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Mirror image mutations reveal the significance of an intersubunit ion cluster in the stability of 3-isopropylmalate dehydrogenase

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Abstract The comparison of the three-dimensional structures of thermophilic (*Thermus thermophilus*) and mesophilic (*Escherichia coli*) 3-isopropylmalate dehydrogenases (IPMDH, EC 1.1.1.85) suggested that the existence of extra ion pairs in the thermophilic enzyme found in the intersubunit region may be an important factor for thermostability. As a test of our assumption, glutamine 200 in the *E. coli* enzyme was turned into glutamate (Q200E mutant) to mimic the thermophilic enzyme at this site by creating an intersubunit ion pair which can join existing ion clusters. At the same site in the thermophilic enzyme we changed glutamate 190 into glutamine (E190Q), hereby removing the corresponding ion pair. These single amino acid replacements resulted in increased thermostability of the mesophilic and decreased thermostability of the thermophilic enzyme, as measured by spectropolarimetry and differential scanning microcalorimetry.

Key words: 3-Isopropylmalate dehydrogenase; Ion cluster; Circular dichroism; Differential scanning calorimetry; Site-directed mutagenesis; Thermostability

1. Introduction

The stability of the three-dimensional structure of native proteins is a result of a delicate balance among large numbers of stabilizing and destabilizing interactions. Our knowledge about protein stability, folding and function is by far not comprehensive. We know that polypeptide chains fold spontaneously under conditions that favor the native state. Most enzymes work near their thermal unfolding temperatures [1]. It was also recognized that proteins handling the same function usually have very similar three-dimensional structures despite their sequence variation and diverse origin.

Thermal adaptation is a good example of how nature can redesign enzymes while preserving their catalytic function. Comparison of related enzymes of different thermal stabilities can teach us how to engineer proteins of increased thermal stability. Since thermal stabilization is achieved by the cumulative effect of small stabilizing and destabilizing contributions, it is hard to sort out and evaluate the effect of individual amino acid replacements in a polypeptide sequence [2–5].

Our strategy was to find a protein which has stability variants (e.g. psychrotrophic, mesophilic, thermophilic) with known three-dimensional structures, do comparative scrutinizing of the structures, identify point mutations that are suspected to contribute to increased stability, create the constructs with single mutations, express and purify them to check the outcome.

(2*R*,3*S*)-3-Isopropylmalate dehydrogenase (IPMDH; EC 1.1.185), an enzyme of the leucine biosynthetic pathway, catalyzing the oxidative decarboxylation of (2*R*,3*S*)-3-IPM into 2-oxicaproate in a NAD-dependent way, fulfills the above requirements. Genes encoding IPMDH have been cloned and sequenced from an extreme thermophile, *Thermus thermophilus* HB8 [6,7], from the mesophilic *Escherichia coli* [8] and from the psychrotrophic *Vibrio* sp. I5 strain [9]. The 3D structures of these enzymes were also determined, in the case of *T. thermophilus* IPMDH by X-ray crystallography [10]. The structures of the *E. coli* and *Vibrio* IPMDHs were first obtained by homology modelling [9,11] and the *E. coli* IPMDH structure was also determined by X-ray diffraction later [12], refining and confirming the results of the homology modelling.

In a comprehensive study comparing all known thermophilic protein structures with their mesophilic homologues, we found that the single property that most frequently differs between mesophilic and thermophilic variants of a protein is the number of ion pairs (publication in preparation). This finding holds for IPMDHs from psychrotolerant, mesophilic and thermophilic sources [9,11,12]. Since IPMDH is a dimeric protein, subunit–subunit interactions are expected to contribute significantly to the overall thermal stability of the dimer. The number of ion pairs between the subunits shows an increase with increasing thermal stability: IPMDH from *E. coli* has six such ion pairs while IPMDH from *T. thermophilus* has 10. There are other differences in the intersubunit interaction patterns as well [13]. Our aim was to assess the contribution of the extra intersubunit ion pairs to the increased stability of the thermophilic IPMDH using site-directed mutagenesis.

