Thermodynamics of a Transition State Analogue Inhibitor Binding to *Escherichia coli* Chorismate Mutase: Probing the Charge State of an Active Site Residue and Its Role in Inhibitor Binding and Catalysis[†,‡]

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ABSTRACT: Electrostatic interactions play important roles in the catalysis of chorismate to prephenate by chorismate mutase. Mutation of Gln88 to glutamate in the monofunctional chorismate mutase from *Escherichia coli* results in an enzyme with a pH profile of activity significantly different from that of the wild type protein. To investigate whether the mutation alters the substrate binding process or the catalysis, we have directly determined the thermodynamic parameters of a transition state analogue inhibitor binding to the wild-type chorismate mutase and its Q88E mutant using isothermal titration calorimetry. The results demonstrate that solvent reorganization and hydrophobic interactions contribute the predominant free energy to inhibitor binding. The charge state of Glu88 in the Q88E mutant was experimentally determined and was shown to be protonated at pH 4.5 and ionized at pH 7.8, consistent with earlier hypotheses. Most surprisingly, inhibitor binding energetics do not exhibit significant pH dependency for both enzymes. Our findings indicate that the charge state of Glu88 has a small impact on inhibitor binding but plays an important role in the catalytic process.

Chorismate mutase (CM)[1] catalyzes the Claisen rearrangement of chorismate to prephenate, which represents the first committed step in the biosynthesis of aromatic amino acids in bacteria, fungi, and higher plants (*1*). Despite sharing the same enzymatic function, CMs from different species demonstrate distinct characteristics. For example, CM from *Saccharomyces cerevisiae* (ScCM) exhibits maximum activity at pH 5.5 but virtually no activity at pH 7.8, whereas the monofunctional CM from *Escherichia coli* (WT) displays similar activities at both acidic and neutral pH (*2*, *3*). Structural information based on X-ray crystallographic analysis has recently been obtained for chorismate mutases from three different species [see the review by Lee et al. (*4*)]. Active site comparisons between WT and ScCM reveal that Gln88 in WT is replaced by Glu246 in ScCM. To maintain the same total charge at the active site, the carboxylate group of Glu246 in ScCM is inferred to be protonated in the active enzyme, and this substitution at the active site has been proposed to account for the different pH profiles for these two enzymes (*5*). On the basis of the structural information, mutagenesis studies of the active site residues have shown that electrostatic and polar interactions play important roles in catalysis (*3*, *6*−*8*). Consistent with the hypothesis of Xue and Lipscomb (*5*), mutation of Gln88 to glutamate in WT results in an inactive enzyme at pH >7.5 but enhances the enzyme activity at pH 4.5 (*3*, *8*).

Notwithstanding structural and kinetic insights, important questions about the pH dependency of CM activity remain unanswered. First, can the proposed charge states of Glu88 in the Q88E mutant of WT (Q88E) and Glu246 in ScCM be experimentally verified? Second, is the loss of enzymatic activities for ScCM and Q88E at pH 7.8 due to the weakened substrate binding or to the inability of the negatively charged carboxylate groups to coordinate catalysis in the two enzymes? Recent thermodynamic studies have shown that inhibitor binding to proteinases is highly pH-dependent and is controlled by the charge states of the active site residues (*9*, *10*). Accordingly, different protonation states of Glu88 in Q88E could affect both substrate binding and chemical conversion.

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[1] Abbreviations: ITC, isothermal titration calorimetry; CM, chorismate mutase; ScCM, chorismate mutase from *Saccharomyces cerevisiae*; WT, monofunctional wild type chorismate mutase from *Escherichia coli*; Q88E, Gln88Glu mutant of WT; TSI, transition state analogue inhibitor.

