Bioscience Reports **4**, *573-579 (1984)*
Printed in Great Britain

Activation of mammalian skeletal-muscle carbonic anhydrase III by arginine modification

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(Received 15 June 1984)

Purified carbonic anhydrase isozymes I, II, and III (CA I, CA II, CA III) from various sources were treated with 2,3-butanedione and their bicarbonate dehydration reactions followed. The specific activities of human and bovine CA I and CA II and chicken CA III were not affected by the butanedione treatment, whereas the activities of human, gorilla, and bovine CA III were rapidly activated. These findings suggest that one, or both, of the two arginyl residues which appear to be unique to the active sites of the mammalian CA III isozymes are modified by butanedione.

The zinc metalloenzyme, carbonic anhydrase (carbonate hydrolyase: EC 4.2.1.1), which catalyzes the reversible hydration of CO_2, occurs in reptiles, birds, and mammals as at least three, genetically distinct, isozymes, termed CA I, CA II, and CA III (cf. Tashian et al., 1983; Hewett-Emmett et al., 1984). The specific CO_2 hydrase and esterase activities of these isozymes and their inhibition by heterocyclic or aromatic sulfonamides and anions can vary substantially (cf. Pocker & Sarkanen, 1978; Lindskog, 1982; Sanyal et al., 1982; Maren & Sanyal, 1983). In particular, the CA III isozymes, which are found at their highest levels in skeletal muscle (Holmes, 1977; Register et al., 1978; Carter et al., 1979; Jeffery et al., 1980), at similar or lower levels in mammalian liver (Carter et al., 1981; Shiels et al., 1984), and at very low levels in mammalian erythrocytes (Carter et al., 1984), differ from the CA I and CA II isozymes in a number of ways: (a) their specific CO_2 hydrase activities are considerably lower than those of

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the CA I and CA II isozymes (e.g, human CA III is about 50 and four times lower, respectively, than the human CA II and CA I isozymes (Sanyal et al., 1982), (b) they exhibit extremely low esterase activity toward *p*-nitrophenyl acetate, a substrate which is actively hydrolyzed by the CA I and CA II isozymes (e.g., rabbit CA III has only about 0.06% the activity of rabbit CA II (Register et al., 1978)), (c) the K_m values (CO_2 hydration reaction) of the CA III isozymes are about 4-8 times higher than those of the CA I and CA II isozymes (Sanyal et al., 1982), and (d) the CA III isozymes are remarkably resistant to inhibition by certain sulfonamides (e.g., acetazolamide, ethoxzolamide) which are specific and powerful inhibitors of the CA I and CA II isozymes (Sanyal et al., 1982; Maren et al., 1983; Holmes, 1977; Register et al., 1978). All of these differences suggest that the CA III isozymes may have a function different from, or in addition to, the reversible hydration of CO_2.

In an attempt to gain an insight into the molecular bases for the sometimes striking differences in the activity and inhibition parameters of the CA III isozymes versus those of the CA I and CA II isozymes, we compared 30 homologous amino acid residues from the CA I, CA II, and CA III sequences of several mammalian species (Tashian et al., 1983; Hewett-Emmett et al., 1984), including ox and human, which were assumed to be homologous to those residues which, on the basis of X-ray-crystallographic studies, have been assigned to the active sites of the human CA I and CA II isozymes (Notstrand et al., 1975). This comparison revealed the interesting findings that five of the putative active-site residues of the CA III isozymes were uniquely different from those at homologous positions in the CA I and CA II isozymes, and invariant at these positions in all CA III isozymes examined (Carter et al., 1984; Riordan et al., 1977). Surprisingly, two of these five unique and invariant residues were arginine and one was lysine (see Table 1). No arginyl residues have as yet been found to occur in the active-site regions of the CA I or CA II isozymes (Tashian et al., 1983; Hewett-Emmett et al., 1984).