Glu-190 in the *T. thermophilus* IPMDH forms an ion pair with Arg-144 in the other subunit. Arg-144 is further engaged in a (weak) ion pair with Glu-142 in the same subunit, thus contributing to an ion triad. In the *E. coli* IPMDH, there is a Gln residue in place of the Glu at this position. There are, however, two positively charged residues in the other subunit that are close enough to this glutamine residue and could form an ion pair with it, if it carried a negative charge (Fig. 1). Thus, changing this glutamine to glutamate appeared a promising way to create intersubunit ion triads and to see

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whether they will stabilize the structure. On the other hand, replacing glutamate by glutamine in the *T. thermophilus* IPMDH provides the means to assess the effect of elimination of the ion triad around Glu-190.

The residue at position 190 (sequence numbering according to *T. thermophilus* IPMDH) in both enzyme variants was replaced by the residue found at the same position in the counterpart structure. In other words, we designed a 'mirror' mutation pair by turning the mesophilic structure, at one location, into the thermophilic one and vice versa. Since the stabilizing effect of residue replacements depends on the structural context, such 'mirror image' mutants are good means to delineate the role of a given residue in different structural contexts.

The mutants were constructed, expressed, purified and their thermal stabilities were compared by differential scanning microcalorimetry, circular dichroism and heat inactivation kinetic measurements.

2. Materials and methods

2.1. Bacterial strains and plasmids

Recombinant DNA experiments were done by standard methods [14]. The *Pst*I-*Hin*cII fragment from plasmid pWally, which encodes the *E. coli leuB* gene, and *Pst*I-*Kpn*I fragment from plasmid pUTL118, which encodes the *T. thermophilus leuB* gene were ligated into the M13mp18 phage vector. The in vitro mutagenesis experiments were carried out by means of the Kunkel method [15] using the following primers: 5′-TTGCACGACCTTAGGAGATA-3′ for the *E. coli*, and 5′-AGCAAGCCCGAGGTGGA-3′ for the *T. thermophilus* mutant *leuB* gene. The DNA sequences of the mutant genes were determined by the dideoxy chain termination method [16] with T7 Sequenase kit (Amersham Life Sciences).

For the production of the wild type and mutated *E. coli* IPMDH the *leuB*-defective *E. coli* strain RDK 1782 (obtained from Gerlind Wallon, Brandeis University) was used. The cells were grown at 30°C in the presence of 100 mg/l ampicillin and 50 mg/l kanamycin, and induced by keeping them at 42°C for 1 h then expression was continued for an additional time of 3 h at 37°C. For the production of the wild type and mutated *T. thermophilus* enzymes the strain BMH 71-18 was used. The cells were cultivated at 37°C with 100 mg/l ampicillin added, and induced in the mid-logarithmic phase with 0.1 mM IPTG for 3 h.

Cells were collected by centrifugation and the enzyme was purified by butyl-Sepharose and DEAE-Sephacell chromatographies using the previously described method [17] with minor modifications.

2.2. Enzyme activity measurements

The catalytic activity of IPMDH was measured in 20 mM potassium phosphate buffer, pH 7.6, containing 25 μl of enzyme solution, 0.4 mM DL-3-isopropylmalate, 0.8 mM NAD, 0.2 mM $MnCl_2$ and 0.3 M KCl in a final volume of 700 μl. Initial rates were determined by monitoring the absorbance of NADH at 340 nm, using a Jasco V-500 spectrophotometer. Measurements were carried out at 37°C for *E. coli* and its mutant, and at 58°C for *T. thermophilus* and its mutant enzymes.

2.3. Spectropolarimetry and differential scanning calorimetry

All circular dichroism (CD) measurements were performed in a Jasco J720 spectropolarimeter in 20 mM potassium phosphate buffer (pH 7.6) containing 0.3 M KCl at 0.4, 0.04 or 0.004 mg/ml protein concentrations. Quartz cuvettes were used for the measurements with a 1 mm, a 10 mm or a 100 mm pathlength depending on the sample dilution. The thermal unfolding of the enzymes was monitored at 221 nm, the heating rate was set to 50°C/h or 100°C/h.

