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Review

Chromatographic and electrophoretic methods related to the carbonic anhydrase isozymes

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Abstract

There are three gene families that encode zinc metalloenzymes that catalyze the reversible hydration of CO_2. The encoded enzymes are termed carbonic anhydrases (CAs). The CA isozymes have been purified from representatives of all types of organisms. Most CAs are strongly inhibited by aromatic sulfonamides. Several chromatographic and electrophoretic methods have been devised to determine binding constants for sulfonamides to CAs, and these compounds have been extensively used for, often single-step, affinity chromatographic separation of CAs from complex matrixes. The purification of different CA isozymes from different organisms is reviewed, as are methods for detection of CAs during chromatography and electrophoresis.

Keywords: Reviews; Carbonic anhydrase; Isozymes; Enzymes

Contents

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1. Introduction and historical aspects

The presence of a substance in hemolysates that increases the rate by which CO_2 reacts was first described by Henriques in 1928 [1]. However, he did not draw the conclusion that there was a catalyst present, but rather that CO_2 reacted with Hb to form carbhemoglobin. A few years later, Stadie and O'Brien [2], and Meldrum and Roughton [3] simultaneously discovered an enzyme that catalyzes the reaction between CO_2 and water. Based on the catalyzed reaction, the enzyme was named carbonic anhydrase (CA). It was known that catalase could be purified from blood by precipitating the hemoglobin with a mixture of organic solvents, and because CA was shown to be stable under similar conditions, this procedure was used to precipitate Hb from erythrocytes. The supernatant was then dried with a fan, extracted with 50% ethanol and stored dry [2]. Alternatively, the CA was precipitated with ether–ethanol [3]. Amazingly, some of the CA activity was preserved during the course of these procedures. Later, more gentle steps, such as ammonium sulfate precipitation [4] and eventually DEAE-cellulose [5], or hydroxylapatite chromatography [6] were added to increase the purity and yield of the enzyme preparations. A very strong binding of HCA II (then called HCA V) to sulfoethyldextran at pH 6.0 was used by Nyman [5] to purify this isozyme from blood.

In the 1960s it became evident that there were isozymes of CA present in the blood of some animals, such as humans [5–8]. These isozymes were termed A, B and C [8,9]. The bovine isozyme A was later shown to be a genetic variant of bovine CA B [10], while the human A probably is a deamidinated form of human CA B [11,12]. To make matters even more complicated, human C is genetically equivalent to bovine B. The CA community has since long decided to use roman numerals instead of capital letters to identify genetically homologous isozymes [13]. Unfortunately, the very confusing capital letter nomenclature is still in use; Sigma, for instance, sells BCA B, which should really be called BCA II.

With the discovery of isozymes of CA, the presence, and amino acid sequence, of CAs in different animals was extensively studied for evolutionary reasons (for reviews, see Refs. [14,15]). Hence, CA I and/or II was prepared from a large number of sources, including blue-white dolphin [16], deer and goat [17], deer mouse and house mouse [18], guinea pig [19], horse [20], moose [21], ox [22], pig [23], rabbit [24,25], rat [26], rhesus monkey (Fig. 1) [27,28] and various other primates [18], sheep [29], wallaby [30], chicken [31], turtle [32], shark [33], frog [34] and face fly [35].

During this period, affinity resins were developed based on the strongly inhibiting aromatic sulfonamides [36,37] and the isozymes I and II can be separated by taking advantage of the differences in sulfonamide and monovalent anion binding of the two isozymes (Fig. 2). The reason BCA II can be purchased so cheaply is that only one isoform of CA is present in bovine blood, and this can be purified in one step from bovine hemolysates using an affinity resin.

In the mid 1970s a third isozyme was discovered in muscle [31,38,39]. Because this form does not bind most sulfonamides very strongly, affinity resins were not available initially. Since the advent of DNA sequencing, and lately genome projects, the number of known CA isozymes have expanded considerably.

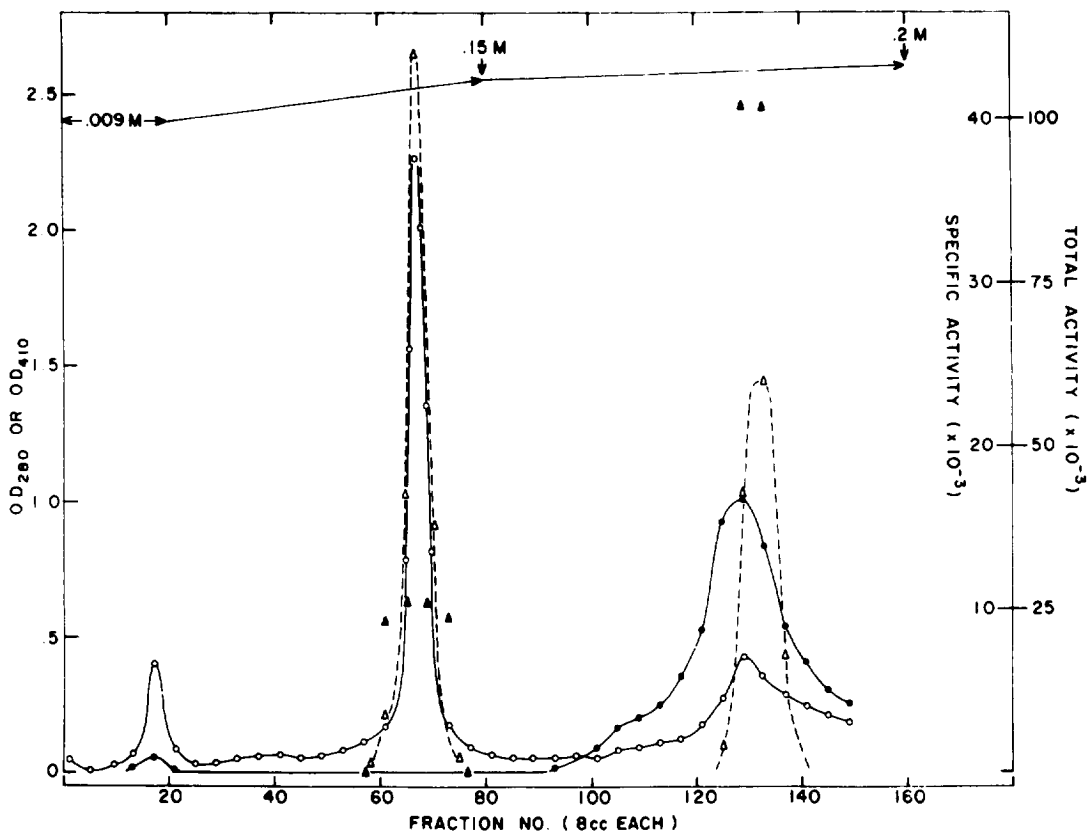

Fig. 1. Chromatographic separation of rhesus monkey (*Macaca mulata*) CA I and II on hydroxylapatite. A 10-ml sample in 9 m*M* phosphate buffer (pH 6.8) was applied to a 16×2.0 cm column. The sample was washed into the column with 160 ml of the same phosphate buffer, followed by elution with two linear phosphate gradients. Fractions of 8 ml were collected throughout. (○) absorbance at 280 nm; (●) absorbance at 410 nm; (△) total activity; (▲) specific activity. Data from Ref. [27]. Note that the high-activity form, CA II, co-elutes with hemoglobin. A specific interaction between HCA II and human hemoglobin has been reported by Backman [63], and HCA II co-elutes with some hemoglobin when purified by affinity chromatography or electrophoresis from human blood [62], indicating that this interaction is present in several species.