The present study was undertaken in order to assess the role of arginine residues in the active sites of the CA III isozymes. Chemical modification with a butanedione-borate system which is known to be selective for modifying arginyl residues (Riordan, 1973; Borders et al., 1975; Riordan et al., 1977) was carried out on CA III isozymes purified from the skeletal muscle of human, gorilla, ox, and chicken, as well as human and bovine CA I and CA II isozymes.

Materials and Methods

Enzyme preparations

Human CA I and CA II and bovine CA II isozymes were purified from hemolysates, and bovine CA I from rumen by column chromatography on sulfonamide-bound affinity columns (Osborne & Tashian, 1975). Human and gorilla CA III were prepared from skeletal-muscle extracts by affinity chromatography (Osborne & Tashian, 1975), eluted with a 0-0.2 M KI gradient in 0.005 M Tris-SO_4 buffer, pH 8.7, and further purified by gel filtration on Sephadex G-75 (Hewett-Emmett et

al., 1983). The bovine and chicken CA III isozymes, which bind only very weakly to the sulfonamide column, were initially separated from the firmly bound CA I and CA II isozymes by passing the muscle extract through an affinity column (Osborne & Tashian, 1975), followed by chromatography on DEAE cellulose and further purification on Sephadex G-75. All stages for the purification of the CA III isozymes contained 0.5 mM dithiothreitol as these isozymes are known to dimerize (Register et al., 1978; Carter et al., 1979).

The identities of the carbonic anhydrase isozymes have been confirmed by amino acid sequencing (see Tashian et al., 1983; Hewett-Emmett et al., 1984). The molecular structures of the human and gorilla CA III isozymes are probably very similar, since of the 98 out of 259 residues sequenced at homologous positions for both enzymes (Hewett-Emmett et al., 1984), only two conservative differences were found, at residues 35 (Ser/Thr) and 77 (Ala/Ser).

Reagents

2,3-butanedione (diacetyl) was obtained from Sigma. All other chemicals were analytical grade.

Modification with butanedione

The purified carbonic anhydrase isozymes were treated with butanedione under conditions known to modify arginine residues (Riordan, 1973; Borders et al., 1975; Riordan et al., 1977). The reaction was carried out in the dark in 10 mM 2,3-butanedione at 25°C in 50 mM borate buffer, pH 8.3. The modifications were initiated by adding a freshly prepared solution of 2,3-butanedione in borate buffer to a solution of the enzyme in the same buffer. Aliquots were withdrawn at specific times and measured for HCO_3^- dehydrase activity.

Enzyme assays

The rate of CO_2 formation from $NaHCO_3$ (dehydration reaction) was followed at 2°C by a pH-stat technique (Hansen et al., 1966; Magid, 1968). The reaction mixture contained phosphate buffer (7 or 10 mM), pH 7.1 or 7.3, 0.5 mM EDTA, and 30 mM $NaHCO_3$. The $NaHCO_3$ solutions were freshly prepared daily. The product, CO_2, was rapidly removed by bubbling pure N_2 through the reaction chamber. The rate of the dehydration reaction is determined as the amount of titrant (1.0 M H_2SO_4) added per unit time to maintain a constant pH. The initial rate of the reaction was calculated from the amount of acid added in a 30-s period, and the carbonic anhydrase activity determined as the difference between the observed initial rate of the enzyme-catalyzed reaction and the initial rate of the spontaneous dehydration reaction (Magid, 1968).

Results

A pronounced activation of the bicarbonate dehydration reaction was found to result from the treatment of human, gorilla, and bovine CA III with 2,3-butanedione (Figs. 1,2); under similar experimental conditions, the human bovine CA I and CA II isozymes were not

