Biochem. J. (1982) **205**, 559–566
Printed in Great Britain

Carbonic anhydrase C in white-skeletal-muscle tissue

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(*Received 13 April 1982/Accepted 2 June 1982*)

We investigated the activity of carbonic anhydrase in blood-free perfused white skeletal muscles of the rabbit. Carbonic anhydrase activities were measured in supernatants and in Triton extracts of the particulate fractions of white-skeletal-muscle homogenate by using a rapid-reaction stopped-flow apparatus equipped with a pH electrode. An average carbonic anhydrase concentration of about 0.5 μM was determined for white skeletal muscle. This concentration is about 1% of that inside the erythrocyte. Some 85% of the muscle enzyme was found in the homogenate supernatant, and only 15% appeared to be associated with membranes and organelles. White-skeletal-muscle carbonic anhydrase was characterized in terms of its Michaelis constant and catalytic-centre activity (turnover number) for CO_2 and its inhibition constant towards ethoxzolamide. These properties were identical with those of the rabbit erythrocyte carbonic anhydrase C, suggesting that a type-C enzyme is present in white skeletal muscle. Affinity chromatography of muscle supernatant and of lysed erythrocytes showed that, whereas rabbit erythrocytes contain about equal amounts of carbonic anhydrase isoenzymes B and C, the B isoenzyme is practically absent from white skeletal muscle. Similarly, ethoxzolamide-inhibition curves suggested that white skeletal muscle contains no carbonic anhydrase A. It is concluded that white skeletal muscle contains essentially one carbonic anhydrase isoenzyme, the C form, most of which is probably of cytosolic origin.

Skeletal muscle has long been thought to be one of the few tissues in which carbonic anhydrase is not present (Roughton, 1935; Maren, 1967). Roughton (1935) concluded that a catalysis of the interconversion

$$CO_2 \rightleftharpoons HCO_3^-$$

by carbonic anhydrase might even be unfavourable for the removal of metabolically produced CO_2 from muscle cells, since the cell membranes can be expected to be highly permeable for CO_2 but not for HCO_3^-. More recently, evidence has been accumulated indicating that carbonic anhydrase does occur in skeletal-muscle tissue and may serve to accelerate the release of CO_2 from muscle tissue and its uptake by blood. Kawashiro & Scheid (1976) showed that a facilitation of CO_2 transport occurs across layers of skeletal-muscle tissue, and it is probable that a muscle carbonic anhydrase is responsible for this enhancement of CO_2 transport. Effros & Weissman (1979) concluded that in skeletal muscle a carbonic anhydrase is accessible from the capillary bed that speeds up CO_2 exchange between blood and tissue. Zborowska-Sluis *et al.* (1974) reported that the space of distribution of HCO_3^- in the blood-free hind limb of dogs is decreased by the specific carbonic anhydrase inhibitor acetazolamide. Thus functional studies show that significant carbonic anhydrase activities must be present in skeletal muscle.

Koester *et al.* (1977) and Carter *et al.* (1978) isolated a new carbonic anhydrase isoenzyme, type A, from red skeletal muscle. Carbonic anhydrase A has an extremely low specific activity and is highly acetazolamide-resistant. It thus appears questionable whether the above-mentioned functional effects of acetazolamide treatment, which were in part achieved with relatively low doses of the inhibitor, can be attributed to the carbonic anhydrase A of red-skeletal-muscle fibres. We have therefore investigated the possibility that a high-activity acetazolamide-sensitive carbonic anhydrase is also present in skeletal muscle, and report in the present paper on the activity and properties of such an enzyme in white skeletal muscle.

0306-3275/82/090559-08$01.50/1

Methods

Preparation of skeletal-muscle tissue homogenate

All experiments were performed with skeletal-muscle tissue from the hind limbs of adult rabbits weighing 2–3 kg. The tissue was rendered blood-free by perfusion with dextran solution. For assessment of contamination of the perfused muscles with erythrocyte carbonic anhydrase, the erythrocytes of the rabbits' circulating blood were labelled with ^{51}Cr before the artificial perfusion was started. Approx. 20 ml of the rabbits' erythrocytes was exchanged for rabbit erythrocytes that had been loaded with ^{51}Cr as described by Gray & Sterling (1950). After 1 h, catheters were inserted into the abdominal aorta and the caval vein, through which the hind limbs were perfused with a 6% (w/v) solution of dextran (Dextran 60000; Knoll A.G., Ludwigshafen, West Germany) equilibrated with CO_2/O_2 (1:9) at 37°C. The perfusion was continued until the outflowing perfusion fluid was free of erythrocytes, usually for several hours. This long perfusion time, during which the hind limbs were almost continuously moved or the muscles were massaged, proved to be necessary to achieve a nearly complete removal of erythrocytes from the tissue. The specific radioactivity of the muscles perfused in this way was 1/20000th to 1/200000th of that of the erythrocytes in the circulating blood before the perfusion was started. This implies that erythrocytes occupy a volume fraction of less than 0.0001 of the perfused muscles (for details see Gros *et al.*, 1980).

