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Reflections on Edsall's carbonic anhydrase: paradoxes of an ultra fast enzyme

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Abstract

John Edsall's investigations of human erythrocyte carbonic anhydrase, a zinc metalloenzyme that powerfully catalyzes the reversible hydration of carbon dioxide, highlighted a conundrum regarding the correct hydration product. The measured kinetic parameters could not be reconciled with the choice of carbonic acid, since its bimolecular recombination rate with enzyme would exceed the diffusion limit. The alternate choice of bicarbonate obviated the recombination rate problem but required that the active site deprotonation exceed the diffusion-limited maximum rate by an even greater extent. This paradox was resolved in favor of bicarbonate when the unsuspected role of buffer species indirectly deprotonating the enzyme was finally proposed, spurring numerous investigations to verify the hypothesis. Edsall's laboratory also reported the accidental discovery of the first competitive inhibitor, imidazole. This opened new avenues to understanding the binding of the CO_2 substrate and stimulated many investigations on this inhibitor. Paramagnetic NMR and crystallographic studies demonstrated that the only other known competitive inhibitor, phenol, apparently shared this unusual binding site. Despite enormous progress since Edsall's retirement, particularly the use of site-directed mutagenesis approaches, the precise interactions of carbon dioxide and bicarbonate with specific active site moieties remain as elusive today as when Edsall first considered these questions.

Keywords: Human erythrocyte carbonic anhydrase; Zinc metalloenzyme; Carbon dioxide; Bimolecular recombination rate; Crystallographic studies

1. Prologue

It has been said that life is an accident, but perhaps it is more like chaos, with some hidden fractal-like order. I still vividly remember the day when our visiting Australian scientist, the smallish Hugh McKenzie, fluttered into the laboratory and tried to impress as usual. He stated that he was invited to visit John Edsall at Harvard and, if I wished, he could recommend his taking me as a post-doctoral fellow. The thought of what to do after graduation was so premature that it had not been discussed with my advisor. Besides, my full attention was on repeating two years' worth of urea denaturation kinetics that mysteriously had changed when we moved into a new building with its own distilled water supply. I curtly told Mc-

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Kenzie that he is welcome to do so. When he returned a few days later claiming that Edsall was interested, this was taken with the usual grain of salt and promptly forgotten. Time passed, but one day my advisor, Walter Kauzmann, walked excitedly into the lab saying that Edsall had just called to inquire about me. Kauzmann's endorsement clearly set the course to follow, but it was McKenzie who had rolled the dice. Many months and delays later, I turned in my dissertation and rushed to join Edsall as his last post-doctoral fellow (along with Friedrich Dorner) from January1968 to September 1970. This new life adventure turned out to include meeting my future spouse and mother-to-be of our two boys, Lilla Csonka, who was working down the hall in the Biological Laboratories. *Note to self*: remember McKenzie a little more kindly; also, credit him with unintentionally proving Gleick's 'Butterfly Effect' [1].

Following an interlude to learn protein NMR, I chose to start my independent academic career at Virginia by returning to the study of carbonic anhydrase to which I was introduced by Edsall. I found it very fruitful to do so for two decades, ending in 1993 when I began my current focus on understanding the role of protein glycation in the development of diabetic complications. I describe here a select few of the many paradoxes presented by this enzyme that were of much interest to Edsall and with which I was involved. The Reader is asked to excuse the personal style of this tribute, which is adopted to give a flavor of Edsall's memorable influence on me. However, it also serves to highlight the significant contributions of his laboratory at the time of his retirement and their considerable subsequent impact on the carbonic anhydrase field.

