Free Activation Energies and Activation Volumes for the Amide Rotation in Some Peptides Studied by High Pressure ^{1}H-High Resolution NMR*

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From the pressure dependence of ^{1}H high resolution NMR spectra of two dipeptides (glycylsarcosine and N-acetyl-L-proline-NH-methylamide in the range 0.1 MPa $\leq p \leq$ 150 MPa the activation volumes $\Delta V^{\neq}$ for the amide rotation are derived. This conformational transition is characterized for glycylsarcosine by $\Delta V^{\neq} = 4 \pm 1$ cm$^3 \cdot$ mol^{-1} and for the proline derivative by $\Delta V^{\neq} = 7.5 \pm 1$ cm$^3 \cdot$ mol^{-1}. From the given results the maximum contribution of proline *cis* $\rightleftharpoons$ *trans* isomerisation to the pressure dependence of the rate of reactivation of proteins can be estimated to $\sim -30\%$ per MPa and proline present.

Introduction

High hydrostatic pressure can have a severe influence upon living organisms. Land living vertebrates can only adapt to rather minute changes of external pressure while marine animals are generally adapted to much higher barotolerance [1, 2]. Quite a few organisms have invaded the abyssal depth of ocean trenches and manage to survive at pressures up to 100 MPa, even at temperatures around 2 °C, under permanent absence of light and under conditions of low oxygen- and nutrient concentrations [2, 3]. In order to evaluate quantitatively the contributions to the pressure effects in organisms it is current practice to study model systems, like enzymes and nucleic acids at elevated pressures [4, 5]. The relevant parameters determining the pressure dependence of the equilibrium and kinetic properties are the reaction volume on one hand and the volume of activation on the other [5, 6].

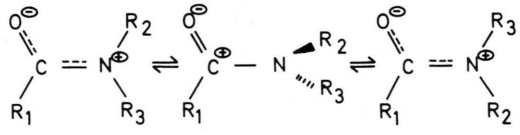
Quantitative data with respect to the volume effects of elementary reactions contributing to the overall volume changes in enzymes or nucleic acids are still scanty. Therefore attempts have been made to study high pressure high resolution nuclear magnetic resonance (NMR) of simple liquid model systems in order to provide deeper insight into the mobility of specific groups in biopolymers.

From the pressure dependence of chemical equilibria, and chemical reactions the reaction volume ΔV and the activation volume $\Delta V^{\neq}$ can be derived:

$$\Delta V = -\left(\frac{RT\,\partial \ln K}{\partial p}\right)_T,$$

$$\Delta V^{\neq} = -\left(\frac{RT\,\partial \ln k}{\partial p}\right)_T. \qquad (1)$$

Among the model systems studied previously by high pressure high resolution NMR are (i) the self association of 9-methylpurine ($\Delta V_{ass} = -(4 \pm 1)$ cm$^3 \cdot$ mol^{-1} [7]), (ii) the pressure dependence of the rate of the *syn* $\rightleftharpoons$ *anti* rotation in a synthetic pteridine-nucleoside analogue ($\Delta V^{\neq} = (5 \pm 1)$ cm$^3 \cdot$ mol^{-1} [8]) and (iii) the activation volume for the rotation in a variety of amides [9–12]. Systematic studies of these compounds included variation of all ligands R (Eqn. (2))



(2)

in order to find out the contributions of the size of the amide ligands to the activation volume [11, 12], and to determine the influence of the carbonyl substituent, and the solvent upon $\Delta V^{\neq}$ [9, 10]. The present experiments complement these studies by investigating the pressure dependence of the amide rotation in some peptides.

* Dedicated to Professor Benno Hess on the Occasion of His Sixtieth Birthday.

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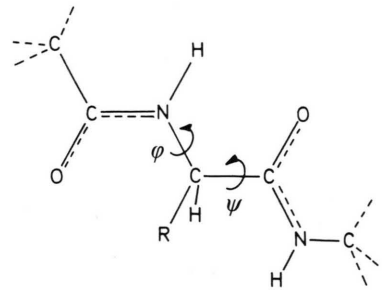
As illustrated in Eqn. (3) the conformational flexibility of a protein consists mainly of the rotational equilibria described by the dihedral angles φ and ψ of the peptide units.



