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Catalytic Efficiency and Some Structural Properties of Cold-Active Protein-Tyrosine-Phosphatase

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A procedure was established for expression and purification of abundant recombinant cold-active protein-tyrosine-phosphatase (RCPTPase), which showed identical enzymatic characteristics to the native enzyme (NCPTPase). The purified RCPTPase showed high catalytic activity at low temperature and maximal activity at 30°C. RCPTPase has a thermodynamic characteristic in that its activation enthalpy was determined to be low, 4.3 kcal/mol, at temperatures below 19.3°C, where the Arrhenius relationship exhibited an inflection point, in comparison with 20.3 kcal/mol above 19.3°C. Also, the thermostability, ΔG_{water}, of the catalytic site in the RCPTPase molecule was increased with a decrease in temperature. It was considered that cold-active protein-tyrosine-phosphatase could maintain its catalytic site in a stable conformation for eliciting high catalytic activity with low activation enthalpy at low temperature.

Key words: catalytic efficiency, cold-active enzyme, kinetics, protein-tyrosine-phosphatase, thermodynamics.

Abbreviations: E_a, activation energy; NCPTPase, native cold-active protein-tyrosine-phosphatase; pNPP, *p*-nitrophenyl-phosphate; PTPase, protein-tyrosine-phosphatase; RCPTPase, recombinant cold-active protein-tyrosine-phosphatase.

Psychrophilic microorganisms and some ectothermic organisms produce enzymes exhibiting high catalytic activity in a low-temperature environment, so-called "cold-active enzymes". Cold-active enzymes show thermal lability (*1–3*), which is presumably attributed to their flexible structures (*4*). It has been suggested from the three-dimensional structures of several cold-active enzymes determined by the molecular modeling method (*5–7*) and X-ray crystallography (*8*, *9*) that this flexibility might be caused by reduction of some structural factors: a low content of proline residues in loops and turns, a decrease in the number of internal electrostatic interactions mediated by arginine residues, and exposure of non-polar groups to the solvent. It remains unknown how cold-active enzymes show high catalytic activity at low temperature.

We previously isolated cold-active PTPase [EC 3.1.3.48] from a psychrophile, *Shewanella* sp., in order to elucidate the mechanism by which cold-active enzymes elicit high catalytic activity at low temperature (*10*). The enzyme is a novel PTPase with histidine as its catalytic residue (*11*) and a conserved amino acid sequence that is observed in many protein-Ser/Thr-phosphatases (*12*). Owing to the low purification yields of this enzyme from psychrophiles, a recombinant enzyme (*gcRCPTP*), expressed in *Escherichia coli* as a fusion enzyme containing glutathione-*S*-transferase, with subsequent elimination of the GST moiety, was obtained as an experimental material (*12*). However, this gcRCPTP was not well suited for investigation of the structural properties responsible for the enzymatic characteristics of a cold-active enzyme because the optimal temperature of this gcRCPTP was higher than that of the native enzyme. Therefore, another expression system for a recombinant enzyme with molecular characteristics identical to the native enzyme is considered essential for further investigation of the structural properties of cold-active PTPase.

In this paper, we describe the establishment of a suitable expression system and a purification procedure for a recombinant cold-active PTPase, and the structural properties of this enzyme that are involved in the high catalytic efficiency at low temperature. We will discuss several adaptation mechanisms of cold-active enzymes to a low-temperature environment.

MATERIALS AND METHODS

Materials—Restriction enzymes were purchased from New England Biolabs (MA, USA). An Original TA cloning kit containing plasmid pCR 2.1 was obtained from Invitrogen (Carlsbad, CA, USA). *AmpliTaq GOLD* DNA polymerase was from Applied Biosystems (Tokyo). The SequiTherm Excel™ Long-Read™ DNA Sequencing Kit and IRD41 infrared dye–labeled primers (M13 forward- and reverse-primers) were from Aloka (Tokyo). Expression plasmids, pET16b and pET22b, and *E. coli* AD494(DE3) were from Novagen (Madison, WI, USA).

