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THE EFFECT OF SULFITE ON THE ATP HYDROLYSIS AND SYNTHESIS ACTIVITY OF MEMBRANE-BOUND H^+-ATP SYNTHASE FROM VARIOUS SPECIES

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SUMMARY The action of sulfite on ATP hydrolysis and synthesis activities is investigated in membrane vesicles prepared from the cyanobacterium *Synechococcus* 6716, chromatophores from the photosynthetic purple bacterium *Rhodospirillum rubrum*, membrane vesicles from the related non-photosynthetic bacterium *Paracoccus denitrificans*, and bovine heart submitochondrial particles. Without any further pretreatment ATP hydrolysis is stimulated by sulfite in all four membrane preparations. Typically ATP synthesis in the cyanobacterial membrane vesicles is inhibited by sulfite, whereas ATP synthesis in chromatophores and the submitochondrial particles is not. These differences in sensitivity of ATP synthesis to sulfite, however, correspond well with the distribution of (photosynthetic) sulfur oxidizing pathways in the remaining three organisms/organelles compared in this study.

The F-type ATP synthase of bacteria, mitochondria, and chloroplasts all share a proton electrochemical potential difference ($\Delta\bar{\mu}_H{}^+$) as the natural activation requirement (see [1]). This activated state decays when $\Delta\bar{\mu}_H{}^+$ is dissipated. The decay is accelerated in the presence of ADP [2]. In the chloroplast system one tightly-bound ADP per molecule F_1 is present after de-energization or isolation [3]. This provides evidence that activation and deactivation of the enzyme are related with release and tight (re)binding of ADP, respectively [4].

Early studies by Moyle & Mitchell (1975) [5] have shown that phosphate and some other oxyanions like sulfite shift the equilibrium between active and inactive ATP synthase towards the active form. In the presence of sulfite tightly-bound ADP is released from the chloroplast ATP synthase [6]. Sulfite thus replaces $\Delta\bar{\mu}_H{}^+$ in activating the enzyme. The exact mechanism of sulfite

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Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll; $\Delta\bar{\mu}_H{}^+$, proton electrochemical potential difference; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitro-salicylanilide; SMP, submitochondrial particles.

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activation is still not known. It is supposed that reaction with a P_i binding site at the enzyme.Mg.ADP complex is the initial event in a series of steps eventually leading to the release of tightly-bound ADP, which results in an activated enzyme [7,8]. There are also indications obtained with yeast vacuolar H^+-ATPase that the stimulatory effects of sulfite may be caused by intrinsic uncoupling of ATP hydrolysis from proton transport [9].

In this study we report on differences in the response to sulfite of ATP hydrolysis and synthesis in membrane preparations of cyanobacteria, purple bacteria and mitochondria. In these membrane preparations of different origin ATP hydrolysis is stimulated by sulfite, but surprisingly, only in the cyanobacterial membrane preparation photophosphorylation is inhibited by this oxyanion.

