Murray, E. D., Jr., & Clarke, S. (1984) *J. Biol. Chem. 259*, 10722–10732.
Murray, E. D., Jr., & Clarke, S. (1986) *J. Biol. Chem. 261*, 306–312.
Nomura, K. (1986) *FEBS Lett. 209*, 235–237.
Orlowski, M. (1983) *Mol. Cell. Biochem. 52*, 49–74.
Pontremoli, S., Melloni, E., Salamino, F., Sparatore, S., Michetti, M., Benatti, U., Morelli, A., & De Flora, A. (1980) *Eur. J. Biochem. 110*, 421–430.
Rupnow, J. H., Taylor, W. L., & Dixon, J. E. (1979) *Biochemistry 18*, 1206–1212.
Schafer, W. (1983) in *Modern Methods in Protein Chemistry* (Tschesche, H., Ed.) pp 337–359, de Gruyter, New York.
Smith, I. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., Ed.) pp 291–307, Pitman, Bath, U.K.
Taylor, W. L., & Dixon, J. E. (1980) *Biochem. Biophys. Res. Commun. 94*, 9–15.
Vandenberg, J. I., King, G. F., & Kuchel, P. W. (1985) *Arch. Biochem. Biophys. 242*, 515–522.
Walter, R., & Yoshimoto, T. (1978) *Biochemistry 17*, 4139–4144.
Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L., & Kerenyi, T. D. (1971) *Science (Washington, D.C.) 173*, 827–829.
Walter, R., Simmons, W. H., & Yoshimoto, T. (1980) *Mol. Cell. Biochem. 30*, 111–127.
Yoshimoto, T., Orlowski, R. C., & Walter R. (1977) *Biochemistry 16*, 2942–2948.
Yoshimoto, T., Kiyokazu, O., Walter, R., Koida, M., & Tsuru, D. (1979) *Biochim. Biophys. Acta 569*, 184–192.

# Synergistic Ligand Protection and Intermediates in the Denaturation of Extremely Thermophilic Glutamine Synthetase†

David J. Merkler,‡ Kotteazeth Srikumar, and Frederick C. Wedler*
*Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802*
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ABSTRACT: Glutamine synthetase (GS) exists in the extreme thermophile *Bacillus caldolyticus* as two regulatory isoforms, E-I and E-II, produced as separate gene products. Thermostabilization of these isoforms was investigated by determining the kinetics of thermal denaturation as a function of temperature, compared to GS from mesophilic *Bacillus subtilis* (BSGS). Apoenzymes exhibited inherent thermostability of the order E-II > BSGS > E-I. Bound substrates and metal ions provided substantial thermostability, but ATP alone rendered each enzyme less thermostable. Analysis of activation parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$) indicated that binding of ligands alters the kinetic barriers between native enzyme and the fully denatured state. The activation energies for certain species or complexes were unusually high: ca. 250 kcal/mol for apo-E-I and ca. 320 kcal/mol for the E-II·MnATP·Glu complex. In addition, strongly synergistic ligand stabilization was observed, especially for the thermophilic enzymes: that is, the sum of the changes in $\Delta G^{\ddagger}$ induced by addition of added L-glutamate or MnATP to apoenzyme was less than the $\Delta(\Delta G^{\ddagger})$ observed when both ligands were added together. The order of relative thermostabilities for fully complexed enzymes was E-II > E-I > BSGS. With few exceptions, the kinetic progress curves for thermal denaturation were biphasic, suggesting formation of a metastable intermediate in the pathway. Circular dichroism studies of equilibrium unfolding/refolding with E-I by guanidine hydrochloride (Gdn-HCl) also clearly indicated an intermediate in the pathway. Refolding of Gdn-HCl-denatured E-I was >90% reversible for this highly oligomeric ($n$ = 12) enzyme.

The concepts of cell thermophily and protein thermostability have intrigued microbiologists and biochemists for several decades. These related problems have proven to be much more complex and subtle than was originally conceived. Comparisons of the size, amino acid content, quaternary structures, and catalytic and regulatory properties of numerous thermophilic vs mesophilic enzymes indicated few obvious differences that correlated with thermostability (Singleton & Amelunxen, 1973; Biesecker et al., 1977; Amelunxen & Murdock, 1978; Wedler, 1978; Wedler & Merkler, 1985). Although it was originally thought that a single, general mechanism might be found to account for the increased thermostability of enzymes and proteins from thermophilic microorganisms (Koeffler, 1957), it now is apparent that this property can result from a variety of minor changes in protein bonding forces (Argos et al., 1979). Since the rate of denaturation at a given temperature is altered 10-fold by a change in $\Delta G^{\ddagger}$ of only ca. 1.4 kcal/mol, changing a single amino acid residue can dramatically alter the thermostability of a protein (Langridge, 1968; Yutani et al., 1978; Gruetter et al., 1979). Consequently, elucidation of factors responsible for thermostability of an enzyme or protein requires detailed physicochemical analysis.

Voordouw et al. (1976) have suggested that a major part of protein stability could be attributed to *activation parameters* (manifested by kinetic effects) rather than *thermodynamic* factors. Furthermore, it was proposed that such factors could be divided into two categories: (a) *intrinsic* properties of the folded polypeptide chain such as the stabilizing energies from

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* Author to whom correspondence should be addressed.
‡ Present address: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461.

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covalent, ionic, and hydrogen bonds, plus hydrophobic bonding, and (b) *extrinsic* effects due to bound ligands, e.g., metal ions, substrates, or effectors. The "total kinetic stability" of a protein was defined as the sum of the contributions from the intrinsic plus extrinsic kinetic effects.

Glutamine synthetase, a key regulatory enzyme of nitrogen metabolism in microorganisms (Stadtman & Ginsburg, 1974), has been characterized from a variety of mesophilic and thermophilic *Bacilli* (Deuel et al., 1970; Wedler & Hoffmann, 1974a; Wedler et al., 1980). Two isofunctional forms of GS,[1] designated E-I and E-II, were discovered in the extreme thermophile *Bacillus caldolyticus*, E-I being the isoform that migrated more rapidly on native PAGE at pH 6.8 (Wedler et al., 1978). These GS isoforms were subsequently shown to differ markedly in their physicochemical, catalytic, and regulatory properties (Wedler et al., 1980, 1981). Preliminary data suggest that E-I and E-II are the products of separate genes. It was also observed previously that GS from *Bacillus stearothermophilus* and *B. caldolyticus* could be substantially stabilized by bound ligands (Wedler & Hoffman, 1974b; Wedler & Merkler, 1985).

Intriguing questions remain unanswered regarding the mechanisms of thermostability and whether denaturation of these enzymes is used as a regulatory mechanism in this extremely thermophilic organism. The present research addresses the first question by a detailed analysis of the thermal denaturation kinetics for both mesophilic and thermophilic GS by means of calculated values for activation parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, $T\Delta S^{\ddagger}$) in the presence and absence of bound ligands. These results provide new insights to the mechanisms of unfolding/refolding and the relative importances of intrinsic and extrinsic stabilization factors for these key enzymes.

## Experimental Procedures

*Materials. Bacillus subtilis* RUB 818, a wild-type strain derived from *B. subtilis* 168, was a gift from Dr. R. Yasbin. Cultures up to 100 L were initiated with a 1% (v/v) inoculum and grown in MG medium (Freeze & Fortnagel, 1969) at 37 °C in a New Brunswick F-130 Fermacell fermentor to 4 h after entering stationary phase. Cell paste was rapidly frozen in liquid $N_2$ and then stored at −20 °C.

*B. caldolyticus* cells were grown and harvested as described previously (Wedler et al., 1980). E-I and E-II forms of GS were purified by modification of previously published procedures [supplementary material (see paragraph at end of paper regarding supplementary material)].

