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Stabilization of creatinase from *Pseudomonas putida* by random mutagenesis*

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Abstract

Creatinase (creatine amidinohydrolase, EC 3.5.3.3) from *Pseudomonas putida* is a homodimer of 45 kDa subunit molecular mass, the three-dimensional structure of which is known at 1.9 Å resolution. Three point mutants, A109V, V355M, and V182I, as well as one double mutant combining A109V and V355M, and the triple mutant with all three replacements, were compared with wild-type creatinase regarding their physical and enzymological properties. High-resolution crystal data for wild-type creatinase and the first two mutants suggest isomorphism at least for these three proteins (R. Huber, pers. comm.). Physicochemical measurements confirm this prediction, showing that the mutations have no effect either on the quaternary structure and gross conformation or the catalytic properties as compared to wild-type creatinase. The replacement of V182 (at the solvent-exposed end of the first helix of the C-terminal domain) does not cause significant differences in comparison with the wild-type enzyme. The other point mutations stabilize the first step in the biphasic denaturation transition without affecting the second one. In sum, the enhanced stability seems to reflect slight improvements in the local packing without creating new well-defined bonds. The increase in hydrophobicity generated by the introduction of additional methyl groups (A109V, V182I) must be compensated by minor readjustments of the global structure. Secondary or quaternary interactions are not affected. In going from single to double and triple mutants, to a first approximation, the increments of stabilization are additive.

Keywords: creatinase; mutagenesis; *Pseudomonas putida*; stabilization

Globular proteins in solution exhibit only marginal free energies of stabilization. The subtle balance of the attractive and repulsive forces within and without the closely packed polypeptide chain yields values for ΔG_{stab} no more than 50 kJ/mol, independent of the mode of denaturation (Pfeil, 1986; Dill, 1990; Pace, 1990; Jaenicke, 1991a). Expressed on a per residue base, this means that the free energy is one order of magnitude below the thermal energy (kT). Thus, on balance, the stability of globular proteins in solution depends on the equivalent of a few hydrogen bonds, hydrophobic interactions, ion pairs, or van der Waals forces. Faced with this experimental fact, it is evident that there must be many ways to accumulate the small surplus of attractive forces, and, consequently, it is highly improbable to deduce general strategies of protein stabilization.

In order to unravel the fundamental mechanisms of protein stabilization, two ways seem to be promising: First, the exploration of "extremophilic proteins" with anomalous stabilities and their comparison with "mesophilic" homologs, and second, studies on point mutations within known three-dimensional protein structures. The first approach turns out to be ambiguous because even functionally and topologically related proteins with high sequence identity show still numerous exchanges within their primary structures (Zuber, 1988; Schultes et al., 1990; Böhm & Jaenicke, 1993; Jaenicke, 1993; Ostendorp et al., 1993). For the investigation of point mutants, lysozyme from bacteriophage T4, a 164-residue stable pro-

* This paper is dedicated to Professor Julian M. Sturtevant on the occasion of his 85th birthday.

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Abbreviations: N, I, U, native, intermediate, and unfolded states, respectively; GdmCl, guanidinium chloride; T_m, transition temperature; 4-AA, 4-aminoantipyrine; TBHB, 2,4,6-tribromo-3-hydroxybenzoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

tein of 18 kDa molecular mass, has been the classical example (Matthews, 1987, 1991; Alber, 1989; Jaenicke, 1991b).

The present work focuses on creatinase (creatine amidinohydrolase) from *Pseudomonas putida*, which is a homodimer with a calculated molecular mass of 90,968 Da. Each polypeptide chain consists of two domains comprising approximately 160 and 240 amino acid residues, respectively. This implies that the subunits are of considerable size, and the domains are approximately twice the size of average.

The relatively unstable structure of the wild-type protein was stabilized by random mutagenesis (Schumacher et al., 1989a,b). Because the three-dimensional structures of the wild-type enzyme and of some mutants are known at high resolution (Hoeffken et al., 1988; R. Huber, pers. comm.), it should be possible to correlate stability properties with defined local changes in the spatial structure of the enzyme.

Creatinase is highly significant in medical diagnostics because creatinine clearance is used to monitor the filtration rate of glomeruli of the kidney (Schumacher et al., 1989b). The enzyme catalyzes the second step in the coupled creatinine assay (Siedel et al., 1984):

$$\text{creatinine} + H_2O \xrightarrow{\text{creatininase}} \text{creatine}; \quad (1)$$

$$\text{creatine} + H_2O \xrightarrow{\text{creatinase}} \text{sarcosine} + \text{urea}; \quad (2)$$

$$\text{sarcosine} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{sarcosine}} \text{glycine} + \text{HCOOH} + H_2O_2; \quad (3)$$

$$H_2O_2 + \text{4-AA} + \text{TBHB} \xrightarrow{\text{peroxidase}} \text{benzoquinone} + H_2O + \text{HBr}. \quad (4)$$

Because of the low specific activity of the enzyme (<15 U/mg) and its relatively low intrinsic stability, the creatinase-dependent second step in the above reaction sequence limits the overall reaction. Thus, applying mutants with improved stabilities may optimize the diagnostic application of the enzyme and, at the same time, provide a better understanding of possible stabilization mechanisms in large oligomeric proteins.

