Permeability of the Mitochondrial Membrane to Bicarbonate Ions

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Osmotic-swelling techniques show that HCO_3^- enters mitochondria by an electrogenic process, effectively HCO_3^- uniport, under non-energized conditions. This mode of translocation accounts for previous reports of non-entry of HCO_3^- in experiments with energy-linked Ca^{2+} uptake. The effects of HCO_3^- on mitochondrial respiration are reported and discussed.

Chappell & Crofts (1966) reported that CO_2 , but not HCO_3^- , supported continued respiration or ATPase (adenosine triphosphatase)-driven swelling of mitochondria in the presence of K^+ and valinomycin or gramicidin, and concluded that CO_2 , but not HCO_3^- , was able to pass across the mitochondrial membrane. Elder & Lehninger (1973) reached a similar conclusion from observations showing that CO_2 but not HCO_3^- supported substantial respiration-driven accumulation of Ca^{2+} by mitochondria. However, since Ca^{2+} ions enter mitochondria by an electrogenic process (Selwyn *et al.*, 1970a), which may be uniport or a $(\text{Ca}_2\text{HPO}_4)^{2+}$ symport as suggested by Moyle & Mitchell (1977), the ion-balancing requirement for energy-linked uptake of Ca^{2+} is not electrogenic uniport of an accompanying anion, but entry of a weak acid such as acetic, or exchange of an anion such as PO_4^{3-} or Cl^- for OH^- (Lehninger, 1974; Stockdale *et al.*, 1970). Ions such as SCN^- , NO_3^- , ClO_3^- or ClO_4^- , which enter by electrogenic uniport, do not support energy-linked accumulation of Ca^{2+} (Lehninger, 1974). Thus the observations of the failure of HCO_3^- to enter mitochondria in the experiments on energy-linked cation (Ca^{2+} or K^+ plus valinomycin) uptake do not demonstrate impermeability of the membrane to HCO_3^- ions. On the contrary, in the course of an investigation of mitochondrial anion permeability with passive osmotic-entry techniques, we have observed that HCO_3^- readily enters mitochondria by an electrogenic uniport.

Experimental

Materials

ADP, antimycin A and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were obtained from Boehringer Corp. (London), Ltd., Lewes, East Sussex, U.K. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. NH_4HCO_3 , KHCO_3 and KCl were of A.R. grade from BDH Chemicals, Ltd., Poole, Dorset, U.K.

Rotenone and soya-bean phospholipid (phosphatidylcholine, type IIS) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. The soya-bean phospholipid (azolectin) was purified by the method of Kagawa & Racker (1971) and stored in solution in chloroform. Acetazolamide [*N*-[5-(aminosulphonyl)-1,3,4-thiadiazol-2-yl]acetamide, sodium salt] was obtained from American Cyanamid Co., Pearl River, NY, U.S.A. and valinomycin from Calbiochem, Ltd., Bishop's Stortford, Herts., U.K. All other reagents were of A.R. grade.

Methods

The preparation of rat liver mitochondria (in 0.25M-sucrose/5mM-Hepes/KOH, pH 7.6), assay of mitochondrial protein, preparation of liposomes with purified soya-bean phospholipid (azolectin) instead of phosphatidylcholine and measurement of light-scattering by liposome or mitochondrial suspensions were as described by Selwyn *et al.* (1970b). Simultaneous recordings of oxygen concentration and light-scattering were made as described by Stockdale *et al.* (1970).

Results

The modes of entry of K^+ in the presence of valinomycin as electrogenic uniport, of NH_3 , rather than NH_4^+ ions, and the ability of uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to act as proton uniporters are now well established.

The anion-permeability of the membranes of vesicles such as mitochondria or liposomes is thus readily investigated by observing light-scattering changes of the vesicle suspensions resulting from osmotic swelling or shrinking of the vesicles in iso-osmotic solutions of ammonium or potassium salts of the anions (Chappell & Haarhoff, 1967; Selwyn *et al.*, 1970b).

Mitochondria suspended in an NH_4HCO_3 medium (Fig. 1a) swell rapidly, as would be expected from

the known permeability of NH_3 and CO_2 , the net effect of this transfer being equivalent to entry of NH_4HCO_3 , since water moves freely across the membrane. Addition of acetazolamide to block carbonic anhydrase activity decreases the rate very markedly (Fig. 1*b*), as under these conditions the rate will be limited by the slow uncatalysed rehydration of CO_2 within the mitochondria. Addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxy-

phenylhydrazone increases the rate of swelling (Fig. 1*c*), which remains rapid in the presence of acetazolamide (Fig. 1*d*), and these results show that HCO_3^- and/or CO_3^{2-} must be able to pass across the mitochondrial membrane, the uncoupler being necessary to maintain electrical and pH balance across the membrane. That HCO_3^- passes across the membrane is shown by the swelling of mitochondria in KHCO_3 medium in the presence of valinomycin

Fig. 1. Swelling of mitochondria in iso-osmotic bicarbonate salts

Mitochondria (2.2 mg of mitochondrial protein) were added to 4 ml of 100 mM-salt solutions adjusted to pH 7.6 at 30°C, containing 1 μg of rotenone and 1 μg of antimycin. Salts and additions were: (a) NH_4HCO_3 ; (b) NH_4HCO_3 plus 25 μM -acetazolamide; (c) NH_4HCO_3 plus 1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; (d) as (c) plus 25 μM -acetazolamide; (e) KHCO_3 , valinomycin, 50 nM, was added at the time indicated by the arrow; (f) as (e) plus 25 μM -acetazolamide. A decrease in absorbance corresponds to swelling of the mitochondria.