2. Edsall's enzyme

The ubiquitous carbonic anhydrase (EC 4.2.1.1) has proven to be of considerable significance to biochemistry in general, beyond any role it has in the survival of the biological organism or in disease. This is in large part due to the pioneering studies of John Edsall in the United States, and Bo Malmström and his student Sven Lindskog in Sweden. The still-exploding knowledge on the structure and function of the carbonic anhydrase family has been recently summarized [2–9], but the status in 1968 is best described in Edsall's Harvey Lectures review [10]. This was a time when polypeptide sequences and three-dimensional structures were being hotly pursued but still needed several more years to yield fruit. *Perhaps more than any other enzyme then or since, its kinetics truly defined carbonic anhydrase*. This was true even before it was ever isolated, for respiratory physiologists predicted its existence when the observed CO_2 escape rates could not be accounted for by the uncatalyzed rates of hydration and dehydration. They inferred a catalyst in blood that can dehydrate bicarbonate and allow it to escape as CO_2 during the short transit time in the airways, paving the way to its isolation [11,12]. Less than a decade later, Keilin and Mann reported seminal findings. They first discovered that zinc is an intrinsic cofactor in the enzyme [13], marking the first biological role for this element. They also deduced that sulfanilamide must be a strong inhibitor of the enzyme [14], thus ushering in pharmacological and medicinal investigations on sulfonamide inhibition of the enzyme. This is best exemplified by the life-long work of the late Thomas Maren, a wonderful mentor and friend, who brilliantly realized his dream of developing topical therapeutic sulfonamide inhibitors for treating glaucoma [15].

It took almost two more decades after the discovery of zinc before carbonic anhydrase fully caught the attention of biophysical scientists from a wider variety of disciplines. As an enzyme with simple substrates, it was recognized as one of the most efficient acid–base catalysts known [16], with simple substrates that made them amenable to theoretical studies [17–19]. As a zinc protein, it became a paradigm for bioinorganic chemists after Lindskog successfully replaced the zinc with cobalt, retaining catalytic activity and introducing an exquisitely sensitive visible spectral probe [20]. The enzyme was subjected to studies of every kind in an effort to understand the coordination chemistry of the intrinsic metal ion and its participation in catalytic roles [20–24]. The mysteries and challenges of carbonic anhydrase proved to be

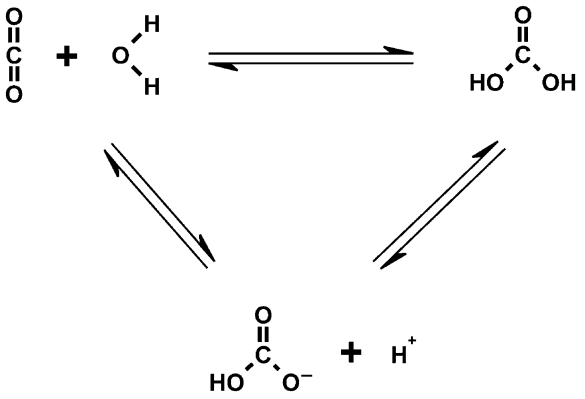

Fig. 1. Reversible hydration and ionization equilibria for carbon dioxide [26].

ideally matched with someone like Edsall, whose broad and rigorous biophysical chemistry approach had always made him truly ahead of his time [25]. *In all this activity, he was not just a pioneer, but also the prime catalyst for the study of the enzyme*.

3. Central dilemmas of an impossibly fast enzyme

3.1. Blurring speed–catalysis on the kinetic edge

Edsall's appreciation of the unusual catalytic power of erythrocyte carbonic anhydrase was enhanced by a deep understanding of the complexity of the uncatalyzed reversible hydration of carbon dioxide [26,27]. The two possible hydration products, carbonic acid and bicarbonate, are coupled in real time through instantaneous ionization (Fig. 1). This makes it impossible to *directly* monitor each product (i.e. reverse substrate) to ascertain if it is the one eliminated first from the enzyme. Normally, substrate or product ionizations are of no particular concern, since they are much more rapid than turnover, but this was not the case here. Furthermore, when products differ from substrates in proton inventory, a reciprocal linkage with ionizations in the active site is established that becomes part of the catalytic cycle. The ambiguity regarding the correct product species actually presented not one but two major dilemmas. Regardless of the choice of product of hydration, i.e. H_2CO_3 or HCO_3^-, the enzyme appeared to exceed the diffusion limit for chemical reactions. William Jencks aptly described such enzymes as being 'impossibly fast'.

