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ISOLATION OF EQUINE MUSCLE CARBONIC ANHYDRASE IN CRYSTALLINE FORM

Toshiho Nishita and Harold F. Deutsch

Department of Physiological Chemistry
University of Wisconsin, Madison, WI 53706

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INTRODUCTION: A basic protein noted by Blackburn et al (1) to be present in rabbit muscle extracts in relatively large amounts was later shown by the same laboratory to possess carbonic anhydrase activity (2). Holmes (3) had previously noted that some of the basic protein components of muscle, liver and lung extracts of various animals and chickens resolved by electrophoresis possessed carbonic anhydrase activity. The protein described by Koester et al (1) appears to be similar to "Protein F" described earlier by Scopes (4).

The basic muscle protein possessing carbonic anhydrase activity is such an enzyme derived from a third genetic locus (2,3,5) and has been designated CA-A (5) or CA-III (6) to distinguish it from the forms usually designated CA-B or CA-I and CA-C or CA-II. The term CA-III is preferable since a common metabolic form of human CA-B has been previously designated CA-A (7).

The ready availability of this enzyme has stimulated a series of further investigations on CA-III isolated from human and gorilla (8-10), sheep, mice, rabbit, chicken (3) and ox (11,12). The sequence of CA-III from the later species has been presented (11,12).

Our laboratory has determined the sequence of the CA-I (13) and CA-II (14) equine enzymes and it appeared of interest to initiate studies on the muscle isozyme. In the present work we report the isolation of equine CA-III in crystalline form and describe some of its properties.

EXPERIMENTAL: The muscles of a fatally nembutalized pony were excised and placed in crushed ice. The tissue was then passed through a fine sieve mechanical grinder and 1 to 2 kg portions placed in a deep freeze. Samples

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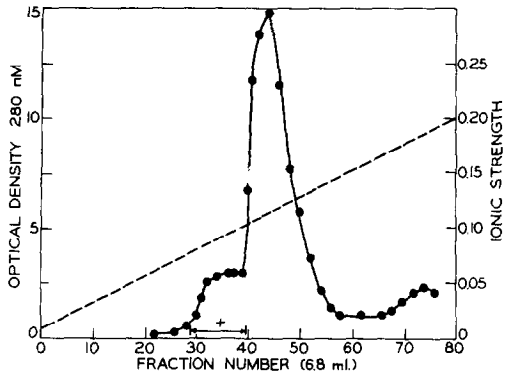
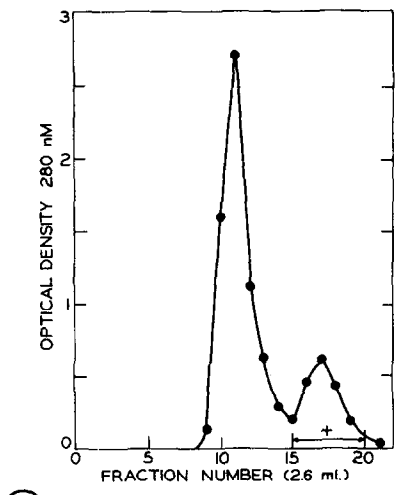


Fig. 1. Chromatographic result for the separation of a horse muscle extract on a 2.8 x 32 cm column of CM-Sephadex. The fractions possessing carbonic anhydrase activity (+) were pooled as indicated.

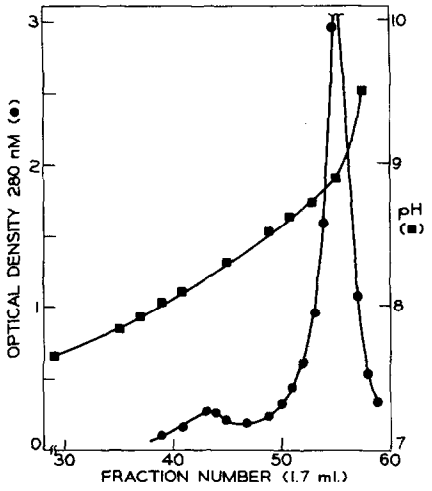
were removed as needed and partially thawed. This material was homogenized with two parts of pH 8.0, 0.01 M Tris-HCl at 0°. The homogenate was centrifuged at 0° for 30 min. at 8000 g and the supernatant filtered through glass wool. This solution was then alkylated by adding iodoacetamide to 0.01 M, the pH being adjusted to 8 if necessary. Dialysis to equilibrium of this solution against pH 6.8, 0.01 M potassium phosphate at 2°-4° was then carried out. The dialyzed material was again centrifuged and the supernatant passed over a column of CM-Sephadex (Pharmacia) equilibrated against the pH 6.8 buffer. The protein was eluted by application of a linear gradient of NaCl in this buffer to 0.2 M. The various fractions eluted were tested for carbonic anhydrase activity by the method of Wilbur and Anderson (15). The chromatographic result obtained for an extract from 200 g of tissue is shown in Fig. 1.

