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Carbonic anhydrase in human platelets

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The carbonic anhydrase activity of human platelets was investigated by measuring the kinetics of CO_2 hydration in supernatants of platelet lysates by using a pH stopped-flow apparatus. An average carbonic anhydrase concentration of 2.1 μM was determined for pellets of human platelets. Analysis of the kinetic properties of this carbonic anhydrase yielded a K_m value of 1.0 mM, a catalytic-centre activity $k_{cat.}$ of 130000 s^{-1} and an inhibition constant K_i towards ethoxzolamide of 0.3 nM. From these values, CO_2 hydration inside platelets is estimated to be accelerated by a factor of 2500. When platelet lysates were subjected to affinity chromatography, only the high-activity carbonic anhydrase II could be eluted from the affinity column, whereas the carbonic anhydrase isoenzyme I, which is known to occur in high concentrations in human erythrocytes, appeared to be absent.

No blood cells other than erythrocytes have so far been reported to contain carbonic anhydrase. As in the course of platelet activation proton movements occur across the plasma membrane (Akkerman *et al.*, 1979), the question arose whether platelets, like other H^+-secreting cells, contain carbonic anhydrase. We have therefore studied carbonic anhydrase activity in the supernatants of human platelet lysates. The results presented here indicate that platelets indeed possess carbonic anhydrase, probably of cytosolic origin.

Materials and methods

Preparation of platelet lysates

Buffy-coat preparations from freshly drawn human blood were obtained from the local transfusion centre. The buffy coats were centrifuged at 150*g* for 10 min to obtain platelet-rich plasma. Platelets were then separated from the plasma by centrifugation at 3500*g* for 10 min. The pelleted platelets were then washed three times in 10 vol. of 0.15 M-NaCl and re-centrifuged at 3500*g* for 10 min. The final pellets were lysed by the addition of 10 vol. of distilled water and subsequent freezing in liquid N_2 and thawing. Thereafter the membranes were removed from the platelet lysates by centrifugation at 30000*g* for 1 h. Contamination of the platelet suspensions with other blood cells was monitored by microscopic investigation of stained smears. The platelet preparations used for determination of carbonic anhydrase activity were essentially free of leucocytes, but did show solitary erythrocytes. The contamination with erythrocytes was quantified by measuring haem concentration, which was less than 3 μM.

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Affinity chromatography of platelet carbonic anhydrase

An affinity gel was used as described by Osborne & Tashian (1975). Prontosil {*p*-[(2,4-diaminophenyl)azo]benzenesulphonamide} was coupled to CM-Sephadex CM-50 (Pharmacia). The affinity column (1.5 cm × 20 cm) was pre-equilibrated with 0.01 M-Tris/H_2SO_4 buffer, pH 8.7. Samples of platelet lysates were dialysed against the same buffer before application to the column. Non-specifically bound proteins were desorbed with a buffer containing 0.1 M-Tris/H_2SO_4 and 0.2 M-Na_2SO_4 (pH 8.7). Then the column was perfused with 0.1 M-Tris/H_2SO_4 buffer containing 0.4 M-KI (pH 7.0). Under these conditions carbonic anhydrase I is known to be eluted when a lysate of human erythrocytes has been applied to the column (Osborne & Tashian, 1975). Subsequently the column was washed with buffer containing 0.2 M-KCN, 0.2 M-Na_2SO_4 and 0.1 M-Tris/H_2SO_4 (pH 7.5). With this buffer, the high-activity isoenzyme II is known to be desorbed from the affinity column. The eluates obtained with both buffers were concentrated, dialysed against 0.01 M-Veronal/0.15 M-NaF (pH 7.8) and then used for carbonic anhydrase activity determinations.

Veronal and NaF were chosen because they have a minimal inhibitory effect on carbonic anhydrase (Maren & Couto, 1979).

