative intensity at 215 nm increases. A drastic shift in the spectra occurred in the narrow change of peptide concentration (between 0.5 and 0.75 mg/ml), suggesting the self-association of Colivelin in water at this peptide concentration, accompanied by the formation of β-sheet structure. As these samples were prepared by dilution of the stock Colivelin solution at 5 mg/ml in water, it is likely that the observed concentration dependence is due at least in part to kinetics of dissociation, if, in fact, the peptide self-associates at 5 mg/ml.

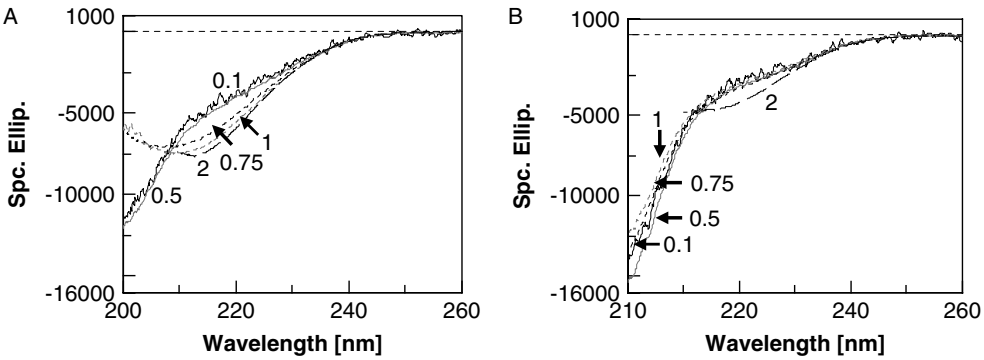

Figure 1 The far UV CD spectra of Colivelin in water (A) and PBS (B). Owing to high absorbance, the 2 mg/ml sample was scanned only to 210 nm. At 0.1 mg/ml (black solid), 0.5 mg/ml (Gray solid), 0.75 mg/ml (black dot), 1 mg/ml (gray dot), and 2 mg/ml (black dash).

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Figure 1(B) shows the far UV CD spectra in PBS. The spectra are nearly identical at 0.1–1 mg/ml and primarily disordered. A small, but significant change occurs at 2 mg/ml. Due to high peptide concentration (2 mg/ml) and hence high absorbance, the scan was stopped at 210 nm. In the absence of the spectra below 210 nm, it is difficult to conclude the type of structure change which occurred at 2 mg/ml. Nevertheless, the observed peptide concentration dependence indicates self-association of Colivelin in PBS, although much weaker than in water (compare the magnitude and concentration range in Figure 1(A)). As the PBS samples were also prepared by dilution of the stock solution in water, kinetics of dissociation may affect the self-association of the sample in PBS as well.

It may be apparent that the secondary structure is similar at low peptide concentration in water and in PBS (compare Figure 1(A) and (B)). Figure 2 more clearly demonstrates this. The spectrum of 0.1 mg/ml is nearly identical in water (black solid) and PBS (gray solid), although small quantitative differences exist. The structure appears to be largely disordered in both solvents. On the contrary, the structure at 2 mg/ml is significantly different in water and PBS. The signal intensity is much more negative in water, suggesting stronger self-association of Colivelin in water.

The effect of temperature was examined for 0.5 mg/ml Colivelin in water and PBS. As shown in Figure 3(A), incubation of the peptide at 37 °C for 30 min leads to a small, but significant downward shift in CD intensity in both water and PBS, suggesting conformational changes most likely due to enhanced hydrophobic self-association. Prolonging the incubation time to 3 h did not cause further change in CD intensity (Figure 3(B)). The observed change by 37 °C incubation was reversible, as the signal returned to the original value as soon as the temperature was brought back to 20 °C.

Having studied the structural characteristics of Colivelin in both water and PBS, it is interesting to compare these characteristics of Colivelin with those of the parent molecules, i.e. ADNF9 and HN analog, AGA-(C8R)HNG17. Colivelin consists in weight of 36% ADNF9 and 64% AGA-(C8R)HNG17 due to their differences in molecular weight. Figure 4(A) compares the CD spectrum in water of 0.1 mg/ml Colivelin (gray) with the spectra of 0.1 mg/ml ADNF9 (black dash) and AGA-(C8R)HNG17 (black dot). It is evident that the Colivelin spectrum closely follows that of ADNF9 in water. Reflecting this and a greater mass contribution of AGA-(C8R)HNG17, the calculated spectrum, which is a simple addition of ADNF9 and AGA(C8R)-HNG17 spectra, is completely different from the experimental one (Figure 4(B)), suggesting that Colivelin carries the structural properties of ADNF9 in water and is completely different from a mixture of ADNF9 and AGA(C8R)-HNG17 structures, although it appeared that Colivelin acquired only the highly active part of ADNF9. However, more important analysis will be in PBS, which was done in Figure 5. In PBS as well, the structural properties of Colvelin more closely follow those of ADNF9. In fact, the far UV CD spectrum of 0.1 mg/ml Colivelin (gray) is nearly superimposable over the spectrum of ADNF9 (black dash) and significantly different from the AGA-(C8R)HNG17 spectrum (black dot). Reflecting this fact, the calculated spectrum (Figure 5(B), black) is considerably different from the experimental one (gray).

