Solvent effects on diketopiperazine formation from N-terminal peptide residues

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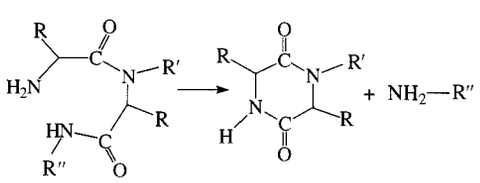
The kinetics of diketopiperazine formation from the peptide H-Ala-Pro-NH_2 with the unprotected amino group in the form of its trifluoroacetate salt have been investigated in a large number of solvents, including aprotic and hydroxylic solvents. The first-order rate constant is considerably affected by the solvent properties, its value spanning more than three orders of magnitude. Moreover, alkylammonium carboxylate salts are efficient catalysts of the reaction. The correlation with Kamlet–Taft solvent parameters shows that the reaction rate is retarded by solvents with a high capacity to stabilise solutes that are charged or dipolar, and that are hydrogen donors and/or acceptors. Solvents with high cohesive energy density values significantly increase the reaction rate. These results are discussed in terms of a proton switch in the rate determining step and of solvation stabilisation of the initial state of the peptide and of the transition state of the rate-limiting step.

Introduction

A variety of spontaneous chemical reactions are known to affect peptides and proteins during their storage and manipulation. Among these, particularly frequent is the cleavage of the peptide bond between the second and third residue with concomitant formation of diketopiperazine (2,5-dioxopiperazine, **DKP**).[1,2] The nitrogen atom of the N-terminal deprotonated amino group can attack the carbonyl carbon atom of the second residue, causing the breakdown of the chain and formation of **DKP**s (Scheme 1). Spontaneous degradation during storage,



R′ = H or an alkyl group

Scheme 1

for instance, has been reported for the undecapeptide substance P,[3] the Tic-containing peptides,[1,4] and the protein recombinant DNA-derived human growth hormone.[5] Moreover, many **DKP**s, encompassing a wide range of biological activities, have been found in a variety of tissues and body fluids.[6]

The role played by the peptide sequence and chirality of the residues involved in the cyclisation has been investigated in detail.[7] The mechanism of the reaction in aqueous solution has recently been described.[8]

However, a thorough investigation of the effects of organic solvents has never been carried out, although this reaction has been reported for several years to occur as a side reaction in peptide syntheses.[7] This information should prove useful in determining conditions for the storage and manipulation of peptides, which, because of their sequence, are particularly prone to give **DKP**s.

In this study, we have investigated the solvent effect on the rate of **DKP** formation from peptides having the N-terminal amino group unprotected, in trifluoroacetate salt form, the form usually obtained from purification of the N-unprotected peptides by HPLC.

The stability of peptide salts is of particular interest because it is widely accepted that the protonation of the N-terminal amino group prevents the formation of **DKP**. That solvents considerably influence chemical reactivity is a well-established phenomenon.[9] A fruitful approach to the analysis of these effects is to treat the logarithm of the observed rate constants by simple or multiple correlation to various parameters characteristic of the solvents.[10] This approach not only provides equations which can also be used to calculate rate constants for solvents not tested experimentally, but by showing the effect of specific solvent characteristics on the reaction rate, the approach also produces useful mechanistic information on reaction pathways and on the structure of the transition state.

A preliminary report has already been published.[11]

Experimental

Materials

The peptide H-Ala-Pro-NH_2 in its trifluoroacetate salt form was synthesised by coupling Boc-Ala-OH with H-Pro-NH_2 using the 1,3-dicyclohexylcarbodiimide–hydroxybenzotriazole method in *N*,*N*′-dimethylformamide.[12] After evaporation of the solvent in vacuum, the crude protected dipeptide was dissolved in water and purified by passing the solution through a mixed bed ion exchange column [Dowex 50WX8 (H^+)/1X8(OH^-)]. After lyophilisation, the dipeptide was Boc deprotected by 100% trifluoroacetic acid (TFA) and then purified by semi-preparative HPLC on a C_{18} column eluted with 0.1% (w/v) TFA in water. ^{1}H-NMR recorded in [$^2H_6$]DMSO at 400 MHz gave δ_H = 1.35 (d, 3H, CH_3, Ala), 1.70–2.30 (m, 4H, $C^\beta H_2$-$C^\gamma H_2$, Pro), 3.45–3.70 (m, 2H, $C^\delta H_2$, Pro), 4.00–4.40 (m, 2H, $C^\alpha H$, Ala and $C^\alpha H$, Pro).

