Experimental Evolution of Enzyme Temperature Activity Profile: Selection In Vivo and Characterization of Low-Temperature-Adapted Mutants of *Pyrococcus furiosus* Ornithine Carbamoyltransferase

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We have obtained mutants of *Pyrococcus furiosus* ornithine carbamoyltransferase active at low temperatures by selecting for complementation of an appropriate yeast mutant after in vivo mutagenesis. The mutants were double ones, still complementing at 15°C, a temperature already in the psychrophilic range. Their kinetic analysis is reported.

Thermodynamics predicts that substantial increases in the thermostability of any enzyme may require only a small number of structural changes (9). However, in most cases it remains difficult to predict which modification will increase stability while keeping the enzyme functional, since no general strategy for thermophilic adaptation appears to exist (10, 22). The same uncertainty prevails for the converse adaptation, psychrophily. Data about cold-active enzymes are still limited but it already appears that efficient catalysis at low temperature may rely on different strategies which are not simply the mirror image of adaptation to heat (4, 7, 21). From the biological point of view the most relevant approach to bypass the problems raised by engineering alterations of the enzyme temperature profile in vitro is experimental evolution, where selection of phenotypes (and not screening) is combined with natural mechanisms of mutagenesis.

Few examples of in vivo selection for a modified enzyme temperature profile have been reported. In most cases, thermostable variants of a mesophilic enzyme were selected in a thermophilic host (1, 8, 16, 23). The selection in a mesophilic host (*Escherichia coli*) for variants of a thermophilic enzyme becoming more active at lower temperatures was described only recently (17). In vivo selection of such mutants may become useful to modify enzymes but mainly, from an evolutionary perspective, to understand how readily and in which ways existing structures can adapt to lower temperatures.

We report the selection of modified ornithine carbamoyltransferase (OTCase) from *Pyrococcus furiosus* (an archaeon with a maximal growth rate at 102°C), which complements a null OTCase mutant of the *Saccharomyces cerevisiae* strain 12S16 (α *ura3* Δ *arg3* *leu2*) at 30 and even 15°C, achieving in one step the largest temperature jump presently obtained by experimental evolution.

OTCase catalyzes the conversion of ornithine and car-

bamoylphosphate into citrulline and inorganic phosphate in arginine biosynthesis. *P. furiosus* catalyzes citrulline synthesis about 35 times faster at 100 than at 30°C (14). The enzyme is a homododecamer, a key feature of its thermostability (24). The corresponding gene, *argF* (20), was amplified by PCR using primers (5'-GCGAATTCATGGTAGTTAGCTTGG C-3' and 5'-GCGAATTCCTAAAGAATAGAGGGTG-3') with extensions carrying *Eco*RI and *Sal*I recognition sites, respectively, and was inserted in two monocopy *E. coli*/*S. cerevisiae* shuttle expression vectors (R&D Systems): pYX112 (with the strong constitutive triose phosphate isomerase promoter) and pYX111 (with the weak constitutive promoter 786). The constructs were made in *E. coli* and transformed into yeast strain 12S16. Complementation of the Δ *arg3* mutation was observed at 40, 30, and 15°C with pYX112 but not with pYX111, which remained negative at all three temperatures, thus providing a suitable material for selection of cold-active *P. furiosus* OTCase mutants.

The pYX111 *argF* plasmid was transformed into the *E. coli* XL1-Red mutator strain (*mutS mutT mutD*), deficient in three primary DNA repair pathways (Stratagene). Plasmid DNA was recovered from a pool of about 30,000 colonies. Fragments bearing only the *argF* gene were separated from mutations in vector DNA by recloning in pYX111, and the resulting library was used to select for 12S16 transformants growing at 30°C in the absence of arginine. Two double mutants (dm1 and dm2) were found to complement the yeast Δ *arg3* strain: TAC to TGC plus GAG to GGG for dm1 (i.e., Y227C plus E277G) and GCT to GAT plus GAG to GGG for dm2 (i.e., A240D plus E277G). They thus share the E277G substitution. All three mutations affect the ornithine binding domain of the OTCase monomer (Fig. 1). The three single mutants still complemented 12S16, though to a lesser extent (see serial dilution tests displayed in Fig. 2). Interestingly, complementation still occurred at 15°C. In liquid minimal medium without arginine, generation times at 30°C were about 6 h for the double mutants and 10 h for the single mutants, compared with 2 h for strain 12S16 in the presence of arginine.

