The Preparation and Characterization of Reduced Methylated Ribonuclease T1

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ABSTRACT: To study the contribution of disulfide bonds to protein stablity, a derivative of ribonuclease T1 (RNase T1) with both cystine residues reduced and methylated has been prepared using an improved procedure adapted from that of Heinrikson [Heinrikson, R.L. (1971) J. Biol. Chem. 246, 4090]. Characterization using urea and thermal denaturation show that the reduced, methylated ribonuclease T1 is about 8 kcal/mol less stable than RNase T1, but is .5 and 1 kcal/mol more stable than reduced carboxyamidomethylated and reduced carboxymethylated RNase T1, respectively. It is suggested that this difference in stability arises from the increase in conformational entropy of the unfolded state and the size and hydrophobicity of the blocking groups.

It was observed thirty years ago that unfolded protein can refold itself in a test tube (Anfinson et. Al., 1961). This work demonstrated that all the information needed to fold a protein is present in the amino acid sequence through the residues' intramolecular and solvent interactions. Unforutnately, we still lack the ability to use this information to predict the folded structure of protein. This inability, termed the protein folding problem, is one of great practical and theortical interest. One way to approach this problem is to use chemical modification or mutagenesis to add or remove an interaction that contributes to the folded stability of protein. One important type of interaction is a disulfide bond between two cysteine residues which serves to cross-link different regions of the protein. Disulfide bonds greatly stabilize folded protein; niether ribonuclease A nor bovine pancreatic trypsin inhibitor can fold when their disulfide bonds are removed. Ribonuclease T1 also unfolds when its two disulfide bonds are broken, but is able to refold completely under stabilizing conditions of low temperature and moderate salt concentrations (Oobatake et. al., 1979). This refolding allowed Pace et. al. (1988) to examine the folding process thermodynamically using derivatives with reduced and blocked disulfide bonds. Unfortunately, these derivatives contain bulky, polar blocking groups which introduce additional

interactions into the folded state of the modified protein and cloud the interpetation of the results. The focus of this work has been to prepare a reduced derivative of RNase T1 with methyl blocking groups. The small size and hydrophobic nature of the methyl function allows any changes in the thermodynamics of the system to be attributed to the loss of the disulfide cross-links alone, giving a clearer understanding of the role of this interaction in the folded protein.

Methods and Materials

Dithiothreitol (DTT) was obtained from either Sigma or Calbiochem. Ammonium bicarbonate was purchased from Baker. Gylcine, acetonitrile, TRIS, EDTA, sulfosalicylic acid, trichloracetic acid, sodium formate, and sodium chloride were obtained from Fisher. Schwarz/Mann Biotech was the sole suppiler of 'ultra pure' urea. Methyl p-nitrobenzenesulfonate (MNBS) was obtained from Kodak. Acrylamide and other gel electrophoresis materials were purchased from Biorad. Ribonuclease T1 was isolated and purified from Escherchia coli as described by Shirley and Laurents (1990). Reduction of the protein's two cystine residues was accomplished by incubation of 15 mg (1.4 umol) of protein in a nitrogen purged, 5ml solution of 20 mg (130 umol) dithiothreitol (DTT), TRIS/EDTA (.1 M and 5 mM, respectively) pH 8.7, 25% acetonitrile, and 6 M urea for 2 hours at 37oC.

A new method of blocking the reduced cystines with methyl functions was determined by variation of pH (6 to 10), denaturant, temperature (22 to 37 °C), reaction time (1 minute to 3 hours), and quantity of the methylating agent, methyl p-nitro benzenesulfonate (MNBS) (80 to 300 mg, 370 to 1400 umol). Because of its size and charge, RM-T1 was assumed to migrate in acrylamide gels at the same rate as reduced carboxyamidomethylated T1. Reaction conditions which increased the population of such a band while decreasing the number and population of other bands were used as selection

criteria. Methylation was stopped by addition of excess thiol. The protein derivative was seperated from organic reagents in the reaction mixture and purified by gel filtration (Sephadex G-25 and G-50, respectively, Pharmacia). The derivative was lyopholyzed and stored at -20oC until use. For some preparations, the reduction and methylation processes were repeated to complete the methylation. Polyacrylamide gel electrophoresis was used to determine the purity of the product. Gels contained 5% (stacker) and 30% (separating) acrylamide and were run at room temperature. Gels were stained with an aqueous solution of 10% sulfosalicylic acid, 10% trichloroacetic acid, and 1% Coomassie Brilliant Blue for twelve hours. An aqueous solution of 25% methanol and 10% acetic acid was used to destain gels.