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Q-Sepharose Fast Flow, the Mono-Q HR5/5 column, pTrc99A, and pKK223–3 were from Amersham Pharmacia Biotech (Buckinghamshine, England). Specially prepared grade-urea was from Nacalai Tesque (Kyoto). Other chemicals used were of analytical grade. Synthetic oligonucleotides were designed for PCR; NPI: 5′-ggatccatgggcAATACTGCAACTGAGTTTGAT-3′, and CPI: 5′-cctcgagTTGCTTACTATCTAGCTTA-3′, corresponding to the N-terminal and C-terminal regions of the cold-active PTPase, respectively. Small letters represent the designed cleavage sites for restriction enzymes.

Expression Systems—For construction of expression plasmids of the recombinant enzyme, the gene encoding the enzyme was amplified with 100 pmol of NPI- and CPI-oligonucleotide primers, 10 ng of the psychrophilic-genomic DNA as a template, and 2.5 units of *AmpliTaq GOLD* DNA polymerase in 100 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2.5 mM $MgCl_2$, and four deoxynucleotides at 0.2 mM each. The amplification conditions were 30 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C after initial denaturation for 10 min at 94°C, followed by a final 10-min incubation at 72°C. The PCR product (about 1.0 kbp) was subcloned into pCR 2.1 with the Original TA cloning kit. On determination of the nucleotide sequence, the inserted DNA was identified as the cold-active PTPase gene. After digestion of the plasmid with *Nco*I and *Xho*I for pET16b and pET22b, *Nco*I and *Eco*RI for pTrc99A, and *Eco*RI for pKK223–3, the DNA fragment, *i.e.* the cold-active PTPase gene, was inserted into the respective plasmid. With regard to the expression plasmid (*pTCPTP*) derived from pET22b, the cold-active PTPase gene was inserted on the 3′-side of the *pel*B signal sequence in order to transfer RCPTPase into the periplasmic space of *E. coli*. The sequence encoding His-Tag was removed from the open reading frame. The constructed expression plasmid was introduced into *E. coli* AD494 (DE3).

For expression of the recombinant enzyme, the transformed cells were inoculated into 5 liters of LB medium [Luria-Bertanis broth: 1.0 % (w/v) trypton, 0.5 % (w/v) yeast extract, and 1.0 % (w/v) NaCl, pH 7.5] containing 500 μg of ampicillin. After incubation for 5 h at 37°C, expression of the recombinant enzyme was induced by the addition of 1.0 mM (final concentration) isopropyl-β-D-thiogalactopyranoside. Then, the induced culture was incubated for 48 h at 15°C.

Purification of the Recombinant Enzyme—Purification of the recombinant enzyme was conducted at 4°C. Transformed cells (wet weight; 15.0 g) from a 5-liter fermentation were frozen at –30°C, thawed, and then suspended in 20 ml of buffer A [25 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 1 M sorbitol, and 1 mM diisopropylfluorophosphate] in order to extract proteins in the periplasmic space of *E. coli*. After incubation for 10 min at 20°C with stirring, the suspension was centrifuged at 11,500 ×*g* for 15 min. The supernatant was applied to a Q-Sepharose FF column (ϕ2.6 × 10 cm) equilibrated with buffer B [25 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$ and 0.5 M sorbitol]. After washing with buffer B, the adsorbed materials were eluted with a linear gradient of 0 to 0.3 M NaCl in buffer B. The active fractions appearing with 0.08 to 0.16 M NaCl were dialyzed against buffer C [10 mM sodium-phosphate (pH 6.8) containing 5 mM $MgCl_2$ and 0.5 M sorbitol], and then applied to a hydroxylapatite column (ϕ1.0 × 10 cm). The adsorbed proteins were eluted with a linear gradient of 10 to 250 mM sodium phosphate in buffer C. The active fractions appearing with 50 to 150 mM sodium-phosphate were dialyzed against buffer B, and then applied to a Mono-Q HR 5/5 column (ϕ5 × 50 mm). The adsorbed proteins were eluted with a linear gradient of 0 to 0.2 M NaCl in buffer B. The active fraction eluted with 0.11 to 0.12 M NaCl was stored at –30°C until use, after the addition of an equivalent volume of ethyleneglycol.

Determination of Phosphatase Activity—Phosphatase activity was determined, at each purification step, in a 100 μl reaction mixture comprising 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 5.0 mM pNPP, and the desired amount of enzyme, pH 7.8. After incubation at 25°C for 3 min, during which the reaction proceeded linearly, 1.0 ml of 0.1 N NaOH was added to stop the reaction, and then the absorbance at 410 nm was measured. The product of the enzyme reaction was quantitated by means of a calibration curve obtained with *p*-nitrophenol. One unit of phosphatase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of pNPP in 1 min at 25°C and pH 7.8. When the characteristics of the recombinant enzyme were examined, the activity was determined in 50 mM MES-NaOH, 2.5 mM $MgCl_2$, 5.0 mM pNPP, and the desired amount of enzyme, pH 6.0, at 30°C.