At the end of the perfusion the muscles of the hind limbs were excised and cut into pieces approx. 5 mm × 5 mm × 5 mm in size. Red-skeletal-muscle and white-skeletal-muscle pieces were separated according to appearance. Only white-skeletal-muscle pieces free of any reddish or brownish tinge were collected, frozen in liquid N_2 and homogenized in a Mikro-Dismembrator (Braun, Melsungen, West Germany). After thawing, the homogenate was centrifuged at 30000 *g* for 1 h without prior addition of buffer. The supernatant was decanted and used for determinations of carbonic anhydrase activity after suitable dilution. In some cases this supernatant was re-centrifuged at 100000 *g* for 1 h, but no significant changes in carbonic anhydrase activity resulted. Haem concentration in these white-skeletal-muscle supernatants was $\leqslant 3\,\mu M$, whereas it was $>30\,\mu M$ in identically prepared red-skeletal-muscle supernatants. This indicates that the present preparation of white skeletal muscle was largely free of haemoglobin as well as myoglobin. To assess carbonic anhydrase associated with the particulate fractions of white-skeletal-muscle tissue, the pellet obtained by centrifugation of the homogenate was again frozen and thawed, then resuspended in 4 vol. of 0.9% NaCl and again centrifuged at 30000 *g* for 1 h. The supernatant was decanted and the procedure repeated five to seven times until no carbonic anhydrase activity was detectable in the supernatant washing solution. The final pellet was incubated in 8 vol. of buffer containing 10 mM-Tris/HCl, 0.3 M-sucrose and 0.5% Triton X-100 (pH 8.0) and stirred for 2 days. The suspension was then centrifuged at 30000 *g* for 1 h. The supernatant was concentrated in an Amicon unit equipped with a PM10 membrane (Amicon, Witten, West Germany), dialysed against the desired buffer, a step that also removed most of the Triton, and was then used for measurements of carbonic anhydrase activity.

Preparation of lysed rabbit erythrocytes

Lysed rabbit erythrocytes were prepared by twice washing rabbit erythrocytes in 0.9% NaCl, lysing them in 3 vol. of distilled water, then adding sufficient NaCl to restore the ionic strength to 0.15 M and centrifuging at 30000 *g* for 30 min to remove erythrocyte 'ghosts'. For determinations of carbonic anhydrase activity, small volumes of the clear lysate were added to the desired buffers.

Determination of carbonic anhydrase activity

Carbonic anhydrase activities were determined by using a rapid-reaction stopped-flow apparatus equipped with a pH-sensitive glass electrode as previously described (Crandall *et al.*, 1971; Gros *et al.*, 1976). Muscle extracts or haemoglobin solutions were appropriately diluted in CO_2-free buffer and rapidly mixed in this apparatus with CO_2 solutions that had been prepared by equilibrating unbuffered 0.15 M solutions of NaCl or NaF with various CO_2/N_2 mixtures. The reaction occurring between the (acid) CO_2 solution and the (nearly neutral) buffer solution is a hydration of CO_2, which is accompanied by a release of protons. The resulting pH change was recorded and the initial slope of the pH trace, together with the buffer capacity of the solution, were used to calculate the initial rate of the CO_2-hydration reaction. By subtracting the independently determined uncatalysed hydration rate, the rate of CO_2 hydration due to carbonic anhydrase catalysis was obtained. The catalysed reaction rate was usually at least 10 times the uncatalysed rate.

The determinations of enzyme activity were done in either 30 mM-imidazole/HCl buffer containing 0.15 M-NaCl or 10 mM-sodium Veronal buffer containing 0.15 M-NaF. Enzymes were characterized in terms of their inhibition constants towards ethoxzolamide, K_i, their catalytic-centre activity (turnover number), $k_{cat.}$, and their Michaelis constant, K_m. Temperature was 25°C throughout.

