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Nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins: A model for the biological role of heat shock proteins

(thermotolerance/protein denaturation/temperature adaptation)

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ABSTRACT **It is demonstrated experimentally that addition of proteins that are themselves resistant to denaturation by heat or ethanol can nonspecifically stabilize other proteins that are ordinarily highly susceptible to inactivation. It is proposed that the diffusion-limited rate with which unfolded protein molecules encounter each other and become irreversibly crosslinked is reduced in the presence of substantial concentrations of an unreactive globular protein. We suggest that one of the functions of heat shock proteins, which are synthesized in large amounts after exposure of cells to increased temperature and other forms of stress, may be to stabilize other proteins kinetically in a similarly nonspecific fashion.**

When exposed to sufficiently high temperatures, cells die (as measured by reproductive assay) at a rate which, after an initial lag period, may be described by a first-order decay law. Although the cause of cell death is not definitely known, various pieces of indirect evidence suggest that the irreversible loss of vital protein structure and associated function is a major factor (1, 2). When cells are pretreated by exposure to increased but nonlethal temperatures or by brief exposure to lethal temperatures followed by a period of recovery, the treated cells exhibit a dramatically enhanced rate of survival upon subsequent exposure to lethal temperatures. This phenomenon is referred to as "acquired thermotolerance" (3).

The mechanism by which cells acquire increased tolerance to thermal stress is unknown, but attention recently has focused on the possible role of heat shock proteins (hsp), a small number ($\leq$16) of specific proteins that are transcriptionally induced and synthesized in great quantity after exposure to elevated temperature ("heat shock") (4–6). The synthesis of hsp has been studied extensively as a model of gene regulation, particularly in *Drosophila* (6) but also in various organisms throughout the phylogenetic tree (4, 7–12). Recently it has been shown that the time scale for the appearance and disappearance of hsp parallels the expression of acquired thermotolerance in various cell types (11–13) and that thermotolerance is not acquired after heat shock if synthesis of hsp is inhibited (8, 12, 14).

The synthesis of hsp is also induced by noxious stimuli other than heat shock, including arsenite (6, 14), oxygen deprivation (4, 6), and ethanol (14). It has been demonstrated that preliminary exposure to ethanol results in subsequently acquired resistance not only to ethanol but also to thermal stress (15). Moreover, thermal pretreatment appears to result in increased tolerance to ethanol (16). These findings suggest that hsp may play a role in a generalized cellular adaptation to stress.

hsp are quite widespread within the cell. Low molecular weight hsp bind predominantly to nuclear chromatin; higher molecular weight hsp are found predominantly in the cytoplasm (4, 7, 17–22). No enzymatic activity of hsp has yet been identified (6, 7).

Here we propose that hsp can contribute to enhanced stress tolerance in cells by nonspecifically stabilizing those proteins in the cell most likely to undergo irreversible loss of structure and function upon exposure to thermal or chemical stress. We demonstrate the feasibility of such a proposal by showing that proteins that are highly susceptible to irreversible inactivation or denaturation by heat and ethanol may be stabilized nonspecifically by the addition of sufficient amounts of other unrelated proteins that are themselves resistant to denaturation by heat and ethanol. A tentative molecular model for the observed effect is presented.

MATERIALS AND METHODS

Calf intestine alkaline phosphatase type I (APase), bovine plasma thrombin, bovine pancreatic ribonuclease A type I-A (RNase), fatty acid-free bovine serum albumin, fetuin type II, chicken egg white trypsin inhibitor type III-O (ovomucoid), sodium *p*-nitrophenyl phosphate, and polyethylene glycol M_r 20,000 were obtained from Sigma. Dextran T-70 and Ficoll 70 were obtained from Pharmacia. Tosylarginine methyl ester, manufactured by P-L Biochemicals, was a gift of J. Gladner (National Institutes of Health). All other chemicals were standard reagent grade.

