REVIEW ARTICLE

The denaturation and degradation of stable enzymes at high temperatures

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Now that enzymes are available that are stable above 100 °C it is possible to investigate conformational stability at this temperature, and also the effect of high-temperature degradative reactions in functioning enzymes and the inter-relationship between degradation and denaturation. The conformational stability of proteins depends upon stabilizing forces arising from a large number of weak interactions, which are opposed by an almost equally large destabilizing force due mostly to conformational entropy. The difference between these, the net free energy of stabilization, is relatively small, equivalent to a few interactions. The enhanced stability of very stable proteins can be achieved by an additional stabilizing force which is again equivalent to only a few stabilizing interactions. There is currently no strong evidence that any particular interaction (e.g. hydrogen bonds, hydrophobic interactions) plays a more important role in proteins that are stable at 100 °C than in those stable at 50 °C, or that the structures of very stable proteins are systematically different from those of less stable proteins. The major degradative mechanisms are deamidation of asparagine and glutamine, and succinamide formation at aspartate and glutamate leading to peptide bond hydrolysis. In addition to being temperature-dependent, these reactions are strongly dependent upon the conformational freedom of the susceptible amino acid residues. Evidence is accumulating which suggests that even at 100 °C deamidation and succinamide formation proceed slowly or not at all in conformationally intact (native) enzymes. Whether this is the case at higher temperatures is not yet clear, so it is not known whether denaturation or degradation will set the upper limit of stability for enzymes.

INTRODUCTION

During the 1980s, organisms were discovered that are capable of survival and growth at temperatures up to 110 °C [1]. Most biochemists accept that an integral feature of such extremely thermophilic organisms are enzymes that are stable *in vivo* at these temperatures. With the discovery of such enzymes that are stable above 100 °C [2,3] (Table 1), it is no longer feasible to confine the study of temperature effects on enzymes to denaturation and the effects of heat on activity. Degradation is likely to play a major role in the loss of enzyme activity above 80 °C, and an increasing number of studies are now describing such effects. The present review will deal with both denaturation and degradation of enzymes at high temperatures, and the inter-relationship of these with each other and with activity. We define denaturation here as loss of tertiary (and often secondary) protein structure not involving covalent bond cleavage, and as being, in principle at least, reversible. Degradation is the loss of primary structure with associated covalent bond cleavage and/or formation, and is irreversible. Because we wish to look at the upper limits of enzyme stability, we will confine ourselves to events taking place readily above 80 °C in aqueous conditions. Much of this work has dealt with the stability of enzymes from extreme thermophiles, since other enzymes stable above 80 °C for useful periods are uncommon. Throughout the review we have attempted to deal with enzyme stability rather than protein stability. While in general we expect the review to apply to all proteins, it is possible that those proteins that have structural, binding or transport functions have a range of structural and dynamic properties which only partially overlap the properties of catalytic proteins, broad though these are.

Table 1 Some enzymes stable above 100 °C

Data from [2,3].

Enzyme	Source	Half-life (h)	Temperature (°C)
Glyceraldehyde-3-phosphate dehydrogenase	*Thermotoga maritima*	> 2	100
Hydrogenase	*Pyrococcus furiosus*	2	100
Amylase	*P. woesei*	6	100
DNA–RNA polymerase	*Thermoproteus tenax*	2	100
Glutamate dehydrogenase	*P. furiosus*	10	100
Cellobiohydrolase	*Thermotoga* sp.	1.1	108
Amylase	*P. furiosus*	2	120

Enzyme denaturation has been the subject of much work over an extended period (e.g. [4,5]), although relatively few detailed studies have been carried out above 80 °C. What is new in this field is the study of enzyme stability at temperatures significantly higher than 100 °C [6,7]. As Figure 1 shows, enzymes are now available which, with or without stabilizing treatments or conditions, have half-lives in excess of 10 min at 130 °C. Furthermore, enzyme activity has been measured at these same high temperatures [8–11]. This is important because many observations of high-temperature enzyme stability have been performed simply by heating the enzyme, rapidly cooling a sample, and assaying for residual activity at a lower temperature (e.g. 95 °C). This procedure is open to criticism because of the possibility that the enzyme may be denatured at high temperature

Abbreviation used: ΔG, the difference in free energy between the folded and unfolded states of a protein.

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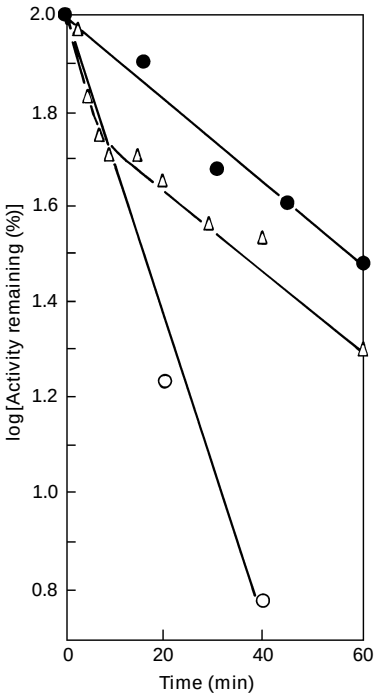

Figure 1 Stability of three enzymes at 130 °C

The plot shows the loss of activity with time of the unpurified amylase from *Pyrococcus furiosus* (○), the α-glucosidase from *Thermococcus* strain ANI in 90% sorbitol (●), and the immobilized xylanase from *Thermotoga maritima* sp. FjSS3BI in molten sorbitol (△). (Data from [8], [11] and [9] respectively.)

and then renatured (thus regaining activity) rapidly enough to display high activity in the cooled assayed sample. While we know of no cases where rapid renaturation of a very thermophilic enzyme has occurred in this way, high-temperature measurements of activity greatly reduce the likelihood that an apparent high-temperature stability is actually caused by reversible denaturation. Data on enzyme stability at high temperatures have been presented by some authors as 'T_{opt}', i.e. the peak temperature on a graph of product produced over a fixed time against temperature. These data are not very helpful in ascertaining enzyme stability since, in addition to the complicating effect of substrate, the T_{opt} will be highly dependent on assay duration (not always given), on stability and on the effect of temperature on activity. The latter may vary from Q_{10} values of 0.4 [i.e. an activity increase of 40% for a 10 °C rise in temperature (for a thermophilic xylanase; C. Aebischer and R. M. Daniel, unpublished work)] to 4.5 (for a thermophilic proteinase [12]). Other temperature-dependent factors such as buffer pK_a, water activity and dielectric constant may also have an effect. Furthermore, such T_{opt} graphs are often derived from measurements of product after a single fixed reaction time, at temperatures where the rate of product formation is almost certainly not linear with respect to time.

Temperature-driven degradation is a newer field of study. Over the time spans we are considering here (minutes to hours), and at neutral pH, such degradation is only significant above 80 °C.