Inhibition constants

These were derived from measurements of initial catalysed CO_2-hydration rates at various concen-

trations of the specific carbonic anhydrase inhibitor ethoxzolamide (Sigma, Munich, West Germany). Results of typical experiments are shown in Fig. 1. K_i values were derived from such data as follows. To a set of data pairs of initial catalytic rate, $v_{cat.}$, and (total) ethoxzolamide concentration, $[I_{tot.}]$, a second-order polynomial of the form

$$v_{cat.}[I_{tot.}] = a + b \cdot v_{cat.} + c \cdot v_{cat.}^2 \quad (1)$$

was fitted. Assuming non-competitive inhibition (Maren *et al.*, 1960; Leibman & Alford, 1960; Maren & Wiley, 1968) it can be shown that the coefficients obtained from a second-order regression analysis have the following meanings:

$$a = k_{cat.}[E_{tot.}][CO_2]K_i/(K_m + [CO_2]) \quad (2)$$

$$b = [E_{tot.}] - K_i \quad (3)$$

$$c = -(K_m + [CO_2])/([CO_2]k_{cat.}) \quad (4)$$

where $k_{cat.}$ is the catalytic-centre activity of the enzyme, $[E_{tot.}]$ its total concentration, $[CO_2]$ the initial concentration of CO_2, and K_m the Michaelis constant of the enzyme for CO_2. By using the fitted values of a, b and c, the total enzyme concentration can be calculated from

$$[E_{tot.}] = b/2 + \sqrt{b^2/4 - a \cdot c}$$

and the inhibition constant can be obtained from

$$K_i = -a \cdot c/[E_{tot.}]$$

Thus inhibition experiments of the type shown in Fig. 1 allowed us to estimate both $[E_{tot.}]$ and K_i. In addition, $k_{cat.}/(K_m + [CO_2])$ as a measure of the specific activity of the enzyme could be derived from the value of the coefficient c and the known CO_2 concentration. It may be noted that this kind of analysis, when a high-affinity inhibitor such as ethoxzolamide is being used, yields reliable estimates of the coefficients b and c, but the coefficient a is obtained with less accuracy because, owing to the low K_i value, it is relatively small. This implies that the values of $[E_{tot.}]$ (note that under the present conditions $a \cdot c \ll b^2/4$) and the specific activity $k_{cat.}/(K_m + [CO_2])$ are generally determined with greater precision than K_i. Another possible source of error for K_i results from the disturbance of the equilibrium between enzyme and inhibitor that is caused by the 1:1 mixing of the ethoxzolamide-incubated diluted supernatant with the enzyme- and inhibitor-free CO_2 solution. After the mixing process in the stopped-flow apparatus the equilibrium between enzyme and inhibitor will be re-established by partial dissociation of the enzyme–inhibitor complex. Kinetic experiments with other sulphonamides (Kernohan, 1966; Lindskog *et al.*, 1971) indicate that this reaction may be slow, with a half-time similar to that of the uncatalysed CO_2 hydration, and thus not complete in the initial stages of the catalysed CO_2 hydration observed in the present work. The degree of enzyme inhibition during the initial reaction phase may therefore be greater than expected from the total concentration of inhibitor present after mixing. It can be shown that this can lead to an underestimation of K_i by at most 50% of the true value but does not affect the estimated values of the total enzyme concentration, the specific activity or $k_{cat.}$.

Michaelis constants and catalytic-centre activities

These were determined from measurements of catalytic rates at different CO_2 concentrations. Plotting $1/v_{cat.}$ versus $1/[CO_2]$ yielded straight lines (see Fig. 2), which allowed us to calculate $V_{max.} = [E_{tot.}]k_{cat.}$ and the Michaelis constant K_m. Inserting values of K_m and $[CO_2]$ together with the value of c (as obtained from an inhibition experiment) into eqn. (4) yielded the catalytic-centre activity $k_{cat.}$.

Affinity chromatography

Carbonic anhydrase isoenzymes B and C were separated by affinity chromatography as described by Siegmund *et al.* (1976). *p*-Aminomethylbenzenesulphonamide (Serva, Heidelberg, West Germany) was coupled to CNBr-activated Sepharose (Pharmacia, Freiburg, West Germany). The affinity column (1.5 cm × 50 cm) was pre-equilibrated with 0.1 M-Tris/HCl buffer, pH 8.9. Samples were dialysed against the same buffer before application to the column. Non-specifically bound proteins were desorbed with 0.1 M-Tris/1 M-KCl buffer, pH 8.9. Carbonic anhydrase B was eluted with 0.1 M-KH_2PO_4/1 M-KCl buffer, pH 6.7, and carbonic anhydrase C was eluted with 0.1 M-acetate/1 M-KCl buffer, pH 5.5. Fractions containing isoenzymes B and C respectively were pooled, concentrated and extensively dialysed against the desired buffer before use for measurements of carbonic anhydrase activity.

