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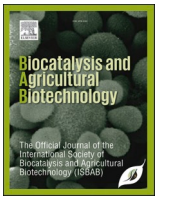

Functional analysis of carbonic anhydrases: comparative methods for hydration and hydrolysis activity

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A B S T R A C T

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes that catalyse the reversible hydration of carbon dioxide and, in some cases, the hydrolysis of esters, phosphates, and sulfates. Their high catalytic efficiency makes them promising biocatalysts for CO_2 capture, conversion, and utilisation in sustainable biotechnological applications. Despite extensive studies on CA structure and medicinal applications, a comprehensive comparison of functional assays for CA activity is lacking. This concise review systematically presents and critically evaluates methods for CA activity analysis, including hydration- and hydrolysis-based approaches such as manometry, gravimetry, protonography, potentiometry, spectrophotometry, isotope exchange, and gas-based detection techniques. Each method is evaluated based on its principles, sensitivity, equipment requirements, applicability across various CA classes, and suitability for high-throughput screening. Special attention is given to methodological challenges affecting accuracy, reproducibility, and the study of novel or engineered CA variants, including those from extremophilic organisms. By consolidating existing techniques and highlighting their advantages and limitations, the review provides a practical framework for selecting and optimising CA assays. Moreover, it supports the discovery, functional characterisation, and industrial application of CAs as versatile biocatalysts, promoting CO_2 valorisation and advancing sustainable processes in biocatalysis and agricultural biotechnology.

1. Introduction

The rapid acceleration of global warming is largely driven by increasing atmospheric carbon dioxide (CO_2) emissions originating from industrial activities, power generation, and transportation. One promising strategy to mitigate these emissions is the utilisation of CO_2 as a feedstock for the production of value-added chemicals and fuels. A range of catalytic technologies is currently being explored to enable such conversions, including biocatalysis, electrocatalysis, photocatalysis, and thermocatalysis (Talekar et al., 2022). Among these approaches, biocatalysis has attracted considerable attention for its potential to provide sustainable, efficient, and cost-effective alternatives to conventional chemical processes, positioning it as an important component of future industrial chemistry. Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloenzymes that catalyse the reversible hydration of CO_2 to bicarbonate with exceptionally high turnover rates in living organisms. Because of their catalytic efficiency, CAs have been proposed as key biocatalysts to accelerate CO_2 capture, conversion, and utilisation processes, thereby contributing to emerging carbon-neutral technologies. CAs are widely distributed across all three domains of life, and eight distinct structural classes have been identified to date (α, β, γ, δ, ζ, η, θ, and ι). Although CA research has been extensively developed in biomedical contexts, its application in industrial biotechnology, particularly for CO_2 capture and conversion, remains at a comparatively early stage. One important factor limiting progress in this field is the lack of robust, standardised methodologies for measuring CA activity. Current enzymatic assays exhibit significant variability in sensitivity,

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environmental dependence, and susceptibility to experimental interference, complicating comparisons of enzymes derived from different sources (Fuchs et al., 2021). In addition, many existing assays were originally designed for mechanistic or physiological studies rather than for enzyme discovery pipelines, creating challenges for high-throughput screening and large-scale biocatalyst development. Carbonic anhydrase activity can be measured using two main reaction types: the physiologically relevant hydration of CO_2 and the hydrolysis of synthetic ester substrates, which is frequently used as a convenient proxy reaction. However, despite the widespread use of both approaches, their methodological differences, advantages, and limitations have not been systematically compared in the literature. In particular, there remains limited critical discussion of the suitability of these assays for high-throughput screening to identify enzymes with enhanced CO_2 hydration activity, the function most relevant to carbon capture and utilisation technologies (M. Verma et al., 2021). To the best of our knowledge, this review presents the first systematic comparison of carbonic anhydrase activity assays based on CO_2 hydration and ester hydrolysis reactions. By critically analysing the methodological characteristics, advantages, and limitations of each approach, this work highlights key methodological gaps, particularly the current lack of efficient high-throughput screening strategies for CO_2 hydration. Addressing these gaps is essential to accelerate the discovery and engineering of improved CA biocatalysts and enable their broader application in industrial CO_2 conversion technologies.

2. Methods for the functional analysis of carbonic anhydrases

CAs are enzymes that catalyse the reversible hydration of carbon dioxide. As CAs are widespread within all living organisms, these enzymes differ in structure, catalytic activity, and distribution (Table 1). As previously mentioned, currently, there are eight distinct classes of carbonic anhydrases found in nature (α, β, γ, δ, ζ, η, θ, and ι) (Jensen et al., 2019). Animals possess only the α-CA class. In addition, this class is also found in plants, algae, and protozoa, while bacteria, fungi, and archaea have β-CAs, and bacteria, cyanobacteria, and archaea have γ-CAs. Marine diatoms have been found to contain the δ, ζ, and θ classes (Aspatwar et al., 2022). Typically, the active site of CAs contains zinc as the most frequently observed metal ion in α, β, γ, and δ-class CAs (Rowlett, 2010). θ-class CA is less described, but it has been demonstrated to have a zinc ion in its active centre (Kikutani et al., 2016). The γ-class can coordinate various metal ions, including Fe^{2+} or Co^{2+}. Meanwhile, ζ-CAs are capable of being active with either Zn^{2+} or Cd^{2+} ions and appear to be coordinated in a fashion similar to certain β-CAs (Amata et al., 2011). η-CAs are reported to coordinate Zn^{2+} metal ions (De Simone et al., 2015). Most recently, ι-CAs have been characterised to have Mn^{2+} (Jensen et al., 2019). Variation of CAs that can operate without a metal ion has also been described (Hirakawa et al., 2021). While the α-, β-, and γ-CAs regulate fundamental processes such as respiration (α), photosynthesis (β), and archaeal metabolism (γ), the more recently evolved δ-, ζ-, and ι-CAs have specialised in marine

Table 1
Comparative analysis of CAs.

