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# IDENTIFICATION OF PSEUDOISOENZYMIC SUBFORMS OF MUSCLE CARBONIC ANHYDRASE

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## Summary

Rabbit muscle carbonic anhydrase III, a recently discovered third isoenzyme (possibly muscle specific) of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) (Register, A.M., Koester, M.K. and Noltmann, E.A. (1978) J. Biol. Chem. 253, 4143—4152) has been subjected to isoelectric focusing. When monomer samples, shown to be homogeneous by both ion-exchange and molecular sieve chromatography, were analyzed by this technique, three subspecies were produced, which were similar in amino acid composition and specific $CO_2$ hydratase activity. In addition to having either monomer or dimer status, the subspecies differed in the extent of oxidation of their sulhydryl groups and in their isoelectric pH values (9.3, 8.8, and 8.4, respectively). Also, the presence of dithiothreitol will affect their relative concentrations. These subforms are therefore designated as pseudoisoenzymes and are considered to be neither genetically nor functionally separate enzyme species.

## Introduction

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), which catalyzes the hydration of $CO_2$ and dehydration of $HCO_3^-$, has been found to occur as two major isoenzymes in erythrocytes and other tissues of numerous species [1,2]. According to Tashian's nomenclature [3], these are designated as carbonic anhydrase I (the low activity form) and carbonic anhydrase II (the high activity form). We have recently identified and characterized a third major isoenzyme, carbonic anhydrase III [4], which is different from the other two major forms and which is possibly muscle specific. This third isoenzyme has 20% of the $CO_2$

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hydratase activity of the low activity carbonic anhydrase I, is comparatively resistant to the highly specific carbonic anhydrase inhibitor acetazolamide, and is immunochemically distinct from the two erythrocyte isoenzymes [5]. It exists in both monomer (mol. wt. 29 000) and dimer (mol. wt. 58 000) forms, the latter being the disulfide oxidation product of the monomer [4].

Many electrophoretic variants of carbonic anhydrase I and II have been observed in mammalian tissues [6—16]. Some of these have been shown to be genetic variants, while others have been demonstrated to be epigenetic modifications or artifacts resulting from preparative procedures or aging of the tissue extract. All of these forms, regardless of their origin, cross-react immunochemically with either carbonic anhydrase I or carbonic anhydrase II, which are in turn immunochemically distinct from each other [17,18].

The present paper describes the generation of sulfhydryl oxidation subspecies of rabbit muscle carbonic anhydrase III by isoelectric focusing, a finding which is particularly relevant since carbonic anhydrase I and carbonic anhydrase II have been reported to exist in muscle, in addition to carbonic anhydrase III, solely on the basis of multiple bands of $CO_2$ hydratase activity appearing on electrophoresis [19,20].

## Materials and Methods

*Preparation of enzyme.* Rabbit muscle carbonic anhydrase III was prepared as described previously [4].

*Assays.* Protein was determined by measurement of the absorbance at 280 nm with use of an extinction coefficient ($\epsilon^{0.1\%}_{280\,nm}$) of 2.32 [4]. $CO_2$ hydratase activity was assayed by the modified [6] method of Wilbur and Anderson [21], as previously described [4,5,16].

*Isoelectric focusing.* Preparative isoelectric focusing was performed in an LKB Model 8101 column (capacity, 110 ml) with the application of a constant potential of 600 V for 96—120 h at 4°C. Sucrose density gradients containing LKB ampholines or Brinkmann pHisolytes (pH range 8—10) were prepared with an LKB Model 8121 gradient mixer. Fractions of 1 ml were collected (flow rate, 30 ml/h) and were assayed for pH, protein concentration and $CO_2$ hydratase activity. For the determination of isoelectric pH values, protein samples of 10—15 mg produced profiles that remained within the narrow part of the pH gradient and which were sharply resolved. For the generation of ample supplies of the modified subspecies for further analysis, the column was loaded with 40—50 mg material.

*Removal of ampholytes.* Ampholytes were removed with Biorad bifunctional resin AG 501-X8 (D) according to the method of Baumann and Chambrach [22], followed by dialysis to remove the sucrose. Alternatively, both sucrose and ampholyte were removed by chromatography on a Sephadex G-75 column similar to that employed for the isolation of carbonic anhydrase III [4], for which the elution profiles of the monomer and the dimer forms had been previously determined by calibration against known standards.