At this date, at least nine genes that are homologous to the first discovered CA have been discovered in mammals [15]. In some cases, the protein has not been purified, or even detected, as is the case for CA VII [40]. Some of the newly described CA-like proteins lack CO_2 hydration activity, and one has only been described in the form of an expressed sequence tag (EST) in GenBank [15]. On top of this, two additional gene families that catalyze the hydration of CO_2 have been described. The original CAs are now known as α-CAs, while plants and bacteria have an additional family designated β-CAs, and in archeabacteria, eubacteria and plants, the γ-CAs have most recently been described. From an evolutionary point of view, it is most interesting to note that some organisms, such as the plant *Arabidopsis thaliana*, contain genes from all three gene families [15].

An excellent review of the procedures for purification of CAs was published by Chegwidden [41]. In this review, he described in detail the procedures for chromatographically purifying CAs from many sources. The present review will briefly describe the purification of the different isozymes, from different sources, especially the recently described isozymes. In addition, the chromatographic and electrophoretic analytical techniques that have been used to study various properties of the isozymes will be discussed.

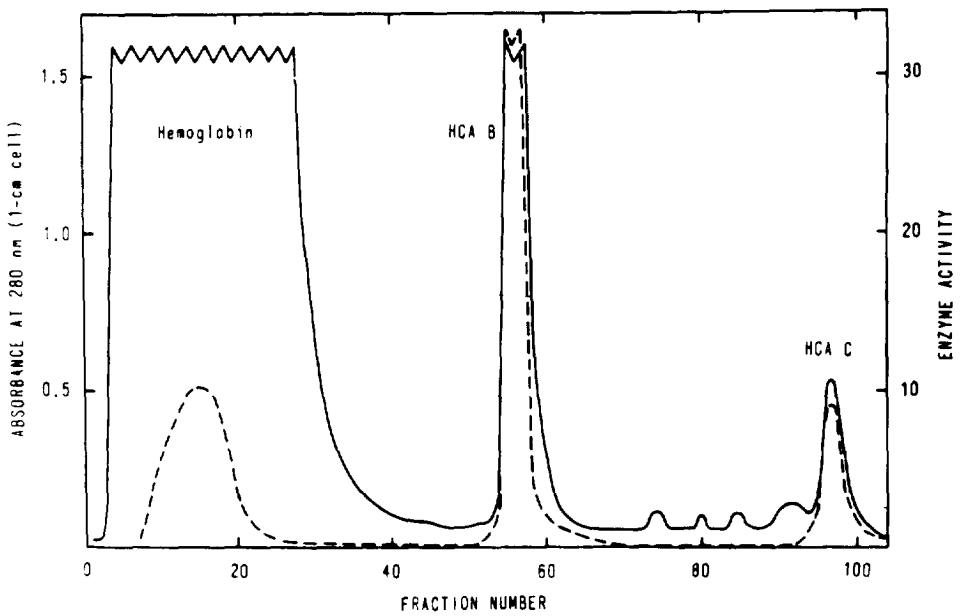

Fig. 2. Chromatography of human hemolysate on sulfanilamide–Sephadex. Erythrocytes were washed in 0.9% NaCl, hemolyzed with distilled water. The hemolysate was dialyzed against 0.1 *M* Tris–sulfate and centrifuged. A sample was applied to the column (12×2.0 cm); the hemoglobin, together with a fraction of the CAs were washed through the column with buffer. HCA I and HCA II were subsequently eluted (4–5 ml fractions) with 0.1 *M* NaI and 0.01 *M* KCNO in Tris–sulfate, pH 7.5. Enzymatic activity (– – –); protein concentration (———). Data from Ref. [36].

2. Detection of CA for chromatographic purposes

Below follows a summary of methods used to detect the activity of CA in solution. Methods to detect CA activity in gels are discussed under the electrophoresis subheading (Section 4).

2.1. CO_2 hydration activity

Physiologically, the CAs catalyze the reversible hydration of CO_2:

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$$

The time-course for the change in pH in a solution when water, saturated with CO_2 is added can be used to detect CAs. In the simplest form this assay can be performed with two small beakers, a magnet, a magnetic stirrer and a stopwatch [8]. This is a very simple method to set up, but the skill and practice of the user will affect the result to some extent.

CA activity can be qualitatively detected using a spot test that was developed by Tashian [42]. In short, aliquots of a few ml is potted on a filter paper that has been soaked in a BTB containing, basic buffer. CO_2 from a gas tank is flushed over the filter paper, and within a few seconds, CA-containing spots will turn yellow. The test can distinguish CA III from I and II by the addition of acetazolamide (that will inhibit CA I and II, but not III) to the BTB buffer.

Rates for the HCO_3^-/CO_2 exchange can be measured from the CA-induced line-broadening of the ^{13}C NMR resonances [43]. The kinetic parameters of the catalyzed reaction has been elucidated to a large part by measuring the pH changes using pairs of buffers and indicators having nearly the same pK_a values [44]. The ^{18}O exchange between H_2O and CO_2 has been used to design methods to measure the CA activity inside cells, and sub-cellular organelles [45–47]. All these methods are extremely elegant, and have been crucial for our present day understanding of the catalytic mechanism and cellular role of CA, but due to the need for rather special equipment, they do not lend themselves very easily

to routine measurements of CA activity in conjunction with chromatography or electrophoresis, and hence they will not be discussed further here.

2.2. Esterase activity

The hydrolysis of esters such as *p*-nitrophenylacetate (pNPA), can very easily be quantified by the absorbance at 348 nm [48]. This wavelength is the isosbestic point of the liberated *p*-nitrophenol, and therefore, the extinction coefficient will not vary with pH. The only drawback of using this ester is that its solubility in water is too low to saturate the CA, and hence one does not measure V_{max} (which is directly proportional to the concentration of enzyme). Instead, one can calculate k_{enz}, the apparent second-order rate constant for the reaction

$$v = k_{enz}[CA] \cdot [pNPA]$$

A practical difficulty with this method is that the pNPA is dissolved in acetone, and it is rather difficult to accurately pipette a few μl of a solution of acetone, because this compound evaporates rather readily. Nevertheless, this is a very accurate technique, that requires no special equipment besides a regular spectrophotometer.

