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Cold Inactivation of L-Threonine Deaminase from *Rhodospirillum rubrum*

Involvement of Hydrophobic Interactions

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Threonine deaminase from *Rhodospirillum rubrum* is subject to reversible cold inactivation at low protein concentrations. The inactivation appears to involve a dissociation of the native tetrameric protein to dimers. The standard free energy of dissociation at 0 °C was +7.8 kcal/mole, while ΔH was −3.2 kcal/mole and ΔS was −41 entropy units. These values suggest that hydrophobic interactions are important in the maintenance of the tetrameric structure.

The labilization of the protein by low concentrations of urea and the protection by high salt concentrations also indicate the importance of hydrophobic bonds in the protein. However, the effects of pH and low salt concentrations on the cold inactivation suggest that ionic bonds do play some role in subunit interactions.

The normal allosteric modifier for threonine deaminase, L-isoleucine, protected the enzyme against cold inactivation. Although the *R. rubrum* enzyme is virtually insensitive to isoleucine inhibition, it is clear that this ligand can induce profound changes in the protein structure.

Current theories of the regulation of enzyme activity by allosteric modifiers [1,2] are based on conformational changes in protein structure mediated through the interactions of subunits. Although sophisticated mathematical models have been developed to describe the effects of ligand binding on such interactions, relatively few data are available for the elucidation of the nature of intersubunit bonds. In general, subunit interactions can be mediated through three types of noncovalent bonds: hydrogen bonds, ionic bonds and hydrophobic bonds. Although particular ionic or hydrogen bonds may play crucial roles in proteins, the high dielectric constant of water along with its high hydrogen-bonding capability considerably reduce the free energy of stabilization that these types of bonds might contribute to the protein structure. In recent years, therefore, increasing attention has been focused on the role of hydrophobic forces in proteins [3—5]. Although quantitative information on these forces has come primarily from studies with model systems [5—7], we believe that the study of cold-sensitive enzymes may provide a direct experimental approach to the study of hydrophobic interactions in proteins.

Enzymes. L-Threonine deaminase has also been referred to as threonine dehydrase and threonine dehydratase. The systematic name is L-threonine hydro-lyase (deaminating) (EC 4.2.1.16). Other enzymes: pyruvate carboxylase (EC 6.4.1.1); 17β-hydroxysteroid dehydrogenase (EC 1.1.1.51); pyruvate kinase (EC 2.7.1.40); glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); and alcohol dehydrogenase (EC 1.1.1.1).

A cold sensitive protein can be defined as one that is less stable at 0 °C than at 25 °C. For a variety of reasons, this phenomenon may be ascribed to the disruption of hydrophobic interactions in the protein [8], and, in many cases examined, the cold inactivation is indeed accompanied by a dissociation of the protein into subunits [9—14]. Thus, a quantitative study of such a cold-induced dissociation could yield direct measurements of the strength of the hydrophobic interactions between protein subunits.

This communication describes the cold inactivation of the threonine deaminase of *Rhodospirillum rubrum* with particular reference to the thermodynamics of the inactivation process and its relationship to current concepts of hydrophobic bonding.

EXPERIMENTAL PROCEDURES

Materials

L-Threonine (*allo*-free) and L-isoleucine were obtained from Calbiochem. Bovine serum albumin was supplied by Sigma Chemical Company. Yeast alcohol dehydrogenase was obtained from Worthington. Urea, purchased from the J. L. Baker Company was recrystallized from 95% ethanol containing 0.1% concentrated HCl. The crystals were washed with 95% ethanol cooled to −15 °C and dried at 40 °C for 48 h. Sephadex G-150 was a product of Pharmacia Fine Chemicals. All other chemicals were of reagent grade.

Preparation of Enzyme

The method used for the preparation of threonine deaminase from *R. rubrum* is described in the preceding paper [15]. The enzyme was homogenous as demonstrated by disc-gel electrophoresis and ultracentrifugation.