High levels of enzyme were achieved by inserting the cognate genes into pYX213, which contained the inducible *gal*I

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FIG. 1. Structural representation of the *P. furiosus* OTCase monomer. The amino acids that have been replaced in the low-temperature-active mutants are indicated.

promoter. OTCase was purified (using successively a monoQ and an arginine-Sepharose column [Pharmacia] [14]) from 2-liter cultures grown in the presence of 2% galactose. From a calibrated Superose P12 HR 10/30 column (Pharmacia), the double mutant enzymes eluted at the same position as the wild-type (WT) OTCase, corresponding to a molecular mass of 400 ± 20 kDa.

The effect of temperature on citrulline synthesis was studied with purified enzyme between 22 and 55°C (Fig. 3), with CP thermolability (13) preventing assays at higher temperatures. Activation energies were, respectively, 42 kJ/mol for mutant Y227C + E277G, 57 kJ/mol for mutant A240D + E277G, and 48 kJ/mol for WT *P. furiosus* OTCase (as for OTCase purified from *P. furiosus* cells [15]).

The $K_{m_{app}}$ for carbamoylphosphate was similar in WT and mutated *P. furiosus* OTCases (about 0.1 mM). For ornithine, however, the affinity varied considerably among mutant OTCases at 55°C (Table 1). Mutation Y227C had only a marginal influence, whereas mutations A240D and E277G resulted in a 10- to 14-fold increase in $K_{m_{app}}$ compared to WT OTCase. In dm2, where both mutations are present, the effect was much more pronounced.

At 30°C, the turnover number (k_{cat}) of the double mutants was about six times higher than for the wild type (eight times higher at 55°C) and for the single mutants, 1.5-fold higher (two to three times higher at 55°C). This explains why only the double mutants were initially selected. Catalytic efficiencies (k_{cat}/K_m) at 55°C decreased 2- to 15-fold, mainly due to the increase in the respective K_m values. Interestingly, higher catalytic efficiencies were observed at 30 than at 55°C for both double mutants (Table 1).

Efficient catalysis at low temperature would appear to require enzymes with a higher k_{cat} and/or k_{cat}/K_m ratio than their

mesophilic or thermophilic homologues in order to compensate for the reduction of reaction rates at low temperature (4, 7). However, it may be difficult to both increase the k_{cat} and decrease the K_m of an enzyme. Moreover, an increase in K_m may actually lower the activation energy of the reaction (4). One may thus expect some enzymes to have adopted this strategy when it was compatible with the requirements of metabolism, as in the present case, when forced accumulation of the metabolic precursor ornithine allows the selection to play mainly on the k_{cat} .

An increase in enzyme activity at low temperature is expected to result from an increase of flexibility, at least at the active site if not in the whole structure. Indeed, the frequent (but not universal [3]) thermolability of cold-adapted enzymes is regarded as a consequence of their flexibility. Double mutants dm1 and dm2 show a dramatic increase in OTCase thermolability (Table 1). The substitutions reported here are located in the ornithine-binding domain of the OTCase monomer (Fig. 1) (15, 24). Glutamic acid 277, which is substituted for in both double mutants, is highly conserved among OTCases (11). This residue interacts with the peptide backbone of histidine 273 by a hydrogen bond. H273 itself lies in the close vicinity of the tetrad H268CLP, which together with the triad SMG defines the ornithine-binding domain of OTCases (25). The absence of this H bond in the E277G mutant may increase the flexibility of the HCLP configuration. This could be the basis of the observed increase in $K_{m_{app}}$ Orn and an important feature of adaptation to lower temperatures.

