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A correlation between protein thermostability and resistance to proteolysis

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The loss of activity due to proteolysis of purified L-asparaginase and β-galactosidase from different sources correlates with the thermal instability of the enzymes. A similar correlation is found when populations of soluble proteins from micro-organisms grown at different temperatures are compared for proteolytic susceptibility and thermal stability. It is proposed that there is a general correlation between the thermostability of proteins and their resistance to proteolysis.

In the course of our research on extremely thermophilic bacteria we noticed that enzymes derived from these organisms seemed to be more resistant to proteolysis than equivalent enzymes from mesophilic organisms. We report here the results of investigations which suggest that this resistance may be a general feature of thermostable proteins.

Experimental

Materials

Escherichia coli β-galactosidase (grade IV), bovine liver β-galactosidase (grade III), *Saccharomyces fragilis* lactase, porcine trypsin (type II), bovine α-chymotrypsin (type II), papain (type II), mercaptoethanol and trichloroacetic acid were obtained from Sigma; *E. coli* L-asparaginase was from May and Baker, Dagenham, Essex, U.K.; *Erwinia carotovara* L-asparaginase was from CAMR, Salisbury, Wilts., U.K. and pilzproteinase P was from Rohm GmbH, Darmstadt, Germany.

Thermus strain 4-1A β-galactosidase (D. A. Cowan, A. M. Martin, R. M. Daniel and H. W. Morgan, unpublished results), *Thermus aquaticus* strain T-351 L-asparaginase (Curran, 1982) and caldolysin [*T. aquaticus* strain T-351 proteinase (Cowan & Daniel, 1982)] were obtained from our own laboratory.

Bacteria

The extremely thermophilic bacteria *T. aquaticus* strain T-351 and *Thermus* strain 4-1A were isolated from the Rotorua thermal area, New Zealand (H. W. Morgan, B. K. Patel, S. Lim and R. M. Daniel unpublished results). They were grown in peptone media at 75°C (Hickey & Daniel, 1979). *Rhizobium japonicum*, *E. coli* and *Bacillus stearothermophilis* were grown as described elsewhere (Daniel & Appleby, 1972; Craven *et al.*, 1965; O'Brien & Campbell, 1957).

Cell extracts

Soluble cell-protein extracts were obtained by ultrasonic cell disruption followed by centrifugation [360000 ***g*** ($r_{av.}$ 6.43 cm), 2 h]. Cell extracts were diluted to 1.4 mg of protein/ml [determined by the biuret assay (Herbert *et al.*, 1971)] with 100 mM-Tris/acetate buffer, pH 7.0.

Thermal denaturation of enzymes

Aliquots of a solution of the specified enzyme (0.1 mg of protein/ml in 100 mM-Tris/HCl buffer, pH 7.0), incubated at the temperature indicated, were removed at intervals. Assays for β-galactosidase or L-asparaginase [performed as described by Ulrich *et al.* (1972) and Curran (1982) respectively] were carried out immediately.

Thermal denaturation of cell-free extracts

To determine the extent of thermal denaturaton in cell-free extracts, triplicate 1 ml samples of each were incubated in sealed Bijou bottles at 60°C and 90°C. After 60 min, the contents were rapidly cooled, centrifuged for 10 min (2800 ***g***, $r_{av.}$ 11.9 cm) and the supernatants assayed for protein content by the method of Bradford (1976).

Proteolysis of enzymes

Proteolytic inactivation of β-galactosidases and L-asparaginases was determined by the incubation of enzyme solutions (2 mg/ml and 0.0125 mg/ml, respectively, in 50 mM-Tris/HCl buffer, pH 7.0) at 37°C in the presence of the specified concentrations

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of proteinase. Aliquots were removed at intervals and immediately assayed. Assay times were less than 5% of the shortest incubation time. In control samples of enzyme (proteinases omitted), negligible activity loss was observed after 1 h, although the *E. coli* β-galactosidase lost 73% of its initial activity during the 22 h incubation.

Proteolysis of cell-free extracts

Volumes (5 ml) of cell-free extract containing 100 μl of water, trypsin (2.5 mg/ml in water), or papain (5 mg/ml in water containing 5 μM-mercaptoethanol) were incubated at 30°C. After 60 min, 1 ml aliquots were removed and precipitated by the addition of 1.5 ml of 5% (w/v) aqueous trichloroacetic acid. Precipitates were removed by centrifugation for 10 min (2800 *g*, $r_{av.}$ 11.9 cm). Undenatured soluble protein was estimated by the Lowry method and protein precipitates by the Bradford (1976) method.

Results and discussion

As judged by the criterion of activity loss (Tables 1 and 2) there is a good correlation between resistance to proteolysis and thermostability in two purified enzymes, L-asparaginase and β-galactosidase, from different sources. Although the proteinases used have different specificities, they have all given a similar pattern of results and we do not believe that differences in their mode of action have significantly affected the results.

