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Archives of Biochemistry and Biophysics
Vol. 363, No. 2, March 15, pp. 349–355, 1999
Article ID abbi.1998.1097, available online at http://www.idealibrary.com on IDEAL®

The Contribution of Acidic Residues to the Conformational Stability of Common-Type Acylphosphatase

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Received October 15, 1998, and in revised form December 14, 1998

Common-type acylphosphatase is a small cytosolic enzyme whose catalytic properties and three-dimensional structure are known in detail. All the acidic residues of the enzyme have been replaced by noncharged residues in order to assess their contributions to the conformational stability of acylphosphatase. The enzymatic activity parameters and the conformational free energy of each mutant were determined by enzymatic activity assays and chemically induced unfolding, respectively. Some mutants exhibit very similar conformational stability, $\Delta G(H_2O)$, and specific activity values as compared to the wild-type enzyme. By contrast, six mutants show a significant reduction of conformational stability and two mutants are more stable than the wild-type protein. Although none of the mutated acidic residues is directly involved in the catalytic mechanism of the enzyme, our results indicate that mutations of residues located on the surface of the protein are responsible for a structural distortion which propagate up to the active site. We found a good correlation between the free energy of unfolding and the enzymatic activity of acylphosphatase. This suggests that enzymatic activity measurements can provide valuable indications on the conformational stability of acylphosphatase mutants, provided the mutated residue lies far apart from the active site. Moreover, our results indicate that the distortion of hydrogen bonds rather than the loss of electrostatic interactions, contributes to the decrease of the conformational stability of the protein.

Key Words: **common-type acylphosphatase; enzymatic activity; conformational stability; urea denaturation.**

The assembly of the native and functional conformation of a protein occurs at the expense of a high entropic cost and must necessarily be driven by enthalpically favorable intramolecular interactions. It is widely accepted that the hydrophobic interactions represent the major driving force to move the energetic balance in favor of the native conformation, whereas the role of hydrogen bonds and salt bridges are at present a matter of dispute (1–10). The importance of each type of interaction occurring within a polypeptide chain has been recently elucidated for a number of proteins by means of site-directed mutagenesis (11–14). In the present study we apply this experimental approach to all the acidic residues of human common-type acylphosphatase (CT AcP).[3] CT AcP is a single-domain protein of 98 residues and constitutes a simple model system for equilibrium and kinetic studies of folding. CT AcP catalyzes the hydrolysis of acylphosphates of physiological relevance such as 1,3-*bis*phosphoglycerate, carbamoylphosphate, and β-aspartylphosphate (15). The biological role of AcP is still uncertain, although there are some experimental indications as to its involvement in the regulation of membrane cation transport through the hydrolysis of phosphorylated intermediates formed during the action of membrane pumps (16, 17). CT AcP shares 55% sequence identity with the highly homologous muscle AcP. ^{1}H NMR and X-ray crystallography have shown that both isoenzymes possess very similar three-dimensional structures consisting of an antiparallel five-stranded β-sheet packed against two antiparallel α-helices (Fig. 1) (18, 19).

The folding of acylphosphatase, investigated by both equilibrium and kinetic experiments, is a typical two-state process where intermediates do not accumulate

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[3] Abbreviations used: CT AcP, common-type acylphosphatase; IPTG, isopropyl thiogalactoside; GST, glutathion *S*-transferase.

