STUDIES OF EQUINE MUSCLE CARBONIC ANHYDRASE (CA- Ⅲ)

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The carbonic anhydrase catalyze the hydration of CO_2 and the dehydration of HCO_3 ($CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$) , a reaction which can occur at extremely high rates. Mammalian carbonic anhydrase is a zinc metalloenzyme which is widely distributed through the body. Two principal isozymes of carbonic anhydrase occur in mammalian erythrocytes ; a high-activity type designated as CA-Ⅱ (C) and a low-activity type designated as CA- Ⅰ(B). A basic protein noted by Blackburn et al. to be present in rabbit muscle extracts in relatively large amounts was later shown by the same laboratory to possess carbonic anhydrase activity. What appear to be similar enzymes have recently been discovered in the skeletal muscle of human, bovine, rat, sheep, chicken, mice and gorilla. This enzyme is the product of a distinct genetic locus and has been designated as carbonic anhydrase Ⅲ (CA-Ⅲ). It constitutes from 1 to 2 % of the soluble protein of muscle and has also been found in liver and lung extracts and in human erythrocytes. The sequence of the equine CA-Ⅰ

and CA-II has been determined by Deutsch et al. and it appeared of interest of initiate studies on the muscle type isozyme. In the present studies, equine CA-III has been isolated in crystalline form and describe its some properties.
The summarized as follows :

1 . Purification of CA-III from equine skeletal muscle

The muscle tissue was excised from a fatally nembutalized pony were excised and placed in crushed ice, passed through a mechanical grinder with fine sieve. The minced muscle (1 Kg) was homogenized with two volumes (2ℓ) of 0.01 M Tris-HCl (pH 8.0) at 4℃ and centrifuged for 30 min at 8,000 xg. Then, iodoacetamide was added to give final concentration of 0.01 M, the pH of the solution was adjusted to 8.0 , and then , mixture was incubated for 30 min under non-denaturing condition. The sample was dialyzed against 0.01 M Tris- HCl (pH 8.0) at 4 ℃. The sample was applied to a column (3.4 X 34 cm) of CM-Sepharose CL-6B equilibrated with 0.01 M Tris-HCl (pH 8.0). After washing the column extensively, the adsorbed proteins were eluted with a linear gradient of NaCl between 0 and 0.15 M in the same buffer. The various fractions eluted were tested for carbonic anhydrase activity. The enzymatically active fractions were pooled and concentrated by precipitation with saturated ammonium sulfate. The precipitate was dissolved in a small amount of pH 8.0, 0.01 M Tris-HCl containing 0.15 M NaCl and passed over a column of Sephadex G-75 (Pharmacia) equilibrated with this buffer. The enzymatically active fraction was dialyzed exhaustively against water , the small amount of precipitate formed was removed

by centrifugation at 4℃ and the supernatant solution subjected to column electrofocusing in a pH 7- 10.5 Ampholyte buffer (Pharmacia). The isoelectric point of CA-Ⅲ was 8.9 . About 300mg of enzyme are obtained per 1 Kg of muscle. A polymorphic form of the equine CA-Ⅲ was designated as CA-Ⅲa and the isoelectric point of it was 8.1. Approximately 15 mg of CA-Ⅲa was obtained from 1 Kg of muscle . A 1 % solution of equine CA-Ⅲ separated by electrofocusing was dialyzed at room temperature against repeated changes of 62 % saturated ammonium sulfate in pH 8.0, 0.05 M Tris buffer. After standing for several days at 4℃, crystals appeared. All three of the equine carbonic anhydrase isozymes have now been prepared in crystalline form . X-ray diffraction studies of them would be of interest to relate secondary-tertiary structural difference to their sequences and variant enzymatic activities and immunological properties. On the other hand equine CA-Ⅲ was poorly retained by p-aminobenzenesulfonamide affinity columns. Koester et al. had previously reported the muscle enzyme to have a relative weak affinity for sulfonamides. Since considerable losses of activity were experienced in the early stages of fractionation author resorted to a somewhat different approach than that used by other investigators. It was based in parton the presence of what appear to be readily reactive cysteines in this enzyme.