A variety of 'physiological' agents can act to stabilize enzymes against heat, although none will stabilize all enzymes. Calcium, for example, is a well known stabilizer of many enzymes at both high and low temperatures. A number of agents (including protein) which act as stabilizers at high concentration may do so by altering the water activity/dielectric content of the medium. In particular, at very low hydration levels (below about 0.4 g of water/g of protein [13]), proteins are greatly stabilized, both as solids [14] and in non-aqueous solution [15]. At high temperatures the most notable stabilizing agents are probably cyclic 2,3-diphosphoglycerate and di-*myo*-inositol 1,1′-phosphate, found in *Methanothermus fervidus* and *Pyrococcus woesei* respectively. In the presence of potassium, these agents increase the half-lives of some enzymes by up to 130-fold at 90 °C [16].

CONFORMATIONAL STABILITY OF ENZYMES

When stable enzymes from thermophiles became available, they were seized upon as tools for the investigation of protein conformational stability. Much of this work compared stable enzymes from extreme thermophiles with less stable enzymes of similar structure and function from mesophiles. Amino acid composition data were often available, sequences less frequently so, and structural information was relatively rare. On the basis of these compositional comparisons a number of fairly specific proposals were made for amino acid changes associated with increased thermal stability, for example an increased arginine/lysine ratio [17,18]. Now enzymes that are stable over an even greater temperature range are available, and more composition (and sequence) data have been obtained, it has become apparent that these 'traffic rules' lack predictive value and statistical significance. The most unequivocal evidence of this comes from a study of the amino acid sequences and compositions of 26 glyceraldehyde phosphate dehydrogenases from organisms growing optimally over the temperature range 20–102 °C, carried out by Bohm and Jaenicke [19] (Figure 2). The data may suggest low serine and glycine and high isoleucine content in the more thermophilic enzymes. However, although the serine content of the *Thermus aquaticus* enzyme is the lowest found (3.5%), the even more thermophilic enzymes have a serine content identical to that of the *Escherichia coli* enzyme; and while the isoleucine and glycine values for the archaeal hyperthermophiles fall outside the mesophilic range, values for the thermophilic bacteria do not [19]. The archaeal origin of the enzymes thus seems to be a more likely cause of amino acid compositional variation than does thermophily.

It is clear that, if there are amino acid changes associated with thermophile enzymes, they are small and probably not universal, and with the number of sequences currently available are not statistically detectable above the background of amino acid changes which are evident for taxonomic or functional reasons. Even in the cases where there is specific evidence bearing on the way a particular amino acid may influence stability, as in the cases of proline, asparagine and cysteine for example (see below), there are enough significant exceptions to cast serious doubt on the universality of any proposed amino acid substitution 'rules' which take no account of the position of the amino acid in the structure.

Structural data give much more specific information. Using a mesophilic ferridoxin structure, Perutz and Raidt [20] compared ferridoxins from mesophiles and thermophiles, and attributed half-life differences of around three orders of magnitude to a quite small number of specific interactions. More recent and detailed structural studies of another low-molecular-mass non-haem iron protein, rubredoxin from the archaeal hyperthermophile *Pyrococcus furiosus* [21,22], indicated that the structure is very similar to that of mesophilic rubredoxins. The authors suggest that additional hydrogen bonds within the β-sheet and a

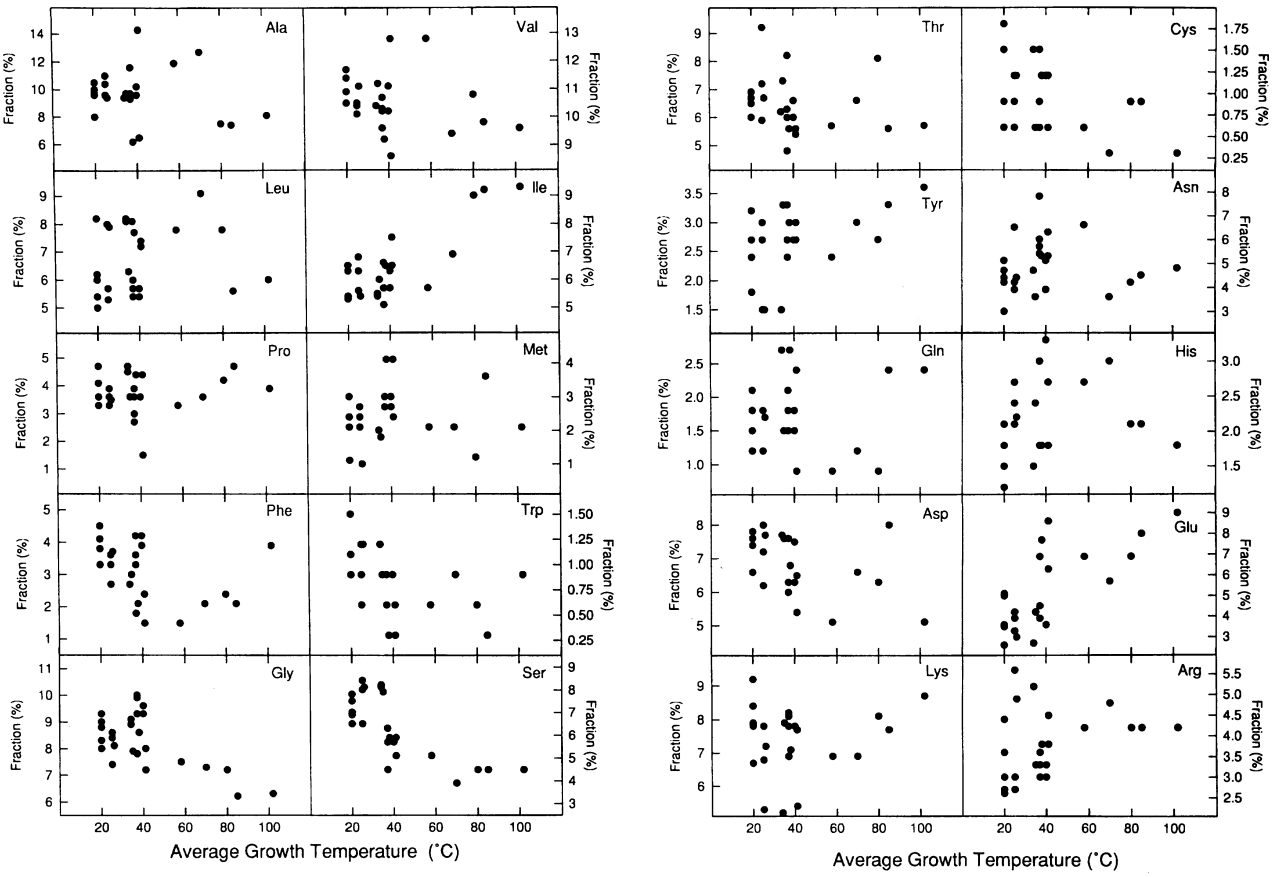

Figure 2 Amino acid occurrences in the database of D-glyceraldehyde-3-phosphate dehydrogenases related to the growth temperature of the enzyme source

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few extra electrostatic interactions at the protein surface may account for the greater stability of the *Pyrococcus* rubredoxin.