0003-9861/99 $30.00

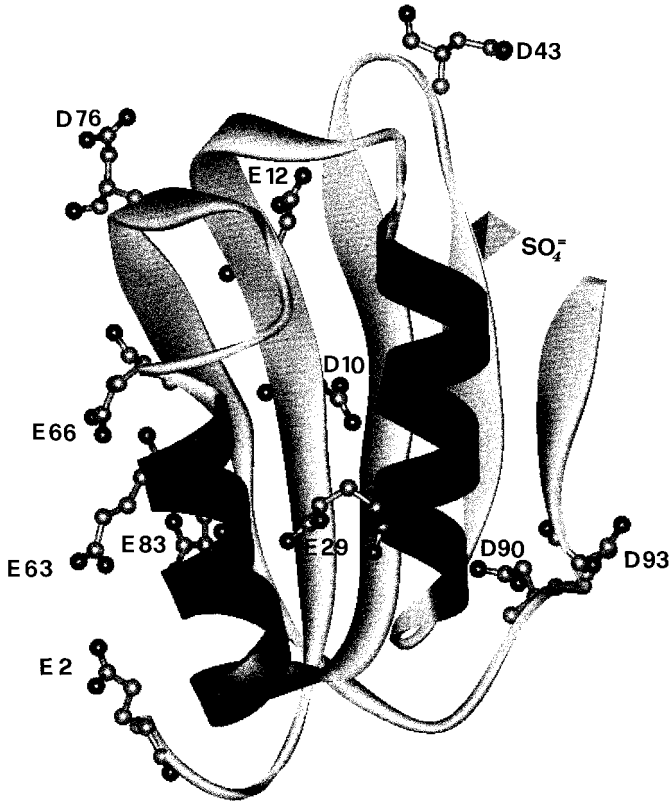

FIG. 1. Schematic representation of common-type acylphosphatase drawn by the program WebLab Viewer 1.0. Atomic coordinates are from the common-type isoenzyme crystal structure (19). The positions of aspartic and glutamic residues and the sulfate ion at the active site are highlighted.

significantly (20–22). Previous studies have also indicated that denatured muscle AcP is characterized by little content of residual structure (23). Therefore, the equilibrium urea-induced unfolding of acylphosphatase can be described as a transition between a fully native and a fully unfolded conformation.

Here we study the enzymatic activity and the conformational stability of single-point mutants of CT AcP where the 11 acidic residues have been replaced by asparagine or glutamine residues. All these residues occupy a superficial position in the wild-type protein and most of them are involved in the formation of hydrogen bonds or salt bridges. The glutamate to glutamine substitution (or aspartate to asparagine substitution) is rather conservative since glutamine and asparagine residues are a little larger than glutamic and aspartic acid residues. Indeed, replacement of aspartate and glutamate residues with asparagine and glutamine residues implies the substitution of a good hydrogen bond acceptor with either a weaker acceptor (carbonyl oxygen) or a hydrogen bond donor (the amide group). Unlike mutations to alanine residues, commonly performed when studying the importance of hydrogen bonding or ionic interactions of charged residues, this particular type of mutation allows the distortions of hydrogen bonding to be investigated.

MATERIALS AND METHODS

Benzoylphosphate was synthesized as previously described (24) and dissolved immediately prior to enzyme activity assay. Glutathione-agarose gel, glutathione, and bovine thrombin were purchased from Sigma. Klenow fragment of *Escherichia coli* DNA polimerase I, T4 DNA ligase, *Taq* polimerase, and restriction enzyme *Bam*HI and *Eco*RI were purchased from Promega; sequenase was from USB; pGEX-KT vector was from Pharmacia; isopropyl thiogalactoside (IPTG) was from Boehringer (Mannheim). Chemically synthesized oligonucleotides were obtained from Pharmacia. Acetic acid and urea were from Merck (Darmstadt, Germany). All other reagents were analytical grade or the best commercially available.

Site-directed mutagenesis. The mutants were obtained directly on the pGEX-KT vector containing the common-type acylphosphatase coding region (25) following the procedure of Deng and Nickoloff (26). Eleven different synthetic oligonucleotides (approx 25 bases long) were used to introduce a single mutation in each mutant. In particular, aspartic acid and glutamic acid residues were replaced by asparagine and glutamine residues, respectively. The mutated genes were completely sequenced by the Sanger's method (27). The pGEX plasmids carrying the synthetic gene of either the wild-type or mutated CT AcP were amplified in *E. coli* strain DH5α.

Fusion protein expression and enzyme purification. The expression of wild-type and mutated acylphosphatase–glutathione *S*-transferase (AcP–GST) fusion proteins was obtained by inducing cell cultures with 0.4 mM IPTG for 2 h at 37°C. The cell cultures were subsequently centrifuged, and the pellet was resuspended in 50 ml of 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, and, finally, sonicated. The AcP–GST fusion proteins were purified from cell lysate using a glutathione–agarose affinity resin as described previously (25). The thrombin cleavage was performed by incubating overnight at 4°C the fusion protein bound to the resin with 1:2000 (w/w) bovine thrombin in 10 ml of 50 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 4 mM $CaCl_2$. The cleaved enzyme was separated from GST and AcP–GST by simple elution. Protein purity was checked by 15% SDS–polyacrylamide gel electrophoresis run with high concentrations of protein.

