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# $HCO_3^-$-STIMULATED ATPase FROM MAMMALIAN PANCREAS

## PROPERTIES AND ITS ARRANGEMENT WITH OTHER ENZYME ACTIVITIES

BERND SIMON AND LOTHAR THOMAS
*Max-Planck-Institut für Biophysik, 6 Frankfurt a.M. (Germany)*
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SUMMARY

Small membrane fragments from mammalian pancreas with different enzyme activities could be separated by gel electrophoresis. In addition to leucine aminopeptidase and alkaline phosphatase, these fragments contain acetazolamide-inhibitable carboxylic esterase and anion-stimulated ATPase activity. Several properties of this enzyme including substrate specificity, dependence on pH and $HCO_3^-$ concentrations are very similar to those of the $HCO_3^-$-ATPase of gastric mucosa.

## INTRODUCTION

In 1965 an $HCO_3^-$-stimulated and $SCN^-$-inhibited microsomal ATPase (EC 3.1.6.3) from gastric mucosa was discovered by Kasbekar and Durbin[1]. Since $HCO_3^-$ is a stimulator and $SCN^-$ an inhibitor of acid secretion, it has been suggested that this enzyme is involved in acid secretion. This assumption has been given additional support by the following facts:

The enzyme is exclusively localized in the acid-secreting cells of this organ and, moreover, a considerable amount of this enzyme is present in smooth surfaced vesicles presumably derived from the secretory tubular system of these cells[2-6].

Recently a particle-bound ATPase was demonstrated in mammalian pancreas which resembles gastric ATPase in the following points[7]: (a) this enzyme is stimulated by $HCO_3^-$ and other oxybases according to a general base catalysis, (b) it is inhibited by $SCN^-$ and $OCN^-$, both of which inhibit pancreatic secretion, (c) it can be solubilized by Triton X-100, (d) it behaves similarly in gel filtration on Sephadex G-200 and 2–22 % sucrose gradient centrifugation, (e) it is associated with alkaline phosphatase, (f) the enzyme can be distinguished from mitochondrial ATPase because of its different location in comparison with mitochondrial markers such as succinate dehydrogenase on the density gradient. These results have been interpreted to mean that pancreatic ATPase may be involved at some critical stage in $HCO_3^-$ transport by this organ.

This assumption demands further characterization of the particle-bound enzyme. Therefore, the dependence of enzyme activity on external $HCO_3^-$ and $H^+$

concentrations, different bivalent cations and nucleotides were tested. It could be shown that many of the properties resemble those of $HCO_3^-$-ATPase from gastric mucosa.

Furthermore, we attempted to determine whether or not $HCO_3^-$-stimulated ATPase is closely associated with other enzyme activities in the particulate fraction. For this purpose, we separated this fraction by gel electrophoresis and found a close correlation of anion-stimulated ATPase with acetazolamide-inhibitable carboxylic esterase, alkaline phosphatase and leucine aminopeptidase. Since the latter two enzymes are known to be located in plasma membranes of several tissues[8-12], the simultaneous presence of acetazolamide-inhibitable esterase activity and $HCO_3^-$-stimulated ATPase supports the hypothesis that this enzyme may play a significant role in transport processes by this organ.

## METHODS

The particulate membrane fraction containing $HCO_3^-$-stimulated ATPase from cat pancreatic tissue was prepared as previously described in detail[7]. In short, the homogenate was layered on a discontinuous gradient which consisted of equal amounts of 15, 25 and 35 % (w/w) sucrose in 10 mM triethanolamine–HCl buffer (pH 7.6), respectively, and centrifuged for 2 h at 105000 × *g*. Most of the $HCO_3^-$-stimulated ATPase activity assembled at the 25 % sucrose value. This fraction was used in all enzymatic and gel electrophoretic studies.

### *Enzyme assay*

The activity of $Mg^{2+}$ and ($HCO_3^-$, $Mg^{2+}$)-ATPase in the particulate fraction was assayed in a medium containing 3 mM sodium ATP, 2 mM ouabain, 0.075 M Tris–HCl buffer (pH 7.6 at 20 °C) with and without 25 mM $NaHCO_3$. Following a 15-min incubation period at 37 °C the samples were heated for 1–2 min in boiling water, chilled and centrifuged (2 min at 14000 rev./min, Microsystem Eppendorf). The amount of $P_i$ liberated was determined in aliquots of the supernatant using the method of Fiske and SubbaRow[13] as modified by Bartlett[14].