Differential scanning calorimetry (DSC) measurements were carried out on a Microcal VP-DSC differential scanning microcalorimeter in 20 mM potassium phosphate buffer (pH 7.6) containing 0.3 M KCl, with a heating rate of 50°C/h. The enzyme samples were dialyzed against the same buffer before analysis then diluted appropriately to have the same (0.4 mg/ml) protein concentration.

2.4. Computer graphics and modelling

We have used the Insight II graphical interface and the Discover molecular simulation program from Molecular Simulations Inc. for the visualization and structure prediction of the mutants. We performed a rotamer library search [18] in order to predict the conformation of the mutated side chain and that of its environment. The most probable conformers of the mutated Glu (Q200E mutant) or Gln (E190Q mutant) side chains were accepted as initial structures and a conjugate gradient energy minimization was performed on the residues in a 6 Å zone of the mutated side chain until the largest gradients reached the 0.25 kcal/Å/mol limit. The accessible surface areas were calculated by the method of Shrake and Rupley [19]. Ion pairs were defined as side chains carrying opposite charges within a distance of 6 Å [20]. Charge clusters are defined as unbroken networks of ion pairs. In this respect, the 6 Å definition seems to be more appropriate for identifying networks of ion pairs than the stricter 4 Å criterion.

3. Results and discussion

3.1. Modelling and site-directed mutagenesis

The role of ion clusters in the conformational stability of enzymes is a difficult and controversial issue. The generally accepted view is that buried ion pairs destabilize the three-dimensional structure [21], though recent calculations show that this may not stand at high temperatures [22]. Surface ion pairs, however, probably have a stabilizing effect, especially when they are cooperatively strengthened by the presence of other ion pairs in a cluster [23–25].

Meticulous structural comparison of IPMDH from psychrotrophic, mesophilic and thermophilic sources pointed to the differences in the number of charged residues involved in ion pairs and ion clusters [9,11,12].

Our attempt was to assess the significance of an ion cluster found around the Arg-144 residue at the subunit–subunit interface in the *T. thermophilus* IPMDH. This ion formation is not found in the *E. coli* enzyme, however, a similar ion cluster can easily be created by replacing glutamine 200 by a glutamate.

The rotameric conformations of the newly introduced Glu-200 residue were modelled in the structural context of the *E. coli* IPMDH, and it was concluded that it is in a position

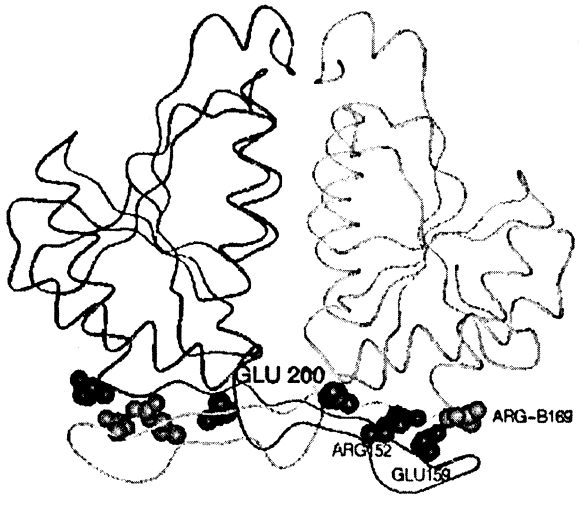

Fig. 1. Ribbon diagram of mesophilic (*E. coli*) IPMDH. The residues visualized by the spacefilling model constitute the ion cluster formed at the subunit interface, upon replacement of Gln-200 by Glu.