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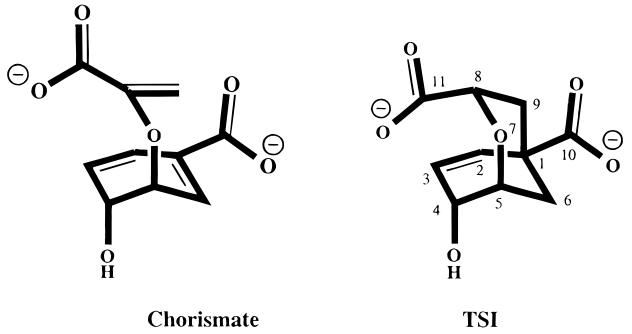
Chart 1



Chorismate **TSI**

Isothermal titration calorimetry (ITC) can be used to measure the proton exchange between the binding complex and the buffer upon inhibitor association (*11*, *12*). To address the role of the protonation state of Glu88 in the catalytic mechanism of CM, we performed an ITC study on the binding of a transition state analogue inhibitor (TSI) (Chart 1) (*13*) to WT and to its Q88E mutant. By comparing the proton exchange for inhibitor binding to WT versus that for the Q88E mutant, we were able to determine the charge state of Glu88 in the mutant enzyme. Moreover, thermodynamic data from different pHs allowed us to characterize the different energetic effects of the protonated and ionized states of the Glu88 carboxylate group on inhibitor binding.

EXPERIMENTAL PROCEDURES

WT and the Q88E mutant were purified from pJS47 and pSZ30 as reported previously (*8*, *14*). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard.

Microcalorimetry experiments were carried out in the appropriate buffer at 20 mM containing 0.1 M NaCl at either pH 7.8 or 4.5. The protein samples were prepared by dialysis against 2 L of the desired buffer solution overnight at 4 °C. Final enzyme concentrations were in the range of 37−92 μM. The transition state analogue inhibitor was dissolved in buffer to a concentration of either 1.4 or 2.5 mM. The titration calorimetry experiments were carried out with an OMEGA titration microcalorimeter from MicroCal, Inc. (*15*). The data were analyzed using the program ORIGIN (MicroCal, Inc.) incorporating equations for multiple independent binding sites (*16*). In all cases, the fitted number of binding sites is consistent with a stoichiometric binding, ranging from 0.85 to 1.1.

RESULTS

Thermodynamics of TSI Binding. Figure 1 shows a typical ITC profile where each peak represents the injection of 8 μL of 2.5 mM TSI into a 43.1 μM WT solution in a 1.4 mL calorimeter cell. At pH 7.8 and 30 °C, the binding of TSI to WT in HEPES buffer is characterized by a ΔH of −3.7 ± 0.1 kcal/mol and a K_d of 3.4 ± 0.5 μM. The temperature dependence of the thermodynamic parameters was determined by carrying out ITC measurements at different temperatures (Figure 2). This analysis gave ΔC_p values of −88 and −181 cal mol^{-1} K^{-1} for WT and Q88E, respectively. The results allowed us to plot the temperature profile of the dissociation constants for inhibitor binding for the two enzymes (Figure 3). With a more profound temperature dependence, Q88E exhibits an equilibrium dissociation constant at least 3-fold greater than that of WT at all temperatures. Thermodynamic binding parameters determined at pH 4.5 and 7.8 for both enzymes are summarized in Table 1. At both pHs, TSI binds to Q88E more weakly than to WT. In terms of ΔG, the mutation destabilizes the binding complex by 0.4 kcal/mol at pH 4.5 and by 1.0 kcal/mol at pH 7.8. The destabilization is the result of an enthalpy

