

## Review

## Marcus rate theory applied to enzymatic proton transfer

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**Abstract**

The hydration of  $\text{CO}_2$  and the dehydration of  $\text{HCO}_3^-$  catalyzed by the carbonic anhydrases is accompanied by the transfer of protons between solution and the zinc-bound water molecule in the active site. This transfer is facilitated by amino acid residues of the enzyme which act as intramolecular proton shuttles; variants of carbonic anhydrase lacking such shuttle residues are enhanced or rescued in catalysis by intermolecular proton transfer from donors such as imidazole in solution. The resulting rate constants for proton transfer when compared with the values of the  $\text{p}K_a$  of the donor and acceptor give Brønsted plots of high curvature. These data are described by Marcus theory which shows an intrinsic barrier for proton transfer from 1 to 2 kcal/mol and work terms or thermodynamic contributions to the free energy of reaction from 4 to 10 kcal/mol. The interpretation of these Marcus parameters is discussed in terms of the well-studied pathway of the catalysis and structure of the enzymes. © 2000 Elsevier Science B.V. All rights reserved.

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**1. Introduction**

Rudolph A. Marcus originally devised the theory which now bears his name to explain the rate of electron transfer between ions in solution (reviewed in [1]). This was a major contribution to efforts of many researchers unfolding over decades to understand the relation between the equilibrium constant and the rate constant for a chemical process. The theory he devised and for which he was awarded the Nobel Prize in 1992 has since been expanded to describe rates not only of electron transfers but of the transfer of protons, deuterons, hydride ions, and in systems extending from ions in solution to complex biological macromolecules. Although the appli-

cation of Marcus theory to electron transfer in proteins has received considerable attention [2–4], the application of Marcus theory to proton transfer in proteins has not developed as rapidly, in part because of the very great complexity of the protein systems in which proton transfers play a role, the difficulty in measuring rates of proton transfer, and the difficulty in identifying the specific proton donors and acceptors. However, current studies on many systems have made significant advances in understanding the possible pathways for proton transfer. These include bacteriorhodopsin [5], cytochrome *c* oxidase [6] and the bacterial photosynthetic reaction center [7]. However, the biological system which has to date provided the most informative subject for application of Marcus theory to proton transfer is carbonic anhydrase, which is a main subject of this review. In carbonic anhydrase, an enzyme which has been well-studied for over six decades, the identity of

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the proton donors and acceptors is known and in many cases their properties are straightforward to measure.

## 2. The Marcus rate theory applied to proton transfer

Many of the fundamental principles that have lead to our current understanding of proton transfer processes were reviewed by R.P. Bell [8], and E.F. Caldin and V. Gold [9] in influential books. Among early advances is the Brønsted equation, a successful attempt to establish a correlation between rate constants and the free energy difference between reactants and products. The Brønsted relation applied to proton transfer correlates rate constants for proton transfer  $k_B$  with the difference in acid or base strength of the acceptor and donor, as shown in Eq. 1 [10].

$$\log(k_B) = \beta[pK_a(\text{acceptor}) - pK_a(\text{donor})] + \text{constant} \quad (1)$$

The slope  $\beta$  of a Brønsted plot of  $\log(k_B)$  vs.  $\Delta pK_a$  can be used to characterize a reaction mechanism and to compare proton transfer processes; there is substantial theoretical and practical support that  $\beta$  provides an estimate of the extent of proton transfer in the transition state [11]. Application of the Brønsted plot to proton transfer in a protein has been limited because of chemical and structural constraints at an enzyme's active site, but significant advances have been made. Toney and Kirsch [12] replaced a lysine residue with alanine in the active site of aspartate aminotransferase and showed that enhancement of catalysis by external amines follows a Brønsted relation. Such a plot for intermolecular proton transfer from a donor site on carbonic anhydrase to buffers in solution [13,14] shows a region of slope near unity at  $\Delta pK_a \ll 0$  and a plateau at the diffusion-controlled limit at  $\Delta pK_a \gg 0$ , very similar to that observed by Eigen [15] for non-enzymic, bimolecular proton transfer between nitrogen and oxygen acids and bases. These studies note the interesting observation of marked curvature in a Brønsted plot, indicating a low kinetic barrier for the proton transfer reaction as in a diffusion-controlled process. This indicates that the transition state in the series of

reactions changes from reactant-like to product-like over a rather narrow range of two or three  $pK_a$  units.

An advantage of Marcus theory is to place these observations more directly in line with chemical events. Marcus originally derived the theory by quantum mechanical approaches to electron transfer between ions [1,16], and applied these ideas later to proton transfers [17]. However, the basic relationship of Marcus theory can also be derived from different models of proton transfer including the Leffler principle and Hammond postulate [18], intersecting parabolas describing the vibrational energy levels of donor and acceptor [19], and solvent polarization [20]. The theory provides a quantitation that defines an intrinsic kinetic barrier for catalysis and the means to separate thermodynamic contributions from the observed activation energy, an excellent description of which is provided by Kresge [21].

The observed overall activation energy for proton transfer  $\Delta G^\ddagger$  is given in Marcus theory by Eq. 2 which expresses  $\Delta G^\ddagger$  in terms of two variables, the standard free energy of reaction  $\Delta G^\circ$  and an intrinsic kinetic barrier  $\Delta G_o^\ddagger$  which is the value of  $\Delta G^\ddagger$  when  $\Delta G^\circ = 0$ ; that is when the transfer is free of thermodynamic influences and represents a pure or 'intrinsic' energy barrier.

$$\Delta G^\ddagger = (1 + \Delta G^\circ / 4\Delta G_o^\ddagger)^2 \Delta G_o^\ddagger \quad (2)$$

This simple form of the Marcus equation is modified to describe proton transfers in which there is a component of the observed activation barrier that does not depend on  $\Delta G^\circ$  for the reaction. This component is called the work term  $w^r$ . In non-enzymic, bimolecular proton transfers, the work term is considered part of the free energy of reaction needed to bring the reactants together, form the reaction complex, and reorganize the solvent structure prior to proton transfer. Similarly,  $w^p$  is the work term required for the reverse reaction.

$$\Delta G^\circ_{\text{obs}} = \Delta G^\circ + w^r - w^p \quad (3)$$

Substituting into Eq. 2 leads to the form of the Marcus equation more useful for proton transfers.

$$\Delta G^\ddagger_{\text{obs}} = w^r + (1 + [\Delta G^\circ_{\text{obs}} - w^r + w^p] / 4\Delta G_o^\ddagger)^2 \Delta G_o^\ddagger \quad (4)$$

Utilization of Eq. 4 assumes that the work terms

$w^r$  and  $w^p$  as well as the intrinsic energy barrier  $\Delta G_o^\ddagger$  do not vary for proton transfer between the series of homologous proton donors and acceptors to which the equation is fit. The observed activation barrier is obtained from  $k_B$ , the experimental rate constant for proton transfer,  $\Delta G_{obs}^\ddagger = -RT \ln(hk_B/kT)$ , where  $h$  is the Planck constant and  $k$  is the Boltzmann constant; and the observed free energy of reaction is obtained from  $\Delta pK_a$  of the reactants,  $\Delta G_{obs}^\circ = RT \ln[(K_a)_{acceptor}/(K_a)_{donor}]$ . Eq. 4 shows that  $\Delta G_{obs}^\ddagger$  is a quadratic function of  $\Delta G_{obs}^\circ$ ; that is, a plot of  $\ln(k_B)$  vs.  $\Delta pK_a$  is predicted to be parabolic with a slope at any point,  $d\Delta G_{obs}^\ddagger/d\Delta G_{obs}^\circ$ , equal to  $\beta$ , the Brønsted coefficient, and the curvature determined by  $d^2\Delta G_{obs}^\ddagger/d(\Delta G_{obs}^\circ)^2 = 1/8\Delta G_o^\ddagger$ .