However, accounting for the difference in stability between two homologous enzymes in this way has not enabled the formulation of systematic mechanisms by which thermostability can be, or even has been, achieved. For this to be the case the structural mechanisms proposed (e.g. loss of surface loops, restriction of N-terminus mobility, stabilization of α-helix dipoles) should be more frequent in the more stable variants of enzymes of similar structure and function, and less frequent in less stable enzymes, and this should be observable in a variety of different enzyme groups. There is not good evidence for this at present. It is possible that more data will resolve this issue, but there currently seems no good reason why a single mechanism or small subgroup of mechanisms should be widely favoured out of the variety of potentially stabilizing structural mechanisms, except in enzymes which have a high degree of sequence and structural identity.

The most structually similar proteins from mesophiles and extreme thermophiles show multiple amino acid differences, and even with structural information it is difficult to identify those changes arising from changed stability rather than from taxonomic or minor functional differences. This problem can be overcome by using point mutations as a probe of stability. In an early study [23], single mutations spread along the β-galactosidase gene of *E. coli* were found to be mostly destabilizing (18 out of 56 decreasing the half-life by more than 10-fold) or had little effect (37 of the 56). Only one mutation increased the half-life by more than 2-fold. The destabilizing mutations tended to be grouped into particular parts of the sequence. Much more detailed research, especially by Matthews and colleagues, who have coupled the use of point mutation with detailed structural studies, has confirmed and greatly extended these earlier results (for reviews, see [24–26]).

A variety of studies [27–31] have shown that the majority of point mutations have little effect on stability. For example, of over 2000 mutations made to T4 lysozyme, 91% had no significant effect on thermal stability [24,25]. Most of these mutations appeared to be at the surface of the protein. However, for those mutations that alter stability, destabilization is a more common effect. For a number of enzymes where enough data have been gathered [28–31], including lysozyme [28] and subtilisin [31], the occurrence of random point mutations which cause stabilization is of the order of 1 in 10000. The destabilizing mutations in lysozyme (about 9% of the total) all occurred at amino acids which have restricted mobility [24,25,32]. These are likely to be in the interior of the protein, and to be stabilized by hydrophobic interactions. This tends to support the view that hydrophobic interactions are the most important interaction in protein conformation [33]. On the other hand, there is a correlation between a large change in the partial heat capacity of unfolding of a protein (which is proportional to the interior hydrophobicity) and low net stability [34]. This may indicate that hydrogen bonding and electrostatic interactions are more im-

portant than van der Waals interactions and the hydrophobic effect.

Conformational stability can be affected by altering the stability of the unfolded as well as of the native form of the protein. Thus a mutation which decreases the conformational freedom of the unfolded state will raise its free energy and increase ΔG (the difference in free energy between the folded and unfolded states), stabilizing the native protein. The substitution of proline for other amino acid residues has been proposed to have a general stabilizing effect [35–37], in some cases due to restriction of the conformational freedom of the unfolded state [36]. However, this is clearly not a universal stabilization rule [19], and the position of proline in the structure is probably a dominating factor [37].

Although the work of Matthews [25] and others has led to great increases in our understanding of the way amino acid substitutions affect structure and stability, variation in protein structure is so great, and the intramolecular interactions so complex, that there has been little success in developing simple and widely applicable amino acid substitution 'rules' by which protein stability may be increased. However, for more tightly defined and specific elements which occur widely in protein structure, e.g. the α-helix, success may be closer. Introduction of a negatively charged residue near the N-terminus of an α-helix has a stabilizing effect due to enhanced electrostatic and hydrogen bonding interactions within the helix [25,33,38–41]. Given the fundamental role played by α-helices, this may be a useful stabilizing strategy, although substitution at the ends of the α-helices may require a very good knowledge of the local stereochemistry to allow such a stabilization strategy to be used reliably.

CONFORMATIONAL STABILITY AT HIGH TEMPERATURES: THEORETICAL CONSIDERATIONS

It has been established for some time that the tertiary structure of proteins is only marginally stable (e.g. [4]). The conformational stability of a protein (defined as the difference in free energy between the folded and unfolded states, ΔG) is the sum of a large number of weak, non-covalent, interactions, including hydrogen bonds, van der Waal interactions, salt bridges and the hydrophobic effect, and the destabilizing forces arising largely from conformational entropy. All of these forces are affected by environmental conditions, including, for example, solvent and temperature, in a complex way.

In an 'average' protein the sum of the stabilizing interactions is large, about 1 $MJ \cdot mol^{-1}$. Destabilizing forces are also large, and ΔG, the difference between the two, is only of the order of 40 $kJ \cdot mol^{-1}$. A single weak interaction, for example, may contribute up to 25 $kJ \cdot mol^{-1}$ [25]. Small changes in the number or strength of the stabilizing interactions will thus have a major effect on stability.

From these considerations (and with hindsight), the very high stability of enzymes from extreme thermophiles does not seem so remarkable. Perutz and Raidt [20] have suggested that, assuming the same ground-state free energy for two similar proteins, the corresponding difference in the free energy of activation, $\Delta G^{\ddagger}$, is given by $\Delta G^{\ddagger} = \boldsymbol{RT}\ln.\Delta t_{\frac{1}{2}}$, where $\Delta t_{\frac{1}{2}}$ is the difference in half-lives of the two proteins. This can be used to approximate the stabilizing energy difference between two proteins. On this basis, comparison of the denaturation rates at 70 °C of two highly similar enzymes, one from a mesophile and one from an extreme thermophile, might well give us a ratio of the order of $1:10^6$, and thus a difference in stabilizing energies of the two proteins of about 40 $kJ \cdot mol^{-1}$ at 70 °C. This could correspond to a rather small number of well placed stabilizing interactions.

Relatively good experimental evidence is available for this contention in terms of destabilization. A number of examples are known [25] where a point mutation has lowered ΔG by more than 20 $kJ \cdot mol^{-1}$. However, such evidence is less readily available in terms of stabilization. We can calculate approximately the sum of the stabilizing interactions responsible for the integrity of the tertiary structure of a particular protein, and the magnitude of some individual interactions in a particular protein, but it is difficult to estimate the extent to which all the amino acids in a protein participate in stabilizing interactions, so we do not know the possibilities for developing additional interactions and thus achieving higher stability by point mutation. On the one hand we have the fact that the majority of point mutations of proteins have little or no effect on stability, suggesting that only a minority of amino acids are involved in significantly stabilizing interactions. On the other hand, quite extensive efforts to use single point mutations to enhance protein stability have been relatively unsuccessful in increasing half-lives by amounts corresponding to stabilizing energy differences (see above and [20]) of as much as 5 $kJ \cdot mol^{-1}$. Nevertheless, accumulated point mutations have led to stability increases of more than 15 $kJ \cdot mol^{-1}$ [42], and there does not seem to be any theoretical objection to proteins of very much greater conformational stability than those currently known.