3.2. H_2CO_3 or HCO_3^-: that is the (not so simple) question

Working in the Gibbs Chemical Laboratory, the famed Harvard kineticist George B. Kistiakowsky carried out the first searching studies of the reversible enzymatic catalysis by carbonic anhydrase [28,29]. His laboratory also pioneered the application of the photometric pH-indicator stop–flow method for kinetic characterization of CO_2 hydration, a now-standard methodology that we were later to refine across the street in the Biological Laboratories [30]. Kistiakowsky was apparently the first to recognize that a bimolecular diffusion limit was encroached if the wrong choice of hydration product was made. Though surely paling in comparison to his construction of the implosion device for the first atomic bomb [31], this was, nevertheless, a landmark achievement for carbonic anhydrase. Working at close to 0 °C before the discovery of the existence of isozymes of differing specific activities, DeVoe and Kistiakowsky obtained sufficient data in both hydration (V_{max} and K_m) and dehydration (V'_{max}, K'_m) directions to reveal the diffusion problem [29]. The first reliable kinetics on the resolved human isozymes I and II (then called B and C) at 37 °C were obtained a decade later in Edsall's laboratory [30], and these data even more strongly supported the original analysis of Devoe and Kistiakowsky.

To understand the implications of the kinetics, one must recall that Peller and Alberty [32] proved that the steady state Michaelis–Menten parameters (K_m and V_{max}) contain sufficient information to establish lower limits for all steps in a catalytic mechanism, regardless of the (unknown) number of enzyme–substrate and enzyme–product intermediates. Thus, the *bimolecular* rate constants for binding substrates (or products in the reverse direction) by the enzyme cannot exceed the corresponding V_{max}/K_m (or V'_{max}/K'_m) ratios. Furthermore, the V_{max} values alone provide lower limits, in the appropriate directions, to any *uni-*

molecular kinetic steps in the enzymatic catalysis, including dissociation of the bound substrates or products. When applied to the dehydration reaction, a computation assuming H_2CO_3 as the reverse substrate led to a rate estimate of its recombination with enzyme that exceeded the diffusion limit by an order of magnitude. The diffusion-limit for a binding of a small molecule to an enzyme can be estimated with the theory of Alberty and Hammes [33], utilizing the diffusion coefficients of the reactants and assuming a reasonable 'reaction radius' (contact distance of closest approach for reactants to become products) of approximately 5 Å. Devoe and Kistiakowsky then demonstrated that this bimolecular rate problem is obviated if the choice is instead made that HCO_3^- is the reverse substrate. Most reasonable biochemists, certainly Edsall [27], were very comfortable with this choice of HCO_3^-. It was well known at the time that the active site bound a variety of anionic inhibitors, with affinity following the Hofmeister series. Thus, there was a good chemical basis for believing that reverse substrate HCO_3^- binds at or close to the metal ion like other anions.

3.3. Microscopic reversibility—a decided lack of balance

The product choice appeared settled for approximately 12 years, but a rare few were reluctant to dismiss H_2CO_3. None were as bold as the physicist Seymour Koenig, who jumped in when the choice of HCO_3^- brought newly recognized difficulties, discussed below. Koenig was convinced that the zinc hydroxide mechanism of Davis that we espoused [30,34] patently violated the principle of microscopic reversibility (also called detailed balance). One expression of this principle is the familiar Haldane relation for a catalyzed reversible reaction, which requires that the V_{max}/K_m ratio for the forward and reverse directions must be equal to the overall equilibrium constant. For physicists, however, this is the same principle of detailed balancing that finds application in spectral transitions (excitation vs. emission probabilities) [35] and is deeply rooted in quantum mechanics [36], e.g. the concept of time reversal. According to Koenig [37], we biochemists had flagrantly violated this most sacrosanct principle when we stated [30] that the basic form of the enzyme (i.e. zinc–hydroxide form) was active in the hydration direction, and the acid form (zinc–water) was active in the reverse dehydration. He took at face value words that were only meant to point out that a different active site ionization form released the product HCO_3^- than that which bound the substrate CO_2. We were only emphasizing the necessity for an active site deprotonation step that must complete the catalytic cycle.