Class	Occurrence	Structural features	Biochemical properties	Confirmed catalytic activity types	Ref.
α-CA	Vertebrates (exclusive in mammals), some bacteria	Monomeric; Zn^{2+} coordinated by three His residues; deep active site crevice	Broad versatility; sensitive to sulfonamide inhibitors; thermolabile	Hydratase (multiple techniques) Esterase, phosphatase, sulphatase, carbonate hydrolase (UV-VIS spectroscopy)	Boone et al. (2014); Supuran (2016)
β-CA	Bacteria, archaea, plants, fungi, algae	Oligomeric (dimer, tetramer, octamer); Zn^{2+} coordinated by three Cys/His residues; active site at subunit interfaces	Thermostability, pH-dependent activity, and unique metal coordination	Hydratase (multiple techniques)	Akocak and Supuran (2019); Supuran (2016)
γ-CA	Archaea	Trimeric; β-helix fold; Zn^{2+} coordinated by three His from adjacent monomers; can coordinate various ion metals (Fe^{2+} or Co^{2+})	Highly thermostable; primitive fold	Hydratase (multiple techniques)	Akocak and Supuran (2019); Aspatwar et al. (2022)
δ-CA	Diatoms	Zn^{2+} ion in the active site	Likely similar to ζ-CA in metal adaptation	Hydratase (multiple techniques)	Aspatwar et al. (2022); Boone et al. (2014)
ζ-CA	Diatoms	Repeated Zn-binding domain; binds Cd^{2+}/Co^{2+}as alternative metals	Adapted to low Zn-environments; metal substitution capability	Hydratase (multiple techniques)	Aspatwar et al. (2022)
η-CA	Protozoa, bacteria	Coordinate Zn^{2+} metal ion	Activated by amines/amino acids	Hydratase (multiple techniques) Esterase (UV-VIS spectroscopy)	Akocak and Supuran (2019)
θ-CA	Unicellular eukaryotes	Zn^{2+} ion in the active site	n.d.	Hydratase (multiple techniques) Esterase (UV-VIS spectroscopy)	Aspatwar et al. (2022); Kikutani et al. (2016)
ι-CA	Diatoms, algae	Metal-independent activity; characterised by Mn^{2+}	Functions without a metal cofactor	Hydratase (multiple techniques) Esterase (UV-VIS spectroscopy)	Aspatwar et al. (2022); Jensen et al. (2019)

carbon fixation. δ- and ζ-CAs optimise CO_2 acquisition in diatoms through surface-associated activity and the use of alternative metal cofactors, respectively. While ι-CA operates independently of metals, it provides ecological adaptability for phytoplankton. Together, these variations highlight how CA classes have evolved to meet the specific environmental demands of their host organisms, underscoring the potential for discovering new CA variants with unique properties and promising biotechnological applications.

The presented comparison shows that all CAs classes are structurally distinct but unified by a single factor: the catalysis of CO_2 hydration. The α, η, θ, and ι classes have been confirmed to display esterase-like activity, while the first identified and most studied α-CAs have been described as catalysing the hydrolysis of specific phosphate, sulfate, and carbonate esters. Other classes lack these activities or have not been thoroughly tested for them. Therefore, the most commonly used methods for CA characterisation encompass CO_2 as the substrate, such as spectrophotometric CO_2 hydration assays employing stopped-flow instrumentation (Khalifah, 1971), exchange of ^{18}O between carbon dioxide and water determined by membrane inlet mass spectrometry (Y. Li et al., 2011; Silverman, 1982), and assays based on the hydrolysis rate of *p*-nitrophenyl acetate as a surrogate (Colombo and Pinelli, 1981). CAs are confirmed as drug targets for various diseases and disorders linked to pH and electrolyte balance, including glaucoma, oedema, acute mountain sickness, epilepsy, cancer, and obesity (Chegwidden, 2021). Therefore, a primary application of CA enzymatic activity assays in drug design is to evaluate the inhibitory properties of new compounds or antibodies. These experiments are conducted under conditions closely mimicking physiological environments, typically at pH 7.0 to 7.5 and with added salts to maintain a constant ionic strength.

The typical CAs in the native state coordinate a hydroxyl ion (OH^-) in equilibrium with its protonated state – water (Fig. 1). Upon the entry of a substrate molecule, CO_2 or an ester, the Zn^{2+} ion acts as a Lewis acid and facilitates the coordination of the ligand through the oxygen atom. Coordination between metal ion and oxygen increases the C=O bond polarisation, making the carbon atoms more susceptible to a nucleophilic attack from the OH^- ion in the vicinity. The formed products undergo an exchange with a free water molecule, returning the enzyme to its native state.