Results and discussion

Wild-type enzyme

As a reference for the various point mutants of creatinase, the wild-type enzyme had to be characterized regarding its structure and stability. In contrast to a previous report where an A-state of the molecule populated at acid pH was investigated (Schumann & Jaenicke, 1993), the present experiments focus on the structure–function relationship under quasiphysiological conditions at neutral pH. Attempts to improve the relatively low stability of the wild-type enzyme under these conditions by extrinsic factors have recently been reported (Schumann et al., 1993).

Point mutants of creatinase

As taken from the crystal structure, the subunits of creatinase consist of two structurally unrelated domains with central antiparallel β-pleated sheets surrounded by α-helices on the outside (Fig. 1). The protein contains neither intra- nor intermolecular disulfide bonds. The domains are loosely connected via 6 hydrogen bonds and 1 ion pair, whereas the subunits establish strong interactions (20 hydrogen bonds plus 4 ion pairs) (Hoeffken et al., 1988). Because the active centers share sites, only the dimer is catalytically active (Coll et al., 1990).

The creatinase gene from *P. putida* has been cloned in *Escherichia coli* (Schumacher et al., 1989a). Making use of random mutagenesis with nitrosoguanidine, creatinase mutants were produced and selected for enhanced thermal stability using a plate assay at elevated temperature and in the presence of detergents (Schumacher & Buckel, 1988; Schumacher et al., 1989b; Stephan, 1989) (Fig. 1).

Purification, homogeneity, and physical properties

The isolation of the mutant enzymes followed the protocol used for wild-type creatinase (see Materials and

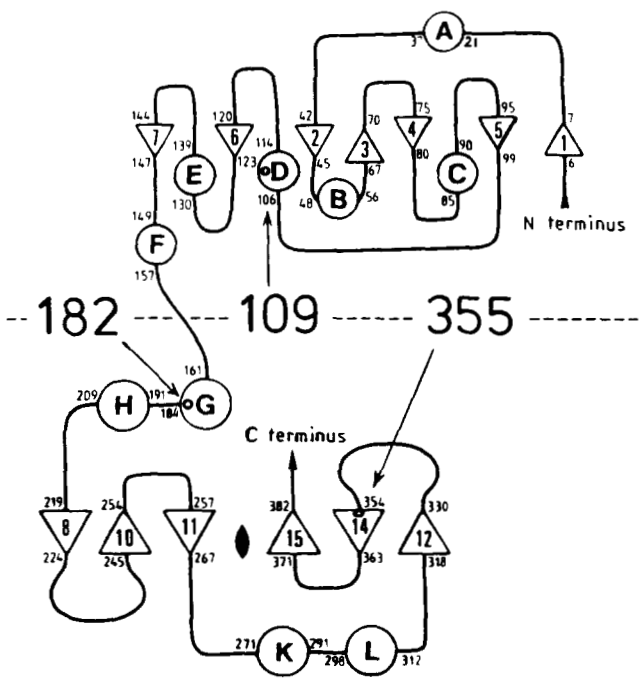

Fig. 1. Topology of creatinase from *Pseudomonas putida* according to Hoeffken et al. (1988). Circles and triangles represent helices and β-pleated sheets, respectively. The upper and lower unit depict the N- and C-terminal domains, connected by the linker (residues 157–161). Arrows point to the residues replaced in the mutants. Mutant 1 (A109V): D helix, N-terminal domain, peripheral; mutant 2 (V355M): β-sheet, C-terminal domain, interior; mutant 3 (V182I): G helix, C-terminal domain, peripheral; mutant 1 + 2 (A109V + V355M): both domains; mutant 1 + 2 + 3 (A109V + V355M + V182I): both domains.