*Amino acid analysis.* Aliquots of each species generated by isoelectric focusing were hydrolyzed for 20 h and analyzed for amino acid content by standard procedures employed in this laboratory [23]. Total half-cystine was

analyzed as cysteic acid after performic acid oxidation according to Moore [24] and free sulfhydryls were determined by the *p*-mercuribenzoate method of Boyer [25].

## Results

*Generation of subforms of carbonic anhydrase III*

Fig. 1 shows the species carbonic anhydrases $III_1$, $III_2$ and $III_3$ generated on isoelectric focusing in the pH range from 8 to 10. The sample applied was from a highly purified carbonic anhydrase III monomer preparation, which eluted as a single peak on both carboxymethyl-Sephadex and Sephadex G-75 chromatography [4]. Some characteristic properties of these subforms are summarized in Table I and the amino acid compositions are shown in Table II. Within the accuracy of the methods used, all three are essentially identical with respect to amino acid composition and specific activity in the $CO_2$ hydratase assay, these parameters showing good correlation with previous data obtained for carbonic anhydrase III [4,5]. They differ only in their states of sulfhydryl oxidation and in their isoelectric pH values.

*Effect of sulfhydryl-reducing agent on the distribution of carbonic anhydrases $III_1$, $III_2$ and $III_3$*

Fig. 2 shows the effect of 10 mM dithiothreitol on the subform distribution pattern. Without dithiothreitol, carbonic anhydrase $III_1$ is 76% of the total protein, carbonic anhydrase $III_2$ is 15%, and carbonic anhydrase $III_3$ is 9%; in the presence of the reducing agent the distribution is 55, 28, and 17% for carbonic anhydrases $III_1$, $III_2$, and $III_3$, respectively. Dithiothreitol therefore

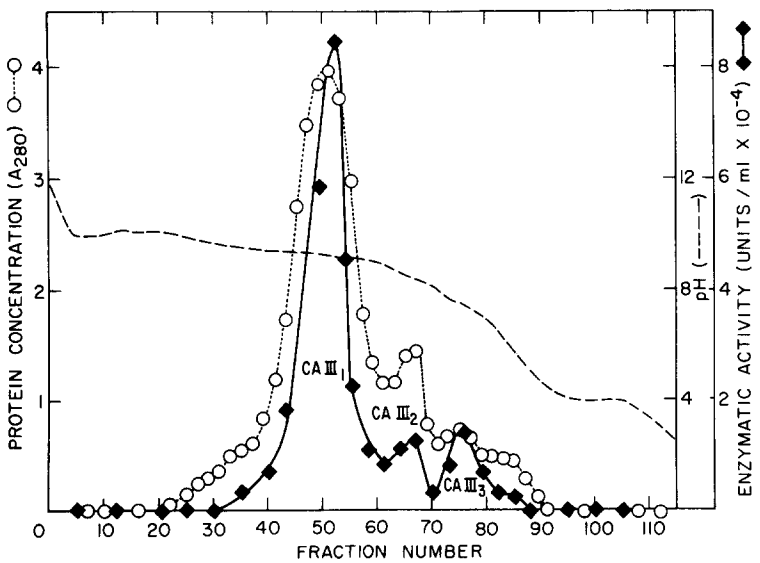


**Fig. 1. Preparative isoelectric focusing of rabbit muscle carbonic anhydrase III in the pH range of 8—10. A protein sample of 40 mg was applied and the focusing experiment was performed without dithiothreitol. Enzyme units are defined as μmol $H^+$ released · $l^{-1}$ · $s^{-1}$ at 0°C. CA, carbonic anhydrase.**

TABLE I

SUMMARY OF CHARACTERISTICS OF THE CARBONIC ANHYDRASE ISOELECTRIC FOCUSING FRACTIONS OF CARBONIC ANHYDRASES $III_1$, $III_2$, AND $III_3$ FROM RABBIT MUSCLE

| | Carbonic anhydrase $III_1$ | Carbonic anhydrase $III_2$ | Carbonic anhydrase $III_3$ |
|---|---|---|---|
| Isoelectric pH * | 9.34 ± 0.06 | 8.76 ± 0.04 | 8.41 ± 0.12 |
| Free sulfhydryls (*p*-mercuribenzoate titration) | 5.08 ± 0.10 (4) ** | 5.51 ± 0.04 (4) | 6.01 ± 0.10 (6) ** |
| $CO_2$ hydratase specific activity ($\mu$mol $H^+ \cdot s^{-1} \cdot \mu$mol protein$^{-1}$) | 1.2 ± 0.2 · $10^3$ | 1.2 ± 0.3 · $10^3$ | 1.1 ± 0.3 · $10^3$ |
| Percent total protein during focusing without dithiothreitol *** | 76% | 15% | 9% |
| Percent total protein during focusing with 10 mM dithiothreitol *** | 55% | 28% | 14% |

