BASIC MUSCLE PROTEIN, A THIRD GENETIC LOCUS ISOENZYME OF CARBONIC ANHYDRASE?

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Summary--Rabbit muscle cytosol extract contains a basic protein which represents about 2% of the total cytosol protein. It contains zinc in a 1:1 stoichiometric ratio, based on a molecular weight of 30,000, and it catalyzes the hydration of CO_2. It is immunochemically distinct from the high and low activity forms of rabbit blood carbonic anhydrase. It has comparatively poor activity as an esterase, and about 20% of the CO_2 hydratase activity of the rabbit blood low activity carbonic anhydrase. This CO_2 hydratase activity is not inhibited by acetazolamide at concentrations which totally inhibit the activity of the blood carbonic anhydrases. The evidence obtained to date, though circumstantial, suggests that this basic metalloprotein is a carbonic anhydrase derived from a third genetic locus with properties considerably different from those of the mammalian carbonic anhydrases heretofore identified.

About five years ago, during the development of preparative procedures for phosphoglucose isomerase, a large protein peak was observed which eluted from CM-Sephadex after phosphoglucose isomerase (1). This "Basic Muscle Protein" was collected and further purified, and initial physical studies indicated its molecular weight to be approximately 30,000. Preliminary investigations have shown that its amino acid composition is similar to that of various carbonic anhydrases (2). It is generally assumed that in mammalian systems there are two types of carbonic anhydrases which are designated CA-I (the low activity form) and CA-II (the high activity form). One or both forms have been found in most tissues (3, 4). Many variants of carbonic anhydrase have been isolated, but all of them (whether they derive from point mutations or epigenetic modifications) crossreact immunochemically with either the I form or the II form, which do not cross-react with each other (5, 6).

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Although many carbonic anhydrases from blood have been studied in some detail (3, 7), little effort has been directed toward the investigation of carbonic anhydrase in muscle tissue, most likely because there does not appear to be an obvious function for this enzyme in muscle. Nonetheless, Holmes recently described bands of carbonic anhydrase activity in crude extracts of muscle in a gel electrophoresis system (8). In the present communication we report some properties of Basic Muscle Protein which has many of the characteristics of known carbonic anhydrases, but whose function in muscle is poorly understood at this time. We have noted that its properties are similar to those of "Protein F" described by R. K. Scopes (9).

METHODS

Protein purification--Frozen rabbit muscle from Pel-Freeze was thawed overnight and extracted according to the procedure used for the isolation of phosphoglucose isomerase (1). The peak labelled X in Figure 6 of Reference 1 was further purified by chromatography in 100 mM sodium phosphate buffer (pH 6.9) on G-75 Sephadex. Rabbit blood carbonic anhydrases (CA) I and II were isolated by a procedure based on that of Armstrong (10), as adapted by McIntosh (11), which involves lysis of the red blood cells followed by chromatography on DEAE-Sephadex. CA-I, CA-II, and Basic Muscle Protein were found to be homogeneous by SDS polyacrylamide gel electrophoresis (12). Protein concentrations were determined by a modified biuret method (13), or by OD_{280} measurement with the use of extinction coefficients (0.1%) of 2.32, 1.82, and 1.74 for Basic Muscle Protein, CA-I, and CA-II, respectively.

Enzyme assays--CO_2 hydratase activity was determined by the Veronal-bromthymol blue assay described elsewhere (14, 15). Units of activity were calculated by the method of Maren et al. (16). Esterase activity was determined colorimetrically on a Beckman DB-G spectrophotometer by the p-nitrophenyl acetate method of Armstrong et al. (10). Acetazolamide was purchased as Diamox from Lederle.

Analytical determinations--Zinc was determined on a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Standard curves were obtained with J. T. Baker's zinc DILUT-IT standards at appropriate dilutions. Attempts to remove the zinc were made by dialyzing the protein exhaustively against 5 mM o-phenanthroline at pH 5.6. Sulfhydryl groups were determined by titration with p-mercuribenzoate according to the method of Boyer (17). Protein molecular weights were determined by SDS polyacrylamide gel electrophoresis with appropriate standards (12).

Preparation of antiserum--One ml of a 1.64 mg/ml solution of Basic Muscle Protein in physiological saline was mixed with one volume of Freund's complete adjuvant and injected into the footpads, thighs, and neck of a male Hartley guinea pig. Further injections were given 3, 6, and 9 weeks later in a similar manner with Freund's incomplete adjuvant and blood was collected by heart puncture 5 days after the last injection. The serum was separated from the clotted cells by centrifugation and stored at -20°C.

Table I. Enzymatic Activities of Basic Muscle Protein and of Rabbit Blood Carbonic Anhydrases.