No such violation had actually occurred in practice. We had expressly written all reaction steps as being reversible [30,34], which automatically prevents a situation where equilibrium is established by unidirectional perpetual loops [35,36]. Koenig could not recognize this. He gained some notoriety as he continued to make these accusations at presentations and conferences. He later went so far as to propose his very own 'principle of charge neutralization in the active site' [38]. This true smoke-and-mirror idea claimed anionic inhibitor binding is pH-independent by postulating that a proton always accompanied an anion upon binding (but where does it sit?). Ergo, there was no active site ionization (read: no zinc–hydroxide). If there is no true ionization involved, then this 'principle' implies that when Cl^- inhibits, it is really HCl that is being bound, and when NO_3^- inhibits, it is HNO_3, etc. Most scientists, surely including Edsall, have been trained to arrive at new principles through an inductive process that synthesizes many independent observations. In contrast, here was a 'principle' that is illogically used to describe a 'hypothesis' for which no support exists except the very data it is supposed to explain. This undeniably circular process is self-deluding, and, regrettably, it is not so rare to find today.

3.4. A stunning proposal

While fomenting this confusion about the zinc hydroxide mechanism and bicarbonate, Koenig 'solved' the diffusion problem for carbonic acid as product by proposing that the 'reaction radius' for the enzymatic catalysis was greater than accepted in chemistry by nearly two orders of magnitude.

Although not explicitly stated as such, he was truly proposing that when the carbonic acid approached the active site within some 200 Å, i.e. *within several enzyme diameters*, it was somehow dehydrated to carbon dioxide [37]. Perhaps Koenig believed in supra-molecular quantum tunneling. I had a slightly different opinion of the reasonableness of his proposal, and the Reader should be clear about this. Life, including the discipline of biochemistry, would be truly different if enzymes catalyzed reactions from such distances. Chemical concepts such as orbital overlap, specificity, specific binding, active sites, acid–base catalysis, approximation, steric control and 'orbital steering' would be utterly irrelevant. Even enzymes would be superfluous. Kistiakowsky's detonation of the first nuclear device would pale in comparison to the impact of Koenig's proposal on Mother Nature. Enough said.

3.5. Proton transfer: from the frying pan and into the fire

A decade after Devoe and Kistiakowsky had rejected H_2CO_3 as hydration product, a different and even more serious dilemma was becoming evident with the alternate choice of HCO_3^- [30,39–41]. If the enzyme is to formally hydroxylate the CO_2 substrate and expel HCO_3^-, then a proton (from the split water) must remain in the active site. To complete the catalytic cycle and be ready for the next catalysis to occur, the deprotonated (basic) form of the active site must be regenerated. The pH dependence of V_{max} indicated that the pK_a of this coupled ionization is in the neighborhood of 7.0–7.5, widely believed to be the ionization of the metal-bound water ligand. Since V_{max} is in the neighborhood of 10^5 to 10^6 s^{-1}, and since this value sets the lower limit for any *unimolecular* step in the catalysis, deprotonation must also be occurring at least this fast. The dilemma occurs because it has been established that ionizing groups of pK_a near 7 can *spontaneously* dissociate a proton with a maximal rate constant of no more than approximately 10^3 s^{-1} [16,42]. Thus, *for the choice HCO_3^- for the product, the kinetics data implies that the diffusion limit is exceeded by two to three orders of magnitude*.

3.6. Heads in the clouds fail to see the deceptive simple trap underneath

Edsall and other biochemists, unlike some physicists, had the instinctive feeling that the enzyme had special ways to deal with facilitating ionizations [16,27,30]. The only problem was trying to figure out how. Evidence was solid from the metal-substitution studies of Lindskog that the enzyme operated by a metal-hydroxide mechanism. Enzymatic hydration thus almost surely produced bicarbonate, not the carbonic acid that had never been observed or isolated as a chemical entity. Is the active site acidic enough to protonate an initial bicarbonate and expel carbonic acid first? To live with the bicarbonate choice, it was common to invoke intramolecular assistance by neighboring ionizable groups in order to achieve active-site (zinc–water ligand) deprotonation at the required rates [27,30]. Somehow this wishful thinking only served to mask our ignorance. The kinetic problem was fully *intermolecular* in nature, since it involved deprotonation to the solvent. An unrealistically large number of facilitating ionizable side-chains would be needed in the active site to make a dent on the intermolecular proton transfer rate.