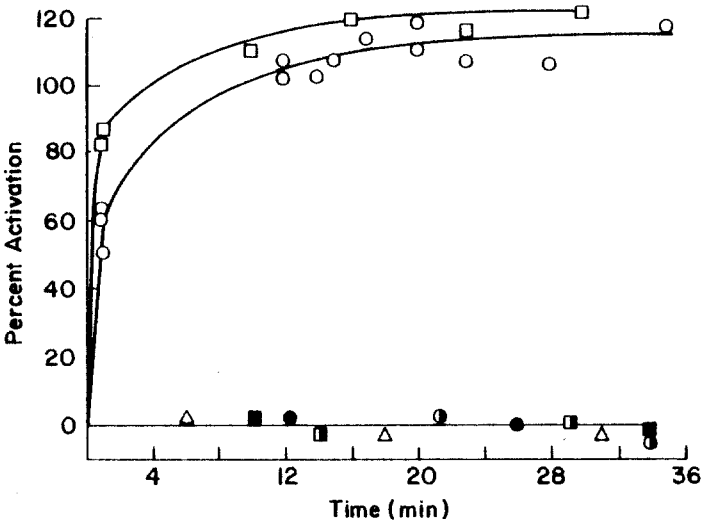

Fig. 1. Effect of 2,3-butanedione on the bicarbonate dehydration reaction of human CA I (◨), human CA II (■), gorilla CA III, (□) bovine CA I (◑), bovine CA II (●), bovine CA III (○), and chicken CA III (△). The enzymes (10 μM) were treated with 2,3-butanedione (10 mM) in 50 mM borate buffer, pH 8.3, at 25°C. The assay mixtures contained 10 mM phosphate buffer, pH 7.3, 0.5 mM EDTA, and 30 mM $NaHCO_3$; final enzyme concentrations: CA Is 0.01 μM, CA IIs 0.001 μM, and CA IIIs 0.05 or 0.1 μM. The control reactions were carried out without butanedione, and the percent activation calculated as the ratio of activity in the activated and control reactions multiplied by 100. Butanedione alone (25 mM) had no effect on the uncatalyzed dehydration reaction.

activated (Fig. 1). As shown in Fig. 2, the treatment of human CA III (14 μM) with 0.25 mM butanedione activated its dehydration reaction approx. 25% in 10 min, and about a 30% activation was produced after incubating the enzyme for only 2 min with 2.5 mM butanedione. This activation could be readily reversed when the butanedione-modified CA III isozymes were diluted with 0.1 M Tris-SO_4 buffer, pH 7.0 (data not shown). This reversibility in the absence of borate is a known characteristic of the butanedione modification of arginine residues (Riordan 1973; Borders & Riordan, 1975; Riordan et al., 1977).

Discussion

The enhancement of the bicarbonate dehydration reaction of the human, gorilla, and bovine CA III isozymes by what appears to be a modification of an active-site residue(s) was unexpected because the chemical modification of arginine residues in the active sites of enzymes has been shown to result in a usually rapid inactivation in its

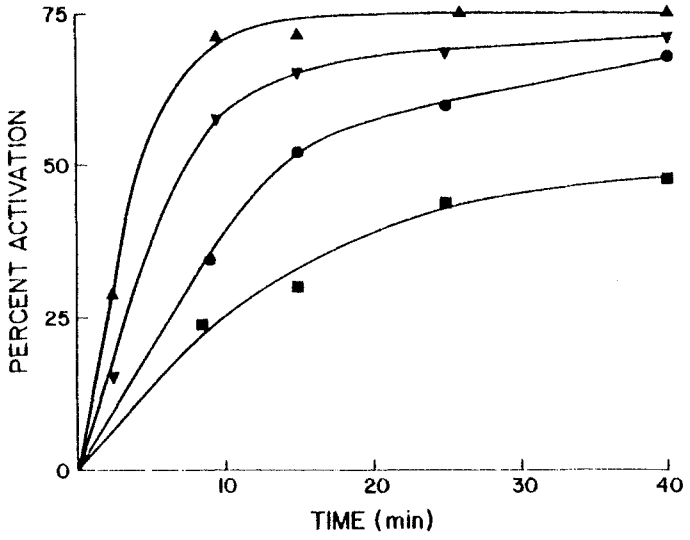

Fig. 2. Effect of increasing amounts of 2,3-butanedione on the bicarbonate dehydration reaction of human CA III. Butanedione concentrations: 0.25 mM (■), 0.5 mM (●), 1.0 mM (▼), 2.5 mM (▲). The enzyme (14 μM) was treated with butanedione in 50 mM borate buffer, pH 8.3, at 25°C. The assay was carried out in 7 mM phosphate buffer, pH 7.1, 0.5 mM EDTA, and 30 mM $NaHCO_3$; the final enzyme concentration was 0.056 μM.

specific activity (cf. Riordan et al., 1977).