Measurement of carbonic anhydrase activity

Carbonic anhydrase activities were determined in platelet lysates and in the fractions obtained by affinity chromatography. The platelet lysates were appropriately diluted in 0.015M-imidazole/0.15M-NaCl buffer (pH7.3), and the fractions obtained from the affinity column were diluted in 0.01M-sodium Veronal/0.15M-NaF buffer (pH7.8). Carbonic anhydrase activity was measured by following the kinetics of CO_2 hydration in a pH stopped-flow apparatus as described by Crandall *et al.* (1971). The initial rate of CO_2 hydration catalysed by carbonic anhydrase was calculated by subtracting the independently determined rate of the uncatalysed reaction from the reaction rate observed in platelet lysates. Catalysed reaction rates were usually 10 times the uncatalysed rates. The average velocity constant for the uncatalysed CO_2 hydration, k_{CO_2}, was determined to be $0.035\,s^{-1}$. The Michaelis constant, K_m, and the maximal catalytic rate, $V_{max.}$, were obtained by measuring catalysed CO_2-hydration rates at various CO_2 concentrations. Inhibition experiments were performed with the specific carbonic anhydrase inhibitor ethoxzolamide (Sigma). Measurements of initial catalysed CO_2-hydration rates at various ethoxzolamide concentrations allowed us to construct an inhibition curve. From the data the inhibition constant, K_i, towards ethoxzolamide, the total enzyme concentration, $[E]_{tot.}$ and the specific activity, $k_{cat.}/(K_m + [CO_2])$, could be derived by a method described previously (Siffert & Gros, 1982). From either K_m or $V_{max.}$ as obtained from a Lineweaver–Burk plot, these data then allowed us to calculate $k_{cat.}$, the catalytic-centre activity (turnover number).

Results

Kinetic properties

Fig. 1 shows a plot of s/v versus s for a platelet lysate. Dilution after mixing in the stopped-flow apparatus was 1 vol. of packed platelets in 80 vol. of 0.015M-imidazole/0.15M-NaCl buffer. CO_2 concentrations were varied between 1 and 5mM. The pH was 7.3 and the temperature was 25°C. Fig. 1 shows the data points along with the calculated regression line. From the regression coefficients K_m for CO_2 is calculated to be 1.0mM and $V_{max.}$ is found to be 0.22mol/s per litre of pelleted platelets (after correction for dilution).

Inhibition by ethoxzolamide

Fig. 2 shows the results of inhibition experiments performed with a platelet lysate (final

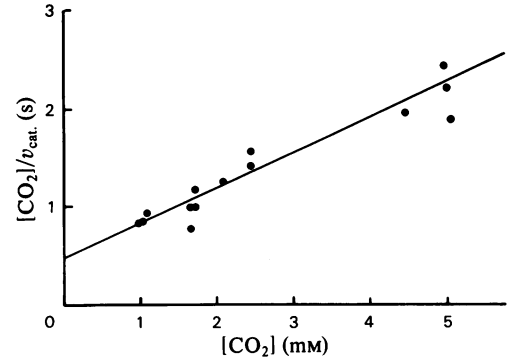

Fig. 1. *Determination of K_m: the ratio of substrate concentrations divided by carbonic anhydrase-catalysed CO_2-hydration rates ($[CO_2]/v_{cat.}$) is plotted versus initial substrate concentrations*

The temperature was 25°C. The rates were measured in 0.15M-NaCl/15mM-imidazole buffer, pH7.3. Final dilution of the platelets in buffer was 1:80. The data points were used to calculate a linear regression ($r = 0.991$), which yielded K_m and V (see the text and Table 1). The line represents the computed regression equation.

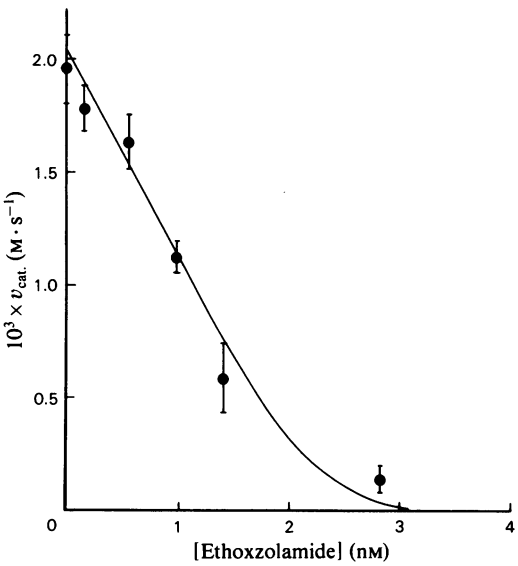

Fig. 2. *Carbonic anhydrase-catalysed CO_2-hydration rates, $v_{cat.}$, in lysates of human platelets as a function of ethoxzolamide concentration*