### 3. Application to carbonic anhydrase

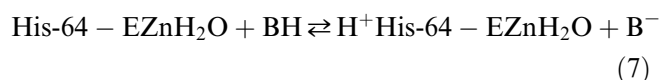
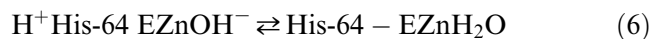
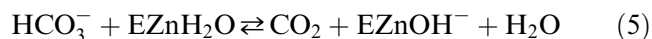
The carbonic anhydrases comprise three genetically distinct classes of zinc metalloenzymes, animal, plant and archaeal, that all catalyze the hydration of  $CO_2$  to produce bicarbonate and a proton [22–25]. Comments here are restricted to the animal or  $\alpha$  class which is by far the most studied. The carbonic anhydrases of the  $\alpha$  class are zinc-containing monomers with a molecular mass generally near 30 kDa which are involved in a number of physiological processes including respiration and formation of secretory fluids [26,27]. Catalysis by carbonic anhydrase is limited by proton transfers, and use of Marcus theory to understand the nature of these transfers has been described in other reviews [28,29].

The application of Marcus rate theory to proton transfer in the catalytic pathway of carbonic anhydrase required a number of preliminary experiments and considerations which are described below: identification of the appropriate proton donor and acceptor for each proton transfer step, measurement of the rate constant for proton transfer, and finally not only the determination of  $\Delta pK_a$  between the donor and acceptor groups but also finding conditions which cause  $\Delta pK_a$  to vary.

#### 3.1. Rate constant for proton transfer

Solvent hydrogen isotope effects (SHIEs) were the first indication that the maximum velocity or  $k_{cat}$  for

the catalysis of the hydration of  $CO_2$  by carbonic anhydrase II (CA II) was limited by an intramolecular proton transfer [30], reviewed in [25]. This and subsequent studies [31,32] showed that this catalysis occurred in two distinct and separate stages, Eqs. 5 and 6, with the proton transfer occurring in the second stage. The first stage in the dehydration direction is the conversion of bicarbonate into  $CO_2$  leaving hydroxide as a zinc-bound ligand (Eq. 5). The second stage is the regeneration of the zinc-bound water through intramolecular proton transfer required for the next cycle of dehydration (Eq. 6). Ultimately, the source of the transferred proton is buffer (BH) in solution (Eq. 7).



It was correctly suggested that His-64 is the intramolecular proton donor to the zinc-bound hydroxide in this catalysis since it is the only residue of the appropriate  $pK_a$  for this function extending into the active site cavity [30]. The most direct evidence for the role of His-64 as a proton shuttle came from its replacement by site-specific mutagenesis; the mutant with the replacement His-64  $\rightarrow$  Ala has  $k_{cat}$  for  $CO_2$  hydration lowered by about 20–50-fold [33]. Crystallographic studies up to 1.5 Å resolution show that there are two predominant side chain conformations of His-64 in CA II, shown in Fig. 1 [34–36]. At pH 8.5, the side chain was pointed into the active site toward the zinc with NE2 9.0 Å from the zinc. At lower pH 6.5, there is a 64° rotation about the side chain torsion angle  $\chi_1$  of His-64 to a conformation pointing out of the active site cavity and NE2 12 Å from the zinc. This orientation at the lower pH may be due to the repulsion between the imidazolium ion and  $Zn^{2+}$  as well as the possibility that the imidazolium ion may be more fully solvated in the outward conformation [37]. The orientation of this side chain was also sensitive to amino acid substitutions in the active site cavity, such as Thr-200  $\rightarrow$  Ser which causes the side chain of His-64 to move to a conformation pointing away from the zinc [38]. These studies do not estimate the energy barrier

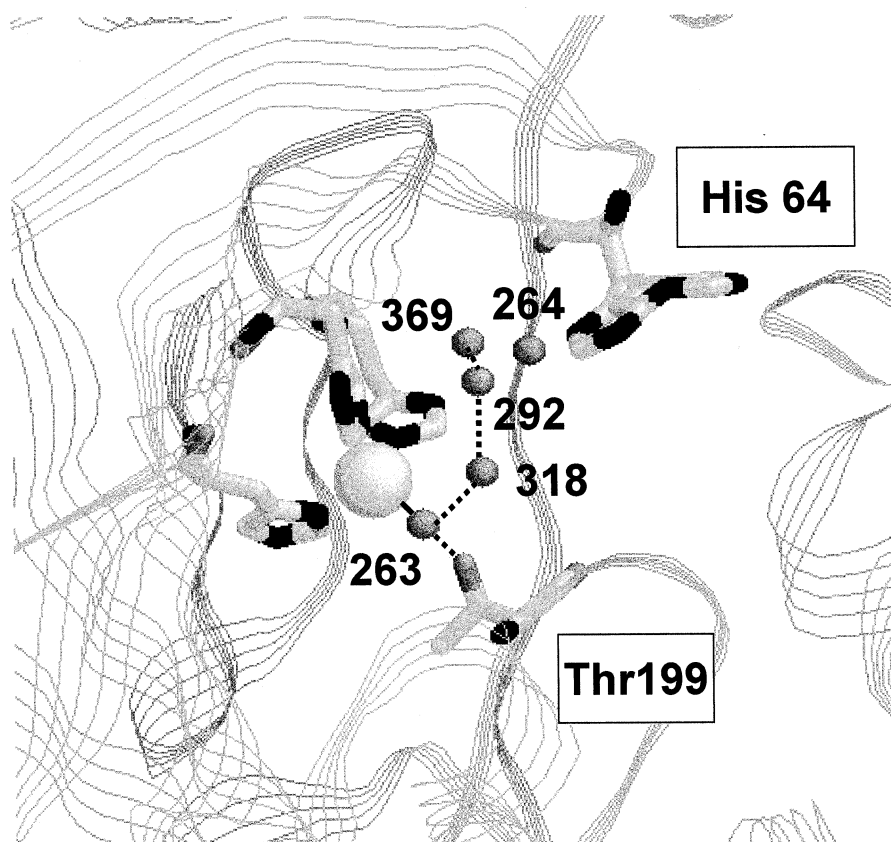


Fig. 1. The active site of human CA II showing the zinc and its three histidine ligands: His-94, -96 and -119. His-64 is shown in both the 'inward' and 'outward' conformations. Water molecules in the active site are numbered and shown as detected in the crystal structure [35].

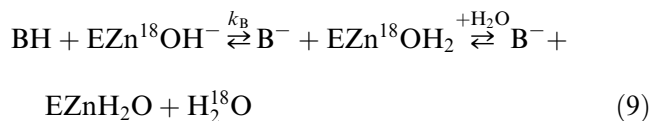
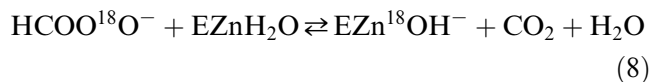
to conformational change of the His-64 side chain, but they do suggest that two conformations exist from which protons may be transferred between the zinc-bound water and buffer in solution. However, several mutants have been studied in which His-64 in the outward position is favored in the crystal structure, T200S, A65S and A65T, and which still show efficient proton transfer pathways [38,39]. Hence, appearance of His-64 in the outward position in the crystal structure does not preclude efficient proton transfer. The crystal structure of human CA II shows an array of apparently hydrogen-bonded water molecules from the zinc-bound water extending out to the position of the side chain of His-64 (Fig. 1). Like many non-enzymic, bimolecular proton transfers in solution [40], this transfer in the active site of carbonic anhydrase proceeds through hydrogen-bonded water bridges.

