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Abbreviations

APS	Sulphuric acid–phosphoric acid anhydride
BR	Bacteriorhodopsin
$C_{carb.}$	Carbonate carbon
CDR factor	Carbon dioxide reduction factor
CH_3-S-CoM	2-(methylthio)ethanesulphonic acid
CODH	Carbon monoxide dehydrogenase
$C_{org.}$	Organic carbon
DGA	Desulphated diglycosylarchaeol
DGC	Diglycosylcardarchaeol
DHA	Dihydroxyacetone
DPE	Distal promoter element
EF	Elongation factor
EOR	Enhanced (or tertiary) oil recovery
F_{420}	Factor 420
F_{430}	Factor 430
FAF	Formaldehyde activation factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GNC	Glucosylnonitolcaldarchaeol
HB	3-Hydroxybutyrate
hMDH	Halophilic malate dehydrogenase
H_4MPT	Tetrahydromethanopterin
HS-CoM	2-Mercaptoethanesulphonic acid/Coenzyme M
HS-HTP	7-Mercaptoheptanoylthreonine phosphate
HV	3-Hydroxyvalerate
LDH	Lactate dehydrogenase
LS	Light scattering
M_2	Molar mass
MDH	Malate dehydrogenase
MPT	Methanopterin
MT	Methyltransferase
NS	Neutron scattering
ORF	Open reading frame
PA	Phosphatidic acid
PAPS	3′-Phosphoadenosine-5′-phosphosulphate
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGC	Phosphodiglycosylcaldarchaeol

PGNC	Glc-nonitolcaldarchaeol-*P*-inositol
PGP	Phosphatidylglycerol phosphate
PGS	Phosphatidylglycerol sulphate
PHA	Polyhydroxyalkanoate
PHB	Poly-3-hydroxybutyrate
PI	Phosphatidylinositol
PM	Purple membrane
PPE	Proximal promoter element
PS	Phosphatidylserine
RuBP	Ribulose bisphosphate carboxylase
S-DGA	Sulphated diglycosylarchaeol
S-TeGA	Sulphated tetraglycosylarchaeol
S-TGA	Sulphated triglycosylarchaeol
SD	Sedimentation velocity and diffusion
SE	Sedimentation equilibrium
TeGA	Desulphated tetraglycosylarchaeol
TGA	Desulphated triglycosylarchaeol
TMP	Trimethylpsoralen
$T_{opt.}$	Optimal growth temperature
TPP	Thiamine pyrophosphate
XS	X-ray scattering
YFC	Yellow fluorescent compound

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Proteins from hyperthermophilic archaea: stability towards covalent modification of the peptide chain

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Introduction

One of the most striking features of the Archaea is their extraordinary thermophilic potential. Thus, hyperthermophiles with optimal growth temperatures above 80 °C are for the most part archaea and all organisms with growth temperatures above 100 °C isolated up to now belong exclusively to the domain of Archaea [1,2].

Life at these high growth temperatures requires provision by the cell of proteins which are stable and biologically active at these extreme temperatures. Although several archaeal proteins exhibiting astonishingly high thermostabilities and high temperature optima of activity have been characterized, only in a few cases are structural data available. The primary structures of only about a dozen proteins from archaeal hyperthermophiles have been analysed. In no case could the three-dimensional structure be resolved, which might have allowed insights into the construction principle of these proteins.

Thus, the question remained unanswered as to how the proteins from these hyperthermophiles are protected against covalent damage at temperatures which induce chemical modifications in 'normal' mesophilic proteins. As has been shown by several authors [3–8] these chemical modifications comprise deamidation of Asn