In order to remove labile contaminants from the three stress-stable proteins (RNase, ovomucoid, and fetuin), concentrated solutions of each of these proteins in the appropriate buffer were immersed in boiling water for 1–2 min, and any precipitate that formed was separated by centrifugation and discarded. The concentration of protein remaining in solution was determined spectrophotometrically. The pH of each incubation medium prepared by mixing buffer with stable solute or with ethanol or with both was adjusted to match that of the corresponding buffer at the temperature of incubation.

Assay for Heat-Induced Coagulation of Albumin. Acetate buffer (0.1 M, pH 5.0 at 64°C) or stable solute/buffer was preheated to 64°C in a thermostatted optical cell (light path, 1 cm). At time 0, a small volume of concentrated solution of albumin in buffer was added and rapidly stirred to attain a final albumin concentration of 0.2–0.5 mg/ml with negligible pH change and minimal dilution of stable solute. The turbidity (apparent absorbance) at 600 nm was measured as a function of time.

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Abbreviations: hsp, heat shock protein(s); APase, alkaline phosphatase.

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Assay for Ethanol-Induced Coagulation of Albumin. Incubation media were prepared by mixing 6 vol of acetate/barbital buffer (0.03 M acetate/0.03 M barbital, 0.1 M Cl^-) with 3 vol of ethanol, adding stable solute if any, heating to 37°C, and adjusting the pH at that temperature to 5.5. No coagulation of stable protein was observed under these conditions. The incubation mixture was placed in an optical cell thermostatted at 37°C, and, at time 0, a small volume of concentrated albumin solution was added and rapidly stirred to attain a final albumin concentration of 0.4 mg/ml. Turbidity was measured as above.

Assay for Thermal Inactivation of APase. In a thermostatted water bath, 0.9 ml of 0.1 M acetate buffer or stable solute/buffer (pH 5.15) was preheated to 56°C. At time 0, 0.1 ml of APase solution in buffer was added and rapidly stirred to attain a final APase concentration of 0.5 mg/ml. At time t, 10 μl of the incubation mixture was removed and transferred to an optical cell containing 1.0 ml of assay buffer (1.0 M diethanolamine/0.5 mM $MgCl_2$/15 mM p-nitrophenyl phosphate, pH 9.8 at 25°C). Recoverable activity at time t was determined from the initial rate of change of absorbance (405 nm) vs. time at 25°C.

Assay for Inactivation of APase by Ethanol. Incubation media were prepared by mixing 6 vol of 0.1 M glycine buffer with 3 vol of ethanol, adding ovomucoid if required, heating to 37°C, and adjusting the pH at the temperature to 9.2. No coagulation of ovomucoid was observed under these conditions. At time 0, 0.1 ml of APase in buffer was added to 0.9 ml of the medium (preheated to 37°C in a thermostatted water bath) and rapidly stirred to attain a final APase concentration of 0.5 mg/ml. Recoverable activity at time t was measured as described above.

Assay for Thermal Inactivation of Thrombin. In a thermostatted water bath, 90 μl of glycine/NaOH buffer (0.1 M glycine) or stable solute/buffer (pH 8.4) was preheated to 54°C in a closed container. At time 0, 10 μl of buffer solution containing 40 National Institutes of Health units of thrombin was added and rapidly stirred to attain a final thrombin concentration of 0.4 unit/μl. At time t, 10 μl of the incubation mixture was removed and transferred to an optical cell containing 1.0 ml of assay buffer (1 mM tosylarginine methyl ester/1.5 mM $CaCl_2$/0.5 M Tris, pH 8.0 at 25°C). Recoverable activity was determined from the rate of change of absorbance (247 nm) vs. time at 25°C.

The following control experiments were carried out in connection with all assays of enzyme activity. (*i*) It was ascertained that the small amounts of stable solutes or EtOH introduced along with the enzyme into the assay buffer (diluted 1:100 from the incubation medium) did not influence the assay. (*ii*) It was ascertained that extended incubation at room temperature in the presence of all stable solutes utilized in the present study did not affect the subsequently measured activity of either APase or thrombin.

