Metabolic channelling of carbamoyl phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus*: dynamic enzyme–enzyme interactions involved in the formation of the channelling complex

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Abstract

Protection of thermolabile metabolites and coenzymes is a somewhat neglected but essential aspect of the molecular physiology of hyperthermophiles. Detailed information about the mechanisms used by thermophiles to protect these thermolabile metabolites and coenzymes is still scarce. A case in point is CP (carbamoyl phosphate), a precursor of pyrimidines and arginine, which is an extremely labile and potentially toxic intermediate. Recently we obtained the first evidence for a physical interaction between two hyperthermophilic enzymes for which kinetic evidence had suggested that these enzymes channel a highly thermolabile and potentially toxic intermediate. By physically interacting with each other, CKase (carbamate kinase) and OTCase (ornithine carbamoyltransferase) prevent thermodenaturation of CP in the aqueous cytoplasmic environment. The CP channelling complex involving CKase and OTCase or ATCase (aspartate carbamoyltransferase), identified in hyperthermophilic archaea, provides a good model system to investigate the mechanism of metabolic channelling and the molecular basis of protein–protein interactions in the physiology of extreme thermophiles.

Introduction

Most of the low-molecular-mass metabolites and coenzymes found in thermophiles are identical to the ones found in mesophiles, yet many are extremely thermolabile, and in some cases their degradation products are toxic to the cell. Thus cells growing at high temperature must have evolved strategies to protect against degradation of thermally unstable metabolites and coenzymes. Thermolabile metabolites and coenzymes can be protected from thermal degradation by mechanisms such as microenvironmental compartmentation, metabolic channelling, increasing catalytic efficiency and deletion or substitution of the thermolabile compound ([1] and references therein).

Metabolic channelling can protect chemically labile intermediates and thus play a critical role in the physiology of thermophiles. Metabolic channelling is the direct transfer of a metabolite between sequential enzymes in a metabolic pathway [2,3]. Several examples exist of enzymes which belong to the same or related biochemical pathways and are organized into functional complexes [4–6]. Within some metabolic pathways one may find stable multienzyme complexes or multifunctional enzymes that catalyse sequential reactions. In addition to those easily identifiable metabolic systems, specific interactions were shown to occur between soluble enzymes acting in sequence [4,7,8]. With the changing view of the interior of the living cell [9,10] and the acquisition of compelling structural and kinetic evidence [5,6], metabolic channelling is now better accepted than it used to be. Nevertheless as stressed a few years ago by Ovádi and Srere [11], there remains a need for experimental systems allowing us to demonstrate the biological relevance and characterize the molecular mechanisms of metabolic channelling. We situate the study of CP (carbamoyl phosphate) metabolism in this perspective.

Carbamoyl phosphate (CP) metabolism

CP, a precursor of the pyrimidine and arginine pathways (Scheme 1), is a very thermolabile intermediate and potentially toxic at high temperature. Besides constituting a significant waste of energy for the cell, since CP synthesis requires ATP, the decomposition of CP in aqueous solutions at high temperatures leads to the accumulation of toxic amounts of cyanate which is a powerful and indiscriminate agent for free amino groups under such conditions [12]. At 100°C, where *Pyrococcus furiosus* reaches its highest growth rates, the half-life of CP is only 2 s [13]. Therefore organisms growing at high temperatures must have evolved strategies to protect CP from degradation. The archaeon *P. furiosus* was

Key words: carbamoyl phosphate metabolism, enzyme–enzyme interactions, hyperthermophile, metabolic channelling, molecular physiology, thermostability.

Abbreviations used: CP, carbamoyl phosphate; CPSase, carbamoyl phosphate synthetase; CKase, carbamate kinase; OTCase, ornithine carbamoyltransferase; ATCase, aspartate carbamoyltransferase.

