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Free Energy of Paired Hydrogen Bonds of 2-Aminopyrimidine*

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ABSTRACT: Complex formation between ϵ-caprolactam and 2-aminopyrimidine in cyclohexane has been demonstrated by ultraviolet absorption spectroscopy. The complex is detected by a shift of the absorption band of 2-aminopyrimidine to longer wavelengths. The equilibrium constant for dissociation of the complex was determined with absorbancy values at a particular wavelength and the slope of a plot of $[A - a_P b(P)_0]/(L)_0$ versus A.

The results are consistent with the formation of a 1:1 complex maintained by paired hydrogen bonds of the type in DNA. The equilibrium constant was used to calculate a $\Delta G°$ of 2.23 kcal mole^{-1} for dissociation of the complex.

An important feature of the structure of DNA is the paired hydrogen bonds between the heterocyclic bases (Watson and Crick, 1953). On the basis of melting experiments with synthetic polynucleotides, Crothers and Zimm (1964) have estimated a free-energy change of 2 kcal mole^{-1} for the paired hydrogen bonds of the adenine-thymine base pair. Although a direct determination of the $\Delta G°$ for the dissociation of these bonds would facilitate the interpretation of experiments on DNA strand separation, measurements of intermolecular hydrogen bonding in polar solvents are seriously complicated by solute-solvent interactions. Attempts to determine $\Delta G°$ values with the purines and pyrimidines of DNA in nonpolar solvents failed because these compounds are not sufficiently soluble to provide stable solutions. On the other hand, simple model compounds may provide valuable thermodynamic data on intermolecular hydrogen bonds.

Hydrogen bonds which are similar to those in DNA should be present in a complex between a pyrimidine derivative of the *cis*-amidine type and a compound of the *cis*-amide type. Preliminary experiments indicated that saturated solutions of 4.3×10^{-3} M 2-aminopyrimidine and 0.14 M ϵ-caprolactam could be prepared in cyclohexane at 25°. The effect of the cyclic lactam on the ultraviolet absorption spectrum of the aminopyrimidine was chosen as a suitable model system for the application of a procedure for the determination of $\Delta G°$ values (Rose and Drago, 1959). This model system is based on the observation that a cyclic lactam in a nonpolar solvent will form dimers which are maintained by paired hydrogen bonds (Tsuboi, 1951; Klemperer *et al.*, 1954). A similar structure has been suggested for the dimer of 2-pyridone (Wigler, 1958; Katritzky and Jones, 1960; Krackow *et al.*, 1965).

Experimental

A sample of 2-aminopyrimidine (Mann Research Laboratories) was purified by vacuum sublimation, mp 126.5–127.0°. The ϵ-caprolactam (Calbiochem Organic Chemicals) was recrystallized from ethyl acetate and dried under vacuum, mp 68.0–68.5°. The cyclohexane (Fisher, reagent grade) was purified by passage through a 6- × 30-cm column of dry silicic acid (Mallinckrodt, A.R.). The solvent was stored over anhydrous sodium sulfate and redistilled from sodium-lead alloy (Baker, Dri-Na) just before use.

The ultraviolet absorption spectra of dilute solutions of 2-aminopyrimidine were determined at 25° in a cylindrical cell of 100 mm light path with a Cary Model 14PM recording spectrophotometer. The spectra of more concentrated solutions were determined with rectangular cells and quartz inserts which provide a minimum light path of 0.5 mm.

Results

The ultraviolet absorption band of 2-aminopyrimidine in cyclohexane is given in Figure 1; two vibronic components are found at 2850 and 2920 A and a minor component is at 3025 A. The spectrum of 2.41×10^{-5} M 2-aminopyrimidine was determined in solutions which contained ϵ-caprolactam at 0.14 M