The activity of GS from *B. subtilis* was assayed in a total volume of 0.2 mL containing 50 mM imidazole–acetate buffer (pH 7.1), 7.5 mM ATP, 7.5 mM $MnCl_2$, 50 mM L-glutamate, and 5 mM $NH_2OH$. The reaction was initiated by addition of sufficient enzyme to produce 0.40 μmol or less of γ-glutamyl hydroxamate within the assay time and was stopped by addition of 2.0 mL of acidic $FeCl_3$ (Deuel et al., 1970). The concentration of γ-glutamyl hydroxamate formed was determined colorimetrically at 540 nm (Shapiro & Stadtman, 1971). A similar assay was used for *B. caldolyticus* GS (Wedler et al., 1980). Protein concentrations were routinely determined with the biuret method of Layne (1957) or the dye method of Bradford (1976), BSA being used as a standard.

HEPES buffer was a product of U.S. Biochemical Corp. All other biochemical reagents were of the highest purity available from Sigma Chemical Co., and inorganic compounds were of ACS analytical grade. Distilled water was further purified with a Barnstead-Sybron Nanopure system.

*Preparation of Metal-Free (Apo) Enzymes.* It was discovered that purified *B. subtilis* and *B. caldolyticus* enzymes contained substoichiometric amounts of Mn(II) ions.[2] This suggested that high-affinity metal binding sites existed for these ions, which necessitated special procedures for the preparation of apoenzymes. Apo-E-I and apo-E-II were prepared by dialysis of 0.1–0.5 mg/mL enzyme vs 10 volumes of 0.2 M imidazole hydrochloride, pH 6.3, for 12–18 h at 4 °C. The protein was then concentrated and dialyzed against 10 mM HEPES, pH 7.0, 0.2 M KCl, and 0.03% (w/v) $NaN_3$. These apoenzymes were stable in this buffer for at least 1 year at 4 °C.

Apo-BSGS was unstable ($t_{1/2} \sim 24$ h), so that slow dialysis of BSGS solutions vs imidazole buffer produced inactive enzyme. Apo-BSGS was prepared and used within 8 h by the following procedure: concentrated (3–5 mg/mL) enzyme stock was diluted 10-fold into 0.4 M imidazole, 0.1 M EDTA, and 0.03% (w/v) $NaN_3$, was immediately applied to a Sephadex G-25 column (0.9 × 30 cm), equilibrated with 10 mM HEPES, pH 7.0, 0.2 M KCl, and 1 mM 2-mercaptoethanol, and was eluted with this same buffer. Each apoenzyme was determined to be free of bound Mn(II) (≤0.1 mol/mol of subunit) by denaturation of protein in 0.1 M $HClO_4$, followed by determination of [free Mn(II)] by ESR. In the absence of added Mn(II) or Mg(II) ions, each apoenzyme was enzymatically inactive, but readdition of metal ions caused regain of the full original activity.

*Thermal Denaturation Kinetics.* Concentrated stock enzyme was diluted ≥10 times into 5 mM DMG buffer, pH 6.7, containing 0.2 M KCl (with or without added ligands), to a total volume of 0.5 mL, in a capped tube to reduce evaporation. Final enzyme concentrations were 0.15 mg/mL for E-I and E-II or 0.25 mg/mL for BSGS. For experiments with BSGS, all solutions also included 1 mM 2-mercaptoethanol. After preincubation for 5 min at 60 °C, the diluted enzyme mixtures were then placed in a Lauda K2 water bath thermostated (±0.02 deg) at the (higher) denaturation temperature. A calibrated Tekmar RA-100 digital thermometer with a microthermistor probe (Hampshire Controls Corp.) inserted directly into the incubation solution indicated that less than 60 s was required for 0.5 mL of enzyme solution to reach the desired temperature in the range of 65–95 °C. At appropriate times, 25-μL samples were rapidly removed from the enzyme incubation solution with a Hamilton syringe and immediately diluted into 0.2 mL of assay mix thermostated at 60 (for E-I and E-II) or 37 °C (for BSGS). The standard assay period was 15 min, but extensively denatured enzymes were assayed for longer times.

*Calculation of Rate Constants and Half-Life Values.* Rate constants were determined by plotting ln (percent activity remaining) vs time. These data were then fit to the best straight line with a linear regression program. For biphasic processes, rate constants for each part of the biphasic denaturation process were obtained by a fit to

$$A(t) = \lambda_f \exp(-k_f t) + \lambda_s \exp(-k_s t)$$

where $A(t)$ is the percent activity remaining at time $t$, $\lambda_f$ is the fast-phase zero-time intercept, and $\lambda_s$ is the slow-phase

[1] Abbreviations: BSA, bovine serum albumin; BSGS, *B. subtilis* glutamine synthetase; DMG, 3,3-dimethylglutamate; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; GS, glutamine synthetase; HEPES, *N*-(2-hydroxyethyl)piperizine-*N*′-2-ethanesulfonic acid; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; TEA, triethanolamine; TES, 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid.

[2] Unpublished results, this laboratory.

Table I: Binding Constants (mM) for Substrates and Metal Ions with *Bacillus* sp. Glutamine Synthetases[a]

| ligand | E-I | E-II | BSGS |
|---|---|---|---|
| Mn(II) | 0.13 (±0.02) [10][b] | 0.03 (±0.01) [1.0][c] | 0.005 (±0.001) [0.2][b] |
| Mg(II) | 6.7 (±1.1) [30][b] | 5.0 [50][d] | 0.04 (±0.005) [50][b] |
| MnATP | 0.5 [5.0][d] | 0.5 [5.0][d] | 0.2 [5.0][e] |
| L-glutamate | 2.2, 38 [50][d,f] | 3.1, 46 [50][d,f] | 0.8 [50][e] |

[a] Errors reported as the 95% confidence levels. The values in the brackets are the concentrations (mM) added during denaturation kinetic experiments. [b] Determined by thermal protection experiments. [c] $K_d$ valued determined by EPR. [d] $K_m$ value from Wedler et al. (1980). [e] $K_m$ value from Deuel and Stadtman (1970). [f] Biphasic binding curves.

zero-time intercept. $\lambda_f$ and $\lambda_s$ were varied independently to find the best values of $k_f$ and $k_s$ that fit the experimental data. For single-exponential kinetics, $\lambda_s$ = zero. For biphasic kinetics, the fast phase was corrected for contribution of the slow phase with

$$A(t)_{cor} = A(t)_{exp} - \lambda_s \exp(-k_s t)$$

where $A(t)_{exp}$ is the experimentally determined percent activity at time $t$. The fast-phase rate constant was then determined by least-squares analysis of a plot of log $[A(t)_{cor}]$ vs time. From calculated values of $k_f$ and $k_s$ the computer program also generated curves of percent activity vs time to check the degree of fit to the experimental data.

For monophasic processes, $t_{1/2}$ values could be calculated explicitly by the relationship $t_{1/2} = (\ln 2)/k$. Since a number of the denaturation processes were biphasic, the time necessary to reach $A(t_{1/2})$ was calculated from

$$dt_{1/2} = X\frac{A(t_{1/2})_{calcd} - A(t_{1/2})}{A(t_{1/2})} + Y$$

where $X = 0.72$ min and $Y = 0.003$ min when $A(t_{1/2})_{calcd}$ was less than $A(t_{1/2})$ or $-0.003$ min when $A(t_{1/2})_{calcd}$ was greater than $A(t_{1/2})$. An initial (estimated) value of $t_{1/2}$ was entered into a computer program that used a reiterative process to fit the experimental data until $A(t_{1/2})_{calcd}$ was within 0.1% of $A(t_{1/2})$. Plots of temperature vs $t_{1/2}$ allowed calculation of the temperatures corresponding to $t_{1/2}$ equaling a specific time, e.g., 300 s.

