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Zero length crosslinking of bovine carbonic anhydrase II and chondroitin-4-sulfate

Rajivinder S. Brar

ZERO LENGTH CROSSLINKING OF BOVINE CARBONIC ANHYDRASE II AND CHONDROITIN-4-SULFATE

by

RAJIVINDER S. BRAR

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Dedication

This dissertation is dedicated to my father, Harmahesh Singh Brar, without whom this work would not have been possible. I would like to thank him for the years of patient guidance in all matters that enabled me to persevere, and for lifting me up onto his shoulders so I could see farther than him.

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Since protein drugs and hormones are usually fragile, complex formulations are usually necessary to preserve biological activity. Despite this, when proteins are delivered to a subject they are exposed to proteolytic digestion and alterations that abolish activity. The majority of proteins will not survive a trip through the digestive system due to harsh pH levels in the stomach and lower intestines thus ruling out oral administration of protein drugs. If proteins were modified so as to be more stable and resistant to such changes, such problems could be circumvented.

The ability to control the release of drugs so as to create a sustained drug delivery system has been largely lacking thus far. Natural and synthetic polymers have been used as carriers of proteins in an effort to achieve sustained drug release (Gombotz et al., 1995; Duncan et al., 1994; Rubinstein et al., 1992). The premise is that the supporting polymer will confer a certain amount of protection to the drug in terms of stability and resistance to denaturants or proteases. The polymers used as carriers are degraded gradually *in vivo* thereby releasing the drug compound enabling it to carry out its biological function. The polymers can either be chemically broken down as a function of time, pH or other factors or be degraded by specific enzymes (Scheline, 1968; Salyers, 1979).

Chemical modification of proteins has been used in laboratories for such purposes as amino acid sequencing, characterization of active site residues and describing the geometry and distances involved between residues that are critical to activity. It has also been used to crosslink protein components that interact directly with each other to form active enzyme complexes. For example, active calmodulin and phosphodiesterase complexes have been created by crosslinking which was further evidence that these two components associate to catalyze a reaction (Kincaid, 1984). Cytoskeletal contractile protein interaction has also

been investigated using crosslinking methodologies (Andreeva et al., 1993). The chemical modification of immunoglobulins has been much investigated. This class of protein has been coupled to many molecules to serve in enzyme assays and joined with molecules, which bind specifically to certain targets, creating tailor made molecules with high specificity for a cellular target (Wong, 1991). Recent advances in vaccine production has also utilized chemical crosslinking to enhance the immune response to bacterial polysaccharides by creating glycoconjugate vaccines (Stein, 1994). In addition, chemically modifying certain standard vaccines increases their resistance to heat, making them more ideally suitable for use in the field (Lemon and Milstein, 1994).

Enzymes that work at extreme temperatures (i.e extremozymes) are also sought for industrial processing. High temperature proteases, amylases and other hydrolases would be beneficial to the food processing industry. These enzymes would serve to prevent contamination of food while fats could be hydrolyzed, proteins digested and fibers modified to make food more palatable and healthier. The production of corn syrup is another example where a high temperature enzyme that converts glucose into fructose could increase yields of fructose. Similarly high temperature enzymes could be used to enhance the flow of oil or gas in drilling operations. In order to facilitate oil flow to the well bore, the surrounding bedrock is pryed open by flooding the well with an aqueous solution of guar gum and sand particles, capping the well and pressurizing the bedrock until it fractures. The polymer solution is viscous and carries the sand through cracks, propping open fissures for oil or gas flow. To enhance the product flow, the polymer solution is chemically thinned by oxidizing the guar gum or by hydrolyzing its sugar linkages with β-mannanases and α-galactosidases. This entire process could be enhanced if a heat stable enzyme was used since conventional

enzymes used have limited stability over 80 ° C. In a deep well, the temperatures can rise to over 120 ° C. Currently, hemicullases from *Thermatoga neapolitana* which hydrolyze guar gum at temperatures of 100 ° C have been isolated (Duffaud et al., 1997; Bauer et al., 1996)

The application of chemically modifying proteins can extend beyond conventional cloning and expression methods in certain ways. For example, through chemical modification a monomeric protein can be turned into a dimer or some other multimer. Simply cloning a gene and expressing its protein product cannot do this. Respiratory pigments have been the focus of such chemical modification studies which resulted in changes of the oxygen binding behavior of these proteins by inducing either intra or intersubunit crosslinking (White et al., 1987; Yang et al., 1991). As a result of some of this work, modified hemoglobins have been produced, which are being developed and presented as artificial oxygen delivery devices to tissues. Other possibilities not easily afforded by molecular biology techniques are the production of multi-enzyme complexes. These conjugates would catalyze reactions which are dependent or related to each other such as the reactions catalyzed by superoxide dismutase and catalase (D'Agnillo et al., 1993).

Most of the work dealing with stabilizing proteins has focused on encapsulating techniques or immobilizing the proteins in some sort of matrix (Lu at al., 1995; Gombotz et al., 1995), which would be biodegraded and thus releasing the protein content. Another strategy is to glycosylate proteins. Since many proteins are glycosylated *in vivo*, and it has been shown that glycosylation affects many of the protein's properties (Wang et al., 1996), alterations of this type could be useful. It has been shown in at least one case that conjugation with a mucopolysacharide polymer stabilized biological activity (Luchter-Wasylewska et al., 1991). Introducing fatty acid moieties that serve to increase the

hydrophobic properties of the protein can alter the solution properties of an enzyme. In this manner, organic drugs can be made more permeable to membranes so they can be more efficiently delivered to target organs such as the brain. Thus it can be seen that chemical modification can help not only stabilize a protein but also confer upon it new characteristics which could be useful.

There are several criteria that are important to consider when modifying a protein with the intent to stabilize the molecule while preserving biological activity. First, no amino acid residue that is involved or essential in the catalytic process can be altered. Second, no immunogenic substance should be introduced to the molecule if the goal is to develop a drug for human consumption. Due to the first reason it is almost essential that an enzyme to be modified be well characterized in terms of structure and function. Ideal candidates for chemical modification would be enzymes whose high resolution crystal structures have been solved. At the very least, the amino acid residues needed for activity must be known.

The availability of specific amino acid residues for crosslinking will determine the choice of chemical crosslinker to be used. There are two general categories of chemical crosslinkers available for use; zero length crosslinkers and bifunctional crosslinkers (Wong, 1991; Wong et al., 1992). The bifunctional crosslinkers are reagents that have two chemical groups available to react with two sites. These two chemical groups can be identical (homobifunctional) or different (heterobifunctional). The overall result is the joining of two molecules with a spacer of variable length in between and thus the introduction of an extra element into the conjugated product. This may be of concern if the application is for human consumption as the extraneous material might be capable of eliciting an unwanted immune response. In order to eliminate the incorporation of extraneous material into the final

product, zero length crosslinking is a feasible method (Wong, 1991). Zero length crosslinking consists of the activation of one chemical group by the zero length crosslinker (usually by the formation of unstable and highly reactive intermediates) and subsequent reaction with the second group and co-elimination of the zero length crosslinker. In this fashion, two molecules are joined together without the incorporation of any other material in the final product (Figure 1).

Carbonic anhydrase is good candidate for use in crosslinking experiments of this nature. Its crystal structure is known to high resolution (Liljas et al., 1972; Eriksson et al., 1988) and though the exact nature of the mechanism remains elusive it has been extensively characterized (Zheng et al., 1992). Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide to form bicarbonate and a proton. In humans, this protein is important in ridding the body of carbon dioxide and also in maintaining a proper acid base equilibrium by converting CO_2 into HCO_3^- in blood at rates that allow efficient CO_2 interchange between blood and tissues. Carbonic anhydrase in the gastric mucosa catalyzes the hydration of CO_2 at a rate sufficient enough to neutralize the excess alkalinity produced by the ionization of water and secretion of hydrogen ions. In the kidney, carbonic anhydrase contributes to the exchange of a Na^+ ion for a H^+ ion across the luminal border of the cell and Na^+ and HCO_3^- diffuse to the opposite side of the cell where the reverse process takes place leading to the appearance of Na^+ in the glomerular filtrate in venous blood. Currently, carbonic anhydrase is thought to play a role in preventing osteoporosis and is used clinically in lowering intraocular pressure in patients with glaucoma. Some clinical manifestations resulting from a decrease or an absence of carbonic anhydrase include renal tubular acidosis, mental retardation, cerebral calcification and growth

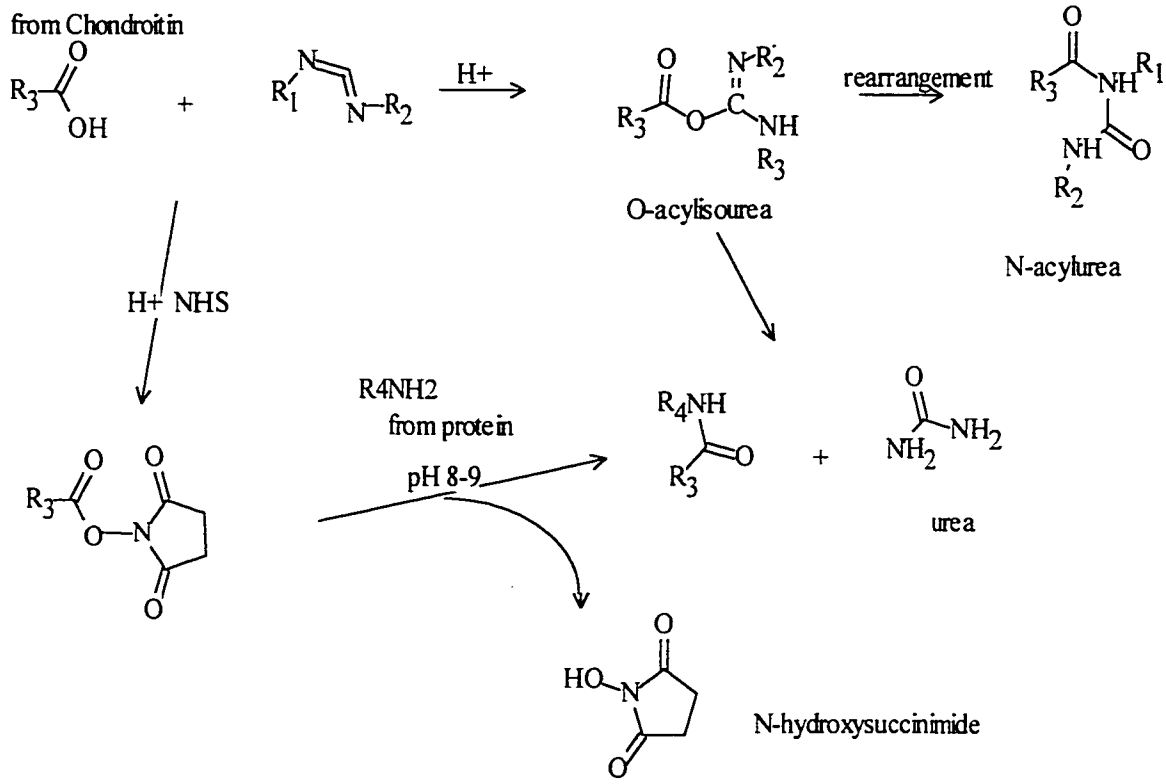


Figure 1. Zero length crosslinking using EDC and NHS. The chondroitin-4-sulfate carboxyl groups are reacted with EDC and NHS. This intermediate undergoes nucleophilic attack by a primary amine from an amino acid side chain from BCA II to form an amide bond between chondroitin-4-sulfate and BCA II.

retardation (The Carbonic Anhydrases, 1991).

In addition to the hydration of carbon dioxide, carbonic anhydrase also is able to hydrolyze esters (Verpoorte et al., 1967) and it has also been demonstrated that it can hydrate acetaldehyde (Pocker and Meany, 1965) though it is unclear what physiological significance such reactions may have. The esterase reaction does have many features in common with the CO_2 hydration reaction such as the essential involvement of the bound zinc ligand and the inhibition by sulfonamides and to a lesser degree anions . Inhibitors of carbonic anhydrase include monovalent anions and sulfonamide compounds as well as natural plasma inhibitors (Wuebbins et al., 1997;Roush et al., 1992; Matuszewski et al., 1994).

The refined crystal structures of carbonic anhydrase I, II and III were determined in the early 1970's. Bovine carbonic anhydrase II is a 30 kDa monomeric protein consisting of 260 residues (Eriksson et al., 1988) and found chiefly in erythrocytes. The protein is roughly globular composed of a 10 stranded beta sheet that halves the molecule (Eriksson et al., 1988). The active site is a cavity 15 angstroms wide and 15 angstroms deep with one half of the cavity being composed by hydrophobic residues. The zinc atom is located at the bottom of the cavity ligated to the ϵ-N of His-94 and His-96, the δ-N of His-119 and a hydroxide ion at high pH (Eriksson et al., 1988). At low pH the fourth ligand is a water molecule and during turnover the fourth ligand can be a bicarbonate ion. The zinc bound ligands are hydrogen bonded to Thr-199, which is itself hydrogen bonded to Glu-106. It appears that this hydrogen bonding pattern is essential for rapid enzymatic catalysis and governs the pKa of the zinc-bound water and inhibitor binding. The presence of the hydrophobic half of the active site is thought to be important for CO_2 recognition (Zheng and Merz, 1992). Finally, there are several ordered water molecules in the active site of CA II

There are several other mechanisms consistent with the above observations. One alternative is similar to the zinc hydroxide scheme except it requires an internal proton transfer of zinc bound bicarbonate. One mechanism which is substantially different yet plausible is termed the proton shuttle mechanism (Kannan et al., 1971). The role of the zinc ion is the same but the proton is not shuttled out of the active site via His-64. Instead a proton relay composed of Thr-199 and Glu-106 is proposed. It should be noted that this scheme requires that the group whose pKa is around 7 to be Glu-106. However, the pKa of glutamic acid is normally around 4.5 though this does not rule out this scheme since perturbed pKa's have been observed in the literature for amino acid residues in proteins (Parsons and Raftery, 1972).

Since the hydration of CO_2 at all pH ranges is extremely rapid and is accompanied by the release of protons, the stop-flow pH indicator technique is an adequate method that can be used to study this reaction. A stopped flow apparatus permits observation of the reaction velocity of two solutions that are forced through a mixing chamber and into an observation cell. The two solutions are initially stored in drive syringes and mixing is induced by operating the pistons by an air-operated plunger or by manually advancing the plungers (Figure 2). After leaving the observation cell, the mixed reactants advance the piston of a stopping syringe, whose piston is brought to a dead stop against the tip of a micrometer. Just prior to stopping, a switch is closed, thereby supplying a signal for triggering an oscilloscope or another appropriate device designed to store the signal. After the reaction trace is stored, the instrument is readied for the next experiment by advance of the micrometer, which displaces it from the end of the stopping syringe, by a fixed increment. Repeated advance of the driving syringes followed by advance of the micrometer

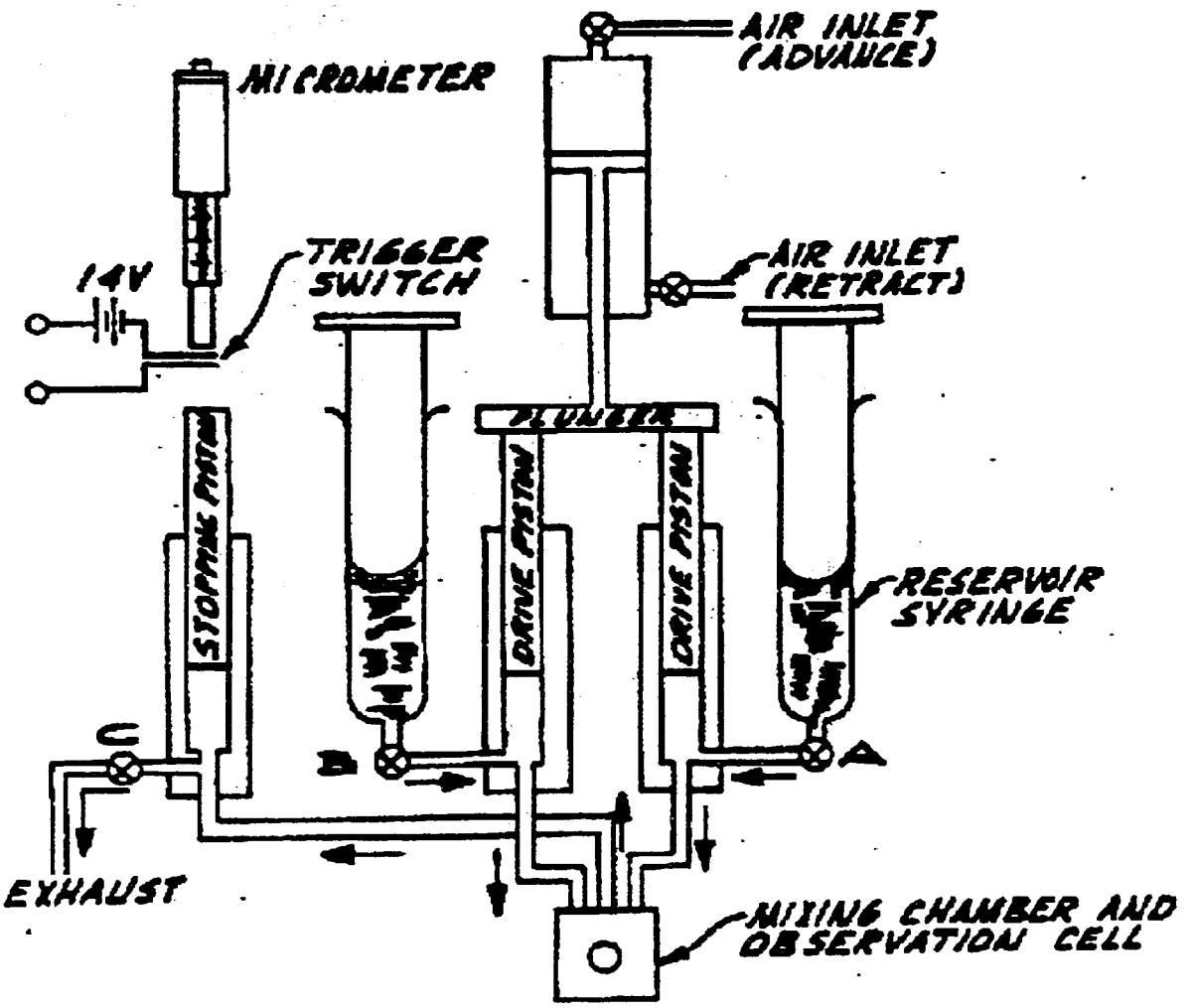


Figure 2. A schematic diagram of a typical stopped flow apparatus.

makes it possible to record several replicates of a reaction velocity before the stopping syringe must be emptied.