2 . Physicochemical properties of equine CA-Ⅲ

The molecular weight of CA-Ⅲ was estimated to be 26,500 by SDS-PAGE , 27,000 by gel filtration, respectively. The molecular weight of CA-Ⅲa was similar to those of CA-Ⅲ. CA-Ⅲa was not a

fragment of CA-Ⅲ.

Equine CA-Ⅲ was dialyzed exhaustively against deionized, distilled water (Milli-Q , Millipore Corp.) and then centrifuged at 8,000 g for 10 min at 4 ℃ to remove a small amount of turbidity . Weighted aliquots of this solution were subject to measurements of dry weight at 105℃, O.D. at 280 nM. Equine CA-Ⅲ was found to have an $E^{1\%}_{280\ nm}$ value of 15.5 . From 40 to 60 % of the expected level of zinc was found in the crystallized enzyme after dialysis into pH 8.4, 0.033 M veronal buffer or distilled water . The relative low enzymatic activity of such a preparation could not be augmented when zinc was added to a level of 1 gm atom per mole of enzyme . The enzymatic activity of these preparations based on their zinc levels appeared to be in the range of that found for the muscle carbonic anhydrases of other species . It is not known whether the enzyme that has had 2 of its 4 cysteine alkylated tends to lose zinc more readily than the native form . Koester et al. have indicated that rabbit CA-Ⅲ contains 1 gm atom of zinc and the metal is only slowly removed in pH 5.6, 100 mM sodium succinate buffer containing 5 mM o-phenanthroline and 1 mM dithiothreitol. Further studies of this problem which make use of the alkylated form of muscle carbonic anhydrases of other species will be required to define any effects of alkylation on the zinc binding activity of this enzyme.

3 . Chemical properties of equine CA-Ⅲ

The amino acid analyses were conducted on hydrolysates

prepared in 5.7 N HCl at 110℃ for 20,40 and 100 hours, a Durrum D-500 apparatus being employed. The levels of labile components were extrapolated to zero time hydrolysis and maximum values for other amino acids were thaken. In cases where no significant level deviations were noted as a function of time , the values were averaged. A total of 257 residues was found when a molecular weightof 27,000 was assumed . The amino acid composition of CA-IIIa was similar to that of CA-III . When CA-III and CA-IIIa were alkylated with iodoacetamide under non- denaturing condition, 2 mol of S-carboxymethylcysteine (CMC) per mol of the enzyme were found , indicating that two cysteine residues may be located on the surface of the molecules and may readily reacts with the iodoacetamide . When CA-III and CA-IIIa were treated with 5 , 5`-dithio-bis (2-nitrobenzoic acid) (DTNB) in the absence of denaturing agent, no sulfhydryl groupwas detected, possibly because the sulghydryl groups of CA-III and CA-IIIa had already been alkylated with iodoacetamide before purification of the enzyme. However , titrations of the enzymes with DTNB in 6 M urea showed that CA-III and CA-IIIa contained 1.7 and 0.9 mol of sulfhydryl group per mol of the enzyme,respectively. It is suggested that one of those cysteines reacted with DTNB whereas the other did not because it bound with some acidic components. The acidic peptide formed upon performic acid oxidation of the protein was isolated as a drop-through product on a Dowex 50-X2 column (0.5X 10 cm) at pH 4.0 following removal of performic acid from the oxidation mixture. A part of this product was hydrolyzed for 20 hr in 6 N HCl at 110 ℃ and applied to the Durrum D-500 analyzer . The remaining part was directly applied to the Durrum D-500 analyzer without hydrolysis.

