PHOSPHORYLATION COUPLED TO MALATE OXIDATION IN ACETOBACTER XYLINUM

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The oxidation of malate to oxaloacetate in A. xylinum has been shown to be irreversible. It is catalyzed by a FAD flavoprotein which is not affected by high concentrations of oxaloacetate and is linked to the cytochrome chain by a vitamin K-like compound (Benziman and Abeliovitz, 1964; Benziman and Galenter, 1964; Benziman and Perez, 1965). In the present communication it is shown that cell-free extracts of A. xylinum couple ATP formation with malate oxidation. The properties of this phosphorylation system are compared to the phosphorylation associated with the oxidation of other substrates by such extracts.

Materials and Methods. Succinate-grown cells of A. xylinum were grown and harvested as described previously (Benziman and Burger-Rachamimov, 1962). Cell free extracts were obtained by treating cell-suspensions (20 mg dry wt/ml) in a French pressure cell under 15000 lb/in^2 followed by centrifugation for 15 min at 18000 x g, discarding the precipitate. Particles were sedimented from extracts by centrifugation for 1 h at 100000 x g. Cells and particles were suspended in a medium containing 0.02 M Tris-HCl buffer (pH 7.3), 0.2 M sucrose, 5mM $MgCl_2$ and 1mM EDTA. Phosphorylation was measured by the incorporation of P^{32} into organic phosphate (Avron, 1960). Oxidation was measured either manometrically or by determining the amount of oxaloacetate formed (Benziman and Abeliovitz 1964).

Results and Discussion. The requirements for phosphorylation coupled to malate oxidation by crude extracts or particles, are given in Table 1. Oxaloacetate was the sole carbon compound formed from malate. Substitution of oxaloacetate or pyruvate for malate did not result in any oxidation or phosphorylation. Thus the oxidation of malate studied was a one-step oxidation reaction. Phosphorylation but not oxidation was dependent on the presence of an ATP-trapping system (hexokinase plus mannose). Omission of ADP or its substitution by AMP greatly decreased phosphorylation without affecting oxidation. Thus our system like other microbial systems (Asano and Brodie, 1965) may be regarded as loosely coupled as it does not seem to be under respiratory control. Incorporation of P^{32} into nucleotides by adenylate kinase, which was present in the extracts, was greatly inhibited by fluoride. The stimulatory effect of bovine plasma albumin is attributed to the binding of endogenous uncouplers. (Racker, 1965). The concentration of Pi did not affect oxidation but phosphorylation increased with increasing concentrations of Pi, with maximal activity at approximately 5mM. Concentrations of ADP above 2mM had little effect on phosphorylation but significantly increased the blank value.

Crude extracts catalyzed oxidative phosphorylation with various substrates (Table 2). The requirements for optimal phosphorylation with all substrates were the same as those described for malate in Table 1. In no case was any phosphorylation observed under anaerobic conditions. Oxidation of $NADH_2$ occured at a rate which

Table 1

Oxidative phosphorylation by A. xylinum extracts

Components	Oxidation (μ atoms O)	Phosphorylation (μ moles Pi)
All	7.5	1.5
- ADP	7.5	0.3
-Hexokinase	7.6	0.2
-Mannose	7.6	0.1
-$MgCl_2$	7.2	1.0
-NaF	7.5	1.6
-Bovine plasma albumin	7.0	0.83
-Substrate	0	0.1

Warburg double side arm flasks contained in the main chamber: 50 μ moles Tris-HCl (pH 7.6), 10 μ moles P^{32} (pH 7.6) (10^4 counts/min/μ mole). 1 mg hexokinase (Sigman, type V), particles (2.5 mg protein) 15 μ moles $MgCl_2$ and 6 mg bovine plasma albumin. In one side arm: 25 μ moles NaF, 2.5 μ moles ADP, 25 μ moles mannose, 20 μ moles L-Malate. Final volume 1.2 ml. In the center well 0.2 ml of 5N NaOH, and in the second side arm 0.1 ml of 3M $HClO_4$ to stop the reaction after 15 min at 30 C. Blank mixtures (minus malate) were run simultaneously for each experiment. The results represent the difference between the two reaction mixtures.

Table 2

Oxidative phosphorylation with various substrates

Substrate	Oxidation (μ atoms O per mg protein)	Phosphorylation (μ moles Pi per mg protein)	P/O
Malate	1.50	0.30	0.20
$NADH_2$	1.60	0.29	0.18
$NADPH_2$	0.76	0.12	0.16
Isocitrate	0.04	0.014	0.35
Isocitrate + NAD^+	0.50	0.10	0.20
Isocitrate + $NADP^+$	0.60	0.10	0.17

Each vessel contained complete reaction mixture as in Table 1, crude extract (4-6 mg protein), substrate 20 μ moles and where mentioned 1.2 μ moles NAD^+ or $NADP^+$. 15 min 30 C. Reaction carried out simultaneously in a Warburg apparatus and in a shaking bath in side arm flasks. In the latter case oxidation was measured by determining oxaloacetate or α-keto glutarate formed, for the malate and isocitrate experiment respectively. For the $NADH_2$ and $NADPH_2$ experiments, aliquotes were withdrawn into neutralized ethanol and changes in absorption at 340 mμ determined. Similar results were obtained by the two methods with a stoichiometry of 1 μ atom oxygen taken up per μ mole of substrate oxidized. Under the essay conditions both the rates of oxidation and phosphorylation were linear with time up to 60 min.

was more than 2 times greater than that of $NADPH_2$, and these oxidations were accompanied by phosphorylation. The P/O ratio obtained was the same in both cases. Isocitrate was oxidized stoichiometrically to alpha keto-glutarate, which was not further metabolized under the experimental conditions used. Thus in this case also the reaction was a one step oxidation. Similar rates of oxidation and phosphorylation were obtained with isocitrate in the presence of either NAD^+ or $NADP^+$.