MATERIALS AND METHODS

Organisms and culture conditions. The cyanobacterium *Synechococcus* 6716 was batch-cultured in BG-11 medium at 50 °C in a 2 litre airlift fermenter as described in detail in [10]. The photosynthetic purple non-sulfur bacterium *Rhodospirillum rubrum* was grown anaerobically at 30 °C according to [11]. *Paracoccus denitrificans* was batch cultured aerobically at 30 °C in mineral medium described by Chang & Morris (1962) [12], with 25 mM succinate as carbon source, and supplemented with a trace element solution described by Lawford et al. (1976) [13].
Membrane preparations. *S.* 6716 membrane vesicles, *Rs. rubrum* chromatophores and *Pa. denitrificans* membrane vesicles were prepared as described in detail by [14],[11] and [15] respectively. Submitochondrial particles (SMP) were prepared as described by Herweijer et al. (1985) [16].
Chlorophyll determination. Chlorophyll *a* concentration in *S.* 6716 membrane vesicles was determined according to Arnon et al. [17]. Bacteriochlorophyll (BChl) in *Rs. rubrum* chromatophores was determined spectrometrically at 770 nm after extraction with acetone/methanol (7 : 2 v/v) according to Clayton (1963) [18].
Protein determination. Protein concentration of *Pa. denitrificans* membrane vesicles and SMP were determined using the Pierce BCA protein assay [19] and the Bio-rad assay [20], respectively.
ATP hydrolysis and synthesis. In all the bacterial preparations ATP hydrolysis or synthesis were measured with a sensitive pH electrode as described by Krab et al. (1993) [1]. The composition of the assay media are described below, and the specific reaction conditions are given in the legends to the figures. In SMP, ATP hydrolysis was determined spectrometrically in a medium with an ATP regenerating system according to Bergmeyer (1970) [21]. ATP synthesis in SMP was determined according to Herweijer et al. (1985) [16].
Assays were started by addition of ATP or ADP. Unless stated otherwise, Na_2SO_3 was added to the assay media either as a buffered 2 M stock solution at pH 8 or 7.5, depending on the conditions during measurements.
Assay media. ATP hydrolysis and cyclic photophosphorylation in *S.* 6716 membrane vesicles were assayed in medium *A* (50 mM KCl, 4 mM $MgCl_2$, 5 mM NaH_2PO_4 and, depending on the required pH range during assay, 1 mM Tricine-NaOH pH 8, or 1 mM HEPES-NaOH pH 7.5). ATP hydrolysis and photophosphorylation in *Rs. rubrum* chromatophores were assayed in medium *B* (50 mM KCl, 50 mM NaCl, 3 mM $MgCl_2$, 5 mM NaH_2PO_4 and, depending on the required pH range during assay, 2 mM Tricine NaOH pH 8, or 2 mM HEPES-NaOH pH 7.5). ATP hydrolysis in *Pa. denitrificans* vesicles was assayed in medium *C* (3 mM Tricine-NaOH pH 8, 10 mM KCl, 50 mM NaCl, 6.7 mM $MgCl_2$, and 400 mM sucrose). Actual pH values of the incubations were determined, averaged over the set of experiments and given in the legends. ATP hydrolysis in SMP was assayed in medium *D* (33 mM Tris-HCL pH 7.5, 1 mM $MgCl_2$, 10 mM KCl, 2 mM phospho*enol*pyruvate, 10 $U.ml^{-1}$ pyruvate kinase, 5,5 $U.ml^{-1}$ lactate dehydrogenase, 0.25 mM NADH, 1 μM S-13, 3 μM rotenone, 83 mM sucrose). ATP synthesis/state-3 respiration in SMP was assayed in medium *E* (10 mM HEPES-KOH pH 7.5, 10 mM potassiumphosphate buffer, 4 mM $MgCl_2$, 3.3 mM AMP, 0.1 mM EDTA, 0.25 M sucrose, 20 mM glucose, 2.6 $U.ml^{-1}$ hexokinase).
Chemicals. ATP, ADP, NADH, phospho*enol*pyruvate and all enzymes were purchased from Boehringer (Mannheim, Germany). AMP, and phenazinemethosulfate were purchased from Sigma (St. Louis, U.S.A.). S-13 was a gift from Dr. P.C. Hamm (Monsanto Company, St.Louis, USA).

RESULTS

In Table 1 the effect of sulfite on the activity of ATP hydrolysis is shown for *S.* 6716 membrane vesicles, *Rs. rubrum* chromatophores and *Pa. denitrificans* membrane vesicles. In all three cases ATP hydrolysis is positively influenced by sulfite. Additionally, in these membrane preparations a basal ATP hydrolysis activity can already be observed in the absence of sulfite.
At pH values of about 8, half-maximal activation is at concentrations ranging from 11 to 14 mM sulfite for all three bacterial preparations. Likewise, activation is maximal from about 30 mM Na_2SO_3. Measurements at lower pH were done for *Rs. rubrum* chromatophores by using an assay medium (weakly) buffered with HEPES at pH 7.5. In that case activation by sulfite is more effective at lower concentration. This can be ascribed to the fact that HSO_3^- (bisulfite) is the ionic species actually responsible for activation [22].

In SMP the ATP hydrolysis in presence of 1 mM ATP was stimulated 1.5 times by 0.25 mM $K_2S_2O_5$ (being equivalent to about 0.5 mM HSO_3^-). However, it was not possible to determine the effect of higher concentrations of sulfite on ATP hydrolysis, as $K_2S_2O_5$ at concentrations beyond 1 mM inhibits the ATP-regenerating system (not shown).