Results

Inhibition by ethoxzolamide

Fig. 1 shows a comparison of inhibition experiments performed with supernatant from white-skeletal-muscle homogenate (dilution after mixing 1 vol. of supernatant and 9 vol. of 30 mM-imidazole/0.15 M-NaCl buffer, pH 7.0) and with lysed rabbit erythrocytes (dilution after mixing 1 vol. of packed erythrocytes and 389 vol. of 30 mM-imidazole/0.15 M-NaCl buffer, pH 7.0). The initial CO_2 concentration after mixing was 2.3 mM, and the temperature was 25°C. Analysis of the data in terms of eqns. (1)–(4) yielded the inhibition constants and specific activities given in Table 1. It is apparent that

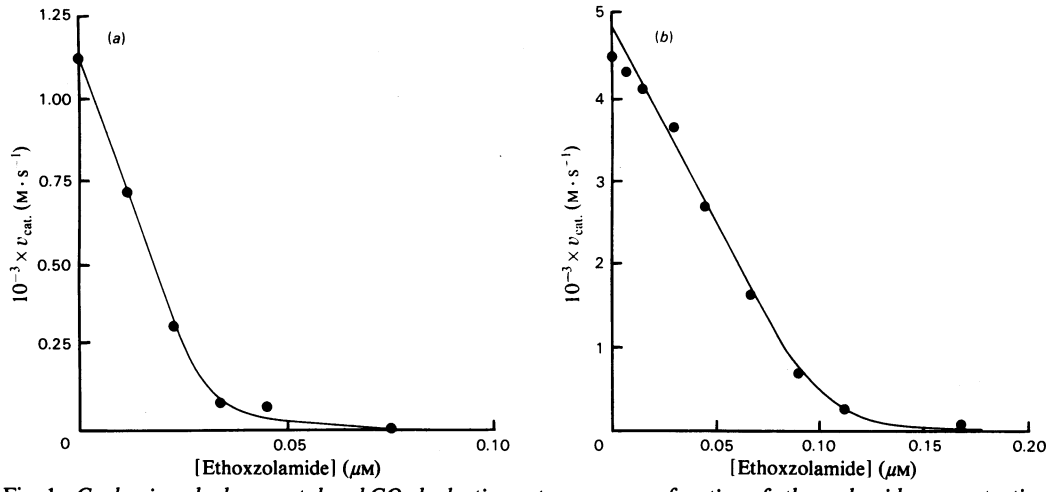

Fig. 1. *Carbonic anhydrase-catalysed CO_2-hydration rates, $v_{cat.}$, as a function of ethoxzolamide concentration*
The temperature was 25°C, and rates were measured in 30mM-imidazole/0.15M-NaCl buffer, pH 7.0, the initial CO_2 concentration being 2.3mM. For full experimental details see the text. Catalysed rates were obtained by subtracting independently determined uncatalysed rates from the total CO_2-hydration rates. The uncatalysed rates yielded an average forward rate constant, k_{CO_2}, of 0.036s^{-1}. (*a*) Supernatant of white skeletal muscle, diluted 1:10 in imidazole/NaCl buffer; $K_i = 0.6$nM. (*b*) Lysed erythrocytes, diluted 1:390 in imidazole/NaCl buffer; $K_i = 0.7$nM. The data points were used to calculate the values of K_i, $[E_{tot.}]$ and $k_{cat.}/(K_m + [CO_2])$ shown in Table 1 from a fit to eqn. (1). The lines are theoretical curves calculated with the fitted constants ($R^2 \geqslant 0.96$).

Table 1. *Inhibition constant, K_i, towards ethoxzolamide, enzyme concentration, $[E_{tot.}]$, specific activity, $k_{cat.}/(K_m + 0.0023$M), Michaelis constant, K_m, for CO_2 and catalytic-centre activity, $k_{cat.}$, of the carbonic anhydrase in the supernatant of white-skeletal-muscle homogenate and in erythrocytes*
The temperature was 25°C, and the initial CO_2 concentration for the inhibition experiments was 2.3mM. For full experimental details see the text. The data indicate that in both tissues only carbonic anhydrase isoenzyme C is active in the presence of 0.15M-Cl^- and 30mM-imidazole.

	White-skeletal-muscle supernatant (30mM-imidazole/0.15M-Cl^-, pH 7.0)	Erythrocytes (30mM-imidazole/0.15M-Cl^-, pH 7.0)
K_i (nM)	0.6 (±0.2)	0.7
$[E_{tot.}]$ (μM)	0.40 (±0.11)	36 (±4)
$k_{cat.}/(K_m + 0.0023$M) ($M^{-1} \cdot s^{-1}$)	1.6 (±0.1) × 10^7	1.9 (±0.3) × 10^7
K_m (mM)	3.2 (±0.4)	4.1 (±1.3)
$k_{cat.}$ (s^{-1})	90000 (±8000)	120000 (±30000)

both constants are almost identical for the muscle and the erythrocyte enzymes.