Enzymatic activity and protein concentration determination. Acylphosphatase activity was measured by a continuous optical test at 283 nm and 25°C using benzoylphosphate as a substrate, dissolved in 0.1 M acetate buffer, pH 5.3, in the presence of 0.1 mg/ml bovine serum albumin. The concentration of substrate ranged from 0.1 to 5.0 mM; the values of K_m and V_{max} were calculated by a nonlinear fitting of the hyperbolic Michaelis–Menten equation by using the computer program Fig.P (Biosoft, Cambridge, UK). The inhibition constant (K_i) for inorganic phosphate (competitive inhibitor of enzyme) was obtained by measuring the rate of the initial hydrolysis of benzoylphosphate in the presence of four different concentrations of inhibitor (ranging from 0.25 to 4 mM). Protein concentration was determined using a BCA kit purchased from Pierce.

Structural analysis. The computer program WHATIF with a default parameter setting was used for calculating the distance between residues and for molecular modeling (28).

Urea-induced denaturation. The conformational stability of the proteins was determined by chemically induced unfolding experiments upon titration with urea in 50 mM acetate buffer, pH 5.3, 25°C. The urea was freshly prepared in a stock solution (9 M) in 50 mM acetate, pH 5.3. Each titration curve was obtained by measuring the fluorescence of at least 30 samples containing 0.02 mg/ml of enzyme preincubated for 1 h in the presence of different concentra-

TABLE I

Main Kinetic Parameters Calculated from Activity Measurement Experiments[a]

	Specific activity (IU/mg protein)	K_m (mM)	K_i (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Wild-type	4500 ± 450	0.15 ± 0.01	0.59 ± 0.04	5610 ± 670
Glu2Gln	4020 ± 400	0.16 ± 0.01	0.44 ± 0.03	4500 ± 530
Glu12Gln	2660 ± 260	0.21 ± 0.02	0.72 ± 0.06	2210 ± 290
Glu29Gln	4570 ± 450	0.14 ± 0.03	0.53 ± 0.05	6000 ± 800
Glu63Gln	4370 ± 440	0.25 ± 0.07	0.64 ± 0.09	3220 ± 550
Glu66Gln	5580 ± 560	0.16 ± 0.03	0.59 ± 0.02	6400 ± 670
Glu83Gln	2880 ± 290	0.32 ± 0.08	0.81 ± 0.07	1650 ± 220
Asp10Asn	4300 ± 430	0.14 ± 0.02	0.61 ± 0.02	5630 ± 600
Asp43Asn	3690 ± 370	0.39 ± 0.004	0.75 ± 0.05	1740 ± 210
Asp76Asn	4500 ± 450	0.15 ± 0.005	0.49 ± 0.03	5410 ± 630
Asp90Asn	4300 ± 430	0.16 ± 0.005	0.53 ± 0.03	4920 ± 560
Asp93Asn	3340 ± 330	0.14 ± 0.003	0.60 ± 0.05	4290 ± 560

[a] One IU is defined as the catalytic activity which hydrolyzes 1 μmol/min of benzoylphosphate, at pH 5.3 and 25°C. K_m and K_i were calculated kinetically using benzoyl phosphate and inorganic phosphate as substrate and competitive inhibitor, respectively.

