New experimental approaches for investigating interactions between *Pyrococcus furiosus* carbamate kinase and carbamoyltransferases, enzymes involved in the channeling of thermolabile carbamoyl phosphate

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Summary A somewhat neglected but essential aspect of the molecular physiology of hyperthermophiles is the protection of thermolabile metabolites and coenzymes. An example is carbamoyl phosphate (CP), a precursor of pyrimidines and arginine, which is an extremely labile and potentially toxic intermediate. The first evidence for a biologically significant interaction between carbamate kinase (CK) and ornithine carbamoyltransferase (OTC) from *Pyrococcus furiosus* was provided by affinity electrophoresis and co-immunoprecipitation in combination with cross-linking (Massant et al. 2002). Using the yeast two-hybrid system, Hummel-Dreyer chromatography and isothermal titration calorimetry, we obtained additional concrete evidence for an interaction between CK and OTC, the first evidence for an interaction between CK and aspartate carbamoyltransferase (ATC) and an estimate of the binding constant between CK and ATC. The physical interaction between CK and OTC or ATC may prevent thermodenaturation of CP in the aqueous cytoplasmic environment. Here we emphasize the importance of developing experimental approaches to investigate the mechanism of thermal protection of metabolic intermediates by metabolic channeling and the molecular basis of transient protein–protein interactions in the physiology of hyperthermophiles.

Keywords: carbamoyl phosphate metabolism, hyperthermophiles, metabolic channeling, protein–protein interactions.

Introduction

Most low molecular weight metabolites and coenzymes found in thermophiles are identical to those found in mesophiles, yet many are extremely thermolabile, and in some cases, their degradation products are toxic to the cell. Although several mechanisms have already been proposed (Daniel and Cowan 2000), detailed information about the mechanisms possessed by thermophiles to protect these thermolabile metabolites and coenzymes is still scarce.

Carbamoyl phosphate (CP), the common precursor of the pyrimidine and arginine pathways, is a highly thermolabile intermediate and potentially toxic at high temperatures (Legrain et al. 1995). At 100 °C, at which temperature *Pyrococcus furiosus* reaches its highest growth rates, the half-life of CP in aqueous solutions is less than 2 s. Therefore, organisms growing at high temperatures must have means to protect CP from degradation.

Isotopic competition experiments with cell-free extracts of *P. furiosus* showed a marked preference of ornithine carbamoyltransferase (OTC) for CP synthesized by carbamate kinase (CK) rather than for CP added to the reaction mixture (Legrain et al. 1995). These results suggested that CK and OTC formed a channeling complex to protect CP from decomposition. Similar results indicated the existence of channeling of CP to both OTC and aspartate carbamoyltransferase (ATC) in *Thermus* ZO5 (Van de Castele et al. 1997).

Evidence for a biologically significant interaction between OTC and CK in *P. furiosus* was provided by affinity electrophoresis and co-immunoprecipitation in combination with cross-linking (Massant et al. 2002, Massant 2004, Massant and Glansdorff 2004). These results suggested that the interaction between the two enzymes was weak or transient, at least in vitro. In the deep sea hyperthermophilic archaeon *P. abyssi*, CP channeling in both arginine and pyrimidine biosynthetic pathways was suggested by isotopic competition experiments and the kinetics of the coupled reactions between CK and both OTC and ATC (Purcarea et al. 1999). However, experimental evidence for a physical interaction between CK and ATC from *P. abyssi* or *P. furiosus* has not yet been obtained. The structures of *P. furiosus* CK (Ramón-Maiques et al. 2000) OTC (Villeret et al. 1998, Massant et al. 2003) and the catalytic trimer of *P. abyssi* ATC (Van Boxtael et al. 2003) have been solved.

The CP channeling complex involving CK, OTC and ATC, identified in *Pyrococcus* species, is a good model system for investigating the molecular mechanisms of thermal protection of intermediates in the physiology of hyperthermophiles, and

for clarifying transient enzyme–enzyme interactions and the molecular basis of metabolic channeling. However, because detailed mechanistic and structural information about metabolic channeling and transient enzyme–enzyme interactions is still scarce, it is important to develop experimental approaches to investigate the nature and dynamics of transient protein–protein interactions.