The temperature was 25°C; the rates were measured in 15mM-imidazole/0.15M-NaCl buffer, pH7.3, and the initial CO_2 concentration was 2.6mM. The final dilution of the platelets in buffer was 1:95. The data points (means of four determinations, ± S.D.) were fitted to the second-order polynomical $v_{cat.}[I]_{tot.} = a + bv_{cat.} + cv_{cat.}^2$, where $[I]_{tot.}$ is the total inhibitor concentration. The coefficients a, b and c were used as described (Siffert & Gros, 1982) to calculate the values of K_i, $[E]_{tot.}$ and $k_{cat.}/(K_m + [CO_2])$ given in Table 1. The line through the data points is a theoretical curve computed with these constants ($r^2 = 0.97$).

Table 1. *Properties of platelet carbonic anhydrase*
Given are the inhibition constant towards ethoxzolamide, K_i, the enzyme concentration $[E]_{tot.}$, the specific activity, $k_{cat.}/(K_m+[CO_2])$, the Michaelis constant for CO_2, K_m, and the catalytic-centre activity, $k_{cat.}$, of the carbonic anhydrase in platelet lysates (±S.E.M.). The temperature was 25°C and the initial CO_2 concentration in the inhibition experiments was 2.6mM. The data indicate that only the high-activity carbonic anhydrase isoenzyme II is active in platelet lysates.

K_i	0.28(±0.06)nM
$[E]_{tot.}$	2.1(±0.6)μM
$k_{cat.}/(K_m+0.0026M)$	$3.6(\pm0.3)\times10^7 M^{-1}\cdot s^{-1}$
K_m	1.0(±0.2)mM
$k_{cat.}$	$1.3(\pm0.3)\times10^5 s^{-1}$

dilution after mixing 1 vol. of packed platelets in 95vol. of 0.015M-imidazole/0.15M-NaCl buffer, pH7.3). The initial CO_2 concentration was 2.6mM and the temperature was 25°C. Analysis of the inhibition data in terms of K_i, $[E]_{tot.}$ and the specific activity $k_{cat.}/(K_m + [CO_2])$ yielded the values given in Table 1. From this specific activity and the above K_m value a catalytic-centre activity, $k_{cat.}$, of $130000 s^{-1}$ is calculated.

Isoenzyme pattern

To study the isoenzyme pattern of carbonic anhydrase in platelets, a lysate of 4ml of packed platelets was subjected to affinity chromatography. Measurement of CO_2-hydration velocities in the two fractions, which were expected to contain carbonic anhydrase I and II respectively, yielded a value of 1:40 for the ratio of the carbonic anhydrase activities per mg of protein in the two fractions. This indicates that it is carbonic anhydrase II that is responsible for the carbonic anhydrase activity in platelets, whereas carbonic anhydrase I is practically absent. Titration of a stock solution of the carbonic anhydrase II fraction with ethoxzolamide indicated that a total amount of 180μg of purified carbonic anhydrase II was obtained from 4ml of platelets applied to the affinity column.

Discussion

Purity of the platelet preparations

The following findings suggest that the carbonic anhydrase activity that we find in platelet lysates is not due to contaminating erythrocytes or leucocytes. (1) It appeared from microscopic investigation that the platelet preparations used for determination of carbonic anhydrase activity were free of leucocytes. (2) Measurements of the haemoglobin concentration in platelet lysates allow us to estimate that less than 0.2% of the observed carbonic anhydrase activities may be due to contaminating erythrocytes. (3) Affinity chromatography of platelet carbonic anhydrase yielded only the carbonic anhydrase II isoenzyme, whereas human erythrocytes contain both carbonic anhydrase isoenzymes, I and II, in a molar ratio of about 8:1 (Osborne & Tashian, 1975; Wistrand, 1981).