Several other esters have been used as substrates as well, and of particular interest was the use of α- and β-naphtyl acetates to detect a mutant form of HCA I that showed and increase in the rate of hydrolysis of these two esters, but no others tested (*p*-nitrophenyl acetate, α-hydroxy-5-nitro-α-toluene sulfonic acid sultone, or the CO_2-hydration activity) when the reaction medium contained added Zn^{2+} [13,49].

2.3. Inhibitor binding

The binding of some sulfonamides to CA results in a change in the spectroscopic properties of the sulfonamide. The emission spectrum of dansylamide (5-[dimethylamino]naphthalene-1-sulfonamide) was shown by Chen and Kernohan [50] to shift from a peak value at 580 nm, to 468 nm, when bound to BCA II. Concomitant with this blue shift of the fluorescence, the quantum yield increases from 0.055 to 0.84. The large blue shift is due to the binding of the dansylamide to the hydrophobic wall of the active site, and the high quantum yield indicates that 85% of the photons absorbed by the seven Trp residues are transferred to the bound dansylamide. The intrinsic Trp-fluorescence of the bovine CA II molecule is heavily quenched as well. These spectroscopic properties, together with the strong binding of the dansylamide to BCA II ($K_d = 0.24$ μM) made it possible to use this sulfonamide to titer the number of active sites, as well as to determine binding constants. One can either follow the decrease in intrinsic Trp fluorescence at 336 nm when the protein is excited at 280 nm, or the increase in ligand fluorescence at 470 nm (ex 320 nm) with the same result (Fig. 3).

Changes in the binding kinetics of dansylamide to BCA II has been used to indicate an interaction between band 3 and BCA II in red cells [51].

3. Chromatographic separation of CA isozymes

There are six active members of the α-CA family (CA I–VI) that have been purified from mammalian sources. Besides these, members of the β- and γ-CA families have been purified from plants and bacteria. The expression pattern and the unique properties of some of the isozymes allow the purification of one family member from the others. CA I and II are for instance the only CAs in appreciable amounts in mammalian blood, CA III is present in muscle, CA IV is bound to membranes, CA V is found inside mitochondria and CA VI is present in saliva. For a review of the expression of CA isozymes, see Ref. [52]. Most CAs bind sulfonamides, the higher the specific activity, the tighter the binding to most sulfonamides. The highly active CA II can, therefore, be purified from the less active CA I on an affinity column, even though both are present in erythrocytes (Fig. 2). Below are brief descriptions of how CA isozymes have been purified from different sources.

3.1. Mammalian CA isozymes

3.1.1. Cytoplasmic CA I and II

The most convenient source for mammalian CA I and II is erythrocytes. In this case, contaminating serum proteins are readily removed by washing the red cells with 0.9% (w/v) NaCl. After lysis of the

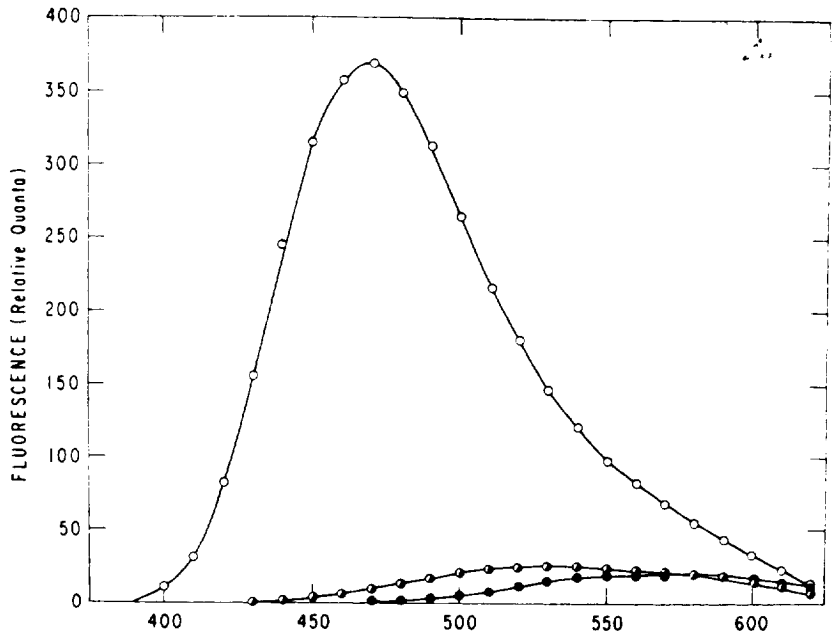

Fig. 3. Corrected emission spectra of DNSA and CA-DNSA. The curves are adjusted so that the areas underneath the lines are proportional to the quantum yields. (○) 1.92×10^{-5} *M* CA and 2.0×10^{-5} *M* DNSA, excited at 310 nm; (●) 1.0×10^{-5} *M* DNSA in 0.02 *M* potassium phosphate buffer (pH 7.4); (□) 1.0×10^{-5} *M* DNSA in 0.01 *M* KOH. Data from Ref. [50].

red cell and removal of the membranes by centrifugation and possibly filtration, one is left with a solution of mostly hemoglobin. The removal of hemoglobin can be accomplished by chloroform–ethanol precipitation [3], differential ammonium sulfate precipitation, or by heat denaturation [53]. An alternative to these is the three-phase partitioning that Pol et al. [54] showed could be used to remove hemoglobin from human hemolysates to purify CA I and II, catalase and superoxide dismutase. The method is based on adding salt, such as ammonium sulfate, to a *tert.*-butanol-water-protein mixture. It was shown by Pike and Dennison [55] that this resulted in denaturation of hemoglobin and myoglobin, and thus it turned out to be useful for removal of hemoglobin to prepare red cell proteins. After adding the organic solvents to the protein solution, Hb precipitated, and was removed. After addition of ammonium sulfate to 90% saturation of the water phase, the remaining proteins were recovered in the gel-like middle phase, and CA, catalase, and superoxide dismutase could be purified from this gel to high yields.