*Calculation of Activation Parameters.* The energy of activation $\Delta G^{\ddagger}$ was calculated at a given temperature from the relationship (Glasstone et al., 1941) $k = (RT/Nh) \times \exp(-\Delta G^{\ddagger}/RT)$. The enthalpy of activation $\Delta H^{\ddagger}$ was obtained from the slope of Arrhenius plots of ln $k$ vs $1/T$ (K): $\Delta H^{\ddagger} = -R[d \ln k/d(1/T)] - RT = E_a - RT$. Once the values for $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$ were obtained, values for $\Delta S^{\ddagger}$ were calculated from the relationship $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$.

In some cases, thermostabilization by added ligand made measurement of rate constants for denaturation of apoenzyme ($k_0$) and ligand-saturated enzyme ($k_{sat}$) at the same temperature impossible. Arrhenius plots for $k_0$ and $k_{sat}$ were extrapolated to the same temperature to obtain the appropriate rate constants, which is valid for linear Arrhenius plots extrapolated over a relatively narrow temperature range.

The contribution of the ligand to the "total kinetic stability" was determined at the same temperature as described by Voordouw et al. (1976):

$$\Delta G^{\ddagger}_{total} = \Delta G^{\ddagger}_0 - \Delta(\Delta G^{\ddagger}_{ligand})$$

$$\Delta(\Delta G^{\ddagger}_{ligand}) = \Delta G^{\ddagger}_0 - \Delta G^{\ddagger}_{total} = -RT \ln (k_0/k_{sat})$$

where $\Delta G^{\ddagger}_0$ and $k_0$ were the values for apoenzyme.

*Equilibrium Unfolding by Gdn-HCl.* Circular dichroism spectra were recorded from 200 to 310 nm on a Jasco J-20 spectropolarimeter with the slit manually adjusted to 2800 μm. The instrument was calibrated to ±2 nm with a 0.06% (w/v) *d*-camphorsulfonic acid solution. Ellipticity was converted to mean residue ellipticity $[\theta]_{222}$ with $[\theta]_{222} = M_0\theta_{222}/lc$, in which $[\theta]_{222}$ is mean residue ellipticity at 222 nm, $\theta_{222}$ is the observed (measured) ellipticity at 222 nm, $l$ is the path length in centimeters, $c$ is the protein concentration in grams per milliliter, and $M_0$ is the mean residue molecular weight, calculated from the data of Wedler et al. (1980) to be 102.2. An aliquot of concentrated enzyme (<20 μL) was diluted to 0.7 mL with buffer (5 mM DMG, pH 6.7, 0.2 M KCl) plus the desired amount of ligand and guanidine hydrochloride, to give a final enzyme concentration of 50 μg/mL. Solutions were incubated for 12–24 h at 25 °C to allow the system to reach equilibrium, after which the CD spectra were determined. Samples were placed in a 1-cm cuvette thermostated at 26 (±0.5) °C. Renaturation and refolding experiments were performed by incubation of a 100 μg/mL protein sample for 12 h at 25 °C, followed by dilution with an equal volume of buffer lacking Gdn-HCl, followed by further incubation for 12–24 h.

## Results

*Binding Constants for Metal Ions and Substrates.* Relatively tight binding of metal ions to *Bacillus* GS was suggested by both kinetics (Wedler et al., 1980) and the observation that several of these enzymes coisolated with substoichiometric amounts of Mn(II).[2] Binding constants for metal ions were subsequently determined by kinetics, ESR, and thermal protection methods (see supplementary material), summarized in Table I. BSGS bound Mn(II) and Mg(II) with relatively high affinity, but $K_d$ values for E-I and E-II were in the range of 30–130 μM for Mn(II) and 5–7 mM for Mg(II). Kinetic saturation curves for L-glutamate were biphasic with both E-I and E-II (Wedler et al., 1980), exhibiting $K_m$ values for the second set of sites near 40–50 mM. In thermal denaturation studies, L-Glu was used at 50 mM (well in excess of the $K_m$ for the first set of sites); higher amounts of L-Glu had little effect on the denaturation kinetics.

*Thermal Denaturation Kinetics.* Typical progress curves for the loss of enzyme activity are shown in Figure 1, plotted in both linear and semilog fashion. The effects of added ligands (substrates and metal ions) on these kinetics were also determined as a function of denaturation temperature.

Progress curves for most of the apoenzymes and enzyme–ligand complexes studied were distinctly biphasic. The simplest explanation for this behavior is that a kinetically isolable intermediate exists on the pathway between native (N) and heat-denatured (D) enzyme forms:

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D$$

This intermediate apparently retains partial activity and hence certain structural elements of the native enzyme. For I to be kinetically observable requires that $k_1 > k_2$.

The only species that did not exhibit biphasic kinetics were apo-E-I at temperatures above 66.3 °C and several of the BSGS binary complexes. This does not mean that an intermediate (I) does not exist on the thermal inactivation pathway for these species. More likely, if $k_1 \leq k_2$, the disappearance of I is faster than its formation, so that I does not accumulate

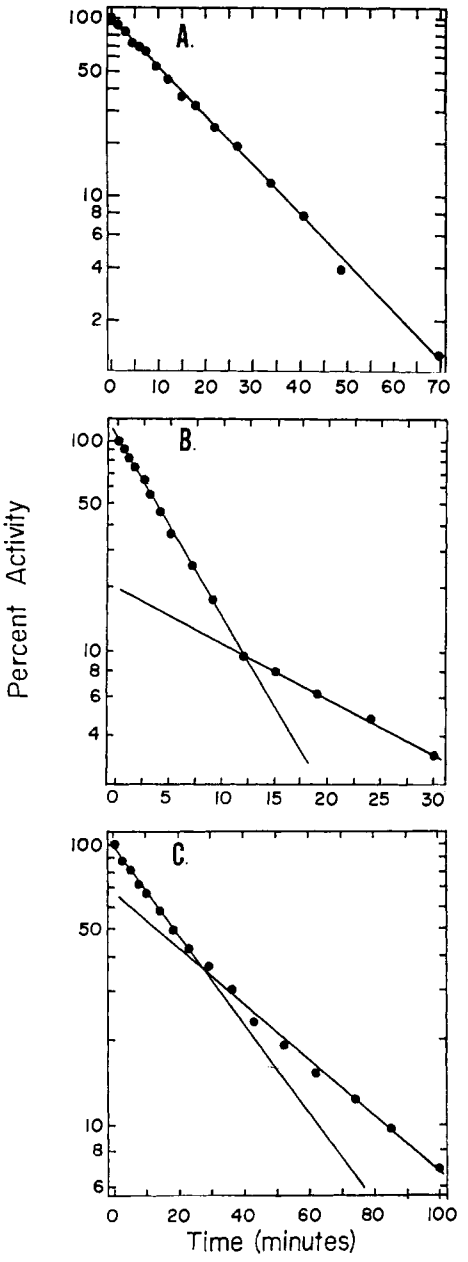


FIGURE 1: Thermal denaturation kinetics for apo-glutamine synthetases: (A) E-I, at 66.5 °C; (B) E-II, at 75.7 °C; (C) *B. subtilis* GS, at 70.1 °C. Slopes of the lines were determined by linear regression analysis (see Experimental Procedures). All experiments were performed in 5 mM DMG buffer, pH 6.7, containing 200 mM KCl and 1 mM 2-ME. E-I and E-II were present at 0.15 mg/mL and BSGS at 0.25 mg/mL protein.

and biphasic kinetics are, therefore, not observed. Thus, disappearance of the biphasic behavior at a certain temperature may indicate a sharp change in the rate-limiting step from $k_2$ to $k_1$. In fact, apo-E-I and the BSGS complexes exhibited biphasic denaturation at lower temperatures, but because the overall rate of thermal denaturation became too slow to observe under these conditions, this biphasic process was not studied in detail.

