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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY · MAY 1974

Impact Factor: 2.78 · DOI: 10.1021/j100604a016

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Conformation of N-Acetyl-L-alanine-N'-methylamide in 1,2-Dichloroethane by Circular Dichroism and Optical Rotatory Dispersion

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Publication costs assisted by the National Institutes of Health

The conformations of *N*-acetyl-L-alanine-*N'*-methylamide in 1,2-dichloroethane have been studied by infrared spectroscopy, optical rotatory dispersion, and circular dichroism as a function of temperature. The results were analyzed in terms of an equilibrium mixture of two conformations: a high-temperature non-hydrogen-bonded form and a low-temperature *intra*molecularly hydrogen-bonded form. By examining the optical activity at a number of temperatures for a number of wavelengths, the $\Delta H°$ for the conformational transition from low T form to high T form was found to be 2570 ± 5 cal/mol and $\Delta S°$ = 6.56 ± 0.01 eu. Although it was experimentally impossible to shift the equilibrium to predominantly one form or the other, it was possible to calculate the approximate ORD and CD of each pure form.

Optical rotatory dispersion (ORD) and circular dichroism (CD) are regularly used methods for observing conformational change and estimating helix content in polypeptides and proteins. Unfortunately, these methods are also sensitive to changes in the solvent and to chemical modification, whether or not these alterations give rise to significant conformational changes. Thus it has been difficult to establish the ORD and CD of different known conformations of the *same* compound under *very similar* conditions in order to unambiguously determine the dependence of the optical activity on conformation alone. For this purpose, *N*-acetyl-L-alanine-*N'*-methylamide ($H_3CCONHCH(CH_3)CONHCH_3$; hereafter referred to as AANMA) was chosen as a very simple, yet optically active, model compound simulating to some extent the behavior of an alanyl residue located in the middle of a polypeptide chain. Similar compounds have been investigated in the past by means of proton magnetic resonance spectroscopy and infrared-absorption spectroscopy[1] and by theoretical energy calculations.[2] Both the experimental and theoretical work showed that in dilute solution with a nonpolar solvent, the favored conformation(s) are those having an *intra*-molecularly hydrogen-bonded seven-membered ring formed by a hydrogen bond in, *e.g.*, AANMA between the acetyl carbonyl and the methyl acetamide NH. In this paper we will first establish that in the case of AANMA in 1,2-dichloroethane (DCE) solution, a significant fraction of the molecules are in such an intra-molecularly hydrogen-bonded cyclic conformation, as was found by Bystrov, *et al.*,[1] for similar compounds in carbon tetrachloride and chloroform. We will then show by examining the infrared (ir) spectrum that the temperature dependence of the ir, ORD, and CD of this solution is due to shifting the equilibrium between the H-bonded form(s) and the non-H-bonded conformations, which are taken together to constitite a non-H-bonded "form." From the temperature dependence of the ORD, $\Delta H°$ and $\Delta S°$ for the conformational transition are determined by fitting a two-state equilibrium model to the experimental data. Furthermore, even though experimental conditions do not permit the equilibrium to be almost completely shifted to one or the other form, the ORD and CD spectra of each pure form can be calculated.

Experimental Section

Materials. Two preparations of AANMA were used: one synthesized in this laboratory and the other purchased as a custom synthesis order from Miles-Yeda Ltd. Since to our knowledge no method of synthesis of this compound has been published, we feel we should describe in some detail our route which is based on the synthesis of *N*-acetyl-L-valine-*N'*-methylamide by Mizushima, *et al.*[3] L-Alanine (Eastman) was converted to acetyl-L-alanine by dropwise addition of acetic anhydride to a constantly stirred solution of L-alanine in 2 *N* aqueous sodium hydroxide, which was kept below 5° at all times. With the temperature maintained between 0 and 5°, the reaction mixture was then acidified with HCl to a strong Congo Blue pH. The acidified solution was evaporated to dryness under vacuum at temperatures between 20 and 30°, and the resulting residue was twice extracted with ethanol by stirring and refluxing for 0.5 hr. Crude acetyl-L-alanine was crystallized from the ethanol solution by addition of a large excess of dry ether and refrigerating overnight. Acetyl-L-alanine is apparently hygroscopic. This was then converted to acetyl-L-alanine methyl ester by adding an ethereal solution of diazomethane dropwise with constant stirring to a *p*-dioxane solution of acetyl-L-alanine. The reaction was apparently complete at the end of the 2-hr addition period. After evaporation of the solvent the remaining yellow viscous oil was extracted with ether to yield the crude methyl ester as both crystals and an oil. All the methyl ester was dissolved in a minimum of methanol and was added to a methanol solution of methylamine. The reaction of the methyl ester to form the *N*-methylamide seemed to be complete after standing for 5 days at room temperature. The methanol was removed and an ethanol-insoluble fraction of the resulting oil was discarded. The oil was further purified by repeated trituration with ethyl acetate and finally dissolved in a 40% ethanol-60% ethylacetate mixture from which the AANMA precipitated suddenly during evaporation under vacuum at room temperature. Recrystallization in ethanol–ethylacetate mixtures gave small white crystals of AANMA, mp 176° (very sharp). The elemental analysis of these crystals for C, H, and N gave percentages that agreed with the calculated values to ±0.2%.

