# Conformational Analysis of XA and AX Dipeptides in Water by Electronic Circular Dichroism and $^1$H NMR Spectroscopy

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We measured the temperature-dependent electronic circular dichroism (ECD) spectra of AX, XA, and XG dipeptides in $D_2O$. The spectra of all XA and AX peptides indicate a substantial population of the polyproline II (PPII) conformation, while the ECD spectra of LG, KG, PG, and AG were found to be quantitatively different from the alanine-based dipeptides. Additional UV absorption data indicate that the ECD spectra of the XG peptides stem from electronic coupling between the peptide and the C-terminal group, and that spectral differences reflect different orientations of the latter. We also measured the $^1$H NMR spectra of the investigated dipeptides to determine the $^3J_{H\alpha NH}$ coupling constants for the C-terminal residue. The observed temperature dependence of the ECD spectra and the respective room-temperature $^3J_{H\alpha NH}$ coupling constants were analyzed by a two-state model encompassing PPII and a $\beta$-like conformation. The PPII propensity of alanine in the XA series is only slightly modulated by the N-terminal side chain, and is larger than 50%. As compared to AA, XA peptides containing L, P, S, K V, E, T, and I all cause a relative stabilization of the extended $\beta$-strand conformation. The PPII fractions of XA peptides varied between 0.64 for AA and 0.58 for DA, whereas the PPII fractions of AX peptides were much lower. From the investigated AX peptides, only AL and AQ showed the expected PPII propensity. We found that AT, AI, and AV clearly prefer an extended $\beta$-strand conformation. A quantitative comparison of AA, AAA, and AAAA revealed a hierarchy AAAA > AAA $\approx$ AA for the PPII population, in agreement with predictions from MD calculations and results from Raman optical activity studies (McColl et al. *J. Am. Chem. Soc.* **2004**, *126*, 5076).

## Introduction

Electronic circular dichroism (ECD) spectroscopy is still one of the most prominent optical tools for the secondary structure analysis of peptides and proteins.[1] While it is generally used to determine the α-helical and $\beta$-sheet content of these biomolecules, another still less prominent conformation has emerged as an ideal target of this spectroscopic technique, namely the left-handed polyproline II (PPII) helix.[1−4] This conformation is generally adopted by *trans*-proline containing peptides. The canonical conformation exhibits dihedral angles of $(\phi,\psi) = (-78°,146°)$ and a 120° angle between consecutive peptide groups.[5] It therefore has an even higher symmetry than the canonical α-helix and is entirely stabilized by its hydration shell and not by interpeptide hydrogen bonding.[6−9] Nearly 30 years ago, Tiffany and Krimm discovered that charged poly-lysine and poly-glutamic acid exhibit ECD spectra similar to that observed for *trans*-poly-proline with a pronounced negative signal at 195 nm and a smaller positive signal at 216 nm.[10] This led the authors to conclude that these peptides adopt at least locally a PPII rather than a random coil conformation, as it was generally believed. This interpretation was later contested by Mattice et al. based on the fact that some blocked alanine-based di-, tri-, and tetrapeptides also exhibit a PPII like ECD signal.[11,12] On the basis of the general belief that their structures are random, it was concluded that this holds true as well for the polypeptides investigated by Tiffany and Krimm. Their hypothesis was reinvestigated only during the last 15 years, mostly by ECD,[1−3] but also by vibrational circular dichroism (VCD)[13] and other optical methods.[14−16] All of these investigations have provided compelling and conclusive evidence that (1) the CD spectra reported by Tiffany and Krimm[10] are indeed indicative of a PPII conformation and (2) the unfolded state of peptides and proteins exhibits, in many cases, a substantial PPII fraction, in contrast to what one would expect from any type of random coil model.[17]

Attempts have been made to quantify the PPII fraction from ECD spectra by using the basis spectrum reported by Sreerama and Woody.[1] However, this renders difficult sometimes because it is unclear how different amino acid side chains contribute to the overall ECD spectrum. A satisfactory theoretical understanding of the PPII spectrum has not yet been achieved, but calculations by Woody indicate that contributions for high-energy $\sigma\rightarrow\sigma^*$ transitions have to be taken into account.[18] In Woody's approach, every residue can contribute to the overall ECD spectrum,[19] which was accounted for by calculating the polarizability tensors for bonds and other groupings. The fact that even dipeptides were found to exhibit an ECD spectrum seems to corroborate this notion,[20] because this would not be expected if the spectrum was due solely to excitonic coupling

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between electronic and magnetic transitions in different peptide groups. It follows from Woody's theory that different amino acid residues affect the CD spectrum differently even if they adopt the same conformation with respect to their dihedral angles.[18]

By measuring and analyzing the ECD spectra of AX, XA, and XG dipeptides, the present study pursued three different goals: (1) checking whether a side chain contribution can be inferred from the ECD spectrum of the peptide backbone depicted between 190 and 220 nm, (2) probing the conformational propensity of different amino acid residues at the C-terminal position, and (3) exploring the influence of the nearest neighbor (N-terminal) residue on the propensity of the C-terminal residue. These investigations can be considered as a continuation of a recently reported spectroscopic study on the respective propensities in AXA peptides.[21] Our structure analysis was further based on the $^3J_{H\alpha NH}$ coupling constant of the respective C-terminal residues, as obtained from the respective $^1$H NMR spectra, and on the temperature dependence of ECD of some XA and AX peptides. We used the combined data obtained from both spectroscopies to perform a thermodynamic analysis based on a simple two-state model, which yielded the enthalpic and entropic differences between the two considered conformations. The results from this study are compared to a very recent study on AcX peptides by Gokce et al.[22] Finally, we reanalyzed the temperature dependence of the recently measured ECD spectra of AAA[15] and AAAA[16] to check the notion that the PPII propensity of alanine depends on the number of consecutive alanine residues.[16,23] Our results are thus important for understanding the determinants of PPII formation in polypeptides that do not contain *trans*-proline.