RESULTS

Coagulation of Bovine Serum Albumin. The dependence of turbidity upon time at 64°C in the presence of varying concentrations of RNase is shown in Fig. 1. In order to compare data obtained under different conditions, the maximal slope of the plot of turbidity vs. time is taken as a quantitative measure of the overall rate of coagulation. At albumin concentrations between 0.2 and 0.5 mg/ml, the dependence of rate upon albumin concentration is well described by rate = Ac_{alb}^n in which c_{alb} is the concentration of albumin and $n = 2.26$. The presence of a heat-stable solute that lowers the overall rate of coagulation (ovomucoid) had no significant effect upon the value of n (Fig. 2).

The effect of several heat-stable solutes upon the rate of albumin coagulation at 64°C is summarized in Fig. 3. RNase, sucrose, and ovomucoid slowed the rate of coagulation; dextran, Ficoll, and polyethylene glycol accelerated it. The effect of ovomucoid upon the rate of albumin coagulation in 30% ethanol (37°C) is shown in Fig. 4. Ovomucoid appears to retard the coagulation of albumin under these conditions even more effectively than at 64°C in the absence of ethanol.

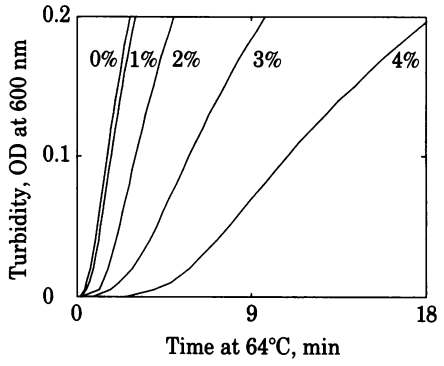

FIG. 1. Appearance of turbidity upon incubation of bovine serum albumin (0.3 mg/ml) in 0.1 M acetate (pH 5.0) at 64°C in the presence of varying concentrations of RNase. Indicated RNase concentrations are equivalent to g/dl.

Irreversible Loss of APase Activity. The dependence of the recoverable activity of APase at 25°C upon the duration of incubation of the enzyme at 56°C is plotted in Fig. 5. The heat-stable solutes reduced the rate of irreversible activity loss at 56°C in the following order of effectiveness per unit weight concentration: ovomucoid > RNase > fetuin > sucrose > sorbitol. The dependence of the recoverable activity of APase at 25°C upon the duration of incubation of the enzyme in 30% ethanol (37°C) in the absence and presence of ovomucoid is plotted in Fig. 6. The degree to which even relatively low concentrations of ovomucoid retarded and possibly halted, the irreversible loss of activity in 30% ethanol is remarkable. The rate of activity loss of APase at 37°C in the absence of ethanol was comparable to that observed in the presence of 30% ethanol and 1.6% ovomucoid (data not shown).

Irreversible Loss of Thrombin Activity. The dependence of recoverable thrombin activity at 25°C upon the duration of incubation of the enzyme at 54°C is plotted in Fig. 7. The presence of RNase seems to have little effect upon the initial rate of activity loss but, at longer times, it appears to halt further loss of recoverable activity. Polyethylene glycol and dextran, on the

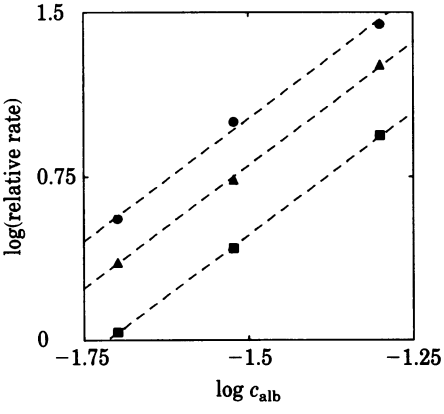

FIG. 2. Dependence of rate of albumin coagulation upon albumin concentration (c_{alb}) in the absence and presence of ovomucoid. Conditions as in Fig. 1. ●, No added ovomucoid; ▲, ovomucoid at 10 g/dl; ■, ovomucoid at 20 g/dl.