The hydrochloride and acetate salts of the peptide were obtained by passing the trifluoroacetate salt through an anion exchange resin, Dowex 2X1, in the appropriate forms. The **DKP**

Table 1 Observed first-order rate constants for diketopiperazine formation from trifluoroacetate salt of H-Ala-Pro-NH_2 at 20 °C

Solvent	k_{obs}/s^{-1}
Acetone	2.1×10^{-6}
Acetonitrile	1.8×10^{-6}
Butan-1-ol	5.8×10^{-7}
Butan-2-ol	8.5×10^{-7}
Butan-2-one	4.9×10^{-6}
Dimethyl sulfoxide	9.5×10^{-8}
Dimethylacetamide	5.8×10^{-7}
Dimethylformamide	4.0×10^{-7}
Ethanol	2.7×10^{-7}
Ethylene glycol	4.0×10^{-8}
Formamide	9.5×10^{-8}
Hexafluoropropan-2-ol	3.2×10^{-9}
Methanol	1.6×10^{-7}
Nitromethane	2.7×10^{-6}
Propan-1-ol	3.2×10^{-7}
Propan-2-ol	3.9×10^{-7}
Propionitrile	1.4×10^{-5}
Propylene carbonate	6.2×10^{-6}
2,2,2-Trifluoroethanol	1.7×10^{-8}
Water	9.1×10^{-8}

cyclo(-Ala-Pro-) was synthesised and characterised as previously reported.[8]

Kinetic measurements

Solutions of the salts of H-Ala-Pro-NH_2, 5 mmol dm^{-3}, were filtered through a 0.45 μm membered filter and then stored in glass microvials in a thermostatted room at 20.0 ± 0.2 °C. At preselected times, an aliquot was removed and analysed by HPLC on a Beckman model system gold using a Beckman C_8 reversed phase column (4.0 × 250 mm, 5 μm particle size). Peptides were detected using a Beckman model 166 variable wavelength monitor at 214 nm with a Shimadzu C-R6A integrating recorder. Elution was achieved using an isocratic elution, 4% of B for 7 min, followed by a linear gradient, 4–15% of B in 15 min: composition of the solution A was 0.1% (w/v) TFA in water, composition of the solution B was 80% (v/v) acetonitrile and 0.1% (w/v) TFA in water. The flow rate was 1.0 cm^3 min^{-1}. The reaction product was identified by comparison with HPLC traces of authentic sample. Side reactions to **DKP** formation were not noted. The rate constants were calculated by least-squares analysis of the time dependence of the peak area corresponding to the starting peptide and/or to the **DKP**, assuming the rate to be first-order. The fitting of the experimental data was satisfactory for all the samples. The values of the rate constants were reproducible within 10%.

The solvents tested (see Table 1) were of the highest available purity and used without additional purification. The results of a few tests carried out in acetonitrile which had been stored overnight on sodium carbonate, distilled from phosphorus pentoxide and stored in nitrogen atmosphere, did not differ significantly from those obtained with the commercially available solvent.

Results

The reaction rate of **DKP** formation was investigated for the peptide H-Ala-Pro-NH_2 in its trifluoroacetate salt form in twenty polar solvents, including aprotic and hydroxylic ones. Apolar solvents could not be used because of the insufficient solubility of the peptide. Kinetic runs in acetonitrile were also performed in the presence of acetic acid, triethylamine and triethylammonium acetate as catalysts, and on the acetate and hydrochloride salts of the peptide. In all the experiments the reaction was first-order and no side-reaction was detected.

Table 1 shows the values of the first-order rate constants (k_{obs}) measured in the solvents used. k_{obs} spans more than three orders of magnitude. Interestingly, the highest k_{obs} values were measured for solvents with aprotic dipolar protophobic character (acetone, acetonitrile, butanone, nitromethane and propionitrile), according to the classification of Kolthoff.[13] Table 2 reports the values of k_{obs} observed in acetonitrile for the reaction from the hydrochloride, trifluoroacetate and acetate salt of the peptide. These data clearly indicate that the reaction rate depends on the acid–base properties of the counterion of the N-terminal ammonium cation. Increasing the basic character of the anion increases the reaction rate. The acetate salt is the most reactive form among those considered. As regards the catalysis of **DKP** formation, Fig. 1 shows the effect of acetic acid, triethylamine and triethylammonium acetate on the observed rate constant in acetonitrile. The salt triethylammonium acetate proves to be a very efficient catalyst, at a concentration of 0.02 mol dm^{-3} the rate constant is increased by a factor of thousand. The shape of the k_{obs} *versus* [triethylammonium acetate] plot, a rapid increase at low salt concentration followed by a region where k_{obs} is constant, is usually taken as an indication of a change of the rate determining step with the buffer concentration.[14] Less marked is the catalytic effect of acetic acid and triethylamine.