tions of urea (ranging from 0 to 8 M). For all samples the fluorescence intensity was measured at 335 nm after excitation at 280 nm using a Shimazdu RF-5000 spectrofluorophotometer. All data obtained from each denaturation curve were fitted using the nonlinear least-squares analysis proposed by Santoro and Bolen (29):

$$y = \{(y_f + m_f[\text{urea}]) + (y_u + m_u[\text{urea}]) \times \exp[-(\Delta G(H_2O)/RT - m[\text{urea}]/RT)]\}/\{1 + \exp[-(\Delta G(H_2O)/RT - m[\text{urea}]/RT)]\}, \quad [1]$$

where y_f, m_f, y_u, and m_u are the slopes and intercepts of the pre- and posttransition regions, respectively, m is a measure of the ΔG dependence on urea concentration, and $\Delta G(H_2O)$ is the free-energy change of unfolding in the absence of denaturant. The determined m value was very similar for the wild-type protein and 7 of 11 mutants, and an average value was calculated using the data obtained for these mutants. Thus, $\Delta G(H_2O)$ was redetermined for each of these mutants as suggested by Pace (30) and Matouschek and Fersht (31) using

$$\Delta G(H_2O) = C_m \times \langle m \rangle \quad [2]$$

where $\langle m \rangle$ is the average m value and C_m the denaturation midpoint of the mutant under consideration. In contrast, the directly measured m value was used for those mutants that exhibit m values significantly lower than the wild-type protein.

RESULTS AND DISCUSSION

Enzymatic Activity and Substrate Binding of Mutated AcP

In order to study the contribution of acidic groups to the conformational stability of CT AcP, all the glutamic and aspartic acid residues were replaced by glutamine and asparagine residues, respectively. The main kinetic parameters of enzymatic activity measured for all mutants are summarized in Table I. No significant differences were found as compared to the wild-type enzyme. While the mutants Glu12Gln, Glu83Gln, Asp43Asn, and Asp93Asn exhibit a slight reduction of the specific activity, the other mutants appear to possess an enzymatic activity comparable to that of the wild-type enzyme. Moreover, the Glu66Gln mutant shows even higher catalytic activity. As far as the K_m values are concerned, slight increases were found for the Glu12Gln, Glu63Gln, Glu83Gln, and Asp43Asn mutants, the other mutants featuring K_m values very similar to that of the wild-type protein. A similar picture emerges as to the K_i values: these are approximately the same as that of wild-type CT AcP, and only the Glu12Gln, Glu83Gln, and Asp43Asn mutants have slightly increased values of K_i. Because both K_m and K_i represent, to a good approximation, an index of the binding capability of the enzyme active site, it is not surprising to find such correlation between the two parameters. On the contrary, the loss of enzymatic activity does not seem to correlate with the loss of binding capability as can be deduced by the K_m (or K_i) and specific activity values reported in Table I. In summary, none of the mutations performed in this study alters dramatically the enzymatic efficiency nor the substrate binding capability of CT AcP. This rules out the involvement of carboxylic groups in the catalysis of this enzyme.

Structural studies based on NMR and X-ray crystallography (18, 19), in combination with site-directed mutagenesis studies (32–34), have allowed the position of the active site to be located at atomic level. This includes the Arg23 and Asn41 residues and the 15–21 loop. In this respect, the relatively high levels of enzymatic activity of the mutated forms of CT AcP are not surprising and reflect the distance from the active site of all the acidic residues. On the other hand, four of the

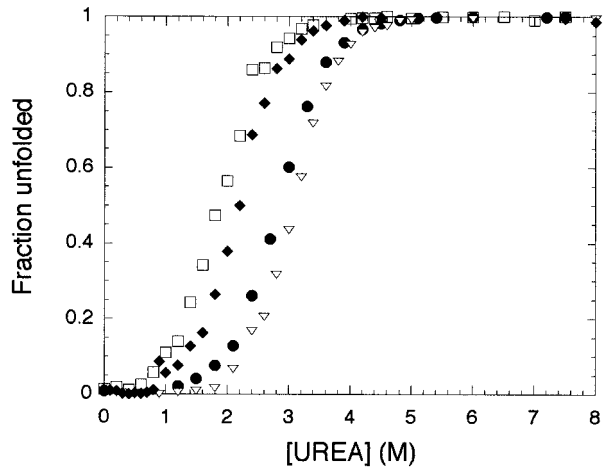

FIG. 2. Urea-induced denaturation of common-type acylphosphatase followed by recording the fluorescence signal at 335 nm as a function of the urea. Wild-type enzyme (●), Glu12Gln mutant (□), Glu83Gln mutant (◆), and Glu66Gln mutant (▽). The curves are normalized to the fraction unfolded (f.u.) = $(y - y_n)/(y_n - y_d)$, where y is the fluorescence observed at a given urea concentration and y_n and y_d are the signals of fluorescence of the native and denatured protein, respectively, at the same urea concentration extrapolated from the pre- and posttransition baselines.