Assay of the Enzyme

Threonine deaminase was assayed by the spectrophotometric method of Davis [16] as described in detail in the preceding paper. In brief, enzyme was added to a reaction mixture containing 100 μmoles potassium phosphate buffer, pH 7.8 and 50 μmoles L-threonine in a final volume of 1.0 ml and the increase in absorbance at 230 nm was followed. The assays were carried out at 25 °C in a cuvette with a 1 cm path length.

Sephadex G-150 Column Chromatography

The molecular weight of the cold-inactivated enzyme was estimated on Sephadex G-150 by the method of Andrews [17]. Sephadex G-150 was allowed to swell in water for several days and the fines were removed by decantation. The gel was packed into a column 77×1.4 cm and equilibrated at 0 °C with 50 mM potassium phosphate buffer, pH 8.0, containing 1.0 M urea. Threonine deaminase (1.8 mg) in 2.0 ml of 0.5 M potassium phosphate buffer, pH 8.0. containing 2 M urea was incubated at 0 °C for 3 h and applied to the column. The enzyme was eluted at 4 °C at a flow rate of 8 ml/h and 1.0 ml fractions were collected. Threonine deaminase was located by measuring the absorbance at 225 nm.

The G-150 column was calibrated by eluting a mixture of blue dextran, bovine serum albumin (1 mg) and alcohol dehydrogenase (400 μg) under the same conditions as above. The blue dextran and the bovine serum albumin were located by their absorbance at 225 nm, while the alcohol dehydrogenase was located by assaying for its activity by the method of Vallee and Hoch [18].

Protein Determination

Protein concentration was determined by the method of Lowry *et al.* [19].

RESULTS

Cold Inactivation and Reactivation

The general features of the cold-inactivation and reactivation of the threonine deaminase are shown in Fig. 1. Cold-inactivation experiments were carried out by diluting isoleucine-free enzyme into 0.50 M potassium phosphate buffer, pH 8.0, containing 0.5 mg/ml bovine serum albumin. The enzyme, in a volume of 1.0 ml in a small test tube (12×100 mm), was assayed immediately after dilution and then placed into an ice bath and stirred manually. At various times after exposure to °0 C, small aliquots were removed with chilled micropipets and assayed at 25 °C using the spectrophotometric assay. No reactivation was observed during the short time of the assay.

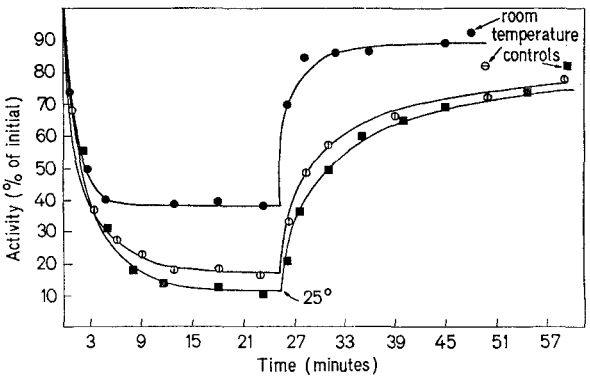

Fig. 1. *Cold-inactivation and reactivation of threonine deaminase.* Immediately after dilution in 0.50 M potassium phosphate buffer, pH 8.0, supplemented with 0.5 mg/ml bovine serum albumin, the enzyme was assayed for activity. The samples were then placed at 0 °C and aliquots were removed at various times and assayed at 25 °C. Enzyme concentrations; 12 μg/ml (■), 24 μg/ml (⊙), and 120 μg/ml (●)

The inactivation rate was very rapid and an equilibrium was achieved within 20 min. This is a true equilibrium, as demonstrated by the near total recovery of enzyme activity upon rewarming. When correction was made for the loss in enzyme activity at 25 °C, the three cold-inactivated samples were found to regain approximately 95% of their activity after 30 min at 25 °C. This reversibility was aided by the inclusion of 0.5 mg/ml of bovine serum albumin in the samples and the controls. Albumin did not have any effect on the rate or on the extent of inactivation. The same results were obtained when serine was used as the substrate in the activity assays.