At low concentrations of external buffer, less than

approximately 10 mM, the intermolecular proton transfer of Eq. 7 becomes rate-limiting for  $k_{\text{cat}}$  [13,14,25]. Hence, there are two rate-limiting proton transfer processes that can be studied in carbonic anhydrase, one inter- and one intramolecular. In well-buffered solutions, the intramolecular proton transfer of Eq. 6 is the rate-limiting step of  $k_{\text{cat}}$ . The rate-limiting step is defined according to Ray [41] as the step in the reaction sequence for which a change in rate constant produces the largest effect in the overall rate. The most sensitive step to isotope effects is clearly the intramolecular proton transfer in CA II; the SHIE on  $k_{\text{cat}}$  for hydration of about 3.8 is more than 95% accounted for by this intramolecular proton transfer [42].

Measurements of the steady-state constant  $k_{\text{cat}}$  are most conveniently made by stopped-flow spectrophotometry in which the rate of change of a pH indicator dye is related the rate of catalysis [43]. These

studies require buffered solutions for pH control, and raise the question of how much proton transfer in the catalysis by carbonic anhydrase is intramolecular and how much is intermolecular via buffer in solution. This can sometimes be addressed by using buffers of large size, such as those identified by their acronyms Mops, Hepes and Taps, that are not efficient proton donors to or acceptors from carbonic anhydrase presumably because they do not fit into the active site of mutants lacking His-64 such as H64A [33]. Another option to avoid the problem of differentiating between inter- and intramolecular proton transfer is to observe the catalysis at chemical equilibrium by measuring the rate of exchange of  $^{18}\text{O}$  between  $\text{CO}_2$  and water [44]. In this case, pH control is not a problem and  $^{18}\text{O}$  exchange studies are carried out using solutions of carbonic anhydrase containing no buffer [45]. This method measures proton transfer in the dehydration direction as shown in Eqs. 8 and 9. The basis of the method depends on the transitory labeling of the zinc-bound hydroxide with  $^{18}\text{O}$ . As labeled hydroxide, this is tightly bound to the zinc; however, after protonation, the  $^{18}\text{O}$ -labeled water readily exchanges with solvent. The rate of distribution of  $^{18}\text{O}$  is continuously measured by membrane-inlet mass spectrometry [44].



Here, BH is buffer in solution and/or a residue of the enzyme acting as an internal proton shuttle. The actual rate constants for proton transfer are determined from the rate of Eq. 9, arising from a solution of the simultaneous kinetic equations for the catalyzed  $^{18}\text{O}$  exchange [32,46]. The rate of release of labeled water in Eq. 9, designated  $R_{\text{H}_2\text{O}}$ , is again rate-limited by proton transfer to the labeled zinc-bound hydroxide as verified by pH rate profiles, SHIEs and enhancements by buffers in solution [44].

### 3.2. Identify the donor and acceptor

In the dehydration direction, the zinc-bound hy-

droxide is the proton acceptor (Eq. 6). The predominant intramolecular proton donor in CA II has been shown to be His-64 [33]. In the mutant containing the replacement His-64 → Ala, there remains maximal turnover  $k_{\text{cat}}$  in excess of  $10^4 \text{ s}^{-1}$ , still appreciable for enzymic proton transfer. This remaining turnover is presumably due to more distant ionizable residues that act as weak proton donors [47]; there is no evidence of  $\text{H}_3\text{O}^+$  acting as donor or  $\text{OH}^-$  acting as acceptor. Residues with ionizable groups have been introduced by mutagenesis, and proton transfer from these sites was identified by the enhanced values of  $k_{\text{cat}}$  and rate of  $^{18}\text{O}$  exchange. The mutants containing a potential proton donor are compared in catalysis with the homologous mutant containing Ala or another non-ionizable residue at the site. Enhancement of  $k_{\text{cat}}$  and  $R_{\text{H}_2\text{O}}$  often by an order of magnitude is observed when a proton shuttle is introduced. This is usually accompanied by little or no change in  $k_{\text{cat}}/K_m$  which is a measure of rate processes of Eq. 5 that contain no rate-limiting proton transfers, a useful control. Moreover, catalysis by the mutant containing Ala or non-ionizable group is capable of enhancement by increasing the concentration of a buffer of small size such as imidazole in solution. Finally, the SHIEs throughout these measurements are of sufficient magnitude, greater than two, to be consistent with rate-limiting proton transfer.

### 3.3. Determine and vary $\Delta pK_a$ between donor and acceptor

The  $pK_a$  of the zinc-bound water is readily obtained from the pH profile of  $k_{\text{cat}}/K_m$  determined either by stopped-flow spectrophotometry [43] or  $^{18}\text{O}$  exchange [44], since this rate constant for dehydration of bicarbonate in the first stage of catalysis, Eq. 5, depends on the fraction of active sites in the zinc-bound water form. In addition, this  $pK_a$  can be determined from the pH dependence of the catalyzed hydrolysis of 4-nitrophenylacetate, for variants of carbonic anhydrase that are able to catalyze this reaction which includes nearly all the isozymes of animal carbonic anhydrase discussed here except wild-type CA III and some but not all of its mutant forms. The  $pK_a$  of the intramolecular proton shuttle is more difficult to determine. This  $pK_a$  can be ob-

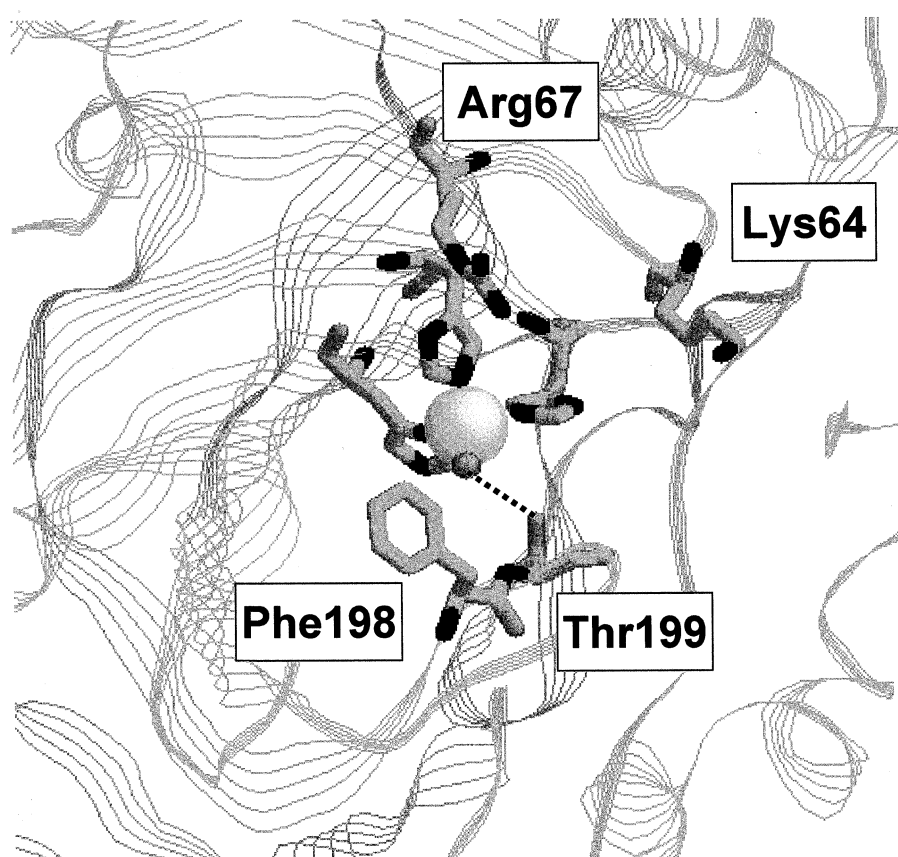


Fig. 2. The active site of bovine CA III showing the three histidine ligands: His-94, -96 and -119. Also shown are residues Lys-64, Arg-67 and Phe-198 that have been subjected to site-specific mutagenesis as described in the text (from [51]).