Determination of the Nucleotide Sequence—The nucleotide sequence was determined by the cycle sequencing method using the SequiTherm Excel™ Long-Read™ DNA Sequencing Kit-LC and IRD41 infrared dye-labeled primers (M13 forward- and reverse-primers) with a LI-COR 4100L DNA sequencer. Analysis and translation of the obtained sequence were performed using the Genetyx Mac 7.3 software package (Software Development).

Determination of the N-Terminal Amino Acid Sequence—The amino acid sequence was determined by automated Edman degradation using a Shimadzu PPSQ-10 protein sequencer.

Treatment of the Enzyme with Urea—After the addition of 5 μl of the enzyme solution to 45 μl of solutions containing various concentrations of urea, the resulting mixtures consisting of 50 mM MES-NaOH (pH 6.0), 2.5 mM $MgCl_2$, 0–8.6 M urea and the recombinant enzyme (6.8 ng, 0.18 pmol) were incubated at varying temperatures for 1 h. When the remaining activity after treatment with urea was measured, the enzyme reaction was initiated by the addition of 50 μl of 50 mM MES-NaOH (pH 6.0) containing 2.5 mM $MgCl_2$ and 10 mM pNPP. The reaction was performed at the same temperatures as those used for the incubation with urea.

The fraction (f_I) of the inactivated enzyme was obtained using the relation $f_I = (y - y_N)/(y_I - y_N)$, where y, y_N, and y_I are the values for the activities of the enzyme treated with the respective concentrations of urea, the native enzyme, and the inactivated enzyme, respectively. The transition equilibrium between approximate inactivated (I) and native (N) states is defined as follows:

$$K_I = [I]/[N] = f_I/(1 - f_I) = \exp(-\Delta G_{denaturant}/RT),$$

where R, T, K_I, and $\Delta G_{denaturant}$ represent the gas constant, absolute temperature, equilibrium constant of the

Table 1. **Purification procedure for RCPTPase.**

Steps	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	-fold
Crude extract	247	93	0.78	100	1
Q-Sepharose	10.5	84.7	8.07	44.1	10.3
HAP	1.94	71.1	36.6	37.0	46.9
Mono-Q	0.45	33.0	73.3	17.1	94.0

inactivated/native enzyme species, and Gibbs free energy for inactivation with the indicated concentrations of urea, respectively.

The Gibbs free energy, ΔG_{water}, for inactivation at zero M urea was determined according to the equation proposed by Santoro and Bolen (*13*).

Determination of the Protein Concentration—The amount of RCPTPase was determined spectrophotometrically on the basis of the molecular absorption coefficient at 280 nm of NCPTPase, 7.75×10^4 cm^{-1}·mol^{-1}·liter (*10*).

SDS–PAGE—SDS–PAGE was performed using 15% gels as described (*14*). Proteins in a gel were visualized by Coomassie Brilliant Blue staining.

Circular Dichroism (CD) Measurements—CD spectra in the far-UV (250–200 nm) region were recorded on a Jasco J-720 spectropolarimeter. CD spectra of the purified RCPTPase (270 μg/ml) in 50 mM MES-NaOH (pH 6.0) containing 2.5 mM $MgCl_2$ were measured at 5°C and 12.5°C. Spectra were obtained 16 times at the scan rate of 50 nm/min, using a spectral bandwidth of 1 nm. The light path length of the cell used was 1 mm. The α-helix content (f_H) was calculated on the basis of the following equation; $f_H = -([\theta]_{222} + 2340)/30300$.

Estimation of Thermodynamic Parameters for the Enzyme Reaction—The changes in thermodynamic parameters, *i.e.*, activation free energy (ΔG), enthalpy (ΔH), and entropy (ΔS), during the transition from the ES-complex to an activated complex were calculated with the following relation as described (*15*),

$$\Delta G = RT \times [\ln(k_B \cdot T/h) - \ln(k_{cat})]$$
$$\Delta H = E_a - RT$$
$$\Delta S = (\Delta H - \Delta G)/T$$

where R is the gas constant (1.9872 cal·K^{-1}·mol^{-1}), k_B the Boltzmann constant (3.298×10^{-24} cal·K^{-1}), h the Planck constant (1.584×10^{-34} cal·s), T the absolute temperature, and E_a the activation energy.