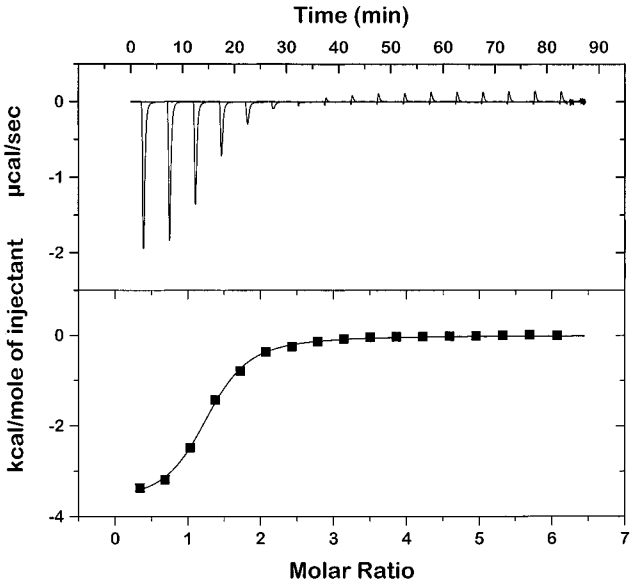


FIGURE 1: Isothermal calorimetric titration of TSI binding to WT at pH 7.8 and 30.0 °C. (Top) Measured heats of injection. Each peak represents the injection of 8 μL of TSI into a solution of WT. The concentrations of WT and TSI were 43.1 μM and 2.5 mM, respectively, in 20 mM HEPES and 0.1 M NaCl at pH 7.8. (Bottom) Calorimetry binding enthalpy vs molar ratio of TSI to WT for each injection. The heats were determined by integration of the injection peak and subtraction of the mixing heat. The solid line represents the best nonlinear least-squares fit. The obtained thermodynamic parameters for this association process were as follows: ΔH = −3.7 ± 0.1 kcal/mol and K_d = 3.4 ± 0.5 μM, with a binding stoichiometry of 1.1 ± 0.1 (TSI:WT enzyme). The error of each parameter represents the error of fitting.

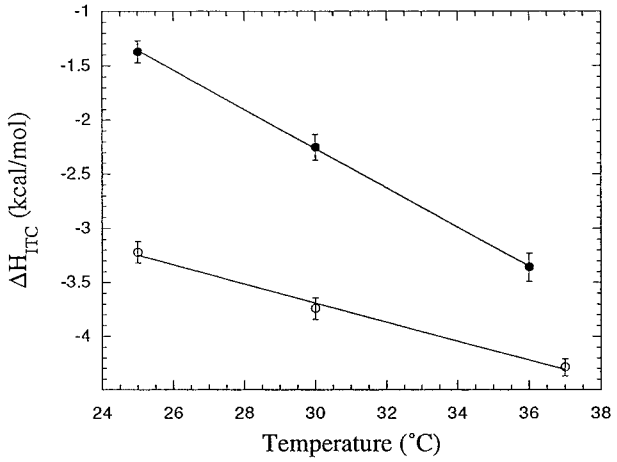


FIGURE 2: Temperature dependence of binding enthalpy. The open and closed circles represent the binding enthalpies of WT−TSI at pH 7.8 and Q88E−TSI at pH 4.5, respectively. Over a narrow temperature range, the temperature dependence of ΔH can be described by $\Delta H = \Delta H_o + \Delta C_p(T - T_o)$, where ΔH_o is the binding enthalpy at an arbitrary reference temperature T_o and ΔC_p is the heat capacity change of binding. The ΔC_p values determined from the slopes of the linear fits were −88 cal mol^{-1} K^{-1} for WT and −181 cal mol^{-1} K^{-1} for Q88E.

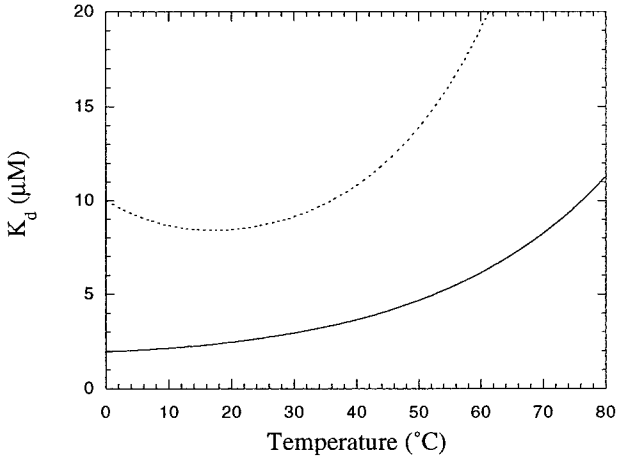


FIGURE 3: Temperature dependence of the equilibrium dissociation constant. On the basis of the data in Figure 2, the K_d values were calculated as functions of temperature for TSI binding to WT at pH 7.8 (solid line) and for Q88E at pH 4.5 (broken line).