tained from the pH dependence of the rate of release of  $^{18}\text{O}$  from the active site [44,45] and from the pH profile for  $k_{\text{cat}}$ , which depends on the ionization state of the proton shuttle [25]. In some cases, the  $\text{p}K_{\text{a}}$  of histidine as a proton donor has been measured from the titration of its proton NMR [48,49].

In order to construct a Brønsted or Marcus plot, of course, one must vary the  $\Delta\text{p}K_{\text{a}}$ . Ideally, this should be done with as little structural change as possible to the donor and acceptor groups. In an enzyme's active site, this is particularly challenging. Achieving a range of  $\Delta\text{p}K_{\text{a}}$  in the case of carbonic anhydrase was possible through the discovery that the  $\text{p}K_{\text{a}}$  of the zinc-bound water in CA III was rather sensitive to the identity of the residue at position 198. This  $\text{p}K_{\text{a}}$  is near 5 in the wild-type CA III with Phe at position 198 (Fig. 2). When this residue was replaced with Leu, which occurs at this site in wild-type CA II and other isozymes, this  $\text{p}K_{\text{a}}$  is 6.9; with Asp at this site, the  $\text{p}K_{\text{a}}$  is 9.2 [50]. This permits a

small range of  $\Delta\text{p}K_{\text{a}}$ . The side chain of residue 198 in bovine CA III is located along the hydrophobic side of the active site cavity with its CZ 8.1 Å from the zinc [51]. This is on the opposite side of the cavity compared with the location of residue 64 (Fig. 2). The appropriate single and double mutants were constructed to examine double mutant cycles [52]. Measurements of  $k_{\text{cat}}/K_{\text{m}}$  and  $k_{\text{B}}$  were, with some exceptions, consistent with an additive or non-interacting relationship between His as a proton donor at positions 64 and 67 and various substitutions at residue 198 [48,53].

### 3.4. Marcus plot for intramolecular proton transfer

Wild-type CA III lacks an efficient intramolecular proton transfer mechanism and initial experiments relied on the activation of human CA III by making the replacement Lys-64 → His. It was anticipated that this substitution would place in CA III a proton

shuttle resembling that of CA II, and this was observed. K64H CA III showed activated proton transfer measured by  $^{18}\text{O}$  exchange consistent with rate-limiting proton transfer from His-64 to the zinc-bound hydroxide [54]. Moreover, this activation by His-64 showed an apparent  $\text{p}K_{\text{a}}$  of 7 with a maximum at low pH consistent with proton transfer from the imidazolium ring of His-64 to the zinc-bound hydroxide. An interesting and significant observation confirmed the role of His-64 and is discussed in the next section:  $^{18}\text{O}$  exchange catalyzed by wild-type CA III in the presence of large concentrations (100–200 mM) of imidazole enhanced proton transfer in the catalysis and mimicked the  $^{18}\text{O}$  exchange catalyzed by K64H CA III [54,55]. The free energy plot of the rate constants for proton transfer  $k_{\text{B}}$  from His-64 to the zinc-bound water for a series of mutants of CA III containing His-64 as well as mutations at 198 showed rather sharp curvature (Fig. 3). The plot is extended in the region of low  $\Delta\text{p}K_{\text{a}}$  by the inclusion of points representing wild-type CA III and K64A CA III; these mutants have no apparent proton donors in the active site cavity but appear to lie on the curve which is a fit of all of the data to the Marcus rate theory, Eq. 4.

The free energy plot of Fig. 3 represents proton transfer between nitrogen and oxygen acids and bases (that is, His-64 and zinc-bound hydroxide), and the value of the intrinsic kinetic barrier  $\Delta G_{\text{o}}^{\ddagger} = 1.4 \pm 0.3$  kcal/mol obtained from this plot [45] is similar to the value near 2 kcal/mol obtained for non-enzymic, bimolecular proton transfers between nitrogen and oxygen acids and bases in solution [21]. It is clear that the intramolecular proton transfer in CA III is dominated by a large work function  $w^{\text{r}} = 10.0 \pm 0.2$  kcal/mol for the dehydration direction and  $5.9 \pm 1.1$  in the hydration direction (Table 1). These results were not altered significantly by the omission in Fig. 3 of the data for wild-type and K64A CA III nor were they altered by the omission of the data for the intermolecular proton transfer to imidazole.

Proton transfer from another similar position in the active site cavity was measured. Position 67 is occupied by Arg in wild-type CA III and has a location in the active site cavity that is similar to that of residue 64 (Fig. 2). That is, the  $\text{C}\alpha$  of position 67 and 64 are nearly equal in bovine wild-type CA III,

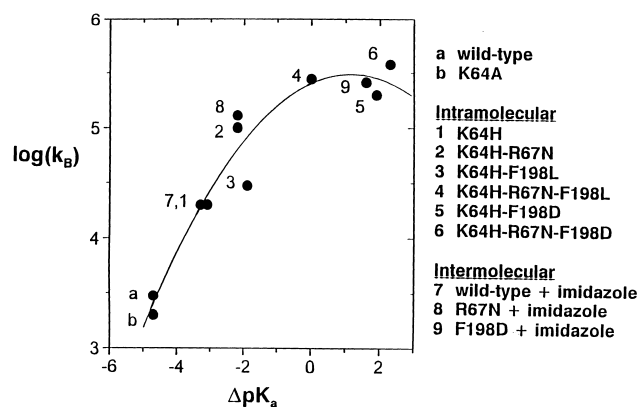


Fig. 3. The logarithm of the rate constant for intramolecular proton transfer  $k_{\text{B}}$  ( $\text{s}^{-1}$ ) from His-64 or exogenous imidazole to the zinc-bound hydroxide in the mutants of human CA III listed on the right. The abscissa is  $\Delta\text{p}K_{\text{a}} = \text{p}K_{\text{a}}(\text{ZnH}_2\text{O}) - \text{p}K_{\text{a}}(\text{donor})$  in which the donor group is His-64 or imidazole added to solution at  $25^\circ\text{C}$ . The solid line is a least-squares fit to the Marcus theory of Eq. 4 with the intrinsic kinetic barrier and work functions given in Table 1 (from [45]).