mutations studied led to the partial loss of enzymatic activity. We have recently produced CT and muscle AcP mutants in which the replacement of amino acid residues falling well outside the active site are responsible for a dramatic decrease of the enzymatic activity (unpublished observations). NMR spectroscopy indicates that these particular mutations act presumably through a slight but extensive reorganization of the structure of the protein that propagates up to the catalytic site of the enzyme (unpublished observations). The decrease of enzymatic activity in four of the mutants in the present study can therefore be attributed to little alterations of the packing of the molecule which originate at the site of the mutation and propagate to the active site. This interpretation agrees with a view that has been demonstrated to be generally valid: a movement of a few Å of residues involved in catalysis would be, in fact, enough to alter significantly the enzymatic activity (35).

Conformational Stability of Mutated CT AcP

To gain insight into the importance of the acidic residues for the conformational stability of CT AcP, we acquired the urea denaturation curves of the 11 mutants (Fig. 2). The equilibrium urea denaturation curves were analyzed as explained under Materials and Methods, and the parameters obtained from the analysis are reported in Table II. The Glu12Gln, Glu83Gln, Asp90Asn, and Asp93Asn mutants appear less stable given their low midpoint of denaturation, C_m (Fig. 2). Interestingly, 4 of the 11 mutants studied have a significantly low m value (Glu63Gln, Asp10Asn, Asp76Asn, and Asp93Asn), while the others have m values similar to that of wild-type CT AcP. It is widely accepted that the m value is linearly correlated to the change of solvent exposed surface area upon denaturation (36, 37). A decreased m value can be found for structurally very similar proteins and is generally explained either by the presence of an intermediate significantly populated at equilibrium during the unfolding reaction or by an increased compactness of the denatured state (38).

Table II reports, together with the $\Delta G(H_2O)$ values for all the mutants, the $\Delta\Delta G$ values defined as the difference between the $\Delta G(H_2O)$ of the mutant and the $\Delta G(H_2O)$ of the wild type. Nearly all mutants display a negative $\Delta\Delta G$ value, indicating that the amino acid replacement of the carboxylic residues of CT AcP leads to a reduction of the conformational stability of the protein. Some destabilizations are very small and perhaps negligible given the experimental error, but some others are undoubtedly significant such as those for Glu12Gln, Glu83Gln, Asp90Asn, and Asp93Asn. Interestingly, two of the mutants, Glu63Gln and Glu66Gln, display a higher C_m value and the Glu66Gln an increased conformational stability compared to the wild-type protein.

TABLE II

Conformational Stability Parameters Derived from Urea Denaturation Experiments[a]

	C_m (M)	m value (kJ mol^{-1} M^{-1})	$\Delta G(H_2O)$ (kJ mol^{-1})	$\Delta\Delta G_{D,25°C}$ (kJ mol^{-1})
Wild-type	2.90	6.3	18.0	—
Glu2Gln	2.85	6.7	17.7	−0.3
Glu12Gln	1.85	6.3	11.5	−6.5
Glu29Gln	2.83	5.9	17.6	−0.4
Glu63Gln	3.16	5.4	17.0	−1.0
Glu66Gln	3.19	6.4	19.8	+1.8
Glu83Gln	2.10	6.3	13.1	−4.9
Asp10Asn	2.71	5.3	14.4	−3.6
Asp43Asn	2.97	5.7	18.5	+0.5
Asp76Asn	2.95	5.1	15.0	−3.0
Asp90Asn	2.28	6.2	14.2	−3.8
Asp93Asn	2.46	4.5	10.8	−7.2

[a] Experimental conditions are described in the text. C_m is the concentration of urea at which 50% of the protein is unfolded. $\Delta G(H_2O)$ is the unfolding Gibbs free energy in the absence of urea; m value is the dependence of $\Delta G(H_2O)$ on denaturant concentration. An average $\langle m \rangle$ value of 6.22 ± 0.60 kJ mol^{-1} M^{-1} has been used for recalculating $\Delta G(H_2O)$ for those mutants that exhibit an m value that is similar to that of the wild-type protein within experimental error. The $\Delta G(H_2O)$ values for the Asp10Asn, Glu63Gln, Asp76Asn, and Asp93Asn mutants were calculated by considering the measured m value. The average error for each m value is 10%, for C_m is 0.1 M, and for $\Delta G(H_2O)$ is 10%.