The discovery and investigation of CA's catalytic properties now spans nearly a century. Over this period, several key studies expanded the range of methods available for CAs' investigations. Starting with the first documentation of CA catalytic properties (Meldrum and Roughton, 1933), authors used manometric CO_2 pressure measurements to examine proteins isolated from mammalian blood. In 1948, Wilbur and Anderson developed a potentiometric method involving CO_2-saturated water and a standard pH electrode. Another breakthrough came from Thorslund and Lindskog (1967), who proved that some CAs can catalyse ester hydrolysis, opening the possibility of employing ester substrates. The last major addition to the CAs activity assays toolbox was the employment of mass spectrometry (MS) to observe isotope exchange rates between different chemical species (Silverman, 1973).

In recent years, increasing attention has been directed toward the development of industrial technologies that use CAs for CO_2 capture and conversion into valuable products. To meet the demands of industrial applications, CAs must exhibit stability and tolerance to high salinity, pH, and thermal fluctuations while maintaining efficiency. As a result, various strategies have been implemented to obtain CAs with these properties. Genetic engineering methods, such as directed evolution and rational design, have been employed to enhance the performance of known CAs. In addition, there is a growing interest in discovering novel CAs with superior catalytic characteristics. A critical component of these efforts is the use of high-throughput screening methods to rapidly identify and characterise active CA variants. For instance, directed evolution relies on constructing a large genetic library using various mutagenesis methods, followed by functional screening. Alvizo et al. (2014) reported a directed evolution study on β-class CA from *Desulfovibrio vulgaris*, in which temperature tolerance was significantly improved to 107 °C in an alkaline environment. Using saturated mutagenesis in combination with the protein sequence activity relationship algorithm ProSAR, large libraries of genetic variants were created. A colourimetric assay was conducted employing potassium bicarbonate as the substrate and phenolphthalein as the pH

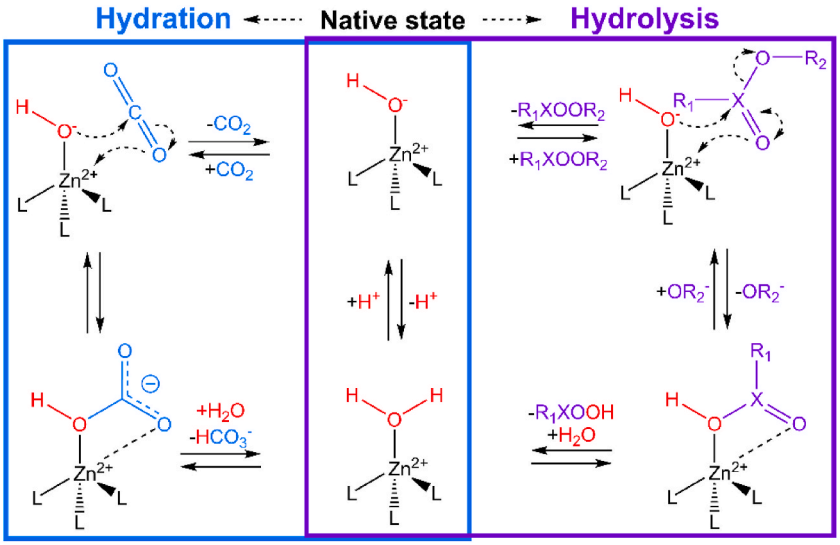

Fig. 1. Simplified reaction mechanisms of hydration and hydrolysis catalysed by the CAs.

Table 2
Techniques for CA functional analysis: assay principles and limitations.

Technique	Activity type	Assay principles	Advantages	Disadvantages	Limitations	Equipment	Ref.
Manometry	Hydration	Changes in CO_2 gas volume/ pressure	• Simple procedure • Inexpensive equipment • Simulation of industrial conditions	• Low sensitivity • High enzyme consumption • Laborious setup	• No high-throughput support	• Manometer • Differential pressure transducer	Karler and Woodbury (1963)
Gravimetry	Hydration	Mass of metal HCO_3^- or CO_3^{2-} salt precipitate	• Simple procedure • Inexpensive equipment • Simulation of industrial conditions	• Low sensitivity • High enzyme consumption • Laborious setup • Long analysis time	• No high-throughput support • Metal ion interference	• Analytical balance	Caulfield et al. (2022)
Protonography	Hydration	Colour change of Bromophenol Blue indicator	• Standard SDS-PAGE equipment • Complex samples • Assessment of enzyme size and oligomerisation • High sensitivity	• Long analysis time • Laborious setup • Short documentation window • Qualitative results	• Inactivation by SDS-PAGE components or procedure • Restricted high-throughput support	• Electrophoresis system • SDS-PAGE documentation system	Del Prete et al. (2015)
Potentiometry	Hydration	Changes in pH values	• Simple procedure • Inexpensive equipment • High sensitivity • Fast analysis	• High enzyme consumption • Specialised setup	• pH meter response time • Protein adsorption on electrode • No high-though support	• pH-meter • Wilbur-Anderson method setup	Wilbur and Anderson (1948)
UV-VIS spectrophotometry	Hydration Hydrolysis	Change in light absorption values	• Simple procedure • Fast analysis • Supports high-throughput screening	• Hydration – cost of stopped-flow setup • Hydrolysis – organic solvents	• Hydration – linear only in indicator transition range • Hydrolysis – not applicable to all CA classes	• UV-VIS spectrophotometer • Plate reader • Stopped-flow setup	Smirnovienė et al. (2017); Uda et al. (2015)
Isotope exchange	Hydration	Changes in isotope distribution between chemical species	• Complex samples • Wide range of conditions • Extreme sensitivity	• Complex results • Expensive equipment and consumables	• NMR – only end-point measurements • Restricted high-throughput support	• Mass spectrometer • Membrane inlet • Nuclear magnetic resonance spectrometer	Silverman (1982)
Gas chromatography	Hydration	Gas detection	• Complex samples • High sensitivity	• Specific equipment	• No high-throughput support	• Gas chromatographer • Gas sample injection system	Iizuka et al. (2024)
Gas FTIR	Hydration	Gas identification through vibration modes	• Simple procedure	• Low sensitivity • Specific equipment	• Moisture interference • No high-throughput support	• FTIR spectrophotometer • Specialised gas cell	Stimler et al. (2012)

indicator to identify improved mutants. This approach avoided time-consuming stopped-flow procedure or error-sensitive pH electrodes, enabling high-throughput evaluation of catalytic activity.