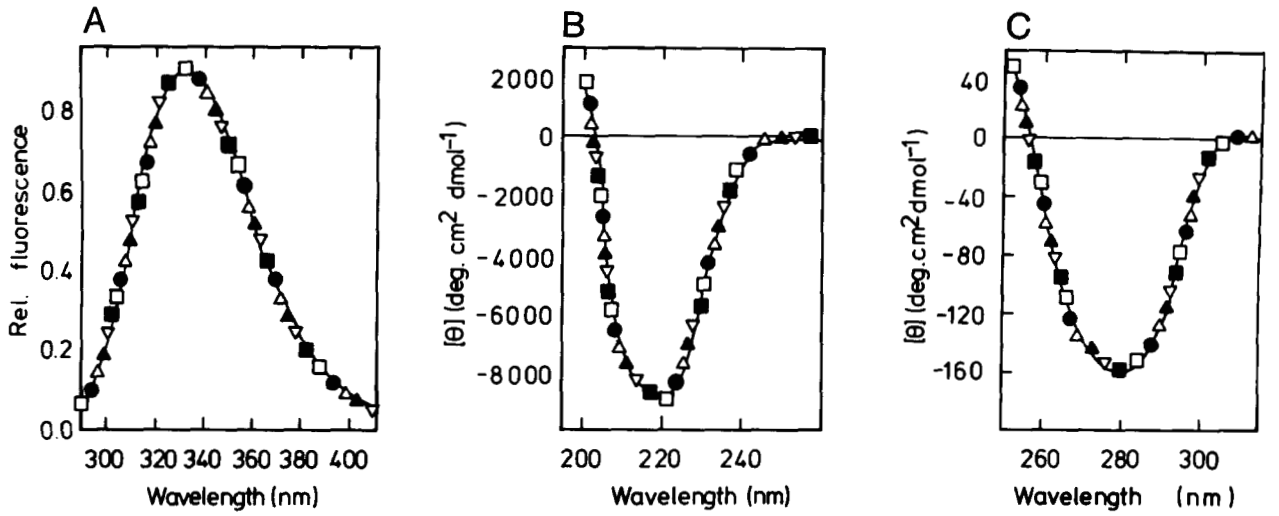

Fig. 2. Fluorescence and CD spectra of creatinase from *P. putida* in standard buffer, pH 8.0, 20 °C. Wild type (●), mutant 1 (▽), mutant 2 (□), mutant 3 (△), double mutant (■), triple mutant (▲). **A:** Fluorescence emission. Wavelength of excitation 280 nm, protein concentration 30 μg/mL. **B:** Far-UV CD. Protein concentration 0.3 mg/mL, 0.1-cm pathlength. **C:** Near-UV CD. Protein concentration 3 mg/mL, 1-cm pathlength.

methods). The purity of the products was better than 98%, as taken from gel-filtration chromatography and SDS-PAGE. Quantitative HPLC gel chromatography (TSK 3000, 20 mM potassium phosphate buffer, pH 7.2, flow rate 0.4 mL/min) yielded elution times of 20.05 ± 0.01 min for all six proteins, corresponding to identical molecular masses of 92 kDa (calculated value 91 kDa). Similarly, no charge differences could be detected: Due to the high negative net charge of creatinase at pH 7 (−26), all six enzymes bind to a Mono-Q anion-exchange column and can be uniformly eluted applying a shallow NaCl gradient. Neither differences in surface charge nor impurities were detectable. Elution with 0–0.5 M NaCl (20 mM potassium phosphate buffer, pH 8.0, flow rate 1 mL/min) yielded single peaks at 25.5 ± 0.2 M NaCl for all six enzymes. Furthermore, SDS-PAGE gave single bands, yielding a subunit molecular mass of 43 kDa (calculated value 46 kDa). Finally, using isoelectric focusing, all six proteins show identical isoelectric points, pI = 4.8. In summary, we may conclude that wild-type creatinase and the mutants used in the present study do not exhibit any detectable differences regarding their molecular masses and their electrochemical properties (Schumann, 1992).

Within the ranges of error, the same holds for the spectroscopic characteristics: absorption (data not shown), fluorescence, and CD data are indistinguishable for both wild-type and all five mutants (Fig. 2). This is what one would predict, because (1) no chromophores or fluorophores are replaced; (2) the replacements are conservative, i.e., there is no change in the polarity involved; and (3) crystallographic evidence (at least for the A109V and V355M mutants) proves that the point mutations cause only local alterations within the spatial structure of the enzyme (R. Huber, pers. comm.; L. Rüssmann, pers. comm.).

Enzymatic properties

The solution properties of wild-type creatinase and its point mutants that have been discussed so far refer to global characteristics only. In contrast, the catalytic function and its activation energy respond to structural alterations in a much more sensitive way. Thus, wild-type creatinase and its point mutants were compared with respect to their specific activities, Michaelis constants (K_m), and activation energies. As shown in Table 1, no significant changes can be observed, except that the limit of temperature where deviations from Arrhenius behavior become important is shifted from 25 °C, for wild-type creatinase, to ca. 32 °C, for the most stable triple mutant (1 + 2 + 3). This reflects the stabilizing effect, which has evidently no effect on the catalytic properties.