Some acidic components were released from the purified CA-IIIa which eluted slightly before cysteic acid when applied to amino acid analyzer without hydrolysis . Amino acid composition of these acidic components was cysteic acid (0.63 nmol) , glycine (0.85 nmol), and glutamic acid (1.56 nmol) suggesting that the components are glutathione. The activity of the equine CA-III was in the range of 350 to 400 Wilbur and Anderson units per mg of zinc containing protein. This is about 14 % , 1.4 % of the specific activity of CA-I , and CA-II , respectively, under identical assay conditions. CA-IIIa is 38 % of the activity of equine CA-III . Equine CA-III has a relatively low esterase activity ascompared to the CA-I and CA-II forms. Equine CA-III has been also found to possess weak alkaline and acid phospatase activities. These studies indicate that equine CA-III is apparently a multi-site,multi-functional enyme. Aickin and Thomas reported that the red muscle fibers has 3 times higher CO_2 buffering capacity than the white muscle fibers and the membrane potential of the former is more sensitive to varying CO_2 levels than that of the latter, suggesting a greater need for CO_2 regulation in the red muscle. Thus, carbonic anhydrase may play a significant role in the regulation of CO_2 concentration and of pH in the red muscle. The physiologic meaning of the polymorphic forms of CA-IIIa remains obscure. The observation on these multiple forms probably provide support for the concept of nongenetic, interchangeable conformation-

al modification of the CA-Ⅲ molecule which has been termed as conformational isozymes.

4 . Immunochemical properties of equine CA- Ⅲ

Antibodies to equine CA-Ⅲ were produced in all the seven rabbits employed . Each rabbit was injected initially with 1 mg of purified CA-Ⅲ emulsified with an equal volume of Freund's complete adjuvant , followed by booster injection of the same amount of the enzyme every week for five times. Rabbits were bled via the auricular vein 10 days after the last injection. The specificity of the antiserum was evaluated by double immunodiffusion. Anti-equine CA-Ⅲ serum reacted with equine CA-Ⅲ, but not with equine CA-Ⅰ and CA-Ⅱ. The antiserum to CA- Ⅲ formed a single preciptin line with crude muscle extract, with purified CA-Ⅲ, with liver extract and also with CA-Ⅲa , and all of the precipitin lines fused completely. On the other hand, anti-serum formed a single weak precipitin line with equine thymus. The cross-reactivity of CA-Ⅲ of several animals using anti equine CA-Ⅲ rabbit serum was investigated. Carbonic anhydrase is a enzyme whose species specificity is rather low. Equine CA-Ⅲ formed a spur over bovine, cat, dog, rat, and pig. The extract of muscle and liver from rabbitand chicken did not cross react with rabbit anti equine CA-Ⅲ sera.

5 . Distribution of immunoreactive CA-Ⅲ in various equine tissues and serum.

A higly sensitive sandwich enzyme immunoassay (EIA) for equine muscle CA-Ⅲ has been developed using microplate as a solid-

phase and peroxidase as a labelled enzyme . CA-Ⅲ levels of tissue extracts were measured by EIA and calculated the values per gram wet tissue. The skeletal muscle, liver and thymus contained about 530 μg, 300 μg and 16.5 μg per gram wet tissue,respectively. But in other tissues examined, CA-Ⅲ levels were only several ng per gram wet tissue. To find the normal serum CA-Ⅲ level, sera from equine were assayed. The mean value was 0 ～27 ng /ml.