The rates of inactivation and reactivation were too rapid, and the method of following them too inaccurate, to allow any analysis of the kinetics of cold inactivation. Therefore, the dependence of the equilibrium levels on protein concentration was analyzed in detail.

Protein Dependency of Inactivation

From the physical characterization of the enzyme [15], it appears that the *R. rubrum* threonine deaminase is a tetramer made up of subunits of 46000 molecular weight. The dependence at 0 °C of the equilibrium activity on enzyme concentration, as shown in Fig. 1, suggests that the cold inactivation involves

a dissociation of the enzyme. Using the treatment of Gawronski and Westhead [20], it is possible to obtain a value for the average number of subunits formed in the dissociation. A general expression for the disosciation is given by,

$$E_4 \rightleftharpoons nE^* \quad (1)$$

where n is the number of subunits formed, E_4 is the active tetramer and E* is the subunit. Since over 90% of the activity was lost when the enzyme was inactivated at a protein concentration of 12 μg/ml, it is likely that the subunits are inactive. The equilibrium constant for this dissociation can be written as

$$K_d = \frac{[E^*]^n}{[E]}. \quad (2)$$

Since the total amount of enzyme, $[E_T]$, is given by

$$[E_T] = [E] + [E^*]/n \quad (3)$$

We can rewrite Equation (2) in terms of $[E_T]$ and [E] as

$$K_d = \frac{[E_T - E]^n (n)^n}{[E]}. \quad (4)$$

This equation can be rearranged and written in logarithmic form to yield the expression

$$\log [E] = n\log [E_T - E] + n\log (n) - \log K_d. \quad (5)$$

Assuming that $[E_T]$ is given by the activity before exposure to 0 °C and that dissociated enzyme is inactive, a plot of log (equilibrium activity) *versus* log (initial activity minus equilibrium activity) should give a straight line of slope n, the average number of subunits. This plot (Fig. 2) has a slope of 1.75, suggesting that at 0 °C the equilibrium for the enzyme can be described as

$$E_4 \rightleftharpoons 2E_2. \quad (6)$$

Since the n-value obtained is only an average value, it is possible that the dissociation is more complex than that indicated in Equation (6). However, the observation of stable dimers on a urea-containing Sephadex G-150 column (see Fig. 8) does suggest that Equation (6) is a valid description of the equilibrium.

Using Equation (6), and assuming a molecular weight of 180000 for the tetramer and 90000 for the dimer [15], the calculated molar equilibrium dissociation constants for the three protein concentrations were: K_d(12 μg/ml)=0.55 μM; K_d(24 μg/ml)=0.5 μM; and K_d (120 μg/ml) = 0.66 μM. The variations in the dissociation constant resulted from the difficulty in obtaining accurate values for the equilibrium activity.

Since the inactivation was reversible, we can apply thermodynamic arguments and calculate a free energy of dissociation for the equilibrium using the equation

$$\Delta F = -RT \ln K_d. \quad (7)$$

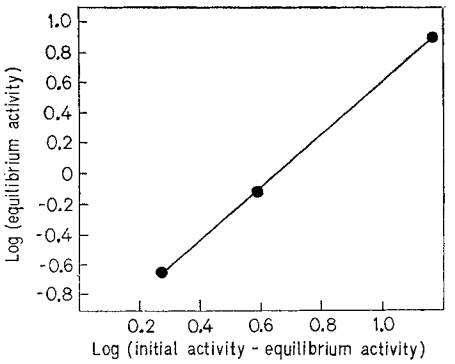

Fig. 2. *Determination of the number of subunits formed in the dissociation.* The data used in this plot are the same as used for the construction of Fig. 1

Protein concentration	Initial activity	Equilibrium activity
μg/ml	μmoles/min	μmoles/min
12	2.11	0.22
24	4.63	0.78
120	20.55	7.87

Assuming an average equilibrium constant of 0.57 μM, we obtain a standard free energy of dissociation at 273 °K of 7.8 kcal/mole. This value indicates that the dissociation is highly unfavored and occurs only at very low concentrations of protein.