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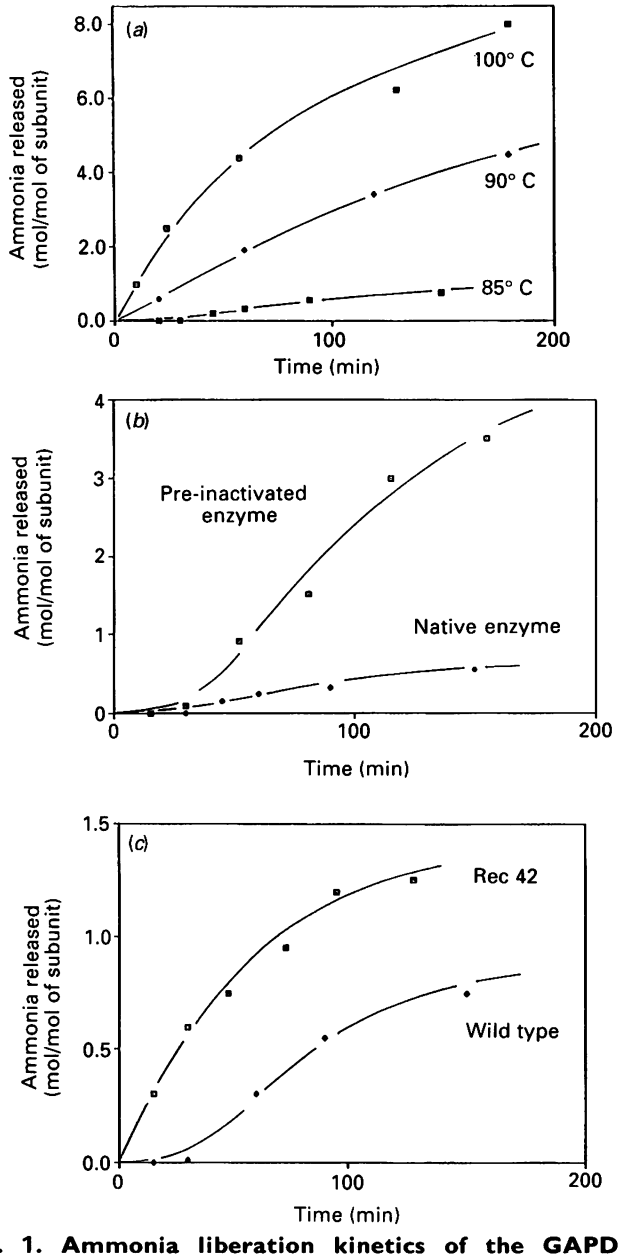

Fig. 1. Ammonia liberation kinetics of the GAPDHs from *M. fervidus* and *P. woesei*. The incubations were performed at pH 7.3 (10 mM-potassium phosphate buffer containing 150 mM-2-mercaptoethanol; protein concentration: 2 mg/ml) in sealed glass capillaries under anaerobic atmosphere. Before the determination of ammonia by an amino acid analyser, the samples (70 μl) were cooled down immediately after incubation and mixed with 210 μl of 0.2 M-citrate buffer, pH 2.2. (*a*)

and Gln, hydrolysis of Asp-containing peptide bonds and Asn-Xaa bonds as well as destruction of cystine bonds.

Here we describe the susceptibility of archaeal hyperthermophilic proteins to thermogenic covalent modifications. The studies were performed with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Methanothermus fervidus* (optimal growth temperature 83 °C [9]) and *Pyrococcus woesei* (optimal growth temperature 100 °C [10]) [11,12]. We have focused on deamidation and hydrolysis of the peptide bonds at high temperatures. To avoid oxidation reactions all incubations were performed under anaerobic conditions in the presence of reducing agents. Destruction of disulphide bonds was disregarded, since no indications for cystinyl cross-links in these proteins are available [12,13].

Results and discussion

Deamidation reactions at high temperatures

To investigate the susceptibility of the GAPDHs from the hyperthermophiles *M. fervidus* and *P. woesei* to deamidation reactions, ammonia liberation from the protein solutions (2.0 mg/ml) was followed at different temperatures. The proteins were incubated at low ionic strength (10 mM-potassium phosphate buffer, pH 7.3), at which the proteins show only a low stability as measured by inactivation kinetics [12,14]. Thus, at the respective optimal growth temperatures of the organisms the enzymes exhibit half-lives of inactivation of 60 min at 83 °C (GAPDH from *M. fervidus*) or of 44 min at 100 °C (GAPDH from *P. woesei*).

As shown in Fig. 1*a* and 1*b*, ammonia liberation can be observed with both enzymes. The GAPDH from *M. fervidus* exhibits, however, a higher susceptibility to deamidation than that from *P. woesei*; at 85 °C the *M. fervidus* enzyme already shows the same ammonia liberation rate as that obtained for the more stable *P. woesei* enzyme at 100 °C. Quite obviously, the enzyme proteins of the hyperthermophilic archaea are not resistant *per se* to chemical modification but must be protected against covalent damage.

Does the native conformation protect the Asn and Gln residues against deamidation?

To investigate whether the Asn and Gln residues are protected in the native conformation, the deamidation reaction was analysed in proteins with destabilized or disturbed conformation.