Table 1: Thermodynamic Parameters for TSI Binding to WT and Its Q88E Mutant at 25 °C[a]

protein	K_a ($\times 10^4$ M^{-1})	K_d (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	ΔC_p (cal mol^{-1} K^{-1})
pH 7.8						
WT	35 ± 2	2.8	-3.2 ± 0.1	-4.4	-7.6	-88 ± 35
Q88E	7.2 ± 1.4	13.9	-0.8 ± 0.3	-5.8	-6.6	nd[b]
pH 4.5						
WT	22 ± 7	4.5	-2.6 ± 0.1	-4.7	-7.3	nd[b]
Q88E	11 ± 2	9.1	-1.4 ± 0.1	-5.5	-6.9	-181 ± 76

[a] The buffers were 20 mM HEPES and 0.1 NaCl at pH 7.8 and 20 mM sodium acetate and 0.1 M NaCl at pH 4.5. [b] nd, not determined.

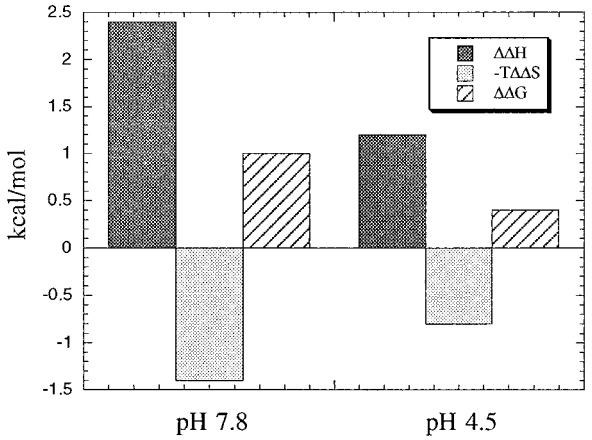


FIGURE 4: Differences in thermodynamic parameters for TSI binding to Q88E and WT at 25 °C. The data were calculated using the calorimetry results in Table 1.

less favorable by 1.2−2.4 kcal/mol that compensates for a slightly favorable entropy (Table 1 and Figure 4). Since formation of a hydrogen bond in water usually has a ΔH of −2.4 kcal/mol and a ΔG of −0.5 to −1.5 kcal/mol (*17*−*19*), in the absence of a mutation-induced conformational change (*3*, *8*), the energetic difference between WT and the mutant complexes at pH 7.8 corresponds to the loss of approximately one hydrogen bond.

As shown in Table 1, the entropy term constitutes 58−88% of the favorable binding free energy in all cases. At 25 °C, the formation of polar interactions has a favorable (negative) enthalpy and an unfavorable entropy, while the burial of nonpolar groups and the concomitant solvent reorganization have a positive enthalpy and a favorable entropy (*20*, *21*). Since the configurational entropy change of the rigid inhibitor should be small, the favorable entropy of inhibitor binding mainly results from the burial of nonpolar groups in the active site and the concomitant release of ordered water molecules due to solvent reorganization. In conclusion, the calorimetry results indicate that, despite the important role of the electrostatic and hydrogen bonding interactions in catalysis (*3*, *8*, *22*), solvent reorganization and hydrophobic interactions are the major driving forces for inhibitor binding. For Q88E, the small contribution of polar interactions to the overall binding energy may explain why TSI binding is insensitive to the charge state of Glu88.

Another interesting finding of the calorimetry data is that the bindings of TSI to both enzymes are relatively insensitive to pH. Between pH 4.5 and 7.8, ΔG varies by only 0.3 kcal/mol. While WT displays similar properties of inhibitor binding and enzymatic activity at acidic and neutral pHs, Q88E exhibits a significant pH dependency for substrate catalysis in this pH range, in sharp contrast to the pH-independent inhibitor binding.

Proton Exchange and the Charge State of Glu88 in Q88E. Upon inhibitor binding, ionizable groups in the reactants could undergo pK_a changes. At an appropriate pH, a pK_a change will result in proton transfer between the ionizable group and buffer molecule. Consequently, the binding enthalpy measured by a single ITC experiment is dependent on the ionization enthalpy of the buffer (*9*, *10*, *23*). The calorimetrically measured enthalpy, ΔH_{ITC}, can be described by

$$\Delta H_{ITC} = \Delta H_{binding} + \Delta n \Delta H_{ion} \quad (1)$$

where Δn is the number of protons transferred upon inhibitor binding, ΔH_{ion} represents the ionization enthalpy of buffer, and $\Delta H_{binding}$ is the buffer-independent binding enthalpy. For the association of TSI with WT and Q88E, $\Delta H_{binding}$ was determined by measuring ΔH_{ITC} for a spectrum of buffers with different ionization enthalpies (Figure 5 and Table 2). At pH 7.8 and 25 °C, neither enzyme undergoes any significant proton transfer upon TSI binding. Since Δn represents the change in charge states of ionizable groups upon inhibitor binding, the difference between the Δn values for WT and Q88E may result from either the different charge states of TSI in the two complexes or the different alterations in the charge states of Gln88 in WT and Glu88 in Q88E upon TSI association.