## Materials and Methods

**Materials.** L-Alanyl-L-glycine (AG), L-glycyl-L-alanine (GA), L-alanyl-L-lysine (AK), L-alanyl-L-leucine (AL), L-leucyl-L-alanine (LA), L-alanyl-L-serine (AS), L-seryl-L-alanine (SA), L-alanyl-L-valine (AV), L-valyl-L-alanine (VA), L-alanyl-L-isoleucine (AI), L-isolucyl-L-alanine (IA), L-alanyl-L-threonine (AT), L-threonyl-L-alanine (TA), L-aspartate-L-alanine (DA), L-alanyl-L-proline (AP), L-prolyl-L-alanine (PA), L-glutamate-L-alanine (EA), L-arginyl-L-alanine (RA), L-alanyl-L-alanine (AA), L-alanyl-L-glutamine (AQ), L-leucyl-L-glycine (LG), L-prolyl-L-glycine (PG), L-lysyl-glycine (KG), and L-glycyl-L-glycine (GG) were purchased from Bachem Bioscience Inc. $D_2O$ and $NaClO_4$ were obtained from Sigma-Aldrich (St. Louis, MO) and were of analytical grade. All of the peptides contained >98% purity and were used without further purification. The pH value of the solutions was adjusted by adding relevant aliquots of DCl (Acros Organics), and the values were converted to pD values by using the method of Glasoe and Long.[24]

**Spectroscopies.** *ECD Spectroscopy*. The UV ECD spectra in the wavelength range of 180−250 nm of all dipeptides listed above were measured with a JASCO J-810 spectrapolarimeter in a 0.1 mm quartz cell with 0.05 nm resolution, at the Drexel University Medical School. The peptides were dissolved in $D_2O$ at a concentration of 10 mM for ECD experiments. We performed the measurements in $D_2O$ rather than in $H_2O$ to allow a comparison with structural data obtained by virtue of vibrational spectroscopies. As recently shown, the PPII propensity of, for example, alanine, is higher in $H_2O$ than in $D_2O$.[25,26] The samples were placed in a nitrogen purged JASCO CD module. The temperature at the cuvette was controlled by means of a Peltier-type heating system (accuracy ±1 °C). For each measurement, the sample was allowed to equilibrate for 5 min at the adjusted temperature prior to acquisition. The spectra were obtained by averaging 10 scans at 10 °C intervals from 20 to 80 °C and were collected as ellipticity as a function of wavelength and converted to molar absorptivities via the equation:

$$\Delta\epsilon = \frac{\theta}{32980 \cdot l \cdot c}$$

where $\theta$ is the ellipticity in [mdeg], $l$ is the path length of the cuvette in [cm], and $c$ is the concentration in [M].

*NMR Spectroscopy*. The $^1$H NMR spectra of all dipeptides were measured with a Varian 300 MHz FT-NMR instrument, at room temperature. The peptides were dissolved in a 1:1 (v/v) $D_2O$:$H_2O$ solution at a concentration of 0.1 M. The mixture of the two was necessary for deuterating the peptide and obtaining a lock for proton NMR to obtain the $^3J_{H\alpha NH}$ coupling constant. The $D_2O$ contained 0.05 wt % of the internal NMR standard 3-(trimethylsilyl) propionic-2,2,3,3-$d_4$ acid (TSP), as a sodium salt. A total of 16 scans were measured and averaged, and the averaged spectra were converted to data files using the program MestRe-C.[27] The $^3J_{H\alpha NH}$ splitting peak was fit using the program MULTIFIT.[28]

*UV Absorption Spectroscopy*. Far-UV absorption spectra of AG and LG were measured with a JASCO J-810 spectrapolarimeter in a 0.1 mm quartz cell with 0.05 nm resolution. The peptides were dissolved in $H_2O$ at concentrations of 0.05 M. The samples were placed in a nitrogen purged JASCO CD module. The temperature of the sample cuvette was held constant at 20 °C, using a Peltier-type heating system (accuracy ±1 °C). A third channel was used along with the CD channel and the high tension voltage channel to record the absorption data. The respective solvent spectra were measured at exactly the same pH, ionic strength, and chloride concentration and subtracted from the absorption spectra.

## Results and Discussion

**Investigation of Dipeptides.** Figures 1 and 2 depict the temperature-dependent ECD spectra of nonaromatic XA and AX dipeptides in $D_2O$, respectively, measured as a function of temperature at acidic pD, where X represents any of the other 20 naturally occurring amino acids. With the exception of AP, all of these spectra show a clearly pronounced couplet with maxima around 216 nm and minima between 190 and 200 nm. Apparently, all XA spectra besides those of DA, RA, and MA exhibit an isodichroic point. Qualitatively, the displayed couplets are indicative of a significant PPII contribution, but the minimum at 195 nm appears significantly reduced as compared to that in a canonical PPII spectrum.[3,10] For the AX peptides, we did not observe an isodichroic point for AS, AV, AT, AI, and AQ, and concomitantly the corresponding 195 nm minimum appears particularly reduced. The only plausible explanation for this observation is that the classical PPII signal in the 190−220 nm region is overlapped by the wing of a strong positive ECD signal below 180 nm, which most likely results from a $\pi \rightarrow \pi^*$ transition of the C-terminal carbonyl group.[29] This notion is corroborated by our finding that the absorption difference spectra of several dipeptides obtained by subtracting the absorption spectrum measured at acidic pH from that observed at neutral pH are clearly indicative of a strong band below 180 nm assignable to the cationic peptide.[30] Apparently, its contribution is even more pronounced in the ECD spectra of most of the investigated AX peptides, thus creating a positive bias for the entire spectral region, which increases with decreasing wavelength. In what follows, we confine the thermodynamic

