The Microwave Spectrum, Structure, and Ring-Puckering of the Cyclic Dipeptide Diketopiperazine

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Received January 3, 2000

Abstract: We have detected the microwave spectrum of the smallest cyclic peptide—diketopiperazine—in the frequency range 48−72 GHz, demonstrating that the molecule does not adopt in isolation the highly symmetric (C_{2h}) planar-ring structure obtained in the solid state via X-ray crystallography. From a comparison of the derived rotational constants (MHz), $A = 4906.4098(44)$, $B = 1582.1420(37)$, $C = 1239.4218(44)$, with those obtained from an ab initio molecular orbital calculation [MP2/6-311++G(d,p) level], the stable form is a boat configuration having C_2 symmetry. Exploration of the ring puckering potential energy surface indicates that this “methylene” boat conformer is the only stable conformer of diketopiperazine. The microwave spectrum deviated from that of a rigid rotor in that all of the measured transitions were members of doublets in which the separation was ~2 GHz. This is attributed to tunneling between two equivalent conformations through a relatively low barrier on the potential energy surface. Our exploration of the ring puckering possibilities via ab initio molecular orbital calculations indicates that the minimum energy pathway linking the two boat (C_2) enantiomeric conformers passes over a barrier of about 470 cm^{-1}. The chair (C_i) conformer is involved at the summit of the barrier. This barrier is significantly lower in energy than the planar ring (C_{2h}) species which appears to be a higher saddle point on the potential energy hypersurface. The calculated energy barrier is plausibly consistent with the tunneling splitting found in the spectrum. A simple empirical modeling of the ring puckering energy of diketopiperazine in terms of peptide linkage torsion and ring-angle deformations represents the ab initio ring flexure energies surprisingly accurately. The fitted torsional energy function is in close agreement with the comparable ab initio ω-torsion in *N*-methyl acetamide and is predominantly quartic. This has implications for protein modeling since this appears to deviate in detail from the form of potential currently included in the molecular mechanics computational models employed for the cis peptide linkage in the theoretical study of protein folding.

Introduction

For many years 2,5-diketopiperazine (DKP, glycine anhydride, 2,5-piperazinedione) has been of interest in connection with the study of the constitution of proteins. It is the simplest cyclic peptide and as such exemplifies the relatively infrequently occurring cis amide peptide linkage.[1]

Diketopiperazines have been used as versatile intermediates in the asymmetric synthesis of amino acid derivatives and related natural products,[2] and the acyclic dipeptide−DKP reaction is thought to play a key role in the kinetics of the decomposition of proteins in fossils.[3] In a recent study[4] electrochemical oxidation of DKP has been used to investigate the general phenomenon of protein oxidation in living systems. In another recent study substituted DKP has been demonstrated to form self-assembled supramolecular “tapes” of two different classes depending on the planarity or nonplanarity of the DKP ring.[5]

Structural investigations on crystals of DKP by X-ray[6−8] and electron diffraction[9] showed that the eight heavy atoms are essentially coplanar. Sletten[10] found by X-ray diffraction that *trans*-dimethylDKP [the cyclic dipeptide from D-alanine with L-alanine] deviates slightly from ring planarity into a chair configuration with the methyls axial while *cis*-dimethylDKP [from L-alanine with L-alanine] is appreciably puckered into a twisted boat conformation with the methyls axial. Karle et al.[11] studied various substituted DKP and found geometries of the six-membered ring ranging from planar to puckered (by up to 40°).

