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High kinetic stability of Zn^{II} coordinated by the tris(histidine) unit of carbonic anhydrase towards solvolytic dissociation studied by affinity capillary electrophoresis

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ABSTRACT

Solvolytic dissociation rate constants (k_d) of bovine carbonic anhydrase II (CA) and its metallovariants (M-CAs, M = Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}, and Cd^{II}) were estimated by a ligand substitution reaction, which was monitored by affinity capillary electrophoresis to selectively detect the undissociated CAs in the reaction mixture. Using EDTA as the competing ligand for Zn-CA, the dissociation followed the unimolecular nucleophilic substitution (S_N1) mechanism with $k_d = 1.0 \times 10^{-7}\ s^{-1}$ (pH 7.4, 25 °C). The corresponding solvolysis half-life ($t_{1/2}$) was 80 days, showing the exceptionally high kinetic stability of t Zn-CA, in contrast to the highly labile $[Zn^{II}(H_2O)_6]^{2+}$, where the water exchange rate (k_{ex}) is high. This behavior is attributed to the tetrahedral coordination geometry supported by the tris(histidine) unit (His_3) of CA. In the case of Co-CA, it showed a somewhat larger k_d value ($5.7 \times 10^{-7}\ s^{-1}$, pH 7.4, 25 °C) even though it shares the same tetrahedral coordination environment with Zn-CA, suggesting that the d^7 electronic configuration of Co^{II} in the transition state of the dissociation is stabilized by the ligand field. Among M-CAs, only Ni-CA showed a bimolecular nucleophilic substitution (S_N2) reaction path in its reaction with EDTA, implying that the large coordination number (6) of Ni^{II} in Ni-CA allows EDTA to form an EDTA-Ni-CA intermediate. Overall, k_d values roughly correlated with k_{ex} values among M-CAs, with the k_d value of Zn-CA deviating strongly from the trend and highlighting the exceptionally high kinetic stabilization of Zn-CA by the His_3 unit.

1. Introduction

Metalloenzymes harness a specific metal ion in their center to utilize its intrinsic properties in redox, hydrolysis, isomerization, and other reactions, which cannot be achieved by other metal species [1–3]. The biochemical environment of the enzyme is very complicated, with matrices including endogenous ligands (e.g. inorganic anions and carboxylic acids), and dynamic, since the central metal ion can be lost by mass transfer. Unless the enzyme is kinetically stable, the central metal ion dissociates from the enzyme, resulting in enzyme malfunction. In general, most of the first-row transition metal ions can be found in the center of metalloenzymes. Among them, some metal species, such as Zn^{II}, are intrinsically more labile than others, like Ni^{II}, as judged by their water exchange rates (k_{ex}), 3×10^7 [4] and $1 \times 10^4\ s^{-1}$ [5] (25 °C), respectively. It is interesting how such labile metal ions are utilized by nature, despite the kinetically unfavorable environment. Being one of the Zn^{II}-containing enzymes, carbonic anhydrase (CA) has been used as a model owing to its ready availability, lesser tendency to denaturation, and profuse information on the structure and physicochemical properties [6]. This enzyme catalyzes the hydration of carbon dioxide and dehydration of carbonic acid. Zinc(II) is retained in a tetrahedral coordination geometry by the tris(histidine) unit (His_3) of CA and one water or hydroxyl ligand. Another advantage of using CA is the feasibility of the preparation of its metal-substituted variants (M-CAs). Considering the abovementioned advantages, we studied the kinetic stability of M-CAs by measuring the solvolytic dissociation rate of the metal center to see whether the His_3 unit or the nature of metal center is the major factor governing kinetic stability. Reaction rates were measured for the ligand substitution reaction with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA) and monitored by affinity capillary electrophoresis (ACE), which can separate M-CA, apo-CA, and trace impurities, such as

Abbreviations: ACE, affinity capillary electrophoresis; C.N., coordination number; CA, carbonic anhydrase; CE, capillary electrophoresis; DPA, 2,6-dipicolinic acid; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate; His, histidine; LFSE, ligand field stabilization energy; M-CA, metallovariants of carbonic anhydrase; MO, methyl orange; PAR, 4-(2-pyridylazo)resorcinol; SA, 1-(4-aminosulfonylphenylazo)-2-naphthol-6,8-disulfonate; SDS, sodium dodecylsulfate; S_N1, unimolecular nucleophilic substitution; S_N2, unimolecular nucleophilic substitution.