Effect of Temperature

When the enzyme was incubated at temperatures greater than 0 °C, the rate of inactivation was slower and the extent of inactivation was decreased. From the equilibrium activity levels achieved at eight temperatures between 0 °C and 15 °C, equilibrium constants were determined and a van't Hoff plot of these data was constructed (Fig. 3). Although it is possible to fit two straight line segments through the experimental points, there is no compelling reason to do so since a linear van't Hoff plot occurs only for a reaction in which there is no change in the heat capacity. Indeed, Brandts has pointed out [21] that when a reaction involves a change in the environment of a number of apolar groups, a difference in the heat capacity between the initial and the final states is expected.

Thermodynamic quantities were obtained by application of the power series [20]

$$\Delta F = A + BT + CT \ln T \quad (8)$$

to the data of Fig. 3. From the ΔF values at three temperatures (273 °K, 280 °K, 288 °K) the three curve-fitting parameters were obtained. These constants are related to the thermodynamic quantities through the expressions $\Delta H = +A - CT$, $\Delta S = -B - C$ [ln$T$ + 1] and $\Delta C_p = -C$. When this

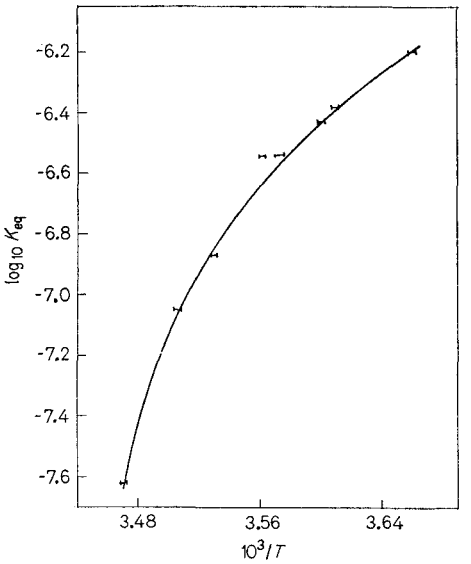

Fig. 3. *Effect of temperature on the cold inactivation.* Inactivation at various temperatures was carried out in 0.50 M potassium phosphate buffer, pH 8.0, supplemented with 0.50 mg/ml bovine serum albumin at an enzyme concentration of 19 μg/ml

analysis was carried out, the values obtained were: at 288 °K: $\Delta F = +10$ kcal/mole, $\Delta H = -65$ kcal/mole and $\Delta S = -261$ entropy units; at 273 °K: $\Delta F = +7.8$ kcal/mole, $\Delta H = -3.2$ kcal/mole and $\Delta S = -41$ entropy units. The heat capacity change, ΔC_p, was -4000 cal $\times$ mole$^{-1} \times$ °K^{-1}.

Although the accuracy of these values is probably not very great, nevertheless we can draw some general conclusions. First, these values are in agreement with accepted concepts of hydrophobic bonding as being favored by the entropy change (thus, there is a loss in entropy in dissociation), but opposed by the enthalpy change. Second, it is clear from Fig. 3 that the temperature of maximum stability of threonine deaminase lies above 15 °C. Also, the thermodynamic quantities demonstrate the mutually compensatory relationship between enthalpy and entropy changes in aqueous solutions. Thus, although ΔH and ΔS vary widely going from 0 °C to 15 °C, the free energy only increases by 2.2 kcal/mole.

Effect of pH

The effect of pH on the equilibrium activity at 0 °C is shown in Fig. 4. This figure shows that as the pH was increased the enzyme became more susceptible to cold inactivation; a result not predicted on the basis of hydrophobic interactions. There are two possible explanations for this behavior. One involves an increase in the net charge of the protein as the pH is increased (the isoelectric point of the enzyme is 5.2 [22]). An increase in the net charge