Several theoretical studies have used empirical force fields to explore the conformational flexibility of the diketopiperazine ring.[12−14] For DKP, Karplus and Lifson[8] found a broad nonplanar minimum separated from a planar ring structure by

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10.1021/ja000042n CCC: $19.00

less than 350 cm^{-1}. Semiempirical CNDO/2 calculations[15] gave larger energy differences between planar and boat form (up to 2000 cm^{-1}). Our own earlier ab initio calculations[16] at levels ranging from HF/3-21G to MP2/6-31G(d,p)//HF/6-31G(d,p) indicated the methylene boat form as the only stable conformer and provided an energy difference of 326 cm^{-1} with respect to the planar ring form at the latter level. More recently as part of their study of the thermodynamics of peptide oxidation Jonsson et al.[4] reported an ab initio structure investigation of DKP using density functional theory at the B3LYP/6-31G(d) level. The resulting equilibrium structure is reported to be a boat form of C_2 symmetry but further structural data is restricted to internuclear distances only. Hirst and Persson[17] reported ab initio theoretical calculations of the molecular structure of DKP using a level of calculation that involved correlation-corrected basis functions [MP2/cc-pVDZ[18]]. They reported a boat shaped minimum-energy structure that was only 2 cm^{-1} lower in energy than the planar ring conformation. However the reported relative energy is the result of a transcription error and has been revised subsequently[19] to 441 cm^{-1}.

Methodology

Experimental. The free-jet expansion, Stark-modulated spectrometer used in the present study is based on a design that has previously been described.[20] DKP solid, obtained from Sigma, was vaporized at 230 °C in a stream of argon at a pressure of ~0.3 atm. Following free-jet expansion with a jet temperature of 260 °C the rotational temperature was found to be ca. 10 K under the conditions employed. The microwave absorption spectrum was first investigated via an extended spectral search scan over a wide frequency range (48−72 GHz). Once located via the wide search scan, each detected line was digitally acquired via repetitively averaged narrow-band scans, and line frequencies were measured by least-squares fitting of a Lorentzian function to each line profile. For the observed transitions, the typical full width at half the maximum line height was ~300 kHz. This corresponded to an estimated experimental uncertainty in the measured line frequencies of approximately 0.03 MHz.

Theoretical. We performed predictive ab initio geometry optimization calculations of molecular structure, conformational energy, and electric dipole moments. These were started from a wide range of assumed structures corresponding to plausible DKP conformers. The calculations were used to assist in the rotational assignment of the observed spectrum and to serve as a guide to the identification of the species present in the expanding jet. Calculations were performed using the GAUSSIAN 98 package[21] at the MP2/6-311++G(d,p) level. This level of basis set was selected because our previous studies[22] of molecules of similar complexity have yielded energy data from which we derived fully satisfactory interpretations of observed jet-spectra of the molecules. Calculations performed with lower levels of basis sets, for example, when diffuse functions were not included, provided less complete, or conflicting, interpretations.

Results and Discussion

Assignment of Spectrum. Our ab initio molecular orbital calculations for DKP provided predictions of rotational constants and dipole moment components. A rotational assignment of the observed strong lines was made on the basis of the qualitative appearance of quadrupole splitting patterns, Stark effects, and the recognition of progressions of strong lines. Attempts to fit the observed spectrum directly to the Watson *S*-reduced Hamiltonian were not successful. Ultimately the spectrum was found to fit a two-vibrational state model in which there was no significant Coriolis interaction, that is, involving only the splitting between the ring-puckering states together with separate sets of rotational spectroscopic parameter values for the upper and lower tunneling state. All of the assigned transitions involved quantum numbers corresponding to a transition dipole in the *c*-direction. Using this two-state model, we obtained a successful fit which included all of the observed transitions.

Table 1. Fitted Spectroscopic Constants of DKP

parameter[a]	value
A/MHz	4906.4098 (44)
B/MHz	1582.1420 (37)
C/MHz	1239.4218 (44)
Δ_J/kHz	0.048 (8)
Δ_K/kHz	1.922 (63)
$\delta(A)$/kHz	−31.4 (11)
$\delta(B)$/kHz	9.8 (13)
$\delta(C)$/kHz	48.9 (17)
$\delta(\Delta_{JK})$/kHz	−0.041 (7)
Δ/MHz	1075.440 (30)
no. of lines	57
rms (O−C)/kHz	31.0

[a] The parameters A, B, C, Δ_J, and Δ_K are rotational and centrifugal distortion constants of the lower inversion state while the parameters preceded by δ are the *differences* between corresponding rotational and centrifugal constants for the upper and lower inversion states. The parameter Δ is the tunneling splitting between the upper and lower inversion state vibrational levels.