In Fig. 1 the effect of sulfite on cyclic photophosphorylation is presented for *S.* 6716 membrane vesicles (A), and *Rs. rubrum* chromatophores (B), showing a remarkable difference. The *S.* 6716 membrane vesicles are inhibited when sulfite is present. Cyclic photophosphorylation catalyzed by *Rs. rubrum* chromatophores on the other hand is not influenced by sulfite in the same concentration range. In the case of *S.* 6716 membrane vesicles, at sulfite concentrations beyond 10 mM, inhibition is most effective at the lowest pH value tested. Complete inhibition is attained at 25 mM and pH 7.7. This is an indication that the HSO_3^- anion (about 6 mM at pH 7.7 as calculated with $pK_a = 7.2$) is again the ionic species responsible for the observed inhibition.

In *Pa. denitrificans* vesicles the effect of sulfite on oxydative phosphorylation was not determined. In the absence of ADP, the electron transfer from 1 mM succinate to O_2 is inhibited for 80 % by 2 mM Na_2SO_3. A similar effect was found for SMP. However, because inhibition of electron transfer was relatively little at low sulfite concentration it was possible to discriminate

Table 1
Activation of ATP hydrolysis activity by Na_2SO_3 in three different membrane preparations. *S.* 6716 membrane vesicles assayed in medium *A*. Initial [ATP] = 3.8 mM, [Chl *a*] = 32 $\mu g.ml^{-1}$, pH 8.2, and t= 37 °C. *Rs. rubrum* chromatophores assayed in medium *B*. Initial [ATP] = 3.5 mM, [BChl] = 9.7 $\mu g.ml^{-1}$, pH values as indicated and t = 35 °C. *Pa. denitrificans* membrane vesicles assayed in medium *C*. Initial [ATP] = 4 mM, [protein] = 110 $\mu g.ml^{-1}$, pH 8.0,and t = 25 °C. ATP hydrolysis is normalized on basis of respectively the chlorophyll a, the bacteriochlorophyll and the protein concentrations of the three membrane preparations.

Membrane preparation	ATP hydrolysis ($\mu mol.min^{-1}.mg^{-1}$)	+30 mM Na_2SO_3	Half max. activation at (mM Na_2SO_3)
S. 6716	0.9	2.6	14
Rs. rubrum	2.5	11.8	11 (pH 8.0) 5 (pH 7.5)
Pa.denitrificans	0.2	0.3	14

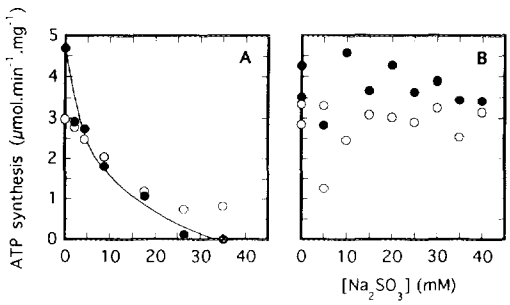

Figure 1. Effect of Na_2SO_3 on ATP synthesis in two different membrane preparations A: *S.* 6716 membrane vesicles assayed in medium *A* with 28 µM phenazinemethosulphate at pH 7.7 (● solid line) and 8.1 (○). Initial [ADP] = 2 mM, initial [NaH_2PO_4] = 5 mM, [Chl *a*] = 30 µg.ml^{-1}, t = 37 °C. B: *Rs. rubrum* chromatophores assayed in medium *B* with 24 µM phenazinemethosulphate and 0.5 mM ascorbate at pH 7.7 (●) and 7.9 (○). Initial [ADP] = 2.4 mM, initial [NaH_2PO_4] = 4.4 mM, [BChl] = 9.8 µg.ml^{-1}, and t = 35 °C. ATP synthesis is normalized on basis of the chlorophyll *a* (A) or the bacteriochlorophyll concentration (B) of the membrane preparations.

between the effects of sulfite on electron transfer and ATP synthesis. In the absence of ADP and in the presence of 0.1 µM S-13 and 30 mM Na_2SO_3 the electron transfer from succinate to O_2 was inhibited for 69%. State-3 respiration is inhibited to the same extend as respiration with uncoupler and in the absence of ADP (not shown). This means that the apparent inhibition of ATP synthesis in SMP by sulfite is clearly due to the inhibition of the state-3 respiration. On the other hand, 38 mM Na_2SO_3 did not inhibit PMS mediated cyclic electron transfer, measured as light induced proton uptake in *S.* 6716 membrane vesicles (unpublished results).