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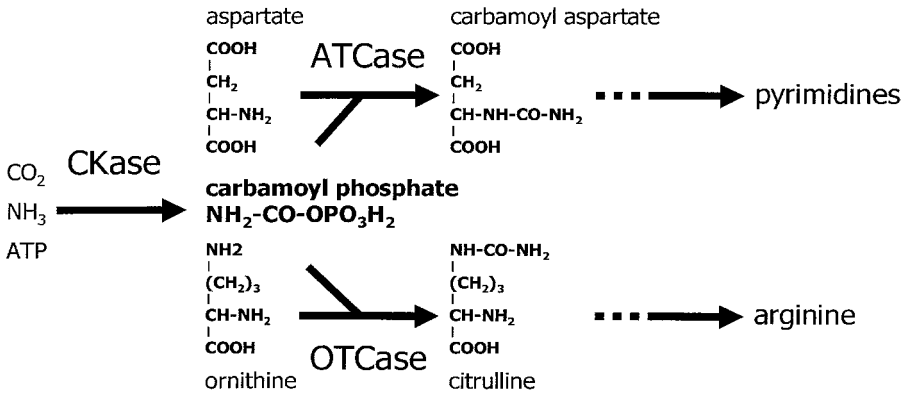
Scheme 1 | CP metabolism in *P. furiosus*



chosen as model system to investigate thermal protection of CP and enzyme–enzyme interactions in hyperthermophiles.

CP is classically synthesized by a CPSase (carbamoyl phosphate synthetase), which in most prokaryotes is a heterodimeric enzyme: a small glutaminase subunit hydrolyses glutamine and the released ammonia is transferred intramolecularly through a tunnel to a large subunit which catalyses CP synthesis in a complex reaction requiring bicarbonate and MgATP [14,15]. In the archaeon *P. furiosus* however, CP is synthesized directly from ammonia [13] and the enzyme from the archaeon is enzymologically and structurally a CKase (carbamate kinase) [16–18]. This CKase from *P. furiosus* is highly thermostable and at ambient temperature is a very slow carbamate kinase, which in the extreme environment of *P. furiosus* exerts the new function of making, rather than using, CP [19]. The enzyme was shown to be able to replace CPSase *in vivo* in *Escherichia coli* [20]. A similar enzymic activity was reported in the related species *Pyrococcus abyssi* [21,22]. This situation is unprecedented in microbial CP biosynthesis. A CPSase gene was found in the genome of *P. furiosus* but not in the genomes of the very related species *P. abyssi* and *Pyrococcus horikoshii* [22], making the situation more complicated but also very interesting from the point of view of evolution.

OTCase (ornithine carbamoyltransferase) carbamoylates ornithine to citrulline in arginine biosynthesis, whereas ATCase (aspartate carbamoyltransferase) catalyses the formation of aspartate and CP into carbamoylaspartate in the committed step of pyrimidine biosynthesis (Scheme 1).

Metabolic channelling of CP

In the extreme thermophilic bacterium *Thermus* ZO5, CP is protected from thermodegradation by channelling towards the synthesis of citrulline and carbamoyl aspartate, as demonstrated by an isotopic competition assay [23]. Likewise, competition experiments with *P. furiosus* cell-free extracts showed a marked preference of OTCase for CP synthesized by CKase rather than for CP added to the reaction mixture [13]. Moreover, the bisubstrate analogue PALO (*N*-*δ*-phosphonoacetyl-L-ornithine) inhibits the formation of citrulline in the CKase–OTCase-coupled reaction much less than in the uncoupled OTCase reaction. These results suggested that CKase and OTCase form a channelling complex to protect CP from decomposition [13].

In the deep sea hyperthermophilic archaeon *P. abyssi*, CP channelling in both arginine and pyrimidine biosynthetic pathways was demonstrated by isotopic competition experiments and the kinetics of the coupled reactions between CKase and both OTCase and ATCase [24]. It was demonstrated that channelling is leaky at ambient temperatures but that channelling efficiency appreciably increased at elevated temperatures. CP was shown to be equally partitioned between pyrimidine and arginine biosynthetic pathways when the enzymes were present in the proportions found in the cell.

Partial channelling of CP was previously suggested to occur in pyrimidine biosynthetic complexes from yeast [25,26], *Neurospora* [27,28] and mammals [29,30] as well as between enzymes of the mammalian urea cycle [31,32]. Metabolic channelling of CP thus appears to be a widespread phenomenon but, especially in thermophiles, it provides an efficient mechanism to protect this thermolabile metabolite from decomposition.