Table 2 Observed first-order rate constants for diketopiperazine formation from H-Ala-Pro-NH_2·HX in acetonitrile at 20 °C

HX	k_{obs}/s^{-1}
HCl	1.3×10^{-6}
CF_3COOH	1.8×10^{-6}
CH_3COOH	3.0×10^{-4}

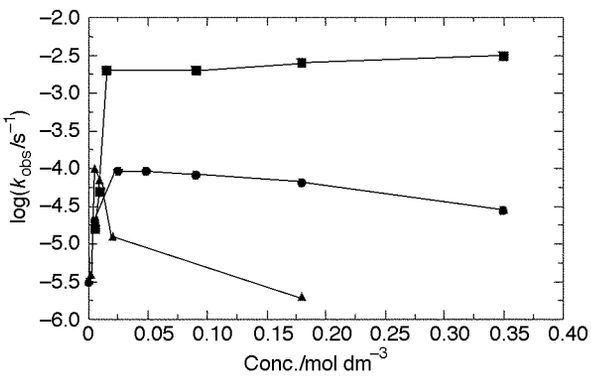


Fig. 1 Dependence of the first-order rate constants for diketopiperazine formation from the trifluoroacetate salt of H-Ala-Pro-NH_2 in acetonitrile on the concentration of triethylammonium acetate (■), triethylamine (▲) and acetic acid (●).

Multiple linear regression analysis

In order to better understand the variations in the rates observed for the title reaction, we treated the kinetic data with the Kamlet–Taft multi-parameter solvent effect equation [15] [eqn. (1)], in which π^*, α and β reflect the dipolarity, the hydrogen

$$\log k = \log k_o + s\pi^* + a\alpha + b\beta + h(\delta H^2) \qquad (1)$$

bond acidity and the hydrogen bond basicity of the solvent, respectively, and δH^2 is the solvent cohesive energy density (cavity term). k_o, s, a, b and h are coefficients depending on the specific reaction and temperature.

Fig. 2 shows the correlation between the rate constants observed and the rate constants calculated by means of eqn. (1) with the coefficients obtained by the fit (Table 3). The fit is sufficiently good; no set of data is further than the others from the straight line. The correlation coefficient is 0.99. The coefficients s, a and b are negative: h is positive.

Table 3 Coefficients of the Kamlet–Taft multi-parameter solvent effect equation [eqn. (2)] for rate constants of diketopiperazine formation from the trifluoroacetate salt of H-Ala-Pro-NH_2 at 20 °C[a]

$\log k_o$	s	a	b	h
−2.0 ± 0.3	−4.5 ± 0.4	−2.1 ± 0.1	−1.4 ± 0.2	0.0043 ± 0.0006

[a] The ranges of the parameters π^*, a, β and δH^2 for the solvents used are 0.41–1.09, 0.00–1.96, 0.00–1.09 and 86–549, respectively.

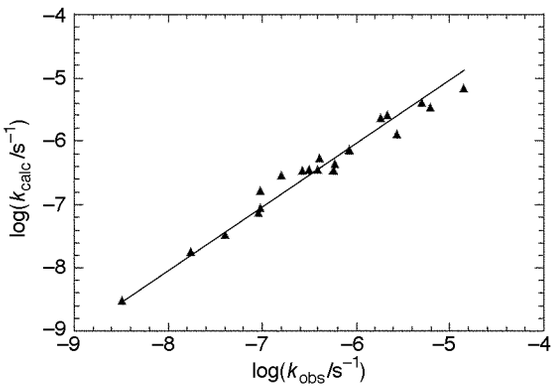
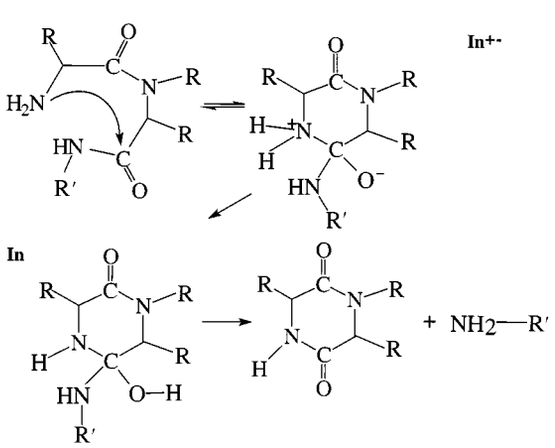


Fig. 2 First-order rate constants for diketopiperazine formation from the trifluoroacetate salt of H-Ala-Pro-NH_2 calculated by the Kamlet–Taft equation with the constants reported in Table 3 against the rate constants observed.