Another commonly used genetic engineering strategy is rational design, which is preferred over directed evolution because it allows targeted modifications at specific sites. The targets are predicted using bioinformatics tools based on the homology screening for protein structures and/or functions available in databases, such as the Protein Data Bank (PDB). For example, the thermostability of α-CA from *Thermovibrio ammonificans* was significantly improved by a rational design strategy that enhanced the protein's structural rigidity (Parra-Cruz et al., 2020). Using molecular dynamics simulations, flexible residues were identified as potential targets for mutations to increase the rigidity of the structure, thereby stabilising enzymes at higher temperatures. Rationally designed mutants were experimentally evaluated for CA thermostability. One variant exhibited a 2.6-fold increase in stability ($t_{1/2}$ of 203 days at 60 °C) compared to the wild-type enzyme ($t_{1/2}$ of 77 days at 60 °C). The evaluation involved assessing CO_2 hydrolysis using the Wilbur-Anderson assay and ester hydrolysis using the *p*-nitrophenyl acetate (*p*NPA) assay. The Wilbur-Anderson assay has its limitations. As this method requires precise low-temperature control, stable CO_2 concentration, and pH monitoring, performing multiple measurements in parallel for large-scale library mutant screening was challenging. In contrast, the *p*NPA assay supports high-throughput screening but is more susceptible to environmental influences and is suitable only for CAs that exhibit esterase activity. The shortcomings of both the Wilbur-Anderson and *p*NPA assays underscore the urgent need for new CO_2-hydration-based assays that can be easily conducted in parallel to screen CA mutant libraries or environmental samples.

Another study by Hsu et al. (2019) introduced a screening platform for assessing CA recombinants, using the Arduino-pH Tracker (ART) in conjunction with a phenol red assay and colony size determination on a plate. The α-CA from thermophilic *Sulfurihydrogenibium yellowstonense* was overexpressed in *Escherichia coli,* and the enzymatic properties were characterised by the ART system. This approach relies on the Wilbur-Anderson assay, in which pH changes are continuously monitored in solution throughout the reaction. The ART tracker enabled real-time monitoring of enzyme kinetics, unlike conventional pH meters, which can only measure pH periodically. Additionally, the phenol red assay for CA activity and the colony-forming unit size of the CA recombinants were evaluated. The phenol red assay is based on the colour change of phenol red, which is an acid-base indicator. During the assay, CO_2-saturated water is used as a substrate for the CA-catalysed CO_2 hydration. Enzymatic activity is monitored by tracking the pH-dependent colour change of phenol red, which is measured at 560 nm using a spectrophotometer. It is essential to note that this method can assess catalytic activity in whole cells without requiring any additional extraction steps, making it convenient for high-throughput screenings. Additionally, the study examined the relationship between the size of the CA recombinant colony on the plate and various CO_2 culture conditions. The authors highlight that a platform employing combined methods could be used for high-throughput screening of active CA recombinants.

Despite the application of genetic engineering to improve existing CAs, there is a high demand for the discovery of novel carbonic anhydrases, particularly those derived from extremophiles. Organisms living in extreme ecological niches often produce proteins that have evolved to maintain functionality under harsh conditions. Hydrothermal vents, hypersaline or soda-alkaline lakes and pools, deserts, and volcanic areas are the primary locations for the discovery of extremophiles (Mesbah and Sarmiento, 2016). However, more than 99% of extremophilic microorganism species cannot be cultured in laboratories using standard protocols (Papudeshi et al., 2017). Thus, metagenomic analyses using high-throughput bioinformatic approaches enable screening for billions of potential enzyme-encoding genes in vast metagenomic datasets (Guazzaroni et al., 2010). However, bioinformatic techniques are usually unable to determine enzyme activity. Currently, most CA enzymatic activity measurement approaches use solution-based colourimetric methods, which are typically used to assess protein extracts from cultured organisms rather than for initial screening. One of the most frequently mentioned methods in the literature for the functional screening of CA-positive organisms is the use of agar plates containing *p*NPA. However, methods using *p*NPA rely on CA esterase activity, which is not present in all CA classes and isoforms (Innocenti and Supuran, 2010). The method for assessing CA hydratase activity was introduced by Ferrer and colleagues (2020), who developed a rapid screening assay to directly detect CA-positive microorganisms in environmental samples. The method uses pH-sensitive strips inside the microcentrifuge tubes that change colour in response to CO_2 gas produced in the CA reaction. Nonetheless, the environmental inferences significantly limit the utility of this method and should be used only as a preliminary indicator, given the lack of alternatives for detecting CA activity. Despite the chosen activity measurement method, there is no universal assay suitable for screening CA-positive microorganisms.

