ADENOSINE TRIPHOSPHATASE FROM RAT LIVER MITOCHONDRIA - EVIDENCE FOR A MERCURIAL-SENSITIVE SITE FOR THE ACTIVATING ANION BICARBONATE

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Summary

The ATPase activity of purified mitochondrial ATPase (F_1) of rat liver is inhibited less than 15% by sulfhydryl reagents when assayed in TrisCl buffer. In Trisbicarbonate buffer the ATPase activity of the enzyme is two- to three-fold higher than in TrisCl. Significantly, the ATPase activity stimulated by bicarbonate can be inhibited by mercurial agents such as p-chloromercuribenzoate. The number of sulfhydryl groups accessible to ^{14}C-p-chloromercuribenzoate is the same in TrisCl and Trisbicarbonate buffers. These experiments suggest that mercurials most likely inhibit bicarbonate-stimulated ATPase activity by blocking a site associated with bicarbonate binding rather than by blocking distinct sulfhydryl-sensitive hydrolytic sites induced by bicarbonate.

Mitochondrial ATPase preparations from rat liver (1 - 3), bovine heart (4), and yeast (5) have been shown to be activated by some oxy anions. Similarly, ATPases associated with the gastric mucosa (6, 7) and the pancreas (8, 9) also have been reported to be stimulated by anions. Among the various anions tested bicarbonate produces one of the most pronounced stimulatory effects. There is considerable interest in the mechanism of anion activation because of the generality of this activation phenomenon among ATPases from different sources, and because there have been suggestions that in some tissues ATPases may be involved in the transport of bicarbonate.

To date, several noteworthy observations have been made in our laboratory (2, 3) and in other laboratories (1, 10, 11) about bicarbonate activation of mitochondrial ATPase. The activation effect is rather specific for ATP hydrolysis (3, 10); it is characteristic of both the membrane-bound and purified enzyme (3, 10, 11); and it significantly alters the kinetics of ATP hydrolysis (3, 10). On the basis of kinetic data, Mitchell and Moyle (11) suggest that bicarbonate and other anions may participate directly in the hydrolytic reaction and Ebel

and Lardy (10) suggest that F_1 contains a distinct binding site for bicarbonate.

Studies described in this report were carried out to determine whether the ATPase activity of F_1 induced by bicarbonate can be specifically blocked without altering the activity observed in nonactivating buffers. The results show that under certain specified conditions this can be done. The results are best interpreted by assuming that F_1-ATPase of rat liver contains at least one bicarbonate binding site which is sensitive to p-chloromercuribenzoate (CMB) and other mercurial agents.

MATERIALS AND METHODS

Purified mitochondrial F_1 of rat liver was prepared exactly as described by Catterall and Pedersen (12) and stored at -20°C in lyophilized form in KP_i until used in assays. Immediately prior to assay, the enzyme was dissolved in water at room temperature and precipitated twice with ammonium sulfate to remove KP_i. The enzyme was dissolved in 0.1 M K_2SO_4, 10 mM TrisCl, pH 7.5 and assayed immediately.

ATPase activity was assayed by following the release of P_i in a 1.0 ml system containing 25 mM TrisCl (or 25 mM Trisbicarbonate), 5 mM $MgCl_2$, 5 mM ATP, pH 7.5. Purified ATPase, 5 μg, was included in the assay. ATP and $MgCl_2$ were added as a 1 : 1 mixture, and the reaction was allowed to proceed for two minutes. The reaction was terminated with 0.1 ml 2.5 M ice-cold perchloric acid, neutralized after 15 minutes with 0.1 ml 2.5 M KOH, centrifuged at 2000 x g for 10 minutes, and assayed for P_i by the procedure of Gomori (13).

ATPase activity was assayed also by the spectrophotometric procedure exactly as described in an earlier report (3) with KCN omitted from the assay buffer.

The number of accessible sulfhydryl groups on F_1-ATPase was determined by the gel filtration method described by Erwin and Pedersen (14) using ^{14}C-labeled p-chloromercuribenzoate.

Soluble protein was measured by the method of Lowry *et al.* (15). The protein was routinely precipitated with 5% trichloroacetic acid and redissolved in 1 N NaOH prior to determination of protein. Crystalline bovine serum albumin was used as standard.

Reagents used in these studies were obtained from the following commercial sources: ATP from P. L. Biochemicals; p-chloromercuribenzoate (CMB), p-chloromercuriphenylsulfonate (CMS), N-ethylmaleimide (NEM) from Sigma; ^{14}C-labeled CMB from Schwarz/Mann; Sephadex G-25 from Pharmacia; and dithiothreitol from Calbiochem. All other reagents were of the highest purity available.

RESULTS AND DISCUSSION

Previous results from this laboratory (3) and from the laboratory of Lardy and co-workers (10) show that the V_{max} of rat liver F_1 in Trisbicarbonate buffer is about 2-to 3-fold higher than in TrisCl buffer. When a coupled assay system is used we usually obtain specific activities of between 50 - 70 and 17 - 27 μmoles ATP hydrolyzed x min^{-1} x mg^{-1}, respectively in the two buffer systems (3), whereas

Ebel and Lardy (10) report values of 27 and 85 μmoles x min^{-1} x mg^{-1}. At ATP levels (1 mM) nonoptimal for hydrolysis the "activation" effect has been reported to be as high as 5.8-fold (10).