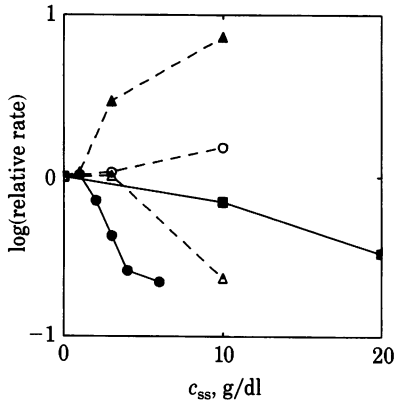

FIG. 3. Effect of concentration of various stable solutes (c_{ss}) on the relative rate of albumin coagulation. Conditions as in Fig. 1. ▲, Polyethylene glycol; ○, dextran or Ficoll; ■, ovomucoid; △, sucrose; ●, RNase.

other hand, strongly accelerated the loss of recoverable enzyme activity at 45°C. After 10 min of incubation in the presence of these solutes under the conditions indicated, recoverable thrombin activity was too low to be measured reliably (<4% of initial activity).

DISCUSSION

The results presented above demonstrate that addition of stress-resistant proteins can slow or halt precipitation or irreversible loss of enzyme activity in other proteins exposed to heat or high concentrations of ethanol. Although we have provided a sufficient variety of examples to indicate that these effects are not specific to a particular protein or pair of proteins, it cannot be inferred that they are completely general. As discussed below, we neither expect nor find this to be the case.

The effect of small molecules upon the stability of proteins has been the subject of extensive research (for example, see refs. 21–23). Some of the effect of a stress-stable macromolecule on the stability of a stress-labile protein no doubt is attributable to small molecule effects arising from local structural features of the added species. In the present study we controlled pH and performed experiments in solutions of substantial and essentially constant ionic strength in order to eliminate additive effects due to variations in these particular environmental parameters. However, two of the three stable proteins used in the present study (ovomucoid and fetuin) have substantial carbohydrate content. It is quite likely that part of the effect these substances have on the stability of stress-labile proteins is due to the well-known effect of simple sugars and polyols on protein stability (21, 24) (Figs. 3 and 5).

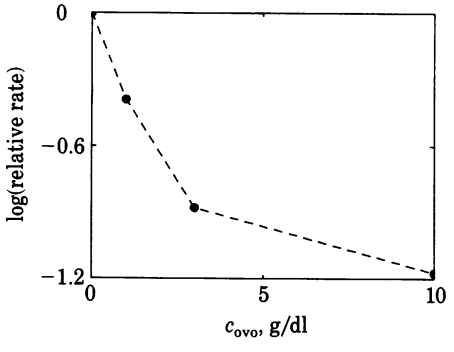

FIG. 4. Effect of added ovomucoid (c_{ovo}) on the rate of albumin coagulation in 30% ethanol (acetate/barbital buffer, pH 5.5, 37°C).

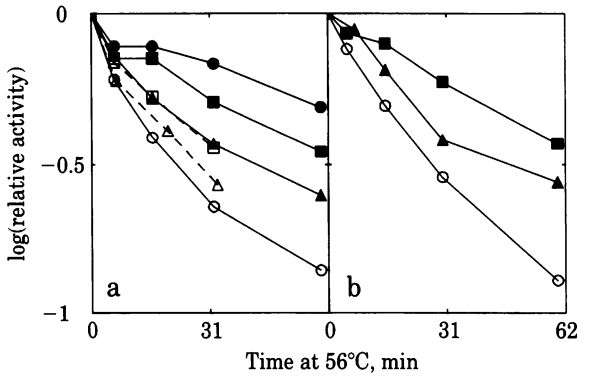

FIG. 5. Dependence of the recoverable activity of APase upon duration of incubation in 0.1 M acetate (pH 5.15) at 56°C, in the absence and presence of stable solutes. (*a*) ○, Control; △, sorbitol at 9 g/dl; □, sucrose, 9 g/dl; ▲, ovomucoid, 1.5 g/dl; ■, ovomucoid, 4.5 g/dl; ●, ovomucoid, 9 g/dl. (*b*) ○, Control; ▲, fetuin, 3.8 g/dl; ■, RNase, 6.3 g/dl.