Inhibition experiments like those shown in Fig. 1 were performed with purified preparations of erythrocyte carbonic anhydrases B and C and muscle carbonic anhydrase (i.e. type C, see below), as obtained by affinity chromatography of lysed erythrocytes and of white-skeletal-muscle supernatant respectively. To avoid the almost complete inhibition of the B isoenzyme by 0.15M-Cl^- (Maren *et al.*, 1976) and its partial inhibition by imidazole (Khalifah, 1971), these experiments were performed in 0.15M-NaF with 10mM-Veronal as buffer. The pH was 7.5, the CO_2 concentration was 2.3mM and the temperature was 25°C. K_i values and specific activities for muscle isoenzyme C and erythrocyte isoenzyme C were calculated from the data and are shown in Table 2. Although the absolute values of K_i and $k_{cat.}/(K_m + [CO_2])$ for these purified preparations deviate significantly from those obtained in dilute haemoglobin solutions and muscle supernatants (see the Discussion section), they indicate clearly that the properties of the white-skeletal-muscle isoenzyme closely agree with the properties of the erythrocyte C isoenzyme but differ markedly from those of the erythrocyte B isoenzyme.

Kinetic properties

Fig. 2 shows Lineweaver–Burk plots for diluted white-skeletal-muscle supernatant and for diluted lysed erythrocytes. The CO_2 concentrations were varied between 1 and 10mM in the presence of

Table 2. *Ethoxzolamide inhibition constants, K_i, and specific activities, $k_{cat.}/(K_m + 0.0023\,M)$, with standard errors, for purified carbonic anhydrase isoenzymes of white skeletal muscle and erythrocytes*

The temperature was 25°C and the initial CO_2 concentration was 2.3 mM. For full experimental details see the text.

	Isoenzyme C (white skeletal muscle) (10 mM-Veronal/0.15 M-F^-, pH 7.5)	Isoenzyme C (erythrocytes) (10 mM-Veronal/0.15 M-F^-, pH 7.5)	Isoenzyme B (erythrocytes) (10 mM-Veronal/0.15 M-F^-, pH 7.5)
K_i (M)	$4\ (\pm 1) \times 10^{-9}$	$6\ (\pm 3) \times 10^{-9}$	$54\ (\pm 13) \times 10^{-9}$
$k_{cat.}/(K_m + 0.0023\,M)$ ($M^{-1} \cdot s^{-1}$)	$3.6\ (\pm 0.3) \times 10^7$	$3.3\ (\pm 0.7) \times 10^7$	$0.9\ (\pm 0.5) \times 10^7$

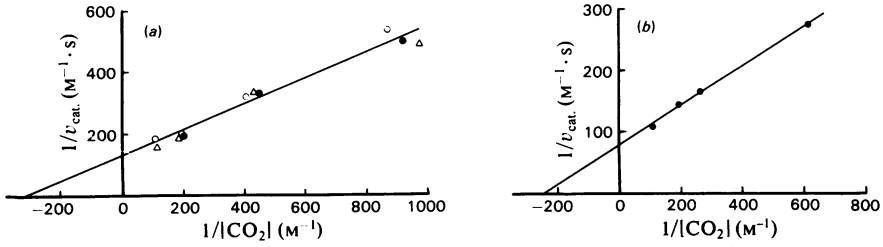

Fig. 2. *Lineweaver–Burk plots of the reciprocals of carbonic anhydrase-catalysed rates ($1/v_{cat.}$) versus reciprocals of the initial substrate concentrations ($1/[CO_2]$)*

The temperature was 25°C, and rates were measured in 0.15 M-NaCl with 30 mM-imidazole buffer (●) or *N*-methylimidazole buffer (△) or phosphate buffer (○), all at pH 7.0. For full experimental details see the text. (*a*) Supernatant of white skeletal muscle, diluted in 0.15 M-NaCl with buffer; $K_m = 3.2$ mM. (*b*) Lysed erythrocytes, diluted in imidazole/NaCl buffer; $K_m = 4.1$ mM. The data points were used to calculate linear regressions ($r > 0.99$), which yielded the Michaelis constants, K_m, which are also shown in Table 1. The lines shown represent the computed regression equations.

0.15 M-NaCl and 30 mM-imidazole buffer (●), the pH was 7.0 and the temperature was 25°C. For both solutions straight lines with correlation coefficients $r > 0.99$ were obtained. From the regression coefficients similar K_m values of 3.2 mM for the muscle enzyme and 4.1 mM for the erythrocyte enzyme were calculated. Fig. 2(*a*) shows that imidazole has no marked effect on the kinetic properties of the muscle enzyme: replacement of imidazole by *N*-methylimidazole (△) or phosphate (○) did not alter the catalytic rates and led to almost identical values of K_m and $V_{max.}$.