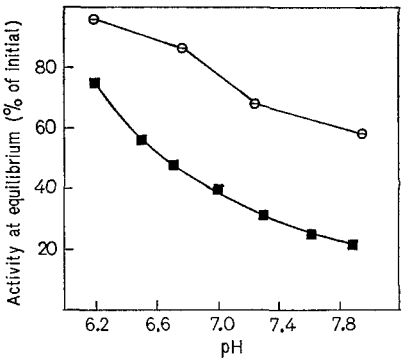

Fig. 4. *Effect of pH on cold inactivation.* Enzyme (19 μg/ml) was inactivated in 0.05 M potassium phosphate buffer (⊖) and in 0.50 M potassium phosphate buffer (■), both supplemented with 0.5 mg/ml bovine serum albumin, until equilibrium had been obtained. Enzyme assays were carried out at pH 7.8

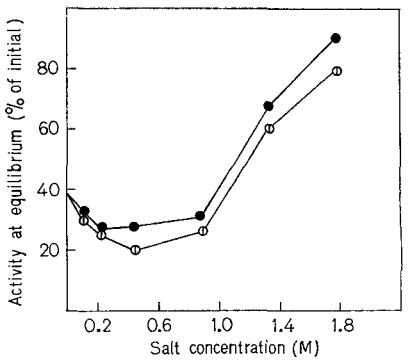

Fig. 5. *Effect of ionic strength on cold inactivation.* Enzyme (15 μg/ml) was incubated at 0 °C in 0.05 M potassium phosphate buffer, pH 8.0, supplemented with 0.5 mg/ml albumin and the salt concentration indicated. Concentrated salt solutions were adjusted to pH 8.0. Each point represents an equilibrium activity level. ●, KCl; ⊖, NaCl

would result in an increase in the electrostatic free energy and would tend to destabilize the tetramer relative to its subunits. However, if the effect of pH were due entirely to electrostatic repulsion, there would have been a greater pH dependence at 0.05 M buffer than at 0.50 M buffer. Since there is little difference between the slopes of the two curves, and since the enzyme was more labile at the higher buffer strength, it seems likely that ionic bonding is also involved in the subunit interactions. This second possibility was studied in more detail by examining the effect of salt on the cold inactivation.

Effect of Ionic Strength

The effect of ionic strength on the equilibrium activity at 0 °C was examined in more detail by adding KCl or NaCl to the incubation mixtures. Fig. 5 shows that as the salt concentration was increased, there

was first a labilization of the enzyme, followed by, at salt concentrations above 1.0 M (total $I = 1.45$), a protection against the cold inactivation. These results suggest the involvement of both ionic and hydrophobic forces in the binding of the subunits. As the ionic strength is increased, ionic bonds are first weakened, resulting in greater inactivation of the enzyme. However, at still higher ionic strengths, hydrophobic bonds will be "strengthened" since apolar groups are "salted out" (*i.e.* maintained in the protein interior), as predicted by Tanford [23]. The standard free energy of dissociation at 0 °C increased from approximately +7.8 kcal/mole at 0.2 M KCl to +10.9 kcal/mole at 1.8 M KCl. Tanford [23], using solubility data, has calculated a free energy of transfer from water to 3 M NaCl for the leucine side-chain to be +440 cal/mole. Unfortunately, these data are for 25 °C and direct comparisons to the results at 0 °C are not possible. The relatively small differences between Na^+ and K^+ suggest that there was no specific cation effect on the cold inactivation.

Effect of Urea

As pointed out by Nozaki and Tanford [7], urea promotes protein denaturation by facilitating the exposure of apolar residues in the interior of the protein to the solvent. If cold-sensitive proteins have a critical dependence on hydrophobic interactions, then urea should be a very effective denaturing agent. Fig. 6 shows the effect of 0.25 M urea on the *R. rubrum* threonine deaminase. In the presence of isoleucine, the enzyme was unaffected by this low level of urea, but in the absecne of isoleucine, urea destabilized the enzyme at 25 °C and increased the extent of inactivation at 0 °C. It should be emphasized that compared to the effect of low levels of urea on the dissociation of human CO-hemoglobin [23] or on the unfolding of β-lactoglobulin [24] the change in the free energy of dissociation of threonine deaminase induced by 0.25 M urea is quite extreme. Higher concentrations of urea (2 M) allowed inactivation at protein concentrations normally too high to display cold inactivation.