9.4 and  $9.7 \text{ \AA}$  from the zinc, respectively, and both side chains extend into the active site cavity [51]. A histidine at residue 67 in human CA III can donate a proton to the zinc-bound hydroxide [48]. This was also found to be true for human CA II in a double mutant in which His-64 was also replaced with Ala (H64A/N67H CA II). This double mutant had 5–20% of the maximal velocity of wild-type containing His-64, depending on conditions [56]. A series of mutants were prepared placing His as a proton shuttle at residue 67 of human CA III and altering residue 198 to change the  $\text{p}K_{\text{a}}$  of the zinc-bound water. A narrower range of  $\Delta\text{p}K_{\text{a}}$  was achieved in this case, but the Marcus rate theory could be made to fit the data (Table 1) [48]. It is clear that the parameters of the Marcus equation are very similar for proton transfer from either His-64 or His-67, the values of  $\Delta G_{\text{o}}^{\ddagger}$  are indistinguishable (Table 1). However, the work function  $w^{\text{r}}$  is greater by approximately 1 kcal/mol for proton transfer from His-67 (Table 1). Proton transfer from His-67 to zinc-bound hydroxide is at best 20% of that from His-64, consistent with the results for proton transfer from His-67 in CA II [56].

Proton transfer from other residues placed at position 64 in human CA III was also measured. The rate constants for intramolecular proton transfer from Asp-64 and Glu-64 were equivalent at  $4 \times 10^4$

$s^{-1}$ , about 20-fold greater than that for wild-type with Lys-64 [57]. Rate constants measured by  $^{18}O$  exchange for proton transfer from Asp-64 and Glu-64 could be described by Marcus rate theory with the parameters of Table 1 [58]. Again, there is an intriguing similarity in these parameters with those for His-64 as proton donor. Although there is more experimental uncertainty, the value of the intrinsic barrier  $\Delta G_0^\ddagger$  appears greater for Asp-64 and Glu-64 as donors than for His-64, and there are differences in  $w^r$  and  $w^p$  compared with wild-type (Table 1).

It is useful to point out that there are positions in the active site cavity from which significant proton transfer is not observed [59]. Asn-62 has its C $\alpha$  12.7 Å from the zinc in bovine CA III and its side chain extends into the active site cavity. Placing a His at site 62 results in no appreciable enhancement of activity [58]. In the mutant of CA II with His-64 replaced with Ala, placing a histidine at position 65 did not increase the rate constant for proton transfer, although placing histidines at sites 62, 67 and 200 was successful in increasing this rate constant [56].

### 3.5. Marcus plot for intermolecular proton transfer

There was a concern that the changes made in the active site of CA III, both introducing proton donors at positions 64 and 67 and altering the  $pK_a$  of the

zinc-bound water through substitutions at 198, were themselves altering catalysis by mechanisms in addition to their effects on the  $pK_a$  values of the donor and acceptors. Double mutant cycles had indicated that the effect on catalysis of most of the changes at 64 and 67 were independent of changes at 198 [48,53]. Nevertheless these residues are prominent in the active site cavity, and the mutants with substitutions at these sites may have been in violation of the rule that requires as few structural changes as possible in the construction of a free energy plot to be analyzed by Marcus theory. To deal with this possibility, another variation of the experiment was carried out relying on the property of buffers of small size to mimic the proton transfer properties of His-64 [45]. In these experiments, a single mutant of carbonic anhydrase was used and the proton donors were buffers, mainly methylated derivatives of imidazole and pyridine, of different acidity to achieve a range of  $\Delta pK_a$  [47]. The enzyme was a double mutant of murine CA V with Tyr-64 and Phe-65 each replaced with Ala to open the active site cavity so there would be less hindrance to the entry of buffers of small size. Each buffer was observed to achieve saturation in the enhancement of catalyzed  $^{18}O$  exchange with apparent binding constants varying in the range from 2 mM to 110 mM. It is at saturation that the properties of intermolecular proton transfer

Table 1  
Marcus theory parameters for proton transfer in isozymes of carbonic anhydrase

System	Proton donor	$\Delta G_0^\ddagger$ (kcal/mol)	$w^r$ (kcal/mol)	$w^p$ (kcal/mol)
Intramolecular				
CA III	His-64 <sup>a</sup>	$1.4 \pm 0.3$	$10.0 \pm 0.2$	$5.9 \pm 1.1$
	His-67 <sup>b</sup>	$1.3 \pm 0.3$	$10.9 \pm 0.1$	$5.9 \pm 1.1$
	Glu or Asp-64 <sup>c</sup>	$2.2 \pm 0.5$	$10.8 \pm 0.1$	$4.0 \pm 1.6$
	His-64 (from SHIE) <sup>d</sup>	$1.3 \pm 0.3$	$(w^r - w^p = 0.6 \pm 0.5)$	
Intermolecular				
CA V	Buffers <sup>e</sup>	$0.8 \pm 0.5$	$10.0 \pm 0.2$	$8.2 \pm 1.0$
CA II	Buffers (from SHIE) <sup>f</sup>	$0.6 \pm 0.5$	$(w^r - w^p = 0.9 \pm 0.5)$	
Non-enzymic	Buffer to buffer <sup>g</sup>	2.0	3.0	

<sup>a</sup>Silverman et al. [45].

<sup>b</sup>Ren et al. [48].

<sup>c</sup>Tu et al. [58].

<sup>d</sup>Silverman et al. [45]. SHIE is the solvent hydrogen isotope effect. Independent measurements of  $w^r$  and  $w^p$  cannot be made by this method, but the results yield the difference  $w^r - w^p = 0.6 \pm 0.5$  kcal/mol.

<sup>e</sup>Earnhardt et al. [47].

<sup>f</sup>Taoka et al. [66].

<sup>g</sup>Kresge [21].



by imidazole match those of His-64 in CA III [54,55]. That is, it appears that these small buffer molecules bind to a region in the active site cavity, probably on the hydrophobic side, and act as proton shuttles from this site. Again, these buffers enhance the proton transfer components of the catalysis, Eqs. 6 and 7, with relatively little effect on  $k_{\text{cat}}/K_m$ . A small inhibition of  $k_{\text{cat}}/K_m$  was observed for some buffers that was taken into account in the analysis of proton transfer [47].

The maximal, pH-independent rate constant for the proton transfer was determined at saturation levels of the imidazole and pyridine type buffers. Again, the rate constants for proton transfer could be adequately described by the Marcus theory (Fig. 4). There were indications from the pH profiles for rate enhancement at saturating buffer concentrations that the  $pK_a$  of the buffers bound to carbonic anhydrase were not altered greatly compared with free buffer; nevertheless, it is the  $pK_a$  of the free buffer that is used in Fig. 4 [47]. The Marcus parameters in this case were similar to those for the cases of intramolecular proton transfer (Table 1). Four of the buffers investigated as proton donors to Y64A/F65A CA V fell significantly below the curve representing the Marcus theory in Fig. 4. These are all pyridine type buffers methylated at position 2. These

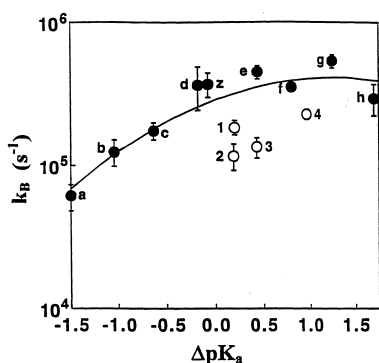


Fig. 4. Dependence of the logarithm of  $k_B$  ( $s^{-1}$ ) for proton transfer from added buffers to Y64A/F65A murine CA V on  $\Delta pK_a$  ( $pK_{a(ZnH_2O)} - pK_{a(donor)}$ ) at 25°C. The added buffers are: a, morpholine; b, 2-methyl imidazole; c, 4(5)-methyl imidazole; d, 1-methyl imidazole; e, 3,4-dimethyl pyridine; f, 3,5-dimethyl pyridine; g, 3-methyl pyridine; h, pyridine; 1 is 2,4-dimethyl pyridine; 2 is 2,6-dimethyl pyridine; 3 is 2,5-dimethyl pyridine; 4 is 2-methyl pyridine. The solid line is a non-linear least-squares fit to the Marcus equation for points 'a'–'h' with values of the intrinsic barrier and work functions given in Table 1 (from [47]).

were neglected in the calculation of the Marcus parameters as having their proton donor sites sterically restricted.