Affinity chromatography purification of the mammalian CA I and II isozymes

Affinity resins for CA were first developed in the early 1970s. Falkbring et al. [36] coupled *p*-aminobenzene sulfonamide (sulfanilamide, pABS; Fig. 4) to cyanogen-bromide activated Sephadex G-150, and Sepharose-2B, -4B and -6B. Using these resins, and sequential elution with Tris–sulfate buffer with 0.1 *M* NaI and 0.01 *M* KCNO, respectively, they separated HCA I and II from human hemolysate (Fig. 2). Later Whitney [37] coupled *p*-aminomethylbenzene sulfonamide (sulfamylon, pAMBS) to CNBr-activated Sepharose-4B. He showed that HCA I and II could be separated by sequential elution with 1 *M* NaCl and 0.5 *M* $NaHClO_4$. Osborne and Tashian [56] reported that the ester linkage formed by CNBr coupling [57] was not perfectly stable, and used a water soluble carbodiimide as described by Hoare and Koshland [58] to couple pAMBS, as well as *p*[(2,4-diaminophenyl) azo]benzene sulfonamide (prontosil) to CM-Sephadex. Using KI and KCN, they purified both HCA I and II from human hemolysate. Later

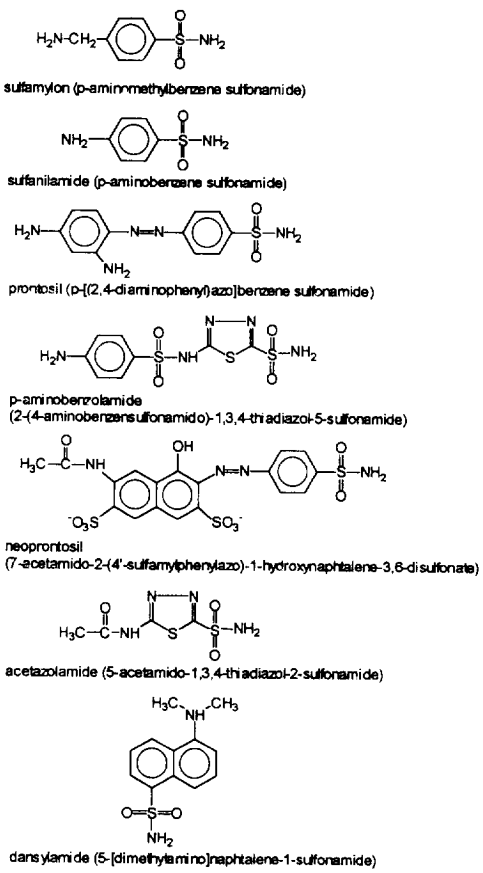

Fig. 4. Structures, trivial and systematic names (in parentheses) of the sulfonamides mentioned in this review.

Khalifah [59] modified this method by using CM-BioGel A, which is more mechanically stable than CM-Sephadex. Recently, Abou-Reybyeh et al. [60] coupled pABS to a epoxy-activated polymer in a stable ether linkage. This affinity resin has a rather long spacer between the matrix and the sulfonamide, and the mechanical stability is very good, which permit the use of this material in an HPLC setup. The use of a spacer between the chromatographic support and the bound inhibitor was also used by Engberg et al. [61] in the purification of BCA III.

Osborne and Tashian [56] pointed out that prontosil has several advantages as a ligand: it binds CA very strongly, and it has a red color, which makes it easy to visually analyze the coupling efficiency. When this resin is stored under buffer, however, it is noticed that some of the prontosil will be cleaved from the resin, giving the supernatant a reddish color. This makes the washing of the resin prior to binding the CA very important, but even so, a fraction of the CA will be recovered with bound prontosil. This does not seem to be the case with pAMBS (Bergenhem, unpublished). One can speculate that this might be due to the aromatic amine in prontosil and the aliphatic amine in pAMBS.

Using an affinity resin to purify HCA I and II from hemolysates, there will be some contaminating hemoglobin [37] especially in the HCA II fraction [62]. The reason for this seems to be a specific interaction between HCA II and hemoglobin [63]. Most of the contaminating Hb can be removed by combining affinity chromatography with electrophoresis [62]. This source of contamination can of course be avoided by expressing HCA II in *Escherichia coli* and affinity purification of the enzyme from this source; however, it must be realized that by doing this, the N-terminus will not be acetylated, and hence the enzyme will not be exactly identical to a preparation isolated from hemolysate [64].

Separation of inactive mutants of human CA II

In the course of investigating the catalytic mechanism, and folding of HCA II several mutants, produced by site-directed mutagenesis with no, or very low catalytic activity of CA have been reported [65–68]. Then, these mutants cannot be excepted to bind to an affinity resin. Since the introduced mutations mostly are found in the active site, other properties of these proteins can be expected to remain constant. Therefore, Alexander et al. [68] used ammonium sulfate precipitation to precipitate impurities at 40% and HCA II at 90% saturation. After dialysis, the CA II fraction was loaded onto a DEAE–Sephacel column, and finally chromatographed on a S-Sepharose to yield $\geq$98% pure HCA II. Some other mutants produced in the same laboratory were purified by chromatography on a S-Sepharose column, followed by gel filtration on a Sephacryl S-100 [66]. The same group also has reported that HCA II mutants can be purified by batch-wise binding to the cation exchanger S-Sepharose at pH 6.2. After washing, the HCA II mutants could be recovered at a purity of 90% [67]

Liang et al. have devised a strategy that is based on anion-exchange on S-Sepharose, hydrophobic

interaction chromatography on phenyl-Sepharose, followed by gel filtration on Sephacryl S-100 [65].

3.1.2. The muscle CA III isozyme

CA III binds sulfonamides weaker than CA I and II, and hence, the first reported purifications was performed with non-affinity techniques [69]. Skeletal muscle was homogenized in buffer, filtered and centrifuged, after which a calcium phosphate slurry was added to the supernatant. The CA activity was recovered from the supernatant after stirring, the solution concentrated and loaded onto a CM-cellulose column. The CA-III-containing fractions were concentrated and loaded onto a Sephadex G-100 column. Finally, pure CA III was recovered after rechromatography an a CM-cellulose, cation-exchange column (Fig. 5) [61,69]. This procedure has been modified by Borén at el. [70] to speed-up the rather time consuming ion-exchange chromatographies. Engberg et al. [61] showed that BCA III binds strongly enough to *p*-aminobenzolamide to use this inhibitor for affinity chromatography if the ligand is bound to a column with a spacer (Affi-Gel 202, Bio-Rad) between the carboxyl function and the matrix. Engberg et al. [61] added pAMBS–agarose to the supernatant from the calcium gel slurry above. This binds any CA I and II in the preparation, and after removal of this affinity resin by filtration, CA III could be bound to the *p*-aminobenzolamide couple to a Affi-Gel 202 (Bio-Rad), washed, and eluted in pure form.

It must be noted, that even though CA III is predominantly found in skeletal muscle, it is present in other tissues, such as male rat liver and fat tissue [52].

3.1.3. The membrane-bound CA IV isozyme

In 1982, Whitney and Briggle reported the successful purification of a membrane-bound form of CA [71]. The enzyme was purified by pAMBS affinity chromatography from bovine lung microsomes by solubilization in 5% SDS, a treatment that denatures, and inactivates all other forms of CA. The membrane bound CA isozyme was not infinitely stable in 5% SDS buffer, but was inactivated within 24 h. The enzyme was shown to be inactivated by DTT reduction, and hence seems to be stabilized by disulfide bonds.