### *Protein assay*

The protein concentration of the fractions was measured according to the method of Lowry *et al.*[15] after precipitation of the protein by 10 % trichloroacetic acid in the cold and dissolution of the precipitate in 1 M NaOH. Bovine serum albumin was used as the standard protein.

### *Separation of the particulate fraction in polyacrylamide-gel electrophoresis*

In order to subfractionate the particulate material, samples of the particulate fractions were run on polyacrylamide gels under different conditions. Therefore, the gel porosity and the buffer system were changed.

To polymerize gels with different porosity, the acrylamide concentration of the gels was varied from 4 to 7 % and the *N,N'*-methylenebisacrylamide concentration from 0.1 to 0.2 %, respectively. Sample gels and stacking gels were omitted.

For separation at different pH values, pH 8.9 gels with the discontinuous buffer system of Davis[16] and pH 7.5 gels with the discontinuous buffer system of

Williams and Reisfeld[17] were used. The actual separating pH of the pH 8.9 gels is 9.5, that of the pH 7.5 gels 8.0 (refs 16 and 17). Before the electrophoretic separation the particulate fraction was homogenized ten times in a Potter–Elvehjem homogenizer and centrifuged for 10 min at 13000 × *g* in order to remove large fragments. Samples containing 50–100 μg protein were layered on the top of the gels. Electrophoresis was carried out at 2–4 °C and 4 mA/gel.

Gels were stained for protein overnight with 0.125 % Coomassie blue and destained in 7 % acetic acid.

*Detection of enzyme activities in the gels*

For detection of enzyme activities the gels were incubated with the appropriate substrates after the gel electrophoretic run. Each enzyme test was carried out on two gels; one was incubated with and the other one without substrate in the reaction mixture. The following assay media were used for:

(*a*) *Alkaline phosphatase*[18]: 0.033 M Tris–HCl buffer (pH 9.5 at 20 °C), 0.025 M sodium α-napthylphosphate, 0.1 % Fast Red TR salt.

(*b*) *Leucine aminopeptidase*[19,20]: 0.050 M acetate buffer (pH 6.5 at 20 °C), 0.060 M NaCl, 0.001 M KCl, 0.001 M L-leucyl-β-naphthylamide, 0.2 % Fast Blue B salt.

(*c*) *Carboxylic esterase*[21]: 0.050 M Tris–HCl buffer (pH 7.2 at 20 °C), 0.001 M β-naphthyl acetate, 0.1 % Echtblau* salt.

In the experiments with acetazolamide the gels were incubated 5 min prior the addition of the substrate in the following buffer medium: 0.050 M Tris–HCl buffer and 0.1 mM acetazolamide (pH 7.2). Thereafter, β-naphthyl acetate and Echtblau salt, dissolved in acetone, were added.

(*d*) *Succinate dehydrogenase*[22]: 0.050 M phosphate buffer (pH 7.6 at 20°C), 0.050 M succinate disodium salt, 0.001 M $CaCl_2$, 0.25 mM $MgSO_4$, 0.030 M $NaHCO_3$, 0.2 mM $AlCl_3$, 0.2 % iodonitrotetrazolium chloride.**

(*e*) *Anion-sensitive ATPase*: To locate the position of anion-sensitive ATPase activity the gels were incubated at 37 °C for 30 min with and without 20 mM $Na_2SO_3$ in the following reaction mixture: 0.10 M imidazole–HCl buffer (pH 7.0 at 20 °C), 0.0025 M $MgCl_2$, 0.0025 M Tris–ATP. Thereafter, excess ATP was removed by intensive washing of the gels in distilled water. The liberated $P_i$, which denotes the position of the enzyme activity in the gels, was detected by immersing the gels at room temperature in a solution, containing 9.4 % $HClO_4$, 1 % ammonium molybdate, 0.25 % reducing mixture (6 parts $Na_2SO_3$, 6 parts $NaHSO_3$ and 1 part 1-amino-2-napthyl-4-sulfonic acid) according to the method of Abrams and Baron[23].