This paradox grated on me for months after I left Edsall, even though I had moved to new and unrelated research. The annoying challenge by Koenig kept bringing the subject up, but it also sharpened our focus. We could not entirely dismiss his utterly unreasonable proposal until we adequately justified the unreasonably high deprotonation rate required by ours. Over the years, the carbonic anhydrase paradox had caught the attention of many distinguished kineticists and enzymologists, such as Eigen, Hammes and Jencks, and was mentioned in some of their reviews. Ironically, the paradox was explainable in the end by data that Eigen, Hammes and others generated on small molecules without recognizing its applicability to this paradox. The information needed for solving the puzzle thus lay naked in all the T-jump data on the ultrafast kinetics of proton transfer between donors (acids) and acceptors

(bases) that were demonstrated to follow Brönsted behavior [16,42]. The Michaelis–Menten kinetic parameters of the 'impossibly fast' carbonic anhydrase that were quoted so often had somehow become too abstracted and disconnected from the context of the experiments that generated them.

The key to the puzzle lay in recognizing how subtly the enzyme kinetics parameters were dependent on the experimental conditions under which they were derived. Buffer components are usually completely glossed over, unless they happen to affect the kinetics. With carbonic anhydrase, standard care had been taken, of course, to utilize non-participating buffers, as judged by the lack of effect of their concentration on the kinetics [30]. *In reality, the buffers used were silent facilitators of the proton transfer from the enzyme, but they had escaped detection since they were used at high enough concentrations that deprotonation was no longer rate limiting* [43,44]. The missing link was simply the coupling of the enzymatic ionization to a simple proton exchange reaction with buffer:

$$EH^+ + B \leftrightarrow E + BH^+ \quad (1)$$

3.7. Finally solving the paradox—a eureka moment

It was the winter of 1972–73, and I was sitting at home in Palo Alto working on an interview seminar to be given in a few days at the University of Virginia. I had decided to feature the kinetics of the enzyme that I had abandoned two years before, rather than present on my current and largely unproductive NMR work. The possibility crossed my mind for the first time that the proton of the enzyme under stop–flow conditions was mostly transferred directly to the basic component of the buffer, rather than spontaneously dissociating. The estimate of the contribution of the buffer to the rate of deprotonation of the enzyme needed only the pencil and paper in my hand. Plugging in the small molecule proton transfer kinetics data of Eigen [16,42] gave the effective enzyme deprotonation rate. Sure enough, the buffer concentration we used was more than enough to satisfy the 10^6 s^{-1} requirement dictated by V_{max} [42,43]. Anyone could have done this trivial calculation decades earlier. My excitement at reaching this conclusion must have been evident during the subsequent (successful) seminar and interviews. I could hardly contain my excitement and impulsively called Edsall from Virginia. I then detoured to Boston instead of flying home to share my revelation with him in person, two years after having left his laboratory. He listened eagerly and then suggested that I stay over the weekend to draft a paper while the thoughts were still fresh. I gladly did so, producing a handwritten manuscript in a record two days time (utterly unbelievable to anyone who has remotely known me since, including the present Editors), which I left for Mrs Nixon to type and for Edsall to submit.