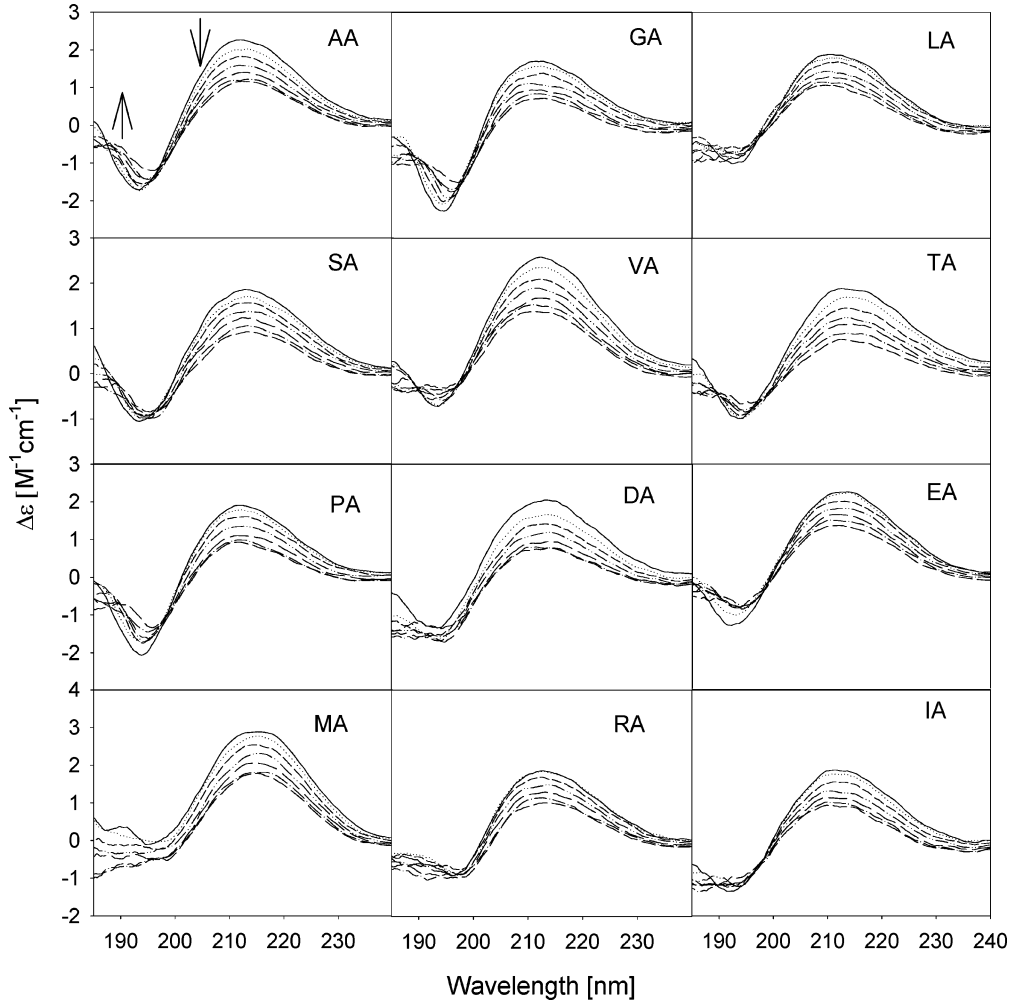


**Figure 1.** Temperature-dependent ECD spectra of a subset of the XA peptides measured between 20 and 80 °C, at intervals of 10 °C. The arrows shown for AA show increasing temperature and are similar for all dipeptide spectra shown.

analysis on those peptides whose spectra show a clearly discernible isodichroic point. Moreover, we only use the peak value of the maximum at 216 nm rather than the combination of the values at 195 and 216 nm earlier employed in our analysis of homo-tripeptides.[8]

We have also measured the $^1$H NMR spectra of the above-mentioned dipeptides to obtain the respective $^3J_{H\alpha NH}$ coupling constants. The NMR spectrum in the region of this proton splitting is depicted in Figure 3 for a few of the investigated dipeptides. The respective splitting values are listed in Tables 1 and 2. The values for XA peptides are in the range between 6.2 Hz (VA) and 6.7 Hz (DA). These are close to the value of 5.8 Hz, which when substituted in the Karplus equation[31] corresponds to $\phi = -75°$, the canonical PPII conformation. If one assumes only one conformation to be present, the Karplus equation yields $\phi$-values between either $-77°$ and $-80°$ or $-160°$ and $-163°$. However, the latter interval can be excluded based on the observed couplets in the ECD spectra, which is not consistent with an extended structure. The $^3J_{H\alpha NH}$ values for the AX peptides are generally higher, that is, between 7.1 Hz (AL) and 8.3 Hz (AT), which would correspond to $\phi$-ranges between $-82°$ and $-89°$ or $-151°$ and $-158°$. Again, the corresponding ECD spectra rule out the latter interval.

The temperature dependence of the ECD spectra indicates the coexistence of two (for those with an isodichroic point) or even more conformations. However, care has to be taken in interpreting the couplets that apparently overlap with the low-energy wing of the high-energy signal assignable to the C-terminal carbonyl group.[29,30,33] The spectra of AS, AT, AQ, DA, and, to a lesser extent, AV and RA suggest that the observed temperature dependence of the 216 nm peak results mostly from a variation of this overlap, which decreases with increasing temperature. The couplet assignable to the peptide's $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions appears nearly temperature independent.[32] This interpretation explains also the absence of an isodichroic point.

Figure 4 shows the ECD spectra of LG, KG, AG, PG, and GG measured at room temperature and acidic pD. These conditions have been chosen to eliminate contributions from the $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ charge-transfer transitions from the C-terminal carboxylate to the peptide group,[33] which would obfuscate the interpretation of the spectra. As expected, no CD spectrum was observed for diglycine. A small positive band appears in the spectrum of AG and PG ($\Delta\epsilon \leq 0.6$ M$^{-1}$ cm$^{-1}$) with their maxima at 209 and 212 nm, respectively. The CD spectra of KG and LG with maxima at 207 and 197 nm, respectively, are more pronounced, which leads us to conclude that the rotational strength correlates to some extent with the bulkiness of the N-terminal side chain. Our AG spectrum is different in magnitude and shape from the much more pronounced one recently reported by Gokce et al.[22] Because these authors measured this spectrum at neutral pH, a direct comparison is difficult because of the likely influence of the above-mentioned charge-transfer transitions.[30,33] We also measured the respective ECD spectra at neutral pH and found them to be identical to those reported by Gokce et al.[22]