Unfolding using urea was followed by intrinsic fluorescence (278 nm excitation, 320 nm emission) with a Perkin-Elmer MPF 44B spectrophotometer. Fluorescent measurements were made in 1 cm2 cuvettes thermostated to 12.5 oC. Thermal denaturations were monitered by change in the opitical rotation at 295 nm of a .1% protein solution in round 1 cm2 cells using a Cary 60 spectropolarimeter. All denaturations occurred in the presence of .25 M sodium chloride and .1 M sodium formate at pH 5.

Results

The following methylation conditions were found to minimize the size and number of electrophoretic impurity bands. Urea was used to denature the protein. Denaturation is necessary to expose the buried 6-103 disulfide bond. To the reaction mixture, 2 ml of a nitrogen purged solution of 2M glycine buffer (pH 10) was added. Immediately thereafter, 200 mg (920 umol) of MNBS in 1.5 ml of purged acetonitrile was added. The reaction was allowed to proceed at 37 oC for five minutes before stopping with 200 mg (1.3 mmol) of DTT in 1 ml water. Gel filtration cleanly seperated the protein from other reagents. Acrylamide gel electrophoresis revealed a product which migrated at a rate equal to that of reduced carboxyamidomethylated ribonuclease T1 (RCAM-T1), and 57% the rate of wild type protein. This is expected since both RM-T1 and RCAM-T1 are unfolded under electrophoresis conditions and will posess a larger effective radius. As neither blocking group is charged, these derivatives have the same net charge. Two faint additional bands were also present on the gels of the reaction product. One, representing less than 5% of the total protein, migrated at a rate only 40% that of native protein and could be removed by repeated gel filtration or methylation. A second faint band or 'halo' immediatedly preceeded the RM-T1 band. Neither gel filtration nor ion exchange chromatography proved effective in removing this

band. Although it is not known whether the halo represents a gel artifact or is a distinct derivative of RNase T1, the affect of this possible impurity upon the results of the denaturation experiments was assumed to be negligible.

Evidence from chemical denaturation data suggest that this assumption is a good one. If the ´halo´ were a distinct derivative, denaturation of a mixture would produce a denaturation profile with two distinct transitions or a broadened single transition. Neither of these is observed. A broadened single transition would also decrease dependence of free energy of folded on denaturant concentration producing a smaller m value. The m value for RM-T1 is slightly higher than that of RCAM-T1, suggesting that no significant transition broadening is occuring. The ´halo´ may represent a distinct by product which unfolds simulataneously with RM-T1 under the conditions used.

The results a of typical urea denaturation of the methylated protein is shown in Figure 1. The analysis of such data has been throughly discussed in the literature (Pace ,1975; Pace et al., 1989), but will be reviewed here. RNase T1 unfolds by a two-state mechanism (J. Thomson et al., 1989; J. Thomson, unpublished observations). By estimating the fluorescence of folded and unfolded protein, Yf and Yd, respectively, in the transition region by linear extrapolation one may estimate the fraction of protein that is unfolded, Fd, by using:

$$Fd = (Yf - Y) / (Yf - Yd) \qquad (1)$$

where Y is a fluorescence value obtained in the transition region. Knowing Fd allows one to calculate the equilibrium constant, K, with the equation:

$$K = Fd / 1 - Fd \qquad (2)$$

In turn, the equilibrium constant may be used to calculate a value for ΔG, the free energy of unfolding using the central equation of thermodynamics:

$$\Delta G = -RT \ln K \qquad (3)$$

Values for Fd, K, and ΔG are calculated for each data point in the transition region. A plot of ΔG versus urea concentration, shown in Figure 2, is a rich source of thermodynamic data. Of great interest is ΔG_{H_2O}, the conformational stability of the protein and the value for G in the absense of denaturant. This value is obtained by linear extrapolation of the ΔG versus denaturant data to zero urea concentration using the equation:

$$\Delta G_{H_2O} = \Delta G - m\ (urea) \qquad (4)$$

where m is the slope of the line and (urea) is the concentration of denaturant in moles per liter. The plot may also be interpolated to find the midpoint of the unfolding curve, (urea)1/2. The m value is also of interest because its magnitude is proportional to the amount of interaction of the newly exposed protein with denaturant. The results of these unfolding curves is given in Table 1. For comparison, values obtained with RCAM-T1, and carboxymethylated T1 (RCM-T1) from Pace et. al. (1988) are also included in Table I. These results reveal that breaking the disulfide bonds decreases the conformational stability of RNase T1 by approximately 8 kcal/mol. Amoung the derivatives, RM-T1 is about .5 and 1 kcal/mol more stable than RCAM-T1 and RCM-T1, respectively. Breaking the disulfide bonds increased the m value from 1205 for native RNase T1 to about 1800 for RM-T1 and RCAM-T1 and 2060 for RCM-T1.

The results from the thermal unfolding of RM-T1 are shown in Figure 3. When followed by optical rotation, values of Fd and K may be calculated for each point in the transition region by the method used in the chemical denaturation studies. Interpolation to Fd = .5, where K = 1, gives the midpoint of the thermal unfolding curve, Tm, which is analogous to (urea)1/2. Calculation of conformational stability from a thermal unfolding experiment requires a value for ΔHm, the enthalpy for folding, and ΔCp, the heat

capacity change for folding. ΔHm may be determined as the slope of the van't Hoff plot of ln K versus 1 / T shown in Figure 4. A value for ΔCp may be calculated by taking the second derivative of the van't Hoff plot. Unfortunately, as shown in Figure 5, the data were not of sufficent quality to a value for ΔCp. ΔCp has been estimated previously for RNase T1 (C.N. Pace and D.V. Laurents, 1989) and more recently by calorimetery (J. Thomson, personal communication) to have a value of 1650 kcal/mol °K. This value is used to estimate the conformational stability of RM-T1. The results from thermal unfolding studies are given in Table II. The differences in stability obtained by urea and thermal unfolding are in excellent agreement. This suggests that the unfolded states for urea and thermal unfolding are equivalent.

Discussion

Gels of methylated protein prepared by the method of Hienrikson revealed the presence of several bands. In addition to a band which tracked with RCAM-T1, the correct product, were a series of bands with decreasing mobility and concentration. A plot of the log of multiples of 11089 daltons, the molecular weight of RNase T1, vs relative mobility at ca. 30 °C was linear, suggesting that this series of bands consisted of unfolded protein dimerized, trimerized, etc by intermolecular disulfide bonds. Bands of mobilities between that of RCAM-T1 and RNase T1 were also

present. These may be protein in which only 1 disulfide bond had reacted. Both types of impurities suggested that the reaction was not proceeding to completion, thus conditions were modified to be more vigorous. It was found that the pH giving the best results was about 9.9. At this pH the thiol groups would be ionized and highly reactive. Methylation above pH 10 was not explored for fear of basic hydrolysis of peptide bonds and possible methylation of tyrosine residues. Urea proved to be the denaturant of choice. MNBS proved to be only slightly soluable in a solution of concentrated guanidinium salt. Carrying out the reaction at 37oC also improved the solubility and reactivity of the MNBS. The quantity of MNBS used was also an important parameter. Hienrikson suggested a two fold equivalent excess of MNBS per thiol, however it was found that under these conditions a 3.5 fold excess was required for most efficent methylation of RNase T1. Even under these optimial methylation conditions, a small amount of ´dimer´ protein was observed on gels. Although this was generally removed by three seperate runs down a gel exclusion column, repeating the methylation procedure entirely was also found to be effective at eliminating this band. Removal by repeating the reduction and methylation steps was less tedious and resulted in a higher yield than repeated gel exclusions. Another possible way to improve the purity of the product would be to conduct both the reduction and methylation under strict anaerobic conditions.

While the solutions used in these reactions were initially purged with nitrogen and generally kept sealed, air was able to contact solutions when seals were broken to add reagents. It is quite probable that entering oxygen is able to oxidize the thiols to form the intermolecular disulfide bonds which require so much effort to remove. The use of septums, nitrogen gas, and addition of reagents through syringe will prevent the entry of air and quite possibly eliminate the dimer band without resort to additional chemistry.