6. CA-Ⅲ immunohistochemical localization in equine skeletal muscle

Conjugation of peroxidase to the specific antibody was done by the periodate method . The tissue was embedded in paraffin and sectioned at 5 μm. For the immunohistochemical localization of CA-Ⅲ direct immuno peroxidase method was used. The sections were pre-treated with 0.3% H_2O_2 - methanol to block endogeneous peroxidase activity and with normal rabbit sera to block Fc receptors. The sections were then examined by the direct immunoperoxidase method using peroxidase conjugated specific anti CA-Ⅲ antibody. As a control, peroxidase labelled normal rabbit Ig-G was used insted . By the direct immuno-histochemical method , the characteristic dark brown peroxidase reaction product was localized mainly inside the muscle fibers but not all of them, consequently producing a mosaic appearance. Moynihan (1977) found the presence of extravascular carbonic anhydrase activity biochemically in rat skeletal muscles. He noted that the red soleus muscle contained a significantly higher concentration of the enzyme compared with the white muscles. To confirm

these data, Ridderstrale (1979) examined the rat skeletal muscles histochemically using a modified Hansson's cobalt phosphate method but could not find any significant difference. Lonnerholm (1980) examined sections of resin-embedded rabbit skeletal muscle with the same method and found that the staining of the cytoplasm of the muscle fiber varied in its intensity, but did not clarify whether carbonic anhydrase-rich fibers were of the so-called red or white type. However,he noticed that the sarcoplasmic staining was at least 1,000 times less sensitive to acetazolamide inhibition than the staining of structures, such as walls of capillaries situated within the muscle bundles. According to the recent studies on CA- Ⅲ, this isozyme shows relatively poor CO_2 hydration activity and is less sensitive to acetazolamide inhibition compared with red cell isozymes. Using the indirect immunoperoxidase method, the localization of CA-Ⅲ in frozen sections of human skeletal muscle was investigated by Shima et al. Human CA-Ⅲ was found to be localized in Type- I muscle fibers when compared with serial sections stained with myosin ATP ase and other reactions. This fact along with other investigators result suggested that cytoplasmic staining could reflect CA-Ⅲ activity. In this study, auther suggested that CA-Ⅲ was indeed localized in Type-I fibers (red muscle type) in accordance with the above mentioned data, but detailed localization of CA-Ⅲ inside the muscle fiber was difficult to ascertain by this method.

7 . Peptide map of CA-Ⅲ

CA-Ⅲ was reduced with 0.05 M dithiothreitol in pH 8.5 , 0.05 M Tris-HCl containing 6 M urea and then alkylated with 0.125 M

iodoacetamid . For citraconylation , 10 mg of reduced and alkylated CA-III were dissolved in 1 ml of 2 % sodium bicarbonate (pH 8.2), which 0.01 ml citraconic anhydride were added three times at intervals of 20 min with stirring. During this process, the pH of the solution was adjusted to 8.2 with 2 N NaOH. The mixture was then allowed to react at room temperature for 3 hours and dialyzed against 0.01 M ammonium bicarbonate (pH 8.0) to remove excess reagents. Citraconylated CA-III was removed from dialysis tube and lyophilized. 2 mg of enzyme were dissolved in 1 ml of pH 8.0, 0.01 M ammonium bicarbonate and heated at 100℃ for 1 min. Digestions of heated denaturing enzymes by trypsin were carried out in pH 8.0, 0.01 M ammonium bicarbonate. Proteolysis was initiated by the addition of an amount of TPCK-treated trypsin equal to 1% of the protein weight. These digeation were allowed to proceed for 6 hours at 37 ℃ with shaking. The digests were then centrifuged to remove any in- soluble, " core peptides ", and the clear supernatant of digestswere lyophilized. Peptide mapping of tryptic digests was carried out on Whatman 3 M chromatograph paper (46 X 57 cm). The sample was completely dried on the chromatograph paper before proceeding. The paper was eqilibrated with the chromatographic solvent by hanging it in the chromatocase. Descending chromatography with a n-butanol-acetic acid - water mixture (40 : 10 : 50) was conducted for 16 hours at room temperature. After chromatography the paper was removed and dried at 60 ℃ for 1 hour in the chromatogram drying oven. The electrophoretic dimension of the peptide map is developed vertically to the chromatographic dimmention at pH 6.5, pyridine-acetatebuffer (10 % pyridine, 0.5 % acetic acid). Electrophoresis