A number of methods for investigating protein–protein interactions qualitatively and quantitatively have been described (for an overview, see Phizicky and Fields 1995). The yeast two-hybrid system is a genetic method that employs *Saccharomyces cerevisiae* and detects transcriptional activity as a measure of protein–protein interactions in vivo (Fields and Song 1989). Gel filtration is a simple way to detect protein complexes and estimate the binding constant (Beeckmans 1999). When the association between the proteins is only dynamic and equilibrates rapidly, the Hummel–Dreyer method of equilibrium gel filtration (Hummel and Dreyer 1962) sometimes allows the interaction to be analyzed both qualitatively and quantitatively (Gegner and Dahlquist 1991). Isothermal titration calorimetry measures the heat released or absorbed upon association of two proteins (Wiseman et al. 1989). The calorimetric data could also allow the calculation of thermodynamic binding parameters.

We have initiated physicochemical studies on the interactions between the enzymes (CK, OTC and ATC) from the CP channeling complex using these different methods; the present data provide convergent and independent evidence, both in vivo and in vitro for the existence of physical interactions between CK and the two carbamoyltransferases in *P. furiosus* and therefore pave the way for more refined studies of this model system.

Materials and methods

Expression and purification of P. furiosus ATC, CK and OTC

The *P. furiosus pyrBI* fragment was cloned into the pET28b expression vector (Novagen, Madison, WI) and transformed into *Escherichia coli* Rosetta(DE3) host (Novagen). Cells of *E. coli* Rosetta(DE3) containing pET28b/*Pfu-pyrBI* were grown in a 13-l batch Biolaftite fermentor in rich 853-medium (Glansdorff 1965) containing 30 $\mu\text{g ml}^{-1}$ chloroamphenicol and 50 $\mu\text{g ml}^{-1}$ kanamycin at 37 °C. Expression of the recombinant enzyme was induced by adding IPTG (isopropyl-thio- α ,D-galactopyranoside).

The purification procedure for *P. furiosus* ATC was similar to the procedure described for *P. abyssi* ATC (Van Boxstael et al. 2003). Frozen cells were thawed in extraction buffer and sonication was performed at 280 K for 30 min. Taking advantage of the thermal stability of the ATC protein, the cell-free extract was heat-denatured at 75 °C for 40 min. Anion-exchange chromatography (ResourceQ and MonoQ, Amersham Pharmacia Biotech (APB), Part of GE Healthcare) was followed by gel filtration (Superdex 200 pg, APB) to separate the ATC holoenzyme (MW = ~300 kDa) from catalytic trimers (MW = ~100 kDa). The ATC holoenzyme fractions were con-

centrated (23 mg ml⁻¹) and determined to be pure by Coomassie staining after SDS-PAGE. Approximately 35 mg of ATC holoenzyme could be purified from 10 g of recombinant cells. In comparison, when using the pTrc99A expression vector in combination with the pSJS1240 bearing *E. coli* C600 strain, only 3 mg of ATC catalytic trimer could be purified from 10 g of recombinant cells (Van Boxstael et al. 2003). Thus, the use of the pET expression vectors in combination with *E. coli* Rosetta(DE3) strain gives a significantly higher yield of ATC. This made biophysical methods to investigate protein–protein interactions feasible.

Pyrococcus furiosus CK was purified from recombinant *S. cerevisiae* cells as described by Uriarte et al. (2001). *Pyrococcus furiosus* OTC was purified from recombinant *S. cerevisiae* cells as described by Legrain et al. (2001) using arginine-Sepharose chromatography for the last purification step. All protein concentrations mentioned are expressed as oligomer (CK dimer, OTC dodecamer, ATC dodecamer) equivalents.

Yeast two-hybrid analysis

Yeast two-hybrid screening was performed with the MATCH-MAKER Gal4 Two-Hybrid System 3 (Clontech, Palo Alto, CA). *Saccharomyces cerevisiae* strain AH109 [*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, Δ *gal4*, Δ *gal80*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*] (James et al. 1996) was the host used to investigate the interaction between CK and OTC from *P. furiosus*. *ArgF*, encoding OTC, and *cpkA*, encoding CK, from *P. furiosus* were inserted in the *NcoI* and *BamHI* sites in the multi-cloning site (MCS) from pGBKT7 and pGADT7. pGBKT7-53 and pGADT7-T (MATCHMAKER Two-Hybrid System 3) were positive control vectors. p53 and large T-antigen are known to interact in a yeast two-hybrid assay (Li and Fields 1993).