Discussion

The results of the multiple linear regression analysis of solvent effects on the title reaction show that solvents with a high ability to stabilise charged or dipolar solutes, by virtue of charge–dipole or dipolar interactions ($s = -4.5$), and to stabilise hydrogen donor or acceptor solutes ($a = -2.1$ and $b = -1.4$) decrease the reaction rate. Although it is not straightforward to draw inferences about molecular processes from macroscopic properties, in our opinion the solvent effect reported here provides some interesting indications of the reaction pathway.

Previous studies[8] of **DKP** formation in aqueous solution have shown that in this medium only the fraction of the peptide with the deprotonated N-terminal amino group reacts at an appreciable rate. The reaction involves the pre-equilibrium attack of the N-terminal amino group on the carbonyl carbon of the second residue, giving a zwitterionic cyclic intermediate[16] in acid–base equilibrium with various forms characterised by different grades of protonation. Breakdown to the product occurs only from the neutral and anionic forms of the intermediate by parallel steps.[9] The step from the neutral form (Scheme 2) is prevalent and rate-determining from acidic to moderately basic pH. This step is catalysed by polyfunctional acid catalysts, as often observed in cases where a proton switch is involved.[17]



Scheme 2

In organic solvents, peptide salts, as any salts obtained from proton transfer from an acid (H–X) to a base (B), are present in several forms in chemical equilibrium. A simplified description of these equilibria, in which complexes higher than 1:1 and specific solute–solvent interactions are not considered, is given in eqn. (2), where the dashed lines represent hydrogen bonds.

$$X^- + H\text{–}B^+ \rightleftharpoons X^- \cdots H\text{–}B^+ \rightleftharpoons X\text{–}H \cdots B \rightleftharpoons X\text{–}H + B \quad (2)$$

In buffered aqueous solution the fraction of the unprotonated peptide is determined by the pH value fixed by the buffer, whereas in pure organic solvents it depends on the solvating capacity of the surrounding medium. Solvents with a low capability to stabilise ionic forms shift the equilibria to the right. As regards the kinetic aspects of **DKP** formation, it is worth noting that proton transfer between oxygen and nitrogen atoms is usually much faster than the rate observed in the present work,[18] and then eqn. (2) represents equilibria that precede the rate determining step (rds). In these cases the effects of solvents on the reaction rate can be analysed in terms of the free energy difference between the transition state of the rds and the initial ionic substrate. The enhanced reactivity observed with triethylammonium acetate suggests that a proton switch occurs in the rds. Taking these facts into account, our experimental results imply that the pathway proposed for the reaction in water also holds in organic solvents, and that the rds is either the transformation of $\mathbf{In}^{+-}$ to **In** or of **In** to the products (Scheme 2). Both possibilities are consistent with the solvent effect reported here. Solvents with a high ability to stabilise charged and hydrogen donor or acceptor solutes (high values of s, a and b coefficients) should stabilise the ionic form of the substrate more effectively than the transition state of the proposed limiting-step, that is expected to have a reduced ability to form hydrogen bonds due to the delocalization of the charges. Therefore, the net effect of polar and hydrogen donor and/or acceptor solvents is an increase of the energy difference between the ionic substrate and the transition state, hence a decrease of the reaction rate. The coefficient h for the cavity term, which reflects the energetic cost of disrupting the solvent–solvent interaction to create or expand a cavity in the solvent, also provides support for the proposed rds. The positive sign is consistent with the expectation that the solvent shell contracts as a consequence of the weakening of the ion–solute interaction, as the initial ionic substrate converts to the neutral product. The cyclisation step to $\mathbf{In}^{+-}$ is ruled out as the rds because no proton switch occurs in this step.

The mechanistic description reported above also gives a sound explanation for the higher reactivity of salts from weak acids (Table 2). In fact, according to our model, decreasing the acidity of X–H [eqn. (2)] should result in a shift of the multi-equilibrium toward the unprotonated form of the peptide, which would reduce the free energy difference between the reactant and the transition state. Finally, it is worth noting that, because of the small size of the **DKP** ring, only peptides with the first peptide bond in the *cis* conformation can react; hence for most substrate molecules the first step of the overall reaction is the *trans*→*cis* isomerization. However, from previous studies on short Pro-containing peptides, it can be excluded that the large solvent effect reported here is due to the solvent influence on the isomerization step. First, the *trans–cis* equilibrium is only slightly affected by solvents in comparison with the effect reported here.[19] Secondly, the *trans*→*cis* rate constant is several orders of magnitude higher than the higher value measured in this work[20] and consequently cannot be rate determining for the overall reaction of **DKP** formation.