TABLE III

Hydrogen Bonds and Salt Bridges Involving Acidic Residues[a]

Residue	H Bonds and salt bridges	Distance in Å
Glu2	Glu2Oε2—Arg59Nη1	3.20
Asp10	Asp10Oδ2—Asn82Nδ2	2.72
	Asp10Oδ1—Gln50Nε2	2.74
Glu12	Glu12Oε2—Thr46Oγ2	2.69
Glu29	Glu29Oε1—Lys32Nζ	3.27
	Glu29Oε1—Trp64Nε	3.78
Asp43	—	—
Glu63	—	—
Glu66	—	—
Asp76	Asp76Oδ1—His74Nε2	2.94
Glu83	Glu83Oε2—Arg59Nη2	3.30
	Glu83Oε1—Gln62Nε2	3.96
Asp90	Asp90Oδ2—Gln52Oε1	2.75
	Asp90Oδ2—Gln52Nε2	3.69
Asp93	Asp93Oδ2—Phe94N	3.09

[a] Data were obtained from the common-type acylphosphatase crystal structure (19). The distance between two residues was calculated using the program WHATIF (28).

The interpretation of these data requires a careful evaluation of the interactions formed by each residue within the native conformation of the protein. Table III reports the hydrogen bonds and salt bridge interactions formed by each residue in the native conformation as deduced by the crystal structure of CT AcP (19). Some of the acidic residues form one or two hydrogen bonds with the side chain or the backbone NH groups of residues of other nature such as arginine, lysine, threonine, phenylalanine, tryptophan, etc. Five of the acidic residues are spatially located near basic residues and presumably participate in the formation of a salt bridge. Because of the superficial location of nearly all carboxylic residues of the protein, the hydrophobic interactions are not considered in our analysis, except where otherwise specified. The loss of a salt bridge is not a major determinant of the observed destabilization caused by amino acid replacement. Indeed, the destabilization observed following elimination of a residue that participates in the formation of a salt bridge is not, on average, higher than that observed for residues that are not involved in ionic bonds (cf. Table II and Table III). By contrast, the loss or reorganization of hydrogen bonds appears to be more important. Figure 3 shows the statistical correlation between changes in conformational stability upon glutamate to glutamine substitution (or aspartate to asparagine) and the number of hydrogen bonds formed by the mutated residue. A significant correlation is found, as inferred by a linear correlation coefficient of 0.75. In some cases it is likely that these amino acid substitutions leave the protein substantially unchanged with the same number of hydrogen bonds and similar mutual distances between the atoms, especially in a molecule such as CT AcP where the acidic residues are largely solvent exposed. Nonetheless, it might happen that the hydrogen bonds network formed in the mutated proteins is somewhat distorted, resulting in a destabilization of the native state. Glu12 has been omitted from the analysis carried out in Fig. 3 because the side chain proximal end of this residue is closely packed to the aromatic ring of Phe14. Such hydrophobic interaction is supposedly very important in determining the contribute of Glu12 to the overall conformational stability of the protein.

Another important aspect is the stabilization of CT AcP generated by the elimination of Glu66. This stabilization can be ascribed to the vicinity of such residue to Glu63 in the wild-type protein (5.8 Å). In a modeling study performed on the structure of CT AcP (see Materials and Methods) the replacement of Glu66 causes the two residues at positions 63 and 66 to be so close (3.3 Å) as to allow the formation of a new hydrogen bond. In this respect, CT AcP has been found to be stabilized upon increasing the ionic strength, suggesting that the alleviation of electrostatic repulsion may give rise to an increased conformational stability of the protein (22).