Table 1 presents the fitted spectroscopic constants. For the rotational part Watson's *A*-reduced Hamiltonian in the I^r representation was used. Table 1S in the Supporting Information lists the assigned transitions with their observed frequencies and (observed − calculated) values. From the spectroscopic data in Table 1 we see that the vibrational energy separating the two vibrational levels is ~1 GHz, similar in magnitude to the 0−1 splitting in trideuteroammonia (1.6 GHz) and considerably smaller than the 0−1 ring-puckering splitting in cyclopentene (27.3 GHz).[23] The rotational constants are less than 1% different from those predicted for the boat-shaped form of DKP by our molecular orbital calculations (see Table 2).

Theoretical Investigation of Molecular Structure and Ring-Puckering. All unconstrained geometry optimizations at the MP2/6-311++G(d,p) level converged to one or the other

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Table 2. Predicted Rotational Constants from ab Initio MP2/6-311++G(d,p) Structure Optimization Calculations

parameter[a]	DKP conformer C_2	C_i	C_{2h}	observed
A /MHz	4924.95	4885.79	4899.93	4906.41
B /MHz	1574.84	1570.55	1564.14	1582.14
C /MHz	1249.45	1210.57	1203.44	1239.42
rms(O−C)[b]	13	22	24	

[a] A complete listing of ab initio predicted geometric parameters is given in Table 2S. [b] Root-mean-square deviation in MHz between the observed and ab initio rotational constants.

of two enantiomeric "methylene" boat conformers of C_2 symmetry. An optimization constrained to be of C_i symmetry converged to a chair conformer ~470 cm^{-1} higher in energy than the boat and with a relatively smaller puckering amplitude. However a frequency calculation at the smaller basis MP2/6-31G(d,p) level showed the C_i form to be a saddle-point on the potential energy surface. Insufficient computational power was available to undertake frequency calculations with the larger basis set, and thus the status of the C_i form at this level remains indeterminate. An optimization constrained to have all of the heavy atoms coplanar, resulting in C_{2h} symmetry, produced an energy ~600 cm^{-1} above the boat conformer. A standard Gaussian 98 frequency calculation at the smaller basis MP2/6-31G(d,p) level (with the structure optimized at this level) showed the C_{2h} form to be a saddle-point of index = 2 on the potential energy surface.

To gain more insight into the puckering PES of diketopiperazine we evaluated the ring-puckering parameters q_2, ϕ_2, and q_3 of Cremer and Pople[24] for the three conformations in Table 2 (see Figure 1). The values, together with the alternative parameters, Q, θ, and ϕ, are listed in Table 3. These confirm that the more stable C_2 conformer is almost pure boat shape ($\theta = 90°$, $\phi = 169.2°$), while the C_i conformer is pure chair ($\theta = 0°$). Because of the observed doubling of the spectrum, we sought the minimum energy pathway corresponding to the inverting of the boat. It was apparent that inversion could be simply achieved by passing through the planar ring C_{2h} form. However alternative lower-energy pathways were considered that avoided passing through this highly strained structure in which both of the methylene CCN bond angles are forced to deviate significantly from the preferred near-tetrahedral value. We therefore explored the potential energy curve by performing constrained optimizations for a series of fixed puckering parameter values running monotonically from values appropriate to the boat conformer to its inverted shape.

Exploratory calculations in which we considered twisting (varying ϕ) indicated that this led to higher-energy forms and thus was not the primary parameter in seeking the lowest-energy valley. We then found that appropriate variations in Q and θ, with ϕ fixed at 169.2°, led to a local energy maximum of about 470 cm^{-1}, illustrated in Figure 2. This relatively small barrier in the energy path from boat to the inverse boat conformation seems compatible with a tunnelling splitting of magnitude such as that observed. Ring conformations in the vicinity of the barrier progress through a half-boat "envelope" form to the C_i chair form and then to the alternative half-boat. In this sequence the methylene CCN bond angles are distorted serially rather than simultaneously as in the planar ring structure.