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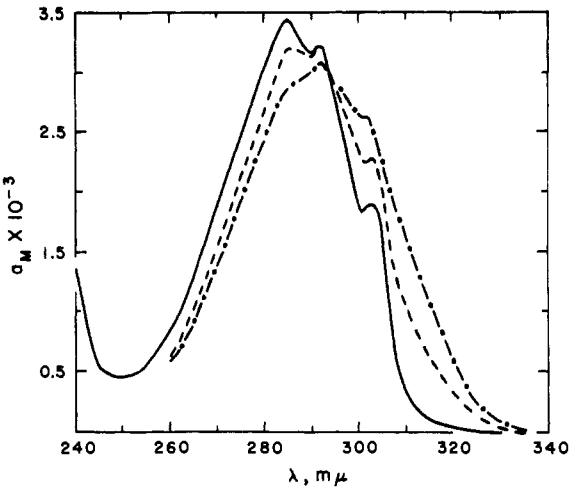


FIGURE 1: The absorption spectrum of 2.4×10^{-5} M 2-aminopyrimidine in cyclohexane in the presence of ε-caprolactam at different concentrations at 25°. ———, lactam = 0; ---------, lactam = 1.8×10^{-2} M; -·-·-·, lactam = 1.4×10^{-1} M.

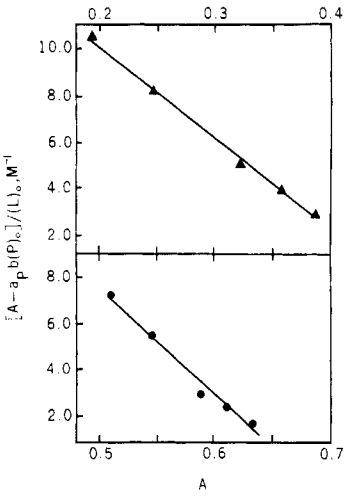


FIGURE 2: A plot of $[A - a_P b(P)_o]/(L)_o$ versus the absorbancy of a 2.4×10^{-5} M solution of 2-aminopyrimidine in cyclohexane with different concentrations of ε-caprolactam. The lines at 3090 A (▲) and 3005 A (●) were calculated by the method of least squares.

and 1.8×10^{-2} M. Although there is no detectable change in the wavelength of the individual vibronic components, a shift to longer wavelengths of the absorption band envelope is observed. A difference spectrum was used to detect an isosbestic point at 2935 A and difference maxima at 3090 and 3005 A. The spectrum of 2-aminopyrimidine in 0.2 M diethyl ether in cyclohexane is similar to the spectrum in pure cyclohexane. The spectrum of the pyrimidine in 2.0 M diethyl ether in cyclohexane, however, is shifted to the red wavelengths. This spectrum resembles the results in solutions of 0.1 M ε-caprolactam.

The spectral changes given in Figure 1 may be used to determine the equilibrium constant from the absorbancy of 2-aminopyrimidine at 3090 and 3005 A, determined with different concentrations of ε-caprolactam. The molar absorbancy of the monomer (a_P) is obtained from the solid-line curve given in Figure 1. The validity of this assumption is supported by the observation that the molar absorbancy of 2-aminopyrimidine in cyclohexane at 2850 A is independent of concentration, within experimental error, from 4.3×10^{-3} to 2.4×10^{-5} M. The reliability of an equilibrium constant determined from spectral data is substantially improved by the selection of wavelengths where a_P is much lower than the molar absorbancy of the complex (a_D) and the absorbancy of ε-caprolactam is zero (Conrow *et al.*, 1964).

In the following equations for a 1:1 complex, K is the equilibrium constant for dissociation, $(L)_o$ and $(P)_o$ are the total concentrations of ε-caprolactam and 2-aminopyrimidine, (D) is the concentration of the complex, A is the absorbancy of the solution, and b is the light path.

$$A = a_P b[(P)_o - (D)] + a_D b(D) \quad (1)$$

$$K = [(L)_o - (D)][(P)_o - (D)]/(D) \quad (2)$$

$$\frac{(D)}{(P)_o} = \frac{A - a_P b(P)_o}{[a_D - a_P]b(P)_o} = \frac{1}{1 + K/[(L)_o - (D)]} \quad (3)$$