Of greater interest to us in the present context is that part of the total effect of macromolecular additives upon protein stability that cannot be attributed to small molecule effects—i.e., deriving specifically from the macromolecular nature of the additive. For example, whereas sucrose and other simple sugars stabilize proteins, the polysaccharides dextran and Ficoll clearly destabilize them. The model described below is presented as an initial attempt to account for these specifically macromolecular effects on the basis of a molecular mechanism and is not expected to be either complete or correct in all of its particulars.

For simplicity we represent all enzymatically active conformations of the protein as a single species denoted N (for "native") and all enzymatically inactive or unfolded conformations as a single species denoted D (for "denatured"). A hypothetical scheme for interconversion between them is written as

$$N \underset{k_R}{\overset{k_D}{\rightleftharpoons}} D \qquad [1]$$

in which k_D and k_R are rate constants for "denaturation" and "renaturation," respectively. The process of coagulation is represented by the irreversible formation of clusters of unfolded or partially unfolded protein molecules:

$$D_m + D \xrightarrow{k_{m1}} D_{m+1}. \qquad [2]$$

These clusters are not specific compounds but probably rep-

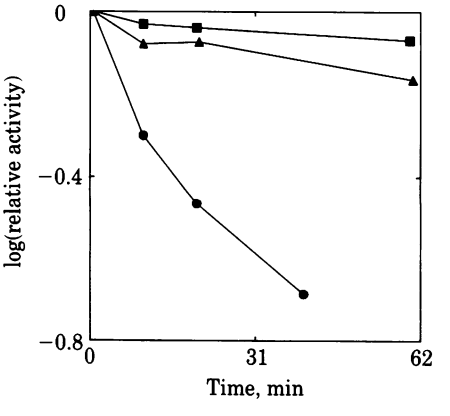

FIG. 6. Dependence of the recoverable activity of APase upon duration of incubation in 30% ethanol (0.1 M glycine, pH 9.2, 37°C) in the absence and presence of ovomucoid. ●, Control; ▲, ovomucoid at 1.6 g/dl; ■, ovomucoid, 4.8 g/dl.

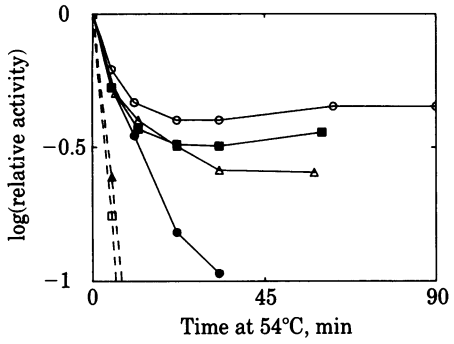

FIG. 7. Dependence of recoverable thrombin activity upon duration of incubation in 0.1 M glycine (pH 8.4) at 54°C in the absence and presence of stable solutes. □, Polyethylene glycol, 9.5 g/dl; ▲, dextran, 9.5 g/dl; ●, control; △, RNase, 3.1 g/dl; ■, RNase, 6.2 g/dl; ○, RNase, 12.4 g/dl.

resent the end product of various nonspecific crosslinking reactions (25), one of which is depicted schematically in Fig. 8*a*. The rate constant for reaction **2** will be equal to the frequency of collision of the two species D_m and D times the probability that, having collided, they will become affixed to each other. Thus, we may write

$$k_{m1} \propto (D_m + D_1)[D_m][D]P_{m1} \qquad [3]$$

in which D_i is the self-diffusion coefficient of species i, $[D_i]$ is the concentration (wt/vol) of D_i, and P_{m1} is the conditional probability of crosslinking upon collision of species D_m and D.

The rate of appearance of turbidity depends in a complex fashion on both the rate of formation of the various clusters D_i and upon the light-scattering properties of each cluster. Our observation that the rate of appearance of turbidity in heated solutions of albumin varies as $c_{alb}^{2.26}$ indicates that, under the conditions at which we studied this reaction, the rate-limiting step or steps involve self-association of protein molecules. (Were reaction **1** rate limiting, the rate of appearance of turbidity would vary linearly with albumin concentration.)