Activity	Basic Muscle Protein	Carbonic Anhydrase I[a]	Carbonic Anhydrase II[a]
p-nitrophenyl acetate hydrolysis (μmoles/min/mg)[b]	0.0031 ± 0.0014	0.60 ± 0.04	5.19 ± 0.17
β-naphthyl acetate hydrolysis (μmoles/min/mg)[b]	---	0.209 ± 0.005	0.027 ± 0.006
CO_2 hydration (μmoles H^+/sec/μmole protein)[c]	$1.0 \pm 0.3 \times 10^3$	$4.7 \pm 0.9 \times 10^3$	$3.3 \pm 1.6 \times 10^4$
Acetazolamide:protein molar ratio necessary to produce 50% inhibition[d]	1100:1	0.56:1	0.56:1

[a]Data for the rabbit blood carbonic anhydrases are from Walther et al. (18).

[b]The esterase assays were performed as described by Armstrong (10).

[c]The CO_2 hydration assay was performed by incubation of 0.5 ml of a 25 mM Veronal buffer solution (pH 8.2, with 0.01% bromthymol blue as a pH indicator) in an ice bath with 5 to 20 μl of protein solution containing 0.2 to 4 mg/ml protein, adding 0.5 ml of ice cold CO_2 saturated water, and timing the reaction to its colorimetric end point with a stop watch. In this series of assays, the uncatalyzed reaction was complete in 150 seconds. The Veronal buffer was titrated with HCl and found to take up 20.7 milliequivalents H^+ per ml buffer. This value was used in the calculation of turnover numbers. The day-to-day variability in this assay is marked, but consistent with that observed by other investigators (15, 16).

[d]The protein concentrations in the assay mixtures were 0.31 μM, 0.39 μM, and 0.39 μM for Basic Muscle Protein, CA-I, and CA-II, respectively.

RESULTS

The enzyme activity assay results are shown in Table I. In the protein concentration range of 0.05 - 0.50 μM, doubling the amount of Basic Muscle Protein in the CO_2 hydratase assay causes the endpoint to be reached in half the time. We have ruled out general protein buffer effects by using

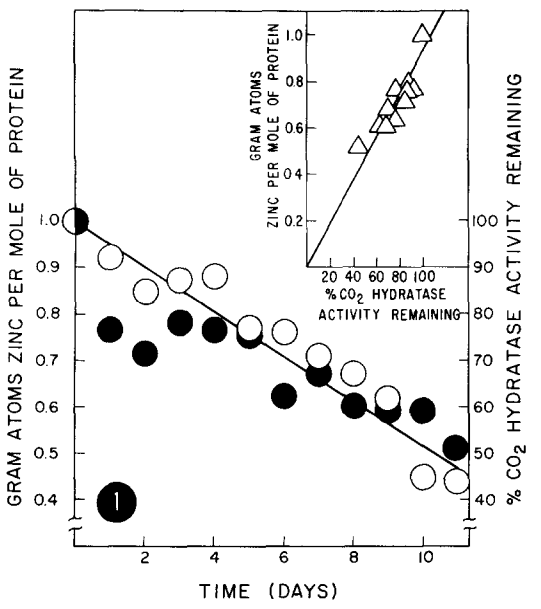

Fig. 1: Parallel loss of zinc and CO_2 hydratase activity as a function of time. The protein was dialyzed for eleven days against 100 mM sodium succinate (pH 5.6), 5 mM o-phenanthroline, and 1 mM dithiothreitol. On each day a one-ml aliquot was removed and dialyzed for 48 hours against a 50 mM Veronal buffer solution (pH 8.2) with 1 mM dithiothreitol, and assayed for CO_2 hydratase activity, protein (biuret method), and zinc content. Closed circles represent the zinc, open circles the activity remaining. The insert shows the loss of activity as a function of the loss of zinc.

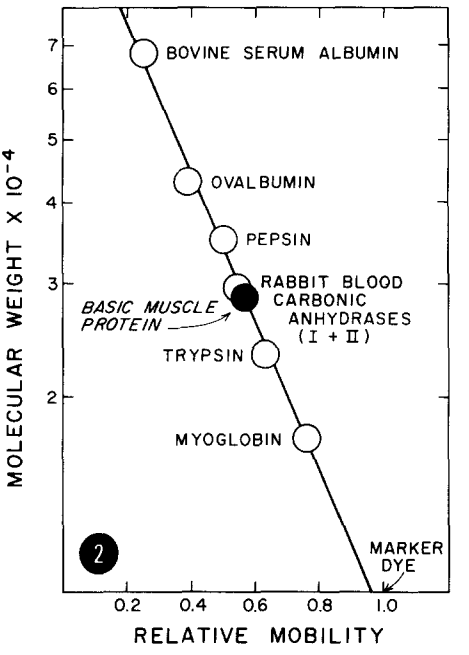

Fig. 2: Molecular weight determination of Basic Muscle Protein by SDS polyacrylamide gel electrophoresis. The basic protein, the rabbit blood carbonic anhydrases, and the indicated protein standards were subjected to electrophoresis for 2.5 hours at 8 ma/gel at room temperature. Approximately 10 µg of each sample were applied to 10% gels. The points shown are averages of several determinations.