Results presented in Table 1 are consistent with previous findings. When a P_i-release assay is used the ATPase activity observed in Trisbicarbonate buffer is 2-fold higher than the activity observed in TrisCl buffer. Although the P_i-release assay gives about 2-fold lower specific activities than the coupled enzyme assay routinely used in this laboratory (3, 12), it was chosen for initial studies which involve inclusion of sulfhydryl reagents in the assay. Such agents interfere with the coupled enzyme assay.

As can be seen in Table 1 a variety of sulfhydryl reagents (CMB, CMS, and NEM) have little effect on the ATPase activity of rat liver F_1 when assayed in TrisCl buffer. These findings are entirely consistent with the earlier observations of Pullman et al. (16) and Senior (17) that the ATPase activity of bovine heart F_1 is insensitive to inhibition by sulfhydryl reagents when assayed in Trisacetate and Trissulfate buffers, respectively. In contrast to the results observed in TrisCl buffer, however, results presented in Table 1 show that ATPase activity of F_1 is inhibited between 50 - 55% by mercurial agents when assayed in Trisbicarbonate buffer. The amount of ATPase activity inhibited by mercurials correlates well with the amount of activity activated by bicarbonate. NEM is without effect on ATPase activity. Consistent with the view that mercurial inhibition may involve interaction of the reagent with one or more sulfhydryl groups, inclusion of dithiothreitol in the assay completely prevents mercurial inhibition of bicarbonate-activated ATP hydrolysis.

The above results are subject to two possible interpretations. First, the effect of bicarbonate might be to bring about a change in the structure of F_1 so as to expose mercurial-sensitive hydrolytic sites. Alternatively, one or more sulfhydryl groups (already accessible on the surface of F_1) might be necessary (directly or indirectly) for interaction of F_1 with bicarbonate. To distinguish between these two possibilities the number of accessible sulfhydryl

TABLE 1

Effect of sulfhydryl reagents on the ATPase activity of purified F_1-ATPase of rat liver mitochondria.

Assay Condition[a]	ATPase Activity[b]			
	In TrisCl	% Inhibition	In Trisbicarbonate	% Inhibition
No inhibitor	61	-	126	-
CMB	53	13	58	54
CMS	54	11	63	50
NEM	61	0	121	4

[a] For assay conditions see MATERIALS AND METHODS. All sulfhydryl reagents were included in the assay at a final concentration of 1.0 mM.

[b] Activity is expressed in nmoles P_i released x min^{-1}.

groups of F_1 was determined in TrisCl and in Trisbicarbonate buffers. Results tabulated in Table 2 show that, although there is some variability in the accessible sulfhydryl content from one F_1 preparation to another, the number of accessible groups on each preparation is the same in the two buffer systems. The results favor the view that one (or more) sulfhydryl groups may be essential for interaction of the enzyme with bicarbonate.

If in fact the above interpretation is correct, it should be possible to show that when F_1 is preincubated with CMB (rather than included directly in the assay as in experiments summarized in Table 1) and then subjected to assay, the usual activity observed in TrisCl buffer will remain unaltered whereas the activity usually stimulated by bicarbonate will not appear. Results presented in Table 3 show that these are the results obtained when F_1 is pretreated with CMB and then assayed in TrisCl and Trisbicarbonate buffer. Assays were conducted at 4 mM ATP and it can be seen that the ATPase activity stimulated by bicarbonate is inhibited about 84%. [When assays are conducted at lower ATP concentrations (2 mM)

TABLE 2

Amount of p-chloromercuribenzoate (CMB) bound to purified F_1-ATPase of rat liver mitochondria.

Preparation Number[a]	Moles CMB Bound/Mole ATPase[b]	
	In TrisCl	In Trisbicarbonate
1	5.1	4.2
2	3.3	3.6
3	5.6	5.1
4	3.9	3.3

[a] The ATPase preparations used were purified by the procedure of Catterall and Pedersen (12).

[b] The amount of CMB bound to the native enzyme was determined exactly as described under MATERIALS AND METHODS.

the amount of bicarbonate-activated ATP hydrolysis is inhibited about 50%.]

Studies reported here were not carried out with the membrane-bound F_1. However, we have reported recently that following preincubation of the membrane-bound F_1 with CMB the ATPase activity of the enzyme (assayed in Trisbicarbonate buffer) is inhibited about 66% (18).

Although the studies reported here suggest that sites on F_1 involved in ATP hydrolysis and in bicarbonate binding are chemically distinct, they do not distinguish between a positive allosteric effect of bicarbonate and a direct participation of bicarbonate in catalysis.

Finally, it seems likely that the effect observed here will be shown to be characteristic of other anions as well. In this regard it should be noted that Pullman *et al.* (16) have shown that the ATPase activity of bovine heart F_1 activated by 2,4-dinitrophenol is inhibited by CMB.

TABLE 3

ATPase activity of F_1 in TrisCl and in Trisbicarbonate buffer following preincubation with CMB.

Preincubation Conditions	Assay Buffer	ATPase Activity[c]
TrisCl[a]	TrisCl	18.5
TrisCl	Tris HCO_3	54.0
TrisCl + CMB[b]	TrisCl	17.3
TrisCl + CMB	Tris HCO_3	23.0

[a] 50 μg of purified F_1, after precipitating two times with ammonium sulfate, was incubated for 2 min at 25°C in a mixture containing 21 mM TrisCl, pH 7.5 and 10 mM KP_i.

[b] Preincubation conditions as above except 17 mM CMB was included in the preincubation medium.

[c] ATPase activity was assayed by the spectrophotometric procedure with KCN omitted from the buffer medium (3). 1.0 μg was taken for assay. Activity is expressed as nmoles ATP hydrolyzed x min^{-1}.

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