PROTEIN METHYLATION IN *CALDARIELLA ACIDOPHILA*, AN EXTREME THERMO-ACIDOPHILIC ARCHAEBACTERIUM

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1. Introduction

The enzymatic methylation of proteins occurs in both prokaryotes and eukaryotes by a group of enzymes highly specific with regard to the amino acid sidechain and protein involved [1].

Three methylating enzymes have been identified so far and characterized on the basis of the methyl-accepting protein moiety: the guanidino-groups of arginine residues are the methyl acceptors for protein methylase I (PM I; *S*-adenosylmethionine: protein–arginine–*N*-methyl transferase, EC 2.1.1.23) [1–3]; protein methylase II (PM II; *S*-adenosylmethionine: protein carboxyl-*O*-methyl transferase, EC 2.1.1.24) methylates the free carboxyl groups of aspartic and glutamic acid residues [1,3,4], while the ϵ-amino groups of lysine residues are methylated by protein methylase III (PM III; *S*-adenosylmethionine: protein ϵ-lysine–*N*-methyl transferase, EC 2.1.1.43) [1,3,5].

Despite the large number of reports on protein methylation in mesophilic organisms, no data are available on the occurrence of these biochemical processes in thermophiles. It is well known that thermophilic microorganisms are capable of proliferating at elevated temperatures and the majority of their marcromolecules are heat stable.

The mechanisms underlying the thermostability of cell constituents and the structural alterations in cellular components which allow the survival at high temperatures are not completely known at present. It is generally accepted that the primary structure of thermophilic proteins is similar to the mesophilic proteins with the same function [6]; however, thermophilic proteins are generally more stable to denaturing conditions than their mesophilic counterparts [6]. Various investigators suggest that the high stability of thermophilic proteins could result from subtle changes in hydrophobicity of a small part of protein molecule [7]. Among the post-translational covalent protein modifications, methylation results in an increased hydrophobicity of the molecule that might induce stereochemical alterations affecting its structure and functions [1,8]. Therefore the methyl modification of proteins could be one of the biochemical strategies employed by the thermophilic cell in adapting to the thermal stress.

We report here evidence on the enzymatic methylation of proteins in *Caldariella acidophila*, an extreme thermo-acidophilic archaebacterium; some peculiarities of protein methylase II are also described. Preliminary reports of this work have been made [9].

2. Materials and methods

2.1. *Chemicals*

S-Adenosyl-L-[*methyl*-^{14}C]methionine (spec. radioact. 50–60 Ci/mol) was purchased from the Radiochemical Centre, Amersham. *S*-Adenosyl-L-homocysteine was supplied from Sefochem Fine Chemicals, Israel. 3,5-Dinitrobenzoyl chloride was from Eastman Kodak Co.; silica gel TLC plates from Merck, Darmstadt.

All other chemicals were from local sources and of the best grade available. A citrate–phosphate buffer (pH 6.0) was prepared as in [10].

2.2. *Cell cultures*

Caldariella acidophila, strain MT4, was isolated from an acid hot spring in Agnano, Naples. The bacterium was grown as in [11].

2.3. *Homogenate preparation*

Washed cells (20 g) were ground with 35 g glass beads and 35 ml 5 mM sodium phosphate buffer (pH 7.5) [11]. The crude homogenate was then centrifuged at 35 000 × *g* for 1 h. The pellet was resuspended in 15 ml 5 mM borate buffer (pH 9.0). The supernatant (S_1 fraction) was used as enzyme source.

2.4. *Enzymatic assay for protein: S-adenosylmethionine methyltransferases (trichloroacetic acid precipitation method)*

The simultaneous assay of protein *N*-methyl transferases (protein methylase I and III) was performed by fractionating the $^{14}CH_3$ incorporated in the trichloroacetic acid-insoluble material as in [12] with minor modifications. The standard assay mixture contained 75 μl citrate–phosphate buffer (pH 6.0), 20 nmol *S*-adenosyl-L-[*methyl*-^{14}C]methionine (100 dpm/pmol) and 1–2 mg *C. acidophila* proteins in total vol. 250 μl. Control samples contain 0.2 μmol *S*-adenosyl-L-homocysteine, concentration which gave 90% inhibition of the reaction. Incubation was performed at 70°C for 10 min in sealed plastic vials.

2.5. *Enzymatic assay for protein methylase II (isoamyl alcohol extraction method)*

A second assay method, specific for PM II involves the extraction of [^{14}C]methanol derived from protein carboxyl methyl esters with isoamyl alcohol as in [1,13]. The assay conditions were the same as the previous method, but the reaction mixture was scaled down to 125 μl.

2.6. *Preparations of methyl-3,5-dinitrobenzoate*

The preparation of 3,5-dinitrobenzoate derivative of methanol obtained from protein methyl esters, was performed according to [14,15].