We have previously observed that ADNF9 is monomeric in PBS [14]. Here we have used sedimentation velocity (Figure 6(A)) and equilibrium (Figure 6(B)) analysis for Colivelin. The velocity analysis showed a main peak at 0.386 S with a small amount at 0.872 S. The observed small sedimentation coefficient (0.386 S) for Colivelin is consistent with the low calculated mass of the peptide (2645). Figure 6(B) shows a representative data that is fitting for the data obtained at 0.25 mg/ml and 40 000 rpm, indicating a good fitting. A global fitting to the nine sets of sedimentation profiles, obtained at different loading concentrations and rotor speeds, gave a molecular weight of 2504 ± 120, which

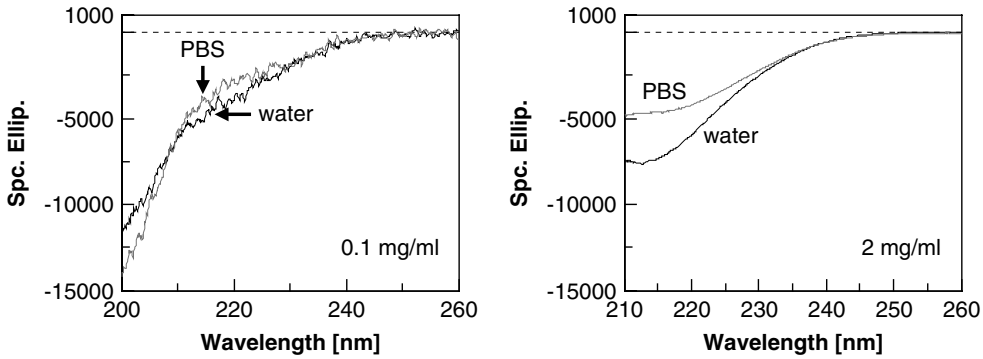

Figure 2 Comparison of the far UV CD spectra of Colivelin in water (black) and PBS (gray) at different peptide concentrations. Due to high absorbance of 2 mg/ml sample, spectral comparison was made between 210 and 260 nm for this sample. At 0.1 mg/ml, left panel; at 2 mg/ml, right panel.

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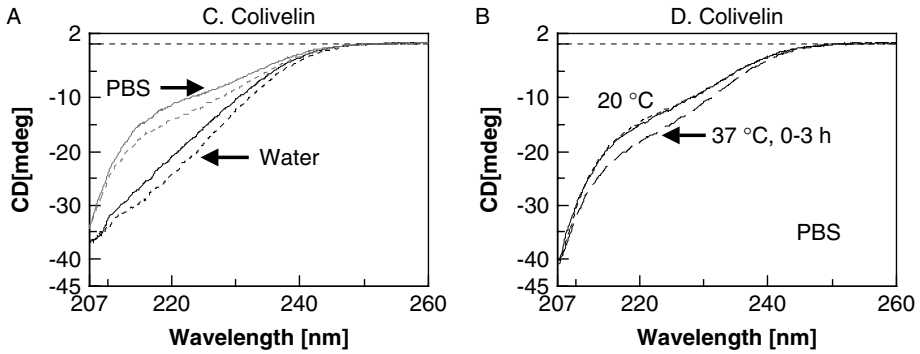

Figure 3 (A) Temperature dependence of the far UV CD spectra of 0.5 mg/ml Colivelin Black line, water; gray line, PBS. Solid line, 20 °C; dotted line, 37 °C (30 min) Solvent spectra are identical for water and PBS at 20 and 37 °C within experimental errors. (B) Reversibility of CD change of 0.5 mg/ml Colivelin in PBS with temperature Solid line, 20 °C before heating to 37 °C; dotted line, 20 °C after heating to 37 °C. Dashed line, 37 °C for 3 h.