### 3.6. Marcus plot for SHIEs

Melander [60] and Westheimer [61] recognized that a maximal SHIE in a proton transfer-limited reaction within a series of homologous acceptors and donors would occur between sites for which  $\Delta pK_a$  is close to zero. This was observed for non-biological systems in which a plot of the isotope effect against  $\Delta pK_a$  was bell-shaped with a maximum near  $\Delta pK_a$  zero [62,63]. This observation is attributed to the expected position of the proton in the transition state midway between proton acceptor and donor for  $\Delta pK_a$  at zero, and hence most susceptible to slower motion by deuterium substitution. This should be a general phenomenon extending to intramolecular transfer in a protein, and was observed in the case of proton transfer from His-64 in mutants of human CA III measured by  $^{18}O$  exchange (Fig. 5). Since the Marcus theory describes the properties of a deuterium transfer as well as a proton transfer with respect to the free energy of reaction, it can also describe the SHIE [64,65].

Application of the Marcus theory to the isotope effects of Fig. 5 yields  $\Delta G_0^\ddagger$  of  $1.3 \pm 0.3$  kcal/mol (Table 1) [45]. Although using the SHIE cannot give independently both  $w^r$  and  $w^p$ , it does give their difference [64]. For the data of Fig. 5,  $w^r - w^p = 0.6 \pm 0.5$  kcal/mol, which is not in good agreement with the value of this difference near 4 kcal/mol determined by the rate constant for proton transfer (Table 1). This may indicate a failure in the approach to explaining these data, or may result from accumulated experimental uncertainties. There are several assumptions required to apply Marcus theory to the SHIE, some of which may not be applicable in the case of carbonic anhydrase. One such assumption is that there is no SHIE in the work terms  $w^r$  and  $w^p$  themselves [64]. The observation that the maximum in the observed SHIEs of Fig. 5 does not appear to occur exactly at  $\Delta pK_a = 0$  is also related to the value of  $w^r - w^p$  [45].

Similar measurements were made based on the SHIEs observed in the enhancement by methylated imidazole and pyridine buffers of catalysis of the

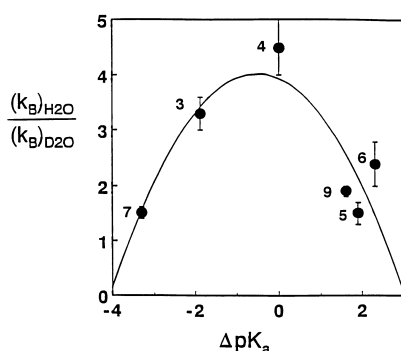


Fig. 5. SHIEs on the rate constants for proton transfer in the mutants of human CA III numbered in Fig. 3 as a function of  $\Delta pK_a = pK_{a(\text{ZnH}_2\text{O})} - pK_{a(\text{donor})}$ . The solid line is a fit to the Marcus equation describing isotope effects, Eq. 10 of [64], with parameters given in Table 1 (from [45]).

hydration of  $\text{CO}_2$  measured in this case by an initial velocity method, stopped-flow spectrophotometry [66]. In this case, the enzyme was a mutant of human CA II containing the replacement His-64 → Ala, removing the internal proton shuttle. The results for CA II were quite similar to those obtained for intermolecular proton transfer with CA V (Table 1).

#### 4. Interpretation of the Marcus plot and parameters

##### 4.1. The Marcus plot

It is useful to point out that the results of fitting the Marcus theory to the rate constants for proton transfer in carbonic anhydrase achieve several goals. First, the proton transfer processes can be expressed in terms of thermodynamic quantities, the work functions  $w^r$  for the dehydration and  $w^p$  for the hydration, and the intrinsic kinetic barrier  $\Delta G_0^\ddagger$ . Second, these data apply to the series of proton transfers for many mutants and can be compared with Marcus parameters in other systems. All of the experimental results accumulated in Table 1 applying the Marcus theory show that the intrinsic kinetic barriers measured for carbonic anhydrase are small with values near or less than 2 kcal/mol, consistent with bimolecular, non-enzymic proton transfers between nitrogen and oxygen acids and bases in solution [21]. They show large work functions  $w^r$  in the range 10–11 kcal/mol. It is this work function that answers

the question of why proton transfer in carbonic anhydrase, which is at most  $10^6 \text{ s}^{-1}$ , is so much slower than the maximal rates of proton transfer near  $10^{11} \text{ s}^{-1}$  observed for example for proton transfer from naphthol-related photo acids to acetate in solution [67]. A major challenge is to determine what processes contribute to the work functions.

It is important to point out that these general conclusions are quantitated by application of the Marcus theory; however, the conclusions follow qualitatively without the Marcus equation from the high extent of curvature of the Brønsted or free energy plots of Figs. 3 and 4. These are in themselves so similar to the case of non-enzymic bimolecular proton transfer (as for example from [21,67]) that we reach the conclusion that the intrinsic kinetic barrier for proton transfer in carbonic anhydrase is small as in the non-enzymic case, with the major part of the observed activation barrier for the enzyme in the work functions. Hence, the qualitative results are not dependent on the applicability of the Marcus rate theory to proton transfer in the active site of an enzyme, although the quantitative results are.

The quadratic form of the Marcus equation, Eq. 4, makes the well-known prediction that as the proton transfer becomes more and more favorable with decreasing  $\Delta G^\circ$ , the observed activation barrier  $\Delta G^\ddagger$  decreases until a point is reached at  $\Delta G^\circ = -4\Delta G_0^\ddagger$  beyond which further decreases in  $\Delta G^\circ$  give less and less favorable proton transfers with increasing activation barriers. That is, the parabolic form of Marcus plots predicts that in the inverted region as the transfer becomes more and more favorable in terms of  $\Delta G^\circ$ , the proton transfer rate constant is decreased. This unexpected prediction of reaction rates for  $\Delta G^\circ < -4\Delta G_0^\ddagger$  forming an inverted region as exoergicity is increased has been observed for electron transfers [68]. However, such an inverted region has not yet been observed for proton transfers, and there is good reason to believe that this prediction of Marcus theory for an inverted region does not apply to proton transfer. A problem in the application of the Marcus treatment of reactivity to proton transfers is the assumption that all the distortions that convert the reactant to the transition state and the transition state to product can be represented by changes in the value of a single collective coordinate  $\Delta G^\circ$ . Real potential surfaces are hyperdimensional,

and the distortions leading to the transition state are not all coordinated. Proton transfer occurs through excited vibrational levels on the ground electronic surface, levels that are sometimes so closely spaced as to be practically continuous. The inverted region may be avoided for proton transfer because the transition state predicted for the inverted region will shift to another place on the surface where the energy barrier is more favorable. Because of the richness of the vibrational spectrum, there are many other modes that can be excited, and will influence the energy of proton transfer, so that in a protein with many breathing modes the problem is amplified.