Membrane-bound CAs have since been found in rat [72,73] and human lung [74], in human kidney membranes [75] and urinary membranes [76], as well as in rat skeletal muscle membranes [73]. At this point there seems to be a controversy as to the

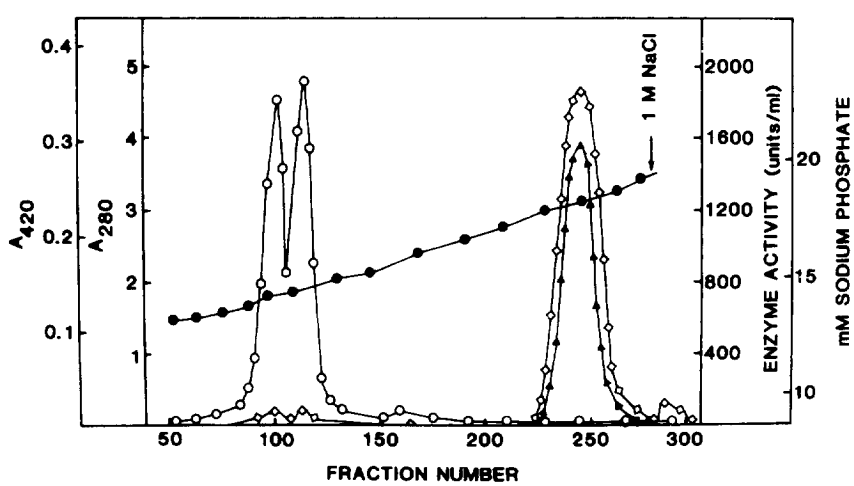

Fig. 5. Final CM–cellulose chromatography of BCA III. A 40×2.5 cm column with CM–cellulose, equilibrated with 10 m*M* phosphate buffer (pH 6.6) was eluted with a linear gradient of 13–20 m*M* of the same buffer. (□) $A_{280\ nm}$; (○) $A_{420\ nm}$; (▲) enzyme activity; (●) buffer concentration. Data from Ref. [61]. The high p*I* of BCA III (8.5 [61]) makes the use of a cation-exchange resin well suited for purification of this isozyme.

true molecular mass of the membrane-bound CAs. The original molecular mass for the purified enzyme from bovine lung was reported to be $52 \cdot 10^3$ by SDS gel electrophoresis [71]. It was first reported that the molecular mass of human renal CA was $68 \cdot 10^3$ [77], while this molecular mass later has been modified to $34.4 \cdot 10^3$ [75]. The enzyme from human and rat lungs have been reported to be linked to the membrane through a phosphatidylinositol linkage, and the enzyme from a number of mammals, with the exception of human, has been shown to be glycosylated; the molecular mass of the non-glycosylated form from human lung has been reported to be $35 \cdot 10^3$ [74]. The controversy regarding the molecular mass, as well as the state of glycosylation of the membrane bound form, could possibly indicated that there are more than one isozyme that are found bound to membranes.

Recently, a CA IV-like membrane bound form of CA has been purified from crab gill [78].

3.1.4. The mitochondrial CA V isozyme

The mitochondrial isozyme, CA V, has been purified from guinea pig liver mitochondria. By initially preparing the intact mitochondria, all cytosolic proteins could be removed. After freeze-thawing the mitochondria, and centrifugation, the soluble mitochondrial matrix was loaded onto an affinity column with pAMBS linked to CM BioGel A. After washing, elution was performed with NaN_3 [79]. This protein has since been cloned, expressed in COS cells and purified by similar techniques as discussed for the guinea-pig liver mitochondria above [80].

3.1.5. The secreted CA VI isozyme

The secreted CA VI was found in sheep saliva by Fernley et al. [81], in sheep parotid gland and saliva. The isozyme from rat has catalytic properties similar to the rat CA II, and binds aromatic sulfonamides with comparable binding constants [82]. This sulfonamide binding has been utilized to purify the isozyme using pAMBS affinity chromatography from the saliva of sheep, ox [83], human [84], rat [82], as well as from mouse, dog and sheep parotid gland [83,85].

3.2. Non-mammalian CAs

3.2.1. Avian

CA II has been purified from the red cells of leghorn hens [86] and from turkey [87]. In both cases, chloroform–ethanol precipitation of Hb, followed by gel permeation on Sephadex G-75 (Fig. 6) and CM–cellulose cation-exchange chromatography was used. Holmes [31] used affinity chromatography to purify CA I, II and III from chicken intestine, erythrocytes and leg muscles, respectively. The same strategy was used by Kadoya et al. [88] to purify CA (probably CA I) from erythrocytes of leghorn hen.

3.2.2. Reptilian

CA I and II has been purified from diamondback terrapin (*Malaclemys terrapin centrata*) by Hall and Schraer [32]. They precipitated the Hb from turtle hemolysate with chloroform–ethanol. The supernatant was dialyzed, concentrated and loaded onto a Sephadex G-75 gel filtration column. The single peak with CA activity was re-chromatographed on a DEAE Sephadex A-50 anion-exchange

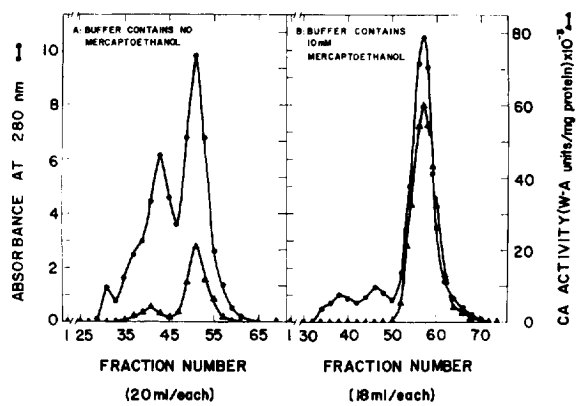

Fig. 6. Sephadex G-75 chromatography of the chloroform–ethanol extract of leghorn hen hemolysate; 600 mg of protein in a volume of 50 ml was loaded onto a 88×5 cm column and eluted with 0.01 *M* potassium phosphate–0.1 *M* KCl (A). The buffer was supplemented with 10 m*M* mercaptoethanol (B). Data from Ref. [86]. Unlike isozymes like HCA II and BCA II with one and none cysteine residue, respectively, the leghorn hen enzyme contains seven cysteine residues [86], and it is evident form the increase in the high-molecular-mass fraction, and the lower recovered activity in panel A compared to panel B, that this enzyme is sensitive to oxidation. A similar behavior has also been reported for turtle CA II [32], frog [34] and shark CA [33].