Relationship between Enzymatic Activity and Conformational Stability

It seemed interesting to evaluate the possible use of enzymatic activity measurements as a probe to gain insight into the effects of the mutations on CT AcP structure and conformational stability. The enzymatic activity of CT AcP is very sensitive to minimal rearrangements of the structure of the protein and thus provides a valuable tool to assess easily whether the effects of mutations are localized or more extended.

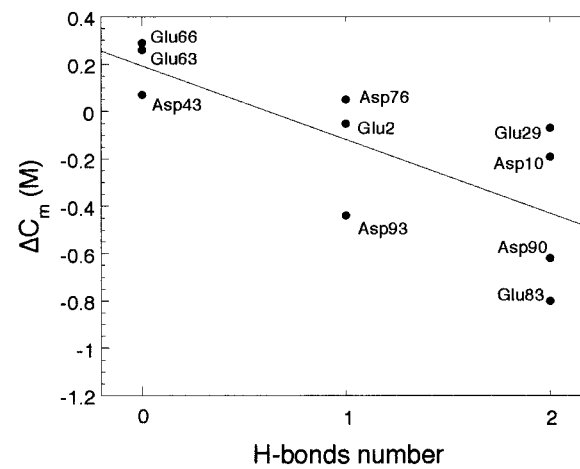

FIG. 3. Linear correlation between ΔC_m and the number of hydrogen bonds.

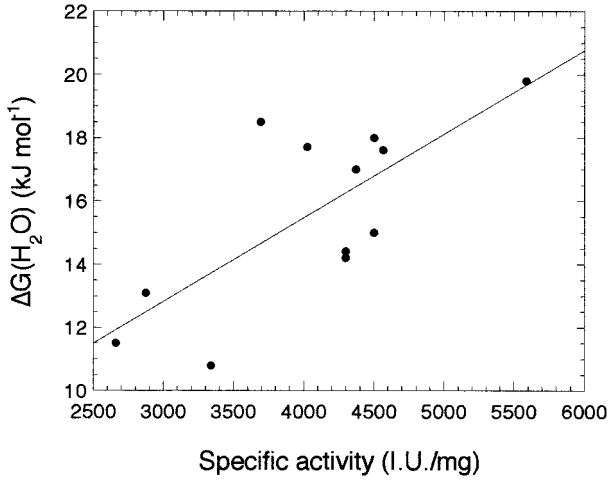

FIG. 4. Relationship between specific activity and $\Delta G(H_2O)$ for both wild type and mutants of CT AcP.

Fig. 4 illustrates the correlation between the specific activity and the free energy change of unfolding (the linear correlation coefficients is 0.75). Since all the mutations are far away from the catalytic site of the protein, this relationship provides further evidence that the structural effects of mutations are not strictly localized and are likely to propagate up to the active site of the molecule. Interestingly, the Glu66Gln mutant features an increase of both stability and enzymatic activity (Table I and Table II). It is tempting to speculate that the elimination of a destabilizing residue such as the Glu66 introduces more favorable interactions giving rise to a higher rigidity of the native conformation and to a better positioning of the groups in the catalytic site.

CONCLUSIONS

While salt bridges do not seem to contribute largely to the conformational stability of CT AcP, the surface hydrogen bonding appears to play a significant role. Although the hydrophobic effect is not the focus of the present work, the largest destabilization in our set of mutants has been found for the mutant of Glu12, a residue partially buried in the hydrophobic core. The importance of hydrophobic interactions and hydrogen bonding in maintaining the native and functional conformation of a protein is now well acknowledged (6, 10). Our study suggests that the surface salt bridges do not play a relevant role in protein stability. In this respect, our data are in close agreement with those emerging from the investigation of other proteins (6, 14, 39–41). Nevertheless, the introduction of conservative mutations and their repercussion on the enzymatic activity address the importance of molecular distortions in determining the causes of protein destabilization.

ACKNOWLEDGMENTS

The work has been supported by funds from Italian CNR (Progetto Finalizzato "Biotecnologie"), from MURST (Progetto "Biologia Strutturale"), and from European Community (Contract ERB BIO4-CT96-0517).

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