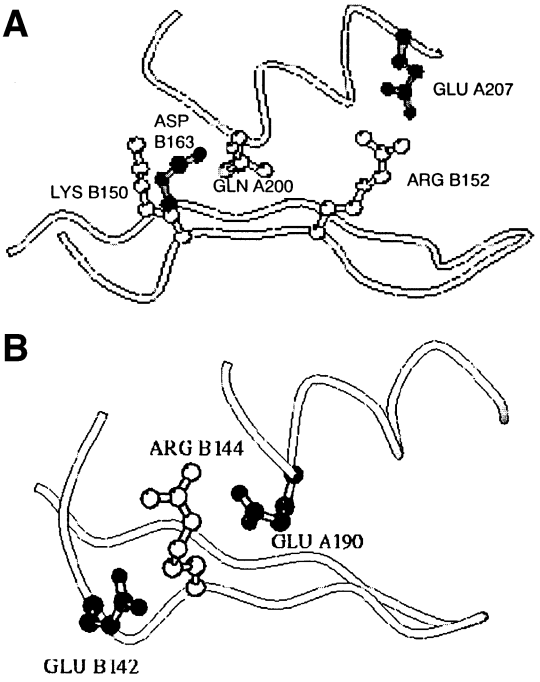

Fig. 2. A: Model of the *E. coli* IPMDH showing the side chain of Gln-200 and some charged residues in its proximity. The ribbon represents the protein backbone. When Gln-200, located at the N-terminus of a helical region, is mutated to Glu (in subunit A), it can form an intersubunit ion pair with either Lys-B150 or Arg-B152, both located in the arm-like region of subunit B. Both these residues themselves form ion pairs with other charged side chains: Lys-B150 forms an ion pair with Asp-B163 in the same subunit (subunit B); and Arg-B152 forms an ion pair with Glu-A207 in the other subunit (subunit A). Glu-A207 is part of a larger ion cluster (not shown). Because of the symmetry of the dimer, the same situation is observed when Gln-200 is mutated to Glu in subunit B. B: Model of the *T. thermophilus* IPMDH showing the side chain of Glu-190 and some charged residues in its proximity. The ribbon represents the protein backbone. When Glu-190 is mutated to Gln (in subunit A), the intersubunit ion pair with Arg-B144 and thus the three-member ion cluster (formed also by Glu-B142) is disrupted.

to form an intersubunit ion pair with either Lys-150 or Arg-152 in the other subunit. In both cases, the resulting ion pair will be part of a larger ion cluster; in the first case an ion triad (GluA200–LysB150–AspB163; A and B denote the subunits) and in the second case a cluster formed by nine residues (GluA200–ArgB152–GluB159–ArgA169–GluA173–ArgA177–GluA215, ArgB152–GluA207–ArgA169–GluA211). We expect Glu-200 to fluctuate between these two conformations. These networks may have a strong stabilizing effect due to cooperativity; therefore, the newly introduced ion pair might contribute significantly to thermostability. Because of the symmetry of the dimer, each extra ion pair is present in duplicate, doubling the stabilizing effect. The residues around the mutation are all solvent accessible according to accessibility calculations. Residue 200 is located at the N-terminus of an α-helix (Glu-200–Glu-215). Lys-150 and Arg-152 are positioned in an arm-like protrusion (consisting of a pair of antiparallel β-strands Lys-150–Arg-163) attached to the other subunit (Fig. 2A).

In the structure of the thermophilic IPMDH, the equivalent of Gln-200 of the mesophilic enzyme is glutamate-190. This residue forms a small ion cluster of three members with Glu-A142 and Arg-B144 (*T. thermophilus* numbering) (Fig. 2B). It can be expected that the disruption of this cluster would destabilize the *T. thermophilus* IPMDH.

Based on these modelling studies we constructed a point mutant of the *E. coli* IPMDH, Gln-200→Glu (Q200E) and the 'mirror' mutant of the *T. thermophilus* IPMDH, E190Q (in which the corresponding glutamate was replaced by glutamine) was also constructed. The mutant enzymes were expressed in *E. coli* and purified to homogeneity.

3.2. Conformation and catalytic activity

According to the near- and far-UV CD spectra, neither of the mutations caused significant changes in the secondary or tertiary structure of the enzyme (data not shown). In addition, the conservation of the global structure is also supported by the fact that the mutant enzymes retained their catalytic activity. The specific activity of the mutant *E. coli* IPMDH has remained essentially unchanged, while that of the 'destabilized' *T. thermophilus* IPMDH has been reduced to about half of the value measured for the wild type enzyme.