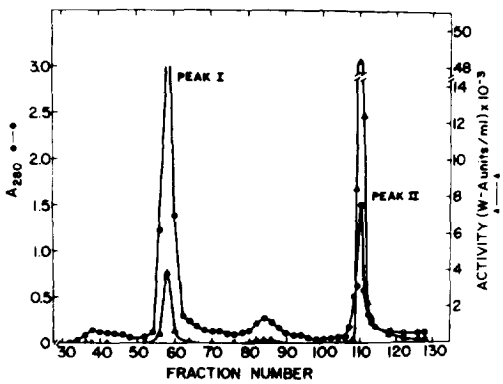

Fig. 7. DEAE Sephadex A-50 separation of the two carbonic anhydrase isozymes in diamondback terrapin turtle hemolysate. The sample (125–150 mg) and the column (50×2.5 cm) were equilibrated with 0.05 *M* Tris–5 m*M* mercaptoethanol (pH 8.7). Peak I (low activity form) was eluted with 0.1 *M* Tris–5 m*M* mercaptoethanol (pH 8.7). Peak II (high activity form) was eluted with 0.1 *M* Tris–0.3 *M* NaCl–5 m*M* mercaptoethanol (pH 8.7); 6-ml fractions were collected. Data from Ref. [32].

column (Fig. 7), and the purity confirmed by chromatography on a CM-cellulose column, as well as by PAGE of the native, and SDS-denatured enzymes. Interestingly, the authors found that the high activity form (CA II) was inactivated by sulfhydryl oxidation, or if β-mercaptoethanol was omitted in the buffers, while low activity form (CA I) was unaffected by oxidation.

3.2.3. Amphibian

CA has been prepared from the blood of bullfrog (*Rana catesbeiana*) [34]. The frog hemoglobin was precipitated with chloroform–ethanol, followed by dialysis. It in noteworthy that DTT or similar reducing agents had to be used during the dialysis to prevent loss of activity. This indicates the frog erythrocyte CA, like the shark [33] and chicken (Fig. 6) [86] equivalents, is sensitive to sulfhydryl oxidation. DEAE–BioGel A was used to further purify the frog CA. As the final step, the eluate from the DEAE BioGel A column was electrophoresed on an PAGE disc gel. Two thin longitudinal slices of the disc were cut, and these were stained for CA activity, with and without acetazolamide, using the bromophenol blue method described by Tashian [42]. The position of the acetazolamide inhibitable band was judged from the two thin slices, and the corresponding position of the disc gel was cut, macerated in buffer, and the CA eluted by diffusion. Using this technique, Bundy and Cheng [34] purified 130 mg CA from a total of three gels.

3.2.4. Elasmobranchs

Maynard and Coleman [33] purified CA from the tiger (*Galeocerdo cuvieri*) and bull (*Carcharhinus leucas*) shark, by chloroform–ethanol precipitation of the hemoglobin, followed by DEAE–cellulose and hydroxylapatite chromatography. The purification of tiger shark CA was later modified using pABS after chloroform–ethanol precipitation by Bergenhem et al. [89]. The tiger-shark enzyme has been shown to contain eight surface-exposed cysteine residues that will readily react with cysteine or glutathione [89]. The many reactive cysteines in the shark enzyme could be the reason for the loss of activity that Maynard and Coleman [33] found during dialysis; hammerhead CA was especially sensitive and lost all activity during dialysis.

3.2.5. Teleosts

The enzyme has been prepared from two teleosts, eel [30] and sheepshead [90]. One form of CA was reported from sheepshead erythrocytes, while a low- as well as a high-activity form was recovered from eel erythrocytes.

3.2.6. Cyclostomes

CA has been purified from two species that belong to the most primitive vertebrate group, the cyclostomes. Carlsson et al. [91] purified CA from hagfish (*Myxine glutinosa*) using a modification of method II from Keilin and Mann [92]. Following hemolysis in distilled water, the hemoglobin was precipitated with chloroform–ethanol, the supernatant was dialyzed and applied to a DEAE anion-exchange column. Interestingly enough, this enzyme lost activity during a subsequent concentration step. The activity could be recovered by addition of Zn^{2+} to the preparation, indicating that hagfish CA binds the zinc ion much weaker than other CAs. The authors note that this is physiologically feasible, due to a some 50-fold higher concentration of Zn^{2+} in hagfish compared to human plasma.

The same laboratory also prepared CA from lamprey (*Lampetra fluviatilis*) [93]. In this case,

pAMBS affinity chromatography was used to purify CA from hemolysate. As in the case with hagfish, a fraction of the enzymatic activity was lost when dialysis was performed, but unlike the hagfish CA, the lamprey equivalent could not be reactivated by the addition of Zn^{2-}.

3.2.7. Invertebrates

The presence of CA in several insects has been reported [94], but it has only been purified from face-fly larvae [35]. The successful procedure involved affinity chromatography, chromatofocusing, as well as gel filtration.

Recently, it was reported that a membrane-bound CA, with properties similar to mammalian CA IV is found in the gills of a crab (*Carcinus maenas*) [78]. The crab gill CA was found to bind sulfonamides strongly, and it could be purified on a pABS resin in the presence of Triton X-100.

3.2.8. Higher plants and algae

The CAs found in the chloroplasts of higher plants are evolutionarily distinct from the α-CAs, and should be designated β-CAs [15]. β-CA has been prepared from the chloroplasts of higher plants, such as pea (*Pisum sativum*) [95] and spinach [96]. It is noteworthy, that two α-CAs have been isolated from the periplasmic space of the algae *Chlamydomonas reinhardtii* [97,98]. Even though there is no evolutionary relationship between these two different CA families, they both bind sulfonamides. The CAs from the periplasm of *C. reinhardtii* have been purified using ammonium sulfate fractionation, followed by pAMBS affinity chromatography [97,98], and the pea β-CA was purified in a pAMBS chromatography step from homogenized and filtered leaves [95].

A non-affinity procedure for purifying spinach CA has been reported by Rowlett et al. [96]. They recovered pure spinach CA by ammonium sulfate precipitation, followed by DEAE–Sephacel, Bio-Gel A and, lastly, a second DEAE–Sephacel chromatography.

3.2.9. Eubacteria

CA was purified from *Neisseria sicca* by Adler et al. [99] using ion-exchange chromatography. The enzyme is released into the medium if the culture is kept at 37°C overnight. A partial sequence of a CA from *N. gonorrhoea* has been deposited in GenBank (entry No. U11547), identified as ORF2 in a genomic clone containing the *uvrB* gene. Interestingly enough, this enzyme is of the α-CA type, while other reported DNA sequences from prokaryotes point to the presence of β-CAs in prokaryotes [100–102].