Table 1. *Enzymatic properties of wild-type creatinase from Pseudomonas putida and its point mutants*

Creatinase	Specific activity (U/mg)	K_m (creatine; 25 °C) (mM)	Activation energy (kJ/mol)
Wild type	15.0	14.3	43
1	16.1	12.5	40
2	15.9	13.9	39
3	14.6	14.7	43
1 + 2	15.2	16.1	41
1 + 2 + 3	18.0	15.6	43

Stability

Kinetics of deactivation

The kinetics of deactivation were measured in order to compare the relative stabilities of wild-type creatinase and its point mutants (Fig. 3). The kinetics are independent of protein concentration, thus proving that loss of activity is attributable to first-order unfolding rather than aggregation, even at high protein concentrations. As has been reported (Schumann et al., 1993), the deactivation profiles may be described by a sequential uni-unimolecular reaction

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D. \qquad (5)$$

Upon shifting the temperature from 20 °C to 47 °C, only the slower of the two first-order rate constants can be resolved (Table 2). Deactivation is accompanied by aggregation (data not shown).

The wild-type enzyme and mutant 3 (V182I) show closely similar behavior. As in the case of the thermal stability (see below), the mutation in the C-terminal domain seems to have a higher impact on the kinetic stability than the one located in the N-terminal portion of the enzyme. Considering the temperature dependence of the two consecutive reactions, it turns out that the mutations have no effect on the activation energies. Both wild-type creatinase and the triple mutant yield identical Arrhenius plots for the N → I and the I → D transitions with activation energies of 176 ± 8 and 390 ± 27 kJ/mol, respectively.

Table 2. *Kinetics of thermal deactivation of creatinase from P. putida*[a]

Creatinase	$k_2 \times 10^4$ (s^{-1})
Wild type	21.0
1	5.9
2	9.0
3	18.0
1 + 2	1.9
1 + 2 + 3	0.6

[a] k_2 represents the slower of the two first-order rate constants in the uni-unimolecular thermal deactivation reaction (cf. Equation 5) at 47 °C.

Deactivation/denaturation transitions

The temperature-induced deactivation transition is evidence for the relatively low intrinsic stability of wild-type creatinase. Deactivation in standard buffer and in the absence of stabilizing additives becomes detectable at temperatures around 30 °C (Fig. 4). The low thermal stability is confirmed by the exceedingly low concentrations of chaotropic agents required for deactivation and aggregation. The mutants, except mutant 3 (with the V → I replacement at the end of the G-helix exposed to the solvent), exhibit enhanced thermal stabilities (Fig. 4). In the

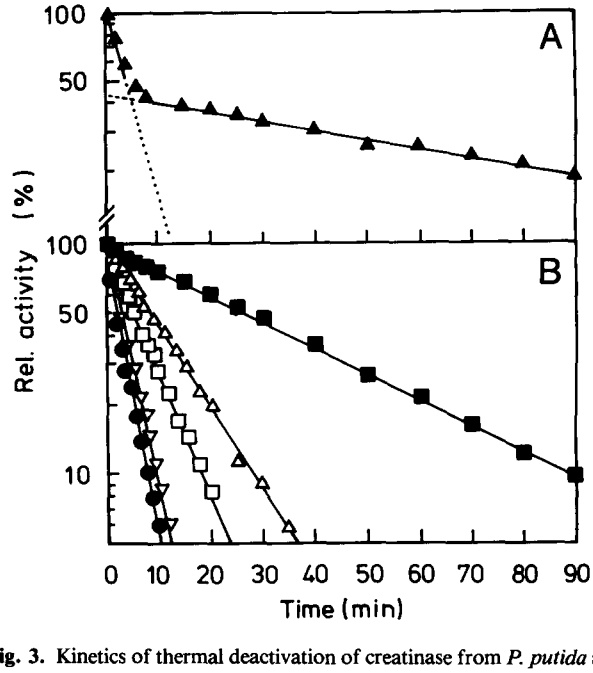

Fig. 3. Kinetics of thermal deactivation of creatinase from *P. putida* and its mutants. Incubation of enzyme solutions (4 μg/mL) in standard buffer, pH 8.0, at 47 °C. Samples were taken after defined times; residual activity was determined at 25 °C. Wild type (●), mutant 1 (△), mutant 2 (□), mutant 3 (▽), double mutant (■), triple mutant (▲). Kinetic analysis according to first-order kinetics, normalized to 100% at initial time ($t = 0$) . **A:** Linearization of the sequential deactivation reaction according to Equation 5. Due to its enhanced stability, the triple mutant is the only one that allows both kinetic phases (k_1 and k_2) to be resolved at the given temperature. **B:** Linearization of the slow phase (k_2 in Equation 5); at 47 °C the precursor reaction (k_1) for wild-type creatinase and mutants 1–4 is too fast to be resolved.