Selection media (SD/–Leu/–Trp, SD/–His/–Leu/–Trp and SD/–Ade/–His/–Leu/–Trp) were prepared from Yeast Nitrogen Base without Amino Acids from Difco (YNB without amino acids), the appropriate dropout (DO) medium from Clontech (DO/–Leu/–Trp, DO/–His/–Leu/–Trp or DO/–Ade/–His/–Leu/–Trp) and agar from Oxoid (Hampshire, U.K.) (for plates only). The media were autoclaved and cooled to ~55 °C before adding additional adenine, except for SD/–Ade/–His/–Leu/–Trp, and glucose. SD/–Leu and SD/–Trp media were prepared from SD/–Leu/–Trp by adding tryptophan or leucine respectively.

X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside) was used to monitor *MEL1* expression directly on nutritional selection plates. For spreading onto premade plates, X- α -Gal was diluted 10 times and 100 μl was evenly spread onto a 10-cm-diameter plate. Cells were streaked on X- α -Gal plates and incubated at 30 °C until blue colonies formed. As α -galactosidase accumulates in the medium, it hydrolyzes X- α -Gal, causing yeast colonies to turn blue. In the liquid α -Gal assay, the catalytic activity of α -galactosidase is monitored colorimetrically by measuring the rate of hydrolysis of the chromogenic substrate, *p*-nitrophenyl- α -D-galacto-

pyranoside (PNP- α -Gal). The α -galactosidase assay was performed following the protocol described in the Yeast Protocols Handbook from Clontech. A small aliquot of cell-free culture media (80 μ l) was combined with a fixed volume of assay buffer (240 μ l) and incubated for 1 h at 30 °C. Assay buffer (two volumes of 0.5 M Na-acetate, pH 4.5 and 1 volume of 100 mM PNP- α -Gal) was freshly prepared before each use. The reaction was terminated by adding 680 μ l of 10 $\times$ Stop solution (1 M Na₂CO₃) and optical density was recorded at 410 nm. The yeast two-hybrid results were based on two independent transformations and screenings.

Chromatography

A Superose 12-prepacked HR 10/30 gel filtration column was used in small zone and Hummel-Dreyer equilibrium chromatography experiments. For small zone chromatography, the column was equilibrated with 20 mM Tris-HCl, pH 8.0 and 100 mM KCl. Injection samples of 100 μ l containing 50 μ M (4 mg ml⁻¹) CK, 8.3 μ M (3.5 mg ml⁻¹) OTC and 16.7 μ M (5 mg ml⁻¹) ATC in the same buffer were separated on the column. For the Hummel-Dreyer method of equilibrium chromatography, the column was equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1.7 μ M (0.5 mg ml⁻¹) ATC. A 100- μ l injection sample contained the same buffer with ATC and CK concentrations as described in the results. The chromatography was performed with a flow rate of 0.25 ml min⁻¹. Absorbance of the eluant was monitored at 280 nm with a Pharmacia UV-1 control unit (Pfizer, New York, NY). Binding experiments were done at room temperature (~25 °C).

An estimate of the binding constant with the Hummel-Dreyer method of equilibrium gel filtration was calculated as described by Gegner and Dahlquist (1991). Therefore, the free ATC concentration must be known. This concentration was determined by varying the ATC concentration in the sample applied to the column containing buffer equilibrated with 1.7 μ M ATC. The amount of free ATC in the sample is reduced by the amount bound to the protein. A trough is observed when the remaining free ATC concentration is less than in the buffer; a peak is observed when this concentration is greater than in the buffer. Trough or peak area was measured (by photocopying and weighing) and plotted against ATC concentration for 5 and 10 μ M CK series. At the point where no trough or peak is observed, calculated by linear regression (Figure 4), the free ATC concentration equals the concentration of ATC in the buffer, and thus, the dissociation constant of the interaction can be calculated. When one assumes an equimolar binding between ATC and CK, the dissociation constant corresponds to $K_d = [\text{CK}_{\text{free}}][\text{ATC}_{\text{free}}]/[\text{CK-ATC}_{\text{complex}}]$. The equimolar binding is only a rough approximation because we do not yet have an idea about the stoichiometry of the interaction (the ATC dodecamer contains six catalytic centers for two catalytic centers in a CK dimer). For the series with 5 μ M CK added to the sample, $K_d \cong (5 - 0.5 \mu\text{M})(1.7 \mu\text{M})/(0.5 \mu\text{M}) \cong 15 \mu\text{M}$, with 10 μ M CK added to the sample $K_d \cong (10 - 1.1 \mu\text{M})(1.7 \mu\text{M})/(1.1 \mu\text{M}) \cong 14 \mu\text{M}$.