* Calculated from 5 or 6 separate values which were obtained under conditions of analytical isoelectric focusing as described under Materials and Methods (see also ref. 4)

** The numbers of free sulfhydryls have previously [4] been calculated to be 5.87 ± 0.12 and 4.94 ± 0.13 per monomer equivalent (mol. wt. 29 000) for the carbonic anhydrase III monomer and dimer, respectively. The numbers in parentheses indicate the number of assays.

*** As illustrated in Fig. 2.

TABLE II

AMINO ACID COMPOSITION OF THE ISOELECTRIC FOCUSING FRACTIONS OF CARBONIC ANHYDRASES $III_1$, $III_2$, AND $III_3$

The values represent an average of the number of analyses indicated in parentheses, hydrolyzed for 20 h in 6 M HCl. The data have not been extrapolated to zero-time hydrolysis or to maximal recovery, and are therefore not directly comparable to reported values [4] for those residues which are readily degraded or incompletely hydrolyzed.

| Amino acid | Carbonic anhydrase | | |
|---|---|---|---|
| | $III_1$ (8) | $III_2$ (6) | $III_3$ (6) |
| Lys | 17.8 | 17.7 | 17.7 |
| His | 11.1 | 11.1 | 10.6 |
| Arg | 12.6 | 13.1 | 12.6 |
| Asp | 28.1 | 28.6 | 28.2 |
| Thr | 10.8 | 10.9 | 11.0 |
| Ser | 18.3 | 18.0 | 17.8 |
| Glu | 18.8 | 18.5 | 18.9 |
| Pro | 22.9 | 22.9 | 22.5 |
| Gly | 18.8 | 18.8 | 18.6 |
| Ala | 14.4 | 14.3 | 14.3 |
| Val | 12.5 | 12.4 | 12.4 |
| Met | 2.1 | 1.5 | 1.7 |
| Ile | 9.1 | 9.4 | 9.2 |
| Leu | 19.3 | 19.8 | 19.1 |
| Tyr | 8.4 | 8.2 | 8.9 |
| Phe | 10.5 | 11.7 | 11.7 |
| Cys-CH * | 5.0 | 5.5 | 6.0 |
| Trp ** | ≈10 | ≈10 | ≈10 |

* Free cysteine was determined independently by titration with *p*-mercuribenzoate [25].

** Values for tryptophan are based on absorbance measurements at 280 nm for which the $\epsilon_{280nm}$, 0.1% values of the three subforms were all within ±3% of the previously reported [4] value of 2.32, which corresponds to 10 residues per molecule of protein prior to separation by isoelectric focusing.

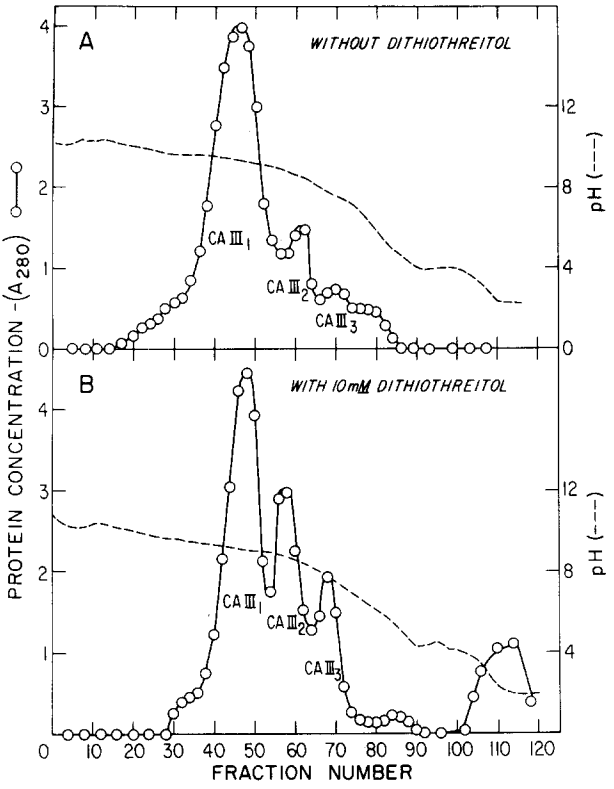


**Fig. 2. Effect of dithiothreitol on the distribution of the carbonic anhydrase subspecies $III_1$, $III_2$, and $III_3$ during isoelectric focusing in the pH range of 8—10. Protein samples of 40—50 mg were applied to each column. A, Focusing without dithiothreitol; B, focusing with 10 mM dithiothreitol. (Occasionally, as in B, denatured protein was produced at the $H_2SO_4$ (0.01 M) anode-sucrose gradient interface.) CA, carbonic anhydrase.**