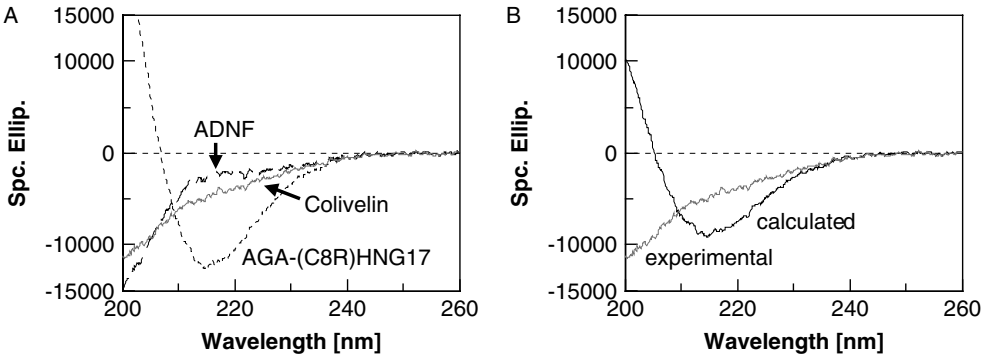

Figure 4 (A) Comparison of the far UV CD spectra of Colivelin with ADNF and AGA-(C8R)HNG17 at 0.1 mg/ml in water. (B) Comparison of theoretical and experimental spectra of Colivelin at 0.1 mg/ml in water. Theoretical spectrum was calculated by 0.36 × ADNF + 0.64 × AGA-(C8R)HNG17.

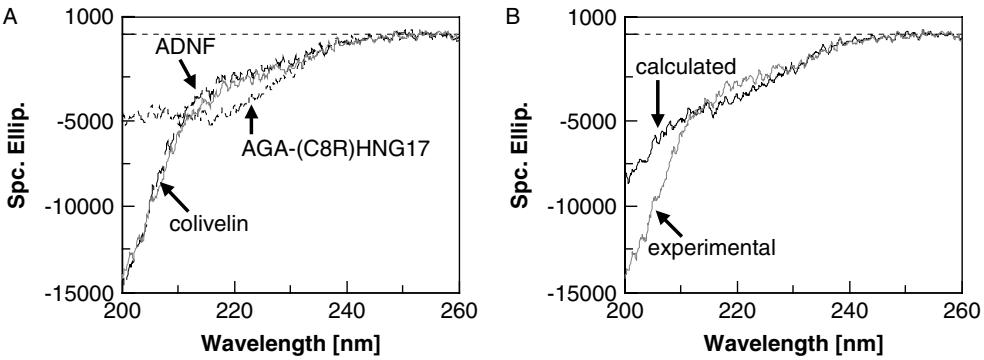

Figure 5 (A) Comparison of the far UV CD spectra of Colivelin with ADNF and AGA-(C8R)HNG17 at 0.1 mg/ml in PBS. (B) Comparison of theoretical and experimental spectra of Colivelin at 0.1 mg/ml in PBS. Theoretical spectrum was calculated by 0.36 × ADNF + 0.64 × AGA-(C8R)HNG17.

is identical, within experimental errors, to the theoretical molecular weight of 2645. There is no systematic deviation of the fitting as shown in the residuals (see Figure 6). Thus, Colivelin is a monomer in PBS, identical to the state of ADNF9 in PBS. However, this is expected from the fact that AGA-(C8R)HNG17 is also a monomer in PBS (manuscript in preparation). A small sedimentation peak at 0.872 S indicated presence of oligomers. It should be noted that these samples were also prepared by dilution of stock solution in water. Both sedimentation velocity and equilibrium experiments take much longer time (over 6 h) than CD measurements and thus may be less affected by kinetics of dissociation.

As previously reported, both ADNF9 and Colivelin are active at fM concentrations [9,10]. Assuming that