Fig. 1 illustrates the simplified reaction mechanisms catalysed by CAs, highlighting their characteristic reversible hydration of CO_2 to HCO_3^-. In addition, certain CA isoforms can catalyse the hydrolysis of carboxylic, phosphoric, and sulfuric esters, as well as carbonate esters.

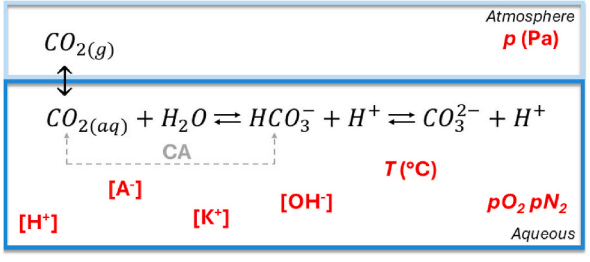

Fig. 2. CO_2 exchange and equilibrium in an air-aqueous biphasic system.

Further, Table 2 outlines a range of established analytical approaches for probing the catalytic performance of CAs. Each technique is distinguished by its specific detection method, experimental requirements, and suitability for different reaction types, including both CO_2 hydration and hydrolytic activities. The table compares the operational advantages and limitations of each method, helping researchers select the most suitable assay for their specific needs.

The overview of techniques used for CA activity measurements presents a wide range of methods with different levels of complexity, sensitivity, and methodological challenges. It also highlights two significant shortcomings that could hinder and frustrate researchers: most assays rely on CA hydration-catalytic activity and are not suitable for high-throughput screening. The substrate in hydration-based methods is the CO_2-saturated water. While its preparation is relatively simple and inexpensive, the gaseous properties of CO_2 may affect measurement reproducibility and accuracy. High-throughput techniques often rely on methods that allow fast scanning of samples in series or single measurements in parallel, e.g., a UV-VIS plate reader. The rapid turnover of CO_2 as a substrate, along with techniques that do not support or are heavily restricted by the analysis time or detection mode, leaves only CAs with hydrolytic activity compatible with high-throughput functional analysis. The presented advantages, disadvantages, limitations and proposed new strategies will be examined in more detail in the following sections.

2.1. Hydration-based assays for measuring CA activity

Medical and pharmacological investigations use CAs as targets for controlling pH and fluid balance-related diseases. Non-medical investigations mainly involve CO_2 fixation and subsequent utilisation (Nguyen et al., 2024). Therefore, it is not surprising that most available activity assays observe and measure the CO_2 hydration process. However, one of the main issues in activity experiments is the CO_2 gas, which is weakly soluble in water (Carroll et al., 1991). To prepare the substrate solution for measurements, dry ice is added or CO_2 gas is bubbled into the water, a process that can take up to 3 h to achieve a fully CO_2-saturated solution. The substrate concentration in a solution can be estimated from phase diagrams, but the actual CO_2 saturation and concentration remain unknown due to the dynamic CO_2 equilibrium illustrated in Fig. 2.

Zeebe and Wolf-Gladrow (2001) have summarised the factors affecting CO_2 equilibrium in their work on CO_2 in seawater. They used kinetic models to show that not only instantaneous pH, but also solution ionic strength, temperature, other gases, and atmospheric pressure influenced CO_2 solubility and hydration. Furthermore, $CO_{2(aq)}$, the substrate for CAs, accounts for only up to 1% of the equilibrium. On top of that, even pressurised solutions slowly leak gas, resulting in a continuously decreasing substrate concentration and shifting of the equilibrium. It is especially problematic in experiments measuring kinetic and inhibition constants for high-affinity compounds, since substrate concentration is one of the key variables. Another challenge with a CO_2-saturated solution comes in then enzyme is immobilised. Depending on the immobilisation method, the substrate has to cross several additional barriers between phases, such as the bulk liquid-to-liquid film on the carrier surface, to access the enzyme's active sites, as outlined in a review on immobilised CA application by Molina-Fernández and Luis (2021). If the immobilised CA retains its high turnover number, mass transfer may become the rate-limiting step, distorting the activity results. To overcome these issues, a possible strategy would be to develop an inert additive that increases CO_2 water solubility or use non-aqueous systems such as ionic liquids or deep eutectic solvents, which exhibit better CO_2 equilibrium stability (Trivedi et al., 2016). An often-overlooked alternative is to use carbonate salts instead of CO_2 to observe the dehydration reaction via a change in pH (Alvizo et al., 2014). Despite the listed limitations, most routine CA assays use CO_2 as the substrate, ranging from simple gas-volume measurements to sophisticated isotope-exchange analyses.

2.2. Physical measurements

Manometry, which measures gas pressure in a closed system, is a simple and direct method for quantifying CO_2 consumption in reactions (Dilmore et al., 2009). It was one of the first techniques used to identify CA in human blood and had an intrinsic role in studying CO_2 metabolism and CA's role in mammalian biochemistry (Karler and Woodbury, 1963; Roughton and Booth, 1946). Although this method has become less popular due to its laborious setup and the need for higher precision, especially in biomedical fields, it is still employed in a similar fashion for CO_2 capturing or immobilised CA research. The enzyme is placed in a flow-through system, such as a bubble or packed-bed reactor, followed by the injection of a known-composition gas mixture. Changes in effluent pressure, volume, or CO_2 fraction in gas-IR analysis allow the estimation of the kinetics of CO_2 absorption and catalysis in systems mimicking real-world conditions (Alvizo et al., 2014; Peirce et al., 2018; Yadav et al., 2010). Similarly, gravimetry can be used to measure the mass of absorbed CO_2 (Trivedi et al., 2016). By conducting the reaction in a solution containing Ca^{2+}, Mg^{2+}, or Ba^{2+} ions, the formation of CO_3^{2-} can be estimated based on the mass of the precipitated carbonate salt (Caulfield et al., 2022; Uygun et al., 2015). This method is often employed to investigate the role and potential applications of CA in biomineralisation and sequestration processes (Mirjafari et al., 2007; Power et al., 2016).