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed using an MCS isothermal titration calorimeter from Microcal (Northampton, MA). Injections of 15 μ l of CK solution were added from the computer-controlled syringe at intervals of 240 s into the OTC solution (cell volume = 1.7 ml), with stirring at 350 rpm. Both enzymes were dissolved in the same 20 mM potassium phosphate buffer, pH 7.4 with 7.5% PEG_{12,000}. The temperature of the cell was set at 55 °C, the temperature of the bath at 40 °C. Titration of CK into a solution of ATC was also performed with injections of 15 μ l at intervals of 240 s (temperature of the cell at 50 °C), but both enzymes were dissolved in 20 mM Tris-HCl, pH 8.0 (at 25 °C, pH 7.8 at 50 °C) with or without 5% PEG_{12,000}.

Electrostatic isopotential contours

Electrostatic potentials were calculated and displayed with the DelPhi module of InsightII (Accelrys, Tokyo, Japan). The electrostatic potential around the protein was obtained by solving the linear Poisson-Boltzmann equation using a 65 $\times$ 65 $\times$ 65 grid, 80% grid fill by solute. The internal dielectric of the protein was set to 2.0, and that of the solvent was set to 80.00. The ionic strength was set to 0.1 M. The individual enzymes were given total net charges using the standard charge file of Delphi of -7.5 for the OTC catalytic trimer (-30 for the OTC dodecamer) and -1 for the CK dimer.

Results

Yeast two-hybrid system

The two-hybrid system (Fields and Song 1989, Chien et al. 1991, Fields and Sternglanz 1994) relies on the modular nature of the yeast GAL4 protein, which consists of a DNA-binding domain (BD) and a transcriptional activation domain (AD). The DNA-binding domain serves to target the activator to the specific genes that will be expressed, and the activation domain contacts other proteins of the transcriptional machinery to enable transcription to occur. The two-hybrid system is based on the observation that the two domains of the activator need not be covalently linked and can be brought together by the interaction of any two carrier proteins. The MATCHMAKER Two-Hybrid System 3 from Clontech was used to demonstrate the interaction between OTC and CK from *P. furiosus* in vivo.

Construction of fusion genes The genes encoding for OTC (*argF*) and CK (*cpkA*) from *P. furiosus* were inserted in either of the fusion vectors (pGBKT7 and pGADT7) of the MATCHMAKER Two-Hybrid System 3. The DNA-BD and -AD fusion constructs were independently transformed into strain AH109 to verify that constructs did not activate reporter genes. The transformants were assayed for *HIS3*, *ADE2* and *MEL1* activation (see Materials and methods); SD/-Trp/ -His, SD/-Trp/ -Ade and SD/-Trp/X- α -Gal or SD/-Leu/ -His, SD/-Leu/ -Ade and SD/-Leu/X- α -Gal for transformants containing the BD- and AD-fusion constructs, respectively (Table 1). No activation of reporter genes was observed.

Table 1. Yeast two-hybrid analysis. Growth of the two-hybrid strain on selection media: + = growth; and – = no growth. Abbreviations: BD = binding domain; AD = activation domain; OTC = ornithine carbamoyltransferase; and CK = carbamate kinase.