Many studies have shown disulfide bonds affect protein stability by two mechanisms. Schellman (1955) and Flory (1956) first considered the effect of a cross-link on protein stability over thirty years ago. Their studies and others (Goldenberg, 1985) have revealed that the addition of a disulfide bond to unfolded protein reduces its conformational entropy. This entropy change, S, can be estimated using an emperical formula given by Pace et. al. (1988):

$$\Delta S = -2.1 - (3/2)\ R \ln n \qquad (5)$$

where R is the gas constant and n is the number of residues in the loop formed by the disulfide. A greater reduction in entropy is obtained by increasing the loop size. This reduction in entropy raises the free energy of the unfolded states. Unless strained, disulfide bonds have little effect on folded protein. Thus, disulfide bonds act by destabilizing the unfolded states of protein. This effect

explains the large decrease in stability of RNase T1 when its disulfides are reduced and blocked.

However, disulfide bond formation can introduce strain into folding protein by forcing residues to adopt less favorable bond lengths and angles. The disulfide bond itself requires a specific geometry which is often strained in folded proteins. Both types of strain are largely relieved upon unfolding and favor that process. Bacause of strain, many attempts to improve protein stability by the introduction of disulfide bonds have failed (Wetyl, 1987). For reduced methylated ribonuclease T1, a different sort of strain, steric replusion, is added to the folded state by forcing the protein to accomidate four methyl groups. With more bulky blocking groups, greater strain is present in folded RCAM-T1. The four carboxymethyl groups of RCM-T1 are sterically and eletrostatically replusive. A favorable electrostatic interaction of these groups is achieved by protonation of the carboxylate functionals, but this is a energetically unfavorable process at pH 5. This loss of stability due to strain is partically compensated for by the release of disulfide angle strain and, especially for RM-T1, the hydrophobic effect. It is probable that these issues account for the differences in conformational stability observed amoung the derivatives.

Consideration of both effects suggest that the largest stabilization of the folded state will result when disulfide bonds cross-link groups that are held rigidly in the folded

state and possess the optimum positioning for disulfide bond geometry.

It is interesting to consider the effects causing the increase in the m value for the derivatives. The m value may be increased if the intermediate folding states are less populated. This suggests that the derivatives approach a two state folding to a better approximation than RNase T1. Since a two state mechanism has been well established for RNase T1 (Thomson et. al., 1989), it is unlikely that this effect is giving rise to the changes in the m value. For two state folding, the m value is dependent on the interaction of unfolded protein with solvent. A larger value of m would be result if breaking the disulfide bonds allowed more interaction of the unfolded states with urea. This seems reasonable. At pH 5.0, unfoldied RNase T1, RM-T1, and RCAM-T1 possess a net charge of -4. The four carboxymethyl functions in unfolded RCM-T1 would increase its net charge at this pH to -8. Increased electrostatic replusion from this larger net charge may result in expanded unfolded states which are more exposed to urea. This would account for the larger m value observed for RCM-T1.

This study confirms the important contributions of disulfide bonds to the conformational stability of ribonuclease T1. Changes in stability of the derivatives arises both from the loss of the disulfide bond and from the interactions of the blocking groups. Of these the loss of the disulfide bond is probably the more important, given the

small differences in stability between the derivatives.

Table Legends

Table I [a] Data for RNase T1, RCAM-T1, RCM-T1 are from Pace et. al. (1988).

Table II [a] Data for RNase T1, RCAM-T1, RCM-T1 are from Pace et. all. (1988).

Table I: Urea Denaturation of RNase T1 and Derivatives

protein[a]	ΔG kcal/mol	[urea]1/2 mol/liter	m cal/mol/liter
RNase T1	10.1	8.42	1205
RM-T1	2.2	1.20	1830
RCAM-T1	1.6	.88	1790
RCM-T1	1.0	.51	2060

Table II: Thermal Denaturation of RNase T1 and Derivatives

protein[a]	ΔG	Tm	Hm
	kcal/mol	°C	kcal/mol
RNase T1	10.2	59.3	95
RM-T1	1.9	24.4	56
RCAM-T1	1.5	21.2	50
RCM-T1	0.9	16.6	46

Figure Legends

Figure 1. Urea unfolding curve for RM-T1 at pH 5.0, .1 M sodium formate, .25 M sodium chloride, 12.5 °C.