When a stable macromolecule, X, is added to the solution containing the denaturing protein, allowance must be made for the possibility that the added species will coprecipitate with the denatured protein. Even stable solutes that do not react chemically with D can be physically crosslinked with it, as illustrated in Fig. 8*b*. The set of reactions represented by reaction **2** therefore may be generalized to

$$D_mX_n + D \xrightarrow{k_{mn10}} D_{m+1}X_n \qquad [4a]$$

$$D_mX_n + X \xrightarrow{k_{mn01}} D_mX_{n+1}. \qquad [4b]$$

By analogy to Eq. **3**, the rate constants may be shown to be proportional to the following factors:

$$k_{mn10} \propto (D_{mn} + D_{10})[D_mX_m][D]P_{mn10} \qquad [5a]$$

$$k_{mn01} \propto (D_{mn} + D_{01})[D_mX_n][X]P_{mn01} \qquad [5b]$$

in which D_{mn} is the self-diffusion coefficient of species D_mX_n, P_{mn10} is the conditional probability of crosslinking upon collision of species D_mX_n and D, and P_{mn01} is the conditional probability of crosslinking upon collision of species D_mX_n and X.

In our study we observed that the rate of coagulation or loss of recoverable activity under denaturing conditions was increased in the presence of the random-coil polymeric additives polyethylene glycol, dextran, and Ficoll, whereas it was decreased in the presence of the stable globular proteins RNase, fetuin, and ovomucoid. We believe that the difference between these two types of additives is due to a requirement that, in order for a physical crosslink to be formed between D and X, both species must be at least partially unfolded. Hence, if X is a polymer, $P_{mn01} > 0$; and if X is a globular protein, $P_{mn01} \simeq 0$. In the latter case, the reaction scheme indicated by Eqs. **4** and **5** reduces to that indicated by Eqs. **2** and **3**.

We attribute the ability of stable globular proteins to decrease the rate of coagulation of D to the effect of total macromolecular concentration on the rate of self-diffusion of D and D_m. The diffusion coefficient of a trace component, D, varies with the fraction of total solution volume occupied by macromolecules, ϕ, in a manner that may be approximated by the expression

$$D(\phi) = D_0 e^{-g\phi}$$

in which D_0 is the self-diffusion coefficient of the trace component in the limit of $\phi = 0$ and g is a coefficient whose value is independent of ϕ but varies with the relative sizes and shapes of the trace component and the predominant space-filling species (26). As an example of the order of magnitude of this effect, it has been found that the self-diffusion coefficients of trace amounts of labeled hemoglobin or bovine serum albumin in buffer are approximately halved by the addition of 10 g of the corresponding unlabeled protein per dl (27, 28).

In preliminary experiments we observed that the rate of loss of recoverable activity of some enzymes (acetylcholine esterase, aryl sulfatase) was not significantly affected by the addition of heat-stable proteins. In the model described above, the reduction of the rate of loss of recoverable activity by the addition of heat-stable proteins is attributed to the effect of these proteins upon the rate of irreversible self-association of D. However, irreversible loss of enzyme activity can occur for reasons other than coagulation. (*a*) The original native conformation is not stable relative to D, but the rate of spontaneous denaturation under normal conditions is very low ($k_D > k_R$, but $k_D \ll 1/t^*$), in which t^* is the span of time over which the protein is observed. (*b*) N is stable relative to D, but the rate of renaturation under normal conditions is very low ($k_D < k_R$, but $k_R \ll 1/t^*$). To the extent that the process of self-association is not rate limiting, macromolecular additives would not be expected to alter the rate of activity loss.