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isozymes [7]. As a result, the highest kinetic stability among the M-CAs ($M = Co^{II}$, Ni^{II}, Cu^{II}, Zn^{II}, and Cd^{II}) was observed for Zn-CA, highlighting the role of the His_3 unit in gaining kinetic stability.

2. Experimental

2.1. Equipment

Capillary electrophoresis (CE) was carried out on an Agilent CE 7100 instrument equipped with a fused silica capillary (inner diameter = 50 μm; outer diameter = 375 μm) supplied by GL-Sciences Inc. A TOA-DKK pH meter (HM-25R) with a combined glass electrode (GST-5425C) was used for pH measurements. A Yamato Coolnics Circulator CTE42W was used for maintaining constant temperature.

2.2. Materials

The CA used throughout the study was isolated from bovine serum (>98% purity, cat. no. C3934) purchased from Sigma-Aldrich (St. Louis, MO, USA). The main constituent of the CA was identified as BCA II by CE of the CA isozyme II from bovine erythrocytes (C2522) purchased from Sigma-Aldrich [8]. Metal-substituted CAs were prepared by mixing the corresponding metal salt solutions with apo-CA, obtained by dialysis of CA with 2,6-dipicolinic acid (DPA, Fig. 1) [9] (see Supplementary data for details). Visking seamless cellulose tubing (UC-24-32-100) was used for CA dialysis. The sulfonamide inhibitor, 1-(4-aminosulfonylphenylazo)-2-naphthol-6,8-disulfonate (SA, Fig. 1), was prepared as reported previously [8]. All other chemicals used were of reagent grade. Deionized water prepared with an Elix Advantage 5 Water Purification System (Merck Millipore) was used throughout the study.

2.3. Ligand substitution reactions

The solutions of native CA, M-CA and the competing ligands (DPA and EDTA) were maintained at 25 °C before the reaction, which was started by adding an aliquot of the competing ligand solution to native CA or M-CA. The mixture was maintained at 25 °C and small portions were occasionally subjected to ACE analysis after a certain reaction time. The compositions of the reaction mixtures were as follows. Native CA-DPA system: 0.3 mg/mL CA, 10 mM NaH_2PO_4, 0.4–2.5 mM DPA, pH 7.4. M-CA-EDTA system: 1.0×10^{-5} M apo-CA, 2.0×10^{-5} M M^{II}, 5 mM NaH_2PO_4, 1.0×10^{-4} M Methyl Orange (MO, as an internal standard), 0.5–5.0 mM EDTA, pH 7.34.

2.4. Electrophoresis

Prior to the ACE run, the capillary (total length $L = 80.6$ cm, effective length $l = 71.9$ cm) was thoroughly washed with a mixture of 0.01 M sodium dodecylsulfate (SDS) and 0.01 M NaOH, and then rinsed with an electrophoretic buffer (20 mM phosphate or 25 mM Tris-phosphate buffer [pH 8.4 each] containing 7.9, 8.8, 44.1, and 441 μM SA, depending on the affinity of M-CA to SA). The sample solution of CA, apo-CA, or M-CA in 25 mM Tris-HCl (pH 8.4) was injected into the anodic end of the capillary by applying a pressure of 50 mbar for three seconds, and separation was then implemented by applying a potential of 30 kV, with absorption detection at the cathodic end carried out at 200 nm.

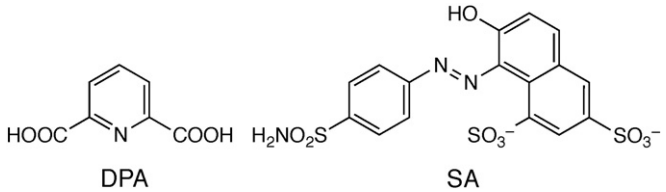

Fig. 1. Structures of DPA and SA.