2.3. Electrometry

During CO_2 hydration, a single H^+ ion is produced, which changes the solution's pH; therefore, a pH electrode can directly measure the pH-decay rate. In fact, Wilbur and Anderson published such a method in 1948, which is now known as the Wilbur-Anderson assay (Wilbur and Anderson, 1948). It has been highly successful to the point that hydration activity units (W-A units) are now named after the authors and serve as the standard descriptor of CA catalytic efficiency. Even spectrophotometric assays, discussed later, express the enzymatic activity through W-A units. Nonetheless, a few aspects need to be considered. Firstly, the Wilbur-Anderson assay requires a specialised setup and laborious preparation, as the measurements are performed at 0 °C using CO_2-saturated water. Additionally, the

pH meter's slow response time can make it difficult to obtain results with high replicability. Secondly, this method needs a relatively large quantity of enzyme because the reaction volume for common pH electrodes is 5-10 mL. Thirdly, protein adsorption on the surface of a typical glass-membrane pH electrode may interfere with measurements. Lastly, measurements of immobilised CA require vigorous mixing to minimise the lag between H^+ diffusion from the enzyme to the electrode surface. Despite that, the characterisation of CA with W-A units remains the industry standard and will likely remain so for the near future.

2.4. Protonography

There is only one qualitative method for measuring CO_2 hydration – protonography. It is a specialised technique used to detect enzymatic activities that shift the pH in the presence of a substrate; i.e., catalysis changes the H^+ concentration. To perform protonography, SDS-PAGE electrophoresis is carried out using a standard protocol with a minor difference – the protein samples are not treated with 2-mercaptoethanol and temperature to avoid denaturation (Del Prete et al., 2015). For the detection of CAs activity, the SDS-PAGE gel is incubated in a buffer supplemented with bromothymol blue pH indicator, then in CO_2-saturated deionised water. The absorbed indicator undergoes a colour transition from blue (alkaline) to yellow (acid) near pH 7. In the gel, the CA catalysis process accelerates the release of H^+ ions, resulting in a green-yellow colour at the enzyme bands. This method provides a simple way to identify CA in protein mixtures, determine their molecular size or oligomerisation, and can even provide a rough comparison of activities. However, protonography has a few minor drawbacks. For instance, a sample treated without 2-mercaptoethanol or without boiling does not guarantee that the proteins will retain their activity. Furthermore, the catalytic activity of CA can be reversibly inhibited by SDS, and the inhibition can be reversed by incubating the gel in a Triton X-100 solution (Del Prete et al., 2015a,b). The indicator colour shift is a rapid process and must be documented within 10-30 s after the gel is placed in CO_2-saturated water. Otherwise, the CA bands begin to blur due to H^+ diffusion, lowering the overall pH and causing a colour transition in bromothymol blue throughout the gel (De Luca et al., 2015). In summary, protonography is a unique method that readily provides valuable information about CAs; however, the lengthy preparation and electrophoresis time, combined with a short time window to obtain decent results, require some practice and experience.

2.5. UV-VIS spectrophotometry

Spectrophotometric methods have been developed to measure CA activity using pH indicators as chromophores. The basic procedure involves mixing a buffer solution with an indicator and the enzyme. The reaction is initiated by adding CO2-saturated water to the mixtures, which catalyses the release of H^+ ions. The spectrophotometric measurement monitors the change in absorbance at the appropriate wavelength as pH decreases. The main drawback of this principle is that colour changes in indicators occur over narrow ranges. For an activity assessment over a wide pH range, multiple indicators are required, as illustrated by Smirnovienė et al. (2017). Khalifah (1971) investigated the use of *p*-nitrosophenol, *p*-nitrophenol, *m*-cresol purple, and chlorophenol red as indicators, but phenol red is the most common (Anandabaskar, 2022). It should be noted that each indicator has a unique molar extinction coefficient; thus, their characterisation must be conducted to normalise the hydration activity results across different indicators. Most spectrophotometric assays employ stopped-flow systems, an automated, extremely accurate and efficient mixing setup with measurement dead time as low as milliseconds, because the CO_2 hydration catalysis rate is extremely high, exceeding 1 M s^{-1} (Wistrand, 1981). Furthermore, stopped-flow systems are convenient for investigating the kinetics and mechanisms of action of inhibition. For more routine activity measurements, Kim and Jo (2022) recently developed a spectrophotometric method using real-time measurements in a cuvette with phenol red as an indicator. Briefly, despite the cost of stopped-flow equipment, spectrophotometric methods are essential for CA investigations and research, but are limited by the indicators used.