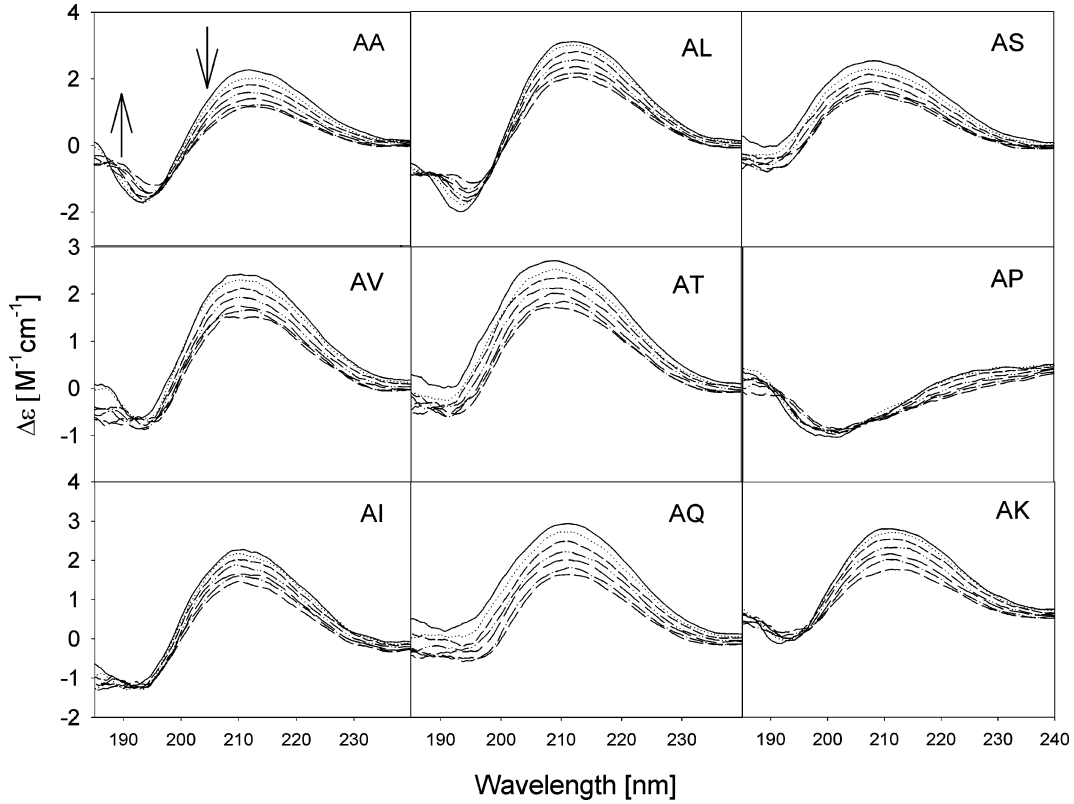


**Figure 2.** Temperature-dependent ECD spectra of a subset of the AX peptides measured between 20 and 80 °C, at intervals of 10 °C. The arrows shown for AA show increasing temperature and are similar for all dipeptide spectra shown.

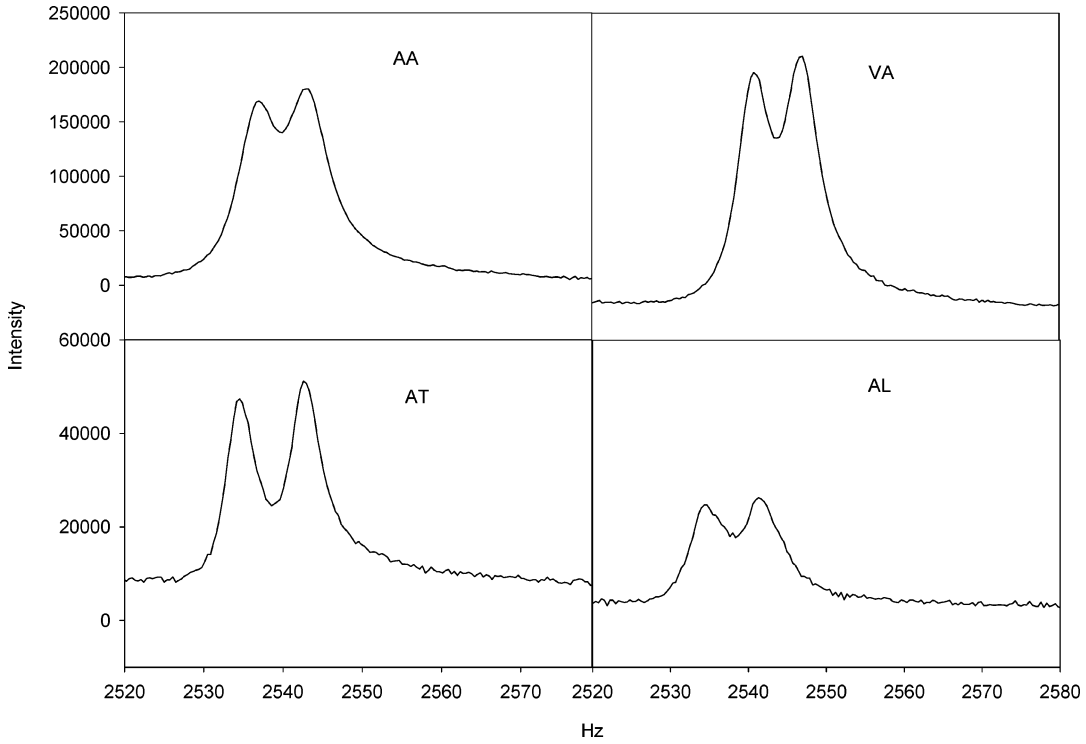


**Figure 3.** NMR spectra of the $^3J_{H\alpha NH}$ splitting for a representative set of the indicated dipeptides, measured at room temperature in a 50:50 mixture of $D_2O/H_2O$.

Before we proceed to the analysis of the temperature dependence of the ECD spectra, we first have to identify means to distinguish between contributions from the N- and C-terminal residues. The XG spectra show that the N-terminal residue alone can give rise to a measurable ECD spectrum for bulky side chains. Because the rotational strength of AG is weak, the contribution of alanine can generally be neglected for all ECD spectra of the AX peptides investigated. On the contrary, the pronounced spectrum of, for example, LG seems to suggest a substantial contribution of L to any dipeptide spectrum with this N-terminal residue. For LA, one would thus expect a substantial downshift of the maximum and the isodichroic point