Fig. 2. Respiration of isolated mitochondria in KCl , KHCO_3 and mixed media

Each assay contained 12 mg of mitochondrial protein, 10 mM-potassium succinate, 10 mM-potassium phosphate, and rotenone 0.2 μg of, in a total volume of 5 ml at 30°C. (a) 0.1 M- KCl /5 mM-Hepes, adjusted with KOH to pH 7.6; (b) 0.1 M- KHCO_3 /5 mM-Hepes, adjusted with KOH to pH 7.6; (c) 0.02 M- KHCO_3 /0.08 M- KCl /5 mM-Hepes, adjusted with KOH to pH 7.6. In each case the upper trace is the oxygen tension, the lower trace is the light-scattering record. The arrows labelled ADP mark the addition of 1 μmol of ADP and those labelled FCCP, the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to give a final concentration of 0.4 μM .

(Fig. 1e), since this is little affected by acetazolamide (Fig. 1f) and does not require the presence of uncoupler. At the pH used, swelling entails net entry of KHCO_3 , and hence entry of K^+ and CO_2 , or K^+ and CO_3^{2-} , would require the presence of uncoupler to maintain electrical neutrality and prevent development of a pH difference across the membrane. Entry of both CO_2 and CO_3^{2-} , although equivalent to entry of HCO_3^- is not a satisfactory explanation, since it would be slowed by inhibition of carbonic anhydrase. It is therefore concluded that direct passage of HCO_3^- across the mitochondrial membrane by electrogenic uniport must occur at a rapid rate.

Similar experiments with liposomes made with soya-bean phospholipids failed to show rapid swelling in NH_4HCO_3 or KHCO_3 media, which suggests that there is some carrier system for HCO_3^- in the mitochondrial inner membrane.

The permeability of the mitochondrial membrane to both HCO_3^- and CO_2 suggests that this system could function as an uncoupler. Replacement of Cl^- by HCO_3^- (Figs. 2a and 2b) results in a marked inhibition of the rates of basal, ADP-stimulated and uncoupler-stimulated respiration. Partial replacement of Cl^- (Fig. 2c) leads to lesser inhibition and, although the respiratory-control index (rate of ADP-stimulated respiration/rate after utilization of ADP) is decreased from 4.2 in the Cl^- medium to 2.5 in the presence of 20mM- HCO_3^- the ADP/O ratio remained unchanged within experimental error at 1.8. The percentage inhibition of the rate before addition of ADP is 42%, which is very similar to the percentage inhibition of the ADP-stimulated rate (38%), whereas the rate after utilization of the ADP is virtually unchanged by the presence of 20mM- HCO_3^- . The inhibition of basal respiration might be a result of an increased mitochondrial-membrane potential produced by inwards diffusion of HCO_3^- or, more probably, since uncoupler-stimulated respiration is also inhibited, it may be a direct effect on one or more mitochondrial enzymes or electron carriers. The failure to observe stimulation of respiration by an uncoupling effect of the $\text{CO}_2/\text{HCO}_3^-$ couple may be due partly to the inhibitory effects masking the stimulation and also to the low concentration of CO_2 in equilibrium with HCO_3^- and H_2CO_3 at pH 7.6, limiting the uncoupling rate.

Discussion

HCO_3^- is a major ionic component of cells *in vivo* and the potentiality for uncoupling and inhibition of succinate oxidation are of physiological interest. However, since the $\text{CO}_2/\text{HCO}_3^-$ is mostly produced in the mitochondria, there will be a concentration gradient that will oppose CO_2 entry into the mitochondria. In the absence of re-entry of CO_2 , energy

loss will occur if the major mode of exit is HCO_3^- rather than CO_2 , but if the H^+/ATP stoichiometry is 2, then the maximal loss would amount to only 10% of the energy conserved as ATP in the case of pyruvate oxidation.

The HCO_3^- movement is driven by the mitochondrial-membrane potential and is thus a mechanism for energy-linked HCO_3^- extrusion which will maintain a low intramitochondrial HCO_3^- concentration. In view of the effect on succinate (3-carboxypropionate) oxidation and the reports of effects of HCO_3^- on the mitochondrial ATPase complex (Ebel & Lardy, 1975; Pedersen, 1976), the present observations suggest that HCO_3^- may have a role in regulating mitochondrial metabolism in relation to the electrical potential and pH differences across the mitochondrial membrane. An alternative explanation for the inhibition of respiration may be that the $\text{CO}_2/\text{HCO}_3^-$ couple decreases the electrical potential and pH differences across the mitochondrial membrane and thereby decreases the intramitochondrial substrate concentration.

A point of experimental importance is that CO_2 and HCO_3^- movements may affect the values obtained in measurements of proton stoichiometry such as those of Moyle & Mitchell (1977), especially as, in their experiments carbonic anhydrase was added to the medium rather than an inhibitor of the intramitochondrial carbonic anhydrase.

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