Properties of human platelet carbonic anhydrase

The properties of human platelet carbonic anhydrase shown in Table 1 can be expected to be those of the isoenzyme II, since carbonic anhydrase I should be fully inhibited under conditions of 0.15M-NaCl (Maren & Couto, 1979; Wistrand, 1981). Therefore comparisons of the data in Table 1 are made with data for various carbonic anhydrase II isoenzymes. The K_i value for ethoxzolamide of about 0.3nM is of the order of that reported by Maren (1967) for the human isoenzyme II and almost identical with the value that we find for carbonic anhydrase II in a lysate of rabbit erythrocytes under the same experimental conditions (Siffert & Gros, 1982). The catalytic-centre activity of $130000 s^{-1}$ given in Table 1 clearly indicates that the enzyme involved is the high-activity carbonic anhydrase. Although this value is only one-quarter of the $k_{cat.}$ value reported by Khalifah (1971) in the absence of the inhibitory Cl^-, it is in close agreement with the $k_{cat.}$ value for rabbit erythrocyte carbonic anhydrase II obtained by us in the presence of Cl^- (Siffert & Gros, 1982). The Michaelis constant, K_m, of 1.0mM reported here is significantly lower than the values reported in the literature for the purified human carbonic anhydrase II isoenzyme: Steiner *et al.* (1975) give a value of 8.3mM for K_m in the presence of 0.05M-*N*-methylimidazole, pH7.0, at 25°C and $I=0.2$ in the absence of Cl^-. Wistrand (1981) found an even higher K_m value of 14mM under similar conditions. However, in the presence of 0.15M-Cl^- we find a value of 3.5mM for K_m in crude lysates of human (W. Siffert, unpublished work) and rabbit erythrocytes (Siffert & Gros, 1982) under experimental conditions identical with those used here. We conclude from these findings that the properties of the carbonic anhydrase that catalyses CO_2 hydration in platelet lysates are comparable with those of the carbonic anhydrase isoenzyme II of human and rabbit erythrocytes. This is in keeping with the results of the affinity chromatography, showing carbonic anhydrase activity in the 'II-peak' but almost none in the 'I-peak'. It may be noted that we have not investigated the presence of the carbonic anhydrase isoenzyme III, which is known to occur in high concentrations in red skeletal muscle (Koester *et al.*, 1977; Holmes, 1977; Carter *et al.*, 1978; Siffert *et al.*, 1980). We have also not

attempted to study the question of whether platelet membranes and organelles contain carbonic anhydrase, but the enzyme described here, being present in the supernatant, is likely to be of cytosolic origin.

Activity and possible physiological significance of carbonic anhydrase activity in human platelets

Assuming that most of the carbonic anhydrase activity found in platelet lysates is of cytoplasmic origin, and using the values for $[E]_{tot.}$, $k_{cat.}$ and K_m of Table 1, one obtains an estimate of about 2500 for the carbonic anhydrase activity in human platelets at 25°C, pH 7.3, and $[CO_2] = 1.2$ mM. It should be noted that this activity refers to whole pellets of platelets and thus may be higher when expressed as cytosolic activity.

Only speculations are possible about the physiological implications of the carbonic anhydrase activity found in human platelets. Horne *et al.* (1981) reported that human platelets on stimulation by thrombin exhibit a rapid increase in cytoplasmic pH of about 0.3 unit within 30 s. This increase in pH_i is probably due to protons being extruded to the extracellular space (Akkerman *et al.*, 1979). Carbonic anhydrase-catalysed CO_2 hydration might be involved in this process by making protons in the cytosol rapidly available, which then can be transferred from the cytoplasm to the extracellular space. Cytosolic platelet carbonic anhydrase may also play a role in the delivery of protons to the H^+ pump that maintains an acid pH within the 5-hydroxytryptamine-accumulating platelet granules (Johnson *et al.*, 1978).

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