This correlation between resistance to proteolysis and thermostability also holds when based on the quite different criteria of heat-induced insolubility and of the proteolysis of populations of proteins (Table 3). Both of these factors correlate well with the growth temperature of the organism. This is not unexpected, since it has been known for some time that proteins from thermophiles are in general more thermostable than those from mesophiles (e.g. Koffler & Gale, 1957; Amelunxen & Murdoch, 1978).

Individually, both of these experiments are open to criticism. The β-galactosidase from *Thermus* 4-1A, for example, has a lower molecular weight than that from the other organisms (D. A. Cowan, A. M. Martin, R. M. Daniel and H. W. Morgan, unpublished work), and differences in the effects of proteolysis on subunit interactions might well be responsible for some of the results. Nor do the results in Table 3 preclude a resistance to both proteolysis and thermal denaturation conferred by soluble cellular factors [although no grounds for the latter were found by Koffler & Gale (1957) in a similar experiment, nor would this view be consistent with overall research in this field (Amelunxen & Murdoch, 1978)]. But, taken together with isolated observations by other workers on the resistance of individual thermostable enzymes to proteolysis [e.g. D-asparaginase (Guy & Daniel, 1982), 6-phosphogluconate dehydrogenase (Veronese *et al.*, 1981), glutamine synthetase (Matsunaga & Nosoh, 1974)] and the demonstration that factors conferring

Table 1. *Comparison between proteolytic inactivation and thermal stability of β-galactosidases from different sources*
Standard deviations of all values were less than 5%. –, Experiment not performed. All proteinase concentrations were 0.05 mg/ml.

Proteinase used (incubation time)	Susceptibility to proteolysis (% of activity remaining after proteolysis) of enzyme from:		
	Thermus strain 4-1A	*Saccharomyces fragilis*	*Escherichia coli*
Organism growth temperature ...	75°C	25°C	37°C
Thermal denaturation (half-life of enzyme) ...	50 h at 75°C	36 min at 50°C	2.3 min at 51°C
Chymotrypsin			
(1 h)	100	–	0
(6 h)	96	–	0
Trypsin			
(1 h)	100	44	3
(6 h)	100	0	2
Papain			
(1 h)	100	91	53
(6 h)	82	0	1

Table 2. *Comparison between proteolytic inactivation and thermal stability of L-asparaginases from different sources*
Standard deviations of all values were less than 5%. –, Experiment not performed. Proteinase concentrations were: chymotrypsin, 0.04 mg/ml; trypsin, 0.04 mg/ml; pilzproteinase, 0.13 mg/ml; caldolysin, 0.07 mg/ml.

Proteinase used (incubation time)	Susceptibility to proteolysis (% of activity remaining after proteolysis) of enzyme from:		
	Thermus aquaticus strain T351	*Escherichia coli*	*Erwinia carotovora*
Organism growth temperature ...	75°C	37°C	25°C
Thermal denaturation (half-life of enzyme) ...	3 h at 80°C	40 min at 60°C	1 min at 60°C
Chymotrypsin			
(2 h)	105	25	2
(6 h)	86	–	–
Trypsin			
(30 min)	106	3	7
(4 h)	77	–	–
Pilzproteinase			
(30 min)	92	40	43
(1 h)	118	4	0
(6 h)	98	0	0
Caldolysin			
(15 min)	117	70	74

Table 3. *Thermal stability and proteolytic susceptibility of total soluble proteins in four bacteria*
Standard deviations were all less than 6%, except for the 90°C value for *P. japonicum* which was 26%. Thermal denaturation is % of soluble cell protein remaining soluble after 60 min at the temperature indicated; proteolysis is % of total trichloroacetic acid-precipitable protein after 60 min hydrolysis and, in parentheses, trichloroacetic acid-soluble protein (mg/ml) released by hydrolysis, with the proteinase shown.

Organism	Growth temperature	Thermal denaturation at:		Proteolysis with:	
		60°C	90°C	Trypsin	Papain
Rhizobium japonicum (A.T.C.C. 10324)	25°C	35	26	35 (0.37)	66 (0.19)
Escherichia coli B (A.T.C.C. 11303)	37°C	42	26	41 (0.29)	66 (0.13)
Bacillus stearothermophilus (N.T.C.C. 10007)	55°C	98	32	77 (0.08)	98 (0.09)
Thermus aquaticus (A.T.C.C. 25104)	75°C	100	54	88 (0.01)	92 (0)

thermostability (e.g. substrate, cofactor, low molecular weight) tend to protect against proteolysis (Amelunxen & Murdoch, 1978; Goldberg & Dice, 1974; Schimke, 1975), we believe the results strongly support in general terms a correlation between thermostability and resistance to proteolysis.