From the above considerations it is evident that structural changes are not a requirement for changed stability. There is also no strong evidence that evolutionary changes in enzyme stability have proceeded by any particular strategy. There is a trend towards an increase in the average hydrophobicity of amino acids in some thermophilic enzymes [17] and, given that the hydrophobic effect is strongest at around 70 °C (and is weaker at temperatures above and below this), this might seem to indicate a broadly applicable stabilization strategy. However, this increase in hydrophobicity is also seen in hyperthermophilic enzymes which are stable above 100 °C [43]. The hydrophobic effect plays a major role in enzyme stability, and it would be surprising if in many very stable enzymes an increase in hydrophobicity could not be detected. Examples of increased enzyme stability caused by other weak interactions are also known [30,41,44].

A variety of strategies have been successful in achieving modest increases in protein stability, in addition to point mutations. These have included the incorporation of metal binding sites [45], elimination of a buried solvent molecule [46] and addition of a peptide β-hairpin [47]. Detailed comparison of mesophilic and extremely thermophilic enzymes has led to suggestions that the elimination of cavities within the enzyme structure and a decrease in the number of surface loops [48] may be ways of achieving greater stability. However, in addition to the difficulty of distinguishing which structural changes are associated with stability rather than, say, activity, comparisons are often further complicated by the likelihood that the extremely thermophilic enzyme will be from an Archaea, which is taxonomically separated from bacteria and possibly more primitive [7,48]. As is the case for amino acid substitutions, there is not yet evidence that any of these mechanisms are of very broad applicability. In structural terms the differences between stable and less stable enzymes are no greater than those found between enzymes of similar stability.

INTER-RELATIONSHIP OF ENZYME STABILITY AND ACTIVITY

Relatively few studies have been carried out on the dynamics of very stable enzymes, but evidence from hydrogen–deuterium

exchange shows that, at a given temperature, thermostable enzymes are less flexible than thermolabile ones [49], and more specifically that enzymes from extreme thermophiles are, at room temperature, less flexible than those from mesophiles [50,51]. Theoretical studies [52] confirm this. However, at the temperatures at which they have evolved to act, the flexibility of enzymes from mesophiles and extreme thermophiles is similar.

There is considerable evidence that enzyme activity is dependent upon enzyme flexibility [53–56]. It is implicit in the concept of induced fit [57,58] that an enzyme must flex over a time scale in keeping with its catalytic-centre activity, and that changes between conformational substates allow catalytic activity. Enzyme activity has been demonstrated at temperatures below the glass transition [59], where mobility does not occur [60–63], but catalytic-centre activities are very low, and the significance of this activity is not yet clear.

Mesophilic enzymes from different sources carrying out the same reaction display a very wide range of specific activities. Once enough thermophilic enzymes have been studied, we may well find their range of specific activities to be large also. It is therefore difficult to validly compare enzymes of similar structure and function from mesophiles and thermophiles in terms of their specific activities. Such a comparison is further complicated because the 'natural' (*in vivo*) growth temperatures of many thermophiles may be different from those found using relatively rich growth media. It is fairly clear that an *E. coli* strain isolated from the human gut and growing optimally *in vitro* at 37 °C has evolved to function at that temperature. However, we do not know with any degree of certainty the temperatures at which most thermophiles (and indeed most mesophiles) have evolved. For example, *Thermus* strains isolated from hot pools at 85 °C may grow optimally *in vitro* at 70 °C. With these reservations, there is not currently good evidence to suggest that enzymes from thermophiles and mesophiles have different specific activities at their respective growth temperatures, apart from those special cases where changes in the susceptibility of the substrate occur at different temperatures. A typical example of this is the high specific activity of thermophilic proteinases when (denatured) mesophilic protein is the substrate, but not when small peptides are the substrate [64].

The activities of very stable enzymes thus correlate well with what is known of their dynamics: at temperatures where their flexibility is similar to that of less stable enzymes, there is no evidence for differences in specific activity. This correlation extends to conformational stability. Although enzymes from organisms growing at high temperatures are more stable than their counterparts from mesophiles, they are still denatured fairly quickly 20 °C or so above the optimum growth temperature for the organism [2]. The stabilities *in vivo* of the enzymes from both mesophiles and thermophiles are generally similar; or, to put it another way, the stabilities are similar at similar levels of molecular flexibility.

We can therefore correlate in general terms conformational stability, flexibility and specific activity, and together with the general structural and functional identity of stable and less stable enzymes, this leads to the view that the instability of enzymes from mesophiles is a functional requirement, rather than because of any restraint on achieving higher stability. It is required so that enzymes have sufficient flexibility to perform their catalytic functions. An additional requirement for instability can be inferred from the finding that, irrespective of whether or not they are denatured, stable proteins are more resistant to proteolysis [65]. Excessively stable enzymes may therefore hinder the normal cellular turnover of enzymes.

While enzyme activity is dependent upon flexibility, a less flexible enzyme will be more stable. Techniques that restrain free movement of the enzyme at the molecular level, such as immobilization and intramolecular cross-linking, tend to stabilize enzymes [66], including enzymes from extreme thermophiles [9,11]. However, excessive flexibility is the first step towards denaturation, so although an enzyme must be sufficiently rigid to have a reserve of stability, it must also have the flexibility needed for effective catalysis. These are of course generalizations, since stability is a global property of an enzyme [25] whereas the flexibility required for activity may well be localized [54–56]. They do, however, explain why enzymes tend in general to be denatured at temperatures not very far above their 'design' temperature. Too much stability would mean not enough flexibility for effective catalysis, whereas too little stability means too short a useful lifetime [7]. Point mutations which increase enzyme stability often lower specific activity [44,67–69], and vice versa. There is, therefore, strong evidence for the contention that enzyme stability, flexibility and activity are closely interrelated, and that a balance between stabilizing and destabilizing interactions is required to meet the conflicting demands of stability on the one hand, and catalytic function and cellular turnover on the other. As a consequence, if we consider only conformational stability, we may postulate that the reason we have not found proteins that are stable much above 130 °C is because we have not found organisms growing above 110 °C (rather than vice versa).

PROTEIN DEGRADATION ABOVE 80 °C

Although conformational stability can be maintained for thermophilic proteins at high temperatures [2,3], irreversible degradative processes can lead to enzyme inactivation [70,71]. In contrast to denaturation, the irreversible processes of protein inactivation arise from changes in covalent bonding. Deamidation of the amide side chain of Asn and Gln residues, succinimide formation at Glu and Asp, and oxidation of His, Met, Cys, Trp and Tyr are the most facile and common amino acid degradations. These mechanisms of protein degradation are greatly accelerated at high temperatures, and can thus play an important role in the thermo-inactivation of enzymes.