The anion-sensitive part of the ATPase activity could be visualized more easily in the gels if the anion $HCO_3^-$ was replaced by $SO_3^{2-}$ because $SO_3^{2-}$ stimulated more effectively than $HCO_3^-$.

## MATERIALS

The centrifugations were carried out in an Omega II ultracentrifuge (Heräus-Christ).

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* Diazotized 4-amino-2,5-diethoxy-benzanilid zinc double salt.

** 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

The analytical acrylamide-gel electrophoresis apparatus was purchased from Shandon, Frankfurt.

All chemical reagents were obtained from Serva, Heidelberg.

## RESULTS

### *Properties of $Mg^{2+}$- and ($HCO_3^-$, $Mg^{2+}$)ATPase*

*Substrate specificity.* It can be seen from Table I that the $HCO_3^-$-stimulated hydrolysis of different trinucleotides decreases in the sequence ATP > GTP > ITP > UTP. No $HCO_3^-$-stimulated dinucleotide and mononucleotide hydrolysis can be detected (($HCO_3^-$, $Mg^{2+}$)-/$Mg^{2+}$-ATPase activity ratio = 1). Glucose 6-phosphate was found to be inactive as substrate.

*Metal ion requirement*: Table II shows that divalent cations such as $Co^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Cd^{2+}$, $Zn^{2+}$ can substitute for $Mg^{2+}$ with different degrees of effectiveness. $Mg^{2+}$ is the most effective cation (expressed as 100 % activity) and of the others tested only $Co^{2+}$ causes a stimulation of comparable magnitude of both $Mg^{2+}$ and ($HCO_3^-$,

TABLE I

SUBSTRATE SPECIFICITY OF PARTICULATE ($HCO_3^-$, $Mg^{2+}$)- AND $Mg^{2+}$-ATPASE

The ratios of the enzyme activities (($HCO_3^-$, $Mg^{2+}$)-/$Mg^{2+}$-ATPase) in the particulate fraction of the discontinuous sucrose gradient are shown for the different nucleotides. The means ± S.D. of 5 experiments are given.

| *Substrate* | *($HCO_3^-$, $Mg^{2+}$)-/$Mg^{2+}$-ATPase activity* |
|---|---|
| ATP | 1.50 ± 0.23 |
| GTP | 1.30 ± 0.16 |
| ITP | 1.24 ± 0.16 |
| UTP | 1.22 ± 0.36 |
| ADP | 1.00 ± 0.46 |
| AMP | 1.00 ± 0.40 |

TABLE II

EFFECT OF DIVALENT CATIONS ON ATP HYDROLYSIS CATALYZED BY PARTICULATE ATPASE IN THE ABSENCE AND PRESENCE OF $NaHCO_3$

The enzyme activities are expressed as percentage of the activities of $Mg^{2+}$-ATPase and ($HCO_3^-$, $Mg^{2+}$)-ATPase (relative enzyme activity). Concentration of divalent cations, 3 mM; concentration of $NaHCO_3$, 25 mM.

| *Cation* | *Relative enzyme activity* | |
|---|---|---|
| | *No $HCO_3^-$* | *$HCO_3^-$* |
| $Mg^{2+}$ | 100 | 100 |
| $Ca^{2+}$ | 75.2 | 45.8 |
| $Co^{2+}$ | 69.2 | 79.4 |
| $Cd^{2+}$ | 53.2 | 7.4 |
| $Mn^{2+}$ | 47.2 | 23.4 |
| $Zn^{2+}$ | 23.7 | — |

$Mg^{2+}$)-stimulated ATP hydrolysis. In the presence of $Mg^{2+}$ addition of other ions resulted in an inhibition of $HCO_3^-$-stimulated ATPase activity. Maximal activity of the enzyme was observed at molar ratios of ATP to $Mg^{2+}$ of 1.0.