Because the minimum energy trajectory involves virtually no change in the twisting of the ring (i.e., value of ϕ), it seems that the linkages along either side of the "boat", that is, the peptide linkages, are relatively resistant to distortion. We explored this by investigating how well a simple modeling of the energetics of the diketopiperazine ring distortions in terms of a two-parameter anharmonic twisting potential for the peptide linkage, plus harmonic potentials for the three types of ring angle deformations, would represent the ab initio puckering energy. We fitted a quadratic/quartic potential for the peptide linkages, and quadratic potentials for the ring angle deformations, to the

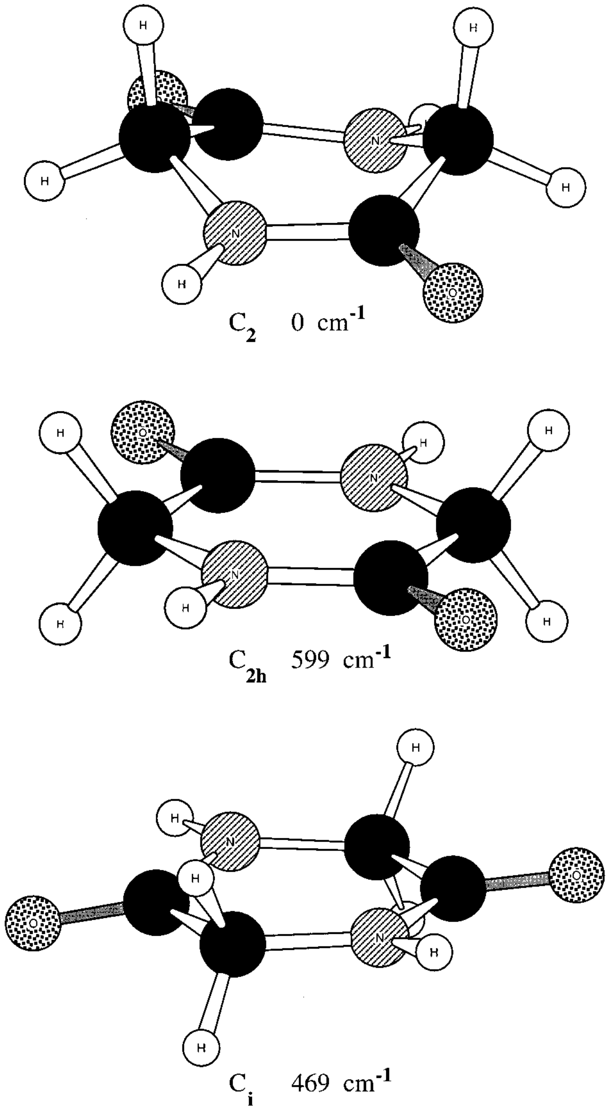

Figure 1. Three conformations of diketopiperazine calculated from Gaussian 98 geometry optimizations at the MP2/6-311++G(d,p) level. Relative energies are shown, calculated at the same level. The C_i and C_{2h} forms were optimized with constrained symmetry and do not represent local energy minima.

Table 3. Cremer−Pople Ring-Puckering Parameters[a]

conformer	q_2	ϕ_2	q_3	Q	θ
C_2	−0.508	169.1	0	0.5084	90
C_i	0	259.7	0.1612	0.1612	0
C_{2h}				0	

[a] Following the definitions given by Cremer and Pople,[24] the parameter Q, the total puckering amplitude, is a measure of the extent of puckering of the ring ($Q = \sqrt{\Sigma z^2}$, z being the perpendicular distance of a ring atom from the mean ring plane); θ ranges from 0° for a chair shape to 90° for a boat shape; ϕ (the subscript 2 is not necessary for a six-membered ring) passes through values for the various boat and twisted boat shapes as it ranges from 0° to 180°.