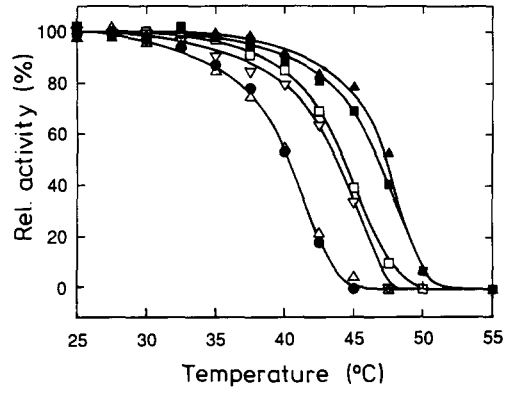

Fig. 4. Thermal deactivation of creatinase from *P. putida* and its mutants in standard buffer, pH 8.0. Residual activity after 30 min incubation at temperatures given; normalized to 100% for activity at 20 °C; enzymatic assay at 25 °C. Protein concentration 4 μg/mL. Wild type (●), mutant 1 (▽), mutant 2 (□), mutant 3 (△), double mutant (■), triple mutant (▲).

Table 3. *Thermal deactivation and denaturation transitions of creatinase from P. putida (standard buffer, pH 8.0)*

Creatinase	Activity[a]		Unfolding[b]	
	$(T_m)_A$ (°C)	$(\Delta T_m)_A$ (°C)	$(T_m)_D$ (°C)	$(\Delta T_m)_D$ (°C)
Wild type	40.0		48.4	
1	43.4	3.4	52.0	3.6
2	43.8	3.8	50.1	1.7
3	40.0	0.0	48.6	0.2
1 + 2	46.5	6.5	53.5	5.1
1 + 2 + 3	47.5	7.5	54.7	6.3

[a] $(T_m)_A$ quantifies the deactivation transition and $(\Delta T_m)_A$ the stabilization of creatinase point mutants relative to the wild-type enzyme.
[b] $(T_m)_D$ quantifies the denaturation transition monitored by differential scanning calorimetry (protein concentration 1.5 mg/mL, heating rate 1 °C/min) and $(\Delta T_m)_D$ the shift in transition temperature relative to wild-type creatinase.

case of the double and triple mutants, to a first approximation, the stability increments are additive (Table 3). Thermal deactivation and denaturation are irreversible and accompanied by aggregation (Schumann et al., 1993). Consequently, spectroscopic measurements are inappropriate to follow the reaction, and thermal analysis does not yield true thermodynamic parameters. However, given the close structural and functional similarities of the various proteins, differential scanning calorimetry yields a useful operational parameter to quantify the stability of the various proteins. Because the heating rate has a strong effect on the N → D transitions (Schumann et al., 1993), identical conditions were applied to determine the apparent transition temperatures T_m. Data at a heating rate of 1 °C/min, and the respective shifts in the transition temperature are included in Table 3. Again, data obtained for the double and triple mutants indicate that the contributions of single point mutations to the thermal stability are additive.

Denaturation by chaotropic agents, such as GdmCl or urea, were used to complement the thermal analysis. Because in this case aggregation only occurs in a relatively narrow range of denaturant concentrations, fluorescence emission and dichroic absorption may be applied to assign conformational transitions to specific chromophores or structural elements within the proteins. Taking, first, deactivation as a measure (Fig. 5A), the differences in intrinsic stability of the wild-type enzyme and its mutants