The changes in the free energy (ΔG_{ES}), enthalpy (ΔH_{ES}), and entropy (ΔS_{ES}) between E + S and the ES-complex were calculated with the following relation,

$$\Delta G_{ES} = RT \cdot \ln(K_m)$$
$$\Delta G_{ES} = \Delta H_{ES} - T\Delta S_{ES}.$$

RESULTS AND DISCUSSION

Selection of Vectors for Expression of Recombinant Cold-Active PTPase—Efficient expression of the recombinant enzyme was examined in several plasmids, including pET16b, pTrc99A, pKK223–3, and pET22b. The optimized expression systems with the respective plasmids derived from pET22b, pET16b, and pTrc99A provided 2.5, 2.1, and 0.93 mg of recombinant enzyme in crude extracts of transformed *E. coli* after 5-liter fermentation. The respective weights of the expressed enzymes were estimated from the specific activity of the native enzyme (*10*). It could be concluded from the respective weights that the expression system involving the plasmid (*pTCPTP*) from pET22b was the most effective for obtaining abundant recombinant enzyme.

Purification of RCPTPase—The crude extract of *E.coli* AD494(DE3) transformed with pTCPTP was subjected to sequential column chromatographies, a peak showing phosphatase activity being yielded 0.11 to 0.12 M NaCl on Mono-Q HR5/5 column chromatography (data not shown). On Mono-Q column chromatography, other fractions (0.08 to 0.11 M NaCl) exhibiting phosphatase activity were obtained (data not shown), which showed maximal activity at 45°C unlike the NCPTPase (*10*), although this enzyme also exhibited high activity at low temperature. Furthermore, this phosphatase could not be obtained with good reproducibility in several experiments. These facts indicated that the enzyme eluted with 0.08 to 0.11 M NaCl was not a satisfactory material for this study.

On 5-liter fermentation, 0.45 mg of phosphatase with a specific activity of 73.3 units/mg was purified to homogeneity, as judged on SDS–PAGE (Fig. 1A and Table 1). The phosphatase showed maximal activity at 30°C and high catalytic activity in the low-temperature range of 0 to 15°C: 10.8 to 33.2% of the activity at the optimal temperature (Fig. 1B). The activity of the purified enzyme exhibited identical temperature dependence to that of NCPTPase (*10*). The N-terminal amino acid sequence of the enzyme protein was Met-Gly-Asn-Thr-Ala-Thr-Glu-

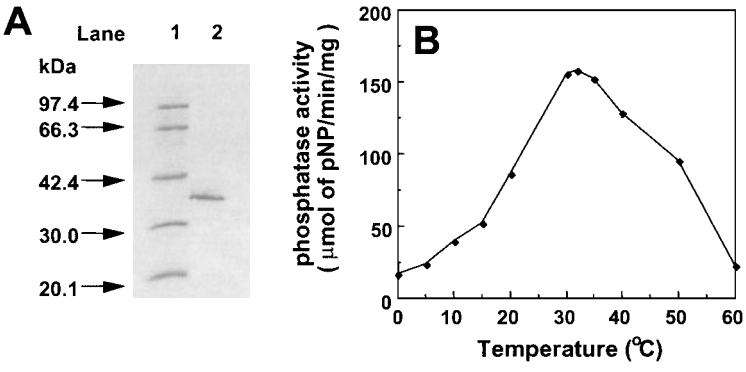

Fig. 1. **Homogeneity of the purified enzyme and temperature dependency of the enzyme activity.** A: The enzyme preparation was subjected to SDS–PAGE on a 15% gel. Lane 1, molecular weight markers. Lane 2, purified phosphatase (1.2 μg). B: The activity of the phosphatase at the indicated temperatures was determined as described under "MATERIALS AND METHODS".