Asparagine residues

Asn residues are labile due to the deamidation of the side-chain amine, which yields Asp (Figure 3). This Asn deamidation in proteins and peptides in aqueous solution can proceed at a much higher rate than is observed for hydrolysis of an amide linkage of a peptide bond [70,72,73], and the increased rate suggests an intramolecular reaction [74,75]. Some deamidation in peptides and proteins is known to occur through intramolecular attack of the carboxy-peptide-nitrogen on the side-chain γ-carbonyl carbon, resulting in the formation of a succinimide ring [72,76]. The succinimide is unstable in aqueous solution and is hydrolysed at either carboxy group, producing a mixture of aspartyl and isoaspartyl peptides (Figure 3). The succinimide can further react by hydrolysis or racemization and yield L- and D-aspartyl and L- and D-isoaspartyl peptides [72].

The mechanism of deamidation in peptides is pH-dependent. Slow deamidation in peptides at pH 1–2 appears to largely bypass the succinimide intermediate [77]. Maximum stability of Asn residues within peptides was observed between pH 2 and 5. Between pH 5 and 12, the reaction proceeds entirely through a succinimide intermediate and is dependent on the concentration of OH^- (nucleophiles appearing to catalyse the reaction).

Furthermore, the identity of the amino acids in the vicinity of the Asn residue can affect rates of deamidation [72], because of

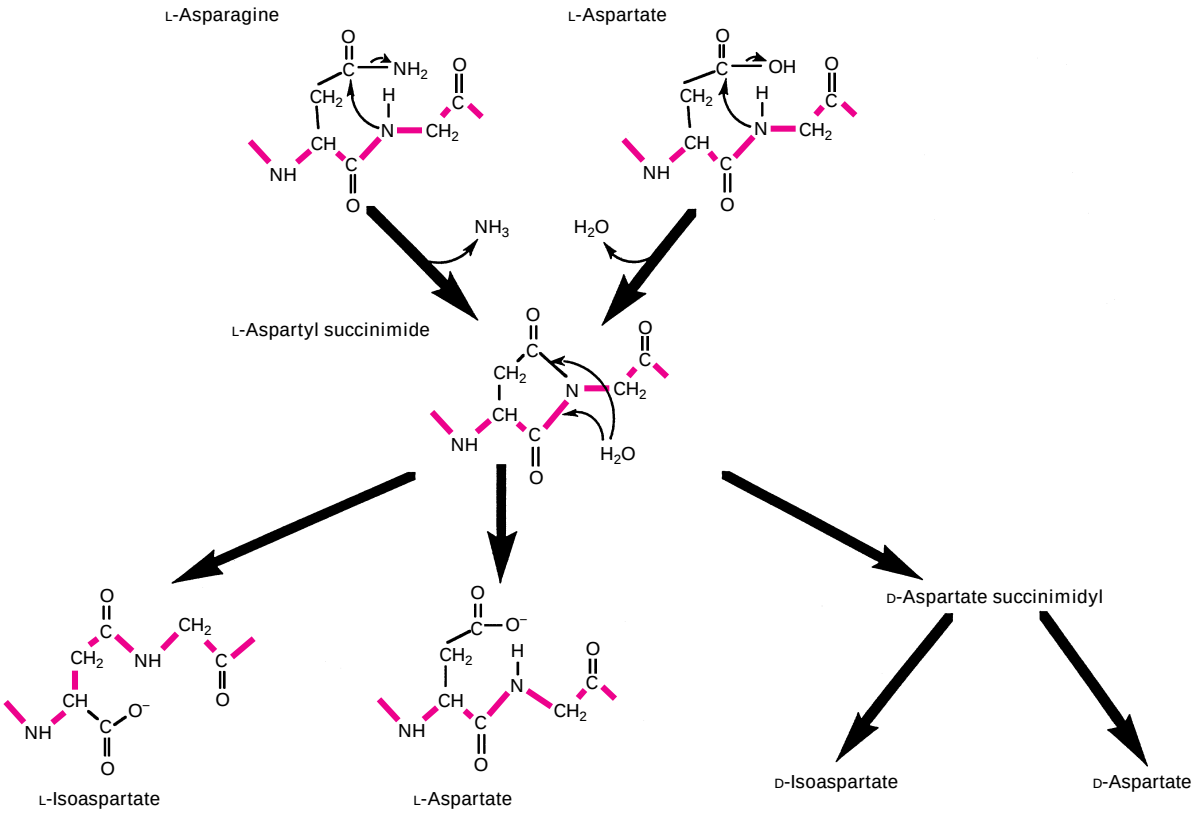

Figure 3 Mechanism for succinimide formation in asparagine and aspartate residues

The coloured bonds indicate the peptide backbone. See [81].

the conformational constraints imposed by the tertiary structure. Trypsin deamidates at three residues, Asn-48, -95 and -115, all of which have similar main-chain and side-chain conformations, characterized by the Oδ1 oxygen of the side chain forming a hydrogen bond with the ($n+2$) peptide nitrogen [78]. This type of interaction is observed in peptide turns where Asn is found in the first position [79]. Modelling of the Asn environments suggests that with minimal main-chain movement ($+20$ ° phi, psi) the side chain can be rotated by 80 ° around the Cα–Cβ bond, which places the amide-carbonyl carbon in an orientation where it is readily accessible to attack by the peptide nitrogen of the succeeding ($n+1$) residue. Additionally, the $n+1$ peptide nitrogen must be accessible and deprotonated. Each susceptible Asn is followed by a Ser with the side-chain hydroxy group in a position to hydrogen-bond to the Asn Oδ1 and Asn Nδ2 hydrogens, or the $n+1$ peptide nitrogen (which may aid in deprotonation of the nitrogen). These hydrogen bonds are thought to facilitate the formation of the cyclic imide intermediate [78].

Conformation alone, however, does not predict the propensity for deamidation. In trypsin, Asn-34 has a similar conformation to Asn-48, -95 and -115 and is followed by a Ser, yet no deamidation is observed. The succeeding peptide nitrogen of Asn-34 is hydrogen-bonded to a carbonyl oxygen. Further, the N34 to O64 and O34 to N64 hydrogen-bond network inhibits conformational change. Thus a combination of steric and hydrogen-bonding interactions prevent productive succinimide geometry at Asn-34. Other Asn residues are stable, since succinimide formation would require rotation of 180 ° around the peptide bond. Structurally constraining hydrogen bonds protect other Asn residues in the highly structured Ca^{2+} binding loop [78]; Asn-34, -79, -97, -143 and -233 all hydrogen-bond to the $n+1$ peptide chain nitrogen (Figure 4), hindering the side-chain geometry required for succinimide formation. Hydrogen bonding of the main chain appears to inhibit deamidation more than hydrogen bonding of the amide side chain. Deamidation may, however, be limited when hydrogen bonding requires the side-chain amide moiety as a donor or where multiple hydrogen bonds are made with side chains [78]. Based on site proximity, the size of the $n+1$ amino acid seems to be more influential in deamidation than the identity of the $n-1$ residue or β-carboxy linkages [78].