*Relative Thermostabilities.* Kinetic progress curves for thermal denaturation as in Figure 1 allow one to derive a useful parameter, the "isokinetic temperature", at which the half-life for the thermal denaturation process was the same, ignoring biphasic behavior. A comparison of these values, called $T_{300s}$, for the apoenzyme forms of E-I, E-II, and BSGS in the presence and absence of added ligands is shown in Figure 2, These data indicate that apoenzyme thermostability was in

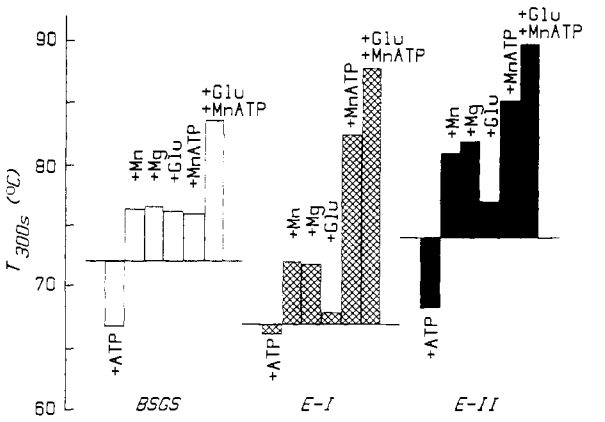


FIGURE 2: Relative thermostabilities of mesophilic and extremely thermophilic *Bacillus* sp. glutamine synthetase and ligand complexes thereof. $T_{300s}$ is the temperature at which the half-life ($t_{1/2}$) for thermal denaturation was 300 s (see text). In each case the *base line* was the value for apoenzyme. Symbols: (open bars) BSGS; (shaded bars) E-I; (filled bars) E-II.

the order of E-II > BSGS > E-I but that in the presence of MnATP plus L-Glu the order became E-II > E-I > BSGS. It is also obvious that added ligands caused dramatic increases in thermostability for all three enzymes, especially E-I. These intriguing effects were explored in greater depth by more detailed kinetic studies described below, in which changes in $T_{300s}$ induced by added ligands were seen to parallel in a quantitative way the observed changes in $\Delta G^{\ddagger}$.

*Effects of Ligands on Denaturation Kinetics: Activation Parameters.* The activation parameters for apoenzymes were calculated from Arrhenius plots of thermal denaturation data (see Experimental Procedures) and are presented in Table II. Similar experiments were performed in the presence of saturating concentrations of various combinations of substrate and the activation parameters calculated from these divalent metal ion ligands.

Due to high values of $E_a$, the rate of denaturation for several of the thermophilic GS species changed from immeasurably slow to immeasurably fast over a very narrow temperature range, usually 3–4 deg. For apo-E-I, $E_a$ = 248 kcal/mol, and for the E-I·MnATP·Glu complex (fast phase), $E_a$ = 320 kcal/mol. Denaturation of these species is therefore apparently a highly concerted or cooperative process. $E_a$ values for denaturation of most other GS species were closer to 100 kcal/mol, which is still greater than those reported previously for other thermophilic proteins.

Apo-BSGS denatured at approximately the same temperatures as apo-E-I, and only in the presence of saturating MnATP plus L-glutamate did E-I exhibit markedly greater thermostability than the *B. subtilis* enzyme.

Table II lists values for the free energies of activation ($\Delta G^{\ddagger}$) for the thermal inactivation process, all typically in the range of 20–35 kcal/mol; the *differences* between $\Delta G^{\ddagger}$ for the apoenzymes vs the enzyme–ligand complexes indicate the relative contribution to the *total kinetic stability* of each metal ion or substrates. Calculated values of $\Delta(\Delta G^{\ddagger})$ are also listed in Table II and are plotted in Figure 3. These data indicate that effects due to addition of single ligands were modest, but there was striking synergism between MnATP and L-glutamate: the *sum* of the $\Delta(\Delta G^{\ddagger})$ values due to addition of MnATP or L-glutamate individually were less than the $\Delta(\Delta G^{\ddagger})$ elicited by addition of MnATP plus L-Glu together, especially for thermophilic GS. Both E-I and E-II also showed substantial stabilization by MnATP, e.g., $\Delta(\Delta G^{\ddagger}) \simeq$ 2–5 kcal/mol, whereas addition of ATP alone caused all three forms of GS to be *less* stable.

Table II: Activation Parameters for Thermal Denaturation of *Bacillus* sp. Glutamine Synthetases

| species | phase | range (°C)[a] | kcal/mol $E_a$ | kcal/mol $\Delta H^\ddagger$ | kcal/mol $\Delta G^\ddagger$ | kcal/mol $-\Delta(\Delta G^\ddagger)$ | $\Delta S^\ddagger$ (eu) |
|---|---|---|---|---|---|---|---|
| (A) E-I[b] | | | | | | | |
| apo-E[c] | fast | 66–69 | 0 | | 23.9 | | |
| | slow | | 248 | 247 | 25.0 | | 802 |
| +ATP | fast | 65–69 | 73 | 25 | 22.7 | −1.2 | 8 |
| | slow | | 151 | 95 | 23.3 | −1.7 | 212 |
| +Mn(II) | fast | 71–76 | 100 | 99 | 25.3 | 1.4 | 218 |
| | slow | | 28 | 28 | 25.3 | 0.3 | 7 |
| +Mg(II) | fast | 69–74 | 156 | 155 | 26.1 | 2.2 | 380 |
| | slow | | 151 | 150 | 27.3 | 2.3 | 362 |
| +MnATP | fast | 79–83 | 110 | 109 | 26.1 | 2.2 | 244 |
| | slow | | 54 | 54 | 29.9 | 4.9 | 82 |
| +Glu[c] | fast | 66–69 | | | | | |
| | slow | | 292 | 291 | 25.4 | 0.4 | 78.4 |
| +MnATP·Glu | fast | 87–91 | 86 | 85 | 28.5 | 4.6 | 166 |
| | slow | | 109 | 108 | 31.0 | 6.0 | 227 |
| (B) E-II[d] | | | | | | | |
| apo-E | fast | 76–79 | 110 | 109 | 24.2 | | 242 |
| | slow | | 0 (±1) | 0 (±1) | 25.3 | | −75 |
| +ATP | fast | 68–72 | 17 | 17 | 22.8 | −1.4 | −17 |
| | slow | | 88 | 88 | 25.3 | 0.0 | 182 |
| +Mn(II) | fast | 80–83 | 146 | 145 | 26.1 | 1.9 | 341 |
| | slow | | 150 | 149 | 27.5 | 2.2 | 347 |
| +Mg(II) | fast | 76–83 | 72 | 71 | 24.9 | 0.7 | 133 |
| | slow | | 104 | 104 | 26.7 | 1.4 | 219 |
| MnATP | fast | 83–87 | 86 | 85 | 25.9 | 1.7 | 169 |
| | slow | | 206 | 205 | 30.8 | 5.5 | 499 |
| +Glu | fast | 74–78 | 65 | 64 | 23.8 | −0.4 | 116 |
| | slow | | 89 | 89 | 25.7 | 0.4 | 180 |
| +MnATP·Glu | fast | 88–92 | 320 | 320 | 35.5 | 11.3 | 811 |
| | slow | | 89 | 88 | 28.3 | 3.0 | 171 |
| (C) BSGS[e] | | | | | | | |
| apo-E | fast | 69–74 | 94 | 93 | 23.7 | | 200 |
| | slow | | 72 | 71 | 24.8 | | 132 |
| +ATP | fast | 64–69 | 67 | 66 | 23.0 | −0.7 | 127 |
| | slow | | 83 | 82 | 24.5 | −0.3 | 170 |
| +Mn(II)[f] | fast | 73–80 | | | | | |
| | slow | | 47 | 46 | 24.9 | 0.1 | 63 |
| +Mg(II) | fast | 72–80 | 32 | 32 | 24.5 | 0.8 | 21 |
| | slow | | 0 | −1 | 25.2 | 0.4 | −75 |
| +MnATP[f] | fast | 73–80 | | | | | |
| | slow | | 48 | 47 | 24.9 | 0.1 | 67 |
| +Glu[f] | fast | 73–80 | | | | | |
| | slow | | 43 | 42 | 25.0 | 0.2 | 49 |
| +MnATP·Glu | fast | 83–87 | 212 | 212 | 30.3 | 6.6 | 522 |
| | slow | | 117 | 116 | 25.7 | 0.9 | 260 |

[a] Beyond these limits denaturation was too fast or too slow to measure accurately. [b] Values calculated for 66.1 °C. [c] Monophasic kinetics above 66.1 °C. [d] Values calculated for 77.3 °C. [e] Values calculated for 74.0 °C. [f] Monophasic kinetics at all temperatures.