*pH optimum.* The effect of pH on ($HCO_3^-$, $Mg^{2+}$)-ATPase and $Mg^{2+}$-ATPase activity is shown in Fig. 1 (left side). Since it is not proven that the $HCO_3^-$-stimulated ATPase and the non $HCO_3^-$-ATPase represent two different enzymes, the dependence of the ratio ($HCO_3^-$, $Mg^{2+}$)-/$Mg^{2+}$-ATPase on the pH is also demonstrated in Fig. 1 (right side). It can be seen that the highest rate of $HCO_3^-$-stimulated ATP hydrolysis is at pH 7.6.

*Effect of increasing $HCO_3^-$ concentration on ATPase acitivity* (Fig. 2). The maximal rate of ($HCO_3^-$, $Mg^{2+}$)-stimulated ATP hydrolysis was reached at a $HCO_3^-$ concentration of 20 mM and remained virtually constant beyond this level. During the pH studies the $HCO_3^-$ concentration necessary for maximal enzyme stimulation could be maintained over the range from 7.0 to 9.0.

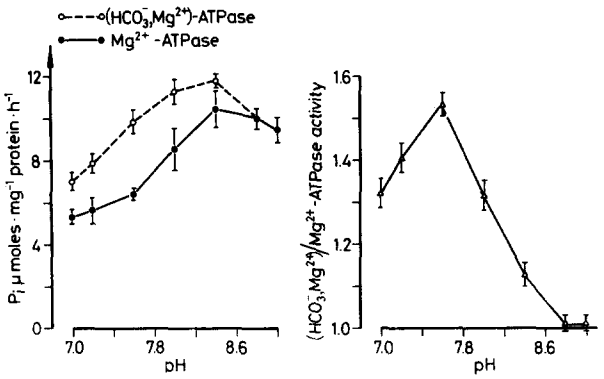


Fig. 1. Effect of pH on particulate $Mg^{2+}$- and ($HCO_3^-$, $Mg^{2+}$)-ATPase activities. The absolute activities of ($HCO_3^-$, $Mg^{2+}$)-ATPase and $Mg^{2+}$-ATPase dependent on pH are shown on the left side. The effect of pH on the ($HCO_3^-$, $Mg^{2+}$)-/$Mg^{2+}$-ATPase activity is illustrated on the right side. The means ± S.D. of 4 experiments are given.

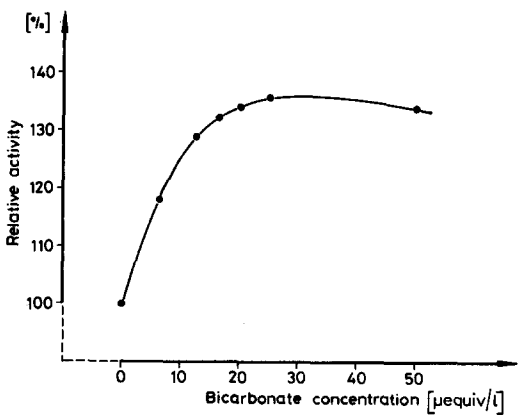


Fig. 2. Effect of $HCO_3^-$ on ATPase activity. The sodium salt of the anion was added to the incubation mixture containing enzyme, 0.003 M sodium ATP, 0.003 M $MgCl_2$, 0.002 M ouabain and 0.075 M Tris–HCl buffer (pH 7.6). Each point represents the mean of 4 experiments.

*Gel electrophoretic separation*

On 4% acrylamide gels at pH 9.5 the particulate fraction separated into 11 protein bands (Fig. 3). A considerable amount of protein remained on top of the gels and the activities of leucine aminopeptidase, alkaline phosphatase, succinate dehydrogenase, carboxylic esterase and anion-stimulated ATPase could be detected near the origin (cathode). During all electrophoretic procedures succinate dehydrogenase could only be visualized on top of the gels.