Figure 2. ΔG as a function of urea concertration. ΔG was calculated using the data in Figure 1. The least-squares fit of the line using eq 2 gave GH20 = -1832 (urea) + 2205.

Figure 3. Thermal unfolding curve for RM-T1 at pH 5.0, .1 M sodium formate, .25 M sodium chloride.

Figure 4. Van't Hoff plot for RM-T1, using the data from Figure 3.

Figure 5. Calculation of ΔCp. The points given are the difference in consecutive values for ΔHm calculated from Figure 4 data.

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FIG 1

FIG 2

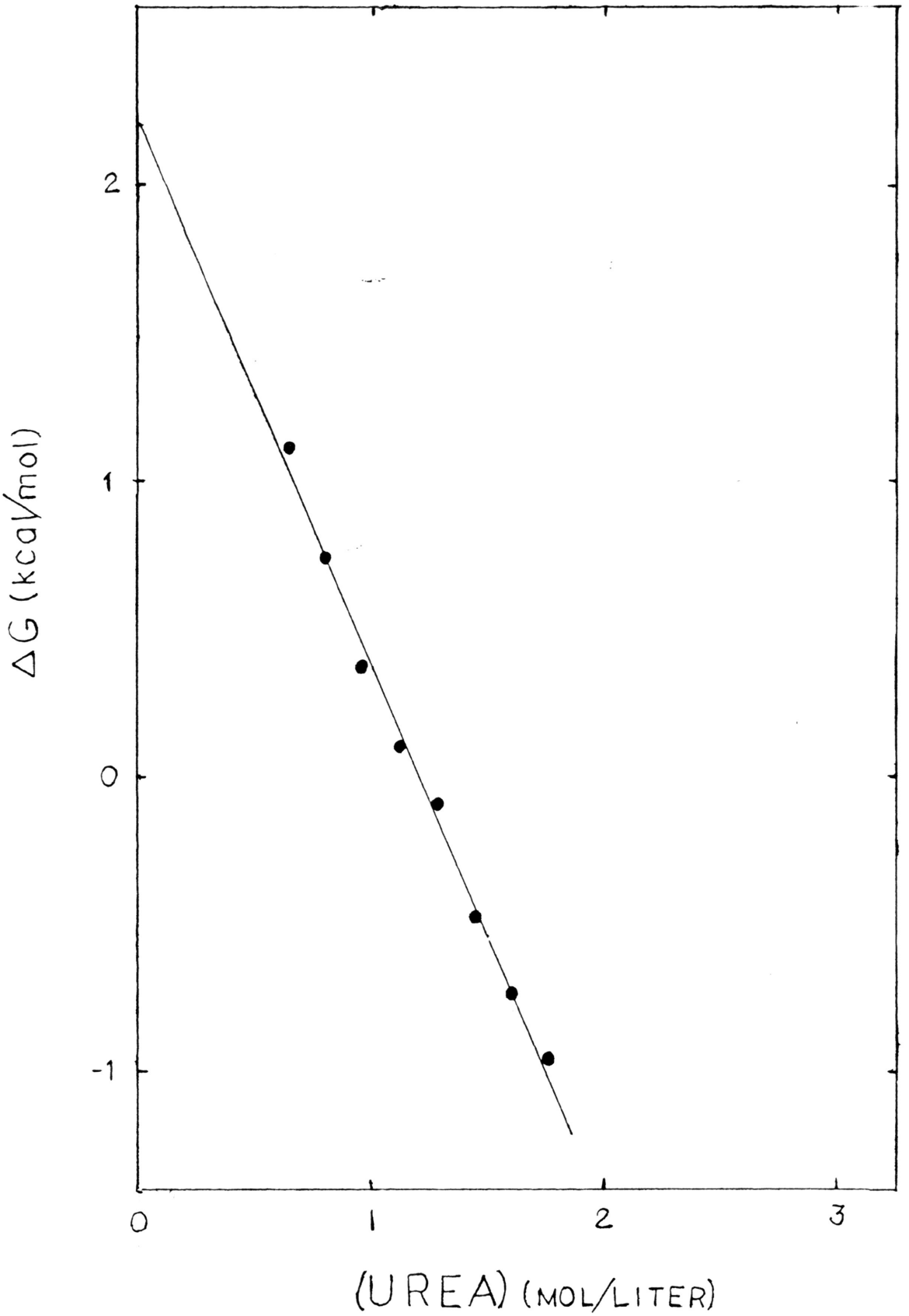

FIG 3

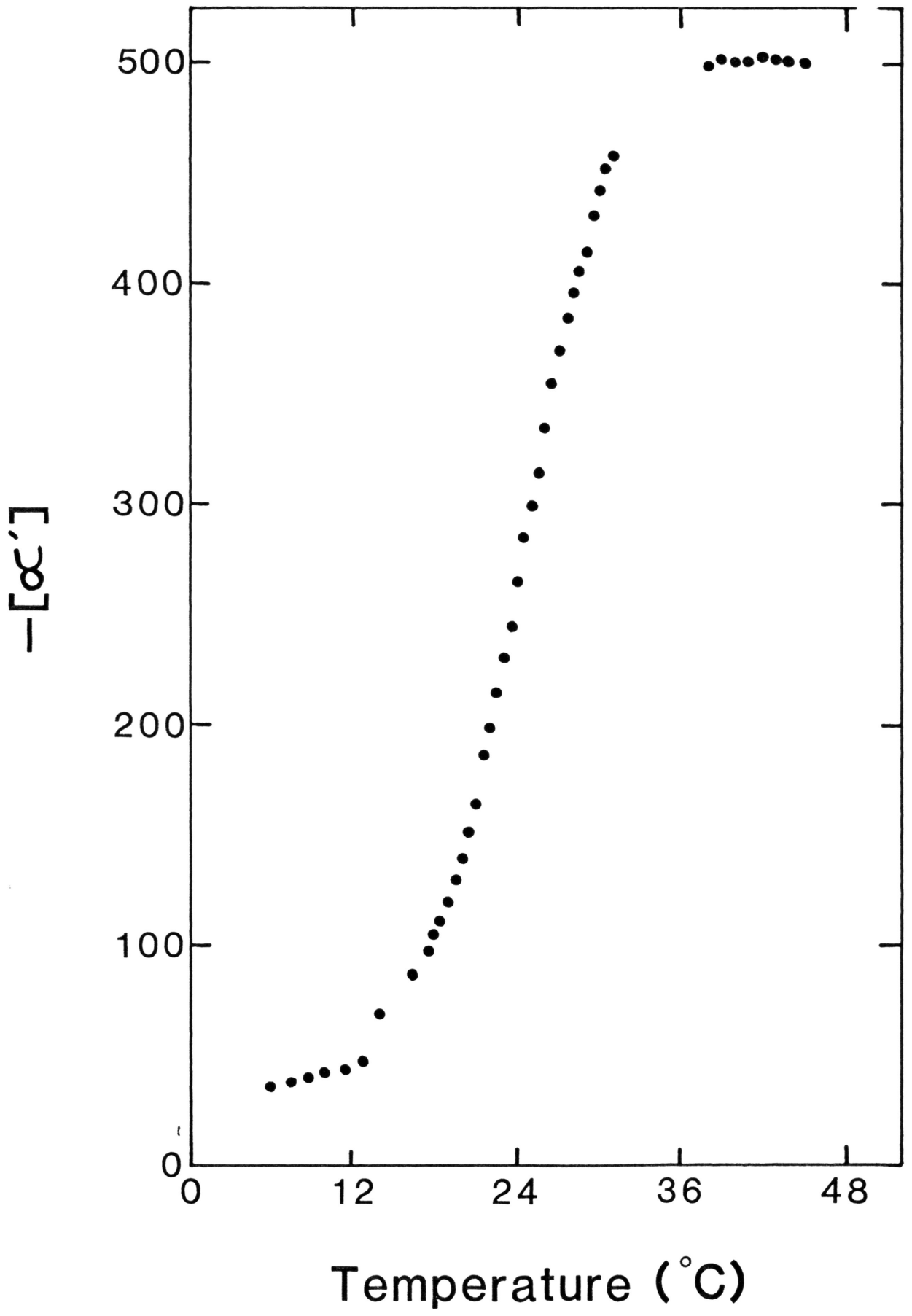

FIG 4

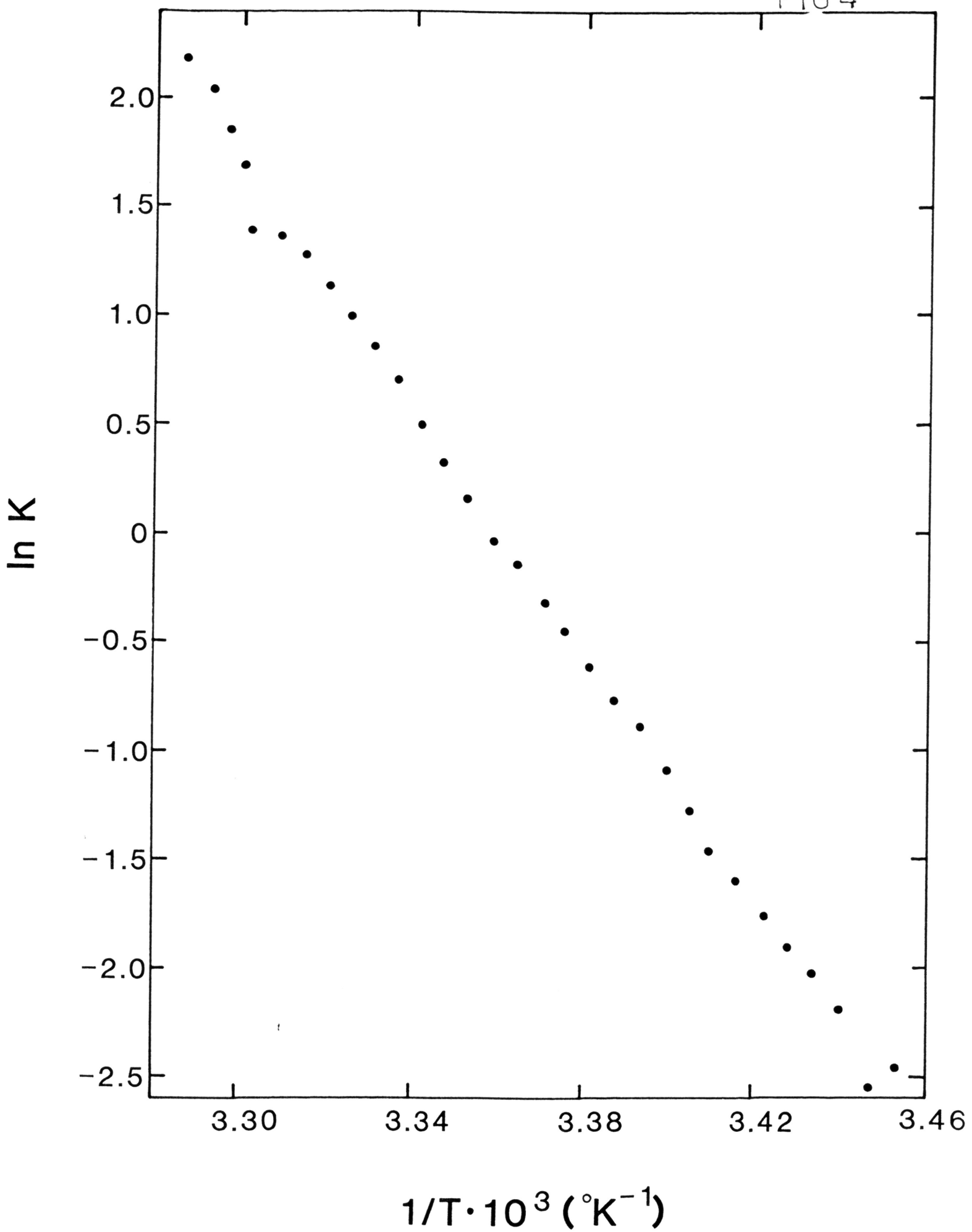

FIG 5