First, TSI contains two carboxylate groups that can undergo pK_a changes that are concomitant with binding to the enzyme. In the crystal structure, these carboxylate

Table 2: Proton Exchange and the Buffer-Independent Inhibitor Binding Energetics at 25 °C[a]

protein	K_d (μM)	$\Delta H_{binding}$ (kcal/mol)	Δn
pH 7.8			
WT	2.8	-3.8 ± 0.2	0.2 ± 0.1
Q88E	13.9	-2.0 ± 0.4	0.1 ± 0.1
pH 4.5			
WT	4.5	-2.2 ± 0.3	-3.0 ± 0.2
Q88E	9.1	-1.3 ± 0.4	-1.8 ± 0.3

[a] $\Delta H_{binding}$ and Δn were the buffer-independent parameters defined in eq 1.

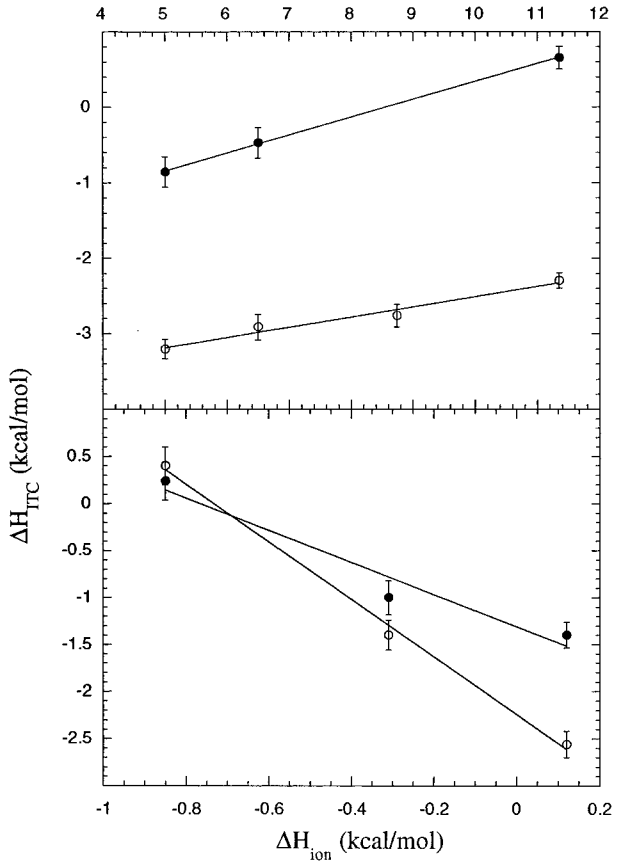


FIGURE 5: Calorimetrically measured binding enthalpy in different buffers. The open and closed circles represent the data for WT and Q88E, respectively. ΔH_{ion} is the ionization enthalpy of buffer: (top) experiments performed at pH 7.8 and 25 °C and (bottom) experiments performed at pH 4.5 and 25 °C. The buffers (sodium salt) and their ionization enthalpies (in kilocalories per mole) at 25 °C are as follows: HEPES (pH 7.8), 5.0; bicine, 6.5; imidazole, 8.7; TRIS, 11.4; acetate (pH 4.5), 0.12; succinate, −0.31; and citrate, −0.85. The ΔH_{ion} values of succinic acid and citric acid were determined in this laboratory, and those of other buffers were taken from the work of H. Fukata and K. Takahashi (personal communication) and from ref *27*. Using eq 1, we were able to determine the buffer-independent binding enthalpy and the number of transferred protons (Table 2).