causes the latter two species to double in relative yield with a corresponding reduction in carbonic anhydrase $III_1$, indicating that the sulfhydryl oxidation state has been affected.

In order to obtain some insight into the identity of the three forms, each species (from Fig. 1) was subjected to chromatography on Sephadex G-75. Both carbonic anhydrases $III_2$ and $III_3$ are predominantly (>90%) monomer, while carbonic anhydrase $III_1$ is 45% dimer and 55% monomer. We have previously [4] shown that carbonic anhydrase III forms a dimer species under oxidizing conditions; however, the dimer concentration was never more than 20% of the total carbonic anhydrase III. Application of a strong electrical potential in a medium of basic pH seems to be much more effective in generating the dimer species, since the carbonic anhydrase $III_1$ form dominates the isoelectric focusing profile. Nonetheless, during the pooling of the sample with ampholytes present and the subsequent rechromatography on Sephadex G-75 in phosphate buffer (pH 6.9), about half of the dimer species reverted to the monomer form. Identification of carbonic anhydrase $III_1$ obtained immediately after isoelectric focusing as dimer is confirmed by *p*-mercuribenzoate titration (Table I).

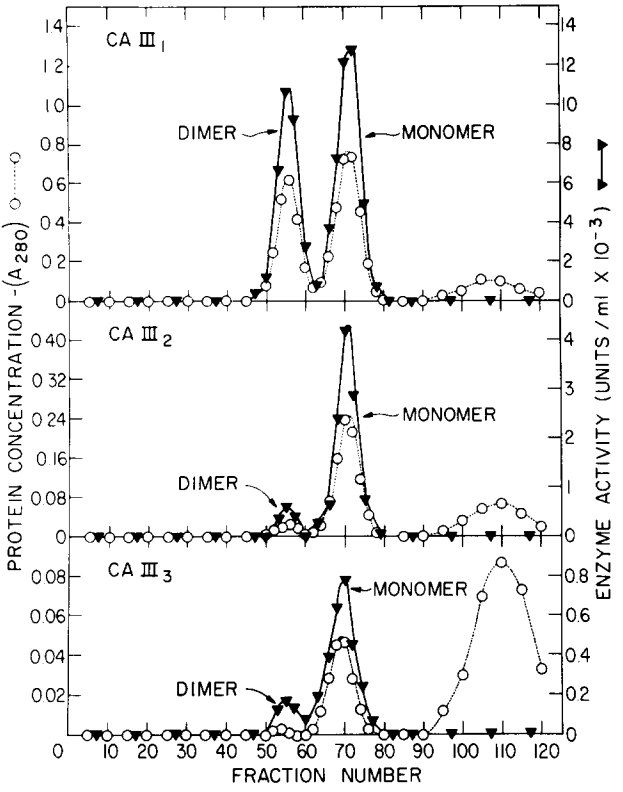


Fig. 3. Rechromatography on Sephadex G-75 of rabbit muscle carbonic anhydrase (CA) subspecies $III_1$, $III_2$, and $III_3$. Chromatography was performed as previously described [4]. The elution buffer was 0.1 M sodium phosphate (pH 6.9) without dithiothreitol present. Carbonic anhydrase $III_2$ and $III_3$ pools (from Fig. 1) were applied directly to the column but, to avoid contamination from the neighboring peak, only the left half of the carbonic anhydrase $III_1$ peak was applied. The $A_{280nm}$ peak at fraction No. 110 has no $CO_2$ hydratase activity. Based on its absorbance at 400 nm, it corresponds to the ampholyte present in the sample applied to the column. Enzyme units are defined as in the legend to Fig. 1.

It is significant to recall in this context that we have previously noted strong oxidizing effects to occur during isoelectric focusing of another muscle enzyme, phosphoglucose isomerase. As in the case of carbonic anhydrase III, the most reduced species of phosphoglucose isomerase was found to dominate the carboxymethyl-Sephadex profile, but the most oxidized species dominated the isoelectric focusing profile [26]. For both enzymes, dithiothreitol cannot prevent the formation of the more oxidized forms during isoelectric focusing but it does inhibit the process of oxidation to some extent.