Steady-state ATP dependent kinetics of ATP hydrolysis are compared for all the membrane preparations discussed here. K_m and V_{max} values are listed in Table 2. From this table it becomes clear that sulfite increases V_{max}, especially in membrane preparations isolated from *Pa. denitrificans* and *Rs. rubrum*. The data further reveal that the apparent K_m for ATP hydrolysis

Table 2
Kinetic parameters for ATP hydrolysis in different membrane preparations in absence or presence of HSO_3^-. for [HSO_3^-] are calculated using the Henderson- Hasselbalch equation. Apparent K_m and V_{max} values are determined with a nonlinear least square fit of the Michaelis-Menten equation to the ATP hydrolysis data. V_{max} values for *S.* 6716 membrane vesicles and *Rs. rubrum* chromatophores are normalized to Chl *a* and BChl concentrations, respectively, those of *Pa. denitrificans* membrane vesicles and SMP are normalized to protein concentrations.

Membrane preparation	[HSO_3^-] (mM)	K_m for ATP (mM)	V_{max} (µmol.min^{-1}.mg^{-1})
S. 6716	0	1.0	1.0
	1.5	0.38	1.3
Rs. rubrum	0	0.20	2.8
	2.2	0.54	15.4
Pa. denitrificans	0	0.28	0.1
	1.0	0.56	0.3
SMP	0	0.05	5.3
	0.5	0.05	8.4

catalyzed by *S.* 6716 membrane vesicles is decreased whereas V_{max} is slightly affected in the presence of sulfite. In SMP sulfite clearly has no effect on K_m. The two other bacterial membrane preparations show an increase of K_m of at least a factor 2 in the presence of sulfite.

DISCUSSION

The results presented in Table 1 and 2 clearly show that in all preparations ATP hydrolysis is stimulated by sulfite. Background ATP hydrolysis activity observed in all membrane preparations, including the SMP implies that a fraction of the enzyme survives the membrane isolation procedure in an active state possibly as a result of the partial loss or displacement of regulatory polypeptides [23]. Additionally, inhibitor studies with *S.* 6716 membrane vesicles showed that a minor part of this background activity is a result of other ATP consuming processes (Van Walraven, unpublished).

The sulfite-induced changes of ATP-dependent kinetics (Table 2) are not very different between the membrane preparations tested. Although K_m may increase or decrease in the different preparations, the changes induced by sulfite are relatively small. The effect in all cases is an increase of V_{max}. But these kinetic data do not reveal clear-cut differences that correspond with the different sensitivities of ATP synthesis to sulfite among the membrane preparations tested.

Inhibition of ATP synthesis by Na_2SO_3 is demonstrated here for *S.* 6716 membrane vesicles, but also occurs in spinach chloroplasts (unpublished results). ATP synthesis catalyzed by *Rs. rubrum* chromatophores is insensitive to sulfite while in SMP the electron transfer is inhibited. Inhibition of state 3 respiration to the same extend shows that in SMP there is no additional effect of sulfite on the ATP synthase itself.

The absence of sulfite inhibition of ATP synthesis, may be related to the occurrence of sulfur metabolism that involves sulfite. This would apply to *Rs. rubrum* [see 24], the related *Pa. denitrificans*, and perhaps (as an evolutionary relict) also to the more distantly related mitochondria. On the other hand, only a few cyanobacterial species are able to gain energy from the oxidation of H_2S (sulfide) to elemental sulfur by anoxygenic photosynthesis in which sulfite does not participate as an intermediate [25]. Therefore, this metabolic difference between the purple bacteria and the cyanobacteria may account for the observed sensitivity of ATP synthesis to sulfite in the latter group. Sulfite may interact with the phosphate binding site [7] on the β subunit of the ATP synthase. The high degree of conservation of the amino acid sequences adjacent to the N-terminal side of the phosphate-binding P-loop in cyanobacteria and chloroplasts, compared with purple bacteria and mitochondria [26,27] may provide a molecular clue to the observed differences in sensitivity to sulfite inhibition.