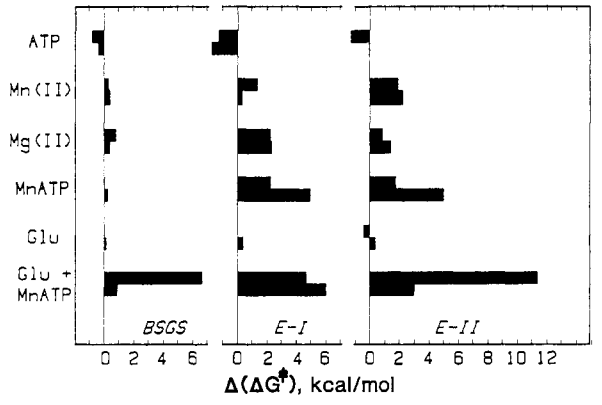


FIGURE 3: Ligand-induced thermostabilization of mesophilic and extremely thermophilic *Bacillus* sp. glutamine synthetase, expressed as $\Delta(\Delta G^\ddagger)$ for thermal denaturation. Each *pair* of bars represents $\Delta(\Delta G^\ddagger)$ values from Table II for the fast (upper bar) and slow (lower bar) kinetic phases of thermal denaturation.

*Equilibrium Unfolding/Refolding.* The possibility that denaturation of GS occurs via formation of one or more intermediates, suggested by biphasic thermal denaturation kinetics, was investigated further by equilibrium unfolding by Gdn-HCl with E-I. Of the three enzymes studied, E-I was available in greatest quantities but showed the least striking biphasic behavior. Thus, observation of an intermediate in equilibrium unfolding of apo-E-I would be considered quite significant. Apo-E-I exhibited a CD spectrum quite similar to those reported previously by Manavalan and Johnson (1983) for proteins with high β-sheet content (see Figure S-3 of the supplementary material). Indeed, in the X-ray crystallographic structure of *Salmonella* GS recently determined by Almassy et al. (1986) there was an extensive "β-barrel" structure near the active site region, plus α-helical structures.

Changes in mean residue ellipticity at 222 nm for apo-E-I and the Mn(II) complex of E-I as a function of Gdn-HCl concentration are shown in Figure 4 (top). Addition of 10 mM $MnCl_2$ or 1 mM β-mercaptoethanol had virtually no effect on the spectrum of E-I at 25 °C in the absence of added Gdn-HCl. At Gdn-HCl concentrations between 1.0 and 3.5 M, the increase in $[\theta]_{222}$ indicated that the protein undergoes an abrupt structural transition. Above and below the transition region, linear changes in mean residue ellipticity were primarily due to solvent perturbation of exposed tyrosine and tryptophan

Table III: Parameters for Fitting of the Two- and Three-State Models to the Observed Transition Curves in Figure 5[a]

| | kcal/mol | | | | | |
|---|---|---|---|---|---|---|
| | $\Delta G^{total}_{H_2O}$[b] | $\Delta G^{1}_{H_2O}$[c] | $\Delta G^{2}_{H_2O}$[c] | $m_1$ | $m_2$ | $Z$ |
| three-state model | | | | | | |
| apo-E-I | 17.8 | 13.9 (±3.4) | 3.9 (±1.0) | 22.4 (±5.6) | 4.1 (±1.0) | 0.53 |
| E-I + Mn(II) | 5.3 | 4.3 (±1.6) | 1.0 (±1.3) | 5.5 (±2.6) | 1.1 (±0.9) | 0.44 |
| E-I + 2-ME | 6.3 | 4.7 (±2.3) | 1.6 (±1.6) | 6.8 (±1.6) | 1.6 (±1.2) | 0.50 |
| two-state model | | | | | | |
| apo-E-I | 3.5 (±0.8) | | | 4.7 (±1.1) | | |
| E-I + Mn(II) | 2.6 n±0.7) | | | 3.1 (±0.8) | | |
| E-I + 2-ME | 2.9 (±0.8) | | | 3.7 (±1.1) | | |

[a] Errors are reported as the 95% confidence limits. [b] Calculated from the relationship $K_{app} = K_{H_2O} \exp[(m/RT)a_{Gdn\text{-}HCl}]$ where $a_{Gdn\text{-}HCl}$ = guanidine hydrochloride activity (Aune & Tanford, 1969a,b). $K_{H_2O}$ is the equilibrium constant in the absence of Gdn-HCl, and $m$ is the dependence of $\Delta G_{app}$ on denaturant concentration (see text). [c] A nonlinear least-squares program was used to fit the data to (Tanford, 1970) $K_{app} = (K_1K_2 + ZK_1)/[1 + (1 - Z)K_1]$, where $K_1 = [I]/[N]$ and $K_2 = [U]/[I]$ at a given Gdn-HCl concentration and $Z = (\theta_I - \theta_N)/(\theta_D - \theta_N)$ where $\theta_I$ was the $[\theta]_{222}$ for the intermediate.

residues (Matthews & Crisanti, 1981).

The unfolding transition was further analyzed by converting the data in Figure 4 (middle) to the apparent fractional change in $[\theta]_{222}$, $F_{app}$, defined by

$$F_{app} = (\theta_{obsd} - \theta_N)/(\theta_D - \theta_N)$$

where $\theta_{obsd}$ was the observed $[\theta]_{222}$ and $\theta_N$ and $\theta_D$ were the values of $[\theta]_{222}$ for the native and unfolded forms, respectively, at a given Gdn-HCl concentration. Values of $\theta_N$ and $\theta_D$ at Gdn-HCl concentrations in the transition region were obtained by linear extrapolation of the base lines as shown in Figure 4.

To characterize these transitions and to obtain thermodynamic parameters for the unfolding process, the $F_{app}$ values were first fitted by a computer program that assumed a model with only two macroscopic states:

$$N \underset{}{\overset{K}{\rightleftharpoons}} D$$

The dependence of the apparent equilibrium constant for unfolding on Gdn-HCl concentration was calculated. Values of $\Delta G_{H_2O}$ (calculated from $K_{H_2O}$ at 26 °C) and $m$ that best described the data for this model are listed in Table III.

As seen in Figure 4 (middle), the fit to the data by the two-state model (dotted line) was poor; therefore, a more complex model had to be considered, namely, one in which an intermediate I was included in the pathway between the native and unfolded forms:

$$N \overset{K_1}{\rightleftharpoons} I \overset{K_2}{\rightleftharpoons} U$$

Values for $\Delta G^1_{H_2O}$ and $\Delta G^2_{H_2O}$, calculated from $K^1_{H_2O}$ and $K^2_{H_2O}$, at 26 °C, as well as values for $m_1$, $m_2$, and $z$ that provided the best fit to the data are shown in Table III. This latter three-state model provided good fit to the CD unfolding data (see Figure 4, middle, solid line). Inclusion of additional intermediates did not significantly improve the fit. These results are consistent with equilibrium unfolding of E-I via formation of an intermediate, although not necessarily the same one that was observed by thermal denaturation kinetics.