For this purpose the velocity of deamidation was determined with Rec 42, an enzyme mutant of the *M. fervidus* GAPDH. This mutant enzyme represents a hybrid enzyme constructed by recombination of the structural GAPDH genes from the thermophile *M. fervidus* and the mesophile *Methanobacterium bryantii* [14]. The hybrid GAPDH consists mainly of the *M. fervidus* GAPDH sequence but exhibits at its *C*-terminus a 42 residue fragment of the *Mb. bryantii* enzyme. This exchange resulted in

M. fervidus wild-type GAPDH incubated at different temperatures. (*b*) Native and pre-denaturated (see text) *P. woesei* GAPDH incubated at 100 °C. (*c*) *M. fervidus* wild-type and mutant (Rec 42) GAPDH incubated at 85 °C.

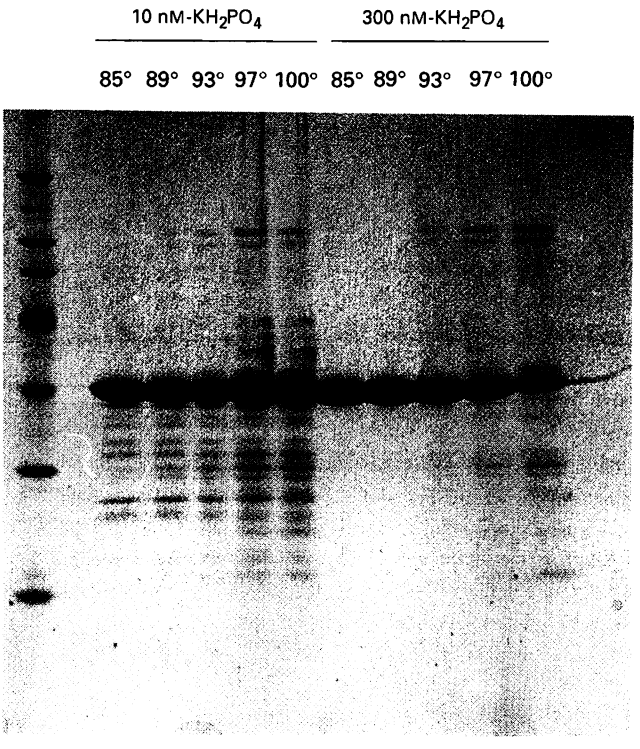

Fig. 2. Hydrolysis of peptide bonds. Electropherogram of *M. fervidus* GAPDH incubated for 1 h at different temperatures at low (10 mM-potassium phosphate buffer, pH 7.3 containing 150 mM-2-mercaptoethanol) or at high ionic strength (300 mM-potassium phosphate buffer, pH 7.3 containing 150 mM-2-mercaptoethanol).

real substitution of only 10 residues [14]. No additional Asn or Gln residues were inserted in the recombinant structure. On the contrary, two Asn residues were substituted and the *C*-terminal Gln residue was eliminated by this recombination.

As documented previously [14], this rather minor exchange greatly destabilizes the protein structure (half-life of inactivation at 85 °C: 0.5 min (Rec 42) as opposed to 20 min determined for the wild-type GAPDH from *M. fervidus*). With the lower stability of the native conformation the susceptibility to deamidation reactions also increases, as shown in Fig. 1*c*. Thus, the retardation of ammonia evolution, characteristic for the wild-type enzyme from *M. fervidus*, disappeared in the case of Rec 42. Quite obviously, the conformational stability of the mutant enzyme is too low to retard the deamidation reaction.

The assumption that the network of non-covalent bonds in the native state protects the peptide chain against deamidation is also supported by deamidation experiments with the *P. woesei* GAPDH disrupted in its native conformation by

Fragment	Sequence	
	M. fervidus	*P. woesei*
1	GLSFN▼SLSN	QVSFVSSSN
2	AIIPN▼PPKL	AIKP.SVTI
3	MHQHN▼VMVE	MHVHSIMVE
4	VVSCN▼TTGL	VVSCNTTGL
5	KGPIN▼AIIP	RGPINAIKP

Fig. 3. Cleavage sites of the non-enzymic hydrolysis of the *M. fervidus* GAPDH and comparison with homologous sequences of the *P. woesei* GAPDH. The positions of cleavage sites (marked by arrows and large type-size symbols) were deduced from the *N*-terminal amino acid sequence of the respective peptide fragments. Positions homologous to the cleavage sites in the *M. fervidus* GAPDH are underlined. Sequencing of the peptides was performed on a gas-phase sequencer (A470 from Applied Biosystems) after electroblotting on glass fibre sheets [18].

pretreatment with 8 M-guanidium chloride at 70 °C (Fig. 1*b*). This irreversibly denatured protein (after denaturation the denaturant was removed by dialysis to avoid interference with ammonia liberation) shows a significantly faster ammonia liberation than the native enzyme, indicating that in the disrupted conformation more residues are susceptible to covalent modification than in the native state. Nevertheless, as in the case of the pretreated enzyme a sigmoidal curvature can be observed, which accounts for a certain conformational stability of the wrongly refolded state.