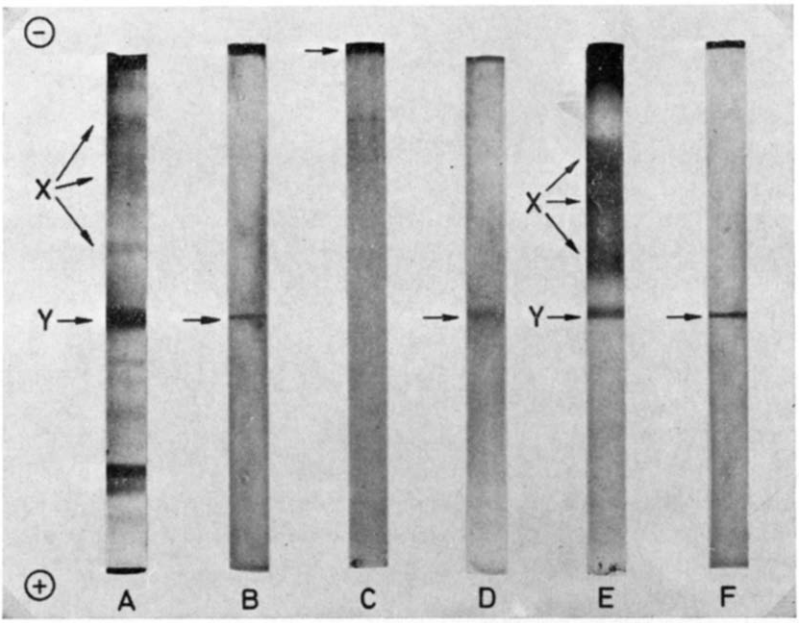


Fig. 3. Particulate fractions were subjected to electrophoresis in parallel on 4% acrylamide gels with 0.1% *N,N'*-methylenebisacrylamide using the pH 8.9 gels described by Davis[16]. In all electrophoretic runs the cathode is at the top, the anode at the bottom. The location of the proteins and the enzymatic activities (indicated by arrows) are shown in Gels A–F: (A) protein pattern (Coomassie stained); (B) $SO_3^{2-}$-stimulated ATPase. $Mg^{2+}$-ATPase is not illustrated; (C) succinate dehydrogenase; (D) alkaline phosphatase; (E) carboxylic esterases (acetazolamide-inhibited esterase activity is not shown); (F) leucine aminopeptidase. All enzyme activities (B–F) can be visualized on top of the gels near the cathode. In the Gels A and E, Arrow X indicates protein bands and the corresponding carboxylic esterase activities which cannot be inhibited by acetazolamide. In contrast, Arrow Y in the same gels indicates the protein band and the corresponding carboxylic esterase-activity (E) which can be suppressed by acetazolamide. The same protein band shows also $SO_3^{2-}$-stimulated ATPase (B), alkaline phosphatase (D) and leucine aminopeptidase (F).

The band near the middle of the gels (indicated by an arrow) shows leucine aminopeptidase, alkaline phosphatase, carboxylic esterase and $SO_3^{2-}$- stimulated ATPase activities.

Carboxylic esterase activity in this band, in contrast to the carboxylic esterase with lower migration rates, is completely inhibited by acetazolamide.

To find out if the four enzyme activities were localized in one fragment, as indicated after the electrophoresis in 4% acrylamide gel at pH 9.5, the electrophoretical conditions were changed as follows: (a) Altering the gel concentration continuously from 4 to 6% caused only a retardation of the relative migration rate.

At a gel concentration of 7 % the complex was not allowed to enter the gel. (b) Changing the actual separating pH values from 9.5 to 8.0 in the 4 % acrylamide gels decreased the migration rate of the enzyme complex, but no dissociation of the enzyme activities occurred.

We therefore conclude that these enzyme activities are localized in the same membrane fragment and are not of mitochondrial origin. However, we do not know whether anion-stimulated ATPase and the other enzyme activities, detected on top of the gels together with succinate dehydrogenase, belong to mitochondrial or to large membrane fragments.

## DISCUSSION

In addition to earlier biochemical studies of particle-bound $HCO_3^-$-stimulated ATPase from pancreatic tissue[7], the results presented in this paper indicate further similarities to microsomal $HCO_3^-$-ATPase of gastric mucosa[1,2]. For example, identical substrate specificity for mono-, di- and trinucleotides, identical $HCO_3^-$ concentration dependence for maximal stimulation, and identical pH optima are observed. There is, however, a discrepancy with respect to the metal ion requirement.