3.3. Thermal stability of the mutant enzymes

To compare the thermal stabilities of the wild type and mutant enzymes the thermal unfolding was followed by CD spectroscopy at 221 nm and by DSC. We stress that T_m' values, listed in Table 1, correspond to the apparent melting temperatures defined as the maxima of the DSC curves or as the inflection point of the unfolding profile obtained by CD measurements. We have studied the enzymes under different experimental conditions, and the unfolding was found to be irreversible in all cases. It is hard to separate the reversible step(s) of the unfolding from the subsequent irreversible ones. Therefore the transitions monitored either by CD spectroscopy or by DSC represent the whole course of denaturation including unfolding and subsequent aggregation of the protein at high temperature.

The T_m' values (Table 1) clearly show that the Q200E mutant melts at higher and the E190Q mutant at lower temper-

Table 1
Apparent transition temperatures of wild type and mutant IPMDHs as measured by CD spectroscopy and DSC

	Heating rate (°C/h)	Concentration (mg/ml)	T_m' (°C)		$\Delta T_m'$ (°C)	T_m' (°C)		$\Delta T_m'$ (°C)
			E. coli wild type IPMDH	*E. coli* Q200E IPMDH		*T. th.* wild type IPMDH	*T. th.* E190Q IPMDH	
CD	100	0.4	72.8	76.2	3.4	88.8	86.9	−1.9
	50	0.4	69.7	73.7	4.0	86.2	83.0	−3.2
	50	0.04	69.8	71.3	1.5	87.2	79.8	−7.4
	50	0.004	67.9	69.8	1.9	85.0	76.8	−8.2
DSC	50	0.4	70.9	72.9	2.0	87.8	83.8	−4.0

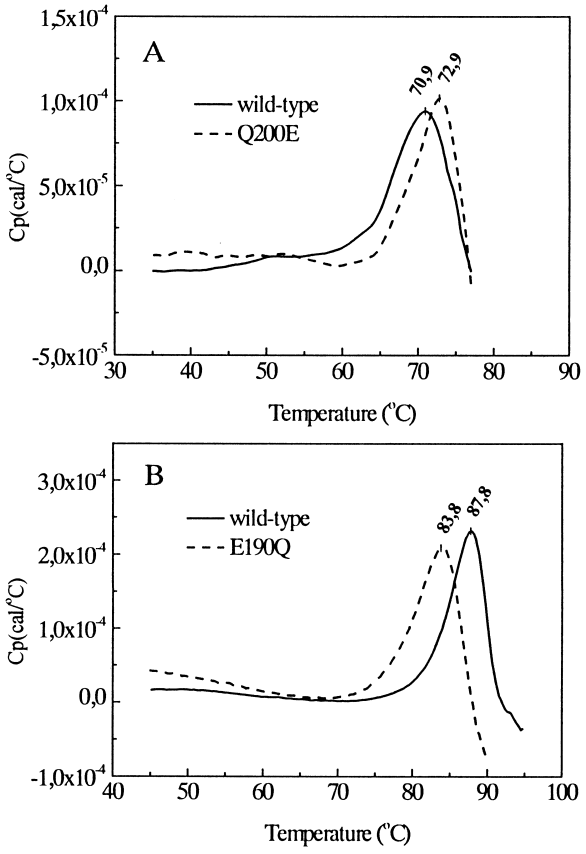

Fig. 3. Partial heat capacity curves of (A) the *E. coli* (solid line, $T_m' = 70.9°C$) and of its Q200E mutant (dashed line, $T_m' = 72.9°C$) IPMDHs and (B) the *T. thermophilus* (solid line, $T_m' = 87.8°C$) and of its E190Q mutant (dashed line, $T_m' = 83.8°C$) IPMDHs. The measurements were carried out with a heating rate of 50°C/h in pH 7.6 potassium phosphate buffer containing 0.3 M KCl, the protein concentration was 0.4 mg/ml. The unfolding was irreversible.

atures than their wild type counterparts, irrespective of the experimental conditions. DSC curves are presented in Fig. 3A,B, and a set of CD curves (obtained at 0.4 mg/ml protein concentration) in Fig. 4A,B. The definite stabilization or destabilization effect unambiguously indicates the significance of the presence or absence of the additional ion pair in the IPMDH structure.