Chondroitin-4-sulfate is a naturally occurring polymer based on a polylactose backbone consisting of alternating N-acetyl D-galactosamine β 1;4 and D-glucoronate β 1:3 residues (Figure 3) (Scott et al., 1995). Normally it is found throughout almost all connective tissues including cartilage, cornea, trachea, vessel walls and skin in the form of a proteoglycan. Sulfation in chondroitin sulfate is variable depending on the tissue type, age and species with the two predominant species being chondroitin-4-sulfate and chondroitin-6-sulfate. The distribution of chondroitin sulfate in tissues has been shown to about 10% of the dry weight of cartilage.

Chondroitin sulfate together with hyaluronate appear to function in space filling capacity perhaps as a pressure resistant filling responsible for the elasticity of cartilage (Scott, 1975). They may also be involved in keeping collagen fibrils apart so as to possibly preserve the transparency of the cornea (Scott et al., 1981). It has been shown that chondroitin sulfate has a preferred configuration in solution stabilized by hydrogen bonds. This solution conformation is no doubt essential to its capacity to fulfill a space filling role and its ability to interact with collagen (Scott and Orford., 1981). A proposed secondary structure of chondroitin sulfate which accounts for the quantitative and qualitative aspects of the NMR spectra of polymers and oligomers has three intramolecular hydrogen bonds per tetrasaccharide unit (Scott et al., 1983). These NMR secondary structures have been analyzed with molecular modeling to determine the geometric and energetic constraints on duplex and higher aggregate formation. The calculations suggest that chondroitin-4-sulfate could not form duplexes while chondroitin-6-sulfate and undersulfated chondroitin-4-sulfate

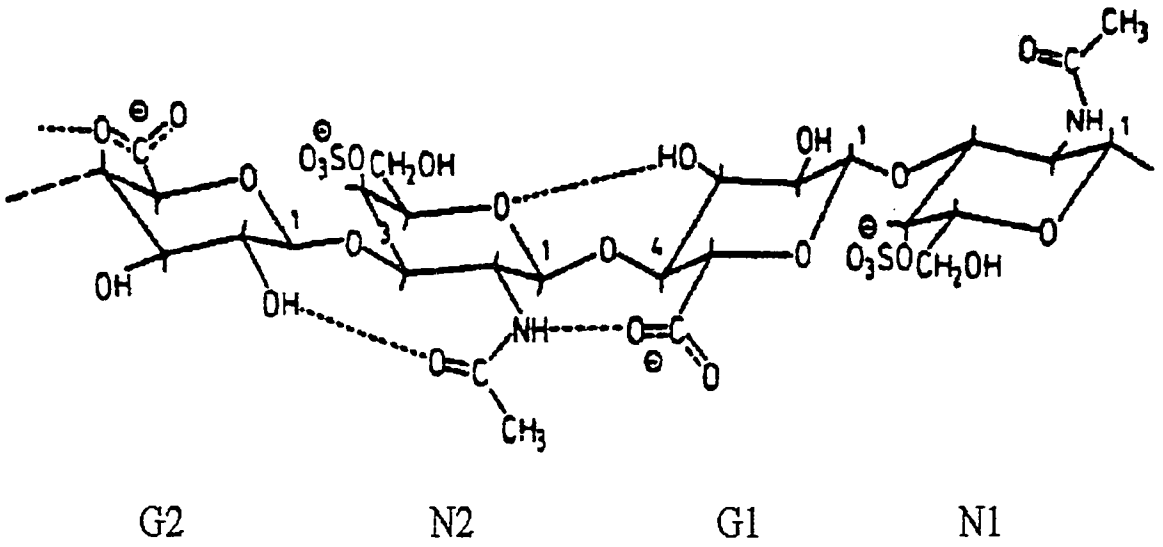


Figure 3. Structure of chondroitin-4-sulfate. From Scott et al., 1982.

could (Scott et al., 1992). Furthermore, water molecules stabilize the two fold helix into twofold helical, flat tape like molecules which aggregate via hydrophobic interactions between the very extensive hydrophobic patches (9 CH units) repeated on alternating sides of the polymer. Thus, the potential to form a secondary structure such as a multihydrogen bond array throughout the molecule involving acetamido, carboxylate, hydroxyl and ring oxygen is present. Chondroitin-4-sulfate isolated from bovine trachea has ~1.0 sulfate group per disaccharide unit and a typical molecular weight of about 20 kDa, corresponding to roughly 40 repeating disaccharide units (Scott et al., 1995). Biopolymers such as this have been shown to stabilize human prostatic acid phosphatase upon crosslinking (Luchter-Wasylewska et al, 1991) and chondroitin has also been proposed as a biodegradable carrier for colon specific drug delivery (Rubinstein et al, 1992).

In this study, zero length crosslinking (using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as the crosslinking agent) will be used to create hybrid species of bovine carbonic anhydrase II (EC 4.2.1.1). It will be investigated whether creating covalently bound dimers of a normally monomeric protein confers any advantages in terms of stability of the protein and activity. Also, chondroitin sulfate, will be conjugated to carbonic anhydrase. This conjugate will also be characterized in terms of stability and activity and for the potential of its use as a sustained release drug carrier. Sensitivity to synthetic and natural inhibitors will also be investigated. Since previous studies have shown that attaching polymers such as chondroitin-4-sulfate to enzymes increases the stability of the protein, the hypothesis is that chondroitin-4-sulfate will confer stability to bovine carbonic anhydrase upon conjugation with EDC.

HIV contamination of blood supplies and the U.S. army's interest in a new short term

battlefield resuscitation solution have spurred the development of numerous types of blood substitutes. Most of these types of developments have focused on delivering oxygen to tissues following massive blood loss by the patient. Some of the efficacy end points of such blood substitutes include reduced allogeneic blood use, lowered cost of care and better tissue oxygenation (Winslow, 1997). In addition, the lack of special storage conditions for these blood substitutes is also inviting. However, it must be noted that a massive loss of blood would require intervention that would not only adequately oxygenate the tissues but also remove the toxic buildup of carbon dioxide. The aim of this study is to show that a stable, heat resistant carbonic anhydrase enzyme can be created that is capable of hydrating and dehydrating carbon dioxide at rates that could allow for the efficient transport of carbon dioxide from tissues while possessing a higher tolerance to denaturation by heat and other chemicals. Ultimately, such a modified carbonic anhydrase enzyme might be used along with artificial oxygen delivery devices to better meet the physiological demands of tissues which would otherwise die due to the accumulation of carbon dioxide.

Materials and Methods

Materials. Acrylamide, sodium dodecyl sulfate (SDS), glycine, tris[hydroxymethyl]amino methane (TRIS), N,N,N',N'-tetramethylenediamene (Temed), N,N' methylene-bis-acrylamide, Coomassie Brilliant Blue R-250, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), Sephadex G-150, Hepes, Mes, phenol red and chlorophenol red were purchased from Sigma (St. Louis, MO). Sodium bicarbonate was purchased from Fisher Scientific. Hanks Balanced Salt Solution was purchased from Gibco BRL. Ficoll Hypaque was purchased from Pharmacia Biotech.

Bovine erythrocyte carbonic anhydrase II (EC 4.2.1.1) was purchased from Sigma (St. Louis, MO) and used without further purification. The purity of the sample was determined with 12% SDS-PAGE analysis. Bovine chondroitin-4-sulfate isolated from trachea was also purchased from Sigma (St. Louis, MO). This preparation consisted of 70% chondroitin-4-sulfate and 30% chondroitin-6-sulfate according to the manufacturers analysis. Gel electrophoresis was done with a Mini-Protean II Electrophoresis System (Bio-Rad, Richmond CA).

Homologous Covalent coupling of bovine carbonic anhydrase. Bovine carbonic anhydrase II was reacted with EDC in double distilled water at pH 4-5 adjusted with 0.1 M HCl. The reaction was allowed to proceed at room temperature for 15 minutes at which time the pH was adjusted to 8-9 with 0.1 M NaOH. The reaction mixture was analyzed by 12% SDS-PAGE after 15 minutes of incubation and after one hour of incubation. The concentration of the enzyme was kept constant at 33 μM while the concentration of EDC was varied from 7.8 mM to 34 mM. The reactions were terminated with the addition of β-

mercaptoethanol.

Covalent coupling of bovine carbonic anhydrase II with chondroitin-4-sulfate. Crosslinking of BCA II with the polymer chondroitin-4-sulfate was carried out using the following protocol. Solutions of chondroitin-4-sulfate (15 mg/ml) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (12 mg/ml) were mixed at pH 5.5 for five minutes at room temperature to reach a final concentration of 6.5 mM and 15 mM respectively with a total reaction volume of 2 ml. This was followed by adding 1 ml of 12mg/ml solution of N-hydroxysuccinimide to make a final concentration of 8.6 mM. The reaction was allowed to proceed on ice while being constantly stirred gently for ten minutes. The pH was then adjusted to 8-9 with 0.1 M NaOH and carbonic anhydrase II was added to the reaction (final concentration of 33 μM). The reaction was then allowed to proceed at 4°C in a cold room while being gently stirred for up to 24 hours. The total volume of the reaction incubation was 4 ml. The molar ratio of enzyme to chondroitin-4-sulfate was 0.005 and the molar ratio of chondroitin-4-sulfate to EDC was 0.43.

BCA II was also crosslinked with chondroitin-4-sulfate in a similar manner as described above except with the final concentration of reactants as follows; 8.2 mM chondroitin-4-sulfate, 26.1 mM NHS, 15.6 mM EDC and 0.216 mM BCA II. The concentration of reaction components were chosen according to recommendations from unpublished graduate work performed in the laboratory of Dr. Hiroshi Mizukami and from other studies on zero length crosslinking (Grabarek and Gergely, 1990; Sehgal and Vijay, 1993; Luchter-Wasylewska et al., 1991).

To evaluate the effect of NHS on the extent of crosslinking, reactions were also

performed as described above except NHS was not included. Similarly, the effects of pH on the extent of crosslinking was investigated by activation of chondroitin-4-sulfate carboxyl groups with EDC at pH 5.5 and adding the enzyme solution without adjusting the pH to 8-9.

Gel Filtration and Absorbance Spectrophotometry. A column (2.5 x 49.5 cm) loaded with Sephadex G-150 gel was equilibrated with 50 mM Hepes buffer at pH 7.5. The reaction mixture was filtered with a sterile disposable cellulose acetate membrane syringe filter (Corning, 0.45 micron) and approximately 4 ml of the filtered sample was loaded onto the column. Separation of the reaction mixture was carried out at room temperature with a flow rate of approximately 40 ml/hour with the same buffer. Fractions were collected in tubes (approximately 15 ml per tube) and the protein content of each tube was measured by reading the absorbance of the samples at 280 nm. The column was calibrated using blue dextran (~ 10^6 Da), yeast alcohol dehydrogenase (150 kDa), met-hemoglobin (68 kDa) and bovine carbonic anhydrase II (30 kDa). In order to estimate the concentration of crosslinked BCA II, serial dilutions were made of a 1.2 mg/ml solution and the absorbance at 280 nm of each dilution was determined. The results were used to create a plot of A_{280} versus concentration and this plot was used to determine the amount of crosslinked protein present in the purified and concentrated fractions from the G-150 column. In addition, the absorption spectra of the unmodified and modified enzymes as well as chondroitin-4-sulfate were taken from 350-200 nm.

Gel Electrophoresis. Aliquots of the original reaction mixture and their column fractionated eluants were separated on 12% or 7.5 % SDS-PAGE under reducing conditions

The spectrophotometer lamp was powered by a HP 6263B DC power supply and a current amplifier (Keithley) amplified the signal. The output from the spectrophotometer was converted to a digital format by a PCL 812G Data Acquisition card (CyberReasearch, Branford CT) controlled with Labtech Notebook software operating on a Hewlett Packard 100 MHz Pentium computer. The sampling rate of all scans was set at 100 Hz. The initial rate was determined by analyzing the linear portion of the reaction trace in the first 20% of the reaction and subtracting the background. The background hydration rate was determined by measuring the rate without any enzyme present. Figure 4 depicts a typical result from a stopped flow experiment using BCA II to hydrate carbon dioxide.

All buffers were maintained at an ionic strength of 0.1 M by adding appropriate amounts of Na_2SO_4. Saturated solutions of CO_2 were prepared by bubbling the gas through a 50 mM sodium sulfate solution in a vessel fitted with a stopcock at room temperature. Portions of this solution were withdrawn carefully with a syringe and diluted in degassed 50 mM sodium sulfate solution in another syringe by coupling the two syringes together and mixing quickly. Measuring the pH of the saturated solution with a pH electrode approximated the concentration of CO_2. The reversible reaction of CO_2 with water to form H^+ and HCO_3^- can be thought of as $K= [H^+] [HCO_3^-]/[CO_2]$ with $K_{eq}= \sim 4.3 \times 10^{-7} M^{-1}$ (Carey 1992). Thus the pH of a saturated solution of carbon dioxide can be approximated using this relationship. In most experiments the pH of the CO_2 solutions was approximately 4.12-4.15. The final concentration of CO_2 was varied from about 6 mM to 1 mM with serial dilutions. The final concentration of bicarbonate for the dehydration reaction was varied from 50 mM to 12.5 mM HCO_3^-. If the proton releasing reaction and the ionization of the buffer and indicator are instantaneous then the rate of absorbance change of the indicator is related to

the rate of proton release by the buffer factor (Q) as follows.

$$dA/dt = (1/Q)\ (dx/dt)$$

Q is a function of the states of ionization of the buffer and indicator and will tend to vary as the reaction proceeds. If this is the case then Q is a function of pH and the time course of the absorbance change is not linearly related to the time course of the actual release of protons by the reaction. The accuracy of the rate determination would be enhanced if Q were independent of pH as in a case if the absorbance kinetics and the actual kinetics were linearly related. Variability in the buffer factor can be diminished by using well buffered systems which allow marginal pH changes during the course of the hydration reaction. This approach however will reduce the sensitivity of the measurement. A more general way to make the buffer factor independent of pH is to use buffers and indicators with identical pK values (Khalifah, 1971). The buffer factor can be easily determined by spectrophotometric titration of the buffer-indicator system used for the kinetic studies. Aliquots of standardized acid or base are added to the buffer system and the resulting absorbance changes are measured. The reciprocal of the slope of such titration plots is Q (Kernohan, 1964; Gibbons and Edsall, 1963).

The output from the spectrophotometer as collected by the A/D card allows the change in absorbance as a function of time (d(OD)/dt or d(mV)/dt) to be calculated as the slope of a zero order plot since the millivolt reading from the spectrophotometer is related to the percent transmission. In order to express the rate in terms of the change in the absorbance of the indicator to the amount of protons liberated by the reaction, it is necessary to take into account the buffering factor Q (units are (mol/l)/mV or M/mV). The buffering factor was measured for each buffer used in the assays by titrating the buffer with a

into 4.5 ml of buffer (50 mM Hepes (pH 7.5), 50 mM sodium sulfate, 80 mM phenol red indicator). The rate was determined at least four times and the average rate at each time incubation was used to determine the percent activity remaining using the control (unheated sample) rate as 100%. All background rates were determined and subtracted from the initial rates. The rate of carbon dioxide hydration by the crosslinked complex was measured in a similar manner. The final concentrations of the unmodified and modified enzymes was 7.92 x 10^{-8} M and 7.28 x 10^{-8} M respectively.

Effects of SDS. Aliquots of carbonic anhydrase were diluted into 0.25 ml of SDS for five minutes before being diluted further into 4.75 ml of buffer (50 mM Hepes (pH 7.5), 50 mM sodium sulfate, 80 mM phenol red indicator). The concentration of SDS was varied from 0.25% to 5% and the substrate concentration was fixed at 6 mM CO_2. The rate of each incubation was determined at room temperature four times to determine the average rate at each concentration of SDS. The percent of carbon dioxide hydration activity remaining was calculated by taking the hydration rate of the control sample (no SDS) as being 100%. The final concentrations of the unmodified and modified enzymes was 7.24 x 10^{-8} M and 7.28 x 10^{-8} M respectively.

Denaturation by guanidine hydrochloride. Aliquots of carbonic anhydrase were diluted into 0.25 ml of guanidine hydrochloride for five minutes before being diluted further into 4.75 ml of buffer (50 mM Hepes (pH 7.5), 50 mM sodium sulfate, 80 mM phenol red indicator). The concentration of guanidine hydrochloride was varied from 1 M to 8 M and the substrate concentration was fixed at 6 mM CO_2. The rate of each incubation was

determined at room temperature four times to determine the average rate at each concentration of guanidine hydrochloride. The percent of carbon dioxide hydration activity remaining was calculated by taking the hydration rate of the control sample (no guanidine hydrochloride) as being 100%. The final concentrations of the unmodified and modified enzymes was 7.24×10^{-8} M and 7.28×10^{-8} M respectively.