Enzyme–enzyme interactions in the CP channelling complex

The *sine qua non* for metabolic channelling is the existence of some sort of structural organization of the enzymes of the metabolic pathway, a 'metabolon'. This organization depends on the way the enzymes interact with each other, whether they form stable complexes or transient associations, possibly metabolite-induced. In such an enzyme complex the intermediate can be transferred by different mechanisms: intermediates can be covalently linked to a swinging arm [33], gated through a tunnel through the interior of the protein [34], or a favourable electrostatic field between the adjacent active sites can be used to constrain the intermediate within the channelling path along the surface of the protein [35,36]. In yeast [26] and mammals [30] channelling of CP in pyrimidine biosynthesis occurs in multienzymic polypeptides. Such multienzymic polypeptides are not present in the thermophilic organisms investigated. Since

CP channelling is nevertheless observed, the enzymes from these thermophilic organisms must form a stable or dynamic complex able to channel CP.

By size-exclusion chromatography a stable complex between CKase and OTCase or ATCase from *P. abyssi* could not be detected [23]. Neither elevated temperature, which promotes more efficient channelling, nor the presence of substrates, which could have induced change in enzyme conformation and thus promote complex formation, led to the formation of complexes detectable by gel filtration. With affinity electrophoresis and co-immunoprecipitation experiments, evidence for physical interaction between CKase and OTCase from the hyperthermophile *P. furiosus* was obtained [37]. This was the first evidence for a physical interaction between two hyperthermophilic enzymes involved in channelling a thermolabile metabolite.

Affinity electrophoresis experiments allow the visualization of a direct interaction between two different proteins or enzymes in conditions that mimic the crowded intracellular environment. In such experiments, purified CKase or OTCase was allowed to diffuse electrophoretically through a strip containing immobilized OTCase or CKase, respectively, and enzyme localization was performed by assaying activity *in situ* and by immunochemical staining. The immobilized CKase was effective in binding the migrating OTCase, thus indicating an interaction between the two enzymes. The resulting association between CKase and OTCase was shown to be specific to the extent that OTCase did not interact with other metabolically unrelated immobilized proteins with a comparable pI value. Further evidence for a biologically significant interaction between CKase and OTCase was obtained by co-immunoprecipitation experiments. When *P. furiosus* extracts were prepared in a gentle way with high protein concentration, the CKase–OTCase complex could be precipitated. Because co-immunoprecipitation can be performed with cell-free extracts, it permits the detection of multienzyme associations in a system where all cellular components are still present. In these experiments, only a fraction of CKase was shown to coprecipitate with OTCase and *vice versa*, suggesting a relatively weak, unstable interaction. This interaction between CKase and OTCase could be captured when the cell-free extracts were incubated with the cross-linking agent formaldehyde prior to immunoprecipitation. Adding excess free enzyme *in vitro*, thereby driving enzyme association, resulted in a better precipitation of the CKase–OTCase complex. When extracts were prepared by sonication, co-immunoprecipitation was not observed; however, when these extracts prepared by sonication were incubated at 60°C for a few minutes prior to co-immunoprecipitation, the CKase–OTCase complex partially reformed and could be precipitated. No influence of the substrates on precipitation of the complex could be observed. Using the IAsys technology [38] we were not able to obtain a more quantitative appreciation of the observed interaction between the two enzymes; the interaction between CKase and OTCase is probably too weak to be detected by this technique.

Recent isothermal titration calorimetry experiments also point to an interaction between CKase and OTCase but the data are still not sufficient to estimate the binding constant. Using the yeast two-hybrid system additional confirmation about the interaction between CKase and OTCase could be obtained. With OTCase from *P. furiosus* fused to the GAL4 DNA-binding domain and CKase fused to the GAL4 activation domain an interaction between the two enzymes could be detected. *Vice versa*, with CKase as the binding domain fusion partner and OTCase as the activation domain fusion partner an interaction between the two enzymes could not be observed. It is frequently encountered that an interaction between A and B may be detected, for example, with A as the binding-domain fusion partner but not with A as the activation-domain fusion partner [39]. The yeast two-hybrid system is a promising method for the future, because it allows molecular characterization of the interaction by analysis of mutants. Finally, preliminary results obtained with the Hummel–Dreyer method of equilibrium gel filtration [40] also gave indications for a physical interaction between CKase and ATCase from *P. furiosus*.