AH109 hybrid strain	Low stringency	Medium stringency	High stringency
	SD/–Leu/–Trp	SD/–Leu/–Trp/–His	SD/–Leu/–Trp/–His/–Ade/X- α -Gal
BD-53/AD-T ¹	+	+	+
BD-OTC/AD-OTC	+	+	+
BD-OTC/AD-CK	+	+	+
BD-CK/AD-CK	+	+	±
BD-CK/AD-OTC	+	+	±
	SD/–Trp	SD/–Trp/–His	SD/–Trp/X- α -Gal
BD-OTC	+	–	–
BD-CK	+	–	–
	SD/–Leu	SD/–Leu/–His	SD/–Leu/X- α -Gal
AD-OTC	+	–	–
AD-CK	+	–	–

¹ BD-53 and AD-T encode fusions between the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively. p53 and large T-antigen are known to interact in a yeast two-hybrid assay (Li and Fields 1993).

Yeast two-hybrid screen Yeast strains containing the pGBKT7/*Pfu-argF* (BD-OTC) and pGBKT7/*Pfu-cpkA* (BD-CK) fusion vectors were subsequently transformed with the pGADT7/*Pfu-cpkA* (AD-CK) and pGADT7/*Pfu-argF* (AD-OTC) vectors. Because OTC and CK were expected to interact weakly or transiently, screening was performed stepwise from low to high-stringency (Table 1). Low-stringency screening involved plating of transformants on SD/–Leu/–Trp medium for selection of DNA-BD and AD vectors containing transformants. This selection step provides an initial phase of growth that maximizes plasmid copy number, which results in greater quantities of fusion proteins. This, in turn improves the chances of detecting AD fusion proteins that interact weakly or transiently with the bait. The amplified yeast cotransformants were plated at high density on medium-stringency medium, SD/–Leu/–Trp/–His, to screen for *HIS3* expression. To eliminate false-positives, growing colonies were further screened on high-stringency medium SD/–Leu/–Trp/–His/–Ade/X- α -Gal. α -Galactosidase activity (*MEL1* reporter gene) was confirmed by a liquid α -Gal assay.

An interaction between OTC and CK was detected when OTC from *P. furiosus* was fused to the GAL4 DNA-binding domain and CK was fused to the GAL4 activation domain (Table 1). Conversely, with CK as the BD fusion partner and OTC as the AD fusion partner, the interaction between the enzymes was less clear (Table 1). The strain containing the BD-CK/AD-OTC and BD-CK/AD-CK hybrids clumped in liquid culture and grew more slowly than the other hybrid strains. Also colonies on plates had an irregular appearance and the BD-CK/AD-OTC hybrid produced only light blue colonies on X- α -Gal indicator plates. It is common to detect an interaction between two proteins, A and B for example, with A as the binding domain fusion partner, but not with A as the activation domain fusion partner (McAlister-Henn et al. 1999). For the BD-OTC/AD-OTC hybrid activation of all the reporter genes was observed, whereas for the BD-CK/AD-CK hybrid no

growth on high-stringency medium was observed. This indicates that the chimeric BD-CK construct is less stable or that the BD-CK and AD-CK hybrids are incapable of association in *S. cerevisiae*. The results obtained with the yeast two-hybrid system demonstrated the interaction between OTC and CK from *P. furiosus* in vivo and thus confirmed earlier results from affinity electrophoresis and co-immunoprecipitation. The two-hybrid system is a promising method, because it allows the molecular characterization of the interaction by analysis of mutants. Because ATC is expressed from the bicistronic *pyrBI* operon, it could not be used in a yeast two-hybrid screen.

Chromatography

The genes *pyrB* and *pyrI* from the hyperthermophilic archaeon *P. furiosus*, which code, respectively, for the catalytic and regulatory chains of ATC, have been expressed at high levels in *E. coli* and the enzyme purified (see Materials and methods). The purified quantity of ATC holoenzyme made chromatographic techniques to study its interaction with CK feasible.

An overview of several chromatographic methods to study protein–protein interactions was given by Beeckmans (1999). In small zone gel filtration, a protein–ligand complex, if the interaction is stable enough, will elute earlier than either protein alone. When the complex is dynamic and equilibrates within a time scale faster than or comparable to the running time, the equilibrium filtration method of Hummel-Dreyer (Hummel and Dreyer 1962) proves to be useful in the detection and characterization of interactions between two proteins.

Small zone gel filtration.