(3)

The restriction in configurational space is caused by the fact that most natural peptides occur only in the given *trans* form (for exceptions *cf.* [13–15]). Incorporation of the amino nitrogen into the proline ring or methylation, as in the case of sarcosine, does reduce the energy difference between the *cis* and *trans* conformation, and leads to comparable concentrations for both rotamers [15–17]. Since it has been shown that the *cis* $\rightleftharpoons$ *trans* isomerization of proline residues is the rate limiting step in the refolding of proteins (after previous denaturation), the pressure dependence of this reaction is of considerable interest. The following experiments are concerned with the dynamics of the amide group in two synthetic dipeptides containing proline and sarcosine.

Materials and Methods

Glycylsarcosine (GlySa) and N-acetyl-L-proline-NH-methylamide (AcProMe) were purchased from Bachem (Bubendorf, Switzerland). GlySa was studied at a concentration of 0.44 molal in a 4:1 (w/w) D_2O/H_2O mixture. The light water was added in order to provide a strong proton-signal for internal locking and shimming. The uncorrected pH at atmospheric pressure was 5.4. AcProMe was studied at 4.7% w/w of the peptide in 47.6% w/w benzene-h_6 (Merck, Darmstadt), 45.7% w/w acetone-d_6 (Merck, Darmstadt) and 2% w/w hexamethyldisiloxane (Fluka, Switzerland). The benzene had to be added, to introduce sufficient chemical shift differences between the proton signals from the various conformers; in addition it provides the proton-signal for internal locking in a spectral region, where no other signals were observed. In order to simplify the spectra the NH-proton was replaced by a deuteron by three lyophilisation cycles from D_2O.

Experimental

Details of the high pressure cell have been published previously [7, 9]. The temperature was controlled to $\pm$ 0.5 K with a miniature thermocouple. The spectra were obtained at 100.1 MHz in the FT-mode (Varian XL-100-15 spectrometer, interfaced to a Varian 620-1-100 computer with interactive disc accessory). During spectra accumulation the spectrometer was locked to an external ^{19}F lock.

Spectra analysis

Simulation of the exchange broadened spectra was performed by application of the non iterative part of the DNMR5-program [18]. The chemical shifts $\delta\nu$ (relative to an internal standard) in the region of chemical exchange broadening were determined from spectra obtained 30 to 50 K beneath the temperatures where the influence of the exchange process becomes observable. $|\partial\delta\nu/\partial T|$ varies between 0.01 and 1 Hz · K^{-1}.

The line width (LW) without chemical exchange, was calculated from the low temperature spectra and a reference line, by assuming $LW^{ref} \cdot LW^{-1}$ independent of p and T. Given the quality of the spectra, errors in $\delta\nu$ and/or LW $\leq$ 0.3 Hz cannot be resolved by comparison of the simulated and experimental spectra. This uncertainty leads to a maximal error for $\Delta G^{\#}$ and $\Delta V^{\#}$ of the order of $\pm$ 1 kJ · mol^{-1} and $\pm$ 1 cm^3 · mol^{-1}, respectively.

Results

Glycylsarcosine

The formulae given in Fig. 1 depict the two conformations of glycylsarcosine (GlySa). The assignment of the ^1H-signals to the single resonances is taken from the literature [19]. Corresponding to the isoelectric point (IP ~ 5.9) the zwitterionic form is practically the only form present in aqueous solutions at pH ~ 5.4 [19]. From a comparison of the partial spectra for the N-methyl group it is evident, that increasing pressure lowers the rate of rotation. The signals resulting from the groups A to D (Fig. 1) overlap strongly; threrefore simulations of

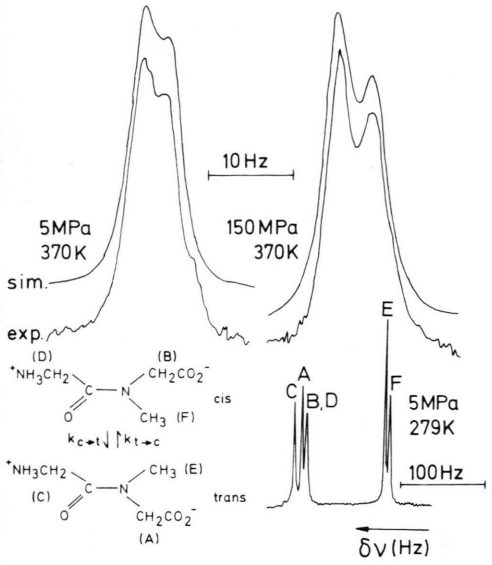
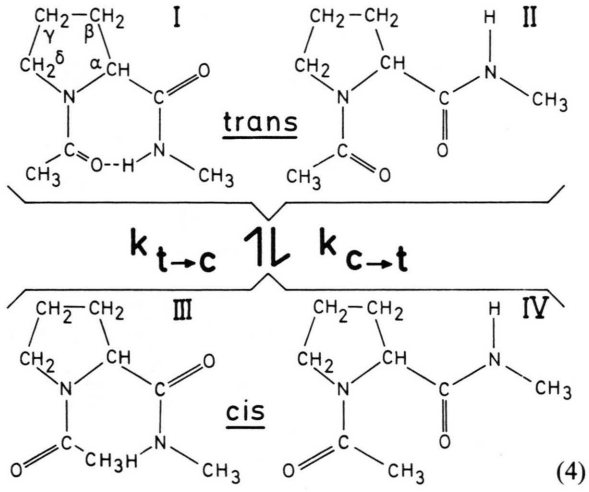