Acknowledgements

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References

1 B. J. Marsden, T. M.-D. Nguyen and P. W. Schiller, *Int. J. Pept. Protein Res.*, 1993, **41**, 313.
2 S. L. Steinberg and J. L. Bada, *J. Org. Chem.*, 1983, **48**, 2298.
3 U. Kertesher, M. Bienert, E. Krause, N. Sepetov and B. Mehlis, *Int. J. Pept. Protein Res.*, 1993, **41**, 207.
4 S. Capasso, F. Sica, L. Mazzarella, G. Balboni, R. Guerrini and S. Sanvadori, *Int. J. Pept. Protein Res.*, 1995, **45**, 567.
5 J. E. Battersby, W. S. Hancock, E. Canova-Davis, J. Oeswein and B. O'Connor, *Int. J. Pept. Protein Res.*, 1994, **44**, 215.
6 (*a*) K. Iénaga, K. Nakamura, M. Kurohashi, T. Nakanishi and T. Ichii, *Phytochemistry*, 1990, **29**, 35; (*b*) J. Møss and H. J. Bundgaard, *J. Pharm. Pharmacol.*, 1990, **42**, 7; (*c*) T. Shiba, H. Uratani, I. Kubota and Y. Sumi, *Biopolymers*, 1990, **20**, 7.
7 M. Bodanszky and J. Martinez, *The Peptides*, ed. E. Gross and J. Meienhofer, Academic Press, New York, 1983, vol. 5, ch. 3.
8 S. Capasso, A. Vergara and L. Mazzarella, *J. Am. Chem. Soc.*, 1998, **120**, 1990.
9 N. S. Isaac, *Physical Organic Chemistry*, Longman Scientific & Technical, 1992, ch. 2.
10 K. A. Connors, *Chemical Kinetics: The Study of Reaction Rates in Solution*, VCH Publishers, New York, 1990, ch. 8.
11 S. Capasso and L. Mazzarella, *Peptides*, 1998, **19**, 1998.
12 M. Bodanszk, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin, 1984.
13 (*a*) I. M. Kolthoff, *Anal. Chem.*, 1974, **46**, 1992; (*b*) C. Reichardt, *Solvents and Solvent effects in Organic Chemistry*, VCH, Weinheim, 1988; (*c*) M. H. Abraham, P. L. Grellier, J. M. Abboud, R. M. Doherty and R. W. Taft, *Can. J. Chem.*, 1988, **66**, 2673.
14 (*a*) E. S. Hand and W. P. Jencks, *J. Am. Chem. Soc.*, 1975, **97**, 6221; (*b*) R. W. Nagorski, T. Mizerski and J. P. Richard, *J. Am. Chem. Soc.*, 1995, **117**, 4718.
15 (*a*) M. J. Kamlet, J. L. Abboud and R. W. Taft, *J. Am. Chem. Soc.*, 1977, **99**, 6027; (*b*) *ibid*., 1972, **94**, 8325; (*c*) M. J. Kamlet, J. L. Abboud, M. H. Abraham and R. W. Taft, *J. Org. Chem.*, 1983, **48**, 2877.
16 (*a*) W. P. Jencks, *Acc. Chem. Res.*, 1976, **9**, 425; (*b*) P. Camilleri, R. Ellul, A. J. Kirby and T. G. Mujahid, *J. Chem. Soc.*, *Perkin Trans. 2*, 1979, 1617; (*c*) A. J. Kirby, T. G. Mujahid and P. Camilleri, *J. Chem. Soc.*, *Perkin Trans. 2*, 1979, 1610; (*d*) J. P. Fox and W. P. Jencks, *J. Am. Chem. Soc.*, 1974, **96**, 1436.
17 (*a*) F. Hibbert, *Adv. Phys. Org. Chem.*, 1986, **22**, 113; (*b*) A. J. Kirby, *Adv. Phys. Org. Chem.*, 1980, **17**, 183.
18 B. H. Robinson, *Proton-transfer Reactions*, ed. E. Caldin and V. Gold, Chapman and Hall Ltd, London, 1975, ch. 5.
19 (*a*) C. Grathwohl and K. Wüthrich, *Biopolymers*, 1976, **15**, 2025; (*b*) C. Grathwohl and K. Wüthrich, *Biopolymers*, 1976, **15**, 2043.
20 (*a*) C. Grathwohl and K. Wüthrich, *Biopolymers*, 1981, **20**, 2623; (*b*) K. B. Wiberg, P. R. Rabblen, D. J. Rush and T. A. Keith, *J. Am. Chem. Soc.*, 1995, **117**, 4261.

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