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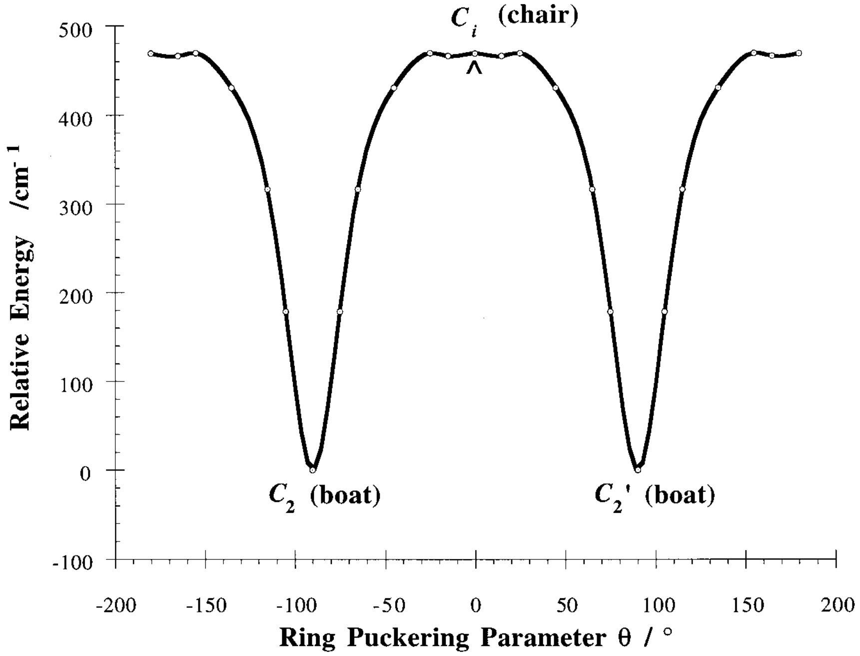

Figure 2. Energy profile for ab initio MP2/6-311++G(d,p) minimum energy pathway for inverting diketopiperazine.

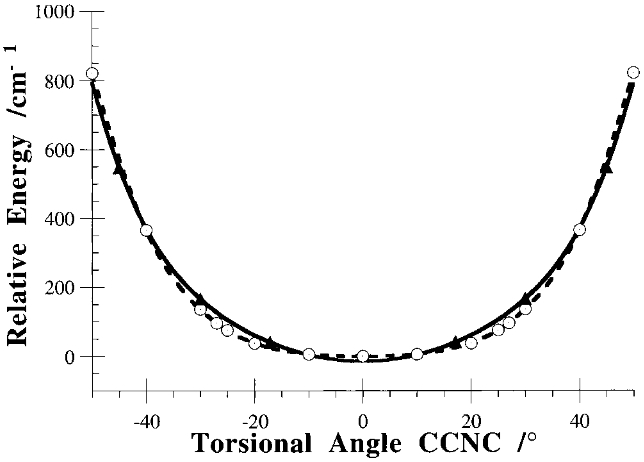

Figure 3. Comparison of the CCNC torsional parts of empirical potential functions for DKP ring distortion (dashed line, open circles) and NMA CCNC torsion (full line, triangles). In each case the empirical potential function was fitted to MP2/6-311++G(d,p) ab initio energies obtained from suitably constrained geometry optimizations.

energy variation and were surprised as to how faithfully this simple approximation reproduced the quantum chemical calculations of the energy variation as diketopiperazine is distorted. The resultant torsional part of this potential is shown in Figure 3.