The results presented in Table 2 show that the ability to form ATP, estimated by the P/O ratios observed with various substrates is significantly lower than that of mammalian systems, suggesting the possibility that

the respiratory chains in A. xylinum may have fewer phosphorylating sites than those of animal tissues or alternatively that the microbial system is much more readily damaged or uncoupled.

Oxidative phosphorylation with the various substrates was affected differently by some inhibitors and uncoupling agents (Table 3). In all cases both oxidation and phosphorylation were inhibited by low concentrations of cyanide. The complete inhibition of phosphorylation by low concentrations of oligomycin is another indication

Table 3

Inhibition of respiration and phosphorylation

	Inhibition					
	Malate		$NADH_2$		Isocitrate	
Inhibitor	ΔO_2	Δ Pi	ΔO_2	ΔPi	ΔO_2	ΔPi
	%	%	%	%	%	%
CN^- (1mM)	100	100	100	100	100	100
DNP (0.8mM)	15	30	0	23	25	50
Dicumarol (0.1mM)	100	100	87	90	85	90
Dicumarol (0.01mM)	40	75	42	80	40	87
Amytal (2mM)	82	90	0	40	0	62
Oligomycin (0.6 γ/mg protein)	4	90	5	86	4	90
Irradiation (45 min, 360 mμ)	50	40	0	0	0	0
K_3(0.1mM)	-175[x]	78	-200[x]	- 20[x]	-200[x]	-18[x]

[x] (-) = Stimulation

Test systems as in Table 2. Isocitrate was supplemented with 1.2 μ moles NAD^+. K_3 and oligomycin were added in methanol. Irradiation was carried out as previously described (Benziman and Perez, 1965)

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that ATP formation proceeds via the reactions of oxidative phosphorylation. The slight inhibition of respiration by oligomycin is in line with the loosely coupled nature of the system (Hujing and Slater, 1961). 2.4 Dinitrophenol uncoupled only slightly phosphorylation in A. xylinum systems at a relatively high concentration . Whereas with malate amytal inhibited both oxidation and phosphorylation, with $NADH_2$ and isocitrate amytal acted as an uncoupler. This observation is in line with the suggestion (Chance et al., 1962) that amytal inhibits energy transfer at the $NADH_2$ and flavin site. The lack of inhibition of $NADH_2$ and isocitrate oxidation suggests different electron transport pathways for malate and $NADH_2$ oxidation, at least in the region assigned for amytal inhibition of electron transfer, namely between flavin and the cytochrome chain (Estabrook, 1957). Rotenone ($2x10^{-5}$M) had no effect in our system on oxidation and phosphorylation with any of the substrates tested. Dicumarol inhibited at high concentrations both respiration and phosphorylation with all the three substrates while at low concentrations it uncoupled phosphorylation. This may indicate that in A. xylinum, dicumarol may have two sites of action: At low concentrations it may affect phosphorylation and at high concentrations it may inhibit a specific respiratory site. The effect of dicumarol was more pronounced in the absence of bovine plasma albumin, due probably to the absorption of the inhibitor on the added protein. Menadione (K_3) stimulated the respiration observed with all the three substrates, but only in the case of malate did it inhibit coupled phosphorylation. This result may suggest that the phosphorylation step(s) of

the respiration chain measured with malate as substrate is not the same as that measured with $NADH_2$ or isocitrate, and that the former is more susceptible to uncoupling by K_3. Extracts of A. xylinum rapidly oxidize reduced menadione (K_3H_2) but without coupled phosphorylation. Thus the effect of menadione on phosphrylation could have been attributed to the leakage of electrons from the respiratory chain to form K_3H_2. However, this seems unlikely considering the observed lack of inhibition of the phosphorylation with $NADH_2$ or isocitrate. A more likely explanation is that K_3 acts as a real uncoupler by reacting with an enzyme necessary for the phosphorylation coupled to malate oxidation. The observation that light irradiation inhibited oxidation and phosphorylation only with malate (Table 3) may serve as an additional indication that in A. xylinum the respiratory chain from malate to oxygen and the phosphorylation associated with it is to a large extent separate from that catalyzing electron transport from $NADH_2$ to oxygen.

References:

Asano A., and Brodie, A. F., J. Biol. Chem. 240 4002 (1965)
Avron, M., Biochim. Biophys. Acta 40, 257 (1960)
Benziman, M., and Abeliovitz, A., J. Bacteriol. 87, 270 (1964)
Benziman, M., and Burger-Rachamimov, H., J. Bacteriol. 84 625 (1962)
Benziman, M., and Galenter, Y., J. Bacteriol. 88, 1010 (1964)
Benziman, M., and Perez, L., Biochem. and Biophys. Research Communs. 19, 127 (1965)
Chance, B., Hollunger, G., and Hagihara, B., Biochem. and Biophys. Research Communs. 8 180 (1962)
Estabrook, R. W., J. Biol. Chem. 227 1093 (1957)
Hujing, H., and Slater, E. C., J. Biochem. (Tokyo) 49 493 (1961)
Racker, E., Mechanisms in Bioenergetics, Academic Press, New York 1965, p. 145