The conclusion that the deamidation requires an unfolding of the chain seems to be plausible considering the reaction mechanism proposed by Clarke [4]. As outlined, the reaction starts with a nucleophilic attack of the peptide amino group on the amide carbon, forming a cyclic intermediate, a succinimide derivative. This reaction pathway requires that the ψ and χ angle assume values of $-120°$ and 120°, which, however, is only possible in an unfolded state.

Thermogenic hydrolysis of the peptide bond

Further hints that conformational stability governs the susceptibility of hyperthermophilic proteins to chemical modifications can be deduced from studies on the thermogenic hydrolysis of peptide bonds in the GAPDHs from *M. fervidus* and *P. woesei*.

As documented in the left part of Fig. 2, incubation at or above 85 °C causes a fragmentation of the peptide chain of the *M. fervidus* GAPDH, however, only at low ionic strength, i.e. under non-stabilizing conditions. At a high phosphate concentration (right part of Fig. 2), known to stabilize proteins from this methanogen [15], the reaction is hindered.

From the sequences of the *N*-termini of the fragments the respective cleavage sites could be deduced. As shown in Fig. 3, hydrolysis occurs exclusively next to Asn

residues. Obviously, the often described cleavage of the peptide bond at Asp residues takes place mainly at acidic pH and is not relevant at physiological (neutral) conditions.

The non-enzymic cleavage at Asn residues has already been described in proteins from mesophilic organisms [7,8]. Like the deamidation reaction, the cleavage also proceeds via a cyclic succinimide derivative, but in contrast with the deamidation reaction, the cyclization starts with a nucleophilic attack of the amide nitrogen on the carbonyl carbon of the peptide bond, thus leading to the cleavage.

As with the deamidation reaction [15a], since the cleavage reaction itself is favoured at high ionic strength, the hindrance of hydrolysis at high ionic strength, as observed in the case of the *M. fervidus* GAPDH, must be due to the extrinsic stabilization of the protein conformation.

Resistance of *P. woesei* GAPDH to non-enzymic hydrolysis: hints for deactivation of the weak links of the peptide chain by substitution or elimination of Asn residues

As one can expect from its higher resistance to deamidation, the GAPDH from *P. woesei* also shows a significantly lower susceptibility to hydrolysis of the peptide bonds. Thus, after incubation for 1 h at 100 °C no, or very few, hydrolysis products are visible in the respective electropherograms (not shown). We assume that, for the most part, the higher conformational stability of this protein causes its higher resistance to non-enzymic hydrolysis.

Additionally, we speculate that in proteins adapted to extremely high thermal conditions, 'hot spots' of chemical modification are avoided, especially in flexible structure elements.

In this regard we interpret the finding that the *P. woesei* GAPDH sequence lacks Asn residues at three positions, which are homologous to the 'fragile' positions in the *M. fervidus* sequence (Fig. 3).

At two 'fragile' positions, however, Asn residues are conserved in the *P. woesei* structure, probably for functional reasons. By analogy with the three-dimensional structure of the *Bacillus stearothermophilus* GAPDH [16], the conserved Asn residue in fragment 4 is the neighbour of the catalytically essential Cys residue, whereas the other conserved Asn residue in fragment 5 is located in the functionally important S-loop. Obviously, these residues are protected by the rigid conformation of the *P. woesei* GAPDH.

Because of their chemical lability we would expect that Asn residues are generally reduced in number in proteins adapted to the higher temperature range (above 80 °C). Comparing the GAPDH and 3-phosphoglycerate kinase sequences from mesophilic and thermophilic archaea [12,17] the expected tendency can be confirmed. Similar trends are also visible in bacterial proteins; strikingly low Asn content was found in enzyme proteins from *Thermus* strains with upper growth temperatures around 85 °C indicating that the requirements for the construction of proteins for the higher temperature range are similar in both domains.

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