oxygens are 5.3 and 5.4 Å from the side chain of Gln88. A model of Q88E suggests that the side chain of Glu88 can be accommodated in a location similar to that of Gln88 in WT (*8*). Consequently, the charge state of the carboxylate groups in TSI should be the same when the inhibitor binds to WT and Q88E. Second, Gln88 in WT is not able to contribute to Δn at either pH 7.8 or 4.5, with or without any pK_a change. Taken together, $\Delta\Delta n$, the difference in Δn between Q88E and WT, must correspond to the change in the charge state of Glu88 in Q88E. As shown in Table 2, there is no difference, within experimental error, between the Δn values of WT and Q88E at pH 7.8. The charge state of Glu88 in the Q88E mutant apparently does not change upon TSI association. Although the active site is buried inside the protein, Glu88 is still accessible to the solvent, suggesting that Glu88 in both the bound and unbound forms of Q88E is deprotonated at pH 7.8.

At pH 4.5 and 25 °C, where WT and Q88E exhibit similar enzymatic activities, the Δn values for WT and Q88E are −3.0 and −1.8, respectively. The negative sign of Δn indicates that upon TSI binding, protons are released from the binding complex and transferred to buffer. To release a proton at pH 4.5, an ionizable group on the reactants must have a lower pK_a in the buried complex. The difference between the Δn values indicates that there is approximately one less proton transferred to the buffer for the binding of TSI to Q88E than for binding to WT. Since $\Delta\Delta n$ corresponds to the change in the charge state of Glu88 in Q88E, these data indicate that the pK_a of Glu88 increases upon TSI binding, resulting in an uptake of a proton that offsets the protons released from common ionizable groups between the two sets of reactants.

DISCUSSION

Titration of active site residues has long been used to establish the pH dependence of enzyme activity. The determination of whether the substrate binding or catalysis step is affected by the titration requires detailed mechanistic studies. A previous kinetic study of the bifunctional T-protein from *E. coli* at different pHs attempted to identify titrated active site residues (*24*). Alternatively, ITC provides a direct measurement of inhibitor binding energetics and protonation state changes, and it can be used to obtain information about the inhibition mechanism. Comparison of the wild type and mutant enzymes can thus reveal differences in the energetics of inhibitor binding and in the active site charge states.

In this study, the binding of TSI to WT and Q88E was measured using ITC at pH 4.5 and 7.8. Almost no proton transfer was observed upon inhibitor binding to either enzyme at pH 7.8. Since Glu88 in the Q88E mutant should be ionized in the uninhibited enzyme, the calorimetry data indicate that Glu88 maintains its negatively charged state in the inhibitor-bound complex. At pH 4.5, 1.2 fewer protons were released from Q88E than from WT, suggesting that Glu88 in Q88E exhibits an increase in pK_a upon TSI binding and gains a proton to become fully protonated in the binding complex. Since the mutant protein is active at pH 4.5 and inactive at pH 7.8, our results show that a protonated Glu88 is required for the enzyme activity, consistent with the earlier structural and enzymatic studies (*5*, *6*, *8*, *22*).

In contrast to the pH dependence of enzyme activity, the thermodynamic parameters (ΔG, ΔH, and ΔS) for inhibitor binding to Q88E as well as to WT show little dependence on pH. Since the inhibitor used in these studies has a charge state similar to that of the natural substrate (chorismate), our results suggest that substrate binding should not vary significantly with pH. This conclusion is at first glance not consistent with the previous kinetic results (*3*, *8*) where the K_m of Q88E is strongly dependent on pH. As noted in those studies, the mutant enzyme could not be saturated by the substrate at pH 7.8, indicating a significant increase in K_m from pH 4.5. The findings that K_d for TSI is independent of pH but K_m for chorismate is pH-dependent may be understood using the standard Michaelis−Menten mechanism depicted in eq 2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_{cat}} E + P \qquad (2)$$