By way of comparison, the AANMA synthesized by Miles-Yeda had a melting range of 186–187°, and the elemental analysis agreed with calculated percentages to ±0.1%. Perhaps a different solvent was used for the final recrystallization of the Miles-Yeda AANMA, which might account for the difference in melting points. The Miles-Yeda batch $[\alpha]^{16}D = -57.0$ for 2% solution, while ours had $[\alpha]^{25}D = -51.2$ in water solution, which we take as evidence that the synthesis did not significantly racemize the L-alanine.

Most of the spectral work (on AANMA) was done with the one Miles-Yeda synthesized lot dissolved in DCE (spectroquality, Matheson Coleman and Bell) from the same bottle.

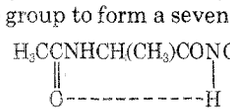
Methods. Infrared spectra were measured with a Beckman IR-9 spectrophotometer, ORD with a Cary 60 spectropolarimeter, and CD with a Durrum-Jasco J-10 spectropolarimeter. Since DCE tends to absorb a few per cent of water upon exposure to the atmosphere, all DCE to be used was first dried by letting it stand over P_2O_5 for a few days. However, because AANMA precipitates from DCE solutions in the presence of desiccant, such as silica gel or P_2O_5, the solutions were prepared with dried, filtered DCE, and then stored in a desiccator containing P_2O_5. It will be shown in the next section how crucial traces of water can be.

Infrared difference spectra were taken using a pair of 0.5-mm path length calcium fluoride cells, which are transparent in the region of interest. Optical activity measurements were made with 1- or 0.5-mm path length quartz cells. Temperature control was effected by circulating thermostatically controlled water baths surrounding the sample cells. For the ir measurements, the cell containing the AANMA solution was held at the desired temperature, but the cell in the reference beam containing pure DCE was always at the same temperature, about 30°.

Computations were performed using Fortran on the CDC 6400 computer at the University of California, Berkeley. Ir spectra were resolved into gaussian bands by means of an analog du Pont 310 curve resolver so that areas of overlapping bands could be compared.

Results

The ir spectrum of AANMA in DCE at a concentration of 4 g/l. with pure DCE as reference shows two partially overlapping peaks at 3400 and 3300 cm^{-1}. On the basis of the work of many other authors, *e.g.*, Bystrov, *et al.*,[1] these are assigned to the NH stretching mode, nonhydrogen bonded, and hydrogen bonded, respectively. The relative areas of the two peaks were unchanged by two-, four-, and eightfold dilutions, which was taken as proof that the hydrogen bonding was *intra*molecular, not intermolecular. Conformational calculations on AANMA[2] and experiments on similar compounds[1] show that intramolecular hydrogen bonding takes place only between the oxygen of the acetyl carbonyl group and the hydrogen of the NH of the methylamide group to form a seven-membered ring



There are two conformations of AANMA in which this can take place, corresponding to different puckerings of the ring, but this study does not attempt to distinguish which (if either) is preferred.