An obvious regularity is observed in the data: the melting (unfolding) temperature decreases with decreasing concentration for all four samples. This pattern is in accordance with the assumption that subunit dissociation is the rate limiting step in the thermal unfolding of IPMDHs in general. The assumed unfolding scheme is:

$$M_2 \rightleftarrows 2M \rightarrow 2U$$

where M indicates the folded and U the unfolded monomer.

The extent of thermal stabilization resulting from the Q200E mutation ($\Delta T_m' = 2$–$4°C$) may seem small but in fact, it is not insignificant given the size of the protein (about 80 kDa for the dimer) and the fact that the residues involved are rather solvent accessible. Actually, surface ion pairs introduced into proteins usually result in very small or undetectable stabilization [26,27]. The stabilization in our case is probably due to the fact that an additional subunit–subunit link is created and the ion pair is strengthened by nearby charged residues.

Aoshima and Oshima [5] created several mutant *E. coli* IPMDHs in order to reveal the role of a five-membered motif in the stabilization of thermophilic IPMDHs. Their mutants include a fourfold mutant where residues 201–204 are all changed to their thermophilic equivalents, and a fivefold mutant derived from the fourfold mutant by introducing the Q200E mutation, which is the subject of the present paper. But while the single mutation, as we just demonstrated, results in increased stability, the fivefold mutant is slightly destabilized compared to the wild type protein [5]. Furthermore, as opposed to the wild type enzyme, the fourfold mutant is not stabilized by the Q200E mutation [5]. These observations show that the effect of a particular local interaction is greatly (and sometimes unpredictably) influenced by the local environment.

Our study revealed that the thermostability of the *E. coli* IPMDH enzyme can be increased by replacing glutamine-200 by glutamate (the corresponding residue in the *T. thermophilus* IPMDH) by creating an extra intersubunit ion pair which is cooperatively strengthened due to the presence of other ion pairs in its vicinity. In accordance with this observation, the

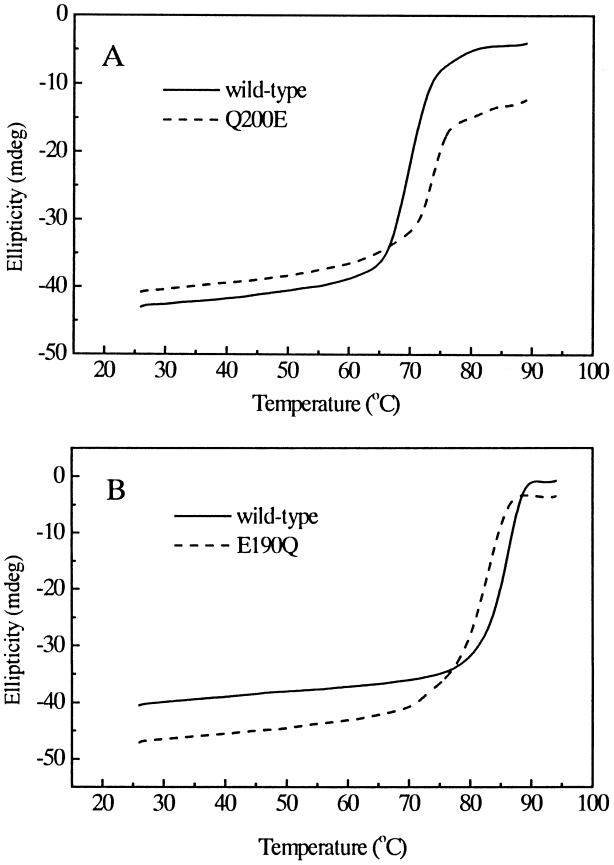

Fig. 4. Unfolding of (A) the *E. coli* (solid line, $T_{tr}' = 69.7°C$) and of its Q200E mutant (dashed line, $T_{tr}' = 73.7°C$) IPMDHs and (B) of the *T. thermophilus* (solid line, $T_{tr}' = 86.2°C$) and of its E190Q mutant (dashed line, $T_{tr}' = 83.0°C$) IPMDHs followed by CD spectroscopy at 221 nm. The raw data were smoothed. The transition temperatures obtained this way show the increased stability of the mutant IPMDH. The measurement was carried out in pH 7.6 potassium phosphate buffer containing 0.3 M KCl, at a protein concentration of 0.4 mg/ml. The heating rate was 50°C/h.

elimination of the corresponding ion pair in the thermophilic structure results in a decrease in thermostability to a similar extent. The results demonstrate that site-directed mutagenesis, based on careful structural analysis and computer simulation, can be used to construct enzymes with increased thermal stability.