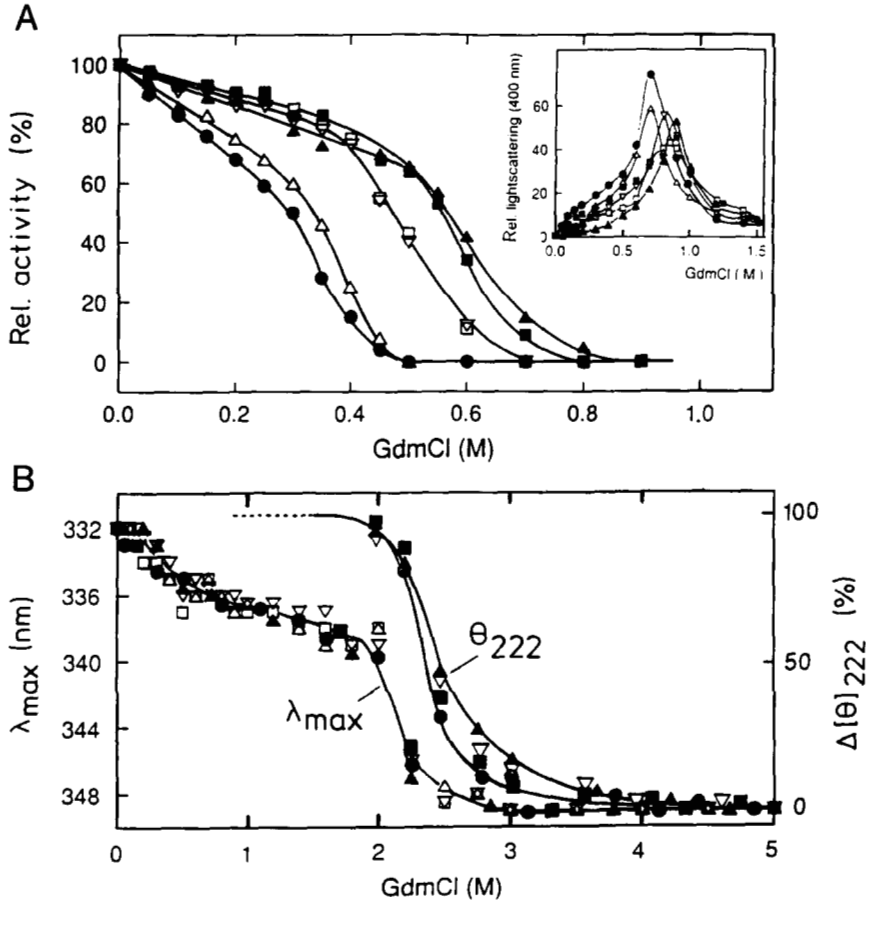

Fig. 5. Deactivation and denaturation of creatinase from *P. putida* and its mutants in GdmCl at 25 °C, standard buffer, pH 8.0. Wild type (●), mutant 1 (▽), mutant 2 (□), mutant 3 (△), double mutant (■), triple mutant (▲). **A:** Deactivation. Residual activity after 24 h incubation, normalized to the activity in the absence of GdmCl = 100%. Protein concentration 6 μg/mL. *Inset:* Aggregation at varying GdmCl concentrations. The GdmCl concentrations where maximum aggregation occurs (as a qualitative measure of protein stability) are: wild type and mutant 3, 0.70; mutant 1, 0.83; mutant 2, 0.85; double mutant, 0.88; triple mutant, 0.88. **B:** Denaturation, monitored by CD ($[\theta]_{222nm}$) and fluorescence emission (λ_{max}), after 24 h incubation. Normalized to $[\theta]_{222nm}$ and λ_{max} = 100% at c_{GdmCl} = 0. Dotted line: range of GdmCl-dependent aggregation. Protein concentration 0.3 mg/mL and 30 μg/mL for CD and fluorescence measurements, respectively.

are reflected by a linear decrease in activity at low c_{GdmCl}, which precedes the cooperative transitions at higher denaturant concentrations. As taken from the light-scattering versus c_{GdmCl} profiles, aggregation increases linearly, starting at lowest GdmCl concentrations (inset, Fig. 5A). Thus, at least part of the linear decrease in the deactivation profiles is attributable to loss of enzyme during equilibration.[1]

Regarding the effect of the point mutations on both the backbone conformation of creatinase and the local environment of its fluorophores, CD and fluorescence emission show clearly that the denaturation transitions of wild-type creatinase and its mutants coincide (Fig. 5B,C). During the first transition, a slight increase in the wavelength of maximum fluorescence emission parallels deactivation. For the second major unfolding transition at high GdmCl concentration, the point mutations do not have any significant effect on either the secondary structure or the packing around chromophores: All transition curves for θ_{222} and λ_{max} coincide within the limits of experimental error.

Comparing urea as a denaturant with GdmCl, the fact that it is nonionic offers the advantage that there is much less aggregation in the transition range below $c_{urea} = 2$ M. As illustrated in Figure 6A, deactivation occurs in a noncooperative fashion over an anomalously wide concentration range, with an apparent half-concentration of ca. 1.2 M. Because urea is a reaction product, this transition may be due to effects on the enzyme mechanism rather than protein stability. It seems unrelated to either of the two conformational transitions monitored by fluorescence or dichroic absorption (Fig. 6A,B). As in the case of GdmCl, again, no significant differences between wild-type and the various mutant proteins can be detected, except for a slight change in $\Delta\lambda_{max}$ for the first urea transition.

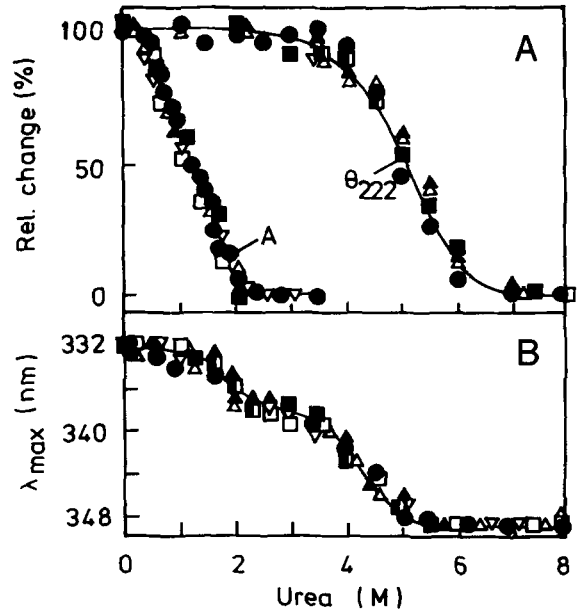

Fig. 6. Deactivation and denaturation of creatinase from *P. putida* and its mutants in urea at 25 °C, standard buffer, pH 8.0. Wild type (●), mutant 1 (▽), mutant 2 (□), mutant 3 (△), double mutant (■), triple mutant (▲). **A:** Deactivation and denaturation, monitored by CD. Residual activity (A) after 24 h incubation was normalized to the activity in the absence of urea = 100%. Protein concentration 6 μg/mL. Dichroic absorption ($[\theta]_{222nm}$), after 24 h incubation. Normalized to $[\theta]_{222nm}$ = 100% at $c_{urea} = 0$. Protein concentration 0.3 mg/mL. **B:** Denaturation, monitored by fluorescence emission (λ_{max}), and, after 24 h incubation, normalized to λ_{max} = 100% at $c_{urea} = 0$. Protein concentration 30 μg/mL.