where

$$K_m = k_{-1}/k_1 + k_{cat}/k_1 \qquad (3)$$

From the Michaelis−Menten formalism, it is obvious that K_m will not in general be equivalent to K_d (k_{-1}/k_1). Since the K_d for TSI binding is pH-independent, we can assume that the K_d for substrate binding is as well. Therefore, one can deduce that the pH dependence of K_m for Q88E is determined by the second term (k_{cat}/k_1). It follows that fluctuations in k_1 as a function of pH must be accompanied by corresponding changes in k_{-1} for K_d to remain constant. It is interesting to note that for Q88E, K_m is much higher at pH 7.8 than at pH 4.5, while k_{cat} has a lower value at pH 7.8. According to eq 3, k_1 should have an even more drastic decrease at higher pH. This type of pH dependency of k_1 is not uncommon. Long-range electrostatic interactions can accelerate association by a factor of up to 10^5-fold in the barstar−barnase complex for example (*25*). The invariance of K_d with pH suggests that net electrostatic interactions leading to TSI binding are largely unaffected at pH 4.5 and 7.8. Consistent with this conclusion, the enthalpy and entropy values for TSI association indicate that, for Q88E, the release of ordered water molecules from the active site and the hydrophobic interactions between the reactants are the driving force of binding, thus explaining why inhibitor binding is insensitive to the charge state of Glu88.

We conclude that the low activity of Q88E at pH 7.8 is not due to a reduced affinity of the substrate, and a protonated Glu88 in Q88E is essential for catalysis, further confirming the hypothesis that Gln88 in WT and Glu88 in Q88E act as hydrogen bond donors in the catalytic mechanism of chorismate mutase (*3*, *6*, *8*, *22*).

We also examined the origin of the unfavorable influence of the Q88E mutation on ligand binding energetics at pH 7.8 (Table 2). Barring any mutation-induced structural change in the enzyme, which seems unlikely since WT and Q88E have superimposable circular dichroism spectra (*8*), the energetic difference between WT and Q88E represents a localized effect of the mutation on inhibitor binding. At pH 7.8, the ΔG and ΔH of TSI binding to Q88E are 1.0 and 2.4 kcal/mol, respectively, less favorable than those for binding to WT. The unfavorable enthalpy change is offset by a more favorable ΔS of 1.4 kcal/mol. We have shown that Glu88 is deprotonated in Q88E at pH 7.8, and Gln88 in WT forms a hydrogen bond with O7 in TSI (*26*). Thus, the measured $\Delta\Delta G$ of 1.0 kcal/mol is an estimate of either the energetics of the added negative charge or the loss of a hydrogen bond at the inhibitor binding site and is consistent with the reported ΔG values of breaking a hydrogen bond in water (0.5−1.5 kcal/mol) (*17*−*19*).

Finally, we analyzed the known crystal structure of the TSI−WT complex for possible groups that are responsible for the observed release of three protons upon TSI binding at pH 4.5. To release a proton, the involved ionizable groups should exhibit decreases in pK_a from a value of >4.5 prior to inhibitor binding to a value of <4.5 after the formation of the complex. In this pH range, the potential candidates are the two carboxylate groups of TSI, as well as the side chains of aspartic acid, glutamic acid, and histidine residues in the enzyme. Because there are three protons released, at least one ionizable group should be located in the enzyme. In the absence of an inhibitor-induced structural change in the protein, these residues are most likely located in the active site. Inspection of three-dimensional structure of the WT−TSI complex (*26*) reveals that the side chain of Glu52 is the only possible candidate. There are no other side chains of aspartate, glutamate, or histidine involved in inhibitor binding. Therefore, under the assumption that there is no inhibitor-induced structural change in the enzyme, the calorimetry results suggest that the two carboxylates in TSI and Glu52 in WT are protonated at pH 4.5 in their unliganded state and become ionized in the enzyme−inhibitor complex. Consistent with this, the crystal structure shows that the carboxylate group of Glu52 forms a hydrogen bond with the C4 hydroxyl group of TSI (*26*), suggesting that Glu52 is charged. Enzymes with mutations of Glu52 to alanine and glutamine exhibit reduced but significant activity at pH 7.5, arguing against an important role of the charge state of Glu52 in catalysis (*3*). Whether the negative charge of Glu52 has impact on substrate and inhibitor binding awaits further investigation.