Samples of 100 μ l were injected onto a Superose 12 (APB) column equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM KCl. The elution profile of each enzyme was obtained by loading samples of 50 μ M CK, 16.7 μ M ATC and 8.3 μ M OTC. The enzymes CK, ATC and OTC eluted at 12.6 ± 0.4 , 10.5 ± 0.4 and 10.2 ± 0.4 ml, respectively. A mixture of CK with either

Figure 1. Small zone gel filtration chromatography. (a) Elution of a mixture of 50 μ M carbamate kinase (CK) (12.3 ml) and 8.3 μ M ornithine carbamoyl phosphate (OTC) (9.8 ml). (b) Elution of a mixture of 50 μ M CK (12.7 ml) and 16.7 μ M aspartate carbamoyltransferase (ATC), c6r6 holoenzyme, c3 catalytic trimer (10.5 ml). Samples of 100 μ l were injected on a Superose 12 (APB) column equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM KCl.

OTC or ATC was applied to the gel filtration column. Purification of CK, OTC and ATC on a Superose 12 gel filtration column gave well-resolved peaks. The elution volumes of CK and OTC or CK and ATC when applied to the column at the same time were the same as when they were applied separately. No peak corresponding to a CK-OTC or CK-ATC complex could be detected by size exclusion chromatography (Figure 1), in accordance with the results obtained for cell-free extracts of *P. abyssi* (Purcarea et al. 1999). These results confirm that CK-OTC and CK-ATC complex formation must be transient or dynamic.

Hummel-Dreyer equilibrium gel filtration

Direct physical interaction between *Pyrococcus* CK and ATC has not been demonstrated. We used the equilibrium chromatography method of Hummel and Dreyer to obtain evidence for such an interaction (Figure 2). In a typical experiment, the

Superose 12 gel filtration column was equilibrated with buffer containing a uniform concentration of 1.7 μ M ATC. When a sample with 5 μ M CK added to the same buffer containing the same ATC concentration was injected on the gel filtration column, a trough was observed at the normal elution time of ATC (Figure 2a). When ATC binding to CK occurs, the amount of free ATC in the applied sample is reduced by the amount bound to CK. A maximum trough was observed when the loaded sample contained no ATC (Figure 2c). No trough was observed when CK was absent from the applied sample. Similarly, when a non-interacting protein such as bovine serum albumin instead of CK was added to the sample, no trough was observed.

By varying the ATC concentration in the presence of 5 μ M (Figures 2 and 3) and 10 μ M CK (Figure 3) in the sample (while maintaining a constant concentration of 1.7 μ M ATC in the buffer) and quantifying the peak area at the ATC elution position, the dissociation constant of the complex was estimated as described by Gegner and Dahlquist (1991). The point at which there is no peak or trough is determined by plotting the trough area against the concentration of ATC in the sample (Figure 3). At this point (approximately 2.2 μ M ATC for 5 μ M CK and 2.8 μ M ATC for 10 μ M CK), the free ATC concentration equals the ATC concentration in the column buffer. When one assumes an equimolar binding between ATC and CK, the dissociation constant corresponds to approximately 16 μ M for the 5 μ M CK series and 14 μ M for the 10 μ M CK series. These preliminary results suggest that CK and ATC interact physically to form a CP channeling complex, with a dissociation constant of the order of 10 to 20 μ M. Further investigation is required to determine the stoichiometry of the interaction and to calculate the dissociation constant, more reliably. The same experiments could be performed with OTC and CK. However, at present, the limited amounts of purified CK and OTC preclude extensive chromatographic experiments.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measures binding interactions by detecting the heat absorbed or released during a binding reaction (i.e. the binding enthalpy change) (Wiseman

Figure 2. Hummel-Dreyer equilibrium gel filtration. A Superose 12 column was equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl buffer containing 1.7 μ M aspartate carbamoyltransferase (ATC). Samples containing 5 μ M carbamate kinase (CK) and various ATC concentrations were applied to the column: (a) 1.7 μ M ATC, (b) 0.9 μ M ATC, and (c) no ATC.

Figure 3. Plot of a series of Hummel-Dreyer protein elution profiles. Trough or peak area was plotted against the aspartate carbamoyl-transferase (ATC) concentration. A Superose 12 column was equilibrated with 1.7 μM ATC in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. Samples containing 5 ($\blacklozenge$) and 10 μM carbamate kinase (CK) ($\blacksquare$) and various ATC concentrations (0, 0.9 and 1.7 μM ATC with 5 μM carbamate kinase (CK), and 0, 1.7, 2.2 and 2.6 μM ATC with 10 μM CK were applied to the column.