Simultaneous solution of equations (1) and (2) gives the equation of Rose and Drago (1959). The application of this equation for the determination of K has been criticized because small errors in $(P)_o$ may lead to a large error in K (Conrow *et al.*, 1964). If experimental conditions are selected, however, to give a negligible (D) in comparison with $(L)_o$ a simplified equation may be derived:

$$K = \frac{(L)_o[a_D b(P)_o - A]}{A - a_P b(P)_o} \quad (4)$$

Since the term $[a_D b(P)_o - A]/[a_D - a_P]b$ is eliminated from this equation for the condition $(L)_o \gg (D)$, a relatively high precision may be attained. Furthermore, equation (4) may be rearranged into linear form; the following equation shows that a plot of $[A - a_P b(P)_o]/(L)_o$ versus A will be a straight line with a slope of $-K^{-1}$ and an intercept of $a_D b(P)_o/K$:

$$\frac{A - a_P b(P)_o}{(L)_o} = \frac{a_D b(P)_o}{K} - \left[\frac{1}{K}\right] A \quad (5)$$

The absorbancy of a 2.41×10^{-5} M solution of 2-aminopyrimidine in cyclohexane was determined at 3090 and 3005 A in the presence of ε-caprolactam from 9.11×10^{-3} to 0.109 M. Linear plots were prepared according to equation (5), and the slopes and intercepts were calculated by the method of least squares. The

TABLE I: Parameters for the Dissociation of the Complex of 2-Aminopyrimidine (2.41×10^{-5} M) and ϵ-Caprolactam (0.0091–0.109 M) in Cyclohexane at 25°.

λ (mμ)	$K \times 10^2$ (M^{-1})	$\Delta G°$ (kcal mole^{-1})	a_P (M^{-1})	a_D (M^{-1})	$(D)/(P)_o$
309.0	2.52	2.18	407	1880	0.27–0.82
300.5	2.13	2.28	1830	2750	0.30–0.86

parameters from the straight lines of Figure 2 are compiled in Table I.

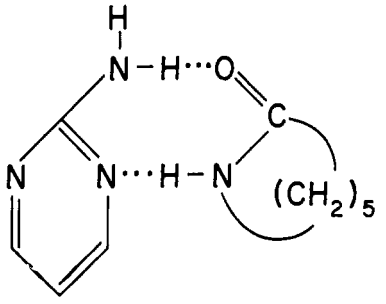
It may be of interest to determine whether the observed absorbancy values are consistent with the formation of a 2:1 lactam–pyrimidine complex. This hypothesis predicts that a plot of $[A - a_P b(P)_o]/(L)_o^2$ versus A should be a straight line. When the A values are treated in this manner, however, the plot is distinctly nonlinear. Thus the data cannot be treated on the basis of a 2:1 complex.

The extent of complex formation, $(D)/(P)_o$, was calculated with absorbancy values and equation (3). The average of the K values of Table I was used to calculate $\Delta G°$ in the usual way; the result is a standard free energy for dissociation of 2.23 kcal mole^{-1}.

Discussion

The effect of 2.0 M diethyl ether on the spectrum of 2-aminopyrimidine is similar to the red shift in the spectrum of aniline in ether solvent (Pimentel, 1957). It has been suggested that the shift to the red wavelengths is due to the hydrogen bond interaction of the amino group with an electron-donor compound. Similar spectral changes are observed with ϵ-caprolactam at much lower concentrations; this finding supports the hypothesis that the complex between the lactam and the aminopyrimidine is maintained by paired hydrogen bonds.

In view of the finding that 2-aminopyrimidine exists largely in the amino tautomeric form (Brown *et al.*, 1955), the following structure for the complex is suggested:



The linear plot of $[A - a_P b(P)_o]/(L)_o$ versus A determined at 3090 A has a slope of −39.6 and a standard deviation of 1.44 (see Figure 2). Thus the 95% confidence interval for the population regression parameter is −35.1 to −44.2 with 3 degrees of freedom. A similar analysis of the data at 3005 A yields a slope of −47.0 with a standard deviation of 3.24 and a confidence interval of −36.7 to −57.3. In addition, an F test for quadratic effects was performed; the F value obtained at 3090 A is 4.99 and the F value at 3005 A is 2.44. The tabulated F value for 1 and 2 degrees of freedom at the 95% confidence interval is larger than the calculated values. The quadratic effects provide a negligible contribution to the regression parameters, therefore, and $[A - a_P b(P)_o]/(L)_o$ is linearly related to A.