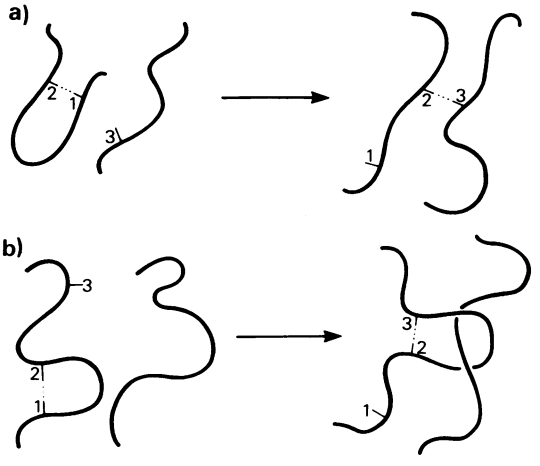

FIG. 8. Irreversible crosslinking via disulfide interchange. (*a*) Chemical crosslinking between two uncoiled protein molecules. (*b*) Physical crosslinking between an uncoiled protein molecule and a chemically inert random-coil polymer.

In order to account for the observation that an added protein can reduce the extent as well as the rate of loss of recoverable enzyme activity (Fig. 7), it is necessary to invoke a nonspecific effect of added protein upon interactions between molecules of N as well as between molecules of D. Excluded volume theory predicts that, as the fraction of solution volume occupied by total protein increases, the tendency of each protein species to self-associate will be greatly enhanced (26). We suggest that thrombin, which is a monomer in the absence of added protein, may be induced to self-associate partially or completely by the addition of sufficient quantities of a globular "inert" protein such as RNase. The oligomeric form of thrombin is likely to be more heat stable than the monomeric form because the presence of stabilizing intramolecular interactions between molecules of N within the oligomer will increase the activation energy for conversion of N to D. Hence the plateau of residual activity at long time seen in Fig. 7 may be due to the fraction of thrombin that was present as oligomer during the period of incubation.

The hypothesis that thermotolerance is mediated at least in part via the nonspecific effects of stress-stable proteins is not intended to account for the complete biological role of all hsp. For instance, it appears that binding of some of the lower molecular weight hsp in *Drosophila* play a specific regulatory role at the level of chromosomes (17). The current model, however, has several attractive features.

(*i*) It explains why hsp are synthesized in large quantity. For example, in yeast the major hsp alone accounts for 2.5% of total cell protein, a 5- to 10-fold increase over the level existing prior to heat shock (9), and in *Drosophila* the combined hsp exceed 10% of total cell protein (5, 17). In *Drosophila* and chicken embryo fibroblasts, hsp account for >50% of protein synthesis after heat shock (6, 10). This degree of protein production, which seems excessive if the function of hsp is enzymatic, is entirely compatible with the larger quantity of protein required for the nonspecific effects proposed in the present model.

(*ii*) The proposed model and supporting data account for the experimental observation that heat-shocked cells develop resistance to subsequent ethanol exposure as well as to heat exposure (16). Regardless of the particular kind of stress imposed upon the cell, an increase in the concentration of stress-resistant globular proteins will tend to stabilize stress-susceptible species.

(*iii*) The model can account for nonspecific stabilization of labile proteins by stress-resistant proteins in linear or planar environments such as chromosomes, cytoskeletal elements, organelles, or membranes as well as in bulk solution. For example, one may regard integral membrane proteins as solutes in a two-dimensional lipid solvent. A model treating volume exclusion and macromolecular diffusion in two rather than three dimensions yields results qualitatively similar to those presented in this paper (unpublished calculations). Such considerations are not incompatible with recent suggestions that the low molecular weight hsp may participate in stabilization of chromatin, whereas high molecular weight hsp may play a role in stabilization of the cytoskeleton (20).

(*iv*) The central feature of the present hypothesis is the physical and nonspecific character of the stabilization afforded by added stress-resistant protein. Because the proposed mechanism in principle can account for the stabilization of a wide variety of different thermal targets against inactivation or denaturation, it may explain the apparently universal occurrence of hsp synthesis throughout the phylogenetic tree (12, 20).

We have proposed that significant amounts of hsp in a physiological compartment is likely to result in the nonspecific lowering of the rate of irreversible loss of vital functions provided by otherwise stress-labile proteins in that compartment. This hypothesis would be greatly strengthened if experiments similar to those reported above were carried out using actual hsp in place of the stress-resistant proteins utilized here. Such experiments must await the purification of large (multigram) quantities of hsp or the development of microassay techniques that would obviate the requirement for large quantities of stress-resistant protein.

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