Figure 4. Isothermal titration calorimetry: (a) 94 μM carbamate kinase (CK) titrated into a solution of 10 μM ornithine carbamoyl transferase (OTC) in macromolecular crowding conditions, 20 mM phosphate buffer with 7.5% PEG_{12,000} at 55 °C; (b) 94 μM CK titrated into 20 mM phosphate buffer with 7.5% PEG_{12,000} at 55 °C.

et al. 1989, Doyle 1997, Pierce et al. 1999, Leavitt and Freire 2001). At present, ITC is probably the most quantitative means available for measuring the thermodynamic properties of protein-protein interactions.

In such ITC experiments, CK was titrated into a solution of OTC or ATC. When 94 μM CK was titrated into a 2 μM OTC solution in 20 mM potassium phosphate buffer, pH 7.4, at 55 °C, no specific heat exchange was observed. On the other hand, the ITC profile of 94 μM CK titrated into a solution of 10 μM OTC in macromolecular crowding conditions, 20 mM phosphate buffer with 7.5% PEG_{12,000} and at 55 °C, showed a profile characteristic for a binding reaction (Figure 4a). Control experiments performed by making identical injections of CK into a cell containing buffer with no OTC showed insignificant heats of dilution, except for the first injection (Figure 4b). These results indicate that the interaction between CK and OTC from *P. furiosus* is measurable with ITC in macromolecular crowding conditions.

The characteristic binding profile was not observed in similar experiments with CK titrated into a solution of ATC. A solution of 144 μM CK was titrated into a solution of 39 μM ATC at 50 °C, in 20 mM Tris-HCl, pH 7.75, with or without 5% PEG_{12,000}. This result means that, in these conditions, the interaction between CK and ATC is probably too weak to be detected by ITC.

Discussion

The first evidence for a physical interaction between CK and OTC of the hyperthermophile *P. furiosus* was obtained by affinity electrophoresis and co-immunoprecipitation (Massant et al. 2002). Here, we show that the stepwise screening of two-hybrids in yeast from low to high stringency medium allowed the detection of the *in vivo* interaction between CK and OTC, which was thought to be weak or transient. The interaction between CK and OTC was detected when OTC was fused to the DNA-BD and CK to the AD. However, with a CK-BD fusion and an OTC-AD fusion hybrid, the interaction between the two enzymes was less clear. Results with the yeast two-hybrid system thus confirmed the evidence obtained by affinity electrophoresis and co-immunoprecipitation and provide an *in vivo* system for molecular characterization of the complex. Deletions could be engineered in the OTC or CK encoding genes to identify a minimal domain for interaction, and point mutations could be assayed to identify specific amino acid residues critical for the interaction (Chien et al. 1991, Li and Fields 1993). Because *P. furiosus* ATC is expressed from the bicistronic *pyrBI* operon, it could not be used in a yeast two-hybrid screen.

Kinetic and thermodynamic constants of the enzyme-enzyme interactions involved in the formation of the CP channeling complex in *P. furiosus* may help explain the mechanism and function of metabolic channeling in a hyperthermophilic cell. We used Hummel-Dreyer equilibrium gel filtration and isothermal titration calorimetry in a first attempt to characterize the enzyme-enzyme interactions kinetically and thermo-

dynamically. In combination with structural information, the study of the energetics of binding can provide a complete description of the interaction and can help in identifying the most important regions of the interface and the energetic contributions.

In the Hummel-Dreyer method of equilibrium gel filtration, both the gel filtration buffer and the sample contained ATC at the same concentration, but only the sample contained CK. Elution of CK through such a column caused a trough in ATC concentration representing ATC that had been removed in the binding. In these experiments, ATC was used to equilibrate the column because this enzyme was available in large quantities (several tens of milligrams are required per experiment). The results from the Hummel-Dreyer chromatography experiments suggest that CK and ATC, assuming an equimolar binding ratio, form a complex with a dissociation constant in the order of 10 to 20 μ M. The influence of CP (and other substrates) on complex formation is worth further investigation. Using Hummel-Dreyer equilibrium chromatography, Yong et al. (1993) found a metabolite modulated enzyme–enzyme interaction: the reversible association between lactate dehydrogenase and α -glycerol-3-phosphate dehydrogenase was shown to occur over a limited range of NADH concentrations.