The enzymatically active fractions were pooled and concentrated by precipitation with saturated ammonium sulfate. The precipitate was dissolved in a small amount of pH 6.8, 0.01 M potassium phosphate containing 0.15 M NaCl and passed over a column of G-75 Sephadex (Pharmacia) equilibrated with this buffer. The results of a typical experiment are presented in Fig. 2.

The enzymatically active fraction was dialyzed exhaustively against water, the small amount of precipitate formed was removed by centrifugation



(2)



(3)

Fig. 2. Chromatographic result for the separation of carbonic anhydrase fractions of Fig. 1 on a 0.8 x 43 cm column of G-75 Sephadex. Fractions possessing enzymatic activity (+) were pooled as indicated.

Fig. 3. The result for a column electrophoresis experiment with the carbonic anhydrase active fractions separated as shown in Fig. 2.

at 0° and the supernatant solution subjected to column electrofocusing in a pH 7-10.5 Ampholyte buffer (Pharmacia). The results of such an experiment are shown in Fig. 3. From 50 to 75 mg of enzyme are obtained per 100 gm of fresh tissue.

The major component of Fig. 3 had an isoelectric point of 8.90 (average of two experiments). These fractions were pooled and dialyzed exhaustively against deionized, distilled water (Milli-Q, Millipore Corp.) and then centrifuged at about 8000 g for 10 min at 0° to remove a small amount of turbidity. Weighed aliquots of this solution were subject to measurements of dry weight at 105°, O.D. at 280 nM and amino acid analyses. The protein was found to have an $E_{280nM}^{1\%,1cm}$ value of 15.5. 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 taken. In cases where no significant level deviations were noted as a

Table I. Amino Acid Contents of Crystalline Equine Muscle Carbonic Anhydrase.

Amino Acid	Residues/ Mole[a]	Integral No. Residues	Amino Acid	Residues/ Mole[a]	Integral No. Residues
Cysteine[b]	3.8	4	Methionine	0.88	1
Aspartic Acid	29.7	30	Isoleucine	9.1	9
Threonine	14.6	15	Leucine	22.5	23
Serine	16.9	17	Tyrosine	9.1	9
Glutamic Acid	20.4	20	Phenylalanine	11.0	11
Proline	20.4	20	Histidine	13.1	13
Glycine	19.2	19	Lysine	17.7	18
Alanine	15.7	16	Arginine	14.2	14
Valine	14.7	15	Tryptophan[c]	9.4	9
				Total Residues	263

(a) A mol. wt. of 27,000 was assumed.
(b) Samples of crystallized protein were alkylated at pH 8.5 in the presence of 6 M guanidine. The same result was obtained when the protein was reduced with 50 mM dithiothreitol prior to alkylation.
(c) Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (16).

function of time, the values were averaged. Amino acid levels obtained are presented in Table I. A total of 263 residues was found when a molecular weight of 27,000 was assumed. Sequence analysis will be needed to determine the exact number of residues.

A 1% solution of the enzyme separated by electrofocusing was dialyzed at room temperature against repeated changes of 60% saturated ammonium sulfate in pH 8.0, 0.05 M Tris-HCl buffer. Small needles formed after several days. The crystalline material was removed by centrifugation, dissolved in a small amount of distilled water and again dialyzed against the above buffer. After standing for several days at 2°-4°, crystals of the type shown in Fig. 4 appeared.

The activity of the equine muscle carbonic anhydrase was in the range of 350 to 400 Wilbur and Anderson (15) units per mg of zinc containing protein. This is from 15 to 20% of the activity of equine CA-I and in the range of activity found for the muscle carbonic anhydrases of other species.