CONCLUSIONS

By monitoring the effect of proton transfer on inhibitor binding, we have shown that Glu88 in Q88E exhibits different charge states at pH 4.5 and 7.8. Although crystallographic data reveal the role of electrostatic interactions in orienting TSI within the active site, thermodynamic parameters for TSI association indicate that solvent reorganization and hydrophobic interactions are the major driving forces for inhibitor binding. In addition, the calorimetry results suggest that the protonation state of Glu88 is unimportant for substrate binding. In the absence of a mutation-induced conformational change (*3*, *8*), the energetic difference between the more stable TSI−WT complex and its TSI−Q88E congener at pH 7.8 corresponds to the loss of approximately one hydrogen bond. These observations may have an impact on designing a novel series of potent inhibitors.

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REFERENCES

1. Haslam, E. (1993) *Shikimic Acid: Metabolism and Metabolites*, Wiley, New York.
2. Schmidheini, T., Mosch, H.-U., Evans, J., and Braus, G. (1990) *Biochemistry 29*, 3660−8.
3. Liu, D. R., Cload, S. T., Pastor, R. M., and Schultz, P. G. (1996) *J. Am. Chem. Soc. 118*, 1789−90.
4. Lee, A. Y., Stewart, J. D., Clardy, J., and Ganem, B. (1995) *Chem. Biol. 2*, 195−203.
5. Xue, Y., and Lipscomb, W. N. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10595−8.
6. Schnappauf, G., Sträter, N., Lipscomb, W. N., and Braus, G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8491−6.
7. Cload, S. T., Liu, D. R., Pastor, R. M., and Schultz, P. G. (1996) *J. Am. Chem. Soc. 118*, 1787−8.
8. Zhang, S., Kongsaeree, P., Clardy, J., Wilson, D., and Ganem, B. (1996) *Bioorg. Med. Chem. 4*, 1015−20.
9. Baker, B., and Murphy, K. (1996) *J. Mol. Biol. 268*, 557−69.
10. Xie, D., Gulnik, S., Collins, L., Suvorov, L., Lee, A., Gustchina, E., and Erickson, J. (1997) *Biochemistry 36*, 16166−72.
11. Murphy, K. P., Xie, D., Garcia, K. C., Amzel, L. M., and Freire, E. (1993) *Proteins: Struct., Funct., Genet. 15*, 113−20.
12. Doyle, M. L., Louie, G., Dal Monte, P. R., and Sokoloski, T. D. (1995) *Methods Enzymol. 259*, 183−94.

13. Bartlett, P. A., and Johnson, C. R. (1995) *J. Am. Chem. Soc. 107*, 7792.
14. Stewart, J. D., Wilson, D. B., and Ganem, B. (1991) *Tetrahedron 47*, 2573.
15. Wiseman, T., Williston, S., Brandt, J., and Lin, L. (1989) *Anal. Biochem. 179*, 131−5.
16. Freire, E., Mayorga, O. L., and Straume, M. (1990) *Anal. Chem. 62*, 950A−9A.
17. Habermann, S., and Murphy, K. (1996) *Protein Sci. 5*, 1229−39.
18. Pace, N. (1995) *Methods Enzymol. 259*, 538−54.
19. Kato, Y., Conn, M., and Rebek, J. J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1208−12.
20. Murphy, K. P., and Freire, E. (1992) *Adv. Protein Chem. 43*, 313−61.
21. Murphy, K. P., and Gill, S. J. (1991) *J. Mol. Biol. 222*, 699−709.
22. Galopin, C. C., Zhang, S., Wilson, D. B., and Ganem, B. (1996) *Tetrahedron Lett. 37*, 8675−8.
23. Xie, D., Gulnik, S., Collins, L., Gustchina, E., Bhat, T. N., and Erickson, J. W. (1997) in *Structure and Function of Aspartic Proteinases: Retroviral and Cellular Enzymes* (James, M., Ed.) pp 381−6, Plenum, New York.
24. Turnbull, J., Cleland, W. W., and Morrison, J. F. (1991) *Biochemistry 30*, 7777−82.
25. Janin, J. (1997) *Proteins: Struct., Funct., Genet. 28*, 153−61.
26. Lee, A. Y., Karplus, P. A., Ganem, B., and Clardy, J. (1995) *J. Am. Chem. Soc. 117*, 3627−8.
27. Christensen, J. J., Hansen, L. D., and Izatt, R. M. (1976) *Handbook of Proton Ionization Heats and Related Thermodynamic Quantities*, Wiley, New York.

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