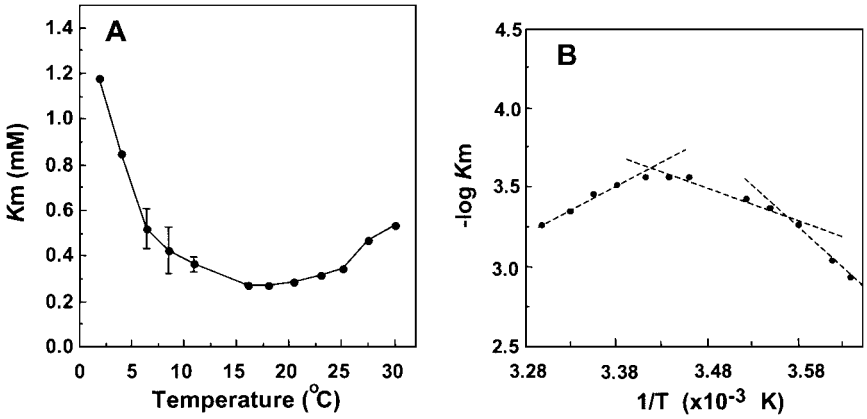

Fig. 2. **K_m values at various temperature and a van't Hoff plot of the K_m values.** A: The K_m values at the indicated temperatures were determined by means of Lineweaver-Burk double reciprocal plots. Data are means ± SE for three separate experiments. B: van't Hoff plot of K_m against the temperature range of 2 to 30°C. Dashed-lines represent theoretical ones.

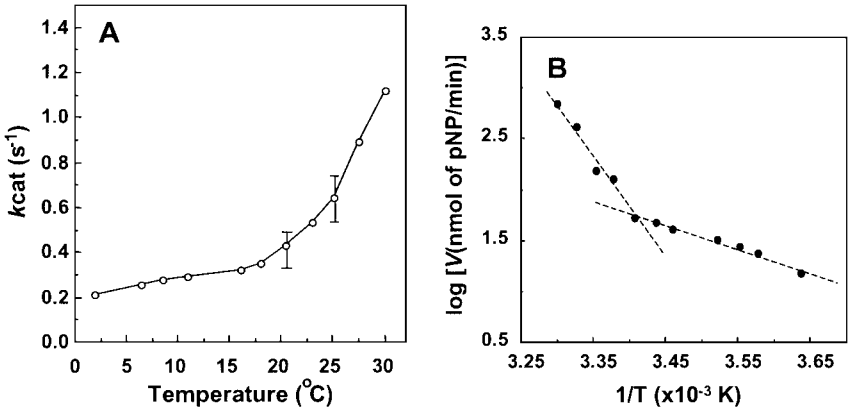

Fig. 3. **k_{cat} values at various temperatures and Arrhenius plots.** A: The k_{cat} values at the indicated temperatures were determined. Data are means ± SE for three separate experiments. B: The maximal velocity (V) of RCPTPase was measured at 2.5°C intervals over the range of 0 to 25°C. Data are means ± SE, which were smaller than the size of the indicated symbols, for three separate experiments. Dashed-lines represent theoretical ones.

Phe-Asp-Gly-. The sequence from Asn to Gly was also identical to that of NCPTPase (*10*). Two amino acids (Met-Gly) were derived from the cleavage site for *Nco*I endonuclease in the NPI-primer, which is required for insertion of the gene encoding the cold-active PTPase into the pET22b plasmid. Therefore, the enzyme eluted with 0.11 to 0.12 M NaCl was used as the recombinant cold-active PTPase (*RCPTPase*) in the following experiments.

Kinetic and Thermodynamic Parameters of RCPTPase—Since high catalytic activity is elicited in the low-temperature range, it followed that RCPTPase must have distinct feature(s) as to the catalytic process. The kinetic parameters, K_m and k_{cat}, of RCPTPase in the temperature range of 2 to 30°C were evaluated by means of Lineweaver-Burk double-reciprocal plots, using 0.05–10 mM pNPP as the substrate, in order to reveal these distinct feature(s). As shown in Fig. 2A, the K_m-value of RCPTPase increased remarkably from 0.28 to 1.19 mM with a temperature decrease from 18 to 2°C.

Some cold-active enzymes, such as *Trematomus centronotus* cold-active lactate dehydrogenase, also show higher K_m values in the low-temperature range in comparison with their mesophilic counterparts, despite a tendency for it to decrease with a temperature decrease (*16*). This contrasts with the fact that other cold-active enzymes, such as trypsin of the Antarctic fish *Paranotothenia magellanica*, exhibit lower K_m values than the mesophilic counterparts (*17*). It is considered that the formation of the enzyme-substrate complex is not a common advantageous process for eliciting the high catalytic activity of all cold-active enzymes at low temperature, assuming that the equilibrium on the binding to substrates of these cold-active enzymes is more rapid than that in the following rate-determining step.