was performed at 2,500 V, for 1 hour using the high voltage electrophorator. After electrophores is the paper was dried completely at room temperature. And then, the chromatogram was dipped in a 3 % pyridine solution in acetone in order to adjust the pH before fluorescamine staining. Peptide were visualized by spraying with a 0.0005 % solution of fluorescamine in aceton. After spraying the fluorescamine, the peptides were distinctly visible under ultra violet illumination(365 nm). The peptide maps prepared by trypsin digestion of equine CA-Ⅲ disclosed the presence of 26 peptides. The spots of peptides were removed by cut off the chromatograph paper and peptides were eluted from paper with 25% acetic acid. The eluted peptides were lyophilized and hydrolyzed in 6 N HCl for 20 hour at 110 ℃ in evacuated and sealed tubes. By amino acid analysis of the eluate from each peptide spot, there were 12 peptides that contained arginine, 13 peptides that contained lysine and 1 peptide that contained both arginine and lysine. The map prepared by trypsin digestion of citraconylated CA-Ⅲ disclosed the presence of 13 peptides. Amino acid analysis of each peptide identified 8 peptides with arginine, 1 peptide with lysine and 4 peptides with both lysine and arginine. Peptide No.21 contained 2 cysteines when CA-Ⅲ was alkylated under nondenaturing condition. This seems to indicate that they were located on the protein surface. Two more cysteines were detected in peptide No.25 when CA-Ⅲ was alkylated with iodoacetamide in 6 M urea. The peptide map of CA-Ⅲa corresponded to that of CA-Ⅲ with the exception of disappearance of peptide No.25. If CA-Ⅲa is associated with the loss of one amide group, a peptide containing either asparagine or glutamine should migrate further to the

anode side on the peptide map. Binding of glutathione to CA-IIIa , has been suggested. It is,therefore, presumed that in CA-IIIa, binding of glutathione to either of the 2 cysteines moieties of peptide No.25 made the peptide insoluble, because that it contains 50 % hydrophobic amino acid, and thus it was precluded from the detection.

8 . Sequence of equine CA-III

Equine CA-III was reduced with a 10-fold molar excess of 2-mercaptoethanol or dithiothreitol in pH 8.5, 0.5 M Tris-HCl buffer containing 6 M guanidine-HCl or urea and 0.01 M EDTA at 50 ℃ for 4 hrs and then alkylated with a one- fold excess of iodoacetamide. Reduce and Alkylation (RA) protein was used in all sequence determinations. Some of the RA-enzyme employed had been also citraconylated. Peptides were isolated from tryptic, chymotryptic digests of enzyme so derivatized. Clevage of the protein was also effected with CNBr. The proteolytic digests were fractionated over columns of Sephadex G-25 equilibrated with pH 8.0, 0.15 M $AmHCO_3$. Peptide fractions resolved by gel filtration were further separated by high pressure liquid chromatography (HPLC). Aliquots of purified peptides were hydrolyzed for 20 hrs. at 110 ℃ in 5.7 N HCl and analyzed for their compositions on a Durrum D-500 analyzer. Peptides were subjected to automated Edman degredation with the Beckman Model 890 C Sequencer using a 1.0 M Quadrol program with the addition of 3 mg Polybrene before the firstcycle was initiated. Cleavage was effected with anhydrous heptafluorobutyric acid (HFBA) and the phenylthiohydantoin (PTZ) derivatives were extracted with butyl