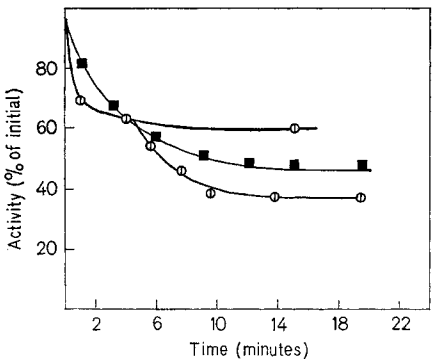

Fig. 6. *Effect of urea on threonine deaminase.* Threonine deaminase was diluted to a concentration of 150 μg/ml in 0.50 M potassium phosphate buffer, pH 8.0. At this concentration, the enzyme lost no activity at room temperature and only 50% of its activity at 0 °C (■). The same dilution into buffer supplemented with 0.25 M urea showed an immediate loss in activity at 25°C (⊙) and a further decrease in activity upon exposure to 0 °C (4 min)

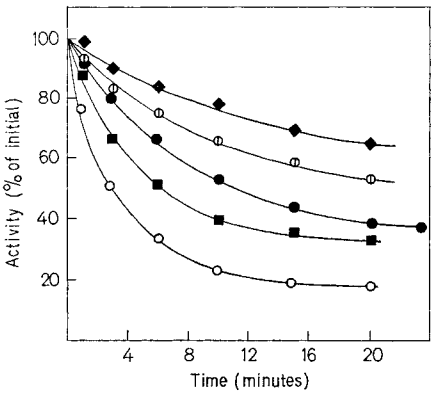

Fig. 7. *L-Isoleucine protection against cold inactivation.* Threonine deaminase (26 μg/ml) was incubated at 0 °C in 0.50 M potassium phosphate buffer, pH 8.0, supplemented with L-isoleucine; ○, no L-isoleucine; ■, 5 μM; ●, 10 μM; ⊙, 20 μM; ◆, 40 μM L-isoleucine. The reversible cold inactivation was essentially complete at the end of 20 min; an additional inactivation, but no greater than about 10% was observed in some cases due to a slow irreversible inactivation phase when diluted enzyme solutions were incubated for periods longer than 20 min (see Discussion)

Protection against Cold Inactivation

The binding of certain ligands to the enzyme protected the protein against cold-inactivation. Enzyme incubated at 0 °C in the presence of 20 mM serine or threonine displayed no inactivation over a 30-min period. As mentioned earlier, L-isoleucine, although unable to inhibit the enzyme significantly was able to protect the enzyme against cold inactivation. The effect of several different concentrations of isoleucine on the extent of inactivation is shown in Fig. 7. From these data, it is possible to calculate that the addition of 40 μM L-isoleucine to the enzyme increased the standard free energy change of dissociation at 0 °C from 7.8 kcal/mole to 9.0 kcal/mole. This indicates that the binding of isoleucine strengthened subunit interactions, but in the absence of information on the number of isoleucine molecules bound to the enzyme, no other information can be derived.

Molecular Weight of Cold-Inactivated Enzyme

Because of the low protein concentrations required for cold-inactivation, it was difficult to obtain direct physical evidence for the dimer-tetramer equi-