If we accept that this is a realistic empirical representation of the distortion potential for the peptide linkage, then we have a valuable input into the empirical computations of protein conformational energies. Many literature discussions of the secondary structure of proteins begin with the view that the amide linkage, because of conjugation, is almost rigidly planar. If one follows the standard backbone dihedral angle notation for protein structures, the $C_\alpha CNC_\alpha$ angle, ω, is said to be either 180° or 0°. (e.g., Allen in a recent comprehensive treatise on this subject[25] quotes the peptide linkage torsional strain energy as equivalent to 1230 cm^{-1} at 20° from planarity). Conformational variation has mainly focused on distortions of the adjacent backbone $NCC_\alpha N$ angle (ψ) and $CNC_\alpha C$ angle (ϕ, which should not be confused with the Cremer–Pople parameter ϕ used earlier to describe ring puckering). In contrast to this prevalent view, our analysis for DKP indicates that in the case of this cis amide peptide linkage, distortion from planarity of ±20° involves an increase in energy of less than 40 cm^{-1} and that the potential function owes its large compliance over angles out to ~±50° to its predominantly quartic dependence.

To see whether the peptide linkage in DKP is significantly influenced by the linkage being part of a ring system we also have studied at the same level of ab initio calculation both the cis and trans minima for the peptide linkage in acyclic *N*-methylacetamide (NMA). We found that the torsional potential was transferable between the two molecules in that the torsional potential function from an ab initio calculation for *cis*-NMA was closely similar to the empirical function fitted to DKP. We fitted the empirical torsional potential function for *cis*-NMA ignoring the ab initio data in the region $\omega = -15°$ to 15° to avoid a small local maximum that presumably was attributable to nonbonded repulsion between the methyl groups. In an ab initio calculation at the same level on *trans*-NMA (which more closely resembles the trans conformation found in most acyclic peptides), there is no central maximum, and the energy rises only about 160 cm^{-1} in distorting the peptide linkage 20° from the planar configuration. Thus, the results imply that both the cis and trans peptide linkages are much more compliant to torsion than is often suggested in the protein literature.

Among the molecular mechanics treatments currently in use for protein modeling the empirical force field MMFF94 appears to be one of the most reliable in terms of its performance on conformational energy differences and single-bond torsion barrier heights.[26] To evaluate its performance in the context of the peptide linkage it was decided to compare results from the MMFF94 force field with our results from ab initio calculations

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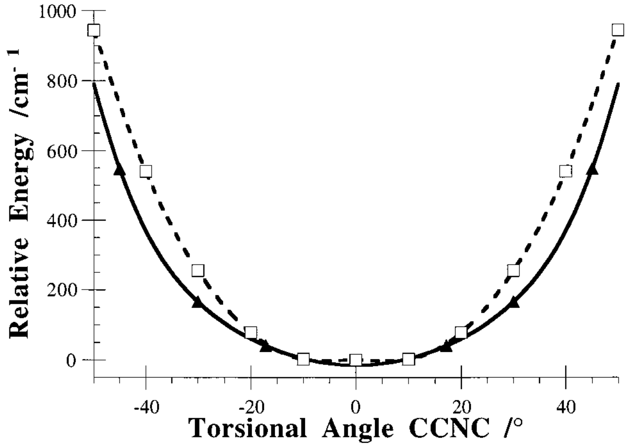

Figure 4. Comparison of the CCNC torsional parts of empirical potential functions for NMA CCNC torsion based on the molecular mechanics empirical force field MMFF94 (dashed line, open squares) and the function derived from ab initio energies (full line, triangles) shown in Figure 3.

for *cis*-NMA. These calculations, involving constrained values of the torsional angle ω for *cis*-NMA, were conducted using the molecular mechanics option within the computational chemistry software package TITAN.[27] As shown in Figure 4, the resultant torsional potential involves a strain energy much less than 1230 cm^{-1} at 20° but nevertheless predicts significantly greater strain energy than the ab initio calculations reported here, at torsional angles greater than 20° (e.g., ~250 cm^{-1} vs ~170 cm^{-1} at 30°).

The implication is that hitherto the most reliable empirical force field calculations of protein structures and underpinning the folding process have employed a significantly overestimated resistance to twisting of the ω torsion angle of the protein linkage. In addition, the thermodynamic calculations that form an important aspect of modern protein folding models, where based on the harmonic approximation for vibrations, are likely to be significantly in error in the case of the rather anharmonic quartic peptide ω-torsion modes. We are currently pursuing further data on the energetics of the distortion of the protein linkage to clarify this important question.