The carbonic anhydrase $III_3$ subspecies can clearly be identified as reflecting the monomeric form of carbonic anhydrase III on the basis of its elution profile on Sephadex G-75 (Fig. 3C) and its free sulfhydryl content. The identity of carbonic anhydrase $III_2$ is, however, not obvious. One possibility is that it may consist of a subform with an internal disulfide bond. Another is that there is a pH-dependent conformational change induced by the isoelectric focusing process itself, a phenomenon which has been described from a theoretical standpoint by Cann and Stimpson [27]. A third is that both sub-

forms $III_2$ and $III_3$ are fully reduced monomers, one of which has been deamidated. It has been previously demonstrated [8] that incubation of carbonic anhydrases I and II in basic solutions can produce an additional series of increasingly deamidated enzyme species which are successively more acidic. However, the pH at which the deamidated forms of carbonic anhydrases I and II were generated was much higher than that employed here. Furthermore, it must be kept in mind that deamidation is irreversible whereas the various subforms of muscle carbonic anhydrase observed by us are at least partially convertible in either direction.

The possibility of amino acid differences among the subforms within the 1—2% analytical error inherent in the amino acid analysis cannot be ruled out unequivocally but is considered to be unlikely due to the homogeneity of the original sample on carboxymethyl-Sephadex chromatography. Ion-exchange chromatography has been shown to readily separate such variants of carbonic anhydrase I and carbonic anhydrase II [8,11,12,16].

## Discussion

Both of the isoenzymes of carbonic anhydrase found in erythrocytes (Type I and Type II) have electrophoretic variants which may originate in vivo or in vitro. These have been shown to arise primarily from point mutations (for example, refs. 9 and 10) and deamidation [8,13]. In addition, 'conformational' isoenzymes have been postulated [28] as well as various unspecified modifications due to harsh preparative procedures [11,14,15]. Sulfhydryl oxidation, though it has been shown previously to result in the production of pseudoisoenzymes (e.g. ref. 26), has not been considered in the past to be the origin of multiple electrophoretic bands of mammalian erythrocyte carbonic anhydrases because of their low cysteine content [2,3]. Nonmammalian carbonic anhydrases of high sulfhydryl content, however, have been shown to exist as multimers [29—31]. Also, dimerization of a carbonic anhydrase II of low sulfhydryl content has recently been reported for a carbonic anhydrase variant in which a cysteine is substituted for the arginine at position 180, this substituted cysteine being highly reactive compared with the one other normally present. As a result, small amounts of carbonic anhydrase II dimer were formed as well as a protein-glutathione derivative [33].

Because of the frequently automatic conclusion that multiple electrophoretic forms of enzymes can be equated with genetically different enzyme species (for discussion, see ref. 32) it is significant to stress that in the case of muscle carbonic anhydrase (as previously shown for another muscle enzyme [26]) the procedure of isoelectric focusing itself generates multiple forms from starting material which consists of homogeneous and fully reduced monomer. Furthermore, the sulfhydryl-reducing agent dithiothreitol alters the proportions of these species. These modified forms are therefore pseudoisoenzymes [26,33] rather than genetically distinct isoenzymes with different primary structures. Holmes [19,20] has claimed that skeletal muscle contains, in addition to carbonic anhydrase III, the isoenzymes I and II and also that carbonic anhydrase III itself is found in tissues other than muscle [19]. The basis for these conclusions has been that multiple carbonic anhydrase activity bands

were observed on electrophoresis in a buffer system of pH 9.2. It would appear that this claim must be substantiated by more rigorous means in view of the lability of muscle carbonic anhydrase III in basic, oxidizing systems which we have demonstrated here. Although changes in the sulfhydryl oxidation state may not account for the presence of all of the observed subspecies of carbonic anhydrase III, the only other likely alternatives (i.e. the potential formation of conformationally different monomers or the remote possibility of deamidation) would also be at variance with the conclusions of Holmes [19,20]. Since all of the species found in our laboratory are identical in specific $CO_2$ hydratase activity and overall amino acid composition, they must be defined as pseudo-isoenzymes of carbonic anhydrase III. They are neither chemically nor functionally distinct from each other and clearly do not appear to be derivatives of carbonic anhydrases I or II.

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