CO_2 hydration in human plasma. Human whole blood was obtained from a volunteer from venipuncture using heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes NJ). The blood was diluted 1:2 with Hanks Balanced salt solution and then centrifuged for 40 minutes at 1500 rpm using a Ficoll Hypaque density gradient. The plasma was pipetted into a sterile test tube for use in kinetic experiments.

The CO_2 hydration of the modified enzyme in human plasma was determined by incubating an aliquot of enzyme solution in 1 ml of human plasma for up to 3 hours at which point 1 ml of a CO_2 saturated solution of 0.9% NaCl was added. The resulting pH change was monitored with a pH electrode connected to a Fisher Model 50 pH meter (Fisher Scientific, Pittsburgh PA) while the reaction mixture was gently stirred. The RS232 output of the pH meter was directly connected to a 386 IBM compatible computer and the data collected was analyzed using SigmaPlot at a sampling rate of 1 Hz. The background rate of the pH change was determined by performing a blank with no added enzyme. All experiments were performed at room temperature.

Results

Homologous Zero Length Crosslinking of Bovine Carbonic Anhydrase II with EDC. To determine if protein-protein conjugates could be formed, BCA II was crosslinked with EDC in the absence of chondroitin-4-sulfate. Protein-protein conjugates with molecular weights of approximately 60 kDa, and 90 kDa have been separated and visualized on 12% SDS-PAGE after 15 minutes of incubation with EDC (Figure 5A) and after 1 hour of incubation with EDC (Figure 5B). Lane 1 contains protein standards and lane 2 contains BCAII with no exposure to EDC. Lanes 3-5 contain BCAII incubated at different concentrations of EDC (7.82 mM, 15 mM, and 34 mM respectively). The concentration of BCAII was kept constant at 33 uM. The reactions were terminated with the addition of β-mercaptoethanol. After 24 hours no gain in yield of crosslinked product is evidenced by 12% SDS-PAGE. Substantial reduction of any crosslinked products occurs if the pH is not adjusted to 8-9 following the 15 minute incubation at pH 4-5.

Zero Length Crosslinking of Bovine Carbonic Anhydrase II with EDC in the Presence of Chondroitin-4-Sulfate. Crosslinking of BCA II with the polymer chondroitin-4-sulfate was carried out under the following conditions. Chondroitin-4-sulfate was mixed with NHS and EDC in double distilled water at pH 4-5 for 5 minutes at room temperature (final concentrations 6.5 mM, 8.6 mM and 15 mM respectively). The pH was then adjusted to 8-9 with 0.1 M NaOH and bovine carbonic anhydrase II was added to the reaction (final concentration of 33 μM). The reaction was then allowed to proceed at room temperature. Aliquots were analyzed on 12% SDS-PAGE after 1 hour and 24 hours. After 1 hour a very

Figure 5. 12% SDS-PAGE analysis of crosslinking reaction with BCA II with no chondroitin-4-sulfate. A: 15 minute incubation, B: 30 minute incubation. Lane 1: protein standards. Lane 2: BCA II control (no EDC). Lane 3: 7.82 mM EDC. Lane 4: 15 mM EDC. Lane 5: 34 mM EDC. The concentration of BCA II in all incubations was 33 micromolar. Sizes of the protein standards are indicated. The crosslinked complexes can clearly be seen in lanes 3-5 at a molecular weight of about 60 kDa.

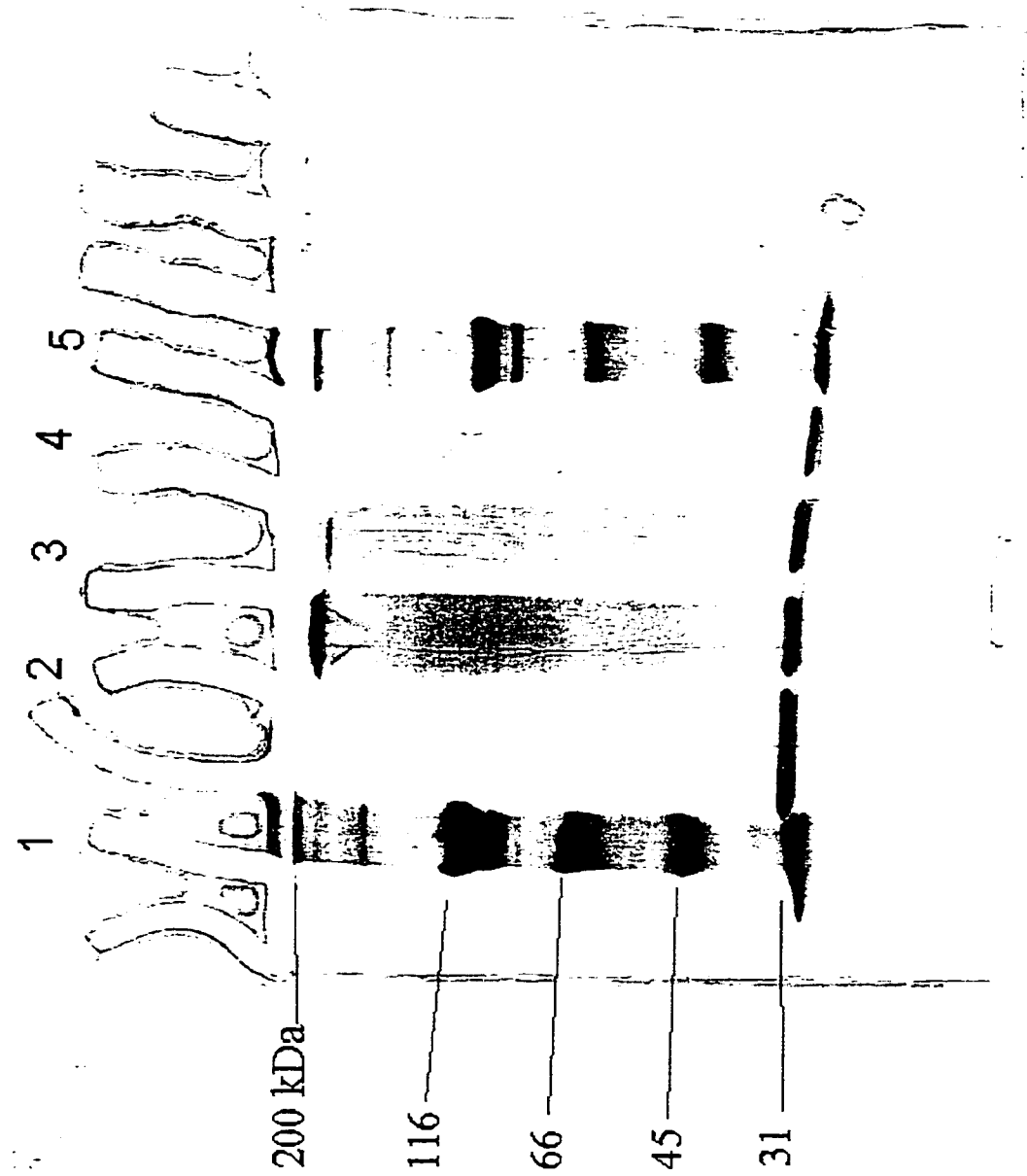
faint band corresponding to a molecular weight of approximately 35 kDa (Figure 6) was detected (which does not correspond to any protein-protein product) suggesting that a fragment of chondroitin-4-sulfate was successfully attached to CA II. This band was much more prominent after 24 hours of incubation. Only a single detectable crosslinked product was obtained. If the pH was changed after the addition of CA II, there were other crosslinked species apparent corresponding to dimers and trimers of CA II. These protein-protein products were completely absent if the pH is changed to 8-9 before the addition of CA II, thus it appears that the reacting nucleophile (most likely the primary amine of a lysine) is very unreactive at pH 4-5. If NHS is left out of the reaction mixture no protein-chondroitin-4-sulfate conjugate is produced. Crosslinking arises from substitution of lysine epsilon amino groups of carbonic anhydrase for the succinimidyl moieties during a 24 hour incubation period.

Bovine carbonic anhydrase was also conjugated under slightly different concentration of reactants in order to create a higher molecular weight complex within a 24 hour reaction time span. Figure 7 shows the result of crosslinking BCAII with chondroitin-4-sulfate with the final concentrations as follows: 15.6 mM EDC, 8.2 mM chondroitin-4-sulfate, 26.1 mM NHS and 0.216 mM BCA II. As can be seen, under these conditions a high molecular weight complex is formed after a 24 hour incubation time. This product is not seen if NHS is left out of the reaction mix. In addition, no other prominent crosslinked species are present under these conditions.

Gel Filtration of Crosslinked Complexes. Sephadex G-150 gel chromatography was used to separate any crosslinked products from unreacted BCA II. After reaction mixtures were

Figure 6. 12% SDS-PAGE analysis of crosslinking reaction with BCA II with chondroitin-4-sulfate after 24 hours of incubation. Lane 1: protein standards. Lane 2: BCA II control (no EDC). Lane 3: 33 micromolar BCA II, 6.5 mM chondroitin-4-sulfate, 15 mM EDC, 8.6 mM NHS, pH was adjusted to 8 before adding BCA II.. Lane 4: same as lane 3 except pH was adjusted to 8 after adding BCA II Sizes of the protein standards are indicated. The crosslinked complexes can clearly be seen in lane 3 just above the band representing BCA II. It is absent in lane 4.

Figure 7. 12% SDS-PAGE analysis of a high molecular weight BCA II : chondroitin-4-sulfate conjugate. Lane 1and 5: protein standards. Lane 2: 20 microliter sample of conjugate. Lane 3: 5 microliter sample of conjugate. Lane 4: BCA II. The reaction conditions for the crosslinking were 0.216 mM BCA II, 8.2 mM chondroitin-4-sulfate, 15.6 mM EDC, 26.1 mM NHS. Sizes of the protein standards are indicated. The crosslinked complex can be seen in lanes 2-3 at the top of the gel slightly below the 200 kDa protein standard.


1
2
3
4
5
200 kDa
116
66
45
31

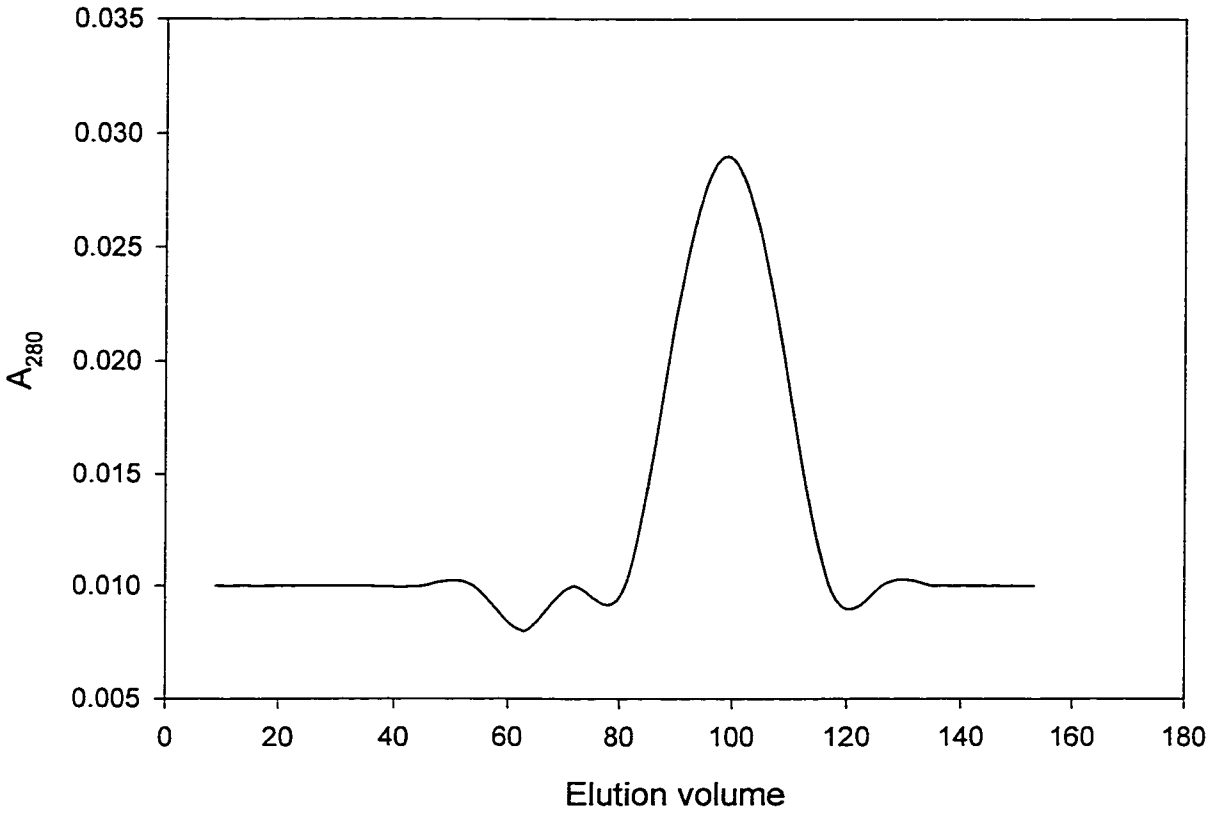
filtered they were loaded onto the column and eluted with 50 mM Hepes buffer at pH 7.5 via gravity filtration. This method proved unsuccessful in separating any products from the reaction mixtures that contained no chondroitin-4-sulfate. Despite the appearance on SDS-PAGE of bands representing dimers and trimers of BCA II no such peaks were eluted from a G-150 column despite four attempts. Figure 8 shows a representative elution profile for such an experiment. It is possible that a very low concentration can be seen eluting before the large peak representing BCA II.

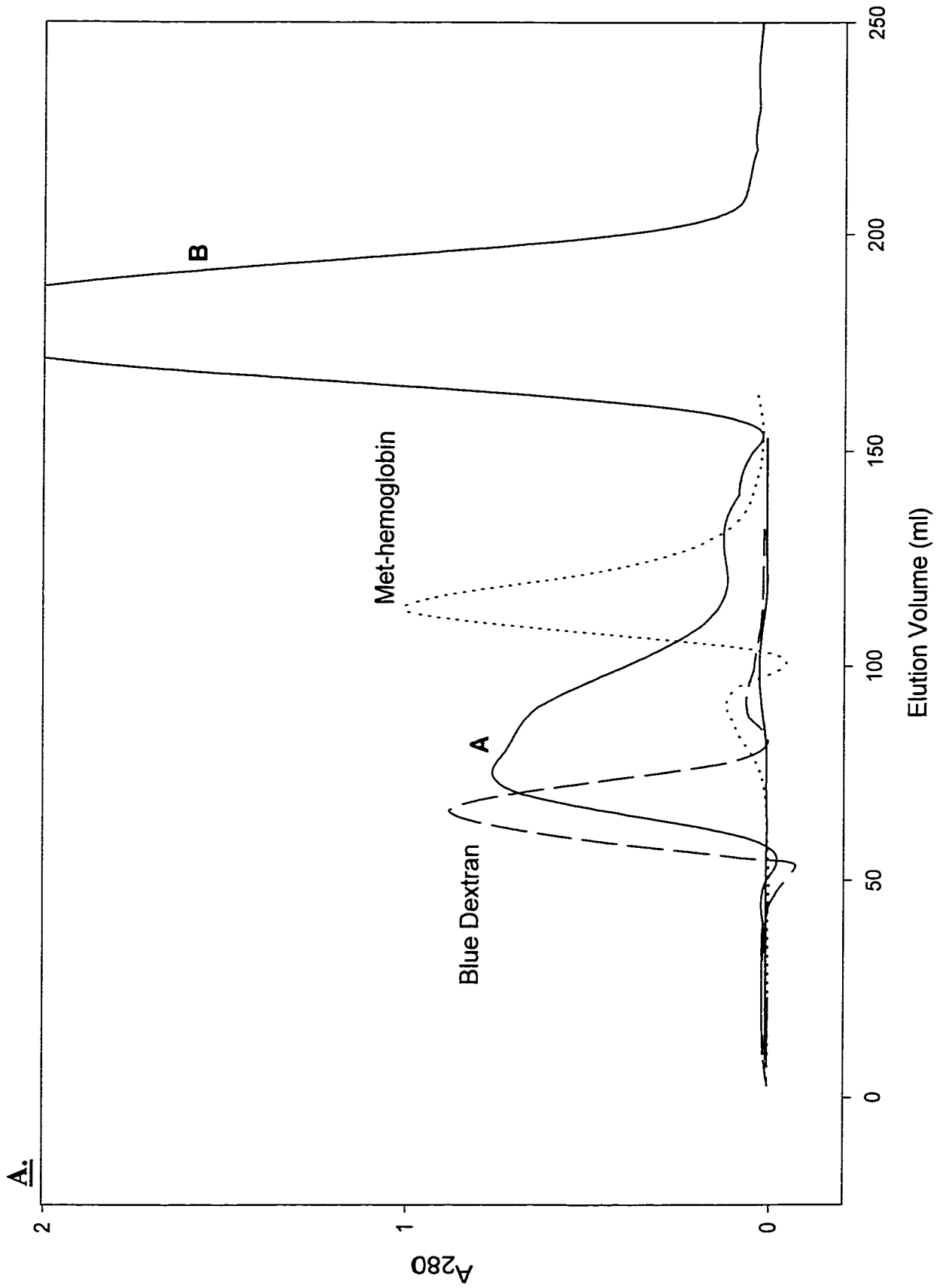
Figure 9 shows the elution profile of a filtered reaction mixture of BCA II and chondroitin-4-sulfate crosslinked with EDC in the presence of NHS. It can be seen that a large molecular weight complex elutes out at the void volume of the column. The column was calibrated with blue dextran, met-hemoglobin, yeast alcohol dehydrogenase and bovine carbonic anhydrase II. The conjugate elutes in between yeast alcohol dehydrogenase (~150 kDa) and blue dextran (~1 million Da) and on SDS polyacrylamide gel the conjugate migrates faster than the 200 kDa molecular weight protein marker. These observations suggest that the chondroitin-4-sulfate:BCA II conjugate is of molecular weight range between 150 kDa and 200 kDa. The amount of conjugate recovered from the column was quantified using the absorbance of the molecule at 280 nm and comparing that to a standardized BCA II concentration curve. This approach was adopted in light of the fact that the conjugate does not differ in its absorbance spectrum from the unmodified enzyme in the region of 350-200 nm. The total amount of BCA II used in the crosslinking reaction was 25 mg, and approximately 15 mg of conjugate was recovered from Sephadex G-150 columns. Thus the yield of the conjugate under the reaction conditions described above was about 60%.