**TABLE 1: Parameters Obtained from Fitting a Thermodynamic Model Described in the Results and Discussion to $\Delta\epsilon_{216}(T)$ of XA Dipeptides[a]**

| dipeptide | $\Delta\epsilon_{216}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{\beta}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{PPII}$ [$M^{-1}$ $cm^{-1}$] | $\Delta H$ [kJ/mol] | $\Delta S$ [J/mol*K] | $\chi_{PPII}$ at room temp | $^3J_{H\alpha N}$ [Hz] |
|---|---|---|---|---|---|---|---|
| AA | 2.3 | −4 | 5.9 | 6.8 ± 0.2 | 18.8 ± 0.7 | 0.64 | 6.46 |
| PA | 1.9 | −4.1 | 5.4 | 6.8 ± 0.3 | 18.5 ± 0.9 | 0.64 | 6.46 |
| GA | 1.6 | −4 | 5.4 | 5.9 ± 0.2 | 17.0 ± 0.7 | 0.59 | 6.63 |
| LA | 1.7 | −5 | 5.4 | 4.8 ± 0.1 | 11.2 ± 0.4 | 0.65 | 6.40 |
| SA | 1.9 | −4.5 | 5.9 | 5.3 ± 0.1 | 14.3 ± 0.3 | 0.61 | 6.56 |
| VA | 2.6 | −4.5 | 5.6 | 7.6 ± 0.2 | 18.8 ± 0.5 | 0.70 | 6.19 |
| IA | 1.9 | −5 | 5.5 | 5.8 ± 0.2 | 14.4 ± 0.6 | 0.65 | 6.38 |
| TA | 1.9 | −4.5 | 5.5 | 6.8 ± 0.1 | 18.4 ± 0.3 | 0.64 | 6.44 |
| RA | 1.7 | −5.1 | 5.4 | 4.9 ± 0.2 | 11.5 ± 0.7 | 0.65 | 6.41 |
| MA | 2.9 | | | | | 0.66 | 6.35 |
| EA | 2.2 | −4.5 | 5.8 | 5.8 ± 0.1 | 14.1 ± 0.6 | 0.66 | 6.37 |
| DA | 2.0 | | | | | 0.58 | 6.67 |

[a] Empty values in the table indicate a peptide with no discernible isodichroic point.

**TABLE 2: Parameters Obtained from Fitting a Thermodynamic Model Described in the Results and Discussion to $\Delta\epsilon_{216}(T)$ of AX Dipeptides[a]**

| dipeptide | $\Delta\epsilon_{216}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{\beta}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{PPII}$ [$M^{-1}$ $cm^{-1}$] | $\Delta H$ [kJ/mol] | $\Delta S$ [J/mol*K] | $\chi_{PPII}$ at room temp | $^3J_{H\alpha N}$ [Hz] |
|---|---|---|---|---|---|---|---|
| AA | 2.7 | −4 | 5.9 | 6.8 ± 0.2 | 18.8 ± 0.7 | 0.63 | 6.46 |
| AK | 2.6 | −2.5 | 8 | 6.8 ± 0.3 | 23.4 ± 0.9 | 0.48 | 7.06 |
| AL | 2.8 | −2.5 | 8.7 | 5.4 ± 0.2 | 19.0 ± 0.5 | 0.48 | 7.09 |
| AS | 2.3 | | | | | 0.36 | 7.57 |
| AV | 2.2 | | | | | 0.35 | 7.61 |
| AI | 2.3 | | | | | 0.39 | 7.45 |
| AT | 2.7 | | | | | 0.19 | 8.25 |
| AQ | 2.9 | 0.42 | 7.31 | | | | |

[a] Empty values in the table indicate a peptide with no discernible isodichroic point.

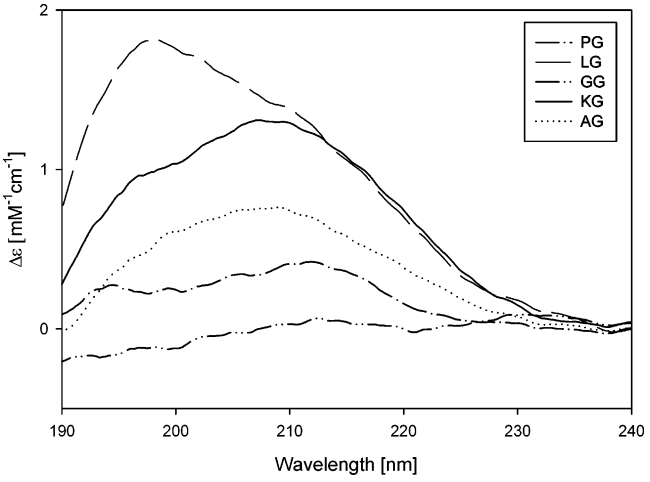


**Figure 4.** ECD spectra of XG peptides, measured at room temperature.

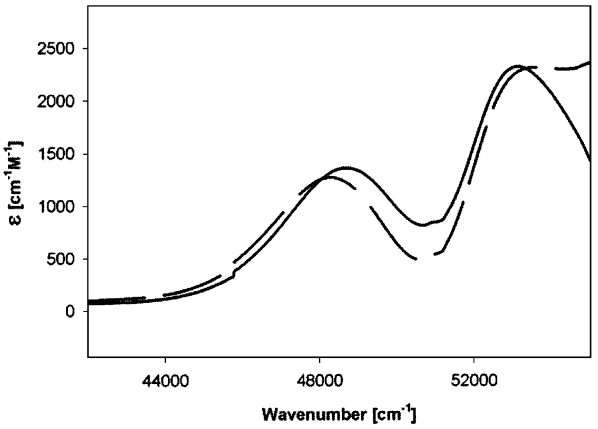


**Figure 5.** UV absorption difference spectra of AG and LG (spectrum at neutral pH − spectrum at acidic pH).

with respect to the AA spectrum. This, however, was not obtained, indicating that the influence of the N-terminal residue is marginal for all XA peptides. A similar argument can be made by comparing PG, PA, and AA. This leads to the question of how the different influence of the N-terminal residue in XG and XA can be rationalized. Woody and co-workers have invoked a direct electronic coupling between high lying $\sigma$-transitions of side chains and the $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions of the peptide group.[18,19] This approach implies that the contribution of the N-terminal residue to the rotational strength of the $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ of XG and XA should be comparable. Alternatively, one can invoke a nearest neighbor coupling between the N-terminal and the C-terminal residue, which yields a preferred orientation of the latter even if the C-terminal residue is G. In this case, the different spectra in Figure 1 would solely reflect different orientational distributions of the C-terminal residue, which would of course alter the electronic coupling between peptide and carbonyl transitions and thus the ECD spectrum. We checked this hypothesis by measuring UV absorption spectra of LG and AG at acidic and neutral pH and subtracted the spectrum at acidic conditions from that at neutral conditions for both peptides. Figure 5 exhibits the UV absorption difference spectra (spectrum at neutral conditions minus spectrum at acidic conditions) to display bands that are assignable to $COO^-\rightarrow$peptide charge-transfer transitions at 49 000 $cm^{-1}$ (204 nm) and at 53 000 $cm^{-1}$ [188.7 nm].[30,33] Differences between the first and second moments of the two bands are apparent. Therefore, electronic absorption data indicate that the N-terminal residue of XG affects the orientation of the carboxylate group. This is consistent with the notion that the ECD spectra of XG peptides result from electronic coupling between the peptide and the C-terminal carbonyl groups and thus reflect the orientation of the carboxylate group. On the basis of this