Fig. 1. Experimental and simulated proton spectra of glycylsarcosine in water. Bottom: Complete experimental spectrum. Assignment as given in the structural formula (left). Top: Partial experimental and simulated spectra of the signals E and F (N-methyl group in *trans* ≡ E).

the exchange-broadened spectra were only performed for the N-methyl groups (E + F) (Fig. 1). The optimal simulation parameters are given in Table I. As taken from Eqn. (1) the activation volume $\Delta V^{\neq}$ may be calculated from the pressure dependence of the rotation rate k: $\Delta V^{\neq} = (4 \pm 1)$ cm^3 · mol^{-1}.

N-acetyl-L-proline-NH-methylamide

The stereochemistry of N-acetyl-L-proline-NH-methylamide (AcProMe) is fairly complicated, due to the presence of the two amide groups, the free rotation around the proline C-α bond, and the flexibility of the proline ring. The structural formulae summarized in Eqn. (4) represent the conformers discussed in the literature [16].



(4)

It is generally accepted, that the N-methyl group is found in *cis* position to the carbonyl group [20]. The rotamer distribution around the C-α bond is stabilized in direction to the *trans*-conformer (**I**) by the formation of an intramolecular hydrogen bond. Solvents which can act as hydrogen bond acceptors (like acetone or water) compete for this hydrogen bond, to the result that the *trans* form is less predominant in such solvents. It is reasonable to assume, that at $T > 300$ K the rotation around the C-α bond is fast on the NMR-time scale [21], and that the chemical inequivalence observed for all proton signals may be ascribed to the rotation

Table I. Optimal simulation parameters obtained for the N-methyl-proton-signals. $\Delta\delta\nu_{cis\text{-}trans}$ [Hz] frequency difference between the signals; LW [Hz] linewidth without chemical exchange; pop_{trans}, mole fraction of the trans form; $k_{trans\to cis}$ [Hz], rate of rotation as defined in Fig. 1.

Spin system $A_3 \leftrightarrow B_3$			signal used: N-CH_3			
	p [MPa]		5	50	100	150
AcProMe	$\Delta\delta\nu_{cis\text{-}trans}$	[Hz]	9.1	9.1	9.1	9.1
335 K	LW	[Hz]	2.2	1.6	1.6	1.8
	k	[Hz]	15.8	14.2	12.5	11
	$\Delta G^{\neq}$ [kJ · mol^{-1}]		74.9	75.1	75.5	75.8
AcProMe	$\Delta\delta\nu_{cis\text{-}trans}$	[Hz]	9.1	9.1	9.1	9.1
332.2 K	LW	[Hz]	1.6	1.6	1.5	1.5
	k	[Hz]	13	11.5	10	9
	$\Delta G^{\neq}$ [kJ · mol^{-1}]		74.7	75.1	75.4	75.8

$pop_{trans} = 0.76$

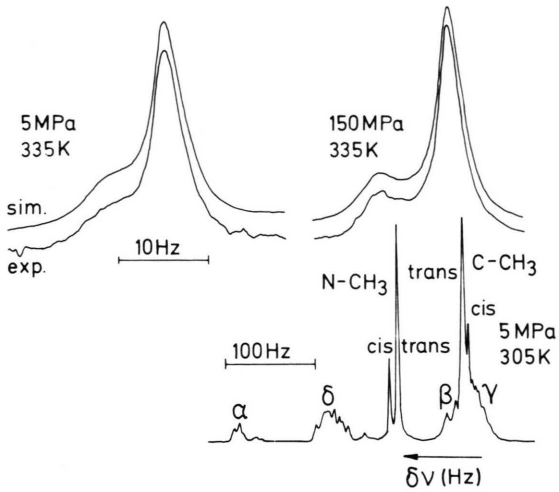


Fig. 2. Experimental and simulated proton spectra of N-acetyl-L-proline-NH-methylamide. Bottom: Complete proton spectrum. Top: Two partial experimental and simulated spectra of the N-CH_3 moiety. Assignment of the ring protons as given in Eqn. (4).