3. Results and discussion

3.1. Experimental design for determining the residual concentration of M-CA

In the past, the rate of Zn^{II} removal from CA by competing ligands was monitored indirectly. For example, an assay of CA enzymatic activity by hydrolysis of *p*-nitrophenyl acetate under controlled temperature conditions can determine the CA residual concentration [10–13]. However, the procedure is laborious: to obtain monoexponential decay data for the metal removal reaction, one must repeat the enzymatic reaction after certain elapsed times. Moreover, this approach is not applicable to M-CAs ($M = Fe^{II}$, Mn^{II}, Ni^{II}, Cu^{II}, Cd^{II}, and Hg^{II}), which have no or little enzymatic activity [14]. Another way to quantify residual CA is the spectrophotometric determination of CA-bound Zn^{II} using 4-(2-pyridylazo)resorcinol (PAR) after chromatographic separation of CA from the Zn^{II} complex of the competing ligand and the excess ligand in the reaction mixture [15]. In contrast to these enzymatic and spectrophotometric methods, ACE is able to determine the concentration of CA in the reaction mixture directly, being able to separate apo-CA and CA on the basis of their electrophoretic mobility differences, which arise from differing affinities to the sulfonamide ligand in the electrophoretic buffer [7]. In fact, we recently applied ACE to determine sulfonamide binding constants (K_b) of M-CAs, which showed significantly larger K_b values than apo-CA [8]. This suggests feasibility of the separation of M-CAs and apo-CA by ACE.

3.2. Choice of the competing ligand

The goal of this study was to estimate the solvolytic dissociation rate (k_d) of M-CA (Eq. (1)) and clarify the dependence of k_d on the metal species, thus figuring out whether the nature of the metal center or the CA skeleton is the major determinant of kinetic stability.

$$M - CA \xrightarrow{k_d} M^{2+} + \text{apo} - CA. \quad (1)$$

This can be regarded as an S_N1 path, aka unimolecular nucleophilic substitution. To selectively observe the dissociation process, the recombination reaction of apo-CA and the free M^{II} (i.e. the reverse of Eq. (1)) needs to be suppressed. For this purpose, a competing ligand (Y) is added to capture the free M^{2+} ions as an $[M^{II}Y]$ complex. If the ligand Y attacks the central metal ion in M-CA directly, as shown in Eq. (2) (i.e. via an S_N2 path, aka bimolecular nucleophilic substitution), the observed rate constant k_{obs} will have a contribution not only from k_d but also from $k_Y[Y]$ (Eq. (3)), where k_Y is the ligand substitution rate constant of the S_N2 path.

$$M - CA + Y \xrightarrow{k_Y} \left[M^{II}Y\right] + \text{apo} - CA. \quad (2)$$

$$k_{obs} = k_d + k_Y[Y]. \quad (3)$$

Thus, the dependence of k_{obs} on the ligand concentration [Y] should provide the k_d value. Here, Y excess to M-CA was used to ensure the pseudo-first order conditions that make k_{obs} constant during the reaction. DPA was initially employed as a competing ligand, because it is known to be a good ligand for the removal the central Zn^{II} ion from native CA by dialysis. After mixing 0.3 mg/mL CA with 2.5 mM DPA in 10 mM phosphate buffer (pH 7.4) at 25 °C, the reaction mixture was subjected to ACE using an electrophoretic buffer containing 7.9 μM SA at pH 7.4, adjusted with phosphate (Figs. 2a and S1). As can be seen, CA and apo-CA showed separate peaks, due to differing electrophoretic mobility, that of CA being larger due to the binding of negatively charged SA. As time elapsed, the CA peak decreased in intensity, and the one of apo-CA increased, suggesting a release of Zn^{II} from CA to form apo-CA. In addition, the CA peak was accompanied by small