**TABLE 3: Parameters Obtained from Fitting a Thermodynamic Model Described in the Results and Discussion to $\Delta\epsilon_{216}(T)$ of AA, AAA, and AAAA**

| peptide | $\Delta\epsilon_{216}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{\beta}$ [$M^{-1}$ $cm^{-1}$] | $\Delta\epsilon_{PPII}$ [$M^{-1}$ $cm^{-1}$] | $\Delta H$ [kJ/mol] | $\Delta S$ [J/mol*K] | $\chi_{PPII}$ at 300 K |
|---|---|---|---|---|---|---|
| AA | 2.3 | −4 | 5.9 | 6.8 ± 0.2 | 18.8 ± 0.7 | 0.635 |
| AAA | 2.8 | −5 | 5 | 5.1 ± 0.3 | 13.0 ± 1.0 | 0.64 |
| AAAA | 8.8 | −5 | 5 | 10.2 ± 0.1 | 26.0 ± 0.4 | 0.795 |

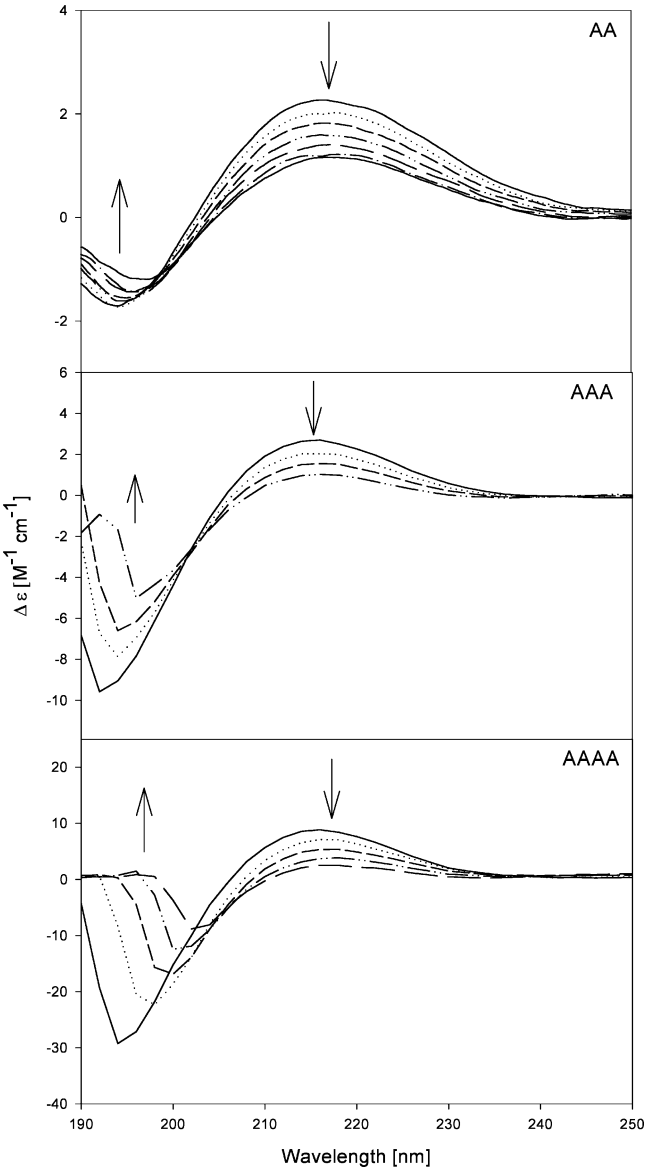


**Figure 6.** Temperature-dependent ECD spectra of dialanine (AA), trialanine (AAA), and tetraalanine (AAAA). The spectra of AAA and AAAA were taken from refs 15 and 16, respectively. The arrows show increasing temperature.

conclusion, our analysis of the AX and XA peptides focuses on determining the conformation of the C-terminal residue.

In a first step, we analyzed the temperature dependence of those dipeptide spectra that depict an isodichroic point. To reduce the number of unknown parameters, we adopted the model of Chen et al., where we identified the two coexisting conformations as PPII ($\phi = -75°$,$^3J_{H\alpha NH} = J_{PPII} = 5.5$ Hz) and an extended $\beta$-strand ($\phi = -145°$,$^3J_{H\alpha NH} = J_{\beta} = 9$ Hz).[34] This model must be considered as minimal in that it does not take into account a possible population of α-helical conformations as predicted by multiple MD simulations.[35−37] Moreover, it does not consider that a conformation like PPII generally represents a group of structures with high similarity and identical macroscopic properties, which are assignable to the trough in the Ramachandran plot.[38] Our thermodynamic analysis, however, is applied solely to the peptides for which we obtained an isodichroic point, for which a homogeneous two-state model is at least a reasonable approximation. For the remaining peptides, we used the model of Chen et al.[34] solely to estimate the PPII/$\beta$-strand population at room temperature. With respect to the obtained numbers, we would put an emphasis on their relative values used for comparing different residues rather than on their absolute values.