Table 2. **Thermodynamic parameters of the enzyme reaction of RCPTPase.**

Temperature (°C)	ΔG_{ES}	ΔH_{ES}	$T\Delta S_{ES}$ (kcal/mol)	ΔG	ΔH	$T\Delta S$
5	–3.4	24.8	28.1	17.0	4.3	–12.7
10	–4.4	10.5	14.9	17.3	4.3	–13.0
20	–4.7	–0.6	4.1	17.7	20.3	2.6

The van't Hoff plot of K_m against temperatures ranging from 2 to 30°C was parabolic in shape with a maximum around 17°C, $1/T = 3.45 \times 10^{-3}$ (Fig. 2B). The parabolic shape indicated the remarkable increase in activation enthalpy, ΔH_{ES}, and the compensatory increase in the activation entropy term, $T\Delta S_{ES}$, at low temperature (Table 2). The resultant Gibbs free energy, ΔG_{ES}, for binding of RCPTPase to substrate pNPP increased slightly, suggesting that the RCPTPase-pNPP complex at low temperature was less stable than that at intermediate temperature. The instability of the complex might be important for RCPTPase due to the influence on the energy level prior to activation of the complex.

The relationship between *k*cat values and temperature in the range of 2 to 30°C, shown in Fig. 3A, exhibited an inflection point around 19°C. The *k*cat values at 2 to 17°C were 20.8 to 29.2% of the value at the optimal temperature, 30°C. In order to maintain the *k*cat value at low

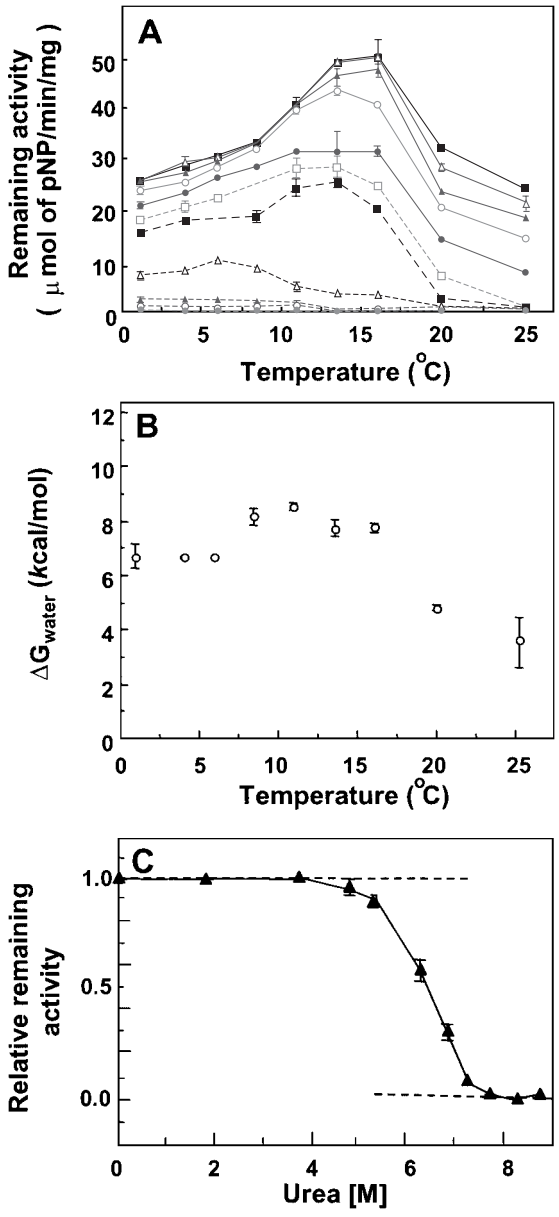

Fig. 4. **Effect of urea on the activity of RCPTPase and the Gibbs free energy for denaturation.** A: Effect of urea on RCPTPase activity was monitored as the remaining activity of RCPTPase (6.8 ng) after 1-h incubation at the indicated temperatures. The symbols on solid- and dashed-lines represent urea concentrations of 0 and 1.9 M (solid square), 3.8 M (open triangle), 4.8 M (solid triangle), 5.3 M (open circle), and 5.7 M (solid circle), and 6.2 M (open square), 6.7 M (solid square), 7.2 M (open triangle), 7.6 M (solid triangle), 8.1 M (open square), and 8.6 M (solid circle), respectively. Data are means ± SE for three separate experiments. B: Temperature-dependency of free energy. The free energy was calculated from the values shown in panel A according to the linear extrapolation method, as described under "MATERIALS AND METHODS". C: Typical dose-dependence of urea as to RCPTPase activity after treatment for 1h with the indicated concentrations of urea at 12°C. A dashed-line represents a theoretical one.