2.7. *Purification of methyl-3,5-dinitrobenzoate by TLC*

The crystals of methyl-3,5-dinitrobenzoate, prepared by the above procedure, were dissolved in acetone and chromatographed on TLC silica gel plates. The elution was achieved with ethyl acetate/*n*-exane (10/90, v/v) using the 3,5-dinitrobenzoate derivatives of methanol, ethanol, *n*-propanol and *n*-butanol as standards. The spots were localized by UV mineral light lamp (254 nm) and the area corresponding to the methyl derivatives was eluted with methanol. This purification procedure was repeated twice. The purified methyl-3,5-dinitrobenzoate, dissolved in pyridine, was crystallized several times with cold water. The specific radioactivity of the methyl-3,5-dinitrobenzoate was evaluated at each purification and crystallization step.

3. Results

In order to investigate the enzymatic methylation of preformed proteins in thermophilic prokaryotes, *C. acidophila* was selected as model system. This microorganism grows at 87°C and pH 3.5 and its enzymes show in vitro high degree of thermophilicity and thermostability [11,16,17].

Initial evaluation of *methyl*-^{14}C incorporation into *C. acidophila* was carried out using crude homogenate as source of enzyme and protein substrates.

The enzymatic mixture was incubated at 70°C for 10 min in presence of *methyl*-^{14}C-labelled AdoMet as methyl donor. The amount of protein *N*-methylations was estimated by measuring the $^{14}CH_3$ incorporated in the protein fraction of trichloroacetic acid precipitate [1,12], while the protein carboxyl-methyl esterification was evaluated by isoamyl alcohol extraction of the $^{14}CH_3OH$ released from the hydrolysis of methyl esterified groups during incubation (see section 2). It is well known, in fact, that protein carboxyl methyl esterification represents a reversible kind of covalent modification [1,3,18]. The formed methyl esters are spontaneously [18] hydrolyzed yielding methanol and the rate of the hydrolysis is strictly dependent from the temperature [19]. The results in table 1 indicate that the methylation of protein free carboxyl groups represents the 81% of the total protein methylation process in this microorganism. The low degree of protein *N*-methylation (7%) compared with carboxyl-methyl esterification is not surprising since in our assay conditions the endogenous methyl acceptor substrates for PM I [1] and PM III [1] are probably already methylated, while the reversibility of carboxyl methylation makes accessible the endogenous substrates for PM II. On the other hand the high temperature of incubation (70°C) prevented the use of the exogenous mesophilic protein substrates normally employed for the in vitro studies of these enzymes [12,20].

The presence of methyl esters in the in vitro methylated *C. acidophila* proteins was confirmed by the detection of methanol, as the hydrolytic product,

Table 1
Enzymatic methylation of *Caldariella acidophila* proteins

Enzymatic reaction	Enzymatic activity $^{14}CH_3$ incorporated (dpm . sample^{-1} . 10 min^{-1})	Specific activity (pmol $^{14}CH_3$. min^{-1} . mg protein^{-1})	% of Total $^{14}CH_3$ incorp.
Protein carboxyl methyl esterefication (PM II activity)[a]	25.300 ± 500	14.05 ± 0.28	81
Protein *N*-methylations (PM I and PM III activities)[b]	1600 ± 33	0.94 ± 0.02	7
Nucleic acid methylations (DNA and RNA methylases activities)[b]	3700 ± 75	2.09 ± 0.04	11

[a] Evaluated by isoamyl alcohol extraction method
[b] Evaluated by trichloroacetic acid precipitation method

The enzymatic assays were performed as described in the text, with 1.8 mg crude homogenate proteins

identified as 3,5-dinitrobenzoate derivative (see section 2). The specific activity of the products of the 3rd and 4th crystallization, of the pre-purified methyl benzoate, was 390 dpm/10 mg and 410 dpm/10 mg, respectively; the melting point of the crystals was 107–108°C, in good agreement with the reported values for the methyl derivative of 3,5-dinitrobenzoate. The radioactivity detectable as 3,5-dinitrobenzoate [^{14}C]methyl ester is calculated to be 23 165 dpm, that represents 90% of the total volatile radioactivity extracted with isoamyl alcohol [1,13] in a parallel assay.

Almost all the enzymatic activity (97%) appears associated with the soluble fraction (S_1) of the homogenate. After ammonium sulphate fractionation, the activity results located in the 0–50% $(NH_4)_2SO_4$ saturation fraction (S_2) while the 50–80% saturation fraction (S_3) contains only methyl accepting proteins. In fact, the addition of varying concentration of S_3 to the incubation mixture, with S_2 as enzyme source, increases the enzymatic activity up to 1.8-fold. On the contrary when S_1 was the enzyme source, the addition of S_3 as methyl accepting proteins, did not significantly affect the reaction rate, indicating that PM II in the homogenate soluble fraction is saturated by its endogenous substrates.