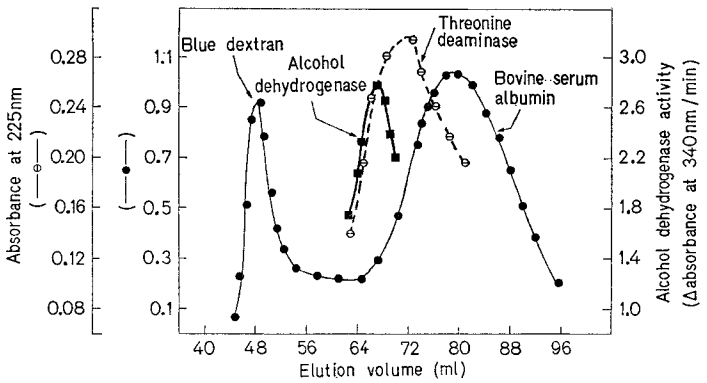

Fig. 8. *Elution profile of inactivated threonine deaminase from urea-containing Sephadex G-150.* Enzyme (0.9 mg/ml) inactivated at 0 °C in 2 M urea was eluted from a Sephadex G-150 column (77 × 1.4 cm) with 1 M urea-containing buffer at 0 °C, as described under Methods. In a separate experiment, blue dextran, alcohol dehydrogenase and bovine serum albumin were eluted from the column under the same conditions. The elution profiles from these column runs are superimposed in this figure to facilitate comparison. ■, alcohol dehydrogenase; ⊖, threonine deaminase

librium. However, higher concentrations of protein could be inactivated if urea were added to the enzyme. Threonine deaminase, incubated at 0 °C in the presence of 2 M urea was applied to a Sephadex G-150 column and eluted at 0 °C with buffer containing 1 M urea. In a separate experiment, blue dextran, alcohol dehydrogenase and bovine serum albumin were chromatographed under the same conditions. The elution patterns of these two columns are shown in Fig. 8. From this figure, it is clear that threonine deaminase migrated as a species of approximately 100000 molecular weight. Thus, this result is consistant with the tetramer-dimer equilibrium indicated by the analysis of the dependence of the equilibrium on protein concentration.

DISCUSSION

In the past few years a variety of proteins have been demonstrated to be cold sensitive, and it is likely that a number of other proteins, heretofore thought to be unstable, will also be recognized as being in this category. Although the details of inactivation vary from protein to protein, several properties seem to be common to all cold-sensitive proteins.

In general, cold-sensitive proteins are multisubunit enzymes; and, in most cases examined, exposure of the protein to low temperatures results in a dissociation of the oligomeric structure [9—14]. In the case of pyruvate carboxylase, Irias *et al.* [25] found that although subunits were formed, the rate-limiting step during inactivation was a conformational change in the oligomer. Cold inactivation of the 17β-hydroxysteroid dehydrogenase was reported to be accompanied by the appearance of high molecular weight species [26], but the dependence of the inactivation on protein concentration suggests that this aggregation may actually follow a preliminary dissociation.

Another property shared by most cold-sensitive enzymes is the large effect on the rate of inactivation exerted by allosteric modifiers. In the case of pyruvate kinase [12], the slow rate of inactivation could be enhanced 1000-fold by the allosteric activator, fructose 1,6-diphosphate. Similarly, ATP, the allosteric inhibitor of glyceraldehyde 3-phosphate dehydrogenase, increased the rate of cold inactivation of this enzyme [14]. In contrast, L-isoleucine, protected threonine deaminase against cold inactivation [27].

Generally, cold inactivation is a reversible phenomenon. However, the extent of reversal usually decreases with increasing time of incubation at 0 °C. Thus, the kinetics of inactivation can be divided into a rapid reversible phase followed by a slower irreversible inactivation.

The property of cold sensitivity has been ascribed to the weakening of hydrophobic "bonds" in the protein. The term "hydrophobic bond" refers to the tendency of nonpolar groups to associate when placed in aqueous solutions. A general explanation for this tendency has been offered by Kauzmann [3] and by Nemethy and Scheraga [6]. They suggest that when a nonpolar group is placed in an aqueous environment, water molecules order themselves around this group. When two nonpolar groups are brought together in solution, the number of water molecules in immediate contact with each groups is decreased, resulting in a decrease in the ordering of the water and thus in an increase in its entropy. This increase in ΔS contributes to a favorable (*i.e.* negative) free energy change for the association of apolar groups. As the temperature is decreased, the contribution of the $T\Delta S$ term to the free energy will decrease. In addition, at temperatures around 0 °C, water will be more structured so that exposure of apolar groups will not lead to large entropy changes for the system. Both of these factors presumably contribute to the

weakening of hydrophobic interactions at low temperatures. It should be emphasized that the term hydrophobic bond is a misnomer. As Hildebrand [28] has pointed out, association of apolar groups is not due to any formal bonds between them, but is due simply to the properties of water itself. However, although van der Waals forces may not be strong enough to account for the association of apolar groups in water, once such association has taken place, these weak forces are aggregated over a large number of interactions and may contribute significantly to the over-all free energy.