The average $\Delta G°$ value of 2.23 kcal mole^{-1}, calculated from the data of Table I, is similar to the value observed for the dissociation of the propionamide dimer in CCl_4 (Badger and Rubalcava, 1954). In addition, the $\Delta G°$ values are close to the estimates for paired hydrogen bonds given by Crothers and Zimm (1964). These authors have suggested that hydrophobic bonds between the stacked bases contribute 7 kcal/mole of the base pairs, and are more important in the stabilization of the DNA helix than the hydrogen bonds.

Acknowledgment

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Binding of Sodium Ions to β-Lactoglobulin*

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ABSTRACT: Measurements on the binding of Na^+ by the allelic forms A and B of β-lactoglobulin reveal no difference in the binding properties of the two forms. No Na^+ is bound at the isoionic point, despite a decrease in *p*H upon addition of NaCl to the deionized crystalline protein at this point. With increasing *p*H, the binding of Na^+ increases to the value of 4.28 per mole of β-lactoglobulin at *p*H 9.48 when the total concentration of sodium ion is 0.06 M. Above *p*H 9.5 the protein becomes irreversibly denatured. A model involving chelation of Na^+ between four carboxyl groups and the four imidazole side chains of the molecule is evoked, and the equation predicting the course of the binding deduced from this model is derived. A representative best fit yields an association constant, k_{Na}, of 150 for the binding of Na^+ to β-lactoglobulin. The calculated curve is compared with previously published optical rotation data in this *p*H region with good fit, from which the conclusion is drawn that both the binding of Na^+ and the configurational change, presumably unfolding, appear to be controlled by the same ionization reaction.

β-Lactoglobulin is one of the few proteins which bind sodium ions. The extent of the binding of Na^+ has been measured previously at *p*H 7.4 (Carr, 1956) and extrapolation of these data to the isoionic *p*H of 5.3 has been used as evidence of the binding of Na^+ or K^+ at the isoionic point as a mechanism for the lowering of *p*H of deionized solutions of β-lactoglobulin upon addition of NaCl or KCl (Nozaki *et al.*, 1959; Tanford and Nozaki, 1959). Studies of other proteins which bind Na^+ or K^+, particularly myosin, have been interpreted by a mechanism involving the chelation of the cation by carboxyl and imidazole or amino groups, thus implying a degree of three-dimensional structural specificity in the protein (Lewis and Saroff, 1957; Saroff, 1957a). In order to investigate this subject in greater detail, the study of the *p*H dependence of Na^+ binding to β-lactoglobulin was undertaken.

Experimental Procedure

Source, Isolation, and Crystallization of Protein. Milk from cows known to be homozygous for either the A or B form of β-lactoglobulin was obtained through the generosity of Dr. C. A. Kiddy of the Agricultural Research Station, Beltsville, Md. The isolation procedure of Aschaffenburg and Drewry (1957) was used, and crystallization was accomplished by deionizing a 0.25% protein solution with a Dintzis (1952) column. Crystals of β-lactoglobulin A formed spontaneously, but crystallization of β-lactoglobulin B was accomplished only after seeding deionized solutions with crystals of this form kindly furnished by Dr. R. Townend of the Eastern Regional Research Laboratory, Philadelphia, Pa. No difference in Na^+ binding was observed between β-lactoglobulin A crystallized twice, as opposed to once, and ultracentrifugation analysis revealed a single symmetrical peak. β-Lactoglobulin B was crystallized only once in view of these results.

Preparation of Protein Solutions. All protein solutions were made by dissolving deionized crystalline protein with dilute NaOH to reach the desired *p*H, and then adjusting the free Na^+ concentration to approximately 0.05 M with NaCl. NaOH solutions were standardized against standard HCl and H_2SO_4 solutions, in turn referred to potassium acid phthalate as a primary standard. NaOH standards were kept in an atmosphere of N_2 and restandardized just prior to each series of measurements. Standard NaCl solutions were made from ACS analytic reagent grade NaCl, dried at 105° for 24 hours. Deionized water was used throughout. Protein concentration measurements were made at 278.5 mμ on a Beckman DU spectrophotometer, using an optical density $E^{1\%}_{1cm}$ value of 9.66, which was determined by dry-weight analysis of three samples of deionized β-lactoglobulin A after exposure to 105° for 24 hours. A

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