equivalent and greater amounts of phosphoglucose isomerase as a control. Phosphoglucose isomerase perturbed the non-enzymatic reaction rate slightly, but to a similar extent irrespective of the quantity of enzyme introduced into the reaction mixture. Basic Muscle Protein has about 20% of the activity of rabbit blood CA-I, and about 3% of the activity of rabbit blood CA-II. In order for Basic Muscle Protein to be inhibited by acetazolamide

to 50% of its original activity, an approximately 2000 times higher concentration is required than for 50% inhibition of the blood carbonic anhydrases. Its esterase activity in the p-nitrophenyl acetate assay is approximately 0.5% of that of the low activity blood enzyme, and sulfonamide inhibition is not detectable within the limits of this assay.

The zinc:protein stoichoimetry has been determined to be 1.06:1. Figure 1 demonstrates that the CO_2 hydratase activity is lost proportionately to the loss of zinc. So far we have not been able to remove more than 50% of the zinc and, under our experimental conditions, the activity can be recovered by no more than 10% with the addition of equimolar zinc sulfate once the zinc has been removed.

Considerable similarity in amino acid composition of Basic Muscle Protein compared with that of various carbonic anhydrases has been noted (2). The most distinct difference is the presence of 6 cysteine residues per 30,000 molecular weight. Our preliminary analyses indicate also that there is a high proportion of basic amino acids. The protein elutes after phosphoglucose isomerase on CM-Sephadex, and therefore its isoelectric pH is probably greater than 8.5 (1).

Figure 2 represents the molecular weight determination of Basic Muscle Protein. In SDS gel electrophoresis rabbit blood carbonic anhydrases I and II show molecular weights of approximately 29,500, with Basic Muscle Protein migrating somewhat faster. It is possible that our protein, being highly basic, moves more rapidly in this system than other proteins of the same size and that its molecular weight may therefore be slightly underestimated. Similar anomalous behaviour of highly charged proteins in the SDS system has been noted by other investigators (19). Although our protein is similar to the rabbit blood carbonic anhydrases with respect to molecular weight and amino acid composition, it is immunochemically distinct from each of them as shown in Figure 3.