Figure 9A. Gel filtration of modified BCA II on Sephadex G-150 column (2.5 x 49.5 cm) eluted with a buffer containing 50 mM Hepes at pH 7.5. The reaction mixture was filtered and then applied to the column and eluted by gravity. The flow rate was approximately 1 ml/min. The protein content was measured by observing the absorbance at 280 nm of the samples collected by the fraction collector. Both peak A (BCA II: chondroitin-4-sulfate complex) and B (BCA II) showed activity and were collected and concentrated further. The molecular weights of the standards used were as follows: blue dextran, ~ one million Da; yeast alcohol dehydrogenase, 150 kDa; met hemoglobin, 68 kDa.

Figure 9B. The elution profile of a dilute sample of yeast alcohol dehydrogenase on the same column as described in Figure 9A.

B.


0.035
0.030
0.025
0.020
0.015
0.010
0.005
A_{280}
0
20
40
60
80
100
120
140
160
180
Elution volume


A.
A280
2
1
0
0
50
100
150
200
250
Elution Volume (ml)
B
A
Met-hemoglobin
Blue Dextran

CO_2 Hydration activity of EDC Crosslinked Conjugates. Samples collected from the column meant to separate the protein-protein conjugates were screened for carbon dioxide hydration activity with the spot test (See Methods section). The results of this experiment were negative for enzymatic activity for any samples collected from such a reaction mixture. In light of this result, no further activity measurements were attempted on any protein-protein crosslinking experiments.

The spot test determination for carbon dioxide hydration yielded a positive result for samples collected from incubations that included chondroitin-4-sulfate. Both the unreacted BCA II peak and the high molecular weight conjugate form (Figure 9) showed the ability to hydrate carbon dioxide. As such, these samples were separately pooled and concentrated for additional kinetic characterization with stopped flow analysis.

Determination of the Kinetic Parameters of CO_2 Hydration. Stopped flow experiments were performed in order to determine the precise parameters of carbon dioxide hydration by BCA II and the BCA II:chondroitin-4-sulfate conjugate. Table 1 summarizes the results of such experiments carried out at various pH values. Table 1A shows the values of the kinetic parameters K_M and k_{cat} for the hydration of carbon dioxide by the unmodified form the enzyme and table 1B shows the values for the conjugated enzyme. Lineweaver-Burk plots were used to generate the kinetic parameters K_M and k_{cat}. Figures 15 and 16 show the Lineweaver-Burk plots for the unmodified enzyme and the conjugate respectively at various pH values. Both the unmodified and modified enzymes display similar kinetic characteristics with the chondroitin-4-sulfate conjugate showing a slight decrease in activity and efficiency of catalysis (Table 1a and 1B). For both enzymes the maximum activity seen is at pH 8.11

Table 1A. Kinetic parameters for the CO_2 hydration carried out by bovine carbonic anhydrase II in a buffer containing 50 mM Hepes, 50 mM Sodium sulfate and 80 μM phenol red indicator at 25° C. [a]

pH	k_{cat} x $10^6(s^{-1})$	K_M (mM)	k_{cat}/K_M x $10^8(M^{-1}s^{-1})$
8.11	3.21	4.15 ± 0.12	7.73
7.51	1.23	3.89 ± 0.31	3.16
7.00	0.796	4.26 ± 0.18	1.86
5.5[b]	0.040	6.72 ± 0.31	0.0595

Table 1B. Kinetic parameters for the CO_2 hydration carried out by bovine carbonic anhydrase II : chondroitin-4-sulfate complex in a buffer containing 50 mM Hepes, 50 mM Sodium sulfate and 80 μM phenol red indicator at 25 ° C. [a]

pH	k_{cat} x $10^6(s^{-1})$	K_M (mM)	k_{cat}/K_M x $10^8(M^{-1}s^{-1})$
8.10	1.87	3.39 ± 0.09	5.51
7.50	0.743	3.26 ± 0.06	2.27
7.00	0.428	2.37 ± 0.12	1.80
5.5[b]	0.061	11.2 ± 0.40	0.0544

[a] The values of K_M were determined from Lineweaver - Burk plots. The value k_{cat} is defined as $V_{max}/[E]_{total}$. The average of 4 trials is shown with standard deviation.

[b] 50 mM Mes buffer, 50 mM sodium sulfate, 80 μM chlorophenol red.

and activity decreases with pH. The K_M values for the conjugated enzyme is slightly lower than the unmodified protein except at pH 5.5, the lowest pH value tested.

The reverse reaction that dehydrates a bicarbonate ion was also examined with stopped flow at pH 5.5. Both forms of the enzyme were able to catalyze the dehydration of bicarbonate ion at essentially equal rates (Table 2). The conjugated enzyme appeared to have a lower K_M than the unmodified form at pH 5.5.

The Effect of Heat on the CO_2 Hydration Activity of Unmodified and Conjugated BCA II. Assays were performed in order to determine if conjugation to chondroitin-4-sulfate conferred any ability to resist thermal denaturation by BCA II. Aliquots of protein samples dissolved in 50 mM Hepes buffer at pH 7.5 were heated in 1 ml microcentrifuge tubes placed in a heating block. Appropriate volumes of protein sample were removed at specific time intervals and assayed for carbon dioxide hydration ability using stopped flow. Table 3 shows a summary of the results of such an experiment on the unmodified form of BCA II and the chondroitin-4-sulfate conjugated protein.

When heated at 65 degrees Celsius the chondroitin-4-sulfate conjugate retains almost 50% of its enzymatic activity after 27 minutes of heating. In contrast the unmodified enzyme loses over 90% of its enzymatic ability. It is evident from the data in table 3 that conjugation of BCAII with chondroitin-4-sulfate confers significant resistance to thermal denaturation leading to loss of enzyme activity.

The Effect of SDS on the CO_2 Hydration Activity of Unmodified and Conjugated BCA II. The effects of a chemical denaturing agent such as SDS on the activity of BCA II was

Table 3. Thermal inactivation of CO_2 hydration at 65°C.

	Unmodified BCA II		Crosslinked BCA II	
time (min)	v x 10^{-2} (M/s)	% Activity Remaining	v x 10^{-2} (M/s)	% Activity Remaining
0	4.14 ± 0.17	100	5.37 ± 0.17	100
2	3.82 ± 0.11	92.3	3.22 ± 0.12	60.0
7	1.20 ± 0.08	29	2.86 ± 0.16	53.3
12	0.582 ± 0.070	14	2.43 ± 0.04	45.3
27	0.582 ± 0.070	7.6	2.36 ± 0.08	43.9

The initial rates of CO_2 hydration were determined by stopped flow in a buffer containing 50 mM Hepes buffer, 50 mM sodium sulfate and 80 μM phenol red at pH 7.5. The final concentration of unmodified enzyme was 7.92 x 10^{-8} M and the final concentration of crosslinked enzyme was 7.28 x 10^{-8} M. The concentration of substrate was approximately 6 mM CO_2 . The numbers shown are the average of four experiments. The background rate has been subtracted from all initial rates determined.

investigated. Protein samples were exposed to different concentration of SDS and assayed for carbon dioxide hydration activity with stopped flow. Table 4 show a summary of the results of these experiments. Unmodified BCA II rapidly loses its ability to hydrate carbon dioxide in the presence of SDS. Even concentrations as low as 0.25% virtually abolished activity. The chondroitin-4-sulfate conjugate however showed a higher ability to resist the denaturing effects of SDS. The conjugate retained higher activity than the unmodified enzyme but this effect was less pronounced at higher levels of SDS.

Effects of Guanidinium hydrochloride on CO_2 Hydration activity of BCA II. The effects of guanidinium hydrochloride on the rate of carbon dioxide hydration by BCA II and the chondroitin-4-sulfate conjugate were determined by incubating the enzyme with various concentrations of the denaturant. Stopped flow was used to determine the rate of the samples after a five minute incubation period. Table 5 summarizes the results of these experiments. The conjugated enzyme retained 72% (± 1.7) of its activity compared to 52% (± 2.3) for the unmodified BCA II when exposed to 1 M guanidinium hydrochloride. At higher concentrations of guanidinium hydrochloride, the protective effect of the chondroitin-4-sulfate was not evident. The unmodified enzyme retained a larger percentage of its original activity (as measured by a control sample not exposed to guanidinium hydrochloride) than the conjugate.

Effects of Acetazolamide, a Carbonic Anhydrase II inhibitor, on CO_2 Hydration. The effects of a known noncompetitive inhibitor, acetazolamide, on the carbon dioxide activity of BCA II and the conjugated enzyme were examined. The enzyme was incubated in a buffer

Table 5. Resistance to denaturation by guanidine hydrochloride.

	Unmodified		Crosslinked BCA II	
[GuHCl] M	v (mV/s)	% Activity Remaining	v (mV/s)	% Activity Remaining
0	46.8 ± 1.4	100	40.9 ± 1.45	100
1	24.5 ± 1.1	52.3	29.5 ± 0.7	72.1
2	20.12 ± 1.31	42.9	16.1 ± 1.0	39.4
4	5.25 ± 0.68	11.2	2.25 ± 0.18	5.5
8	5.46 ± 0.48	11.6	2.44 ± 0.11	6.0

The initial rates of CO_2 hydration were determined by stopped flow at room temperature in a buffer containing 50 mM Hepes buffer, 50 mM sodium sulfate and 80 μM phenol red at pH 7.5. The final concentration of unmodified enzyme was 7.24×10^{-8} M and the final concentration of crosslinked enzyme was 7.28×10^{-8} M. The concentration of substrate was approximately 6 mM CO_2 . The numbers shown are the average of four experiments. The background rate has been subtracted from all initial rates determined.

consisting of 50 mM Hepes, 50 mM sodium sulfate and 80 μM phenol red indicator at pH 7.5, for five minutes at room temperature, and then assayed for activity using stopped flow. The concentration of inhibitor was varied from 1.17×10^{-8} to 9.36×10^{-8} M (Pocker and Bjorkquist, 1977) and the concentration of BCA was kept constant at 1.2×10^{-7} M. The inhibitor constant, K_i, for the unmodified enzyme and the crosslinked enzyme was 4.62 (± 0.23) $\times 10^{-8}$ M and 3.87 (± 0.16) $\times 10^{-8}$ M respectively (Figure 10 and 11). These results indicate that the crosslinked BCA II is slightly more sensitive to the effects of acetazolamide under the conditions described above.

The Effect of Incubation in Human Plasma on the Rate of Carbon Dioxide Hydration. The activity of the unmodified and modified BCA II was examined at room temperature after incubation in human plasma for up to 3 hours. Figure 12 shows the results of such an experiment for the unmodified protein and figure 13 shows the results for the crosslinked enzyme. The concentration of the unmodified BCA II in each assay was 3.3×10^{-8} M and the concentration of the crosslinked BCA II was 2.5×10^{-8} M. A sample with no added enzyme served as a control for the background rate of pH change.

Although the actual rate constants are not easily determined, at high concentration of water it is possible to estimate the relative rate of reaction as a pseudo first order rate constant. Thus the relative reaction rates can be compared by measuring the initial slope of the curves in figures 12 and 13. Table 6 and figure 14 show the change of relative rates of both proteins. As shown in figure 14, it is evident that the crosslinked protein loses its activity steadily the longer it is incubated in plasma, however, the loss of activity is not as great as that of the unmodified enzyme. The unmodified enzyme had lost approximately 92

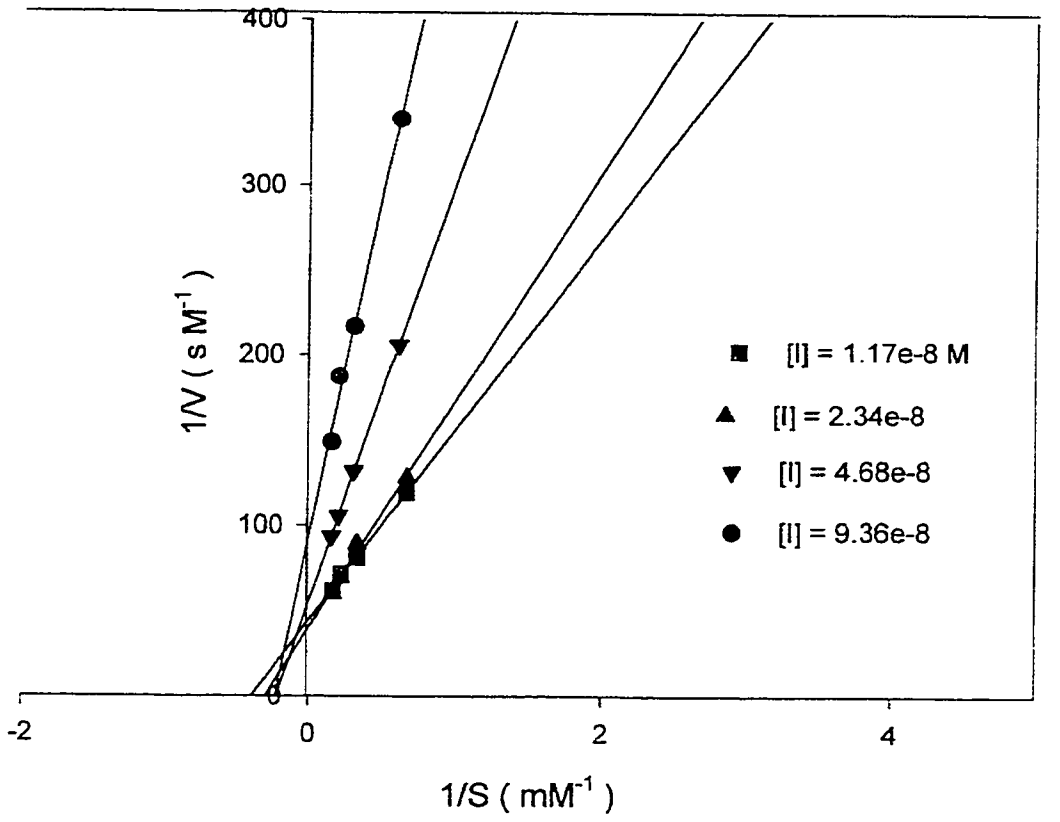


Figure 10. The Lineweaver - Burk plots for the unmodified BCA II catalyzed hydration of CO_2 in the presence of varying concentrations of acetazolamide. Enzyme was incubated for five minutes at room temperature with the inhibitor in a buffer containing 50 mM Hepes, 50 mM sodium sulfate and 80 μM phenol red indicator at pH 7.51. Initial rates were determined with the stopped flow method. The concentration of BCA II in each experiment was 1.28×10^{-7} M. The average of four trials are plotted for each curve.

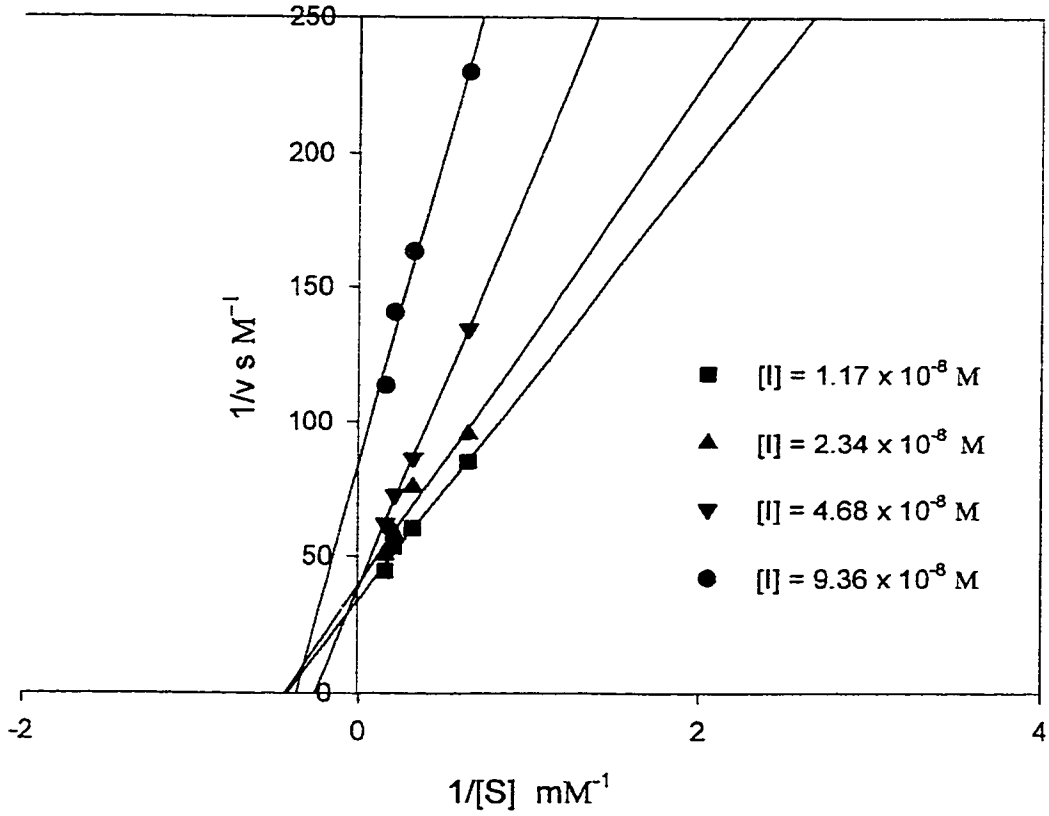


Figure 11. The Lineweaver - Burk plots for the modified BCA II catalyzed hydration of CO_2 in the presence of varying concentrations of acetazolamide. Enzyme was incubated for five minutes at room temperature with the inhibitor in a buffer containing 50 mM Hepes, 50 mM sodium sulfate and 80 μM phenol red indicator at pH 7.51. Initial rates were determined with the stopped flow method. The concentration of BCA II in each experiment was 1.27×10^{-7} M. The average of four trials are plotted for each curve.