3.2.10. Archaebacteria

Recently, a third, evolutionary distinct family of CA isozymes has been discovered in the archeaon *Methanosarcina thermophila* [103]. The CA activity in this bacteria was known to increase when the growth medium was switched from methanol to acetate, which lead Alber and Ferry to purify the enzyme to study its properties. Since this enzyme binds sulfonamides rather weakly, the purification strategy was designed around conventional chromatographic techniques. The enzyme was purified to homogeneity by DE-52 anion-exchange chromatography, phenyl-Sepharose, phenyl-Superose, Mono-Q anion-exchange chromatography, Superose 12 gel-filtration chromatography, and lastly affinity chromatography of the almost pure enzyme on a pAMBS–agarose. Even though the purification scheme contained a rather large number of chromatographies, the enzyme was purified at a high yield (22%) and at great purity. The acetazolamide binding of the purified enzyme was shown to be 3×10^{4}-fold less than that of HCA II, which obviously makes the use of sulfonamide affinity chromatography difficult. Nevertheless, the authors could use an affinity resin for the final purification of the enzyme. It is very interesting to note that even though this enzyme is evolutionarily completely distinct from the α- and β-CAs, it still binds the same inhibitors, albeit more weakly. DNA sequences for proteins homologous to the CA from this archeon have been deposited in GenBank, and this family of CAs should be named γ-CA [15].

3.3. Chromatographic techniques used for analytical purposes

Osborne and Tashian [104] used Sephadex G-25 Superfine in a Waters HPLC system to determine binding constants for neoprontosil. CA was chro-

matographed without any sulfonamide, and the absorbance at 280 nm was recorded; the area under this peak corresponds to the total amount of enzyme (E_t). The column was equilibrated with sulfonamide at 0.025–15 μM, and the absorbance at 370 nm was recorded; the area under this peak corresponds to the amount of bound inhibitor (I_b), which equals the amount of bound enzyme (E_b). Since the concentration of free neoprontosil is known, the dissociation constant for neoprontosil, and the CA in question can be calculated as: $K_d = [I_{free}] \cdot ([E_t] - [E_b])/[I_b]$. This technique is useful in all cases where the absorption spectrum, or for that matter fluorescence, differs between the enzyme and the inhibitor.

High-performance gel-permeation chromatography was used by De Felice et al. [105] to study the aggregation of BCA II during storage at −20°C. They found that about 45% of BCA II aggregated to form dimers during the first 3 h of freezing, when the protein was stored at a concentration of 0.1 g/ml in 0.6 *M* NaH_2PO_4, 0.6 *M* NaCl, 0.15% (v/v) NP-40 and 2.5% (v/v) glycerol. Interestingly enough, they could not detect a difference in enzymatic activity towards the ester triacetin between the monomer and the dimer. They continued to show that dimerization did not take place in the presence of 50% glycerol, or if the protein was stored at −80°C.

4. Electrophoresis

Bovine CA II, with a molecular mass of $29 \cdot 10^3$ and a p*I* of 5.9 (Sigma) is inexpensive to purify from bovine erythrocytes and, furthermore, it behaves as expected during electrophoresis, which probably accounts for this protein being present in almost all electrophoretic standards on the market.

4.1. SDS–PAGE: non-reducing and reducing

SDS–PAGE is a very convenient way of determining molecular mass with reasonable accuracy [106]. As has been mentioned above, some CA isozymes from sources such as shark [89], and turtle [32], have very reactive cysteine residues. In the case of turtle, oxidation causes an inactivation, which may be easily assayed. The molecular mass for shark CA was originally reported to be about $35 \cdot 10^3$ [33]. In the same paper it was reported that all cysteine residues were present in disulfide linkages. By using non-reducing, as well as reducing SDS–PAGE and gel filtration chromatography, Bergenhem et al. [89] showed that the molecular mass of the shark enzyme is $28 \cdot 10^3$ (as for most other CAs), that there are no intra-molecular disulfide linkages and that the high molecular mass is due to cysteine and glutathione residues bound to eight surface-exposed cysteine residues. The redox potential in the erythrocytes is expected to be low enough to prevent any disulfide formation in the cytoplasm, but it is interesting to note that the same, completely oxidized enzyme was recovered with two different preparation techniques [33,89], and that S-thiolation of CA III in liver has been suggested to be indicative of oxidative stress [107].

4.2. Electrophoresis to detect active isoforms

CA activity can be detected in gels after electrophoresis by several techniques. The CO_2-hydration reaction is very sensitive, and the quickly fading, yellowish or green spots can easily be detected in the following way [42]. The gel is covered with a filter paper with a BTB solution. After 5–10 min, a sufficient amount of the BTB has been absorbed into the gel. The gel is then placed on ice, and a stream of CO_2 gas is flushed over the gel. Any CA activity in the gel will soon be visible as a yellowish spot where the pH drops more rapidly than in the rest of the gel. Naphtyl acetates can be used to produce a more permanent record. Unfortunately, esterases other than CA will hydrolyze this ester, but by performing the analysis in the presence of a CA inhibitor, such as acetazolamide, CA-containing bands can be distinguished by the specific inhibition of this enzyme.

Techniques such as the one mentioned above have been extensively used in the past to detect enzymatic variants and polymorphisms [42,108]. Presently, most of the genetic studies are performed at the DNA level, since this will very easily detect any mutation, whether this changes the electrophoretic mobility of the protein product or not.

Dansylamide fluorescence has also been utilized to detect active CA isozymes in polyacrylamide gels [109]. The CAs were detected after removal of most of the Hb from hemolysate with gel filtration.

4.3. Isoelectric focusing: analytical and preparative

Brady et al. [110] used isolectric focusing, followed by transfer to a nitrocellulose membrane by electroblotting, binding of anti-CA I, to detect CA I with a peroxidase-conjugated secondary antibody in gels.

Skipski and Scott [111] used preparative isoelectric focusing in sucrose gradient, using a LKB 8100 column to prepare CA from toad (*Bufo marinus*) lens. From the three detected p*I* values (6.1, 5.7 and 5.4) they concluded that it had to be CA II. They suggested that the different isoelectric points arise from deamidation of Gln and Asn residues, and they went on to show that the more acidic (deamidinated) forms increase during the preparation. This demonstrates the power of isolectric focusing to detect this sort of secondary modification, which has been implicated to take place in the aging lens [112].

Isoelectric focusing was used by Engberg and Lindskog [113] to analyze the expected decrease in p*I* that is brought about by the reaction of 5,5′-[dithiobis(2-nitrobensoic acid)] with reactive sulfhydryls in bovine CA III.

Chai et al. [114] used isoelectric focusing to characterize the S-thiolation of CA III from rat liver, which has been implicated to be involved in oxidative stress in the liver [107]. Later the same group went on and showed that by using IEF to separate cellular lysates for Eastern blotting, this technique could be successfully used to investigate the S-thiolation in vivo [115].

Preparative isoelectric focusing was employed by Bergenhem and Carlsson [116] to show that only one form of soluble CA was present in spiny dogfish (*Squalus acanthias*) blood and muscle.

4.4. Affinity electrophoresis

The combination of affinity chromatography and electrophoresis has been rather extensively utilized to investigate the kinetic, as well as equilibrium constants for binding of inhibitors to CA. Chu et al. [117] used polyacrylamide slab-gels with several sections of varying concentration of aryl sulfonamides to determine the binding constants. They found that a spacer between the gel and the sulfonamide of at least 20 Å was necessary for maximal binding of the inhibitor. Furthermore, they showed that the technique is useful for determining if a protein in a mixture binds to a particular ligand. This was demonstrated by the binding of BCA II in bovine hemolysate to the immobilized inhibitor. The described technique has the advantage of not requiring any special equipment, but any slab-gel apparatus will suffice. Another advantage is of course the very small amount of protein that is necessary to determine the binding constant (0.1 μg if silver staining is used to visualize the proteins).