The differing rates of succinimide formation between similar sequences in peptides and proteins may be explained by the precise positioning of relevant functional groups in rigid regions of the protein [76]. By constraining reactive groups to a limited range of orientations, the rate of deamidation can increase by as much as 10000-fold [80]. The optimal alignment of the peptide nitrogen and side-chain carbonyl oxygen in a three-dimensional protein structure could allow succinimide formation in sequences that are rarely deamidated in peptides [76]. Conversely, rigid suboptimal alignment might be expected to decrease the rate of succinimide formation. The orientation of both Asn and Asp residues in proteins is generally such that succinimide formation does not occur. However, residue combinations known from peptide studies to be susceptible to deamidation are found in proteins as frequently as less reactive combinations [81]. The effect of protein conformation is apparent when considering that

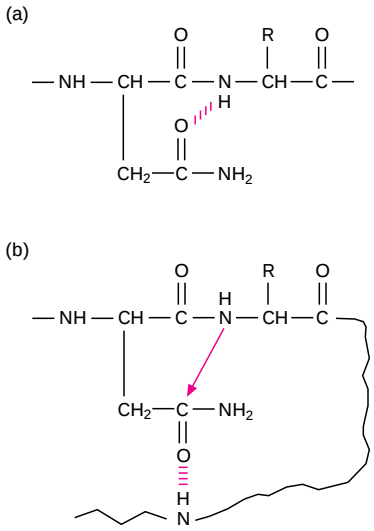

Figure 4 Hydrogen bonding of the Asn side-chain oxygen

Hydrogen bonding is shown to (**a**) the $n+1$ peptide-chain nitrogen (which hinders succinimide formation), and (**b**) a more distant peptide-chain nitrogen, which leaves the $n+1$ peptide chain nitrogen available for succinimide formation (coloured arrow).

the deamidation of Asn-67 in native ribonuclease A at 37 °C and pH 8 was found to be more than 30 times slower than that in the reduced and unfolded protein [82]. Furthermore, the presence of trifluoroethanol, known to stabilize secondary structures in proteins, suppressed deamidation of lysozyme at pH 6 and 100 °C [83].

The closely related structure of Gln is also able to deamidate, but at a much lower rate than for Asn. Gln deamidation is thought to proceed via analogous intramolecular reactions as for Asn, except that a six-membered ring leads to a glutarimide. The less favourable six-membered ring is expected to be formed approx. 10 times more slowly than the analogous five-membered succinimide [84]. However, as discussed above, deamidation in proteins is dependent on conformation; thus Gln deamidation could be a significant process producing glutamyl, isoglutamyl or γ-glutamyl products [85].

Although the most common mechanism for degradation at Asn is deamidation, Asn succinimide formation may cause cleavage of polypeptides under physiological conditions, and is known to occur in peptides at physiological pH [72,86]. The side-chain amide nitrogen attacks the peptide bond, releasing the carboxy-flanking peptide and forming a C-terminal succinimide (Figure 5). Asn cleavage has been noted in α-crystallin, where an Asn has the correct orientation to be both cleaved and de-amidated [87].

The reason for the greatly increased rate of Asn deamidation at high temperatures may be the activation energy, which was found to be 88.7 kJ·mol^{-1} for deamidation of peptides at pH 7.4 [72], indicating a marked temperature-dependence. The high energy of activation suggests that deamidation will occur at higher temperatures and explains the observation that, above 90 °C, deamidation is a major form of irreversible thermo-inactivation in both lysozyme (pH 4, 6 and 8) [70] and ribo-nuclease (pH 6 and 8) [71].

Aspartate residues

Asp is known to form succinimide intermediates which may be hydrolysed to yield a break in the peptide chain and thus lead to irreversible degradation of the protein structure. Although succini-mide formation at Asp is thought to occur by a similar mechanism to that at Asn (Figure 3), the rate of Asp succinimide formation at 37 °C and pH 7.4 was measured in peptides and found to be 13–35 times less than that for the corresponding Asn peptide [76]. However, in proteins a significant fraction of succinimide forma-tion may originate from Asp residues. Indeed, this is the case in lysozyme [70] and ribonuclease [71], where significant irreversible thermo-inactivation was noted at pH 4 above 90 °C due to Asp hydrolysis. In calmodulin, succinimides are formed preferentially from Asp residues rather than Asn residues. At pH 7.4 the Asp side chain has only 1 in every 3000 carboxy groups protonated and thus available for succinimide formation [88]. The rate of succinimide formation for a protonated Asp would, therefore, approach that of an ester and would be much more rapid than the amide [76]. Thus not only conformation, but also the relative degree of protonation, will affect Asp succinimide formation [76].

Investigations of Asp and Asn degradation in calmodulin showed that, when Ca^{2+} was present, only Asp-2 and Asp-78 formed succinimides despite the presence of six Asn residues [89]. Two of the stable Asn residues form Gly-Asn sequences [89], which are labile in peptides [72,75] but not in protein structure. Succinimide formation at Asp and Asn is thus highly regulated by three-dimensional structure, due to the requirement that the peptide nitrogen atom be in a position to attack the side-chain carbonyl of the Asx residue [72]. The Ca^{2+}–calmodulin crystal structure indicates that the peptide-bond nitrogen of Thr-79 is not in a position to attack the side-chain carbonyl carbon of Asp-78 and form a succinimide [90]; however, for calmodulin, the crystal structure [90] and solution studies [91] indicate that Asp-78 and Thr-79 are in flexible regions, allowing succinimide formation. The location of the labile Asp-2 is the flexible N-terminal region of calmodulin [89]. In the absence of Ca^{2+}, the Ca^{2+} binding sites are less constrained and the Asx residue can form succinimides. The removal of Ca^{2+} may allow new contact between Asn and neighbouring residues that may catalyse succinimide formation [89].

Studies at 37 °C of human growth hormone indicated that isoaspartate formation at Asp-130 and Asn-149 occurs when followed by Ser. However, another Asn-Ser sequence does not form isoaspartate, suggesting that the structure of the protein influences isoaspartate formation [92]. In the X-ray crystal structure of the pig analogue of this protein, which is known to have extensive sequence identity to the human hormone, amino acids 128–151 are known to lack well defined structure [93], and thus there may be considerable freedom of rotation around that portion of the peptide backbone. Furthermore, when peptides were constructed to investigate rates of isoaspartate formation, Asp-130 had a $t_{\frac{1}{2}}$ value similar to that of the peptide, indicating similar conformational flexibility. The $t_{\frac{1}{2}}$ of Asn-149 was twice that of the peptide, suggesting that the conformation of the protein prevents succinimide formation in some cases [92].