3.8. Setting the record straight—once, twice, thrice

As required by the Proceedings of the National Academy of Science at the time, Edsall solicited expert comments prior to submission. To my relief, William Jencks was very positive and assured us that we were on the right track. His helpful comments not only sharpened the hypothesis, but they also eliminated one erroneous suggestion for an experimental test. To leave no doubt, Edsall also solicited comments from Gordon Hammes, who, like Jencks, had also puzzled over the paradox in the past. He too responded favorably, believing that the arguments were sound though still wondering whether an active site residue could sufficiently facilitate the proton transfer to solvent. Unbeknownst to me, Sven Lindskog in Sweden had arrived at this same juncture months earlier and was in the process of preparing a similar manuscript for Edsall to submit. My friend Lindskog was understandably very disappointed to learn of our paper, which appeared in print two months before his [44]. Edsall made sure we cross-referenced each other in galleys. Most importantly, both of us felt that we closed the book on Koenig's H_2CO_3 proposal and silenced his criticisms regarding microscopic reversibility. Edsall wrote me with obvious delight and satisfaction: 'I think you have pretty well demolished that argument'.

While still another independent report on the topic appeared afterwards [41], the true ending of the story came several years later. I happened to

browse an old article on enzyme kinetics by Robert Alberty in the 1958 Brookhaven Symposium proceedings [45], a volume that had been in my possession since graduate school days. It included transcripts of questions and answers that followed the talks, a very useful though nowadays extinct practice. I was stunned to read his response to a question by Charles Tanford regarding explicitly including enzyme ionizations in kinetic schemes. Alberty stated in the clearest terms how a buffer-mediated ionization (protonation in this case) can maintain an enzymatic cycle during acid–base catalysis [46]:

> At pH 8, let's say, the hydrogen ion concentration is 10^{-8}, and, even if the bimolecular rate constant is 10^{10} M^{-1} s^{-1}, the reaction of a proton with the site is not going to happen very fast. What is really happening in a step like this is that ES reacts with the acid form of the buffer HA to form ESH plus A, and this reaction, as Eigen's work has shown, is usually a diffusion controlled reaction. This step, then, can occur very rapidly because the concentration of the acid constituent of the buffer is much greater than the concentration of free protons.

It was humbling to read this passage, which predated our 'revelation' by more than a decade and anticipated the situation of carbonic anhydrase even before the kinetics were measured and the paradox recognized. Eigen and Hammes had also noted this buffer mechanism [16] without making the connection to the carbonic anhydrase paradox. But, in no way, did all this diminish my pleasure at having had a 'eureka moment' to share with Edsall. The buffer-mediated proton transfer should now always be referred to as the 'Alberty mechanism'.

The experimental verification of the carbonic anhydrase buffer hypothesis was left for others to accomplish [47–53]. Before long, David Silverman detected a buffer effect on the enzyme-catalyzed rate of exchange of ^{18}O from labeled CO_2 to the solvent at chemical equilibrium [47,52], while Lindskog demonstrated the buffer effect in stop–flow kinetic studies [48,53]. Both of these exceptional scientists also proved that an *intramolecular* proton transfer step is an essential additional element in the rapid catalysis by isozyme II, with the side-chain of His-64 functioning as a surrogate donor group that communicates with the solvent [49–54]. Due to their innovative approaches and their masterful application of site-specific mutagenesis, the critical proton transfer steps of carbonic anhydrase are arguably better understood now than for any other enzyme. Ultimately, they succeeded in detecting buffer effects because the extremely rapid enzyme turnover delicately leaves proton transfer close to the rate limiting pathways.

4. The dilemma of substrate binding

4.1. The elusive CO_2 substrate

In contrast to proton transfer aspects of the catalysis, our understanding of the binding of substrates in the active site cavity has remained to this day speculative at best. Edsall had devoted much energy and thinking on this subject, frequently noting that the physical properties, solubility and geometry of carbon dioxide were virtually identical to those of the isoelectronic and linear nitrous oxide (NNO) molecule [27]. At the time I started, a highly publicized infrared study appeared by Jui Wang, claiming the observation of the asymmetric stretch band of the bound CO_2 substrate. Based on a small frequency shift, he reported that this substrate was loosely bound in a hydrophobic part of the active site [55], which was plausible but not entirely accurate [27,30]. Wang also reported that nitrous oxide competed with the CO_2 and bound with equal affinity to that site. When we rushed to confirm this 'inhibition' by the stop–flow kinetic technique, we discovered that there was no effect whatsoever on the kinetics [30], even at four times the highest concentration of nitrous oxide that Wang used. The conclusion was inescapable that Wang had observed non-productive binding in the active site. This was reinforced by the report that the CO_2 he saw was displaced by nitrate and azide; neither inhibitor was competitive in our CO_2 assays. In actuality, Wang observed two sites, with the alternate site he neglected having better concordance with our inhibition patterns. The innovative infrared approach was later revisited by Carol Fierke and collaborators [56]. However, despite the great technical advances made in the intervening two decades, the more recent experiments proved equally