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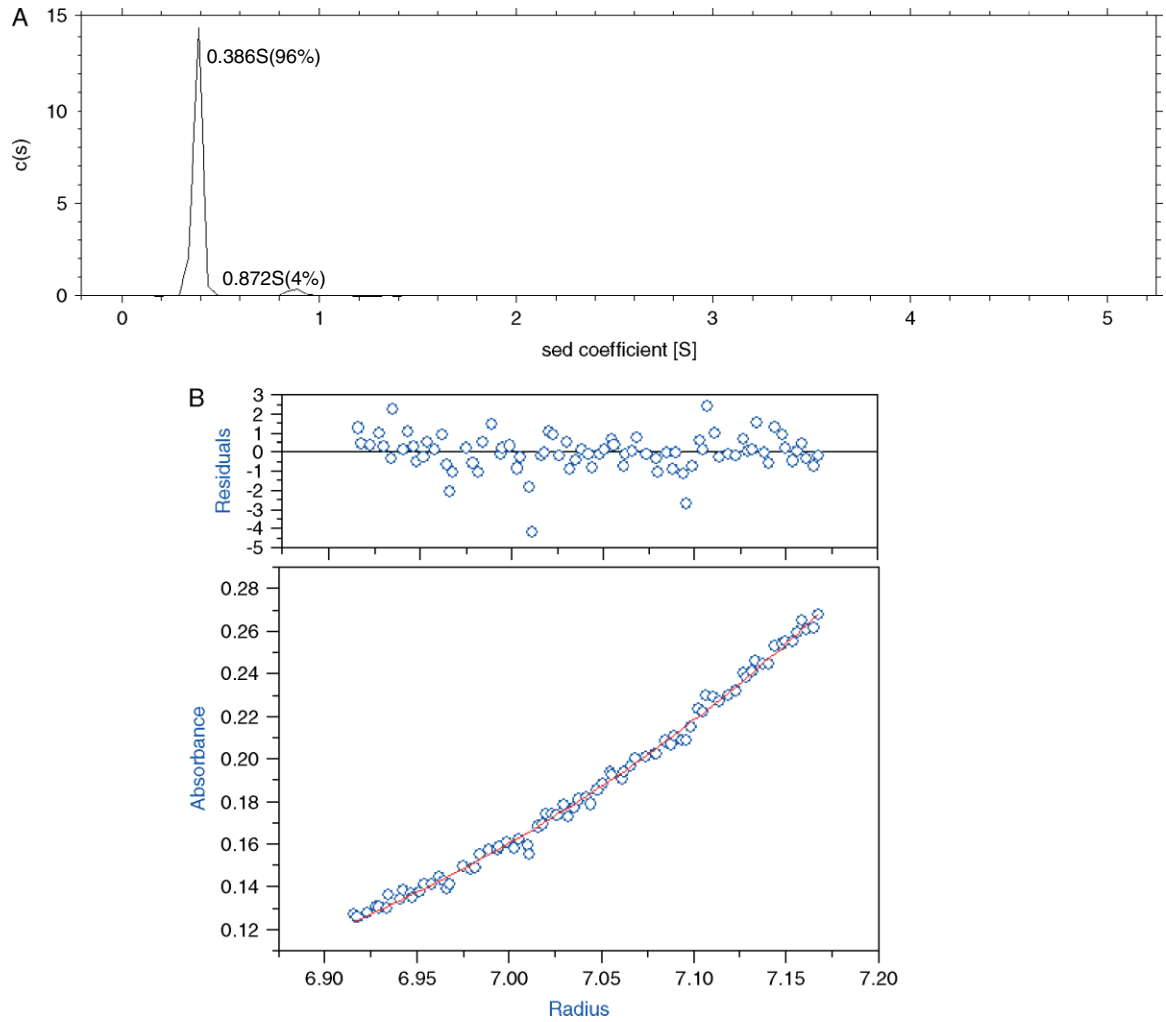

Figure 6 Sedimentation velocity (A) and equilibrium analysis (B) of Colivelin in PBS. The equilibrium data (B) shown is obtained at 0.25 mg/ml and 40 000 rpm.

one can translate fM activity to fM affinity (dissociation constant), such strong binding is rarely observed even for protein–protein or protein–ligand interactions. An exceptional case is the binding between avidin and biotin, which does confer fM affinity [17,18]. The unusually strong reversible binding of biotin by avidin is due to cooperative multiple hydrogen bonds between the two highly structured molecules [18]. We have previously addressed the problems of explaining the fM activity for ADNF9, whose structure is, unlike avidin, disordered [14]. We have clearly shown here that Colivelin closely follows the property of ADNF9 in PBS and hence that the same discussion applies for Colivelin. It is difficult to conceive at this point how such a short disordered and monomeric structure of Colivelin can afford the observed fM affinity. Furthermore, even if Colivelin can bind the target molecules with such high affinity, the situation is completely different from the binding of biotin with avidin. For Colivelin, such a binding event must be translated to the cellular function. The fM means that there are only 6×10^5 molecules of Colivelin per ml at 1 fM. If there are 10^5 cells per ml in the biological system, then there are only six Colivelin molecules per cell, which appear to be too few to exert the biological function. A more rigorous quantitative analysis for the effects of Colivelin as well as ADNF9 on the cell growth appears to be essential to solve this unusual biological activity.

As described above, the structure of Colivelin closely follows that of ADNF9. Nevertheless, there is a distinct difference between the two peptides in biological activity. Colivelin does not lose activity at higher peptide concentration, at which ADNF9 does. The structure analysis of these peptides was done at concentrations far above their physiological concentrations. There may be unexpected factors, which alter their structural properties at much lower peptide concentrations, leading to the observed difference in the activity.

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