Figure 12. CO_2 hydration activity of unmodified BCA II at various times of incubation in human plasma. The concentration of BCA II was 3.3×10^{-8} M. A typical result after each time of incubation is shown. In order to obtain statistically significant relative rates of reaction, the experiment was repeated 4 times. For each incubation time the slope was determined at t = 2 to t = 3 seconds. It is clear that the rate of pH change between 2 and 3 seconds is steadily decreasing the longer the enzyme is incubated in human plasma. This is reflected by a decrease in the slope of the line. The extent to which the pH is lowered by the enzyme's activity is also correlated inversely with incubation time. The total difference in pH end point for the reaction between the control sample and 3 hours of incubation is slightly less than 1 pH unit.

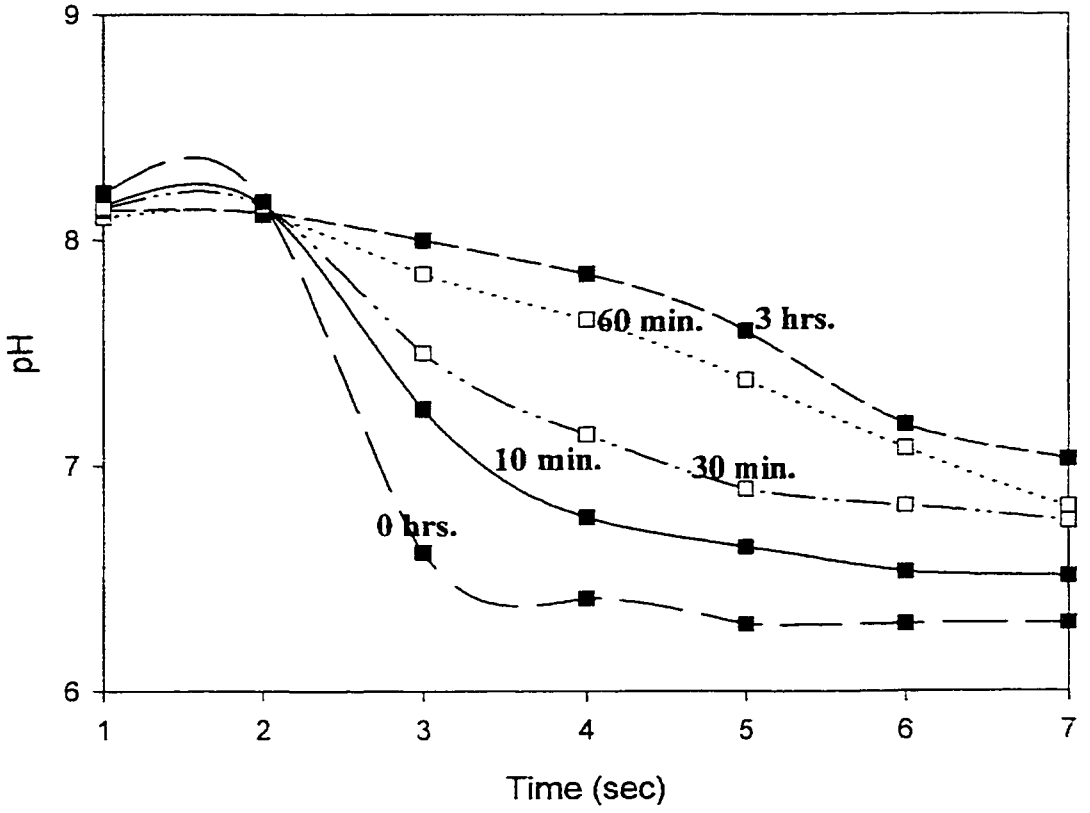

9
8
7
6
pH
1
2
3
4
5
6
7
Time (sec)
60 min.
3 hrs.
10 min.
30 min.
0 hrs.

Figure 13. CO_2 hydration activity of crosslinked BCA II at various times of incubation in human plasma. The concentration of crosslinked BCA II was 2.5×10^{-8} M. A typical result after each time of incubation is shown. In order to obtain statistically significant relative rates of reaction, the experiment was repeated 4 times. For each incubation time the slope was determined at t = 9 to t = 11 seconds. Similar to the unmodified enzyme, the crosslinked product also experiences effects in human plasma that serve to diminish the rate at which carbon dioxide is hydrated. The extent to which the pH is lowered at the end of the reaction does not seem to differ much for incubation times of up to an hour. After 3 hours of incubation the inhibitory effects are most pronounced. The total difference in pH end point for the reaction between the control sample and 3 hours of incubation is approximately 0.5 pH units. Thus the overall extent of pH decrease is largely unaffected for up to 1 hour of incubation in human plasma, while the rate of change of pH with respect to time is decreases.

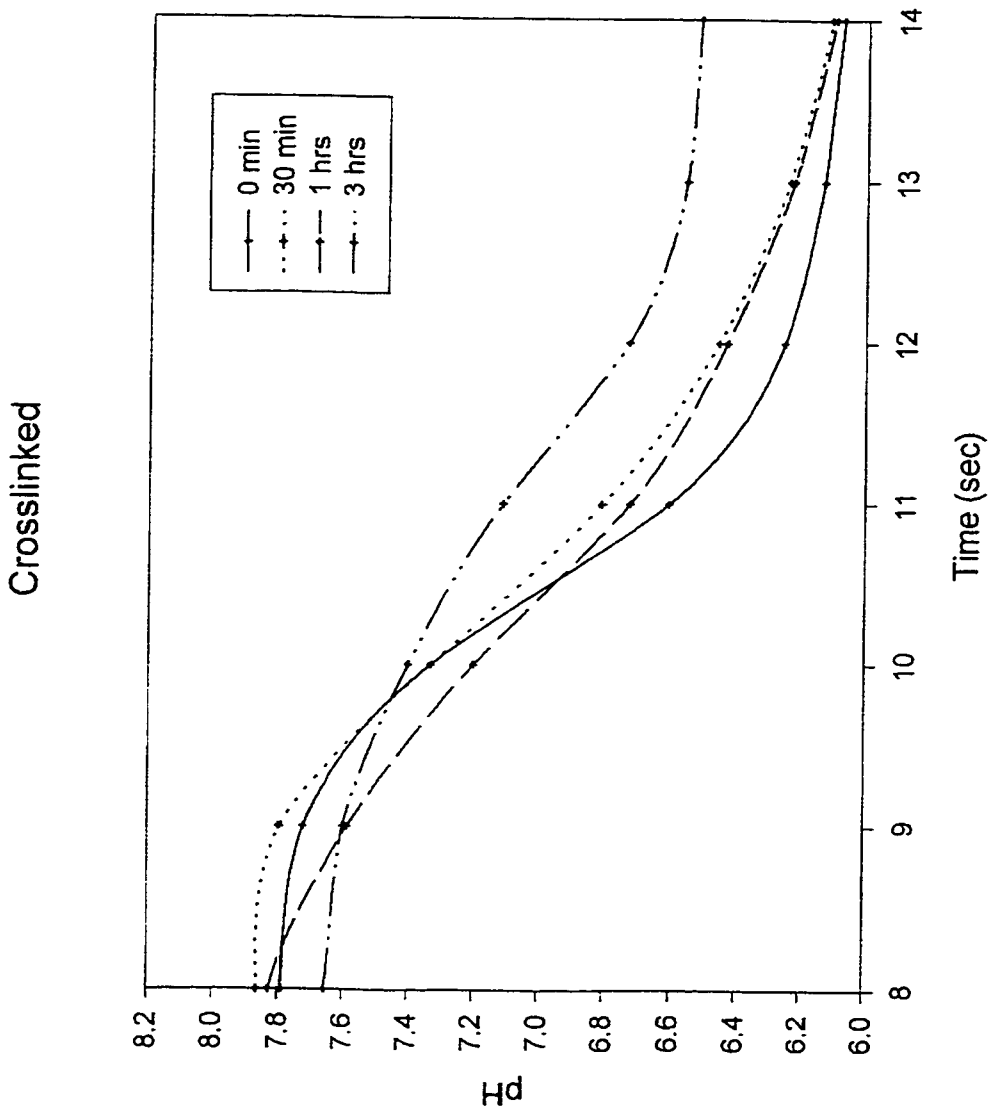
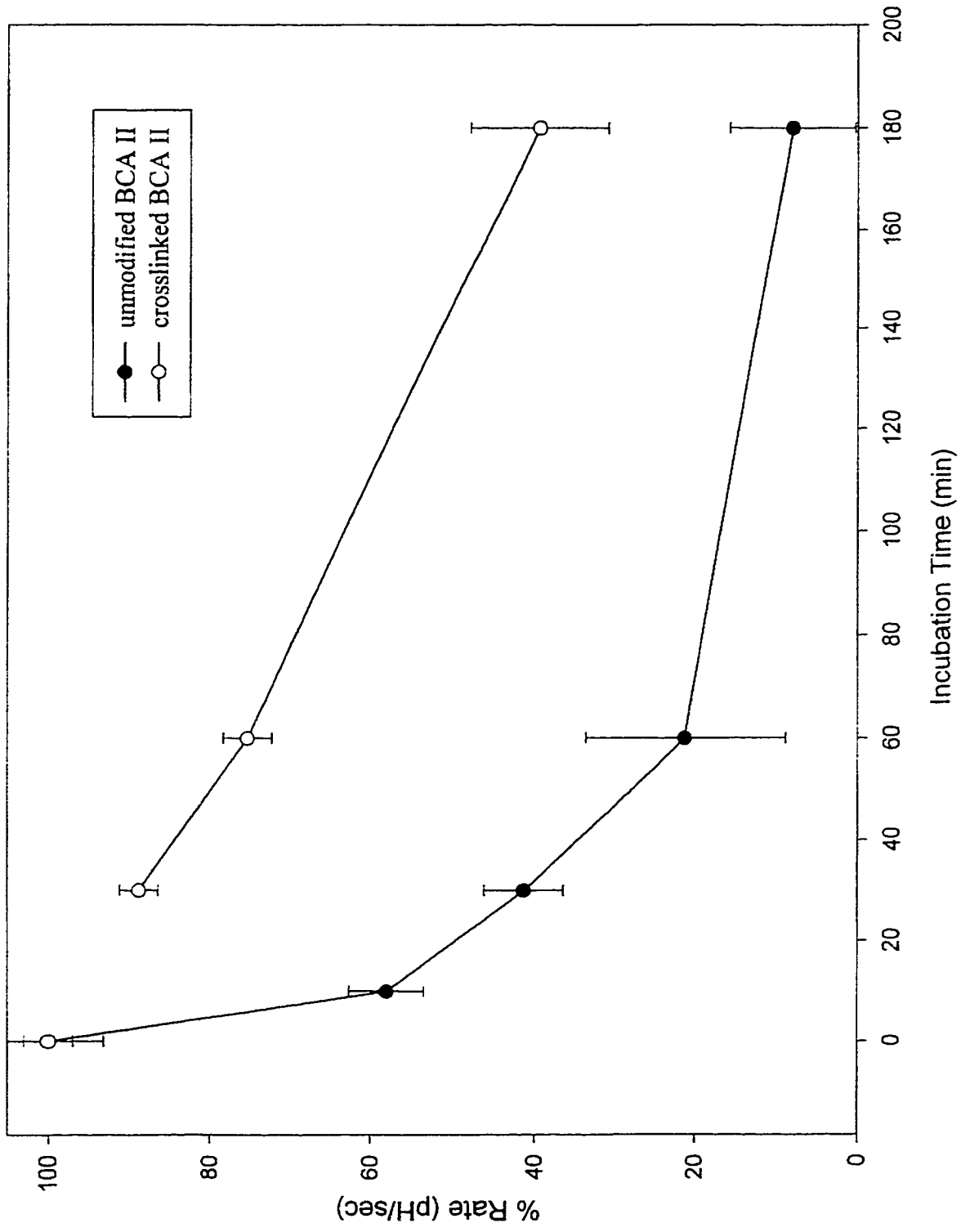

Crosslinked
0 min
30 min
1 hrs
3 hrs
pH
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
8
9
10
11
12
13
14
Time (sec)

Table 6. The effect of incubation in human plasma at room temperature on the rate of carbon dioxide hydration. The average of 4 runs at each incubation time is shown with standard deviation. N.D. : Not Determined.

Incubation Time (min)	Rate (pH/sec)	
	Control	Crosslinked BCA II
0	1.50 ± 0.01	0.545 ± 0.016
10	0.876 ± 0.04	N.D.
30	0.623 ± 0.03	0.485 ± 0.012
60	0.321 ± 0.04	0.410 ± 0.014
180	0.129 ± 0.01	0.215 ± 0.018

Figure 14. The effect of incubation in human plasma on the rate of carbon dioxide hydration of unmodified and crosslinked BCA II at various times of incubation in human plasma. Table 6 and Figures 12 and 13 provided the data for this figure which is normalized for easier comparison of the crosslinked enzyme and the wild type enzyme. The time it takes for the protein to lose one half of its ability to hydrate carbon dioxide is significantly increased after crosslinking with chondroitin-4-sulfate. Extrapolating from the graph, the wild type enzyme has lost 50 percent of its activity after about 20 minutes of incubation. The crosslinked product has lost 50 percent of its activity well after an hour of incubation in plasma, possibly as late as 140 minutes of incubation time. After the maximum time of incubation examined, 3 hours, the wild type has lost 90 percent or more of its ability to hydrate carbon dioxide. After the same exposure period, the crosslinked product has lost only about 60 percent of its enzymatic activity, as measured by the assay. An exact measure is not possible from this data, but it is clear that whatever the inhibitory effects of human plasma are on bovine carbonic anhydrase II, they are less pronounced upon the crosslinked protein.


unmodified BCA II
crosslinked BCA II
% Rate (pH/sec)
Incubation Time (min)
0
20
40
60
80
100
0
20
40
60
80
100
120
140
160
180
200