difficult to execute and quantify. The excessively narrow linewidths for the CO_2 band that were obtained by spectral subtraction diminish confidence in the derived conclusions [56], as do some of the inhibition patterns observed. Another promising approach was ^{13}C paramagnetic NMR relaxation utilizing cobalt and copper substitutions for zinc, as applied by Robert Henkens [57] and Ivano Bertini [58,59]. Their studies attempted to measure the distance of the bound CO_2 from the active site metal, a task made extremely difficult by the unfavorable equilibrium ratio of CO_2 to HCO_3^- that is dictated by the overall first ionization equilibrium [26].

4.2. Chasing the competition

Entirely different approaches to identifying substrate binding sites arose from the accidental discovery of imidazole as the first competitive inhibitor of CO_2 in isozyme I. This unique inhibition emerged literally from my very first kinetics experiment in Edsall's laboratory. My lab predecessors left a note to purify the imidazole buffer being used in the stop–flow studies, since it apparently contained inhibitory impurities. Extensive purification by recrystallization and ion-exchange chromatography did not impact the inhibition, convincing me that it was intrinsic to imidazole. This set the stage for full kinetic characterization, which led to the demonstration that imidazole was a unique competitive inhibitor of CO_2 [30]. A decade later, Lindskog reported the discovery of a second competitive inhibitor in studies on the other major isozyme II, and that was phenol [60]. The imidazole discovery stimulated many spectroscopic, magnetic resonance, calorimetric and crystallographic studies to define the uniqueness of binding [61–67]. Studies on phenol were also carried out with the hope of discovering some commonality that may shed light on substrate location. I have summarized the synthesis of these complex pH-dependent findings and their relation to crystallographic investigations on the imidazole complex elsewhere [68].

Our subsequent ^{13}C NMR paramagnetic studies on the phenol binding permitted accurate distance measurements between the active site metal ion and each carbon in phenol [69]. These were clearly incompatible with metal coordination but were consistent with a face-on orientation similar to what Kannan had crystallographically found for imidazole in the other isozyme [67]. Subsequently, a crystal structure of the phenol complex was reported [70] that was fully consistent with our predictions and our simple molecular modeling [69]. The two unique inhibitors clearly share this unique binding mode at the appropriate pH, with binding being off the metal and without displacing its solvent ligand, as depicted in Fig. 2. The picture that emerges from these competitive inhibitors reinforces the feeling that carbonic anhydrase binds the CO_2 substrate with great specificity and in a defined mode, as originally suggested to account for the enzyme's ability to distinguish between the virtually identical CO_2 and N_2O molecules [30]. However, it must be sadly acknowledged that many investigators today still believe in a diffuse

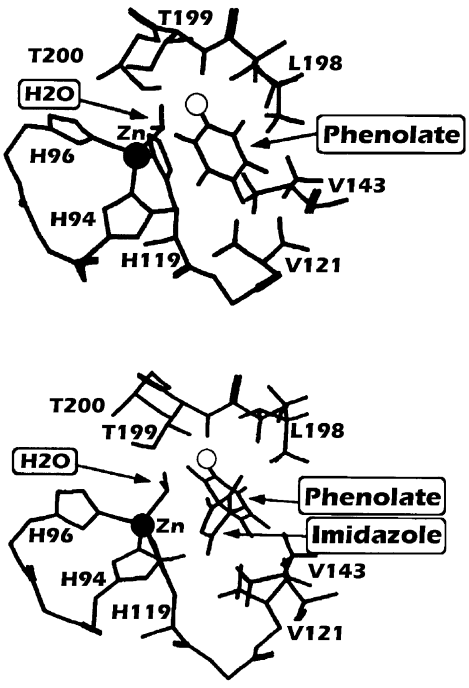

Fig. 2. (Top) Model of the competitive inhibitor phenol in CA II based on paramagnetic C-13 NMR studies of the cobalt enzyme. (Bottom) Comparison of phenol binding with the crystallographic orientation of imidazole oriented as in CA.