From these K_m values and the specific activities $k_{cat.}/(K_m + 0.0023\,M)$ as derived from inhibition curves, the catalytic-centre activities were estimated to be 90000 s^{-1} for the white-skeletal-muscle enzyme and, not significantly different, 120000 s^{-1} for the erythrocyte carbonic anhydrase. We conclude that there is good agreement between the kinetic properties of the muscle enzyme and the erythrocyte carbonic anhydrase (whose activity under the present conditions of 0.15 M-Cl^- is constituted solely by the type-C isoenzyme; see the Discussion section).

Isoenzyme pattern

To study the distribution of carbonic anhydrase isoenzymes, white-skeletal-muscle supernatant and lysed erythrocytes were subjected to affinity chromatography. Determinations of carbonic anhydrase activity on the peaks of isoenzymes B and C as eluted from the affinity column showed that about 20% of the total carbonic anhydrase activity in erythrocytes is contributed by isoenzyme B and 80% by isoenzyme C when the inhibitory Cl^- is absent. With the specific activities of isoenzymes B and C as given in Table 2, this leads to a molar ratio of 1:1 for [B isoenzyme]/[C isoenzyme] in rabbit erythrocytes, a value in excellent agreement with the findings reported by McIntosh (1970). In contrast, in white-skeletal-muscle supernatant only approx. 1% of the total carbonic anhydrase activity is due to isoenzyme B and 99% originates from isoenzyme C.

Similarly, the carbonic anhydrase isoenzyme A, which has been shown to occur in high concentrations in red skeletal muscle (Koester *et al.*, 1977; Siffert *et al.*, 1980), appears to be absent from white skeletal muscle. This is evident from Fig. 1(*a*), which shows that carbonic anhydrase activity in white-skeletal-muscle supernatant is almost completely inhibited at 0.1 μM-ethoxzolamide, whereas inhibitor concentrations of 0.4 mM for acetazolamide (Koester *et al.*, 1977) and 60 μM for ethoxzolamide (W. Siffert & G. Gros, unpublished work) are necessary to achieve 50% inhibition of carbonic anhydrase A. We conclude that carbonic anhydrase

isoenzymes A and B are practically absent from white-skeletal-muscle cytosol of the rabbit and that the carbonic anhydrase activity detected there is almost solely due to the isoenzyme C. In rabbit erythrocytes, on the other hand, isoenzymes B and C are both present to an appreciable extent.

Carbonic anhydrase associated with particulate fractions

Carbonic anhydrase activity in the particulate fractions was determined by using the pellet obtained by centrifugation of white-skeletal-muscle homogenate. After freezing and thawing and subsequent extensive washing of this pellet to remove all free carbonic anhydrase (see above), carbonic anhydrase could be extracted by treating it with Triton. Activity measurements in this extract showed that about 10–15% of the total carbonic anhydrase activity of white-skeletal-muscle tissue seems to be associated with the particulate fractions. No attempt was made to identify the subcellular structures responsible for this enzyme activity, and it should be noted that some activity may also have been extracted from unbroken cells rather than from membranes and organelles. The enzyme was not characterized in terms of inhibition constants or kinetic properties, but it was found that the carbonic anhydrase of the pellet extract was almost fully inhibited by 0.2 μM-ethoxzolamide, which rules out isoenzyme A as the form present in the particulate fractions.

Discussion

Properties of rabbit erythrocyte carbonic anhydrases

The properties of erythrocyte carbonic anhydrase shown in Table 1 are those of the C isoenzyme, since the B isoenzyme can be expected to be fully inhibited under conditions of 0.15 M-Cl^- (Maren *et al.*, 1976). This is confirmed by the excellent fit achieved by applying eqn. (1) (which uses only a single value of K_i and $k_{cat.}$) to the inhibition data of Fig. 1(*b*), and thus suggests the presence of only one active isoenzyme. Comparisons of the present findings are therefore made below with other data for various C isoenzymes. Literature data on rabbit carbonic anhydrase suitable for comparison exist only for K_m. McIntosh (1970) reported for the erythrocyte C isoenzyme at 45 mM-Cl^-/5 mM-phosphate, pH 7.0 and 0°C a K_m value of 6.2 mM, which agrees reasonably well with the present value of 4 mM, obtained at 0.15 M-Cl^-, pH 7.0 and 25°C, if K_m can be assumed to be independent of temperature (Sanyal & Maren, 1981). The K_i for ethoxzolamide shown in Table 1 is of the order of that reported by Maren (1967) for the human C isoenzyme. It is 30–40 times lower than the K_i value that we obtained for acetazolamide with the same erythrocyte lysate under identical conditions (24 nM). This latter value is in excellent agreement with the K_i for acetazolamide found by Skipski & Scott (1980) for the rabbit C isoenzyme and by several workers for the C isoenzymes of other species (see Maren, 1967).