The relative areas of the two ir bands are a function of temperature. The experimentally practical upper limit for temperature is 60° because DCE boils at 83°, and the lower limit for the 4 g/l. AANMA solution is 10° because the AANMA crystallizes out below that point. At 54°, the ratio of the area of the 3400-cm^{-1} peak to that of the 3300-cm^{-1} peak is 64:34, while at 9° it is 47:52. However, if the solution contains a few per cent water, as indicated by significant peaks at 3670 and 3580 cm^{-1} in the difference ir spectrum, then the ratio at 54° is 78:20, and at 9° it is 70:30. In other words, the ir of a wet solution is essentially independent of temperature, whereas in a dry solution the area of the 3400-cm^{-1} peak (non-H-bonded) increases with rising temperature at the expense of the 3300-cm^{-1} peak (H bonded). At no experimentally accessible temperature does one peak disappear, so the molar extinction coefficients of the H-bonded and non-H-bonded NH bands cannot be unambiguously determined. Consequently, it is impossible to determine anything quantitative about this conformational change from the ir evidence alone. However, a qualitative picture does emerge: namely, that of an equilibrium between a low-temperature "form" A of AANMA consisting of all intramolecularly hydrogen bonding conformations and a high-temperature "form" B consisting of an average over all other conformations. Thus at any temperature, T, we can consider the equilibrium

$$A \rightleftharpoons B \tag{1}$$

to have equilibrium constant K, where

$$K = [B]/[A] = e^{-\Delta G^\circ/RT} \tag{2}$$

where $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ is the Gibb's free energy for the reaction 1. Then

$$[A] = C/(1 + e^{-\Delta G^\circ/RT})$$

and

$$[B] = Ce^{-\Delta G^\circ/RT}/(1 + e^{-\Delta G^\circ/RT}) \tag{3}$$

where $C = [A] + [B]$ = total concentration of AANMA. We have assumed the activity coefficients to be unity, that the experimental situation is simply a two-state equilibrium, and that the ΔH° and ΔS° of the reaction are independent of temperature over the range being considered.

As might be expected, the ORD (Figure 1) and CD (Figure 2) spectra also vary with temperature for a dry solution. On the other hand, while the small pertubation of solvent entailed by the addition of a few per cent of water does not affect the ORD spectrum (±2%) at 50°, yet the temperature dependence of the ORD is completely eliminated (±1%). This is to say that the spectrum of a wet solution remains at all temperatures the same as the spectrum of a dry solution at high temperatures. Consequently, it is not necessary to correct the data for thermal expansion of the solvent. Neither is it likely that the dependence of ORD on temperature for the dry solution is due to solvation changes leading to perturbations of the chromophores.

In general, the ORD above 300 nm is monotonic, negative, and weak. Above 240 nm there is little temperature dependence, and below 214 nm the DCE solvent absorbs too strongly. For wavelengths λ between 214 and 240 nm, the optical rotation was noted at 2-nm intervals at T = 53, 43, 33, 23, and 14°.[4] For any λ and T, the optical rotation α was assumed to be the linear superposition of the rotations of the two forms α_A and α_B

$$\alpha(\lambda, T) = [A](T)\alpha_A(\lambda) + [B](T)\alpha_B(\lambda) \tag{4}$$

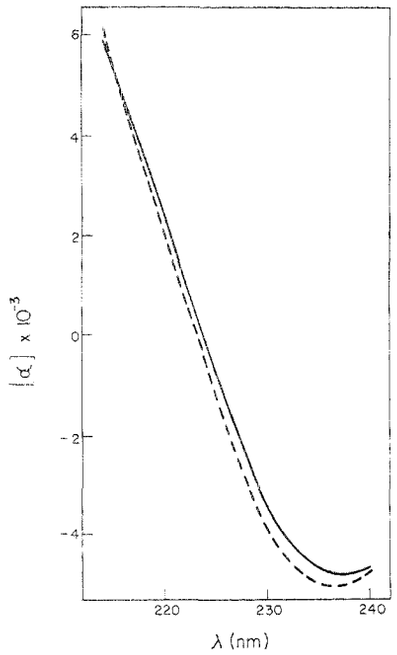


Figure 1. The specific rotation $[\alpha]$ of AANMA in DCE as a function of wavelength λ: 53° (——); 14° (- - - - -).