The results presented in this communication support the notion that the breaking of hydrophobic bonds is involved in the cold inactivation of the threonine deaminase of *R. rubrum*. It is proposed that the tetrameric protein dissociates into inactive dimers at 0 °C. The n-value of 1.75 from Fig. 2 may indicate that there is a small amount of undissociated, but inactive, tetramer present. Although the use of Equation (6) to describe the equilibrium might introduce some error in the calculation of the equilibrium constant, because the free energy is a logarithmic function of K_{eq}, this error should not have a large effect on the values of the thermodynamic parameters. In agreement with the current concepts of hydrophobic bonding, we found that in the dissociation of the protein, there was a net loss in the entropy of the system. This suggests that apolar groups are being exposed to water in the dissociation. At 0 °C, the entropy loss was −41 entropy units, while at 15 °C, dissociation of the protein resulted in an entropy loss of −261 entropy units. This difference can be ascribed to the fact that water is more structured at 0 °C than at 15 °C, so that exposure of apolar groups results in less of an over-all change in entropy. The standard enthalpy change (−3.2 kcal/mole at 0 °C, −65 kcal/mole at 15 °C), on the other hand, favors the dissociation. The enthalpic contribution is probably due to the greater number of hydrogen bonds formed when water is ordered. Again, at low temperatures the water already has a great number of hydrogen bonds and thus the enthalpic change upon dissociation is less than it is at 15 °C.

Quantitative approaches to the strength of hydrophobic interactions have been taken by Nemethy and Scheraga [6] and by Tanford [7] utilizing the transfer energies of amino acids from organic solvents to water. Although the results of the above two laboratories vary somewhat, the average loss in entropy for the breaking of a hydrophobic pair is approximately 10 entropy units at 25 °C. Applying this value to the ΔS obtained at 15 °C we can calculate that at a minimum, 26 hydrophobic interactions are broken in the dissociation of threonine deaminase. If the ΔS is higher at 25 °C than it is at 15 °C the above number would underestimate the number of "interactions" broken. Although no great accuracy can be claimed for the values presented, they do represent an initial attempt toward gaining some insight into the strength of interactions between protein subunits.

The effect of pH and ionic strength on the cold inactivation of threonine deaminase suggested that ionic bonding was also involved in the maintenance of the quaternary structure. The labilization of the protein as pH was increased was probably due both to increased electrostatic repulsion between subunits and to the weaking of ionic bonds between subunits. The addition of 0.2 M salt to the protein (Fig. 5) resulted in a decrease in the standard free energy of dissociation of 700 cal/mole, probably due to the weakening of the ionic bonds. However, raising the salt concentration to 1.8 M resulted in a 3 kcal/mole increase in the standard free energy. This finding strongly indicates the importance of hydrophobic interactions in the protein. This result can be contrasted with the effect of salt on the dissociation of human CO-hemoglobin. With that protein, Tanford [23] found that NaCl promoted dissociation at all concentrations tested up to 3 M salt.

An important aspect of this work is its significance for the study of allosteric interactions, and of how the binding of a small molecule can affect forces between protein subunits. The dramatic effect of L-isoleucine on the cold inactivation of threonine deaminase indicates that the binding of this ligand strengthens the intersubunit forces in the protein. This effect could come about through a conformational change in the protein which increases the number of apolar groups between the two subunits or through the introduction of ionic bonds between the subunits. If these ionic bonds were formed within a hydrophobic environment, we would expect them to be considerably stronger than they are in water. Further studies on the nature of the changes induced in intersubunit forces by the binding of allosteric ligands should give us a greater understanding of how conformational changes are mediated in a large protein molecule.

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