temperature, a decrease in ΔH and/or an increase in ΔS might be essential for RCPTPase. The decrease in ΔH should result from a reduction in E_a, which below 19.3°C was 4.80 ± 0.37 kcal/mol and above 19.3°C was 4.4-fold higher with 21.0 ± 1.7 kcal/mol (Fig. 3B). The obtained ΔH value decreased to 4.3 kcal/mol at the low temperatures of 5 and 10°C, which corresponded to 21% of the value (20.3 kcal/mol) at the intermediate temperature of 20°C (Table 2).

Other cold-active enzymes also exhibited lower ΔH values than those of their counterparts at low temperature (*15*). The transition of the enzyme-substrate complex to the activated form, therefore, might be an important characteristic process in catalytic reactions with cold-active enzymes.

RCPTPase possessed several features that were different from those of other cold-active enzymes in the catalytic reaction. First, the Arrhenius-plot for the maximal velocity of RCPTPase exhibited an inflection point around 19.3°C, $1/T = 3.42 \times 10^{-3}$, as shown in Fig. 3B, indicating that the conformation of the catalytic center in the enzyme molecule might change at around 19.3°C to a form with a low ΔH. One finding in support of this possibility is that the Arrhenius relationships for protein kinase A and protein kinase C of hibernating animals, bats, also showed an inflection point at 10°C. At this temperature, a conformational change of these enzymes into a form with low catalytic efficiency might occur (*18*, *19*). Second, the $T\Delta S$ values of RCPTPase at 5 and 10°C were negative (–12.7 and –13.0 kcal/mol, respectively), whereas the value at 20°C was +2.6 kcal/mol (Table 2). The remarkable decrease in $T\Delta S$ suggested that RCPTPase at 5 and 10°C might differ slightly from that at 20°C in the number of the interactions among amino acids located around the catalytic site.

Effect of Urea on the Activity of RCPTPase—A change in any interaction(s) should influence the stability of the catalytic site of RCPTPase, and the stability might affect the level of remaining activity after treatment with a chemical denaturant such as urea. The susceptibility of the enzyme to urea in the temperature range below 12.5°C was low compared to that above 12.5°C. The remaining activity below 12.5°C remained almost 100% up to 5.3 M urea (Fig. 4A). The Gibbs free energy, ΔG_{water}, for the stability of the catalytic site of RCPTPase was evaluated by the linear extrapolation method (*13*), as shown in Fig. 4B. The obtained ΔG_{water} values are similar, 6.6 kcal/mol, in the range of 1 to 6°C, and about 8.0 kcal/mol at temperatures of 8 to 16°C, whereas the values at 20 and 25°C were strikingly reduced. The far-UV CD spectra of RCPTPase obtained at 5 and 12.5°C were identical, and the α-helix content (f_H) was 0.31, indicating that the slight reduction of ΔG_{water} below 6°C might not reflect a change in the secondary structure.

The ΔG_{water} values for mesophilic enzyme proteins tend to decrease with a reduction in temperature due to reversible unfolding of their conformations (*20–22*). On the contrary, it has been suggested that the catalytic sites of RCPTPase could maintain a stable conformation at low temperature through any type of interaction such as hydrogen bonds, which are easily disrupted by urea.

In conclusion, RCPTPase maintains its conformation for eliciting high catalytic activity with lower activation enthalpy at low temperature. The stable conformation at low temperature could result from an increase in any interaction in the structure of the catalytic center of the RCPTPase molecule. The relationship between the

increased interaction(s) and the retainment of the catalytic activity of RCPTPase at low temperature should be a subject of further study. Whether or not the structural properties of RCPTPase are common features of cold-active enzymes might be determined on the accumulation of information on many cold-active enzymes.

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