From the parameters listed in Table III, the fractions of the native ($f_N$), intermediate ($f_I$), and unfolded ($f_U$) forms at the various Gdn-HCl activities could be derived. The relevant equations for the calculation of these fractions were according to Matthews and Crisanti (1981). The relative amounts of the different species for each unfolding transition are shown in Figure 4 (bottom). $I_{max}$ was the Gdn-HCl concentration at which the greatest fraction of I occurred. For apo-E-I, $I_{max}$ = 1.8 M, but the addition of 10 mM $MnCl_2$ shifted $I_{max}$ to 2.2 M. Calculated values of $\Delta G^{total}_{app}$ for apo-E-I and Mn·E-I are presented in Table IV.

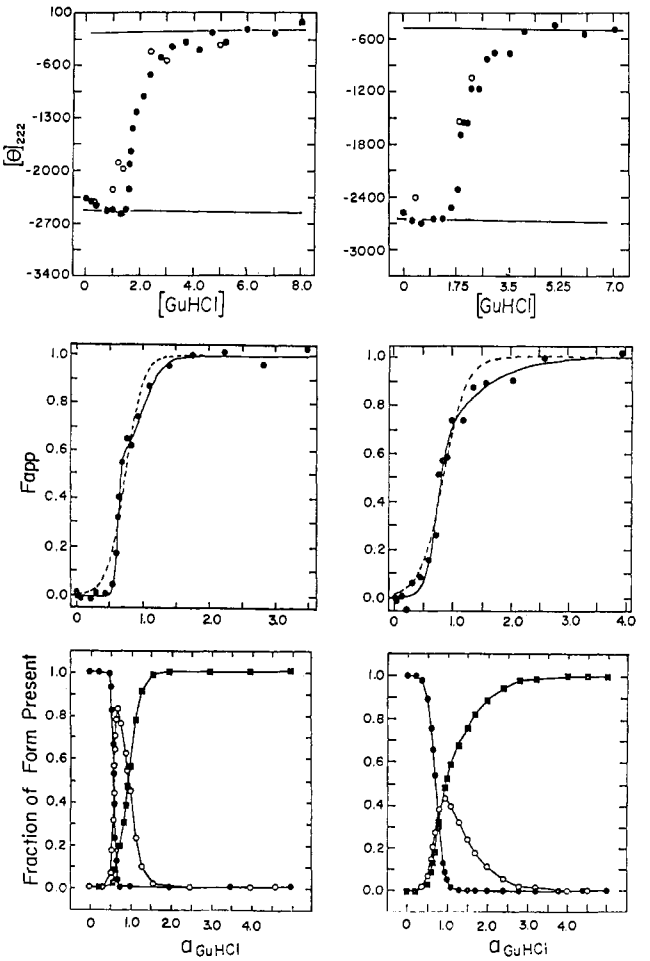


FIGURE 4: Unfolding/refolding of *B. caldolyticus* GS, isoform E-I, by Gdn-HCl, observed by circular dichroism in 5 mM DMG buffer, pH 6.7, containing 200 mM KCl, at 25 °C (see text and Experimental Procedures). (A) Apo-E-I; (B) apo-E-I plus 10 mM $MnCl_2$. (Top) Mean residue ellipticity as a function of [Gdn-HCl]: (○) unfolding data; (●) refolding data. (Middle) Normalized amplitudes for unfolding/refolding, $F_{app}$, as a function of the activity, $a$, of Gdn-HCl. Data fit for two-state (dashed line) and three-state (solid line) models (see text). (Bottom) Fractions of native N (●), intermediate I (○), and denatured D (■) forms of apo-E-I as a function of Gdn-HCl activity. Values were calculated according to $\exp[-\Delta G_1/(RT)] = K_1 = f_I/f_N$ and $\exp[-\Delta G_2/(RT)] = K_2 = f_U/f_I$, where $f_N + f_I + f_U = 1$.

Data in Figure 4 indicate that Gdn-HCl denaturation was apparently reversible, as judged by almost the total regain of ellipticity at low [Gdn-HCl]. Significantly, it was also found that >90% of the original enzymatic activity was regained upon removal of Gdn-HCl by dialysis in the presence of 1 mM β-mercaptoethanol.

Table IV: Values of $\Delta G_{app}^{total}$ at Different Concentrations of Guanidine Hydrochloride[a]

| [Gdn-HCl] (M) | $a_{Gdn\text{-}HCl}$ | $\Delta G_{app}^{total}$ (kcal/mol) | |
|---|---|---|---|
| | | apo-E-I | E-I + Mn(II) |
| 0.88 | 0.25 | 11.22 | 3.60 |
| 1.41 | 0.50 | 4.61 | 1.94 |
| 1.86 | 0.75 | −2.01 | 0.29 |
| 2.26 | 1.00 | −8.62 | −1.37 |

[a] Calculated from $\Delta G_{app}^{total} = \Delta G_{H_2O}^{1} + \Delta G_{H_2O}^{2} - a_{Gdn\text{-}HCl}(m_1 + m_2)$.

## Discussion

Glutamine synthetase from *B. caldolyticus* and *B. subtilis* has a dodecameric structure, with subunits organized as two eclipsed hexagons (Deuel et al., 1970; Wedler et al., 1980). Kinetic studies revealed both positive and negative cooperativity in binding of substrates or modifiers at 37 or 70 °C, respectively (Wedler & Eismann, 1976; Wedler et al., 1980). Whether the negatively cooperative effects are strong enough to consider this protein a hexamer of dimers is not yet proven, however. The existence of divalent metal binding sites with relatively high affinities suggested by previous kinetic data (Wedler et al., 1980) is proven by the present equilibrium binding studies by ESR and thermal protection.

One of the striking features of the equilibrium unfolding/refolding studies with E-I by Gdn-HCl was that the fully denatured form could be reconstituted almost quantitatively upon removal of 7–8 M Gdn-HCl in the presence of β-mercaptoethanol. This represents one of the few cases reported to date of complete renaturation of such a highly oligomeric enzyme (Jaenicke, 1984).

The most important observations and conclusions from this study are that as follows:

(a) The *intrinsic* thermostability of E-II was greater than that of E-I, which was comparable to that of BSGS. The latter mesophilic GS exhibited surprisingly high inherent thermostability. Thus, BSGS may be another example of a protein from a mesophilic source that exhibits unusual intrinsic thermostability (Amelunxen & Murdock, 1978). Perhaps related to these findings are recent studies of the cross-reactivity of antibodies against each of these three enzymes, in which BSGS was found to be more similar to E-II than to E-I and E-I and E-II were more similar to BSGS than to each other (Wedler et al., 1987).

(b) The *extrinsic* thermostablization was provided by bound metal ions and substrates to a substantial extent for all three GS species, so that the relative order of thermostability became E-II ≈ E-I > BSGS.

(c) The calculated values of $\Delta G^{\ddagger}$ for thermal denaturation of E-I, E-II, and BSGS were quite similar to previously reported valves for other proteins, in the range of 20–35 kcal/mol (Stearn & Eyring, 1937; Voordouw et al., 1976; Shibuya et al., 1982).

(d) *Synergistic* thermostabilization by ligands occurred, especially by MnATP with E-I and by MnATP + L-Glu with E-II and BSGS, with maximum kinetic stability in several cases attained upon binding the full complement of substrates and metal ions. The MnATP·Glu complexes of E-I and E-II required temperatures above 90 °C for denaturation, close to the maximum growth temperature for *B. caldolyticus* in its native habitat (Heinen & Lauwere, 1981). The BSGS·MnATP·Glu complex denatured at ca. 85 °C, far above the maximum growth temperature (55 °C) of *B. subtilis* (Buchanan & Gibbons, 1974).