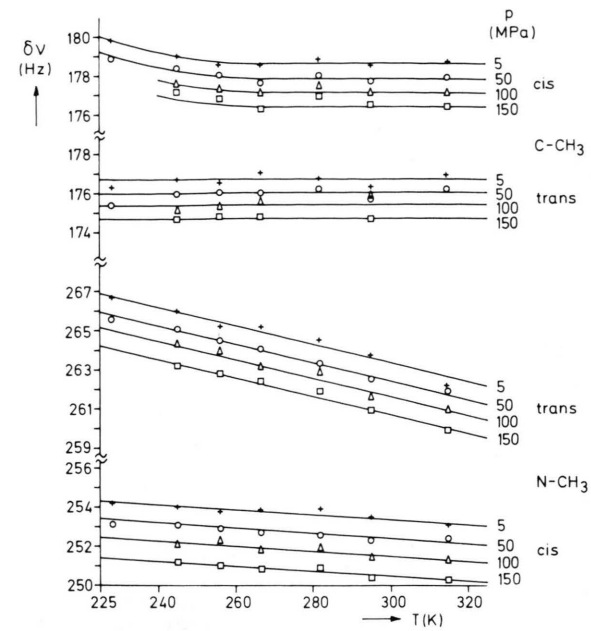


Fig. 3. Temperature and pressure dependence of the proton chemical shifts of C-CH_3 and N-CH_3 in N-acetyl-L-proline-NH-methyl-amide. Assignment according to ref. [22]. Internal standard: hexamethyldisiloxane (2% w/w).

Table II. Glycylsarcosine. Abbreviations as indicated in Table I, except that in this case $k_{trans \to cis}$ is given.

Spin system $A_3 \leftrightarrow B_3$			signal used: N-CH_3 (E, F)			
	p [MPa]		5	50	100	150
GlySa	$\Delta\delta\nu_{trans\text{-}cis}$	[Hz]	4.5	4.55	4.6	4.85
370.5 K	LW[a]	[Hz]	1.5/1.8	1.5/1.8	1.5/1.8	1.5/1.8
	pop_{trans}		0.51	0.515	0.52	0.53
	$k_{trans\text{-}cis}$	[Hz]	6.0	5.7	5.3	4.95
	$\Delta G^{\neq}_{trans \to cis}$ [kJ · mol^{-1}]		85.9	86.1	86.3	86.5

[a] First entry: signal E of Fig. 2 (N-CH_3 *cis*); second entry: signal F of Fig. 2 (N-CH_3 *trans*).

Table III. $\Delta G^{\neq}$ and $\Delta V^{\neq}$ for the amide rotation in the two peptides.

Compound	% w/w	Solvent	% w/w	$\Delta G^{\neq}_{5MPa}$ [kJ · mol^{-1}]	$\Delta V^{\neq}$ [cm^3 · mol^{-1}]
AcProMe	5	Benzene-h_6	50	75	7 ± 1
		Acetone-d_6	43	*cis* → *trans*	
		Hexamethyl-disiloxane	2		
GlySa	5	Water (80% w/w deut.)	95	86 *trans* → *cis*	4 ± 1

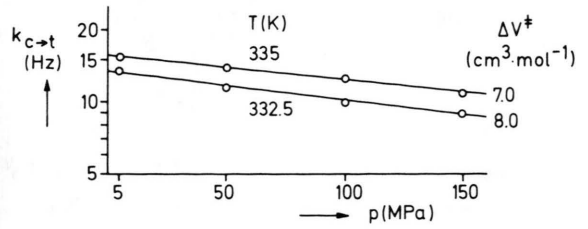


Fig. 4. Pressure dependence of the *cis-trans* rotation rate in N-acetyl-L-proline-NH-methylamide.

around the acetyl-proline bond (**I**). Fig. 2 gives the experimental and simulated spectra. Because of severe overlap of the different signals and poor definition of the spectra only the proton signals from the $-N-CH_3$ group have been simulated. Table I summarizes the fit parameters obtained. In the region suitable for spectral simulation (330–335 K) the population of the two rotamers is found to be independent of temperature and pressure: *cis*:*trans* = 0.31:1.