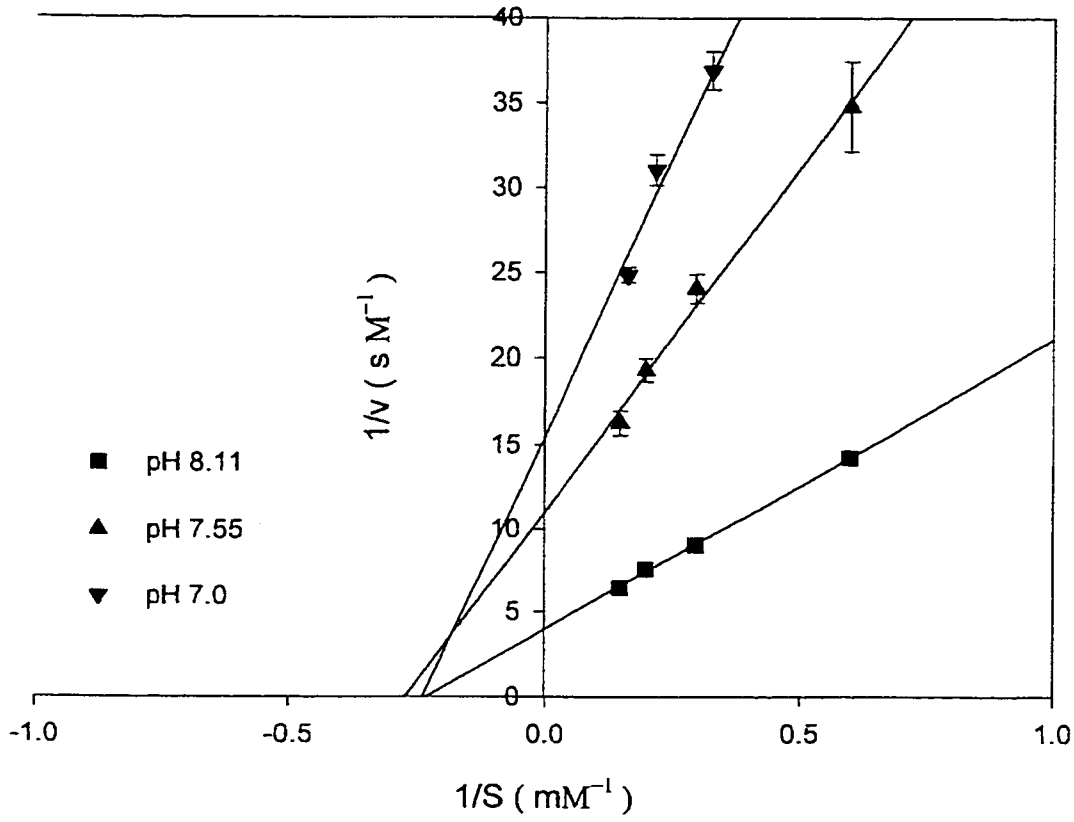
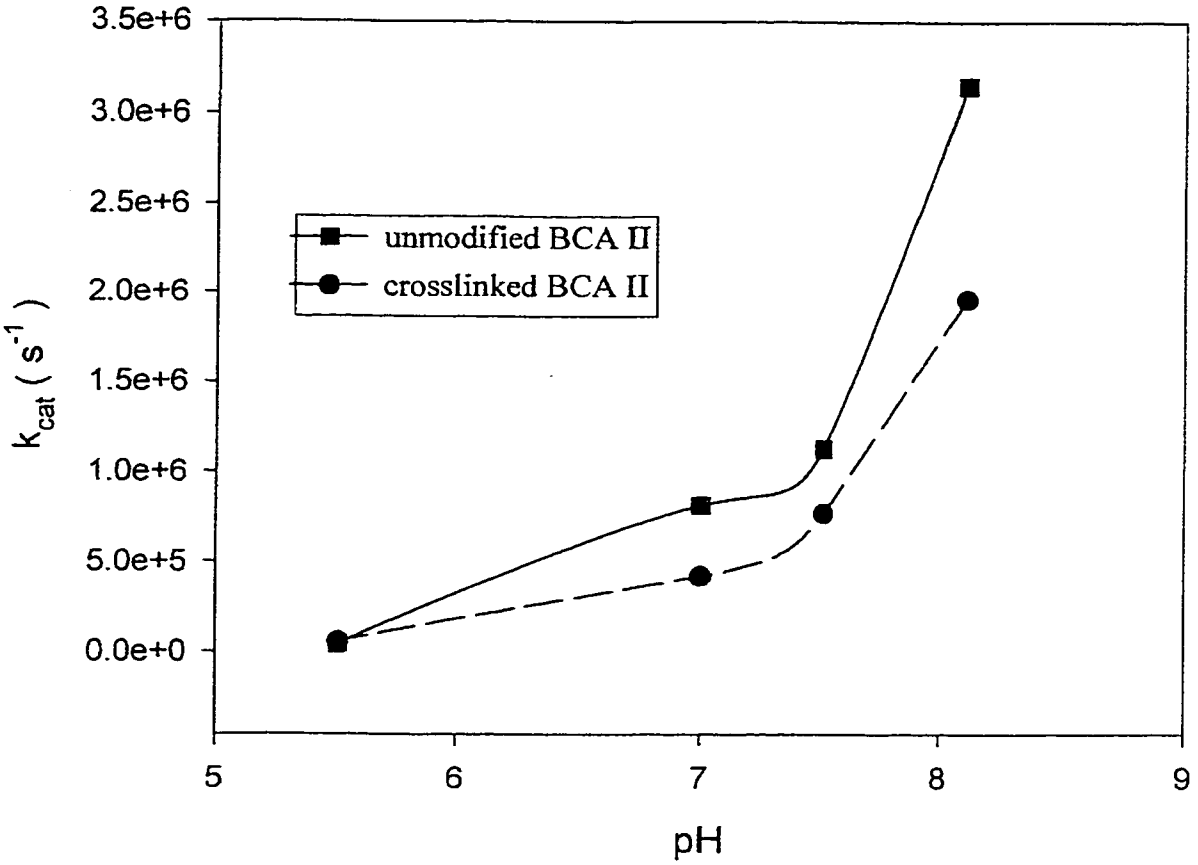


Figure 15. The Lineweaver - Burk plots of unmodified BCA II in a buffer containing 50 mM hepes , 50 mM sodium sulfate and 80 μM phenol red indicator at different pH values obtained by adjusting the concentrations of CO_2 from 6 mM to 1 mM. The average of 4 runs and standard deviation at each substrate concentration are shown.

Figure 17. The effects of pH on the kinetic parameters k_{cat} and k_{cat} / K_M. The values of K_M and V_{max} were determined from Lineweaver-Burke plots. The value k_{cat} is defined as $V_{max} / [E]_{total}$. For pH 5.5, 50 mM Mes buffer, 50 mM sodium sulfate, 80 μM chlorophenol red was used, for all other pH values Hepes buffer was used with 80 μM phenol red as the indicator. The enzyme concentration was 1.2×10^{-7} M. All initial rates were measured using the stopped flow method.


3.5e+6
3.0e+6
2.5e+6
2.0e+6
1.5e+6
1.0e+6
5.0e+5
0.0e+0
k_{cat} (s^{-1})
unmodified BCA II
crosslinked BCA II
5
6
7
8
9
pH

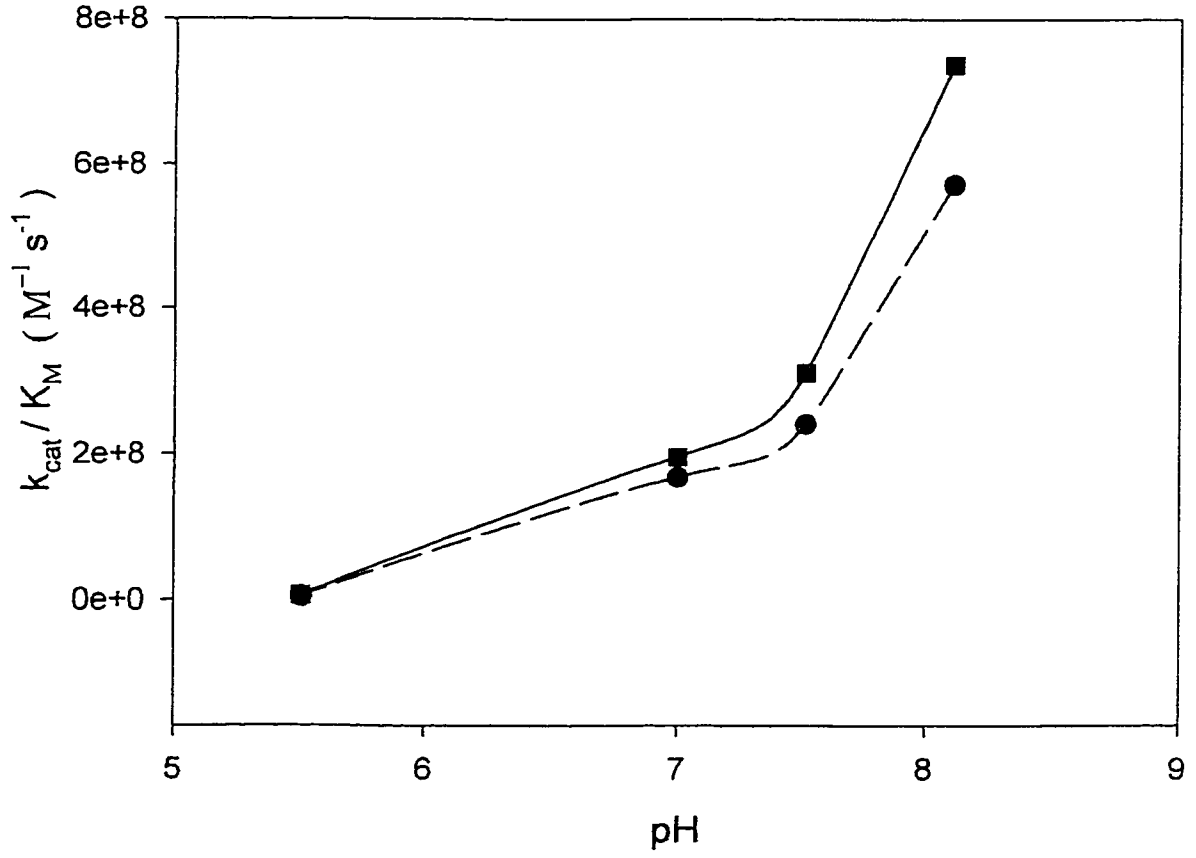
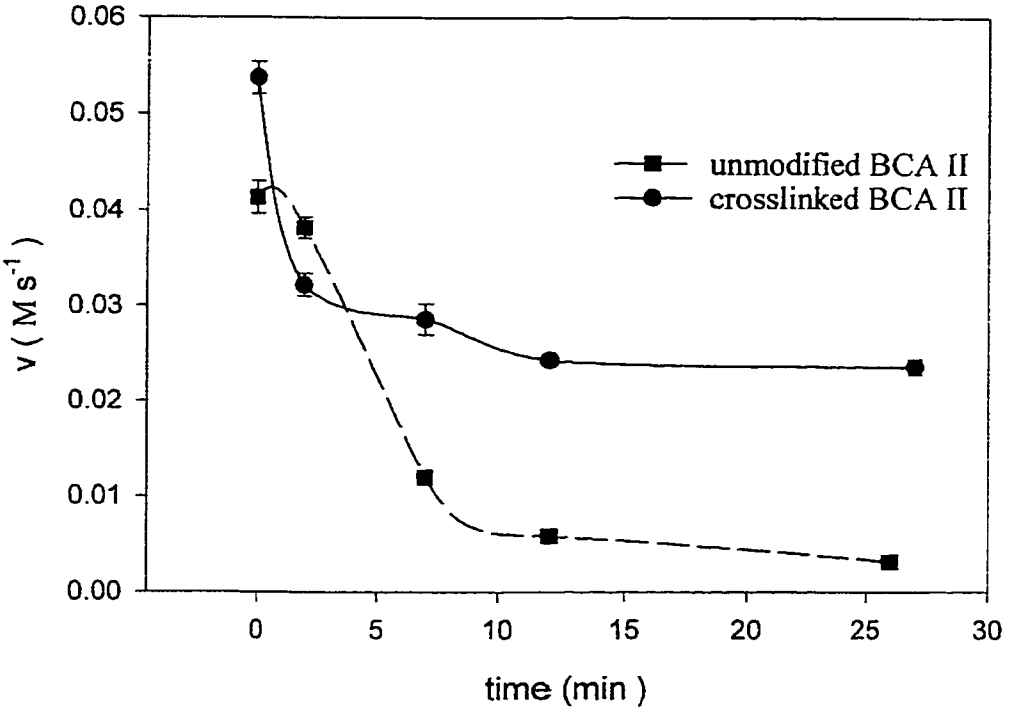
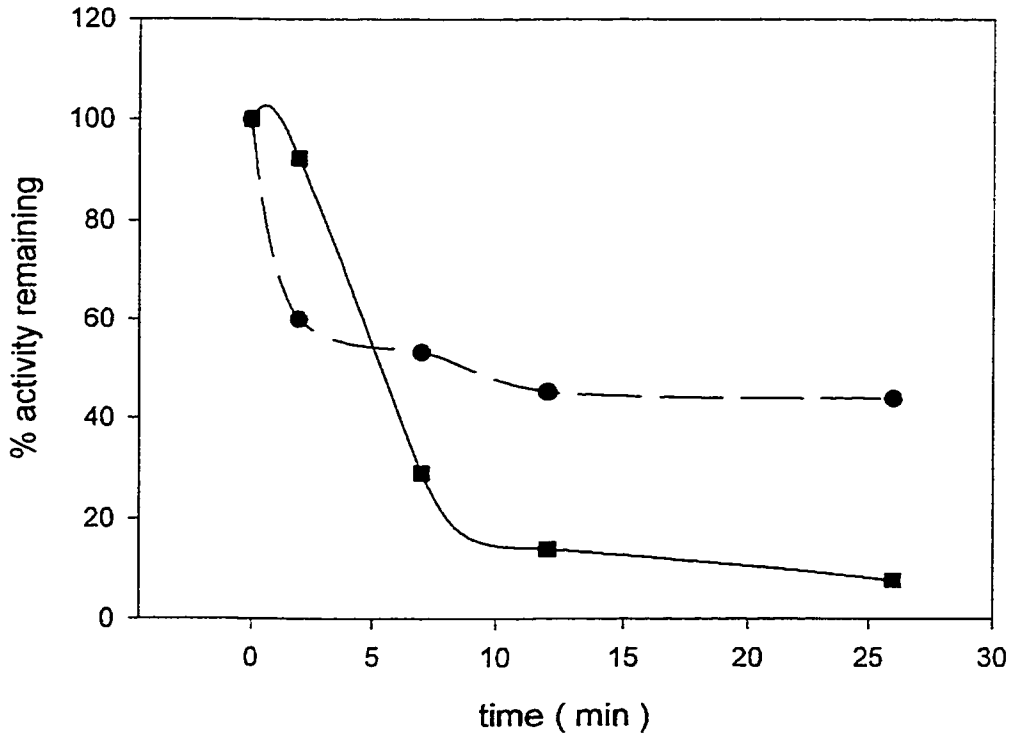
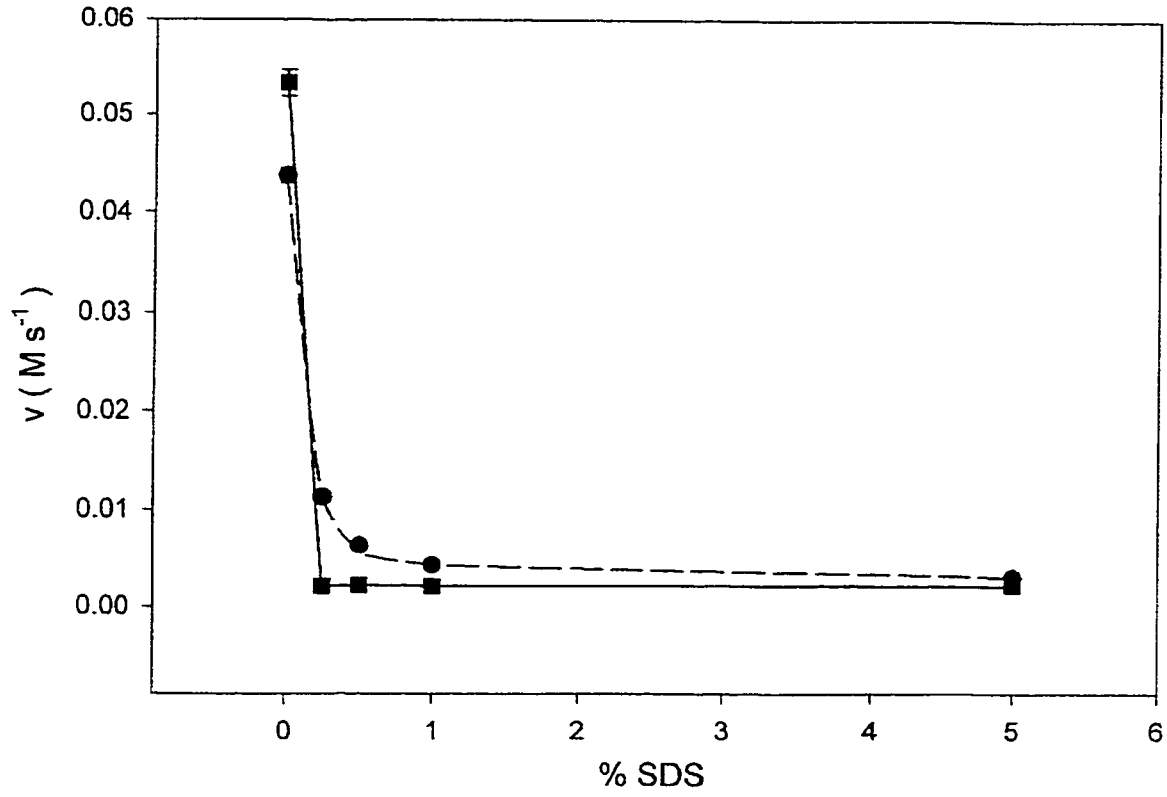

8e+8
6e+8
4e+8
2e+8
0e+0
k_{cat} / K_M (M^{-1} s^{-1})
5
6
7
8
9
pH

Figure 18. The effect of temperature on the rate of CO_2 hydration in a buffer containing 50 mM Hepes, 50 mM sodium sulfate and 80 μM phenol red at pH 7.55. The enzymes were incubated at 65 degrees Celsius for the times indicated and then diluted into 5 ml of buffer and the initial rate was determined using stop flow. The final concentration of CO_2 was approximately 6 mM. The final concentration of unmodified BCA II was 7.92 x 10^{-8} M, and the final concentration of the crosslinked BCA II was 7.28 x 10^{-8} M. The average of four trials and the standard deviation is shown.