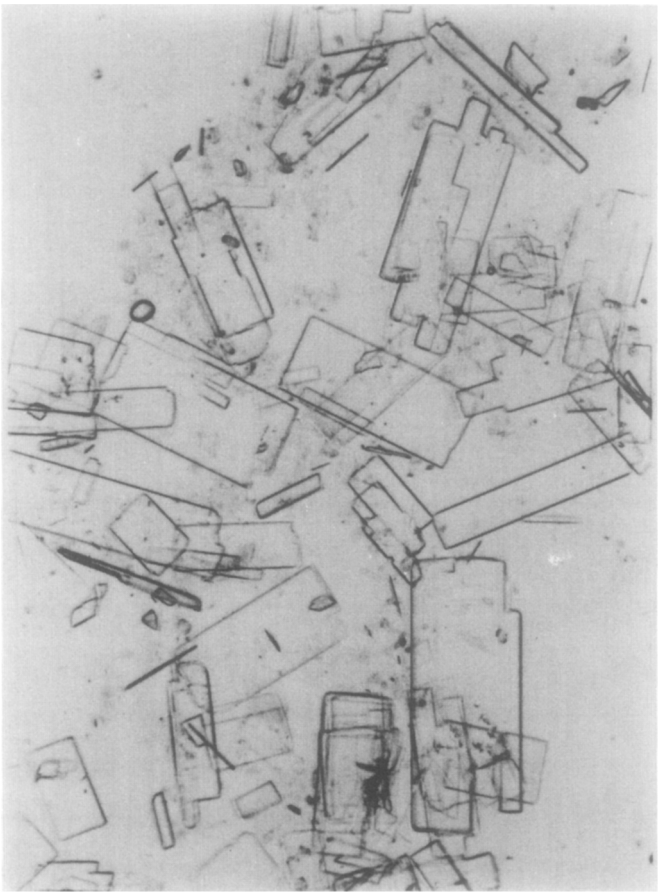


Fig. 4. Twice crystallized equine CA-III. Magnification 325 X.

DISCUSSION: In preliminary experiments we found that equine CA-III was poorly retained by p-aminobenzenesulfonamide affinity columns. Koester et al (2) 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 we resorted to a somewhat different approach than that used by other investigators. It was based in part on the presence of what appear to be readily reactive cysteines in this enzyme.

The number of cysteine residues per mole of CA-III has been reported to be 6 for the rabbit (17), 3 for the ox (12) and 4 for the human (10) proteins. The amino acid composition of equine CA-III appears to most closely resemble that of the human enzyme (10) since 4 cysteines and 1 methionine were also

found. Some of the sulfhydryls in CA-III appear to reside on the surface of the protein and lead to some formation of dimers during isolation (10,17). The inclusion of a reducing agent such as dithiothreitol in all stages of its purification has been utilized by Tashian et al (12). To avoid the use of such reagents we resorted to alkylation of the muscle extract. Such a procedure has been previously found to be quite effective in converting a labile form of an equine mutant CA-II (18) and of human superoxide dismutase (19) to one which could readily be isolated in a form that is readily crystallizable. This proved to be also true for equine CA-III in the present study. Alkylation of the muscle extract by the thiol-disulfide exchange reaction of Grassetti and Murray (18) using a substance such as 6,6'-dithionicotinic acid would permit ready regeneration of the active cysteines following purification.

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. Equine CA-I has been shown to exist in many polymorphic forms (13) and the effects of these pleomorphic substitutions would be of particular interest. It is to be noted that Koester and Noltmann (21) have shown that the conformational properties of rabbit CA-III differ markedly from those of the CA-I and CA-II forms.

The crystalline protein prepared in this manner was found to contain 1.8 residues of S-carboxymethylcysteine. Two additional cysteines could be derivatized when alkylation was carried out with iodoacetamide in the presence of 6M guanidine-HCl at pH 8.5.

From 40 to 60% of the expected level of zinc was found in the crystallized protein after dialysis into pH 8.4, 0.033 M veronal buffer. 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 protein. 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 (2) 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.

SUMMARY: A relatively simple procedure for the isolation of equine muscle carbonic anhydrase (CA-III) in crystalline form has been developed. An important aspect of the method, which should find ready application to this enzyme from other species, is the alkylation of the readily reactive cysteine residues prior to starting the fractionation.

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