Structural characterization of the channelling complex

Detailed mechanistic and structural information about transient enzyme–enzyme interactions is still scarce. The CP channelling complex involving CKase, OTCase and ATCase, identified in hyperthermophilic Archaea, provides a good model system to investigate the molecular basis for the specificity of protein–protein interactions in the physiology of extreme thermophiles. The molecular analysis of the protein–protein interactions assembling the channelling complex requires a detailed structural study of the protein partners. High-resolution structures of the binding proteins can be used in docking simulations to get an idea of possible complex conformations.

The crystal structure of *P. furiosus* CKase has been determined at a resolution of 1.5 Å (Protein Data Bank accession no. 1E19) [41]. The enzyme is a homodimer with the active sites of each subunit located in a large crevice between the N- and C-terminal domains. An ADP molecule bound to this high-resolution structure provided insight into substrate binding. Thermostability of the enzyme results from extensive hydrophobic intersubunit contacts and from the large number of exposed ion-pairs, some of which form ion-pair networks across several secondary structural elements in each enzyme subunit.

In our laboratory, the structure of *P. furiosus* OTCase [42] has been refined to a resolution of 1.87 Å (Protein Data Bank accession no. 1PVV) [43]. The identification of a sulphate molecule at the active site allowed the identification of residues involved in binding of CP. The improved quality of the model resulted in a better definition of several loops and the different interfaces. While most mesophilic OTCases are trimeric, the hyperthermophilic protein is dodecameric, composed of four catalytic trimers disposed in a tetrahedral manner. Extreme thermal stability of *P. furiosus*

OTCase is mainly the result of strengthening of inter-subunit interactions in a trimer and oligomerization of trimers into a dodecamer [43,44]. Interfaces between monomers in a catalytic trimer are characterized by an increase in ion-pair networks compared with mesophilic OTCases. However, the interfaces between catalytic trimers in the dodecameric oligomer are mainly hydrophobic and also involve aromatic–aromatic and cation–π interactions.

The ATCases from *P. furiosus* and *P. abyssi* are also dodecamers but composed of three regulatory dimers and two catalytic trimers, similar to the *E.coli* ATCase [45,46]. ATCase from *P. furiosus* has been purified and is being crystallized. For the ATCase from *P. abyssi* the structure of the catalytic trimer complexed with the bisubstrate analogue PALA (*N*-*δ*-phosphonoacetyl-L-aspartate) has been determined at a resolution of 1.8 Å (Protein Data Bank accession no. 1ML4) [46]. Besides the shortening of several loops, an increased number of charged residues and a concomitant increase of salt links at the interface between the monomers, as well as the formation of an ion-pair network at the protein surface, are the main factors contributing to thermostability.

The structures of the catalytic trimers of ATCase and OTCase are very similar; the structures of the holoenzymes, however, are not. This is interesting in respect to the formation of a channelling complex with CKase.

Final remarks

Evidence for a physical interaction between CKase and OTCase and the preliminary results indicating an interaction between CKase and ATCase support predictions made from kinetic analysis. New technologies and techniques developed for studying protein–protein interactions will have to be used and adapted to characterize more quantitatively the interactions between CKase and OTCase and ATCase respectively. High-resolution structures of the different enzymes involved in channelling of CP provide a basis for detailed mechanistic and structural information about the dynamic enzyme–enzyme interactions in the CP channelling complex of *P. furiosus*. Crystal structures of these enzymes in complex with different substrates should help to identify possible docking conformations. In conclusion, metabolic channelling of CP in *P. furiosus* provides a good model system to investigate the molecular basis of dynamic protein–protein interactions in the physiology of extreme thermophilic organisms.

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