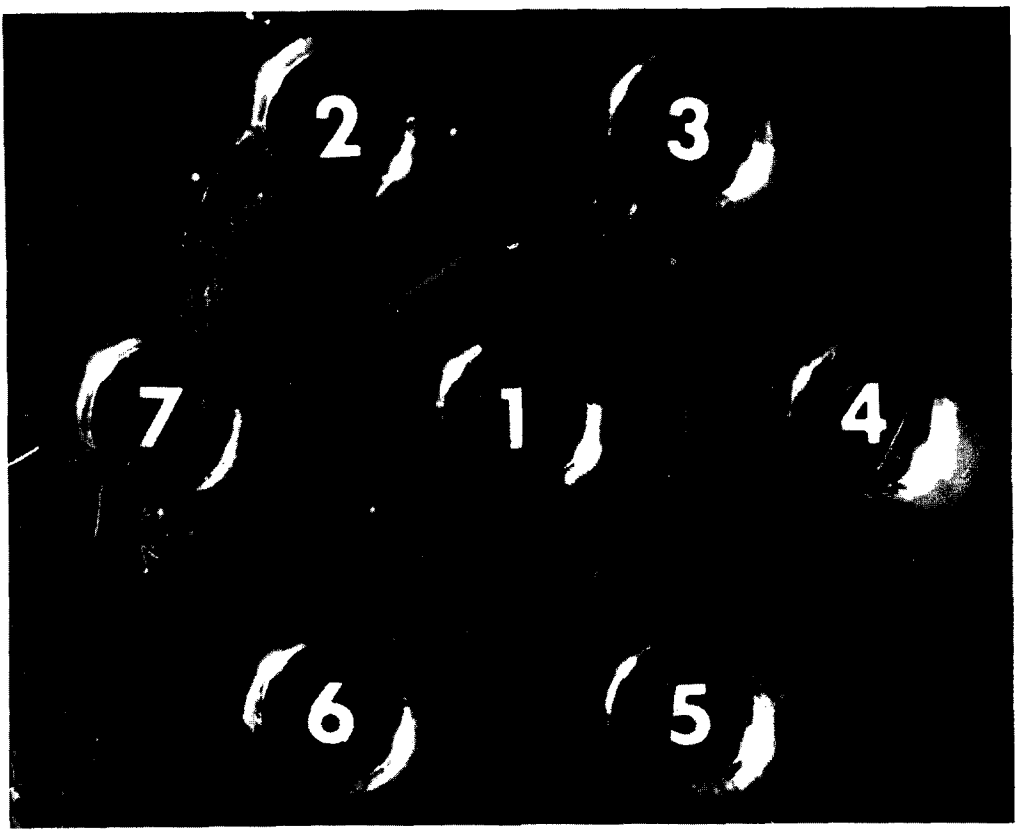

Fig. 3: Double diffusion in Agarose. Immunodiffusion was performed at room temperature in 1.2% Agarose in 0.05 M borate buffer (pH 8.2), 0.05 M potassium chloride, and 0.02% sodium azide. The wells contained (1), guinea pig antiserum to Basic Muscle Protein; (2), (4), and (6), 2.0 µg of the metalloprotein; (3), 1.9 µg rabbit blood CA-I; (5), 1.9 µg rabbit blood CA-II; and (7), 1.8 µg of a subform of CA-II isolated from rabbit blood in this laboratory (18).

DISCUSSION

The basic zinc metalloprotein which we have isolated has many of the characteristics of carbonic anhydrase. It differs from mammalian carbonic anhydrases in the following ways. (1) Enzymatic activity--Basic Muscle Protein has a much lower CO_2 hydratase activity than the blood carbonic anhydrases, and very little activity as an esterase. (2) Inhibition by acetazolamide--Acetazolamide inhibits mammalian carbonic anhydrases with a K_i of approximately 10^{-8} M in both CO_2 hydration and ester hydrolysis (20).

Basic Muscle Protein is relatively sulfonamide resistant. If K_i is presumed to be roughly proportional to $K_{50\%}$, then its acetazolamide K_i would be in the range of 10^{-5} to 10^{-4} M. (3) Lability of the zinc--Under conditions similar to ours, apoenzymes with native conformation can be produced from mammalian carbonic anhydrases (21). We conclude that either the zinc in our protein is considerably less labile than that of known mammalian carbonic anhydrases, or that the protein itself is more susceptible to denaturation at acidic pH than the mammalian enzymes. (4) Amino acid composition--Mammalian carbonic anhydrases ordinarily have 0 to 2 cysteine residues (3) and they contain basic residues in such proportions that their isoelectric points range from 4.50 to 8.12 (22). Our protein has 6 cysteine residues and an isoelectric point which is probably greater than 8.5. Holmes' carbonic anhydrase activity Band A from muscle extracts is also very basic, migrating toward the cathode at pH 9.0 (8), and Scopes estimates that the isoelectric pH of his Protein F is between 8.5 and 9.0 (9). Our immunochemical results support the hypothesis that Basic Muscle Protein is the product of a third genetic locus for carbonic anhydrase.

Although our protein has some properties unusual for a mammalian carbonic anhydrase, they are not unprecedented. Parsley carbonic anhydrase has 7 cysteine residues; it is inactive as an esterase; its CO_2 hydratase activity is poorly inhibited by acetazolamide; and its zinc has so far not been removed without denaturing the protein (23). Spinach carbonic anhydrase has been reported to be inhibitable by acetazolamide with a K_i of about 10^{-4} M (24). Pocker and Ng have proposed that since the plant enzyme is much less susceptible to acetazolamide inhibition and also much less active toward its larger substrates, its active site crevice is smaller than that of the mammalian enzymes (24). It is conceivable that this may also be true for our enzyme.

At this time, we do not have specific enzymological evidence for an *in vivo* functional hypothesis. We believe, however, that a protein which

comprises 1 to 2% of the total muscle cytosol extract must be important in muscle function. It is most interesting to note that Zborowska-Sluis et al. have suggested that carbonic anhydrase must be present in skeletal muscle in order to account for the facilitated CO_2 diffusion which they observe in their perfused muscle system (25). It could be involved in facilitating CO_2 transport out of the mitrochondria. It could also have some role in the regulation of muscle pH. Maren has proposed that the presence in muscle of a carbonic anhydrase might inhibit the transport of CO_2 from muscle to blood (7). However, the preliminary kinetic results presented here indicate that our protein is considerably less active than the high activity form of blood carbonic anhydrase. Therefore, we believe that it could perform its function(s) in muscle without blocking the net outward flux of CO_2.

Although present in high concentrations in muscle, our protein may not be limited to that tissue in mammals. A low activity, sulfonamide resistant carbonic anhydrase has been found in liver (26), and Holmes has observed basic carbonic anhydrase activity bands in liver and lung tissue extracts (8). Whatever its function in muscle or other tissues, we think there is a strong probability that Basic Muscle Protein is a third major isoenzyme of mammalian carbonic anhydrase, with properties which are of genetic, mechanistic, and physiological significance.

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