Conclusions

Creatinase, a homodimer of 45 kDa subunit molecular mass, was investigated regarding the effect of single, double, and triple mutations on its intrinsic stability. In order to avoid artifacts caused by alterations in the gross structure of the enzyme, a detailed analysis of the isomorphism of the wild-type and mutant proteins had to be performed. Evidence from hydrodynamic, electrophoretic, and spectroscopic data proves all five mutants to be indistinguishable from each other and from the wild-type protein. This result is not surprising, considering the positions and the character of the replacements: (1) The maximum difference in molecular mass is below the limit of detection of the methods used for size determination. (2) Aromatic or charged residues are neither eliminated nor replaced. (3) There are no amino acid exchanges in the subunit contact area so that there is no interference with the authentic quaternary interactions.

Regarding their positions within the overall structure, the mutations are equally distributed over the whole molecule, one in the small N-terminal domain and two in the larger C-terminal one. This topological consideration may be the explanation for the fact that, to a first approximation, the increments of stabilization caused by the single replacements are additive (Wells, 1990). The fact that the mutations do not involve amino acid residues engaged in the catalytic mechanism of the enzyme is in accordance with the observation that the enzymatic properties of the mutants are closely similar to those of the wild-type enzyme; obviously, the increase in stability does not affect the flexibility of the active site.

Considering the mutations more closely, it is obvious that all replacements involve additional groups that have to be accommodated in the surface or in the interior of the protein. In the case of the exchanges A109V and V182I, additional methyl groups are inserted in helix D in the N-terminal domain, and at the end of helix G in the C-terminal domain (cf. Fig. 1). Because both residues are

[1] That the transition does not represent an equilibrium becomes clear from the fact that creatinase is inaccessible to renaturation by any available method (Schumann et al., 1993).

exposed to the solvent, we have to assume that the hydrophobic surface is increased by the replacements. In this case, one would expect a decrease rather than an increase in stability. However, adding a more hydrophobic side chain at an exposed site will increase the stability of the enzyme if it can partially bury itself, thus contributing to the hydrophobic core.

In the case of the A109V mutant, the hydrophobic increment of the neighboring lysine residue seems to be of importance, since the valine residue forms hydrophobic contacts to the methylene groups of lysine 5. The structure of the V182I mutant is not available yet. Therefore, computer modeling was applied to fit the altered polypeptide chain into the wild-type coordinates: no analogy to the A109V situation is immediately obvious. However, slight movements of the end of the helix or a minute movement of the entire helix may be visualized as an explanation for the marginal stabilization observed for this mutant. In connection with the V355M exchange, the stabilization may be attributed to the sulfur atom in the linear hydrocarbon chain that replaces the branched valine side chain. Residue 355 is positioned at the beginning of a β-pleated sheet in the interior of the enzyme. The structure in this region is relatively loosely packed and can accommodate the methionine side chain without interfering with neighboring amino acids. Additional (hydrogen) bonds do not seem to be involved because there are no H donors available within the critical distance. Thus, the mutant with the maximum stabilizing effect among the single point mutations gains stability from its improved local packing density, without forming significant additional bonds. This mechanism of stabilization has been described as one important strategy in connection with the adaptation of proteins to high temperatures in hyperthermophilic microorganisms (Wrba et al., 1990; Jaenicke, 1991b, 1993). In the case of small model proteins such as T4 lysozyme or ribonuclease, improved local packing has been investigated in detail (Matthews, 1987, 1991; Alber, 1989; Karpusas et al., 1989; Dao-pin et al., 1990; Dao-pin, 1991; Eriksson et al., 1992; Kimura et al., 1992).