#### 4.2. Intrinsic kinetic barrier

The intrinsic barrier  $\Delta G_0^\ddagger$  for the proton transfer represents a purely kinetic barrier with thermodynamic components absent. The range of values for  $\Delta G_0^\ddagger$  determined from Marcus theory extend as high as 8–10 kcal/mol for proton transfer reactions that involve considerable electron relocalization, such as in the protonation of aromatics or ionization of carbonyls [21]. At the lower extreme is non-enzymic, bimolecular proton transfer between normal nitrogen and oxygen acids and bases involving a minimum of electron reorganization with  $\Delta G_0^\ddagger$  near 2 kcal/mol. A similar value of  $\Delta G_0^\ddagger$  has been determined for the proton transfer from the excited state of photo acids such as naphthols to carboxylic acids present at high concentrations, for example 8 M acetate [67]. At this high concentration of proton acceptor, the donor and acceptor are assumed to be in a reactive complex; hence, the measurement of the proton transfer avoids a preliminary diffusion step. In this case, an intrinsic barrier to proton transfer near 2.5 kcal/mol was found for many photo acids with a range of  $\Delta pK_a$  11. The intrinsic kinetic barrier for proton transfer may itself be dominated by solvent reorganization [69,70]. It may be significant that the intrinsic barrier for proton transfer near 2.5 kcal/mol is also the value for the activation enthalpy found from the temperature-dependent far-infrared spectrum for the reorientation of water dipoles in liquid water [71]. There is no evidence of proton tunneling in the intramolecular proton transfer accompanying catalysis by carbonic anhydrase of  $\text{CO}_2$  hydration as determined by a measurement of the temperature dependence of

H/D isotope effects (C.K. Tu, personal communication).

For the intramolecular proton transfer in carbonic anhydrase, there appears not to be a significant barrier arising from proton transfer through an incompletely formed hydrogen bond or  $\Delta G_0^\ddagger$  would be larger [72]; the barrier for proton transfer in carbonic anhydrase is strongly dependent on the N–O distance between the His-64 and bridging water [73]. It is an open question whether there is formation of a full hydronium ion intermediate that would arise by the stepwise transfer of protons through a water chain. On the one hand, this charged species might be incompletely solvated in the active site cavity and its formation would not be energetically favorable based on the positively charged active site of many of the CA III mutants (Lys-64, Arg-67, Arg-91). On the other hand, the intrinsic barrier for bimolecular proton transfer across a preformed hydrogen-bonded water bridge between, for example, acetic acid and aniline was found by two-dimensional Marcus theory [74] to be stepwise; in this calculation, the rate-limiting step was the transfer of a proton from acetic acid to an intervening water with formation of a hydronium ion accompanied by an intrinsic barrier near 1 kcal/mol [75]. Previous studies had established that such stepwise proton transfer through hydrogen-bonded water could be rapid enough to account for the rates observed in these processes [76]. For interpretation of the results with carbonic anhydrase, it is necessary that an accurate model exists for proton transport through water chains [77,78].

Some of the values of  $\Delta G_0^\ddagger$  of Table 1 are quite small, although proton transfer is associated with substantial reorganization of charge. It is possible that the active site of carbonic anhydrase might lessen the energy associated with such reorganization, just as it lowers the energy of the transition state of the catalyzed  $\text{CO}_2$  hydration compared with the uncatalyzed reaction. This intramolecular proton transfer in carbonic anhydrase has similarities and differences with the classic description of a proton wire [79] such as exists in the transmembrane gramicidin channel. The similarity is that proton transfer is conducted through the array of hydrogen-bonded water molecules and that in carbonic anhydrase as in the gramicidin channel, the formation and breaking of these hydrogen bonds may be the limiting factor

in the rate of proton translocation [80]. However, unlike the gramicidin channel in which hydrogen-bonded water rearranges without being displaced from the channel, in carbonic anhydrase, the departure of bicarbonate must displace much of the hydrogen-bonded water structure that serves as a proton wire, and this wire must then reform in each catalytic cycle.

It is a feature of the data of Table 1 that proton transfer from histidine at two sites in the active site cavity, from 64 and 67, and from Glu or Asp at position 64 have nearly identical values of the intrinsic kinetic barrier  $\Delta G_0^\ddagger$ . Moreover, even proton transfer from bound buffer molecules proceeds with almost the same  $\Delta G_0^\ddagger$ , although the value is somewhat lower for the case of proton transfer from buffers (Table 1). This is likely a reflection of the rather equivalent distances for these proton transfers and also of the flexibility of the ensemble of hydrogen-bonded water structures in the active site cavity. This cavity is roughly conical of depth 15 Å opening at the surface of the protein and with its apex at the zinc. The proton donors at sites 64 and 67 used in these studies have their origins at residues about equidistant from the zinc. Judging from the saturable increase in proton transfer upon addition of imidazole buffer, the imidazole is bound to the enzyme in a Michaelis-like complex from which the proton transfer occurs. This may be on the hydrophobic side of the active site cavity in the vicinity of His-64; indeed, the crystal structure of CA II shows the imidazole group of His-64 near Trp-5, Gly-6, Tyr-7 and Phe-231 in its out conformation [35], a hydrophobic site to which these aromatic buffers might bind. Histamine binds to CAII with its imidazole ring at a distance from the zinc about equivalent to that of His-64 [81].

#### 4.3. The thermodynamic or work functions

The thermodynamic work functions are contributions to the free energy of reaction for the proton transfer that do not depend on  $\Delta pK_a$ . The magnitude of the work functions for non-enzymic proton transfer between nitrogen and oxygen acids and bases in solution is near 3 kcal/mol [21], larger than can be accounted for in terms of simple encounter of donor and acceptor. These functions have been proposed to

contain energy needed to orient these reactant molecules as well as surrounding solvent prior to the proton transfer itself [65]. It is in this context that the values of the work functions obtained for the catalysis by carbonic anhydrase are viewed (Table 1).

There can be two extreme and rather divergent views of the work functions. The most stringent interpretation of the work function  $w^r$  is that this is the free energy that must be added to  $\Delta G^\circ$  so that the observed free energy of activation can be made to fit the Marcus equation. In this view, the work function is a correction factor that describes the inadequacy of the theory to fit the observations, a factor that must be used to make the Marcus equation applicable. In this context, the rather large and dominating values of the work functions (Table 1) indicate that catalysis by carbonic anhydrase is not very suitable for description by Marcus theory and that a rather small component of the observed activation energy varies with the free energy of reaction  $\Delta G^\circ$ . The other extreme is that the work function, in analogy with the interpretation of non-enzymic transfers, represents an energy required to orient the donor residue with the zinc-bound hydroxide and the intervening hydrogen-bonded water chain prior to facile proton transfer. However, this interpretation has cautions as well. In a study of model hydride transfers between analogs of  $\text{NAD}^+$ , the work functions were not found to represent any identifiable structure or local energy minimum on the reaction surface [82]. This emphasizes that there does not need to be any clear separation between the parts of the reaction attributable to  $\Delta G_0^\ddagger$  and that attributable to  $\Delta G^\circ$  and  $w^r$ . Clearly, the large value of  $w^r$  found for catalysis by carbonic anhydrase does not necessarily indicate the presence of a high energy intermediate or specific active site conformation.