The highest specific activity of $HCO_3^-$-stimulated ATPase from pancreatic tissue could be found in the nuclear ($10^4$ $g \cdot$min) and in the crude mitochondrial ($10^5$ $g \cdot$min) fractions, but with density gradient centrifugations it was located in a particle of identical density to the gastric mucosal vesicle[7]. This density indicates localization of pancreatic ATPase in membrane fragments.

However, little is known about the structure, morphology and biochemistry of plasma membranes from pancreatic cells. Therefore no data exist about the arrangement of enzyme activities within these membranes. The isolation of plasma membranes is complicated by the high content of DNA in the low-speed fraction, where most of these membranes are sedimented along with the greater amount of endoplasmatic reticulum and adherent fibrillar material[24].

Plasma membrane fragments from hepatic, renal tubular and intestinal cells, which are well characterized with respect to their associated enzyme activities, contain $Mg^{2+}$-ATPase[9,25,28-30], alkaline phosphatase[8-11,26-29] and leucine aminopeptidase[8-12,25].

Density gradient centrifugations of pancreatic homogenate have shown that the particulate fraction contains leucine aminopeptidase and alkaline phosphatase as well as $HCO_3^-$-stimulated ATPase[7]. To find out whether these activities which assemble at the same sucrose concentration, are arranged in one or in different membrane fragments, we separated the particulate fraction by gel electrophoresis.

Enzyme activities, not arranged in a common fragment, should show different electrophoretic behaviour, if the mobility of the proteins is varied by altering the charge and the molecular sieving. Neither changing the actual separating pH nor altering the pore size from 100 to 50 Å caused a dissociation of the enzyme activities suggesting that all these enzymes are arranged in one fragment.

This enzyme complex, detected near the middle of the gel, does not belong to membrane fragments derived from mitochondria because the succinate dehydrogenase activity could be detected only on top of the gels. This finding emphasizes that anion-stimulated ATPase (visualized on the top) may be identical with mito-

chondrial ATPase described by Racker[31], which also shows some sensitivity to $HCO_3^-$.

We believe that the enzyme complex is a constituent of plasma membranes. The assumption is consistent with histochemical studies done by several groups[33-36] showing alkaline phosphatase and leucine aminopeptidase to be located in the excretory duct system near the luminal cell border. Additional support comes from the studies of Sachs *et al.*[6] who demonstrated that $HCO_3^-$-stimulated ATPase and alkaline phosphatase were in close connection with plasma membranes of oxyntic cells. The findings of Meldolesi *et al.*[32] who could not detect alkaline phosphatase in their plasma membrane preparations, can be explained by the different source of pancreas and especially by the fact that pancreatic tissue consists of quite different cell types which, due to their different function, may also vary in the enzymatic content of their plasma membranes.

Nevertheless, these results can only be related to the small fragments of the particulate fraction with molecular weights not higher than $2 \cdot 10^6$ (ref. 37), since larger ones are not allowed to enter the gel.

In contrast to the several carboxylic esterases detected at lower relative migration rates the carboxylic esterase activity in the enzyme complex is completely suppressed by acetazolamide.

$\beta$-Napthyl acetate is one of several ester substrates[38-40] which are known to be hydrolyzed by carbonic anhydrase of erythrocytes in man and other species[38,41]. Like the $CO_2$ hydrase activity of this enzyme, its carboxylic esterase activity can be suppressed by acetazolamide[42]. But the carbonic anhydrase of other tissues may not be the same as that found in red cells, since some $CO_2$ hydrase activities have been observed that are incapable of splitting $\beta$-naphtyl acetate[42,43].

This emphasizes the need for caution when attempting to detect carbonic anhydrase activity with $\beta$-napthyl acetate as substrate in tissues other than red cells.

The finding, however, of an esterase activity in the common fragment which can be inhibited by acetazolamide hints at the carbonic anhydrase character of this enzyme. Therefore, attempts are presently being made to demonstrate sulfonamide-inhibitable $CO_2$ hydrase activity in the protein band which contains acetazolamide-inhibitable esterase activity.

The simplest interpretation of the above results is that pancreatic $HCO_3^-$-stimulated ATPase is arranged with other enzymes in a complex of about 100 Å size presumably derived from plasma membranes and resembles in many of its chemical properties microsomal ATPase from gastric mucosa.

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