In interpreting the stabilization in terms of specified local interactions, one has to consider that the free energy of stabilization of globular proteins in solution is a marginal difference of large numbers. These result from the vast number of attractive and repulsive interactions within the densely packed polypeptide chain and its solvent environment. In this context, one has to keep in mind that the mean square deviation in high-resolution X-ray structures is on the order of 0.2 Å; the one obtained from energy minimization is ca. 0.5 Å. Thus, important contributions coming from small rearrangements of side chains or structural elements may escape detection. Including the present knowledge base of known high-resolution protein structures by making use of correlation functions (Böhm & Jaenicke, 1992), the comparison of the various homologous creatinases allows the following conclusions: (1) The stability increases in spite of the fact that the local hydrophobic surface area of the mutants is slightly increased. (2) The total surface area is reduced for both the single subunits and their contact area in the native quaternary structure. (3) Some of the mutants show enhanced packing density in the inner core and a parallel decrease in the solvent volume excluded by the protein. (4) The number of hydrogen bonds to the solvent is increased. How these properties contribute to the net free energy of stabilization is still unpredictable (Jaenicke & Buchner, 1993).

In considering the denaturation characteristics of the mutants compared to their parental wild-type enzyme, it has to be mentioned that the denaturation of all six proteins is found to be irreversible. Therefore, stability data in terms of thermodynamic parameters are inaccessible. The overall denaturation reaction of creatinase is at least biphasic, with an irreversible formation of an intermediate which, in a second step, undergoes a reversible denaturation transition:

$$N \rightarrow I \rightleftharpoons D \qquad (6)$$

(Schumann & Jaenicke, 1993; Schumann et al., 1993). The physical properties of the intermediate I differ depending on the denaturant. In all the biphasic profiles observed for the various mutants, the second transition was found to be identical, coinciding with the transition of the wild-type enzyme. This means that the stabilization refers only to the first transition where catalytic activity breaks down. This may be due to the selection method used for the random mutagenesis, where catalytic function at increased temperature was applied as a screening parameter. Whether the differences in the $N \rightarrow I$ transitions are attributable to the domains where the mutation took place, or to the domain contacts, or to the entire molecule has still to be resolved.

Materials and methods

Enzyme

Creatinase (creatine amidinohydrolase, EC 3.5.3.3) from *P. putida* and its mutants were cloned and overexpressed in *E. coli* as described by Schumacher and Buckel (1988). The enzyme represents about 50% of the total protein of the host cells, without forming inclusion bodies. Therefore, the enzyme can be isolated in pure form applying classical purification procedures: lysis of the cells with lysozyme, separation of DNA by polyethylene-imine, binding to DEAE-Sephadex, ammonium sulfate precipitation, and DEAE-Sepharose chromatography. Protein concentrations were monitored spectrophotometrically at 280 nm, with $A_{1cm} = 1.11$ for a 0.1% solution. Enzyme activity was determined in the coupled assay in 0.1 M potassium phosphate buffer, pH 7.8, 25 °C, A_{505nm} (Siedel et al., 1984; Schumann, 1992).

Physical properties

Spectroscopy

Spectroscopic measurements in 20 mM sodium phosphate/boric acid buffer, pH 8.0 (standard buffer), were performed in a Cary 1 double-beam spectrophotometer (1-cm pathlength), fluorescence emission spectra (buffer-corrected, $\lambda_{exc} = 280$ nm) and light scattering (400 nm), in a Perkin-Elmer LS 5B fluorescence spectrophotometer (1-cm pathlength, 30 μg/mL, 20 °C), and CD spectra, in a Jasco J-500 spectropolarimeter (far-UV: 0.1-cm pathlength, 0.3 mg/mL; near-UV: 1-cm pathlength, 3 mg/mL; mean residue molecular mass 112.9).

Molecular mass

Molecular mass determinations made use of HPLC gel chromatography using a TSK 3000 column.

Stability

Thermal deactivation (in standard buffer, 25 °C) was measured as residual activity after 30 min incubation at varying temperatures. For kinetic measurements, the residual activity was determined in aliquots taken after varying incubation times at 47 °C. Reactivation under the given test conditions could be excluded. Thermal aggregation, monitored either by light scattering at 400 nm or by turbidity at 320 nm made use of a thermoprogrammer with a heating rate of 1 °C/min. Kinetic measurements at 30 μg/mL were performed by shifting the temperature rapidly from 35 to 45 °C, using light scattering for detection.

Thermal analysis of denaturation transitions (at 1.4–1.7 mg/mL protein concentration) made use of an adiabatic differential scanning calorimeter DASM-4 according to Privalov et al. (1975).

Denaturation transitions were determined in 20 mM standard buffer, after 24 h incubation at varying GdmCl concentrations and 20 °C. Denaturation was monitored making use of (1) deactivation at a protein concentration of 6 μg/mL; (2) aggregation, i.e., light scattering at 400 nm; (3) shifts in the wavelength of maximum fluorescence emission at 30 μg/mL; and (4) changes in the amplitude of the molar ellipticity at 222 nm at 0.3 mg/mL.

Computer modeling

Homology and structural modeling were based on the program INSIGHTII version 2.0 and DISCOVER version 2.8 (Biosym). Calculations of surface and packing density properties made use of the program "pqms" (M.L. Connolly, New York). All programs applied were installed on a Silicon Graphics IRIS 4D/70 GTB workstation running IRIX 3.2.

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