The catalytic-centre activity of 120000 s^{-1} given in Table 1 is clearly indicative of a type-C isoenzyme. It is, however, about only one-quarter of the $k_{cat.}$ value obtained by Khalifah (1971) for the human C isoenzyme at pH 7.0 in the absence of inhibitory anions such as Cl^-. Part of this discrepancy is probably due to the presence of 0.15 M-Cl^- in our erythrocyte lysates, a Cl^- concentration that is known to cause approx. 40% inhibition of the human C isoenzyme (Maren *et al.*, 1976). Indeed, the specific activity, $k_{cat.}/(K_m + [CO_2])$, given in Table 2 for the isolated erythrocyte C isoenzyme, is almost twice the value found in the crude lysate (see Table 1). This higher value is very probably due to the absence of inhibitory anions and buffers, and to the somewhat higher pH value in the purified enzyme preparation as compared with the crude lysate.

The rabbit erythrocyte B isoenzyme exhibits a specific activity one-quarter of that of the C isoenzyme under the present conditions. At pH 8.2 and 0°C, Koester *et al.* (1977) reported an even more pronounced difference in specific activity of rabbit B and C isoenzymes, that of the former being one-seventh of that of the latter. Another marked difference between the B and C isoenzymes is their affinity towards ethoxzolamide. The K_i of the erythrocyte B isoenzyme is almost 10 times that of the C isoenzyme. This finding is at variance with Maren's (1967) observation of identical K_i values of human B and C isoenzymes for ethoxzolamide, but in keeping with the properties of many other sulphonamides, which show an approximately 10-fold greater affinity towards the C isoenzymes as compared with the B isoenzymes.

It may be noted that the inhibition constants obtained for the isolated C isoenzymes (Table 2) are significantly higher than those obtained for the same isoenzyme in the crude erythrocyte and white-skeletal-muscle preparations (Table 1). At least part of this discrepancy seems to be due to the difference in pH between the crude preparations (pH 7.0) and the purified enzyme solutions (pH 7.5). This is suggested by the finding of a 3-fold increase in K_i when the pH of the erythrocyte lysate was raised to 7.5.

Carbonic anhydrase of white-skeletal-muscle tissue of the rabbit

The present results show that, in contrast with earlier conclusions (Roughton, 1935; Maren, 1967),

carbonic anhydrase C is present in white-skeletal-muscle tissue. Most of this enzyme appears to be cytosolic, and only a minor part may be associated with membranes or organelles. The identification of this carbonic anhydrase as a type C isoenzyme derives from the following findings. (*a*) Inhibition constants and kinetic properties of the carbonic anhydrase of crude white-skeletal-muscle extracts are identical with those of lysed erythrocytes (Figs. 1 and 2 and Table 1); the latter are known to contain only one active isoenzyme, carbonic anhydrase C. (*b*) The catalytic-centre activity of the muscle carbonic anhydrase (Table 1) is significantly higher than those reported for B isoenzymes in the presence (McIntosh, 1970; Maren *et al.*, 1976) and even in the absence of inhibitory ions (Khalifah, 1971; Sanyal & Maren, 1981). (*c*) Affinity chromatography of white-skeletal-muscle homogenates yields a type-C isoenzyme, but only insignificant amounts of carbonic anhydrase B. Furthermore, the present data show that the carbonic anhydrase isoenzyme A is absent from white-skeletal-muscle tissue. This follows from the full inhibition that is obtained with 0.1 μM-ethoxzolamide (Fig. 1), a concentration that is known to leave carbonic anhydrase A almost unaffected (Koester *et al.*, 1977; Siffert *et al.*, 1980). We conclude that white-skeletal-muscle tissue of the rabbit contains essentially one carbonic anhydrase isoenzyme, the C form.

A crucial assumption, that the carbonic anhydrase that we find in muscle homogenate is not due to erythrocytes still present in the muscles after perfusion, forms the basis of this conclusion and deserves discussion. The 100-fold higher concentration of carbonic anhydrase that we find in erythrocytes than in muscle homogenate (Table 1) implies that the presence of 1% (v/v) erythrocytes in the perfused muscle could produce the C-isoenzyme activities that we find in the white-skeletal-muscle supernatants. There are three lines of evidence to suggest that erythrocyte carbonic anhydrase indeed contributes negligibly to the measured muscle enzyme activities. (*a*) The determinations of ^{51}Cr radioactivity in white-skeletal-muscle homogenates indicate that only 0.01–0.001% (v/v) of erythrocytes were present in the muscles after perfusion (for details see Gros *et al.*, 1980). Thus at most 1% of the present muscle enzyme activities should be due to residual erythrocyte carbonic anhydrase. (*b*) Measurements of haemoglobin concentrations in the supernatants argue in a similar way against the presence of significant amounts of erythrocytes in these muscles. From the A_{410}, the haem concentration in the supernatants was estimated to be 3 μM. Assuming that all of this haem represents haemoglobin, a maximum of 0.015% for the volume fraction of erythrocytes in the perfused muscles can be inferred. (*c*) The isoenzyme patterns in erythrocytes and in white skeletal muscle are different. Although B-isoenzyme activity is almost absent from white-skeletal-muscle homogenates, it contributes about 20% of the total enzyme activity in erythrocytes when inhibitory ions are not present. These findings taken together appear to support strongly the conclusion that the C isoenzyme found in white skeletal muscles originates from this tissue and not from erythrocytes.