2.6. Isotopic methods

Both spontaneous and CA-catalysed hydration reactions are reversible, meaning that the formed HCO_3^- is exchanged into CO_2 and vice versa. Isotope analysis works by calculating the exchange rates for non-catalysed reactions *vs* catalysed reactions. For example, Simonsson et al. (1979) developed a method to generate $^{13}CO_2$ from $Ba^{13}CO_3$ in a controlled and sealed system. The catalysed exchange rates of ^{13}C atoms at various pH and buffers were measured by ^{13}C NMR spectroscopy. Such feats are not possible for spectrophotometric methods, as pH indicators are only effective in limited pH ranges (Gurd et al., 1974). Similarly, Silverman (1982) described an approach in which ^{18}O-labelled water was used in isotope-exchange experiments. The analysis was performed using mass spectrometry (MS) to differentiate between and measure the levels of CO_2, $CO^{18}O$, and $C^{18}O_2$ (Delacruz et al., 2010). Of course, the specific isotopes are not limited to the presented techniques and can be used in a variety of ways – ^{13}C for MS (McConnell et al., 2007) or ^{17}O for NMR (Rose and Bryant, 1980). In 2020, the first *in vivo* application of ^{13}C NMR on CA catalytic activity in the brain was published by Tomar and Shen (2020), reaching a milestone not only in the medical field, but also in enzyme functional analysis. In summary, NMR and MS are standard techniques for analysing enzyme catalysis with high sensitivity and are not restricted by buffers or

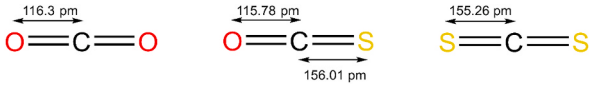

Fig. 3. Molecular structures of CO_2, COS and CS_2.

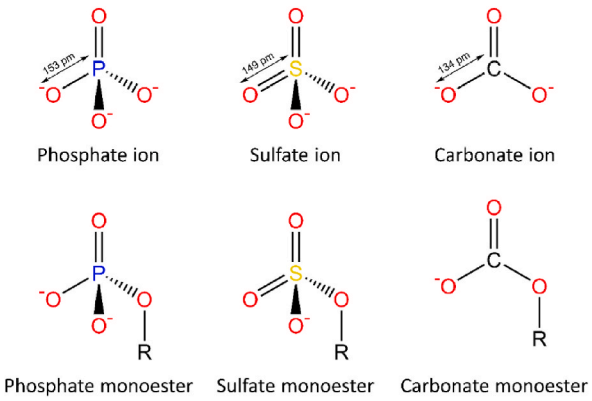

Fig. 4. Molecular structure of phosphate, sulfate and carbonate ions, and their corresponding monoesters.

pH. However, the requirements and cost of isotope-labelled compounds and specialised equipment make them less accessible for CA investigations than other methods.

2.7. Hydration of non-CO_2 substrates

CO_2 is the primary molecule involved in the CA catalytic cycle, but there are a few other molecules that can also participate in the same hydration reaction. Carbonyl sulfide (COS) and carbon disulfide (CS_2) are two such molecules that are structurally similar to CO_2 but with oxygen atoms substituted for sulphur (Fig. 3).

When these molecules undergo hydration, they produce CO_2 and H_2S. The formation of CO_2 can be monitored using gas-IR spectroscopic measurements (Stimler et al., 2012), while H_2S is observed in gas MS (Miller et al., 1989), gas chromatography (Iizuka et al., 2024; Protoschill-Krebs et al., 1996), qualitative PbS precipitate gravimetry (Alterio et al., 2021), or methylene blue formation (Haritos & Dojchinov, 2005). The exact biological function of this activity has not yet been determined; therefore, a broader range of methods is required for further research.

2.8. Hydrolysis-based assays for measuring CA activity

The second type of catalytic activity expressed by CAs is hydrolytic activity. It covers the hydrolysis of esters containing -OH functional groups and carboxylic acids for esterase-like activity, phosphate for phosphatase-like activity, sulfate for sulphatase-like activity, or carbonic acid for carbonate esterase activity.

The CA's esterase-like activity is best characterised by using *p*-nitrophenyl acetate. Its assay utilises UV-VIS spectroscopy to monitor the liberation of the *p*-nitrophenolate (pNP) anion at 400-405 nm (Peng et al., 2016). Research has shown that the substrate hydrolysis rate strongly correlates with the alcohol group pK_a: a lower pK_a results in a higher catalytic rate (Lopez et al., 2011). Because *p*NP has one of the lowest pK_a values for a simple phenol ($pK_a = 7.14$) and exhibits a high molar extinction coefficient above pH 7.5 ($\varepsilon > 10$ mM^{-1} cm^{-1}), it is one of the best chemical moieties for the UV-VIS assay. Longer acid alkyl chains result in slower hydrolysis rates, as demonstrated by investigations on other *p*NP esters, such as propionate, butyrate, valerate, and caproate (Höst et al., 2006). Unlike CO_2, the typical pNP acetate hydrolysis turnover is usually within the 10^1-10^3 s^{-1} range (Verpoorte et al., 1967), making it easy to handle in a standard spectrophotometer cuvette or plate reader, and to work with immobilised proteins (Yoshimoto et al., 2018). Therefore, *p*NP acetate is an excellent choice for investigating and screening potential inhibitors (Uda et al., 2015). On the other hand, *p*NP acetate is an unselective substrate as it is used for activity measurements of esterases (G. Li et al., 2020), lipases (De Caro et al., 1986), and other proteins (Schoonen et al., 2017; Taylor et al., 1963; S. K. Verma and Ghosh, 2010). Furthermore, due to its low water solubility, organic co-solvents, such as acetonitrile or dimethyl sulfoxide, are employed in the assays, which can interfere with enzymatic catalysis. Other substrates, like indoxyl acetate, have also been used for high-throughput screening in well plates (Baliukynas et al., 2020). Meanwhile, mandelic acid esters have been used to explore CA enantioselectivity (Chênevert and Létourneau, 1990). Interestingly, research has also shown that the human CA hydrolyses potential inhibitors based on carbohydrate sulfamates (Lopez et al., 2011). In summary, *p*NP acetate is one of the best-known substrates used in esterase assays, including those involving CAs that express ester-hydrolysis activity. Its wide applicability, low cost, and established methodologies make it an excellent choice for assessing CA activity; however, *p*NP acetate may be a limiting substrate in thermostable or highly alkaline enzyme research due to its high rate of autohydrolysis (Fredslund et al., 2018). In addition to CO_2 hydration, some lesser-known CA activities involve the hydrolysis of substrates such as phosphate, sulfate, or carbonate esters (Fig. 4).