Fig. 3 illustrates the temperature and pressure dependence of the chemical shifts for the various methyl protons. The small p and T dependence of the signals is comparable to the effects observed in conformationally rigid amides in similar solvent mixtures [10, 11].

In Fig. 4 the pressure dependence of the rate constants at two fixed temperatures is given. The corresponding activation volume calculated from the slope is $\Delta V^{\neq} = 7.5 \pm 1\ cm^3 \cdot mol^{-1}$. The results of the analysis for both compounds are compiled in Table III.

Discussion

The dipeptide GlySa represents an amide carrying two charged groups. The activation volume, $\Delta V^{\neq} = 4\ cm^3 \cdot mol^{-1}$, connected with the amide rotation in this compound is found to be relatively small, especially if one compares it to the value $\Delta V^{\neq} \sim 9\ cm^3 \cdot mol^{-1}$ observed in uncharged N,N-dimethylamides [10]. These are characterized by a lower steric asymmetry than present in GlySa. In aqueous solutions of N,N-dimethylamides, a significant decrease of $\Delta V^{\neq}$ is observed which may be ascribed to the formation of an open hydration shell around the dimethylamino moiety. It is improbable, that this explanation can also be applied to the zwitterionic GlySa since the presence of the two charges must destroy any open structure in the immediate vicinity of the ionic charges due to the strong Coulomb-dipole interactions.

In studying activation volumes for N-dialkylated amides in a systematic way, it has been found, that $\Delta V^{\neq}$ decreases with the mass and moment of inertia of the N-substituents [23]. The conclusion drawn from these observations is, that the single 180°-flips of the amide rotation become slow compared to the translational diffusion of the solvent molecules, thus permitting the solvent molecules to rearrange continuously around the rotating group, and eliminating the need for a larger solvent-free space.

$\Delta V^{\neq} \sim 8\ cm^3 \cdot mol^{-1}$ for the AcProMe amide rotation is similar to the $\Delta V^{\neq}$ observed in the N,N-dimethyl-amides proving that neither the flexibility of the proline ring nor the presence of the free rotating NH-methylamide moiety does have an observable effect upon $\Delta V^{\neq}$.

It has been claimed, that the rate determining step in the refolding of denatured proteins, like ribonuclease after treatment with 6 M guanidine · HCl, is the amide rotation of the prolines into their native *cis* or *trans* conformation [15–17, 24–26]. From the given $\Delta V^{\neq}$ result one would predict that the influence of pressure upon a refolding process containing consecutive *cis* ⇌ *trans* isomerization steps at 100 MPa would be a slowing down of the rate of refolding by ~ 30% per proline present.

In this context, it is interesting to note, that for simple amide rotation, the observed $\Delta G^{\neq}$ and $\Delta V^{\neq}$ appear to be independent of the length of the N-alkyl substituents. *E. g.* the activation parameters for N,N-di-*n*-octyl-l-naphthamide ($\Delta V^{\neq} = 6.5 \pm 1.0\ cm^3 \cdot mol^{-1}$, $\Delta G^{\neq} = 76\ kJ \cdot mol^{-1}$) are found to be almost identical to the values observed for the corresponding N,N-dimethyl-l-naphthamide ($\Delta V^{\neq} = 7.5 \pm 1.0\ cm^3 \cdot mol^{-1}$, $\Delta G^{\neq} = 74\ kJ \cdot mol^{-1}$) [22]. Therefore it is not to be expected, that the dynamics of the neighbouring units will severely influence the dynamics of the proline moiety.

The absence of any significant influence of high hydrostatic pressure on the *cis* ⇌ *trans* equilibrium in the given amides, and also in several other model compounds studied previously [11, 12, 23] is quite remarkable and appears at the moment to be a general feature of this kind of rearrangement. It proves that the apparent molar volumes of the two

conformers must be closely similar ($\Delta V \leq \pm 0.5$ $cm^3 \cdot mol^{-1}$). At the same time one has to keep in mind that the extrapolation of results obtained for the monomer to the polymer has to be done with care since it is well established, that poly-L-proline, which at atmospheric pressure is present in a helical form made up of *trans*-prolines, is transformed into an all-*cis* helical form upon applying high hydrostatic pressure [27].

Acknowledgements

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