Possible localization and physiological significance of white-skeletal-muscle carbonic anhydrase

The present data show that most of the white-skeletal-muscle carbonic anhydrase is of cytosolic origin and only 10–15% is associated with the particulate fractions. Which tissue components and subcellular structures are possible sources of this carbonic anhydrase? In the recent literature, the following structures have been implicated as possible sites of carbonic anhydrase activity in muscle tissue: mitochondria, endothelial cells and sarcoplasm.

(1) *Mitochondria.* Dodgson *et al.* (1980) obtained evidence for the presence of carbonic anhydrase in the matrix of skeletal-muscle mitochondria of guinea pigs. It is possible that the carbonic anhydrase that we find in the particulate fractions actually is associated with the mitochondria. However, since electron microscopy of the homogenates obtained with our homogenization procedure (R. Dermietzel, personal communication) revealed a significant number of damaged mitochondria, it is possible that mitochondrial carbonic anhydrase has been unmasked and is present in the muscle supernatants. The following rough calculation attempts to estimate how much carbonic anhydrase can be expected to appear in the supernatant when all mitochondrial enzyme is set free. By using the 'standardized catalytic constant' (intramitochondrial catalysed rate constant per mitochondrial protein concentration), as determined by Dodgson *et al.* (1980) for guinea-pig muscle, $0.067\,s^{-1}\cdot mg^{-1}\cdot ml$, and a value of $200\,mg\cdot ml^{-1}$ for the mitochondrial protein concentration (B. T. Storey, personal communication), one calculates an intramitochondrial rate constant for the catalysed CO_2 hydration of $13.4\,s^{-1}$. Assuming that white skeletal muscle contains 3% (v/v) mitochondria (Weibel, 1979), one would expect a rate constant of approx. $0.4\,s^{-1}$ for the whole tissue if all mitochondria are disrupted. With rate constant $= [E_{tot.}]\cdot k_{cat.}/(K_m + [CO_2])$, the physiological CO_2 concentration $[CO_2] = 1$ mM, and K_m and $k_{cat.}$ from Table 1, one then estimates a maximal concentration of 17 nM for mitochondrial carbonic anhydrase in the supernatant. This is only 3.5% of the enzyme concentration that we actually find in white-skeletal-muscle supernatant. Although this calculation is valid for guinea-pig rather than rabbit

skeletal muscle, it appears probable that mitochondrial carbonic anhydrase, if present in the supernatants investigated in the present work, can account for only a minor part of the enzyme actually detected there.

(2) *Sarcoplasm and endothelial cells.* Indirect evidence suggests that a major part of the intracellular space of skeletal-muscle tissue contains carbonic anhydrase (Zborowska-Sluis *et al.*, 1974). However, the muscles studied by these authors were of the mixed type, and it is thus possible that the effects they observed are due to the isoenzyme A, which is known to be present in high concentrations in red fibres (Koester *et al.*, 1977; Carter *et al.*, 1978; Tashian *et al.*, 1980; Siffert *et al.*, 1980). Indeed, histochemical evidence obtained by Lönnerholm (1980) suggests that carbonic anhydrase C is not present in muscle fibres but occurs in high concentrations in capillaries, an observation also reported by Ridderstrale (1979). Although the specificity of the histochemcal method employed by Ridderstrale (1979) and Lönnerholm (1980) has been questioned (Muther, 1977; Gay, 1980), the cytoplasm of endothelial cells must be considered as a possible location of the cytosolic C isoenzyme. It may be noted that Effros & Weissman (1979), on the basis of $H^{14}CO_3^-$–$^{14}CO_2$-wash-out curves, hypothesized that muscle carbonic anhydrase may be associated with the endothelial plasma membranes and serve to accelerate CO_2 equilibration in the capillary. Besides the mitochondria, the endothelial cell membrane may thus constitute a source of the carbonic anhydrase that can be extracted from the particulate fractions. A definite answer to the question of isoenzyme-C localization in white skeletal muscle has to await further physiological and histochemical studies.

We are indebted to Dr. R. E. Forster and Dr. B. T. Storey (Philadelphia) for letting us know the details of their determinations of mitochondrial carbonic anhydrase and for helpful discussions. We thank Dr. R. Dermietzel (Essen) for electron-microscopic investigation of the muscle homogenate.

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