chloride. The phenylthiohydatioin (PTH) derivitives were formed by heating the dried extracts with 1 N HCl at 50℃ for 15 mins. Identification of the later utilized the IBM Model LC/ 9533 Liquid Chromatograph in conjunction with a Waters Wisp 710 B automated sampler. The sequence of equine CA-Ⅲ has been determined. To provide for homology with the erythrocyte carbonic anhydrases, the first residueis numbered 2 and gap is introduced at residue 126. The extent of homology with the bovine muscle enzymeand with CA-I (low activity) and CA-Ⅱ (high activity) equine erythrocyte forms is caluculated. A strong sequence homology to other mammalian carbonic anhydrase exists, and 86.6% of the residues in the equine and bovine CA-Ⅲ are identical. Although CA-Ⅲ shows strong sequence homologies to the higher activity erythrocyte forms, they vary in many properties. The latter enzymes usually contain a single cysteine, although bovine CA-Ⅱ has none and one minor form of equine carbonic anhydrase and one of equine carbonic anhydrase Ⅱ has 2 such residues. In contrast, equine CA-Ⅲ has 4 cysteines, rabbit has 6, chicken has 7,and bovine has 3 .

9 . Chemical modification of equine CA-Ⅲ

Equine CA-Ⅲ shows a much weaker esterase activity for p-nitrophenyl acetate than the type I and Ⅱ erythrocyte isozymes. The components formed by reaction with the aromatic esters and carbamoyl phosphate possessed more anodic charges at alkaline pH than the starting enzyme. Equine CA-Ⅲ behaves in undergoing extensive acylation of Nε-lysine residues upon reacting with p-nitrophenyl esters. To determine the extent of such derivatization , native

enzyme and the different charged components isolated by electrofocusing were dinitrophenylated with an excess of fluorodinitrobenzene (FDNB) at pH 9.0. After removal of reagents, the protein was hydrolyzed for 20 hrs. at 110 ℃ in 5.7 N HCl and the amino acid compositions determined with a Durrum D-500 Amino Acid Analyzer. An acylated amino acid, i.e. Nε-lysine would not react with FDNB. The level of free lysine in the samples reacted with the ester , i . e. acylated protein, over that in the enzyme controls, would be a measure of the number of lysines that had been derivatized since the acyl groups but not the FDNB derivatives would be removed during hydrolysis. The experiments with carbamoyl phosphate, which appeared to result in the carbamylation of Nε-lysine to form homocitrulline, were more difficult to quantitate due to the back hydrolysis of some of the homocitrulline to lysine. The modification of from 6 to 7 lysine residues results in the production of a series of more anodic electrophoretic components. The derivatization of lysine residues leads to a marked decrease in the enzyme's ability to hydrate CO_2. No such modification was noted for equine CA-Ⅱ. A small amount , i.e. less than 10 % , of equine CA-Ⅰ was converted into a more anodic form by its reaction with PNPA . Thus, the extensive alkylation of CA-Ⅲ during its hydrolysis of aromatic esters appears to be a unique property of the CA-Ⅲ. The ability of the enzyme to auto-acylate some of its Nε-lysine residues is inttriguing but also seeks a physiological meaning. The reactions of CA-Ⅲ with carbamoyl phosphate are also of interest, particulary so since this compound is fairly rapidly converted to cyanate. Carbamoyl phosphate

at a level of 3mM has been reported to almost completely inhibit all three isozymes of carbonic anhydrase (Carter et al) . The experiments of these investigators would appear to be complicated by the formation of cyanate which has been shown by Maren and Conto (1979) and Maren and Sanyal (1983) to be a strong inhibitor of both the hydration of CO_2 and dehydration of bicarbonate. In this experiments which employed carbamoyl phosphate at about 3 mM levels for a long period of time led to extensive carbamoylation of CA-III. However, the individual components isolated still showed considerable hydration activity. The components after extensive dialysis and isolation by electrofocusing for 24 hrs. would not be expected to contain cyanate and the inhibition noted must be due to the carbamylation. The inhibitory results obtained by Carter et al. (1984) would be anticipated to be due to a specific effect of carbamoyl phosphate. The variable and extensive acetylation of the lysine residues of CA-III is of interest but no significant biological meaning is apparent as yet. Further studies of the correlation of the effects of derivatization of muscle carbonic anhydrase on various of its biological activities may eventually lead to a better understanding of its true physiologic function.