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References

[1] Závodszky, P., Kardos, J., Svingor, A. and Petsko, G.A. (1998) Proc. Natl. Acad. Sci. USA 95, 7406–7411.
[2] Jaenicke, R. (1991) Eur. J. Biochem. 202, 715–728.
[3] Jaenicke, R. and Závodszky, P. (1990) FEBS Lett. 268, 344–349.
[4] Akasako, A., Haruki, M., Oobatake, M. and Kanaya, S. (1995) Biochemistry 34, 8115–8122.
[5] Aoshima, M. and Oshima, T. (1997) Protein Eng. 10, 249–254.
[6] Tanaka, T., Kawano, N. and Oshima, T. (1981) J. Biochem. 89, 677–682.
[7] Kagawa, Y., Nojima, H., Nukiwa, N., Ishizuka, M., Nakajima, T., Yasuhara, T., Tanaka, T. and Oshima, T. (1984) J. Biol. Chem. 259, 2956–2960.
[8] Kirino, H., Aoki, M., Aoshima, M., Hayashi, Y., Ohba, M., Yamagishi, A., Wakagi, T. and Oshima, T. (1994) Eur. J. Biochem. 220, 275–281.
[9] Wallon, G., Lovett, S.T., Magyar, C., Svingor, A., Szilágyi, A., Závodszky, P., Ringe, D. and Petsko, G.A. (1997) Protein Eng. 10, 665–672.
[10] Imada, K., Sato, M., Tanaka, N., Katsube, Y., Matsuura, Y. and Oshima, T. (1991) J. Mol. Biol. 222, 725–738.
[11] Magyar, C., Szilágyi, A. and Závodszky, P. (1996) Protein Eng. 9, 663–670.
[12] Wallon, G., Kryger, G., Lovett, S.T., Oshima, T., Ringe, D. and Petsko, G.A. (1997) J. Mol. Biol. 266, 1016–1031.
[13] Akanuma, S., Qu, C., Yamagishi, A., Tanaka, N. and Oshima, T. (1999) FEBS Lett. 410, 141–144.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Clonin: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Yamada, T., Akutsu, N., Miyazaki, K., Kakinuma, K., Yoshida, M. and Oshima, T. (1990) J. Biochem. 108, 449–456.
[18] Ponder, J.W. and Richards, F.M. (1987) J. Mol. Biol. 193, 775–791.
[19] Shrake, A. and Rupley, J.A. (1973) J. Mol. Biol. 79, 351–371.
[20] Szilágyi, A. and Závodszky, P. (1995) Protein Eng. 8, 779–789.
[21] Waldburger, C.D., Schildbach, J.F. and Sauer, R.T. (1995) Nature Struct. Biol. 2, 122–128.
[22] Elcock, A.H. and McCammon, J.A. (1998) J. Mol. Biol. 280, 731–748.
[23] Horovitz, A., Serrano, L., Avron, B., Bycroft, M. and Fersht, A.R. (1990) J. Mol. Biol. 216, 1031–1044.
[24] Yip, K.S., Stillman, T.J., Britton, K.L., Artymiuk, P.J., Baker, P.J., Sedelnikova, S.E., Engel, P.C., Pasquo, A., Chiaraluce, R. and Consalvi, V. (1995) Structure 3, 1147–1158.
[25] Goldman, A. (1995) Structure 3, 1277–1279.
[26] Sun, D.P., Sauer, U., Nicholson, H. and Matthews, B.W. (1991) Biochemistry 30, 7142–7153.
[27] Sali, D., Bycroft, M. and Fersht, A.R. (1991) J. Mol. Biol. 220, 779–788.