With these cautions in mind, it is nevertheless useful to discuss the various active site processes that could contribute to the work function  $w^r$  of 10 kcal/mol for proton transfer from the donor groups including those of Table 1 to the zinc-bound hydroxide in catalysis by carbonic anhydrase. One possibility is rotating the side chain of His-64 into the 'in' position, starting from the 'out' position; both of these side chain conformations are observed in the crystal structures [35]. This rotation involves breaking H-bonds between the side chain and water in the 'out'

position, rotating the side chain about  $60^\circ$  about the  $\chi_1$  torsional angle, and displacing some water in the cavity to fit the side chain in the 'in' position. His-64 has its side chain protonated and hence charged for productive proton transfer to zinc-bound hydroxide; so there may be some cost of moving this side chain in the electrostatic fields of the solvated active site cavity.

To account for the large value of  $w^{\ddagger}$  in terms of hydrogen-bonded water, it is necessary to comment that among many possible arrangements of hydrogen-bonded water in the active site cavity, relatively few are in a configuration that allows proton transfer between His-64 and the zinc-bound water. Simulations of the active site of CA II extending over 50–100 ps determined that the probability of forming a hydrogen-bonded water bridge between His-64 and the zinc-bound water is 1–4% [37]. This corresponds to a free energy barrier of 2–3 kcal/mol for bridge formation in which two water molecules extend the bridge from the zinc-bound water to His-64 [37]. Along with the calculated estimates of 8–10 kcal/mol for the proton transfer itself [73], this gives a view of the proton transfer process from *ab initio* calculations and molecular dynamics simulations. This latter value for the proton transfer is similar to the value near 10 kcal/mol calculated for CA I using the valence bond theory [83]. It is interesting that these calculations also allow for the case of proton transfer involving a bridge of a single water molecule. In this case, the formation of the water bridge is less favorable at 4–5 kcal/mol but the barrier for the proton transfer is more favorable at 3–4 kcal/mol than the proton transfer through a bridge of two water molecules [37]. This comes closer to the values determined from application of Marcus theory to the experimental data. These comparisons suggest that the water bridge that is productive in catalysis is a structure with a low probability of formation. Another comment from these calculations is that other water molecules in the cavity, molecules not involved directly in the proton transfer, can enhance the stability of the hydrogen-bonded water chain [73]. That is, the work function may represent not only the formation of the hydrogen-bonded waters that form the direct proton transfer pathway but also an entire array of water in the cavity including perhaps specific orientations of side chains. There is no

evidence of backbone conformational change required for proton transfer.

Of course, there will be considerable solvent reorganization accompanying the proton transfer itself, especially since the transfer in the dehydration direction for K64H CA III or wild-type CA II eliminates positive charge on His-64 and also eliminates (a delocalized) negative charge of the zinc-bound hydroxide when it is converted to zinc-bound water (Eq. 6). But these energies are most likely represented in the intrinsic energy barrier  $\Delta G_0^{\ddagger}$  and not in the work function itself. Since the rate of intramolecular proton transfer in the catalysis is apparently dependent on the assembly of a hydrogen-bonded water array, it is interesting that the entropy of activation of  $k_{\text{cat}}$  for hydration catalyzed by CA II is very low; nearly all of the observed activation barrier is enthalpic [84]. Presumably this accounts for hydrogen bond formation in the assembly.

More details of the proton transfer pathway in CA II have been obtained from the crystal structure and catalysis of a number of mutants at residue 65 [39,85]. This position is adjacent to His-64 and extends into the active site cavity. Perturbations of the water structure in the active site cavity caused by bulky substitutions at site 65 (A65F, A65H, A65L) correlate with decreased proton transfer efficiency, suggesting that water molecules numbered 292, 369 and 264 (Fig. 1) participate in the proton transfer pathway in CA II. That is, the displacement of these water molecules decreases the proton transfer efficiency either by decreasing the mobility of the side chain of His-64 or by disrupting the water bridge forming the proton wire. These same substitutions at position 65 also decrease the enhancement of catalysis by external buffers such as imidazole; this indicates that these same active site water molecules are also involved in intermolecular proton transfer in which small buffers bypass His-64 or activate H64A CA II and deliver protons to the zinc-bound hydroxide [85].

Yet another approach to understanding the data of Fig. 3 questions the initial assumptions of the Marcus theory as applied to proton transfer. The Marcus theory as expressed in Eq. 4 can be derived by considering the intersection of two parabolas, representing the proton donor and acceptor. Using the valence bond approach, Warshel et al. [86] intro-

duced a third energy function resulting in two energy barriers involving the formation of the hydronium ion in the cavity and its deprotonation to yield product. The intersection of two parabolas may be adequate for describing electron transfer reactions where the mixing in a valence bond approach between the reactant and product state is small, but Warshel et al. [86] suggest that in a proton transfer, a more realistic approach is to utilize a model that consists of three states, that is three parabolas, including a parabolic free energy function for an intermediate hydronium ion. Such an approach leads to very low values of  $w^r$  and larger values of the intrinsic barrier consistent with the 10 kcal/mol found from the valence bond approach to CA I [83] (A. Warshel, communication).

#### 4.4. Conclusion

Certainly the result that the intrinsic kinetic barrier  $\Delta G_0^\ddagger$  for proton transfer in carbonic anhydrase (Table 1) is very similar to that observed for the analogous non-enzymic bimolecular cases is satisfying. It would be rather surprising to discover that the properties of proton transfer carefully worked out in classic experiments of physical organic chemistry do not apply to proton transfers in active sites. Marcus theory is based on a very simplified model; some of its success lies in the empirical adjustment of the parameters. In its application to carbonic anhydrase, a rather significant adjustment in the form of large work terms or thermodynamic components is needed to fit Marcus theory to the data. It is intriguing that the predominant component of the activation barrier is a thermodynamic component  $w^r$ . Since it is difficult to envision construction of a hydrogen-bonded water chain costing 10 kcal/mol, even when it occurs in the partially hydrophobic environment of the active site cavity, the large value of  $w^r$  suggests intramolecular proton transfer as a property that requires reorganization of the active site cavity including waters not directly along the pathway and nearby side chains. It is a problem to deduce exactly which processes are in  $\Delta G_0^\ddagger$  and which are in  $w^r$ ; this is clearly not answered. In addition, it certainly is not a strong point for this review that after many careful studies of proton transfer in catalysis by carbonic anhydrase one deduces that a predominantly large fraction of

the activation barrier for catalysis cannot be described by Marcus theory at all, that is, catalysis is dominated by  $w^r$  which has no dependence on  $\Delta pK_a$ .

These are comments that pertain to specific data on proton transfer in carbonic anhydrase. Unfortunately, there is not another protein system for which Marcus theory has been applied to the extent that it has for carbonic anhydrase. It is certainly a weak point that the information we have concerning application of Marcus theory to proton transfer in proteins comes from studies from one source concerning one enzyme. And in these studies, the range of  $\Delta pK_a$  is rather small, about four or five pH units, which has been extended by including some mutants that appear to fall on the parabolic curve describing Marcus theory but for which the proton donor is not clearly identified. Possible means of extending the range of  $\Delta pK_a$  is through chemical modification with proton donors at specific sites in the active site cavity, recently applied to carbonic anhydrase [87], or of course through introduction of unnatural amino acids. It will be extremely interesting and revealing to extend the Marcus theory to proton transfer in other protein systems which involve proton transfer across significant distances spanned by hydrogen-bonded water bridges.

#### 5. Note added in proof

Peters et al. [88] have observed an inverted region in proton transfer within benzophenone/*N,N*-dimethylaniline contact radical ion pairs.

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