In 1978, Pocker and Sarkanen demonstrated that CA can exhibit phosphatase-like activity (Pocker and Sarkanen, 1978). They compared the kinetics of hydrolysis of dimethyl 2,4-nitrophenyl phosphate and 2,4-dinitroacetate. Following this, Koester et al. (1981) investigated this activity of human CAIII using *p*-nitrophenyl phosphate (*p*NP phosphate) as a substrate. These discoveries prompted several discussions and investigations into whether the phosphatase activity is induced by glutathiolation (Cabiscol and Levine, 1996)

or is an intrinsic part of the enzyme (Kim et al., 2000). Carbamoyl phosphate was used to examine differences in mammalian CA phosphatase activities (Nishita and Deutsch, 1986). Additionally, it has been demonstrated that the α but not β, γ or ζ class CA's exhibit this activity, using *p*NP phosphate and diethyl *p*NP phosphate as substrates (Innocenti and Supuran, 2010). Despite these findings, the overall biological role and the mechanisms underlying CA phosphate activity remain poorly understood.

CA also exhibits a sulfatase-like activity, as evidenced by its ability to hydrolyse a variety of methanesulphonates (Kazancıoğlu et al., 2012) and cyclic diol monosulphonates (Çavdar et al., 2012). Interestingly, CAs have been shown not to catalyse the hydrolysis of pNP sulfate (Innocenti et al., 2008). Not much else is known or explored about CA's sulfatase activity.

The bicarbonate ion produced by CA catalysis is part of an acid-base equilibrium and has a structure similar to that of the carbonate ion. While their esters would be great for enzyme activity measurements, both bicarbonate and carbonate monoesters are highly unstable. Even the synthesis of a simple ester, like monoethyl carbonic acid, requires complex, multi-step, and air-sensitive reactions (Bernard et al., 2017), making these compounds commercially unavailable and practically inapplicable. Additionally, there is evidence of CA inhibition by mono-alkyl carbonates (Pocker and Deits, 1984). This leaves only diesters of carbonic acid, referred to as carbonate esters, as suitable substrates. Pocker and Guilbert conducted monumental kinetic studies in 1972 and 1974 on CA-catalysed carbonate decomposition. They used methyl *p*NP carbonate, bis-*p*NP carbonate, and three isomers of pyridine methyl carbonates to gain insights into the catalytic properties of CA, including inhibition mechanisms (Pocker and Guilbert, 1972, 1974). Surprisingly, no further works have been published on CA characterisations involving carbonate esters. This catalytic property appears to have fallen into obscurity to the point that even more recent publications listing CA-catalysed reactions do not include carbonate ester hydrolysis (Kazancıoğlu et al., 2012). There could be a few reasons, such as the absence of suitable compounds. In addition, the primary synthesis methods for these esters rely on phosgene, an extremely toxic and dual-use gas, which complicates the production of tailor-made substrates, even on a laboratory scale.

3. Concluding remarks

This review summarises the principal methods used to assess carbonic anhydrase activity, from classical techniques such as manometry and gravimetry to advanced analytical approaches including isotope exchange and gas-FTIR spectroscopy. While most assays focus on CO_2 hydration, several methods also evaluate hydrolytic activity, each presenting distinct advantages and limitations in terms of sensitivity, instrumentation requirements, and compatibility with different CA isoforms and reaction conditions. Together, these approaches constitute a valuable toolkit for CA functional characterisation, although their limitations highlight the need for more sensitive, scalable, and high-throughput methodologies. Future progress in CA activity assay development will likely rely on integrating microfluidic high-throughput screening, real-time biosensors, and AI-assisted activity monitoring for accelerated enzyme discovery and design (Pairoh et al., 2025). Microfluidic droplet platforms could enable rapid screening of large CA variant libraries, while biosensor-based assays may allow continuous monitoring of CO_2 hydration kinetics under industrially relevant conditions. An ideal next-generation CA activity assay should therefore combine ultra-high throughput, real-time kinetic measurements, robustness under elevated temperature and pressure, and compatibility with automated, data-driven enzyme engineering workflows. Such advances align with the goals of the new EU Bioeconomy Strategy (European Commission, 2025), which promotes the utilisation of biogenic CO_2 as a renewable carbon feedstock, further highlighting the potential of CAs for sustainable biotechnological CO_2 transformation.

CRediT authorship contribution statement

Justinas Babinskas: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Tautvydas Kojis:** Writing – review & editing. **Ieva Gaubė:** Writing – review & editing, Investigation. **Lina Baranauskienė:** Writing – review & editing, Investigation. **Inga Matijošytė:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

No data was used for the research described in the article.

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