It is of interest to note that MMFF94 calculations predict that the minimum energy form of DKP is a C_2 boat and that the planar ring C_{2h} form is higher in energy by ~1900 cm^{-1}. This value is nearly 4 times greater than the ab initio prediction and would be inconsistent with the substantial tunneling splitting energy observed in the microwave spectrum.

The energy difference of 441 cm^{-1} between the C_2 and C_{2h} forms of DKP reported by Hirst and Persson[17,19] based on calculations at the MP2/cc-pVDZ level agrees within expected limits with the value of 599 cm^{-1} from our own higher level ab initio calculations. The basis set employed[18] by these workers is different in that it lacks radially diffuse functions and is equivalent to the more restricted double-ζ radial dependence rather than the triple-ζ basis of the 6-311G class of split-valence function. Our own preliminary calculations[16] with a wide range of smaller basis sets of the split-valence type, and with or without allowance for electron correlation, consistently yielded energy differences greater than 100 cm^{-1} between the C_2 and C_{2h} forms of DKP.

Conclusions

We have detected the microwave spectrum of diketopiperazine and have shown that it consists of a rotation−inversion spectrum characterized by a tunneling splitting parameter of 1 GHz.

From ab initio molecular orbital calculations at the MP2/6-311++G(d,p) level the lowest energy form is an enantiomeric pair of boat configurations having C_2 symmetry. Exploration of the ring puckering potential energy surface indicates that this pair of "methylene" boat conformers is the only stable conformer of DKP. The theoretical rotational constants for this species are in close agreement with the corresponding values derived from analysis of the observed spectrum.

The ring-puckering potential energy surface was explored via a series of ab initio molecular orbital calculations. These indicated that the minimum energy pathway linking the two boat (C_2) enantiomeric conformers passes over a barrier consisting of a plateau elevated in energy by ~470 cm^{-1}. This pathway involves a sequence of structures progressing from one of the two boat (C_2) conformers through a half-boat form to a chair (C_i) conformer and then to the mirror-image boat via a second half-boat form. Along this pathway the boat and half-boat forms show negligible twisting and retain the two methylene carbon atoms as the bow and stern. The C_{2h} species in which the ring atoms are coplanar is not involved in the inversion pathway but exists as a further saddle point higher up on the potential energy hypersurface. The energy barrier of 470 cm^{-1} separating the two equivalent boat conformers is of an appropriate size to result in the tunneling splitting found in the measured spectrum.

A simple empirical modeling of the ring-puckering energy of diketopiperazine in terms of peptide linkage torsion and ring-angle deformations suffices to represent the ab initio ring flexure energies quite accurately. This fitted torsional energy function is in close agreement with the comparable ab initio ω-torsion in *N*-methyl acetamide and is predominantly quartic. This result supports the assumption of transferability of the peptide torsional potential between differing peptide linkages that underlies the empirical force field methods in wide use for protein modeling. However, the quartic potential well appears to deviate significantly from the harmonic or sinusoidal torsional potential currently included in the leading molecular mechanics computational models. This may have implications for improving the accuracy of the theoretical modeling of protein folding.

Acknowledgment. The research was supported by a grant from the Australian Research Council.

Supporting Information Available: This collection of Tables 1S, 2S and Figures 1S, 2S contains all of the measured and assigned microwave transition frequencies used to derive the rotational constants and the tunneling splitting reported in this work together with the complete ab initio optimized geometries, energy and dipole moment for the C_2, C_{2h}, and C_i structural forms of DKP. Figure 1S provides the atom numbering scheme used in the tabulated ab initio data, and Figure 2S illustrates the quality of the least-squares fit between our empirical DKP torsion-bending potential and the ab initio energies for a wide range of constrained puckering coordinate values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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