By using capillary electrophoresis it is possible to determine, not only the binding constants, but the kinetic constants as well. Avila et al. [118] determined the binding constants for various charged sulfonamides from the changes in migration times, when BCA II binds these charged inhibitors. They analyzed the peak shapes by assuming two forms of CA (free and bound), and simulated the electropherograms to obtain the on and off rates for the inhibitors. This same group later went on and showed that the effect of the electroosmotic flow, that will transport solutes independent of charge, can be compensated for by analyzing the migration of the non-charged compound mesityl oxide [119].

Chu et al. [120] used a different technique to determine binding stoichiometries using capillary electrophoresis. For strongly binding ligands, like biotin-streptavidin, they electrophoresed increasing amounts of fluorescein-labeled biotin with avidin. In this case, all fluorescein migrated with the avidin until there was an excess of biotin. In the case of BCA II, they electrophoresed a constant amount of BCA II with increasing amounts of inhibitor. The running buffer contained a constant amount of sulfonamide, and the binding stoichiometry was determined from a plot of the area of the ligand peak (Fig. 8).

Lee and Yeung [121] were able to determine the hemoglobin and CA content in individual erythrocytes by capillary zone-electrophoresis with laser-induced fluorescence detection. In short, they diluted erythrocytes on a microscope slide, immersed the injection end of the capillary in the droplet and sucked one cell into the injection end. The cell was lysed by osmotic shock, and the proteins separated in the capillary zone-electrophoresis apparatus. Hemo-

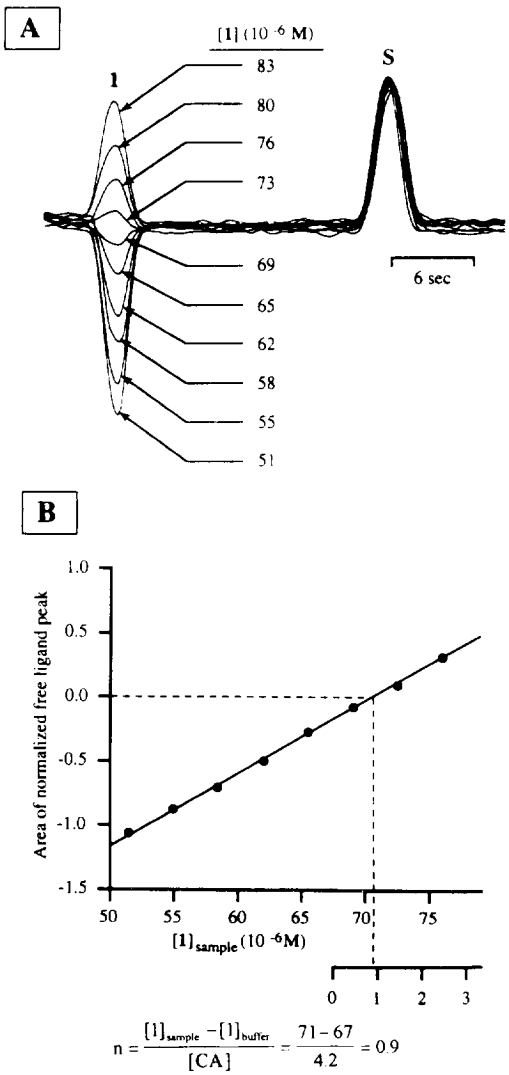

Fig. 8. Capillary zone-electrophoresis to determine binding stoichiometry for arylsulfonamides–BCA II. A constant concentration of BCA II, with varying concentrations of sulfonamide (I) was used for affinity capillary electrophoresis in buffer with a constant concentration if the same sulfonamide. 2-Iodobenzoic acid (S) was used as internal standard. The stoichiometry (n) was determined from a graph of the normalized area of the free ligand peak vs. the concentration of the sulfonamide in the sample, as shown in panel B. Data from Ref. [120].

globin and CA was shown to be present at sufficiently high concentrations to be detectable by the fluorescence after excitation at 275.4 nm by an argon ion-laser.

Capillary affinity-electrophoresis has developed into a very useful analytical technique. On a preparative scale, we have purified human CA I and II from blood by a combination of affinity chromatography and electrophoresis [62] (Fig. 9). CA in human hemolysate was batch adsorbed to a pABS coupled to BioGel A. The affinity gel was packed into a water jacketed condenser to facilitate cooling during the electrophoretic step. By applying a voltage across the column, the hemoglobin migrated out of the column, enabling a very high purity CA I and II preparation.

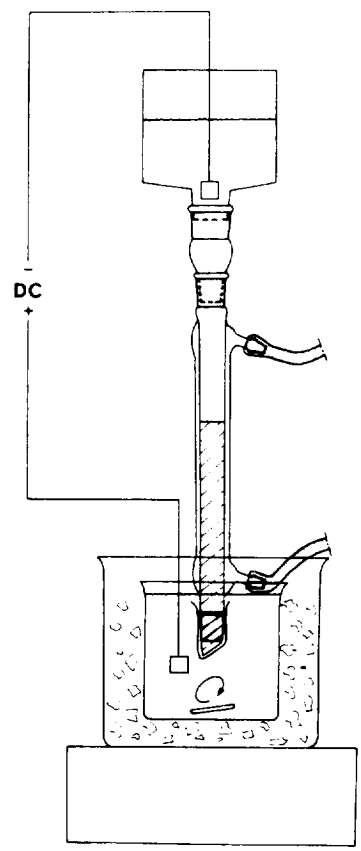

Fig. 9. Preparative affinity electrophoresis setup. HCA I and II were batch-wise adsorbed to pAMBS–Bio-Gel A. Most of the hemoglobin was removed by washing the gel on a Büchner funnel. The gel was subsequently packed into West condenser with a nylon net covering the bottom. A flask with buffer was attached at the top, the bottom was submersed in buffer, and an electric field of 400 V was applied over the column. After 40 min HCA I was eluted with 0.2 *M* KI in 0.1 *M* Tris–sulfate, and HCA II was eluted with 0.4 *M* NaN_3 in the same buffer. Data from Ref. [62].

5. List of abbreviations

BCA	Bovine CA
BTB	Bromthymol blue
CA	Carbonic anhydrase
HCA	Human CA
pABS	*p*-Aminobenzene sulfonamide
pAMBS	*p*-Aminomethylbenzene sulfonamide
PAGE	Polyacrylamide-gel electrophoresis
pNPA	*p*-Nitrophenylacetate
SDS	Sodium dodecyl sulfate

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