We calculate the respective Gibbs energy between the two states, $\Delta G = G_{\beta} - G_{PPII}$, at room temperature by utilizing the equation:

$$\Delta G = -RT \ln[(J_r - J_{PPII})/(J_{\beta} - J_r)] \quad (1)$$

with the experimentally obtained coupling constants denoted as $J_r$ ($R$, gas constant; $T$, absolute temperature). Next, we calculated $\Delta\epsilon$ at 216 nm by employing:

$$\Delta\epsilon_{216} = \frac{\Delta\epsilon_P + \Delta\epsilon_{\beta} e^{-\Delta G/RT}}{Z(T)} \quad (2)$$

where $\Delta\epsilon_{\beta}$ and $\Delta\epsilon_{PPII}$ are intrinsic $\Delta\epsilon$ values assignable to the $\beta_{strand}$ and PPII conformations, respectively. The partition sum $Z(T)$ is written as:

$$Z = 1 + e^{-\Delta G/RT} \quad (3)$$

We inserted the $\Delta G$ values obtained from eq 1 and estimated the $\Delta\epsilon_{\beta}$ and $\Delta\epsilon_{PPII}$ values by fitting eq 2 globally to the $\Delta\epsilon_{216}$ value of all XA and AX peptides that exhibit an isodichroic point. Next, we used them as fixed parameters while fitting eq 2 to the remaining $\Delta\epsilon(T)$ values to obtain $\Delta G(T)$. Finally, we plotted $\Delta G$ as a function of temperature to obtain $\Delta H$ and $\Delta S$. We allowed only a very limited variation of 2.0 $M^{-1}$ $cm^{-1}$ for $\Delta\epsilon_{\beta}$ and $\Delta\epsilon_{PPII}$, to reduce the uncertainty of these parameters. While this procedure yielded a consistent fitting of the $\Delta\epsilon_{216}(T)$ data, the thus obtained negative $\Delta\epsilon_{\beta}$ values for the XA peptides were too large (between −12 and −14 $M^{-1}$ $cm^{-1}$) as compared to the recently obtained values for tri- and tetraalanine.[15,16] This prompted us to modify our analysis in that we assumed a slightly "distorted" PPII conformation with $^3J_{H\alpha NH} = 5.0$ Hz corresponding to $\phi = -70°$ to obtain the mole fractions and parameters listed in Tables 1 and 2.

For the XA dipeptide series, the $^3J_{H\alpha NH}$ coupling values suggest very similar mixings of PPII and $\beta$ at room temperature. We calculated the average value for the XA samples to be 6.44 ± 0.12 Hz. All peptides exhibiting $^3J_{H\alpha NH}$ values within the ±0.12 Hz interval can be considered as identical with respect to their conformational mixture. This subset includes AA, PA, LA, SA, IA, TA, RA, MA, and EA. Only GA (6.63 Hz), VA (6.19 Hz), and DA (6.67 Hz) differ significantly from the average value. This indicates that the structural propensity of the C-terminal alanine can be context dependent. The somewhat higher $^3J_{H\alpha NH}$ value of GA is interesting, because this dipeptide can certainly be considered as a reference system for the absence of any residue−residue interaction. Our data therefore suggest

that besides D, all X-residues investigated have some influence on the C-terminal residue in XA dipeptides, by shifting its equilibrium toward PPII. The underlying interaction seems to be mostly independent of the choice of X. Interestingly, only valine, which has been shown to exhibit a high propensity for an extended $\beta$-conformation,[15,21,26] causes an above-average stabilization of the PPII conformation of its neighbor, by reducing its Gibbs energy by another 0.5 kJ/mol (at room temperature), which mostly results from an enthalpic contribution to the Gibbs energy (Table 1). This observation is in qualitative agreement with recent DFT calculations on Ac−VA−NHMe in vacuo, which indicate a significant influence of valine on the alanine conformation.[39] The $\Delta\epsilon_\beta$ and $\Delta\epsilon_{PPII}$ of the XA peptides are rather consistent and in good agreement with what one would expect from earlier ECD experiments on AAA[15] and AAAA.[16]

On a first view, our results seem to differ somewhat from what Chen et al. have recently obtained for the PPII propensity of alanine in a series of $AcGGXAXGGNH_2$ peptides by NMR and ECD measurements.[34] They found that X = I caused a higher $^3J_{H\alpha NH}$ value for the alanine residue than X = A. This difference is modest at room temperature, but becomes significant at high temperatures. However, these changes occur only if both X residues flanking A are isoleucine, whereas IAA appears just indistinguishable from AAA. This is in perfect agreement with our results on AA and IA. Generally, the $^3J_{H\alpha NH}$ values obtained for the central alanine residue in the above peptide are substantially lower than those obtained for our XA peptides, indicating a higher PPII fraction for the latter.

The $^3J_{H\alpha NH}$ values of the AX peptides are considerably and systematically larger than those obtained for the XA series, indicating different $\phi$-angles or different mixtures of PPII and $\beta$-strand. Unfortunately, only the spectra of AK and AL showed an isodichroic point and could therefore be subjected to a thermodynamic analysis. Additionally, we used the above-introduced modified two-state model to infer the PPII fraction from the respective $^3J_{H\alpha NH}$ coupling constants. Apparently, only K, L, and Q exhibit some PPII propensity, although it is substantially less than that of A. The remaining residues investigated (i.e. S, V, I, and T) exhibit a clear tendency toward a $\beta$-strand conformation. This is particularly the case for threonine (T), a residue that has not yet been investigated in unfolded state experiments.

**Comparison of AA, AAA, and AAAA.** Figure 6 compares the temperature-dependent ECD spectrum of AA with the recently reported spectra of AAA[15,26] and AAAA.[16] It should be noted that while the $\Delta\epsilon$ values of the temperature-dependent ECD spectra of di-, tri-, and tetraalanine were originally reported in units of [$mM^{-1}$ $cm^{-1}$], the $\Delta\epsilon$ are, in fact, in units of [$M^{-1}$ $cm^{-1}$], as reported herein. All spectra have in common that they are indicative of a substantial PPII fraction, which decreases with increasing temperatures. All spectra exhibit a clear isodichroic point, with the exception of the AAAA spectrum measured at 0 °C, which appears somewhat blue-shifted.[16] Interestingly, the 196 nm minimum of AAA and AAAA is much more pronounced than that of AA, which is indicative of reduced influence of the overlap with the band assignable to the C-terminal $\pi\rightarrow\pi^*$ transition.