Preliminary characterization of PM II was performed using the S_1 fraction as enzyme and substrates source. The enzyme shows an optimum pH of 6.0, with 75% of enzymatic activity at pH 5.5–6.7. This optimum pH is the same as reported for the PM II purified from eukaryotes [21], while for the *Escherichia coli* enzyme is reported an optimum pH of 7.4 [22].

As reported in fig.1 the reaction rate is markedly increased by temperature elevation, exhibiting a maximum at 80°C. At 65°C the reaction rate was 50% of the maximal value, while no activity was detectable at 37°C. The decrease of enzymatic activity observable at the maximal assayed temperature, 90°C, is probably related with the termal lability of the methyl donor substrate, i.e., AdoMet, more than to the enzyme inactivation. The optimum growth temperature of *C. acidophila* is, in fact, 90°C and other enzymes purified from this microorganism show a higher degree of thermophilicity [11,16,17].

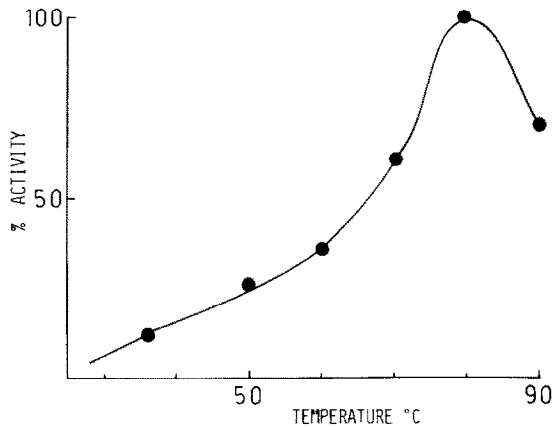

Fig.1. Effect of temperature on PM II activity. The enzymatic assay was performed with isoamyl alcohol extraction method as in section 2 with 1.5 mg S_1 proteins.

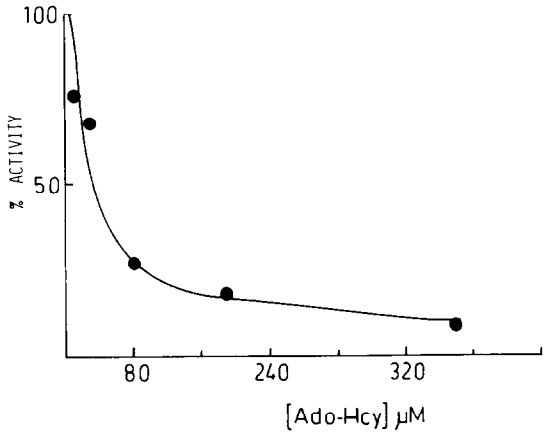

Fig.2. Effect of Ado-Hcy on PM II activity. The enzymatic assay was performed with isoamyl alcohol extraction method, except that AdoMet was 32 μM.

PM II from *C. acidophila* is significantly inhibited by *S*-adenosylhomocysteine (AdoHcy), the demethylated product of the reaction. In fig.2 is reported the effect of this thioeter on the reaction rate: 50% inhibition is observable with AdoHcy concentration equimolar with the methyl donor substrate. This observation confirms the regulatory role of AdoHcy on macromolecules methylation as reported in other systems [23,24].

4. Discussion

We report here an active post-translational protein modification in thermophilic prokaryotes; preliminary observations suggest that the adenylation of glutamine synthetase may also occur in these microorganisms [25]. Our results indicate that the methylation of proteins, largely operative in eubacterial cells and in eukaryotes, is also present in the oldest forms of life, the archaebacteria [26]. The alteration of the protein primary structure may be economical from the standpoint of cellular energetics, since the expenditure of ATP and GTP for the synthesis of polypeptide chains is conserved and yet proteins of different structure are formed. This consideration is important for *C. acidophila* and other archaebacteria, living in a primordial environment with very low levels of chemical energy.

Protein carboxyl-methyl esterification represents a reversible kind of covalent modification: this reaction has recently attracted a great deal of attention, mainly because it plays a crucial role in bacterial chemotaxis. The methylation of glutamic acid residues in *E. coli* [27,28] and *S. thyphimurium* [29,30] methyl-accepting chemiotactic proteins, represents a control mechanism in the behavioral response of bacteria to the chemical stimuli. The presence of a primordial motile apparatus in *C. acidophila* [17,31] justifies further investigation on the occurrence of an AdoMet-dependent chemiotactic response in this microorganism.

The prevalence of hydrophobic residues in a number of proteins from thermophiles is one of the factors responsible for their thermostability. The high levels of protein methylation reported in a thermophile could play a primary role in the adaptation of this microorganism to high temperatures, by increasing hydrophobicity of its protein structures. It could be worthwhile to investigate the relationship between growth temperatures and protein methylase activities.

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