binding mode in the hydrophobic cavity without any particular orienting interaction. This is no doubt influenced as much by the failure to directly observe substrate binding by crystallography as by the results of molecular dynamics simulations that substitute trajectories for energy-minimized defined structures [71,72].

4.3. Binding of the HCO_3^- substrate

The identification of HCO_3^- as the hydration product (reverse substrate) and the known ability of the active site zinc to bind anionic inhibitors implicate the metal in binding of this substrate. However, anion affinity follows a Hofmeister series rather than ligand field strength, and the precise mode of binding of this substrate to the native enzyme is still in doubt. Crystallographic studies have been carried out on various bicarbonate complexes of variants of the enzyme [73–75], with no less than three different binding modes being seen. In some interpretations, the oxygen atom bearing the proton in bicarbonate has been proposed as a ligand to the metal [76]. Such a hypothesis has little, if any, precedents in the coordination chemistry literature. As the case with CO_2, it is likely that the binding mode of this small substrate will remain speculative well into the near future, a reflection of limited resolution as well as the general difficulties of studying very small substrates that lack multiple attachment points in active sites.

5. Conclusions

Edsall retired three decades ago at a time when the study of carbonic anhydrase was about to enter a golden era. The achievements of his laboratory that were highlighted above proved influential and provided the stimulus for a large amount of subsequent research in a number of laboratories. Yet, many of the questions that his research focused on are still unanswered. Why then is the mechanism of the enzyme not better understood, particularly since a staggering number of carbonic anhydrase crystal structures, some 175 at last count, have been solved [3,7,76,77]. One should not be afraid to admit that serious deficiencies frequently undermine the mechanistic speculations that invariably accompany crystallographic structures. Crystal structures are inherently ‘static’ on the time scale of catalysis by most enzymes. Crystallography over-interprets mobility or alternate conformational states, since it cannot tell anything about the *rates*, and hence relevance, of such processes. Several carbonic anhydrase structures have also been derived from crystallizing solutions containing the strong active site inhibitor azide, leading to a recognizable electron density whose consequences are either ignored or dismissed. Another area of concern is the fact that the crystal structures of the uniquely important imidazole and phenol complexes have not yet been deposited in the Protein Data Bank. Further attempts at observing enzyme–substrate complexes have been too readily ruled out. We should question whether the lack of success in observing true enzyme–CO_2 complex by crystallography is really attributable to its being ‘too short lived’ [7], when we know that the enzyme is fully equilibrated with the reactants of this reversible reaction and lifetime is not an issue.

Fresh approaches, not more structures, are needed to reach the next level of understanding of the mechanism. Neutron diffraction can perhaps best put ‘mechanistic protons’ to the test. Is the solvent ligand a water or a hydroxide at high pH? Does the Thr199 side-chain donate or accept a hydrogen bond involving the exchangeable ligand of the zinc? Is the coordinated oxygen of the bound bicarbonate substrate really protonated? Carbonic anhydrase catalysis is subtle and enzyme control is indirect, modulating the properties of the coordinated water ligand and gently orienting weakly bound substrates. Key functional players may surprise, such as the peptide NH of Thr199 that cannot be modified by the usual site-specific mutation approaches. To those who profess to understand the mechanism of this unique enzyme, the challenge can be put in a different way: *why is carboxypeptidase A, a zinc enzyme with an ionizable water ligand and a hydrophobic binding pocket, not a catalyst for the hydration of CO_2, and can we make it so?* Until we can answer such questions, I believe that Edsall’s carbonic anhydrase will retain significant mysteries that will

continue to challenge future researchers for decades to come.

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