$\Delta(\Delta G^{\ddagger})$ indicates the contribution of each ligand or set of ligands to the *total kinetic stability* of an enzyme (Voordouw et al., 1976). Binding of Mn(II) or Mg(II) alone caused increases in $\Delta(\Delta G^{\ddagger})$ for all three GS forms studied, an effect reported previously for other proteins with divalent metal ions (Delange & Lazdunski, 1967; Shapiro & Ginsburg, 1968; Voordouw & Roche, 1975; Zahnley, 1981). The binding of ATP alone to apoenzymes had the opposite effect, reflected by $\Delta G^{\ddagger}$ below that of apoenzyme and hence a negative $\Delta(\Delta G^{\ddagger})$. This effect was not duplicated by EDTA or other reagents that chelate divalent metal ions. If metal ions bridge between negatively charged protein side-chain groups and the negatively charged polyphosphate of ATP in the active site, binding of ATP in the absence of Me(II) ions could cause charge repulsions, making the protein less stable. ATP destabilization of *Escherichia coli* glutamine synthetase that could not be attributed to metal ion chelation was also reported previously by Shapiro and Stadtman (1967).

$\Delta H^{\ddagger}$ values for denaturation of apo-BSGS were lower than for the thermophilic GS isoforms and were more typical of values observed for other proteins. In contrast, $\Delta H^{\ddagger}$ values for denaturation of E-I and E-II were higher than those determined previously for a number of other thermophilic proteins (Pohl, 1968; Norris & Fowden, 1973; Voordouw et al., 1976; Shibuya et al., 1982; Malikkides & Weiland, 1982). For example, thermolysin and thermomycolase both exhibited $\Delta H^{\ddagger}$ values of 60–70 kcal/mol (Voordouw et al., 1976).

Because values of $\Delta H^{\ddagger}$ were unusually large but $\Delta G^{\ddagger}$ values were not, calculated values of $\Delta S^{\ddagger}$ were generally large and positive. Exposure of buried hydrophobic groups to solvent upon denaturation would make a negative $\Delta S^{\ddagger}$ contribution (Franks & Eagland, 1975), as would aggregation of denatured protein via these exposed groups (Rudolph et al., 1979; Mozhaev & Martinek, 1982). In contrast, loss of native tertiary and secondary structure would be expected to make a positive contribution to $\Delta S^{\ddagger}$ (Tanford, 1968, 1970). Of those species for which small negative activation entropies were calculated, the data contained sufficient error to prevent eliminating the possibility that $\Delta H^{\ddagger}$ was small but positive. If this were true, then the actual $\Delta S^{\ddagger}$ value would also be small and positive, as seen for the slow kinetic phase for the Mn·E-II complex.

Data for both thermal denaturation kinetics and equilibrium unfolding/refolding with Gdn-HCl are consistent with formation of a least one intermediate in the overall pathway:

$$N \rightleftharpoons I \rightleftharpoons D$$

It is not suggested that I is necessarily the same species in these very different sets of experiments, especially since the conversion of I to D is irreversible in the thermally driven process. Biphasic thermal denaturation kinetics also have been reported for malate dehydrogenase (Kristjansson & Ponnamperuma, 1980), heavy meromyosin (Kimura et al., 1980), and pyridine nucleotide transhydrogenase (Voordouw et al., 1982). Light scattering and electron microscopy studies indicated that thermal denaturation of E-I, E-II, and BSGS did not produce detectable levels of hexamers, dimers, or monomers (Merkler, 1985). It is possibile, however, that low steady-state levels of these lower molecular weight species actually do form in the course of overall thermal denaturation but ultimately yield highly aggregated amorphous structures.[3] Thus, I could

[3] Differential scanning microcalorimetry studies of E-I and E-II at slow scan rates (0.25 K/min) revealed moderate endothermic profiles for both enzymes. At a scan rate of 1.0 K/min, however, the DSC thermogram for E-I showed a sharp endotherm followed by an equally large exothermic trough. One interpretation of this latter observation is that rapid endothermic formation of an intermediate species occurs, perhaps aggregated protein, followed by slower exothermic denaturation (Merkler et al., 1983).

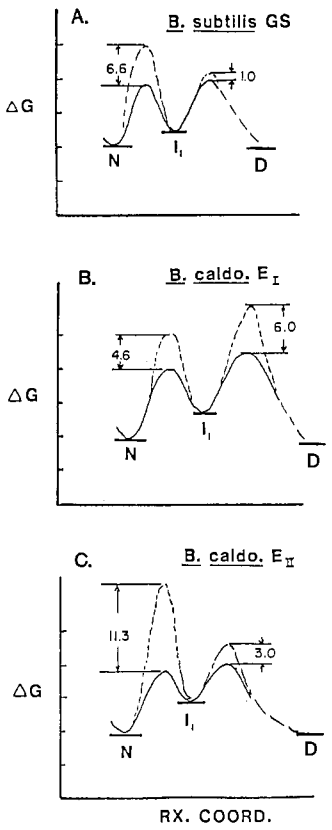


FIGURE 5: Changes in $\Delta G^{\ddagger}$ for reaction profiles of thermal denaturation with mesophilic and thermophilic *Bacillus* sp. glutamine synthetases, caused by addition of saturating concentrations of MnATP + Glu (dotted line) to apoenzymes (solid line)—data from Table II.

represent a partially unfolded, partially active subunit still *within* the native dodecamer.

The fact that partial enzyme activity was retained in the intermediate obtained during thermal denaturation may reflect a protective mechanism to allow survival of GS activity under conditions of thermal stress, allowing for refolding and renaturation. Bound ligands in vivo may serve to raise the kinetic barriers between the native (N) and intermediate (I) forms of the enzyme and the final irreversibly denatured (D) form, as shown in Figure 5.

In contrast to thermal denaturation, unfolding by Gdn-HCl is primarily a dissociative process, so that I may be a species of lower molecular weight (hexamer, dimer) as in

$$N_{12} \rightleftharpoons I_n \rightleftharpoons \rightleftharpoons I_1 \rightleftharpoons \rightleftharpoons D$$

where subscripts indicate the number of subunits. Unfolding via metastable intermediates has been observed in recent years for a number of other enzymes both monomeric and multimeric, e.g., tryptophan synthase (Crisanti & Matthews, 1981; Matthews & Crisanti, 1981), ribonuclease (Krebs et al., 1985; Nall et al., 1978), and phosphoglucomutase (Hermann & Jaenicke, 1985). Fully reversible refolding of oligomeric enzymes is relatively unusual, having been observed for only a limited number of cases (Hermann & Jaenicke, 1985; Hermann et al., 1983; Jaenicke, 1984; Jaenicke et al., 1986; Martel & Garel, 1984; Zettlmeisl et al., 1982).

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## SUPPLEMENTARY MATERIAL AVAILABLE

Revised procedures for purification of *B. subtilis* and *B. caldolyticus* GS and methods and data for Mn(II) binding to each enzyme by ESR or thermal protection methods (three figures) (8 pages). Ordering information is given on any current masthead page.