0.06
0.05
0.04
0.03
0.02
0.01
0.00
v (M s-1)
unmodified BCA II
crosslinked BCA II
0
5
10
15
20
25
30
time (min)


120
100
80
60
40
20
0
% activity remaining
0
5
10
15
20
25
30
time (min)


0.06
0.05
0.04
0.03
0.02
0.01
0.00
v (M s-1)
0
1
2
3
4
5
6
% SDS

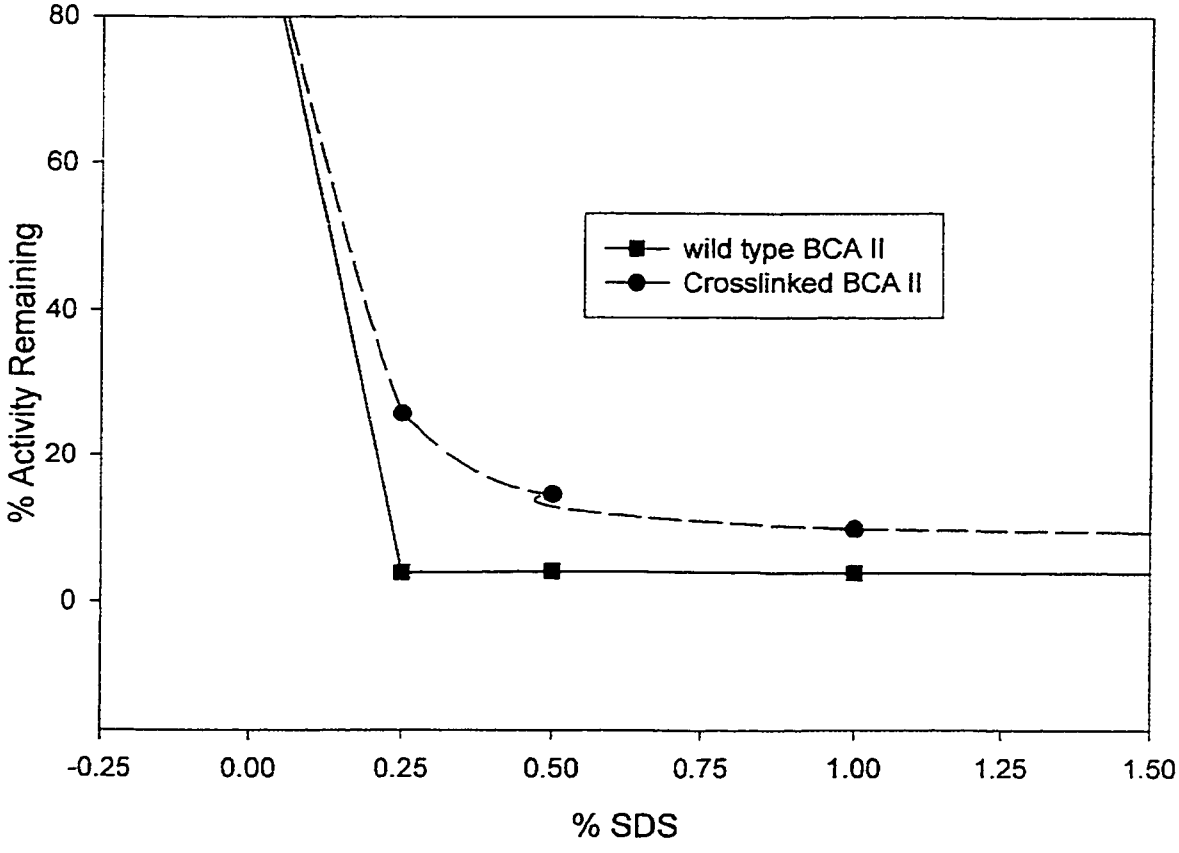
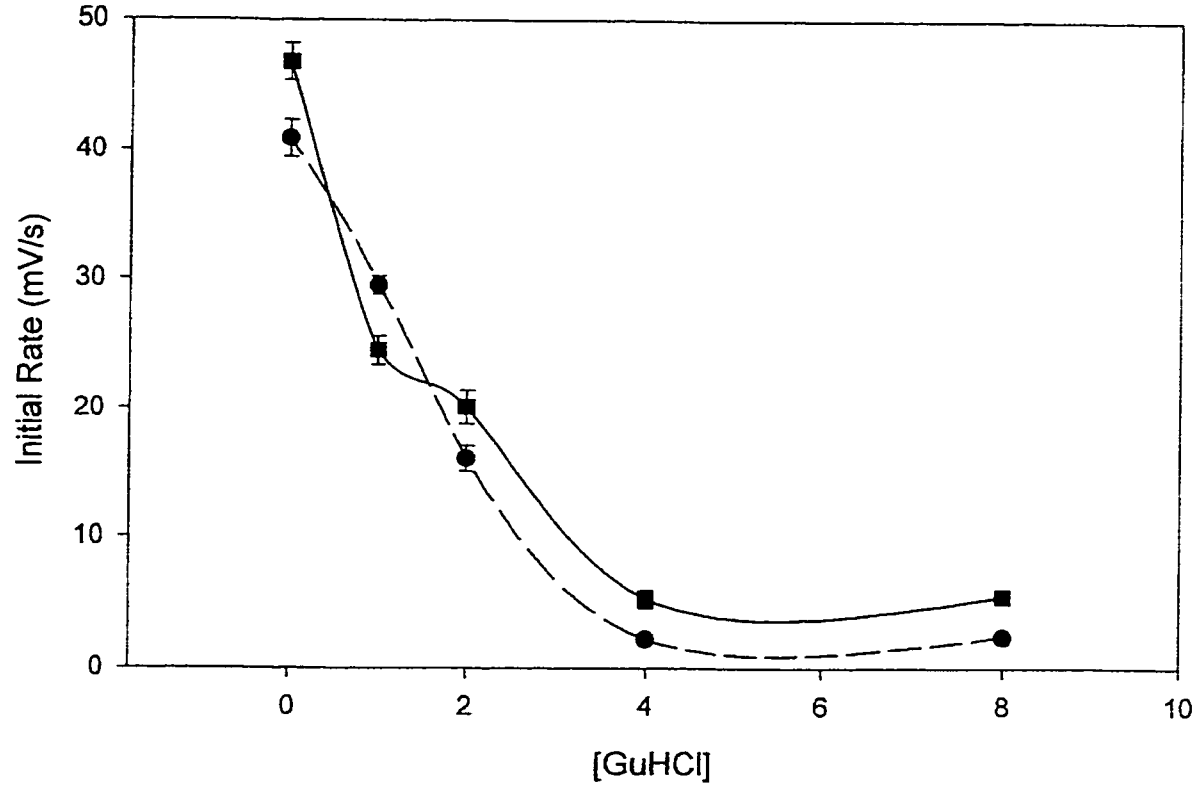
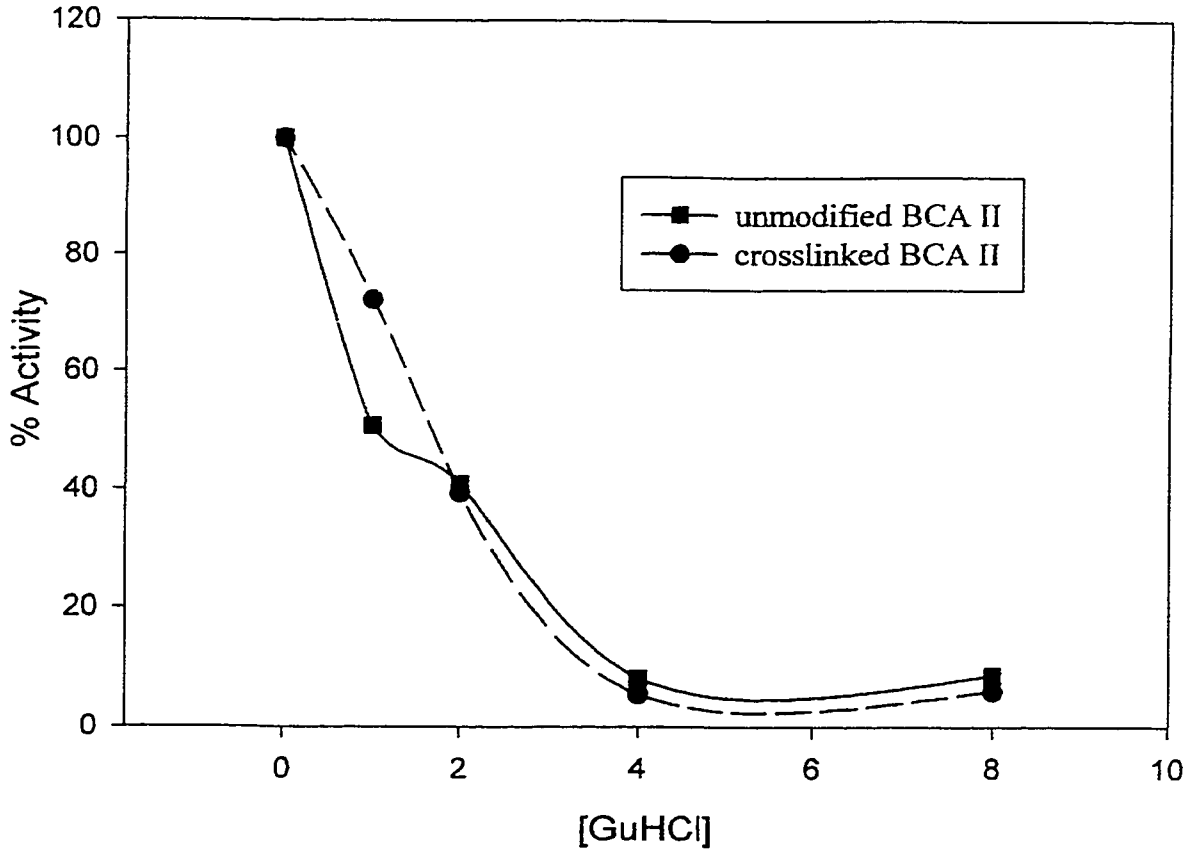

80
60
40
20
0
% Activity Remaining
wild type BCA II
Crosslinked BCA II
-0.25
0.00
0.25
0.50
0.75
1.00
1.25
1.50
% SDS

Figure 20. The effect of guanidine hydrochloride on the rate of CO_2 hydration in a buffer containing 50 mM Hepes, 50 mM sodium sulfate and 80 μM phenol red at pH 7.55. The enzymes were incubated in 0.25 ml guanidine hydrochloride for 5 minutes and then diluted into 5 ml of buffer and the initial rate was determined using stop flow. The final concentration of CO_2 was approximately 6 mM. The final concentration of unmodified BCA II was 7.24×10^{-8} M, and the final concentration of the crosslinked BCA II was 7.28×10^{-8} M. The average of four trials and the standard deviation is shown.


Initial Rate (mV/s)
50
40
30
20
10
0
0
2
4
6
8
10
[GuHCl]


% Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
[GuHCl]
unmodified BCA II
crosslinked BCA II

scheme. Coupling reactions using EDC together with NHS leads to the formation of active aminoacyl esters under facile conditions. These stable esters hydrolyze slowly in aqueous media compared with their rates of reaction with amino groups and ultimately can lead to enhanced coupling efficiencies of carbodiimides for conjugating carboxylated compound with primary amines (Sehgal and Vojay, 1994; Grabarek and Gergely, 1990). Studies performed on the NHS-ester containing compound dithiobis(succinimidyl-propionate) indicate that the half life of hydrolysis for NHS-esters is 4-5 hours at pH 7.0 in an aqueous environment free of extraneous amines. The sulfo-NHS containing compound, sulfosuccinimidyl propionate, has a half-life of hydrolysis reported at 1-2 hours, in an aqueous buffer free of amines at pH 7.4. In contrast, the estimated rate constant for the hydrolysis of O-acylisourea intermediates is of the order of 2-3 s^{-1} (Hoare and Koshland, 1967). At neutral or alkaline pHs, the amino group on a particular ligand will react with the NHS ester to form a stable, amide bond, releasing N-hydroxysuccinimide as a byproduct.

Specific chemical modification of human carbonic anhydrase in order to characterize the active site, the binding of inhibitors and the binding of the zinc atom has been previously carried out. Amidination of all the lysyl residues of the human enzyme with methyl acetimidate hydrochloride caused no loss of activity (Whitney et al., 1967). Thus it is likely that lysine residues play no part in the catalytic event which is supported by current theories concerning the mechanism of the BCA II catalyzed hydration of a carbon dioxide molecule. It should be noted however that the above mentioned study used the catalytic hydrolysis of ρ-nitrophenyl acetate in order to determine activity. Even though carbonic anhydrase has the capacity to hydrolyze certain ester bonds in addition to the reversible hydration of carbon dioxide, it is not clear if both activities are equally affected upon modification of lysine

employed to create conjugates of different sizes. Figure 6 shows the results of one such experiment. The smallest protein-protein conjugate that could feasibly be formed is of molecular weight 60 kDa, thus it is likely that this new band represents some sort of chondroitin-4-sulfate-BCA II complex. The average molecular weight of chondroitin-4-sulfate is approximately 20 kDa (Scott et al., 1995) though it is possible to have fragment of smaller molecular weights (Wasteson, 1970). It is likely that the band seen in figure 6 in lane 3 is composed of a small fragment of chondroitin-4-sulfate (molecular weight ~10 kDa) attached to a molecule of carbonic anhydrase. Allowing the reaction to proceed for longer than 24 hours did not affect the yield of this new product nor did it produce any higher molecular weight products.

Purification of the conjugated bovine carbonic anhydrase as described above was carried out with a Sephadex G-150 column with 50 mM Hepes buffer at pH 7.5. Despite repeated attempts no separation was attained with only the recovery of carbonic anhydrase itself possible. It was determined that the incubation mixture did have the ability to hydrate carbon dioxide as evidenced by the spot test. Unlike the protein-protein conjugation experiment this procedure did not abolish the enzymatic activity of BCA II. However, the conjugated product was not of sufficient size difference to be separated by Sephadex G-50, G-75 and G-150.

Using different concentrations of EDC, NHS, chondroitin-4-sulfate and carbonic anhydrase it was possible to create a high molecular weight conjugate of chondroitin-4-sulfate and BCA II which retained activity and was resolvable with Sephadex G-150 column chromatography. Figure 7 shows the results of such an experiment after a 24 hour incubation period. After separation on a 12% SDS polyacrylamide gel it can be seen that a high

molecular weight conjugate is formed with a molecular weight of slightly less than 200 kDa (Figure 7, lane 2 and 3) as compared to the protein standards (Lanes 1 and 6). The final concentrations of the reactants in this experiment was as follows: 15.6 mM EDC, 8.2 mM Chondroiting-4Sulfate, 26.1 mM NHS and 0.216 mM BCA II, and the total reaction volume was 4 ml. A spot test for carbon dioxide hydration ability was positive after 24 hours of incubation time. These results indicate that conjugation of BCA II with chondroitin-4-sulfate does not abolish activity as was the case with the protein-protein conjugation described earlier. It is likely that conjugating with chondroitin-4-sulfate does not significantly perturb the protein's three dimensional structure as the polymer is most likely limited to attachment to readily available surface accessible lysine residues.

To determine the rate at which the chondroitin-4-sulfate:BCA II conjugate hydrates carbon dioxide stopped flow experiments were performed. The manner in which pH effects the rate of the reaction was determined by varying the pH of the buffer and monitoring the release of protons in solution spectrophotometrically as the change in absorbance of the indicator dye. It is important to note that in this method a limiting factor is the concentration of CO_2 than can be used. Water saturated with carbon dioxide at 25°C at 1 atm pressure contains roughly 33 mmoles of CO_2 per liter (Kernohan, 1965; Gibbons and Edsall, 1963; Harned and Davis, 1943). If such a solution is used in the stopped flow apparatus then the amount of substrate available in the hydration reaction is about 16 mM. This concentration is only sufficient to give a reaction rate equal to about 60% of the maximum rate observed when the enzyme is saturated with substrate (Kernohan, 1965). Water saturated with CO_2 at higher pressures than 1 atm cannot be easily used in the stopped flow apparatus as the gas tends to come out of solution causing bubbles to form in the observation chamber, making

it difficult to get a satisfactory reaction trace. In this study, the amount of CO_2 was measured by directly determining the pH of the CO_2 saturated solutions with a pH meter. In general, the highest amount of CO_2 delivered as a reactant in the stopped flow apparatus ranged from 10 mM to 12 mM.

The kinetic parameter, V_{max} was determined by generating Lineweaver-Burk plots from hydration reactions which were performed by varying the substrate concentrations at a fixed enzyme concentration (Figure 15 and Figure 16). The values for V_{max} and K_M were taken from the best fit line of the data points. The results are summarized in table 1A and table 1B for both the unmodified BCA II and the chondroitin-4-sulfate conjugate.

The effect of pH on the turnover number for both forms of BCA II is quite evident from table 1 and figure 17. For both forms of the enzyme, the rate is the highest at pH 8.11 with a marked decrease as the pH is lowered. Nonetheless, the turnover number is characteristically high as is the case for carbonic anhydrase II, being as high as 10^6 per second at pH 8.11, and even as high as 10^4 s^{-1} at pH 5.5. The Michaelis-Menten parameter, K_M, for the unmodified BCA II enzyme stayed around 4 mM (Table 1A) and increased slightly to 6.72 (± 0.31) mM at pH 5.5. The K_M of the chondroitin-4-sulfate conjugate increased to 11.2 (± 0.40) mM at pH 5.5 from about 3 mM at pH 7-8 (Table 1B). The conjugated BCA II complex shows a modest decline in K_M indicating a higher affinity for substrate as compared to the unmodified carbonic anhydrase. However, at pH 5.5 (where the dehydration of carbon dioxide is more favorable) the affinity for carbon dioxide is markedly decreased for the conjugated carbonic anhydrase when measuring the hydration reaction (Table 1B).

With regards to the turnover number, it can be concluded that the addition of the

significantly affect the protein's ability to either hydrate CO_2 or dehydrate HCO_3^{-1}. This is reflected in the turnover numbers, and in the efficiency as measured by the rate constant k_{cat}/K_M, for both forms of the enzyme. The pH dependence of k_{cat} and k_{cat}/K_M also indicates that crosslinking did not alter the active site environment significantly. In fact, the K_M values for the crosslinked BCA II in the forward reaction indicate that the lowered rate is probably not due to a lower affinity to substrate. This is also true for the dehydration of bicarbonate ion at pH 5.5, though the rate is almost identical for both forms of the enzyme in this case.

Gel filtration results indicate that one species of conjugate formed is approximately between 150-200 kDa in size. The average preparation of chondroitin sulfate contains both chondroitin-4-sulfate and chondroitin-6-sulfate, with the typical molecular weight of about 20 kDa. This would mean that the typical size of a chondroitin-4-sulfate is composed of about 40 repeating disaccharide units representing 40 carboxyl groups which could undergo a nucleophilic attack by a lysine residue from BCA II. If we assume a molecular weight of roughly 170-180 kDa for the complex, and that no inter protein-protein bonds have been formed, the resulting complex could be composed of one protein molecule covalently linked to 5-7 chondroitin-4-sulfate molecules attached by a carboxyl anywhere on the length of the chain. Alternatively, it is also possible that 5 enzyme molecules are bound to a single molecule of chondroitin-4-sulfate. It is likely that it is one of these cases since protein-protein coupling is largely ruled out due to the fact that the coupling reaction is initiated by activating carboxyl groups at pH 4-5 followed by an increase to pH 8-9 prior to adding the protein to the mix. Thus, in order for a covalent bond to be formed between 2 protein molecules, the respective carboxyl groups would have to be activated at pH 8-9, which is highly unlikely, as EDC is mostly quenched at that pH.