The evidence is strongest when based upon comparisons between proteins having large differences in thermostability and proteolytic susceptibility (Tables 1–3); it may not apply where these differences are small. Furthermore, it rests mostly upon the results of a comparison between proteins from thermophiles and mesophiles. Confirmation would best come from a comparison of thermostability and proteolytic susceptibility among homologous proteins, including mutants, derived from organisms whose 'natural' growth temperature is similar. Although a comparison of a large number of individual non-homologous proteins attacked by a variety of proteinases should bear out the correlation, the former test would tend to avoid exceptions which we would expect to arise in the latter case: for example from the presence or absence of stabilizing or destabilizing factors, from subunit and aggregation effects, and from the fact that some proteins can retain activity after considerable proteolysis, whereas others lose activity and/or aggregate after very limited proteolysis. It would also avoid the complication of the tem-

perature-induced conformational changes which occur within the native state when, for example, thermophilic proteins are cooled to 37°C (e.g. Amelunxen & Murdoch, 1978; Matsunaga & Nosoh, 1974).

As indicated above, there are already some indications of a correlation between the thermostability of a given protein and its resistance to proteolysis. It is well known that denatured proteins are particularly susceptible to proteolysis (Anson, 1938), but increased temperature will also increase the susceptibility of a protein to proteolytic attack in its undenatured, active, state; although studies of this type are complicated by the variation in activity and autolysis of the proteinase with temperature, and by the kinetic characteristics of the proteinase (Brandts, 1969) they have been used to follow temperature-induced conformational changes in proteins (Matsunaga & Nosoh, 1974; Lapanje, 1978; Privalov, 1979).

The correlation proposed here applies between proteins as well as to individual proteins. It predicts that some or all of the factors which enhance or decrease thermostability will have a similar effect on the resistance of the protein to proteolysis. It would imply that, at the temperature of proteolysis, thermostable proteins have a sufficiently different exterior surface to confer this resistance to proteolysis. Since protein unfolding is known to increase proteolytic susceptibility (Anson, 1938; Lapanje, 1978; Privalov, 1979), it may be that these thermostable proteins have a somewhat 'tighter' or more folded structure and thus fewer proteolytically-susceptible conformational substrates (Frauenfelder, 1981). However, these structural changes are likely to be subtle since it now seems that no single special mechanism operates to confer thermostability in proteins and that quite minor amino acid differences can considerably enhance or decrease thermostability (Langridge, 1968; Matthews *et al.*, 1974). For example, a single amino acid substitution in lysozyme decreased the 'melting' temperature by 14°C, while changes in the three-dimensional structure were less than a few hundredths of an nm (Grutter *et al.*, 1979). Furthermore, there is no evidence that molecular flexibility, such as is required for allosterism for example, is significantly reduced in thermophilic enzymes, even at temperatures as low as 37°C (Amelunxen & Murdoch, 1978; Curran, 1982).

References

Amelunxen, R. E. & Murdoch, A. L. (1978) *Crit. Rev. Microbiol.* **6**, 343–393

Anson, M. L. (1938) *J. Gen. Physiol.* **22**, 79

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254

Brandts, J. F. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N. & Fasman, G. D., eds.), pp. 213–290, Dekker, New York

Cowan, D. A. & Daniel, R. M. (1982) *Biochim. Biophys. Acta* **705**, 293–305

Craven, G. R., Steers, E. & Anfinsen, C. B. (1965) *J. Biol. Chem.* **240**, 2468–2477

Curran, M. P. (1982) M.Sc. Thesis, University of Waikato, New Zealand

Daniel, R. M. & Appleby, C. A. (1972) *Biochim. Biophys. Acta* **275**, 347–354

Frauenfelder, H. (1981) *Biophys. Struct. Mech.* **7**, 226–227

Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–869

Grutter, M. G., Hawkes, R. B. & Matthews, B. W. (1979) *Nature (London)* **277**, 667–669

Guy, G. R. & Daniel, R. M. (1982) *Biochem. J.* **203**, 787–790

Herbert, D., Phipps, P. J. & Strange, R. E. (1971) in *Methods of Microbiology* (Norris, J. R. & Ribbons, D. W., eds.), vol. 58, pp. 210–336, Academic Press, London and New York

Hickey, C. W. & Daniel, R. M. (1979) *J. Gen. Microbiol.* **114**, 195–200

Koffler, H. & Gale, G. O. (1957) *Arch. Biochem. Biophys.* **67**, 249–251

Langridge, J. (1968) *J. Bacteriol.* **96**, 1711–1717

Lapanje, S. (1978) *Physiochemical Aspects of Protein Denaturation*, pp. 74–75, Wiley, New York

Matsunaga, A. & Nosoh, Y. (1974) *Biochim. Biophys. Acta* **365**, 208–211

Matthews, B. W., Weaver, L. H. & Kester, W. R. (1974) *J. Biol. Chem.* **249**, 8030–8044

O'Brien, R. T. & Campbell, L. L. (1957) *Arch. Biochem. Biophys.* **70**, 432–441

Privalov, P. L. (1979) *Adv. Protein Chem.* **33**, 167–241

Schimke, R. T. (1975) in *Intracellular Protein Turnover* (Schimke, R. T. & Katunuma, N., eds.), pp. 173–186, Academic Press, New York

Ulrich, J. T., McFetters, G. A. & Temple, K. L. (1972) *J. Bacteriol.* **110**, 691–698

Veronese, F. M., Boccu, E. & Fontana, A. (1981) *Proc. Conf. Enzyme Engineering 6th*, abstract 2–4