## REFERENCES

Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H., & Eisenberg, D. (1986) *Nature (London) 323*, 304–309.
Amelunxen, R. E., & Murdock, A. L. (1978) *CRC Crit. Rev. Microbiol. 6*, 343–393.
Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G., & Tratschin, J. D. (1979) *Biochemistry 18*, 5698–5703.
Aune, K. C., & Tanford, C. (1969a) *Biochemistry 8*, 4579–4585.
Aune, K. C., & Tanford, C. (1969b) *Biochemistry 8*, 4586–4590.
Biesecker, G., Harris, J. I., Thierry, J. C., Walker, J. E., & Wonacott, A. J. (1977) *Nature (London) 266*, 328–333.
Bradford, M. B. (1976) *Anal. Biochem. 72*, 248–254.
Buchanan, R. E., & Gibbons, N. E., Eds. (1974) *Bergey's Manual of Determinative Bacteriology*, 8th ed., p 533, Williams & Wilkens, Baltimore, MD.
Crisanti, M. N., & Matthews, C. R. (1981) *Biochemistry 20*, 2700–2706.
Delange, M., & Lazdunski, M. (1967) *Biochem. Biophys. Res. Commun. 28*, 390–394.
Deuel, T. F., & Stadtman, E. R. (1970) *J. Biol. Chem. 245*, 5206–5213.
Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E., & Stadtman, E. R. (1970) *J. Biol. Chem. 245*, 5195–5205.
Franks, F., & Eagland, D. (1975) *CRC Crit. Rev. Biochem. 3*, 165–219.
Freeze, E., & Fortnagel, U. (1969) *J. Bacteriol. 99*, 745–756.
Glasstone, S., Laidler, K. J., & Eyring, H. (1941) *The Theory of Rate Processes*, McGraw-Hill, New York.
Gruetter, M. G., Hawkes, R. B., & Matthews, B. W. (1979) *Nature (London) 277*, 667–669.
Heinen, W., & Lauwers, A. M. (1981) *Arch. Microbiol. 129*, 127–128.
Hermann, R., & Jaenicke, R. (1985) *Biochemistry 24*, 1817–1821.
Hermann, R., Rudolph, R., Jaenicke, R., Price, N. C., & Scobbie, A. (1983) *J. Biol. Chem. 258*, 11014–11019.
Jaenicke, R. (1984) *Angew. Chem. 23*, 395–413.
Jaenicke, R., & Rudolph, R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 525–548, Elsevier, Amsterdam.
Jaenicke, R., Rudolph, R., & Feingold, D. S. (1986) *Biochemistry 25*, 7283–7287.
Kimura, I., Arai, K., Takahashi, K., & Watanabe, S. (1980) *J. Biochem. (Tokyo) 88*, 1703–1713.
Koeffler, H. (1957) *Bacteriol. Rev. 21*, 227–240.
Krebs, H., Schmid, F. X., & Jaenicke, R. (1985) *Biochemistry 24*, 3846–3852.
Kristjansson, H., & Ponnamperuma, C. (1980) *Origins Life 10*, 185–192.
Langridge, J. (1968) *J. Bacteriol. 96*, 1711–1717.
Layne, E. (1957) *Methods Enzymol. 3*, 447–454.
Malikkides, C. O., & Weiland, R. H. (1982) *Biotechnol. Bioeng. 24*, 1911–1914.
Manavalan, P., & Johnson, W. C., Jr. (1983) *Nature (London) 305*, 831–832.
Martel, A., & Garel, J.-R. (1984) *J. Biol. Chem. 259*, 4917–4921.

Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784–792.
Merkler, D. J. (1985) Ph.D. Thesis, The Pennsylvania State University.
Merkler, D. J., Wedler, F. C., Pendleton, T. P., & Sturtevant, J. M. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, Abstr. 2046.
Mozhaev, V. V., & Martinek, K. (1982) *Enzyme Microb. Technol. 4*, 299–309.
Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.
Norris, R. D., & Fowden, L. (1972) *Phytochemistry 12*, 2109–2121.
Pohl, F. M. (1968) *Eur. J. Biochem. 7*, 146–152.
Rudolph, R., Zettlmeissel, G., & Jaenicke, R. (1979) *Biochemistry 18*, 5572–5575.
Shapiro, B. M., & Stadtman, E. R. (1967) *J. Biol. Chem. 242*, 5069–5079.
Shapiro, B. M., & Ginsburg, A. (1968) *Biochemistry 7*, 2153–2167.
Shapiro, B. M., & Stadtman, E. R. (1971) *Methods Enzymol. 17*, 910–922.
Shibuya, H., Abe, M., Sekiguchi, T., & Nosoh, Y. (1982) *Biochim. Biophys. Acta 708*, 300–304.
Singleton, R., Jr., & Amelunxen, R. E. (1973) *Bacteriol. Rev. 37*, 320–342.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755–807.
Stearn, A. E., & Eyring, H. (1937) *J. Chem. Phys. 5*, 113–124.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Voordouw, G., & Roche, R. S. (1975) *Biochemistry 14*, 4659–4666.
Voordouw, G., Milo, C., & Roche, R. S. (1976) *Biochemistry 15*, 3716–3724.
Voordouw, G., DeHaard, H., Timmermans, J. A. M., Veeger, C., & Zabel, P. (1982) *Eur. J. Biochem. 127*, 267–274.
Wedler, F. C. (1978) in *Biochemistry of Thermophily* (Friedman, S. M., Ed.) pp 325–343, Academic, New York.
Wedler, F. C., & Hoffmann, F. M. (1974a) *Biochemistry 13*, 3207–3214.
Wedler, F. C., & Hoffmann, F. M. (1974b) *Biochemistry 13*, 3215–3221.
Wedler, F. C., & Eismann, K. (1976) *Biochem. Biophys. Res. Commun. 69*, 1003–1010.
Wedler, F. C., & Merkler, D. J. (1985) *Curr. Top. Cell. Regul. 26*, 263–280.
Wedler, F. C., Kenney, R. M., Ashour, A. E., & Carfi, J. (1978) *Biochem. Biophys. Res. Commun. 81*, 122–126.
Wedler, F. C., Shreve, D. S., Kenney, R. M., Ashour, A. E., Carfi, J., & Rhee, S. G. (1980) *J. Biol. Chem. 255*, 9507–9516.
Wedler, F. C., Shreve, D. S., Fisher, K. E., & Merkler, D. J. (1981) *Arch. Biochem. Biophys. 211*, 276–287.
Wedler, F. C., Srikumar, K., Moore, K. S., & Premalatha, R. (1987) *Biochim. Biophys. Acta 912*, 144–146.
Yutani, K., Ogasahara, K., Suzuki, M., Sugino, Y., & Matsushiro, A. (1978) in *Biochemistry of Thermophily* (Friedman, S. M., Ed.) pp 233–249, Academic, New York.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1982) *Biochemistry 21*, 3946–3950.

# Thermal Stability of Proteins in the Presence of Poly(ethylene glycols)†

Lucy L.-Y. Lee and James C. Lee*
*Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104*
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ABSTRACT: Thermal unfolding of ribonuclease, lysozyme, chymotrypsinogen, and β-lactoglobulin was studied in the absence or presence of poly(ethylene glycols). The unfolding curves were fitted to a two-state model by a nonlinear least-squares program to obtain values of $\Delta H$, $\Delta S$, and the melting temperature $T_m$. A decrease in thermal transition temperature was observed in the presence of poly(ethylene glycol) for all of the protein systems studied. The magnitude of such a decrease depends on the particular protein and the molecular size of poly(ethylene glycol) employed. A linear relation can be established between the magnitude of the decrease in transition temperature and the average hydrophobicity of these proteins; namely, the largest observable decrease is associated with the protein of the highest hydrophobicity. Further analysis of the thermal unfolding data reveals that poly(ethylene glycols) significantly effect the relation between $\Delta H°$ of unfolding and temperature for all the proteins studied. For β-lactoglobulin, a plot of $\Delta H$ versus $T_m$ indicates a change in slope from a negative to a positive value, thus implying a change in $\Delta C_p$ in thermal unfolding caused by the presence of poly(ethylene glycols). Results from solvent–protein interaction studies indicate that at high temperature poly(ethylene glycol) 1000 preferentially interacts with the denatured state of protein but is excluded from the native state at low temperature. These observations are consistent with the fact that poly(ethylene glycols) are hydrophobic in nature and will interact favorably with the hydrophobic side chains exposed upon unfolding; thus, it leads to a lowering of thermal transition temperature.

Organic solvents have been utilized extensively in various aspects of studies on macromolecules, especially proteins. Empirically, polyhydric alcohols and sugars are introduced into the solvent medium in order to stabilize biological macromolecules in solution (Ball et al., 1943; Boyer, 1945; Bradbury & Jakoby, 1972; Frigon & Lee, 1972; Gerlsma, 1968; Neucere & St. Angelo, 1972). However, as a result of the detailed and

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