In earlier studies, we employed a simple "all or nothing model" as expressed by eq 1 to describe the temperature dependence of ECD spectra.[16,26] In principle, this is an oversimplification, which does not take into account the presence of mixed conformations in a peptide chain. In the case of a tripeptide, for instance, the sequences $\beta\beta\beta$, $\beta\beta$p, $\beta$pp, and ppp ($\beta$, $\beta$-strand; p, PPII) coexist. Each of these sequences can be expected to contribute differently to the measured ECD signal. Because many of the tripeptide ECD spectra depict an isodichroic point with respect to their temperature dependence, it is likely that the spectra of the mixed sequences can still be approximated by a superposition of PPII and $\beta$-strand basis spectra. We therefore made the simplifying assumption that

$$\Delta\epsilon = \sum_{i=1}^{N}\Delta\epsilon_i \tag{4}$$

where $\Delta\epsilon_i$ is the dichroism of the $i$th residue in a peptide with N-amino acid residues. The summation runs from the N- to the C-terminal residue. This leads to the following equation for $\Delta\epsilon(T)$ for AAA:

$$\Delta\epsilon_{A_3}(T) = \frac{2\Delta\epsilon_p + (\Delta\epsilon_p + \Delta\epsilon_\beta)e^{-\Delta G/RT} + 2\Delta\epsilon_\beta e^{-2\Delta G/RT}}{Z_{A_3}} \tag{5}$$

with

$$Z_{A_3} = 1 + 2e^{-\Delta G/RT} + e^{-2\Delta G/RT} \tag{6}$$

and for tetraalanine (AAAA)

$$\Delta\epsilon_{A_4}(T) = [3\Delta\epsilon_p + (2\Delta\epsilon_p + \Delta\epsilon_\beta)e^{-\Delta G/RT} + (\Delta\epsilon_p + 2\Delta\epsilon_\beta)\Delta\epsilon_\beta e^{-2\Delta G/RT} + 3\Delta\epsilon_\beta e^{-3\Delta G/RT}]/Z_{A_4} \tag{7}$$

with

$$Z_{A_4} = 1 + 3e^{-\Delta G/RT} + 3e^{-2\Delta G/RT} + e^{-3\Delta G/RT} \tag{8}$$

where we assumed that the N-terminal residue does not contribute to the ECD signal and that $\Delta\epsilon_p$ and $\Delta\epsilon_\beta$ are not position-dependent. We used the $\Delta\epsilon$-values obtained from the analysis of AA and could reproduce the temperature dependence of $\Delta\epsilon_{216}$ by using solely $\Delta H$ and $\Delta S$ as free parameters. Thus, we found that the PPII fractions of AA and AAA at room temperature are practically identical, even though the respective thermodynamic parameters are different. With respect to enthalpy, AAA is less stabilized than AA, but this is compensated by a reduced entropic stabilization of the $\beta$-strand conformation. Data for di-, tri-, and tetraalanine are presented in Table 3. In agreement with earlier results, we obtained that PPII is significantly stabilized in AAAA, which results from a large enthalpic contribution.[16] This seems to be supportive of the MD calculations of Garcia, which indicated a PPII stabilization for tetraalanine because of an optimal organization of the hydration shell.[40] That PPII is generally favored by enthalpic contributions has recently been reported by Hamburger et al.[41]

## Conclusions and Comparison with Literature

Electronic CD spectroscopy has become a very prominent tool not only for the identification of classical secondary structures such as α-helices and $\beta$-sheets, but also of the PPII conformation in so-called random coil conformations of peptides and proteins.[1−4] While Woody and his associates clearly showed that the asymmetric couplet with the strong minima at 195 nm is diagnostic of PPII,[2,3] the Creamer group started an effort to identify the individual propensities of amino acid residues, a goal similar to that addressed in the current paper.[42,43] The authors focused on proline containing peptides into which they

inserted a short nonproline sequence to study their propensity in a PPII preferring context. Thus, they obtained comparatively high propensities for A, Q, and L, and low ones for V and I. A comparison of our data with theirs suggests that these differences are somewhat more pronounced in a nonproline context, which is expected. Our recent investigation of AXA peptides by vibrational spectroscopies suggests comparable PPII propensities for A and L, while S is more inclined toward the $\beta$-strand.[21] That ionized lysine has significant PPII propensity follows from the work on some polylysine peptides.[44] The current study shows that the earlier obtained propensities generally apply also to C-terminal residues, even though their context is somewhat different from a nonterminal residue, which is likely to be responsible for the somewhat lower PPII propensity of lysine. Moreover, our study adds threonine to, and confirms serine as a member of, the list of investigated amino acids, suggesting that it heavily favors the extended $\beta$-strand conformation. Generally, it can be concluded that residues with bulky, $\beta$-branched side chains exhibit a clear tendency toward the $\beta$-strand conformation. That tendency is clearly reflected by the higher $\beta$-strand propensity of T as compared to S, in that the former can be considered as an S with one of its $\beta$-protons substituted by a methyl group. We agree with the notion of Chellgren and Creamer, which suggests that bulky side chains prevent an optimal backbone solvation by water, thus reducing the PPII propensity.[43] Our results are somewhat at variance with predictions from recent calculations of Tran et al.[45] In agreement with our result, they also obtained very low PPII propensities for V and I, but they found substantial sampling of $\alpha_R$ and a distorted PPII like conformation (called $P_{hyp}$) with $\psi$ angles between 100° and 140° and only very limited sampling of the $\beta$-strand region. The low PPII and high $\beta$-propensity of S and T are not reproduced at all by their calculations.

The negligible contribution of the N-terminal residue to the ECD spectrum, as inferred from the experiments on XG peptides, suggests that a residue alone (comprising the side chain and the next peptide unit) does not produce any signal in the region between 190 and 200 nm. This suggests that dipole coupling between two adjacent units with $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions is a prerequisite for a signal to occur and that side chains can modulate, although not create, an ECD signal. These experimental results should guide further modeling of ECD spectra of peptides.

The $^3J_{H\alpha NH}$ values obtained in this study differ considerably from those of Bundi and Wüthrich reported for various residues in GGXA.[46] Generally, their values are considerably lower than ours (with the exception of A!), which indicates either different $\phi$-angles for the involved conformations or a higher PPII propensity in the context of their tetrapeptide. The latter possibility is more likely because the energy landscape of a distinct residue can be expected to be context-independent in the absence of nonlocal interactions.

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