The well known rapid hydrolysis at Asp is thought to proceed by a similar mechanism to hydrolysis at Asn, with rapid cleavage after formation of a five-membered ring [94] (Figure 5). The observation that Asp is the only amino acid released when proteins are heated in weak acids has long been known [95], and results of hydrolysis in dilute HCl at pH 4 of cytochrome *c*, wool proteins and egg yolk apovitellenins suggest that the majority of Asp bonds are cleaved within 2 h. This cleavage is dependent on the pH, as this affects the proton donor ability of the Asp side-chain carboxy group. Asp hydrolysis accounts for approx. 80 % of the irreversible thermoinactivation of ribonuclease at pH 4

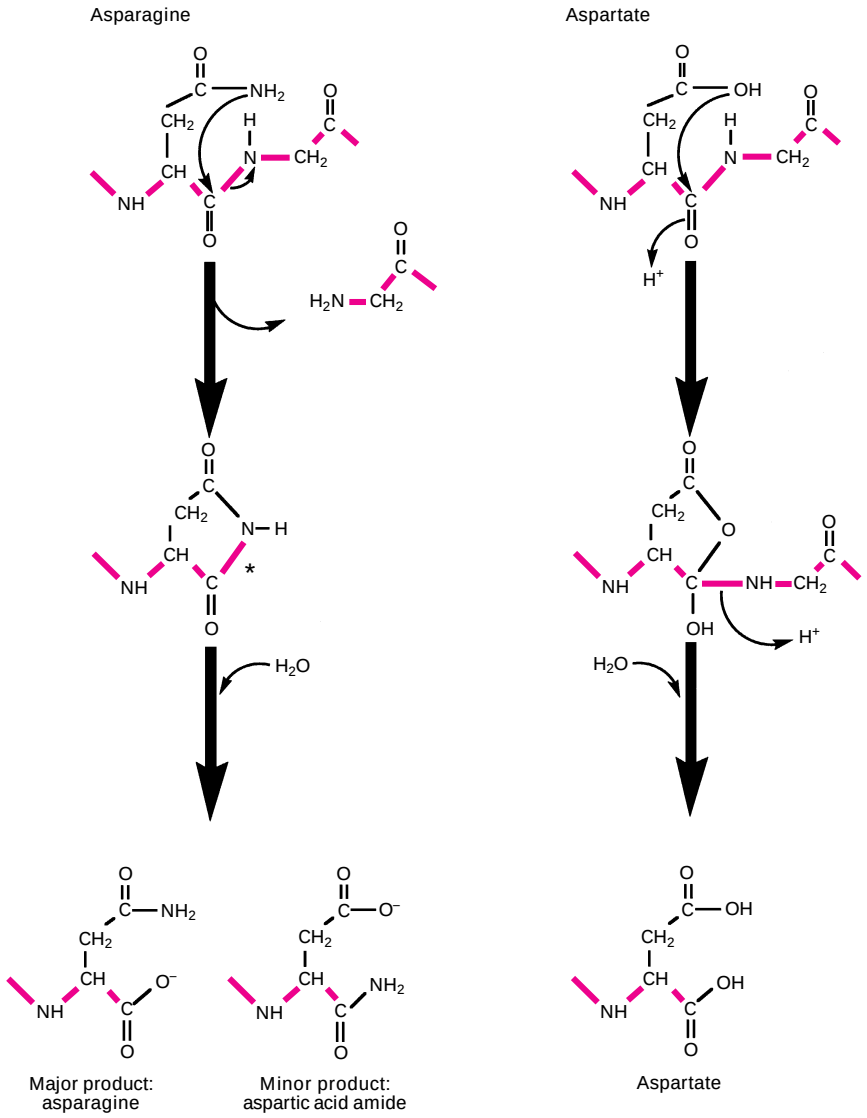

Figure 5 Ring formation and subsequent cleavage of asparagine [87] and aspartate [94] residues in proteins

The coloured bonds indicate the peptide backbone. The asterisk indicates the site of preferential hydrolysis.

and 90 °C [71], and has been noted in lysozyme at pH 4 and 100 °C [70].

Other residues

Oxidation of amino acid side chains also leads to irreversible thermo-inactivation in proteins. Potential sites of oxidation in proteins are the side chains of His, Met, Cys, Trp and Tyr residues [96]. The reactivity of a given Met residue towards oxidation seems to be dependent on its position. For example, human growth hormone Met-170 was found to be completely resistant to oxidation by hydrogen peroxide [97]. The thiol group of cysteine can also be oxidized. Even molecular oxygen will oxidize thiols in the presence of trace amounts of metal ions [98]. The nature of neighbouring groups greatly influences the oxidation of thiols [99]. The side chains of His, Tyr, Met, Cys and Trp residues can be oxidized via photo-oxidation in the presence of dyes [96].

Thermal destruction processes involving disulphides and thiol groups are also important in both structure and catalysis. β-Elimination of cystine under alkaline conditions forms dehydroalanine, which may go on to react with a nearby Lys residue [100]. β-Elimination is not limited to Cys; Ser, Thr, Phe and Lys can also be degraded in alkaline conditions [96]. Cys residues are also susceptible to disulphide exchange, in which disulphide bonds are reduced and later re-form between different Cys residues.

Aggregation of denatured proteins remains an important mechanism of irreversible thermo-inactivation, but often requires substantial disruption of the tertiary structure. The other mechanisms discussed above may occur at discrete sites within the protein while the conformation remains intact or nearly so.

In summary, numerous reactions which irreversibly change protein structure and thus inactivate enzymes are facilitated by increased temperatures (for specific examples, see Table 2). Clearly, the mechanisms of degradation vary dramatically in

Table 2 Major degradative processes determined by experiment in selected enzymes

Enzyme	Conditions	Prevalent degradative processes	Ref.
Cellobiohydrolase I (*Trichoderma reesei*)	70 °C, pH 4.8	Aggregation, deamidation	101
Lysozyme	100 °C, pH 6	Deamidation	83
Ribonuclease A	90 °C, pH 4	Deamidation, hydrolysis at Asp	71
α-Amylase (*Bacillus amyloliquefaciens*)	90 °C, pH 8	Deamidation	102
α-Amylase (*B. stearothermophilus*)	90 °C, pH 8	Oxidation of Cys	102

different enzymes since they are mediated by protein structure and can be highly dependent upon local flexibility and neighbouring amino acid residues.