Like gel filtration experiments, ITC characterizes binding interactions with native unmodified forms of proteins in solution. Titration results indicate that the interaction between CK and OTC is detectable in macromolecular crowding conditions, but not the interaction between CK and ATC, at least under our conditions. Further titration experiments should be performed to determine a binding constant and to analyze the influence of the macromolecular crowding agent. We have yet to perform these titrations because, at present, we have insufficient protein for these experiments.

One of the greatest challenges to structural biologists is unraveling transient interactions of protein complexes. So far co-crystallizations of CK and OTC or CK and ATC have been performed without success (Massant 2004). Based on the structures of CK and OTC, a mode of association that could allow channeling of CP has been suggested by Ramón-Maiques et al. (2000). Corresponding to the relative sizes and shapes of both enzymes, it has been proposed that the formation of the channeling complex is nucleated by an OTC dodecamer associating with six CK dimers in such a way that the molecular two-fold symmetry axes coincide.

Docking simulations with the structures of the individual enzymes have been initiated with the MolFit program (Katchalski-Katzir et al. 1992, Ben-Zeev et al. 2003) in collaboration with Miriam Eisenstein from the Weizmann Institute, Israel (results not shown; Massant 2004). These docking simulations did not provide evidence for the kind of complex formation suggested by Ramón-Maiques et al. (2000). Neither did they provide a clear alternative for the structure of the channeling complex. Because docking simulations take no account of conformational changes or flexibility, they provide only rough approximations. In particular, the SMG-loop of OTC can change conformation upon substrate binding.

Electrostatic isopotential contours of the OTC catalytic trimer and the CK dimer, as calculated with the Delphi program (Figure 5) and the preliminary indications obtained by *in silico* docking, suggest the possibility of the formation of a CP-channeling complex guided by electrostatic forces. A role for electrostatics was also suggested by the co-immunoprecipitation and affinity electrophoresis experiments (Massant et al. 2002).

Besides guiding the formation of the channeling complex, electrostatic forces may play a role in guiding the negatively charged CP molecule between the active sites of CK and OTC. An analogous situation has been described for the channeling of oxaloacetate in pig mitochondria (Vélot et al. 1997). Many questions about the formation and the structure of the CK-OTC CP channeling complex remain to be answered.

A feature of particular interest in the present system is that the two carbamoyltransferases, OTC and ATC, have very different quaternary structures. Although both enzymes contain a similar trimeric catalytic subunit, they differ in their oligomeric construction. Although most mesophilic OTCs are trimeric, the hyperthermophilic protein is dodecameric, composed of four catalytic trimers arranged in a tetrahedral manner (see Villeret et al. 1998 and Massant et al. 2003 for further refinement to a resolution of 1.87 Å). The ATCs from

Figure 5. (a) The ornithine carbamoyltransferase (OTC) catalytic trimer and (b) the carbamate kinase (CK) dimer. Blue areas on the molecular surface represent electrostatically positive regions and red areas electrostatically negative regions. Circles indicate one of the CP binding sites of OTC (a) and CK (b). Electrostatic isopotential contours around the OTC trimer (c) and CK dimer (d), viewed from the same position as in (a) and (b). The blue surface connects all points having a potential of +1.0 kT. The red surface connects all points having a potential of -1.0 kT. Electrostatic potentials were calculated and displayed with the DelPhi program.

P. furiosus and *P. abyssi* are also dodecamers, but are composed of three regulatory dimers and two catalytic trimers, similar to *E. coli* ATC (Lipscomb 1994, Purcarea 2001, Van Boxstael et al. 2003).

In conclusion, the present work provides additional concrete evidence for physical interactions between CK and OTC, the first direct evidence for an interaction between CK and ATC, and a first rough estimate of the binding constant between CK and ATC. It also provides an operational framework for a more detailed study of this model system, keeping in mind that the unraveling of these interactions will have to show how the same enzyme (CK) can interact with two carbamoyltransferases of quite different quaternary structures. Interactions in multienzyme complexes may have played an important role in molecular evolution in general (McConkey 1982) and in adaptation to thermophily in particular.

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