The rapid activation of the dehydration reaction by treatment with 2,3-butanedione suggests that one, or both, of the arginyl residues (positions 67 and 91) assumed to reside in the active-site regions of these enzymes has been modified (Table 1). Preliminary tryptic digestion and amino acid sequence studies on human CA III treated with butanedione indicate that the arginine residues at positions 67 and 91 have been modified (D. Hewett-Emmett and C.R. Chegwidden, unpublished work). Other arginine residues which may be unique to the CA III isozymes occur at positions 80 and 189; however, because these residues are located on the surface of human CA I and CA II away from the active site (Notstrand et al., 1975), it appears unlikely that the experimental conditions of the present study would favor their rapid modification. Just why the probable modification of Arg-91 and/or Arg-67 results in an activation of the dehydration reaction remains obscure. However, it does suggest that one or both of these arginine residues are important to the normal catalytic mechanism of the CA III isozymes of mammals. In theory, if the 'true' substrate for CA III were used in assaying its activity, then blocking an active-site arginyl residue might be expected to result in an inactivation, rather than an activation, of its 'normal' activity (Riordan et al., 1977). In this respect, the fact that a low acid phosphatase activity has been demonstrated for the CA III isozymes of rabbit, ox, and pig, but not their CA I and CA II isozymes (Koester et al., 1981),

Table 1. Basic residues at the active site of human, gorilla, and ox CA III isozymes compared to residues which are different at these positions in the CA I and CA II isozymes*

Isozyme	Residue**		
	64	67	91
CA I	His (H,O)	His (H,O)	Phe (H,O)
CA II	His (H,O)	Asn (H,O)	Ile (H) Val (O)
CA III	Lys (H,G,O)	Arg (H,G,O)	Arg (H,G,O)

*Positions of active-site residues based on the three-dimensional structures of human CA I and CA II isozymes (Notstrand et al., 1975).

**From Hewett-Emmett et al. (1984) and references therein; human (H), gorilla (G), ox (O); numbering of CA II and CA III residues based on homology with human CA I sequence.

suggests that the arginyl residues of these CA III isozymes could bind substrates with negative charges (e.g., phosphate groups) and/or phosphorylated coenzymes (Riordan et al., 1977). This indicates that in addition to its CO_2 hydration activity, mammalian CA III may also function in an as-yet-unknown phosphorolytic or phosphoryl-transfer reaction. It would obviously be of interest to study the effect of arginine modification on the acid phosphatase activity of the CA III isozymes.

Limited sequence studies of the chicken CA III indicate that either arginine or lysine is present at positions 67 and 91 (Hewett-Emmett et al., 1984). The apparent absence of activation for the chicken CA III isozyme suggests that if arginyl residues occur at positions 67 and 91, their modification does not influence the dehydration reaction, or that they are shielded from ready modification.

Acknowledgements

One of us (R.E.T.) gratefully acknowledges the cordial support of Professor Martin Ottesen as well as the staff of the Chemistry Department of the Carlsberg Laboratory during his stay there. He also thanks Dr. B.O. Turbeck for the use of the facilities of the Clinical Chemistry Laboratory (Kommunehospitalet, Copenhagen). The authors thank Ya-Shiou L. Yu and Dr. David Hewett-Emmett for preparing the enzyme samples used in this study. We are also especially grateful to Dr. David Hewett-Emmett for his many welcome suggestions throughout this investigation. This work was supported in part by U.S. Public Health Service grant GM-24681 to R.E.T.

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