INTERACTIONS BETWEEN DEGRADATION AND DENATURATION

The susceptibility of a protein to high-temperature degradative reactions seems to be dependent upon the conformational integrity of the protein at that temperature. The chemical mechanisms for irreversible degradation in proteins require a certain local molecular flexibility. For example, at 37 °C the rate of deamidation has been shown to be higher for small peptides with high flexibility than for proteins when comparing the same amino acid sequence [103], and higher in denatured proteins [82]. A survey of environments around Asp and Asn residues in known three-dimensional protein structures suggests that the rigidity of the folded protein greatly decreases the intramolecular imide formation necessary for further degradation. In the numerous X-ray crystal structures studied, the peptide-bond nitrogen could not approach the side-chain carbonyl carbon closely enough to form the succinimide ring [81].

Experimental evidence has linked the resistance to degradation of a protein with its conformational integrity. Thermally stable proteins have been used for these studies, so that the conformation is known to be retained for significant periods at the high temperatures at which degradative reactions occur. Hensel and colleagues have shown that the rate of deamidation for a thermostable glyceraldehyde phosphate dehydrogenase from *Pyrococcus woesei* at 100 °C is increased after denaturation of the enzyme [104] (and after dialysing away the denaturing guanidinum hydrochloride). Furthermore, for the same enzyme from *Methanothermus fervidus* deamidation occurs more readily at 85 °C in a less stable form of the enzyme in which the protein is formed from a recombinant DNA comprised of both thermophilic and mesophilic genes. Similarly, the addition of phosphate, known to stabilize the conformation of the dehydrogenase, decreases the rate of peptide bond hydrolysis at temperatures ranging from 85 to 100 °C [104].

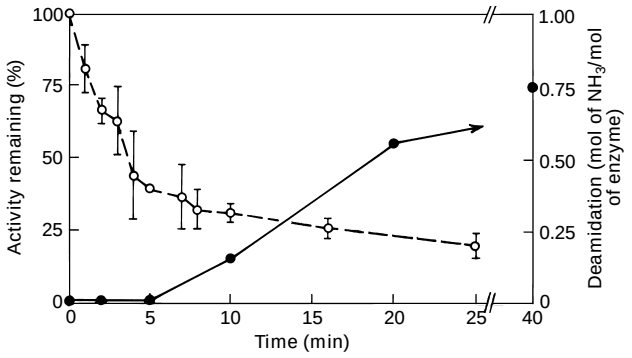

Figure 6 Denaturation and degradation of xylanase from *Thermotoga maritima* sp. FjSS3B1 at 100 °C

The plot shows activity loss (○) (means ± S.D.) and deamidation (●) (C. Aebischer and R. M. Daniel, unpublished work).

Support for the view that the loss of conformation precedes irreversible degradative reactions comes from studies of deamidation (ammonia release) and loss of activity of the very stable xylanase from *Thermotoga* strain FjSS 3B1 in the range 95–110 °C (C. Aebischer and R. M. Daniel, unpublished work). Both the onset and progress of deamidation occurred later than those of activity loss, consistent with a dependence of deamidation upon loss of conformation (Figure 6).

Studies on peptide bond hydrolysis in native and denatured myokinase at 95 °C (M. Dines, H. H. Petach and R. M. Daniel, unpublished work) give similar results. The rate of peptide bond hydrolysis is always lower than the rate of activity loss, although agents which affect the rate of activity loss such as SDS plus mercaptoethanol (faster) and substrate (slower) have a similar effect on peptide bond hydrolysis. These correlations are consistent with a dependence of degradation on loss of conformation.

The irreversible loss of activity of glucose isomerase at high temperatures may also be dependent upon protein unfolding. The temperature-dependence and large activation energy observed for the activity loss of the isomerase are uncharacteristic of covalent reactions. Thus Volkin and Klibanov [105] suggest that the deamidating residues may be located in the centre of the protein, and thus unfolding precedes deamidation, giving rise to large activation energies.

CONCLUSION

Until comparatively recently, an assumption that loss of enzyme activity at 100 °C was simply a more rapid version of that taking place at 60 °C would have seemed very reasonable. However, during the 1980s it became clear that at 100 °C and above a number of irreversible reactions, including deamidation and peptide bond cleavage, occur in addition to denaturation [70,71]. Although it is difficult to place a theoretical upper temperature limit on the stability of proteins to denaturation, because of their universal applicability these irreversible degradative reactions would seem to limit protein stability to temperatures below, say, 120 °C. This prediction would be in keeping with the failure of extensive efforts to find living organisms growing optimally at temperatures above 110 °C, since our concept of life rests heavily on the functioning of enzymes. However, enzymes have been found that have significant half-lives at 130 °C [8,10], and there is growing evidence that the degradative reactions to which proteins are subject are slower or do not occur in conformationally intact proteins. In other words, the upper temperature limit for protein stability may after all be determined by the conformational integrity of the protein, although we must bear in mind that little work on conformational or degradative stability has taken place above 100 °C.

If this is so, and since there is no obvious upper temperature limit on conformational stability, why have more stable proteins not been found? The answer presumably lies in the requirement for sufficient flexibility (instability) for activity and turnover, coupled with the lack of living organisms found growing above 110 °C.

So what is the upper temperature limit for enzyme stability? Experiments carried out at 250 °C indicate that the half-life of some peptide bonds is only a few minutes at this temperature, giving rise to a calculated protein half-life of about 1 s [106]. Even though this work was carried out on small peptides, it is difficult to envisage proteins that are stable enough to withstand this temperature. The most stable proteins found so far have half-lives in excess of 10 min at 130 °C [8], and greater stabilities have been shown for very stable enzymes immobilized and in the presence of stabilizing agents [9,11], so that prospects exist for characterizing denaturation and degradation up to 140 °C or so. Such stable proteins could also be starting points for attempts to engineer higher stability. A variety of strategies are available [107]. Conformational stability can be increased by rigidifying the enzyme structure by such means as cross-linking and immobilization [66], but such a loss of flexibility is also likely to reduce enzyme activity. Thus the most useful approach might be to rigidify the enzyme outside the active-site region, and thus leave the active site flexible enough for high activity. Unfortunately, this may leave the active region of the enzyme as the most susceptible to degradation. Strategies are also available for reducing degradation, for example by mutating susceptible residues such as Asp to the less labile, but structurally similar, amino acid Glu [108,109].

However, the suggestion that enzyme activity is possible below the glass transition [59] (i.e. in a rigid protein structure), if confirmed, may indicate that enzyme activity does not absolutely require a flexible structure and thus one of limited stability. If so, this could hold out prospects of stabilizing the whole enzyme without too large a loss of catalytic activity. Few attempts have been made to further stabilize very stable enzymes. The stabilizations so far achieved in less stable proteins by point mutation and other techniques, while individually relatively small, should, when applied in concert to enzymes stable at 120 °C, be quite sufficient to confer stability at temperatures raised by 30 °C or so. Overall, it does not seem at all unreasonable to expect to see within the next 10 years enzymes that are active and stable at around 150 °C.

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