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REFERENCES

1. Krab, K., Bakels, R.H.A., Scholts, M.J.C., Van Walraven, H.S. (1993) *Biochim. Biophys. Acta 1141*, 197-205.
2. Vulfson, E.N., Drobinskaya, I.E., Kozlov, I.A., Murataliev, M.B. (1984) *Biol. Membranes (USSR) 1*, 696-708.
3. Bruist, M.F., Hammes, G.G. (1981) *Biochemistry 20*, 6298-6305.
4. Schumann, J., Strotmann, H. (1981) in *Photosynthesis II. Photosynthetic Electron Transport and Photophosphorylation* (G. Akoyunoglou, Ed.) pp. 881-892, Balaban, Philadelphia.
5. Moyle, J., Mitchell, P. (1975) *FEBS Lett. 56*, 55-61.
6. Larson, E.M., Umbach, A., Jagendorf, A.T. (1989) *Biochim. Biophys. Acta 973*, 78-85.
7. Guerrero, K.J., Xue, Z., Boyer, P.D. (1990) *J. Biol. Chem. 265*, 16280-16287.
8. Du, Z., Boyer, P.D. (1990) *Biochemistry 29*, 402-407.
9. Kibak, H., Vaneeckhout, D., Cutler, T., Taiz, S.L., Taiz, L. (1993) *J. Biol. Chem. 268*, 23325-23333.
10. Lubberding, H.J., Offereijns, F., Vel, W.A.C., De Vries, P.J.R. (1981) in *Photosynthesis II. Photosynthetic Electron Transport and Photophosphorylation* (G. Akoyunoglou, Ed.) pp. 779-788, Balaban, Philadelphia.
11. Krenn, B.E., Van Walraven, H.S., Scholts, M.J.C., Kraayenhof, R. (1993) *Biochem. J.* 294, 705-709.
12. Chang, J.P., Morris, J.G. (1962) *J. Gen. Microbiol. 29*, 301-310.
13. Lawford, H.G., Cox, J.C., Garland, P.B., Haddock, B.A. (1976) *FEBS Lett. 64*, 369-374.
14. Bakels, R.H.A., Van Walraven, H.S., Scholts, M.J.C., Krab, K., Kraayenhof, R. (1991) *Biochim. Biophys. Acta 1058*, 225-234.
15. Witholt, B., Van Heerikhuizen, H., De Leij, L. (1976) *Biochim. Biophys. Acta 443*, 534-544.
16. Herweijer, M.A., Berden, J.A., Kemp, A., Slater, E.C. (1985) *Biochim. Biophys. Acta 809*, 81-89.
17. Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y., Wada, K. (1974) *Biochim. Biophys. Acta 357*, 231-245.
18. Clayton, R.K. (1963) in: *Bacterial Photosynthesis* (H. Gest, A. San Pietro, L.P. Vernon, Eds.) pp. 495-500, Antioch Press, Yellow Springs,Ohio.
19. Smith, P.K., Krohn, R.I., Hermansson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985) *Anal. Biochem. 150*, 76-85.
20. Bradford, M. (1976) *Anal. Biochem. 72*, 248-254.
21. Bergmeyer, H.U. (1970) *Methode der Enzymatische Analyse*. Verlag Chemie, Weinheim, Germany.
22. Larson, E.M., Jagendorf, A.T. (1989) *Biochim. Biophys. Acta 973*, 67-77.
23. Racker, E., Horstman, L.L. (1967) *J. Biol. Chem. 242*, 2547-2551.
24. Brune, D.C. (1989) *Biochim. Biophys. Acta 975*, 189-221.
25. Cohen, Y., Jørgenssen, B.B., Revsbech, N.P., Poplawski, R. (1986) *Appl. and Env. Microbiol. 51*, 398-407.
26. Van Walraven, H.S., Lutter, R., Walker, J.E. (1993) *Biochem. J. 294*, 239-251.
27. Falk, G., Hampe, A., Walker, J.E. (1985) *Biochem. J. 228*, 391-407.