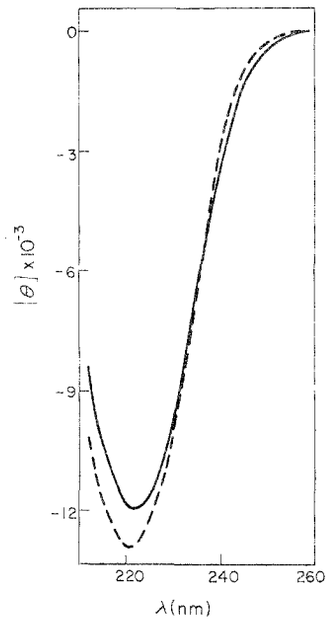


Figure 2. The molar ellipticity $[\theta]$ of AANMA in DCE as a function of wavelength λ: 51° (——); 14° (- - - - -).

where [A](T) and [B](T) are given by eq 3. Then the ORD data, $\alpha(\lambda T)$, for all wavelengths and temperatures were fit to the two-state model by adjusting $\Delta H°$, $\Delta S°$, $\alpha_A(\lambda_i)$, and $\alpha_B(\lambda_i)$ for all $214 \leq \lambda_i \leq 240$ nm by minimizing

$$F(\Delta H°, \Delta S°, \alpha_A, \alpha_B) = \sum_{j=1}^{n_T} \sum_{i=1}^{n_\lambda} \left| [\alpha(\lambda_i, T_1) - \alpha(\lambda_i, T_{n_T})] \times \left\{ \frac{[A]\alpha_A(\lambda_i) + [B]\alpha_B(\lambda_i) - \alpha(\lambda_i, T_j)}{|\alpha(\lambda_i, T_j)| + 0.00001} \right\} \right| \quad (5)$$

The expression in the curly brackets is the per cent deviation of the calculated spectrum from the experimental, while the expression in the square brackets weights the data for each wavelength according to how much the optical rotation changes at that wavelength over the whole temperature range (T_1 = lowest temperature, and T_{nT} = highest temperature). The constant 0.00001 in the denominator is merely to avoid dividing by zero even if $\alpha(\lambda_i, T_j) = 0$.

The function F was minimized with respect to $\Delta H°$, $\Delta S°$, $\alpha_A(\lambda_i)$, and $\alpha_B(\lambda_i)$, $i = 1, \ldots, n_\lambda$ by starting at some initial value for all variables and sequentially varying each individually in the stated order until F was minimized with respect to the one variable, and then proceeding on to the next variable. This whole procedure was iterated some 50 times until F was minimized simultaneously with respect to all variables. Changing one variable was done in discrete steps with an arbitrary but small step size chosen in advance. The final minimum in F was refined with smaller step sizes. The above minimization procedure is extremely crude and slow but also extremely reliable, even in this unstable case where some of the variables, such as $\Delta H°$ and $\Delta S°$, are in exponents. More elegant minimization algorithms might have been more time consuming in the long run.

The result of the fit was that $F = 0.02$ when $\Delta H° = 2570$ cal/mol (at step size of 5 cal/mol) and $\Delta S° = 6.56$ cal/mol deg (step size = 0.01). This value of F corresponds to roughly an average fractional deviation of the calculated α from the experimental α of 9%. The calculated values of $\alpha_A(\lambda)$ and especially $\alpha_B(\lambda)$ were reasonable but very sensitive to experimental error since, for example, [B] = 33% at T = 53° and [B] = 23% at T = 14°, while α(236 nm, T) changed about 4%. In other words, small shifts in the relative concentrations of the two forms were being called upon to explain shifts in ORD which were subject to substantial error. To summarize the calculated spectra, $[\alpha_A]_\lambda$ had a minimum value of −5320 at λ 235 nm and $[\alpha_B]_\lambda$ had a minimum of −4580 at 238 nm, whereas the experimental minimum specific rotation at 53° was −4830 at 237 nm. The "melting temperature," the temperature at which $\Delta G° = 0$, is 119°, which is considerably higher than the boiling point of the solvent.

Using the foregoing equations and the same $\Delta H°$ and $\Delta S°$, it is possible to calculate the CD of the two forms from the CD spectra at only two temperatures, 51.5 and 14°. Here the region of interest is from the absorption cutoff at 212 nm to the tail of the trough at 260 nm. At 51.5°, the molar ellipticity, $[\theta]$, has a single minimum of −12,000 at 222 nm, while for form A the minimum is −15,000 at 220 nm and for B it is −10,000 at 226 nm.