In wild-type OTCase Y227 probably forms an internal aromatic interaction with Y159 since both phenyl ring planes are perpendicular and separated by 4.5 Å (24). A reduction in aromatic interactions may be related to protein adaptation at low temperatures (2). Therefore, the Y227C substitution could

FIG. 2. Effects of mutations on cell growth. Serial tenfold dilutions of yeast cells were plated on minimal medium with or without arginine and grown at 30°C for 3 days or at 15°C for 7 days.

FIG. 3. Temperature dependence of *P. furiosus* OTCase dm1 and dm2 compared to the WT. Open square ($\square$), WT; full square ($\blacksquare$), dm1; triangle ($\blacktriangle$), dm2.

TABLE 1. Kinetic behavior and stability of WT and mutant OTCases

Temperature	Parameter	WT	dm1 (Y227C+E277G)	dm2 (A240D+E277G)	m1 (Y227C)	m2 (A240D)	m3 (E277G)
55°C	K_{mapp} Orn (mM)	0.1	1.6	13	0.3	1.0	1.4
	k_{cat} (s^{-1})	500	3,500	4,300	1,100	1,100	1,600
	k_{cat}/K_m^a	5,000	2,200	330	3,700	1,100	1,140
30°C	K_{mapp} Orn (mM)	0.1	0.8	2	0.2	0.5	0.5
	k_{cat} (s^{-1})	370	2,200	2,900	560	560	560
	k_{cat}/K_m^a	3,700	2,750	1,450	2,800	1,120	1,120
75°C	$t_{1/2}^b$	>10 h	1 min	14 min	45 min	>10 h	10 min
95°C	$t_{1/2}^b$	8 min ^c				16 min	

^a Catalytic efficiency.^b Measure of resistance to irreversible heat denaturation.^c Measurements were done with 0.15 μ g of pure enzyme in piperazine-*N,N'*-bis(2-ethanesulfonic acid)(PIPES) buffer. Earlier experiments (14) gave a value of 20 to 40 min at 100°C but were done with a 20 times higher enzyme concentration and in Tris buffer.

partly destabilize OTCase. Moreover, the shorter side chain of the cysteine residue introduces a cavity within the core of the protein, which could also lower enzyme stability by increasing flexibility (19). Note that a shortening of side chain is also observed in the E277G substitution.

Mutation A240D is less readily interpretable without further studies. Indeed, it slightly increases the thermoresistance of OTCase and, when present in dm2 (A240D plus E277G), enhances the thermoresistance of the enzyme with respect to mutation E277G alone. A240, a nonconserved residue, is situated in a turn exposed to the solvent (24); the substitution gives an additional acidic surface residue. This could improve solvent interactions which, as in class C β -lactamase from *Psychrobacter immobilis* A5 (5), appear to be a determinant of flexibility. On the other hand, the negative charge of the aspartate residue could result in a stabilizing electrostatic interaction with R244, which in the wild-type enzyme interacts with E276, in the close vicinity of the residues involved in ornithine binding (24). Consequently, ornithine binding could be affected in this mutant and the new electrostatic interaction could increase thermoresistance. Thus in the case of A240D, a possible local gain of flexibility by an increased interaction with the solvent would appear compatible with an increase of thermoresistance. Narinx and coworkers also reported a mutant of a cold-active subtilisin which, besides improved specific activity at low temperatures, displayed increased stability as well (18). A twofold increase in low temperature catalysis of *P. furiosus* β -glucosidase CelB was also obtained without loss of stability (12). Interestingly, the two single mutants Y227C and A240D differ dramatically in thermostability despite their similar kinetic behavior. Catalytic activity and resistance to thermodenaturation can thus be separated in *P. furiosus* OTCase, implying that they can involve different regions of the protein. Indeed, the two residues are located in distinct parts of the protein, Y227 being in the interior and A240 at the surface of the enzyme.

The very possibility of modifying so extensively the temperature activity profile of an enzyme by only two mutations is interesting from the point of view of evolution. Moreover, if adverse temperature could, like other kinds of physiological disturbances (6), transiently increase the rate of mutation, it would become conceivable that a succession of moderate temperature stresses could lead to the accumulation of mutations in a number of critical, limiting steps at a rate sufficient to bring

global adaptation to another temperature range within closer reach than generally assumed.

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