Modifying bovine carbonic anhydrase as described above results in the liberation of protein molecules as the samples age. Samples of conjugated BCA II were stored at 4°C and were analyzed on 12% SDS polyacrylamide gels. After approximately 2 months of storage it could be seen that the conjugate was starting to degrade as evidenced by the appearance of a new band (carbonic anhydrase) at a molecular weight of 31 kDa. This illustrates the fact that chondroitin-4-sulfate can be used as a kind of sustained release device. Previous studies have indicated its usefulness in such a manner though it is apparent that the factors controlling release of such coupled ligands is varied. In the present study, the ability of the complex to break down into its component parts was slow, working over the course of several weeks. It is possible that the time course of the release of the protein would be greater *in vivo* where the presence of a variety of enzymes might facilitate such an event. This might be accomplished either by digestion of the chondroitin-4-sulfate polymer or by cleavage of the amide bond holding the polymer to the enzyme.

It is has been shown that chondroitin-4-sulfate cannot form any kind of high order structure in solution unlike chondroitin-6-sulfate which can form duplexes (Scott et al., 1992). The potential to form a secondary structure such as a regular multihydrogen-bond array throughout the molecule is present. Such interactions would involve the acetamido, carboxylate, hydroxyl and ring oxygen (Scott et al., 1992). Based on NMR studies performed in water and in dimethyl sulfoxide, it has been proposed that chondroitin-4-sulfate adopts a stiff chain like conformation stabilized by 3 hydrogen bonds per tetrasaccharide unit (Figure 3).

Having established that crosslinking of chondroitin-4-sulfate to bovine carbonic anhydrase II has little effect on the hydration of CO_2, the effects of acetazolamide, a specific

carbonic anhydrase II inhibitor, was investigated. Acetazolamide belongs to the sulfonamide class of strong carbonic anhydrase inhibitor. Human carbonic anhydrase II has been studied in complex with acetazolamide at 1.9 angstrom resolution (Vidgren et al, 1990). It is thought that the sulfonamide group is bound close to the zinc ion, presumably with its negatively charged amino group liganded to the zinc (Chen and Kernohan, 1967, Kanamori and Roberts, 1983). In the native structure, the amino group of acetazolamide has the same position as the zinc bound hydroxyl ion. Thus, the binding of acetazolamide retains the tetrahedral zinc coordination. A number of additional perturbations are also evident. The NH group from a hydrogen bond to Oγ1 of Thr-199, and one of the sulfonamide oxygen's displaces the deep water molecule and makes a hydrogen bond to the peptide amide of Thr-199. The second sulfonamide oxygen makes no direct contact with the enzyme and is about 3 angstroms away from the zinc. Any additional interactions with the enzyme are dependent upon the nature and location of the substituents of the sulfonamide. The ring structure of acetazolamide is held against the hydrophobic portion of the active site cavity, in close proximity to Gln-92, Val-121, Leu-198, and Thr-200. A hydrogen bond is also formed between the oxygen of the acetamide group and the side chain nitrogen of Gln-92.

In this study, the inhibitor acetazolamide, was premixed with the bovine carbonic anhydrase. It has been pointed out that these conditions lead to noncompetitive inhibition for the BCA catalyzed hydration of carbon dioxide, regardless of whether it is or not (Kernohan, 1966; Lindskog and Thorslund, 1968). This is the result of the rate of dissociation of the EI complex being much slower than the breakdown of the ES complex. As a result, the binding of CO_2 appears to be unaffected by the presence of acetazolamide.

The inhibition of bovine carbonic anhydrase and its chondroitin-4-sulfate conjugate

appears to be noncompetitive (Figure 10 and Figure 11). The inhibition constant, K_i, for the hydration of CO_2 at pH 7.51 for the unmodified enzyme is 4.5×10^{-8} M compared to 3.5×10^{-8} M for the crosslinked protein. It can be seen from the Lineweaver-Burk plots (Figures 10 and 11) that the acetazolamide does seem to affect both K_M and the apparent V_{max}, though the effect on K_M is marginal. Modification of BCA II with chondroitin-4-sulfate appears to have hardly any significant effect upon the binding of acetazolamide to the enzyme. If K_I is seen as a measure of the ability of the inhibitor to bind and inactivate its target, then the crosslinked enzyme has a slightly higher affinity for the inhibitor. This seems consistent with the fact that the conjugated BCA II binds carbon dioxide, which binds in nearly the same location in the enzyme active site, with a higher affinity (Table 1) than the unmodified protein. The presence of the chondroitin-4-sulfate molecule somehow facilitates either the binding or diffusion of the substrate and inhibitor into the active site.

To assess any changes in protein stability brought about by the crosslinking of chondroitin-4-sulfate with BCA II, the ability of the conjugate to hydrate CO_2 at pH 7.5 in the presence of chemical denaturants such as SDS and guanidinium hydrochloride was determined. The effect of heating the proteins at a temperature of 65° on the hydration rate was also determined. The chemical basis of protein stability is currently thought to be the result of the favorable interactions of solvent and protein side chains resulting in the formation of stable secondary and tertiary structures. A major factor of protein stability is hydrophobicity. Most globular proteins such carbonic anhydrase tend to sequester nonpolar amino acids in the interior regions, forming a hydrophobic core of a sorts. In this manner a tight packing is accomplished. For proteins that associate with other components, these hydrophobic clusters are usually found localized on the surface of such molecules. These

hydrophobic patches serve to induce energetically favorable hydrophobic interactions with other proteins, membranes or any other hydrophobic molecule. In essence, the shielding of nonpolar residues from the aqueous medium reduces the unfavorable entropy of the system and thus increases protein stability.

The maintenance of secondary structures such as α-helices and β-sheets is brought about largely by the numerous hydrogen bonds found in proteins. Hydrogen bonds also provide an interface for the interaction of polar side chains with water. The result of such interactions is the hydration sphere of the molecule. The dipole-dipole interaction does not contribute but a small amount of energy, however, the large number of such interactions makes it significant. Electrostatic interactions such as salt bridges between nearby oppositely charged side groups contribute a significant amount to the overall stability of the protein. The loss of such a salt bridge may help explain the denaturation of proteins and pH extremes. If the pH is enough to break these salt bridges the repulsion between numerous similarly charged groups would help to further destabilize the protein (Mathews and van Holde, 1994). Van der Waals interactions also contribute significantly to the stability of a protein. The interior of a globular protein is usually tightly packed facilitating the contact between side chain atoms.

Proteins are often found in a glycosylated state and recent studies have shown that the carbohydrate moiety has a stabilizing influence on protein stability (Wang et al., 1996). Studies of deglycosylated proteins suggested that the carbohydrate groups may prevent the unfolded or partially folded proteins from aggregation. In addition, studies of the reversibility of heat denaturation showed that deglycosylated proteins have a poorer thermal reversibility. It has been suggested that one function of protein glycolysation is the

stabilization of the mature glycoprotein (Li and Sharon, 1993). One possible way for the attached carbohydrate moiety to stabilize the protein structure is to form hydrogen bonds with the polypeptide backbone. Also, the carbohydrate attachment may stabilize the structure by steric interactions with adjacent peptide residues (Gerken et al., 1989; Rudd et al., 1994). An important observation to keep in mind is that heavily glycosylated proteins, either N-glycosylated or O-glycosylated, are thermally destabilized by carbohydrate removal (Wang et al., 1996).

Bovine carbonic anhydrase II at pH 7 is stable for at least 30 minutes at 50° C (Engberg et al., 1985). At 60° C the enzyme retains 40% of its enzymatic activity for 15 minutes (Kisiel and Graf, 1972). At a temperature of 65° Celsius, bovine carbonic anhydrase II steadily loses its ability to hydrate CO_2 (Table 3). After almost 30 minutes of heating the enzyme has lost almost all of its activity with only 7% remaining. In contrast, the chondroitin-4-sulfate conjugated enzyme had over 40% of its activity after 27 minutes of heating. After the first two minutes of heating the unmodified enzyme had only lost about 8% of its activity compared with a 40% loss for the conjugated enzyme. After two minutes of heating however, the crosslinked protein only lost 10-15% more of its activity while the unmodified protein steadily continued to lose its activity. Figure 18 clearly shows that the crosslinking of chondroitin-4-sulfate to BCA II resulted in a definite increase in the thermal stability for the hybrid enzyme. Though chondroitin-4-sulfate is not a typical carbohydrate polymer, it is similar enough that this result lends support to the notion that carbohydrate moieties act to stabilize protein structure especially towards the effect of heat. It is unclear how exactly the presence of an attachment such as chondroitin-4-sulfate may increase the thermal stability of bovine carbonic anhydrase. It is possible that the chemical crosslinks

joining the enzyme and chondroitin-4-sulfate serve to anchor the protein in its active state. When faced with conditions that unravel the protein, such as high temperature, the chondroitin-4-sulfate may reticulate the molecule to reinforce the structure (Wong and Wong, 1992).

Naturally occurring enzymes which operate at extremely high temperatures have been the focus of numerous studies. The driving force behind the polymerase chain reaction is a heat stable DNA polymerase originally isolated from the bacterium *Thermus aquaticus* growing at temperatures of 70°C and higher. Studies on enzymes that operate under unusual and harsh conditions have revealed clues as to how these biological catalysts maintain their structural integrity in the face of high temperatures, extreme pH and salt concentrations. Studies of a psychrophilic protease have suggested that the protein has a higher number of negatively charged residues, lacks any specific intramolecular aromatic and ionic interactions and has more extensions in several polar surface loop regions (Davail et al., 1994). These changes would result in increased solvent interactions, reduced compactness and increased flexibility, factors that would destabilize the protein. It is believed that catalysis at low temperature is facilitated by increased flexibility. For the enzyme to be functional at high temperatures, decreasing the interaction with the solvent would be desirable and it had been proposed as early as the mid-1970s that an increase in the number of ionic pairs on the surface of a protein can increase thermal stability (Perutz and Raidt, 1975). Studies of naturally occurring enzymes such as these indicate that increases in stability arise from numerous and subtle interactions, including more ionic bridges, smaller surface loops, and perhaps a decrease in the surface to volume ratio.

It is conceivable that the attached chondroitin-4-sulfate polymer, which bears a

still retained up to 25% of its original activity (Table 4). At higher concentrations of SDS (Table 4 and Figure 19), the conjugated enzyme gradually lost its activity but still outperformed the unmodified protein.

Exposure to guanidinum hydrochloride did not reveal any clear cut advantage for the conjugated enzyme. The hydration of CO_2 at room temperature, pH 7.5, showed that upon incubation in 1 M GuHCl for 5 minutes the conjugated enzyme retained 72.1% of its activity compared to 52% for the unmodified enzyme (Table 5). This protective effect is absent at higher concentration of GuHCl (Figure 20) where the unmodified enzyme appears to retain slightly higher amounts of hydration activity than its conjugated counterpart.

The ability of SDS to denature a protein is attributed to the disruption of hydrophobic interactions that lead to protein unfolding. The nonpolar portion of SDS unravels the protein interacting with hydrophobic regions, disrupting hydrogen bonds, and ultimately the protein chain is coated with SDS molecules. Guanidinium hydrochloride increase the solubilities of both polar and nonpolar molecules in rough proportion to their accessible surface areas and decreases the magnitude of the hydrophobic interaction by up to one-third. It is likely that GuHCl interacts more favorably with polar and nonpolar surfaces than water since its effect on water is to increase surface tension, which should stabilize a protein.

The sera of many animals, including dogs (Rispens et al., 1985), cats (Booth, 1938), sheep (Leiner et al., 1962) and rabbits (Hill, 1986), have been reported to contain inhibitory factors for carbonic anhydrase though no such factor has been observed in human plasma (Hill, 1986; Booth, 1938). Using a combination of anion-exchange chromatography and CA II affinity chromatography a porcine plasma inhibitor with a very high affinity for CA II has been isolated and purified (Roush and Fierke, 1992). Most recently, this plasma protein has

been cloned, sequenced and expressed (Wuebbens et al., 1997) and shown to be a novel member of the transferrin family. The CO_2 activity of the chondroitin-4-sulfate:BCA II conjugate in human plasma was determined by incubating the enzyme in 1 ml human plasma and monitoring the change in pH upon mixing with 1 ml of carbon dioxide saturated saline solution. Figure 14 shows unmodified BCA II has activity though the rate of hydration is diminished the longer it is exposed to human plasma. The crosslinked enzyme also has activity in plasma but the rate is not diminished as rapidly as the native enzyme (Figure 14). In addition, it can be seen in figure 12 that the extent of the pH change is also decreases, whereas the crosslinked protein is essentially the same except after 3 hours of exposure to plasma (Figure 13). The relative reaction rates can easily be measured by determining the initial slopes of the curves in figures 12 and 13. It is clear that the rate of hydration for both the unmodified and crosslinked protein declines with longer times of exposure to human plasma (Figure 14 and Table 6). However, the rate of hydration of carbon dioxide for the crosslinked protein is clearly affected less by the plasma (Figure 14). The reason for this is not precisely clear. It may be that the crosslinked enzyme is more resistant to proteolytic digestion by enzymes in the plasma. Another possibility is that the crosslinked enzyme has a lower affinity for inhibitors present in the plasma, though as of yet, no inhibitor for CA II have been described in human plasma. These results are encouraging for use of the crosslinked enzyme *in vivo*.

Conclusions

In this study chondroitin-4-sulfate was covalently coupled to bovine carbonic anhydrase II using the zero length crosslinker EDC. The enzyme was also coupled to itself using EDC, resulting in the formation of protein-protein complexes covalently linked. The CO_2 hydration activity was measured with stopped flow and quantified for both the unmodified enzyme and the conjugates. In order to assess what structural benefit might accrue from the addition of the chondroitin-4-sulfate molecule, the CO_2 hydration activity was measured at 65^{0}C and in the presence of SDS and guanidinium hydrochloride.

The conclusions from the above described experiments can be summarized as follows:

1. Homologous crosslinking of BCA II with EDC does not result in any conjugates with the ability to hydrate CO_2.
2. Under specific reaction conditions crosslinking of BCA II with chondroitin-4-sulfate results in the formation of a complex that is approximately 170 kDa in size as evidenced by SDS-PAGE and gel filtration column chromatography.
3. The 170 kDa chondroitin-4-sulfate:BCA II conjugate retains the ability to hydrate CO_2.
4. Stopped flow analysis of the conjugate reveals that the k_{cat} is very pH dependent for the hydration of CO_2 . The k_{cat} values are the highest at pH 8.11 and steadily decrease as pH decreases. The k_{cat} and K_M of the conjugate are slightly lower than those of the unmodified enzyme, however, the k_{cat}/K_M values approach the diffusion controlled rate constant.
5. The chondroitin-4-sulfate:BCA II conjugate also has the ability to dehydrate HCO_3^-

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Abstract

ZERO LENGTH CROSSLINKING OF BOVINE CARBONIC ANHYDRASE II AND CHONDROITIN-4-SULFATE

by

RAJIVINDER S. BRAR

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Advisor: Hiroshi Mizukami, Ph.D.
Major: Biological Sciences (Biophysics)
Degree: Doctor of Philosophy

Bovine carbonic anhydrase II (EC 4.2.1.1) was covalently linked to chondroitin-4-sulfate (β-glucoronic acid-[1-3]-N-acetyl-β-galactosamine-4-sulfate-[1-4]) from bovine trachea. A zero length crosslinking procedure which reacts the protein amino groups with preactivated carboxyl groups of chondroitin-4-sulfate was carried out with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide as coupling agents. The complex remained enzymatically active, was water soluble, and appeared to be about 170 kDa as analyzed on SDS-PAGE and column chromatography on Sephadex G 150. It is likely that 5 protein molecules (30 kDa each) are linked to 1 chondroitin-4-sulfate molecule (20 kDa). The CO_2 hydration activity of the complex was generally slightly less than the unmodified protein with both forms having highest activity at a pH 8 and the lowest activity at pH 5.5. The same was true for the kinetic parameters k_{cat} and k_{cat} / K_M. The pH

dependence of k_{cat} was not affected upon conjugation with chondroitin-4-sulfate. The K_M appeared to be slightly less for the modified form of the enzyme for the hydration of CO_2 at all pH values used. For the dehydration of bicarbonate at pH 5.5 (room temperature), the K_M was also slightly less for the modified enzyme (K_M = 21.7 mM) as compared to the unmodified form (K_M = 24.2 mM). The complex also appeared to be capable of hydrating CO_2 in human plasma and seemed to be more resisitant of inhibitory effects of plasma as compared to the unmodified enzyme. The effects of a noncompetitive inhibitor, acetazolamide, were similar between the crosslinked complex ($K_I = 3.87 \times 10^{-8}$ M) and the unmodified enzyme ($K_I = 4.62 \times 10^{-8}$ M). However, the modified enzyme was more resistant to denaturation by guanidine hydrochloride, SDS and heat. Covalent coupling of chondroitin-4-sulfate to enzymes may present a method with which the stabilization of biological structures can be achieved with a minimal (if any) loss of enzymatic activity. The BCA II : chondroitin-4-sulfate complex produced in this work may be of use as an artificial blood substitute designed to transport CO_2 molecules *in vivo*.

Autobiographical Statement

Rajivinder Singh Brar

Date and Place of Birth

Ludhiana, India, July 24, 1967.

Education

Oakland University, B.Sc., Biochemistry, 1992.
Wayne State University, Ph.D. Biological Sciences (Biophysics), 1998.
Midwestern University Arizona College of Osteopathic Medicine, D.O., 2002.

Society Memberships

The American Association for the Advancement of Science.
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