Discussion

The simple two-state model proposed here may well be an oversimplification, but the data from the ORD experiments are certainly not precise enough to justify a more complicated model, even though the experimental precision was better than 1%. The thermodynamic parameters calculated are of the same order as those found in similar studies (*e.g.*, $\Delta H° = 3100$ cal/mol and $\Delta S° = 9.8$ eu[1]), and the calculated specific rotations and molar ellipticities of the two forms are reasonable in that they have about the same magnitudes and shapes as the experimental curves (which are themselves typical for small peptides). In addition, the calculated curves are the same as the experimental ones at wavelengths where the optical activity is independent of temperature. As often occurs, the CD was more sensitive to the conformational change than the ORD. The temperature dependence of the ORD and CD

must be due strictly to a conformational change, first because of the shifts in area of the ir bands at 3300 and 3400 cm^{-1} with temperature, and second because the addition of a few per cent water gives a spectrum which is the same as that of the high temperature dry solution (±2%), yet the wet solution shows no temperature dependence. This at least clearly rules out the possibility of a spectrum change due directly to temperature. It also illustrates how important interaction with the solvent can be in determining the conformation. Apparently equimolar amounts of AANMA and water in DCE preferentially associate with each other in such a way as to make intramolecularly hydrogen-bonded conformations unfavorable. Considering the rather small differences in the ORD and CD spectra of the two forms, it would seem unlikely that one could detect such hydrogen-bonded rings in a protein or polypeptide when only a few per cent of the residues are in that conformation. Apparently, in general, optical rotatory methods are much more sensitive to the interaction of large numbers of peptide chromophores, as in the case of a helix–coil transition, than to the interaction of only two chromophores, as in this study.

Acknowledgments. We wish to thank Professor I. D. Kuntz for the use of the Beckman IR-9 and related infrared equipment and Dr. H. Yamamoto for his aid in the synthesis of AANMA. This work was supported by NIH Research Grant No. GM-10880 from the National Institute of General Medical Sciences and No. HL-06285 from the National Heart and Lung Institute.

Supplementary Material Available. A listing of $[\alpha]_\lambda$ at these wavelengths and temperatures will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy of $2.00 for microfiche, referring to code number JPC-74-1127.

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(4) See paragraph at end of text regarding supplementary material.

Flash Photolysis of Aqueous Solutions of Cysteine

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Publication costs assisted by the National Research Council of Canada

Cysteinyl radicals (CyS·) produced by flash photolysis of dilute aqueous solutions of cysteine at pH >6.5 form the cystine radical anion ($CySSCy^-$). At low concentrations ($<6 \times 10^{-7}$ *M*) this transient decays by a first-order reaction ($k = 2 \times 10^{-3}$ sec^{-1}) independent of pH and cysteine concentration and not involving dissociation to CyS· and CyS^-. At higher concentrations the transient decays by the well-studied second-order process which is pH and cysteine concentration dependent. The yield of $CySSCy^-$ is reduced by the addition of acrylic acid or allyl alcohol but the subsequent first-order decay is little affected. A new reaction between $CySSCy^-$ and cystine has been observed ($k = 3 \times 10^6$ M^{-1} sec^{-1}). Pulse radiolysis studies confirm these results.

Introduction

The pulse radiolysis of aqueous solutions of mercaptans and disulfides has been extensively studied since certain of these compounds behave as radiobiological protection agents. A focus for many of these studies has been the formation and decay of the transient disulfide radical anion ($RSSR^-$).[1-3] This species is generally formed either by the addition of a hydrated electron to a disulfide[4]

$$e_{aq}^- + RSSR \longrightarrow RSSR^- \quad (1)$$

or by the oxidation of a mercaptan (RSH) or its conjugate base (RS^-) yielding a thiyl radical which takes part in the equilibrium

$$RS\cdot + RS^- \rightleftharpoons RSSR^- \quad (2)$$

The decay kinetics for noncyclic $RSSR^-$ formed by reaction 1 in the absence of RS^- are always first order with rate constants of 10^5–10^6 sec^{-1} and can be related to the dissociation[4]

$$RSSR^- \longrightarrow RS\cdot + RS^- \quad (3)$$

However, when equilibrium 2 is set up the lifetime of the transient is increased and its decay has been found in pulse radiolysis experiments to be purely second order.[5] In apparent contradiction, Caspari and Granzow[6] found a first-order decay for $RSSR^-$ formed by flash photolysis of aqueous solutions of cysteine, cysteamine, and benzene-