Pyruvate-Supported Nitrogen Fixation by Cell-Free Extracts of *Bacillus polymyxa*

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Grau & Wilson (1963) described a method for obtaining cell-free nitrogen fixation with cell-free lysozyme lysates of *Bacillus polymyxa* strain Hino using pyruvate as the source of both electrons and ATP. The nitrogen-fixation activity was low, and the results could not be confirmed by Witz, Detroy & Wilson (1967), who used an extract prepared by crushing the bacteria in a French pressure cell. However, positive results were readily obtained with the assay method described by Bulen, Burns & LeCompte (1965), which uses an ATP-generating system of creatine phosphate and creatine phosphokinase (ATP-creatine phosphotransferase, EC 2.7.3.2) and the electron source sodium dithionite. Thus the cell-free preparation reported by Carnahan, Mortenson, Mower & Castle (1960) remained the only system where a physiological substrate, pyruvate, could be used to support nitrogen fixation. Owing to the discrepancy between the results obtained in our laboratory, we have re-examined this question and have confirmed the earlier report. In addition, we have found that if the pyruvate is used only as a source of electrons nitrogen fixation by cell-free extracts of *B. polymyxa* is equal to that obtained with the ATP-generating system and sodium dithionite.

B. polymyxa was grown as described by Witz *et al.* (1967) and lysates were prepared by resuspending 14g wet wt. of cells in 50ml of degassed

(O₂ replaced with N₂) 25mm - 2 - (N' - 2 - hydroxyethylpiperazin-N-yl)ethanesulphonic acid, pH 7.55, containing 100mg of lysozyme (mucoprotein N-acetylmuramylhydrolase, EC 3.2.1.17) and 1mg of deoxyribonuclease (deoxyribonuclease oligonucleotidohydrolase, EC 3.1.4.5). The mixture was incubated in N₂ gas (1atm) for 1h at 30°C, and then centrifuged at 30 000g for 30min to remove unlysed cells and cellular debris. The supernatant fluid (25–30mg of protein/ml), which contained all the components necessary for pyruvate-supported nitrogen fixation, was stored under liquid N₂ until used.

Nitrogen fixation was assayed in essentially the same way as described by Bulen *et al.* (1965). The reaction mixtures contained 30μmol of creatine phosphate, 0.2mg of creatine phosphokinase, 2.5μmol of ATP, 5μmol of MgCl₂, 20μmol of sodium dithionite, 25μmol of 2-(N'-2-hydroxyethylpiperazin-N-yl)ethanesulphonic acid and protein in a total volume of 1ml at pH 7.55. The reactions were started by injection of the protein and stopped by the addition of 1ml of saturated K₂CO₃. The ammonia was distilled by microdiffusion into a drop of 2.5M-H₂SO₄ and assayed colorimetrically by means of Nessler's reagent. In the assay of pyruvate-supported nitrogen fixation 90μmol of pyruvate was substituted for creatine phosphate, creatine phosphokinase and sodium

Table 1. *Pyruvate-supported nitrogen fixation by cell-free extracts of B. polymyxa*

Nitrogen fixation was assayed as described in the text, with in Expt. I 6.0mg of protein/assay (15 min incubation), in Expt. II 5.2mg of protein/assay (30 min incubation) and in Expt. III 5.0mg of protein/assay (30 min incubation).

	Electron source	ATP source	N fixed (μg)
Expt. I	Pyruvate (90 μmol)	Same	2
	Pyruvate (90 μmol)	ATP (2.5 μmol)	7
	Dithionite (20 μmol)	ATP-generating system	18
Expt. II	Pyruvate (90 μmol)	ATP (2.5 μmol)	21
	Pyruvate (90 μmol)	ATP-generating system	36
	Formate (50 μmol)	ATP-generating system	12
	Dithionite (20 μmol)	ATP-generating system	34
Expt. III	Pyruvate (90 μmol)	Same	5
	Pyruvate (90 μmol)	ATP (2.5 μmol)	26
	Formate (50 μmol)	ATP-generating system	11
	Dithionite (20 μmol)	ATP-generating system	28

dithionite. When pyruvate or formate was used as only the electron source, the sodium dithionite was replaced by 90 μ mol of pyruvate (or 50 μ mol of formate) and 2.5 μ mol of ATP (or the ATP-generating system) was added.

Table 1 summarizes pyruvate-supported nitrogen fixation in the lysates. When pyruvate is used as the source of both electrons and of ATP the fixation is small but definitely positive. The quantity fixed was increased about fourfold by the addition of 2.5 μ mol of ATP. A time-course experiment explained this enhancement. Initially the rates of fixation were equal; however, fixation ceased after 10 min on pyruvate alone but continued in the presence of the added ATP. In some, but not all, experiments the addition of ATP resulted in activity equal to that obtained with the dithionite plus ATP-generating system. Moreover pyruvate plus the ATP-generating system consistently gave specific activities equal to that obtained when

dithionite was used as the source of electrons. The same was true with extracts prepared in the French pressure cell. Probably the discrepancy between the two earlier studies in our laboratory arose from the difference in endogenous ATP in the two types of extracts. H_2 gas, NADH, NADPH or reduced lipoic acid did not replace sodium dithionite; sodium formate did, but was only one-third as effective as pyruvate or dithionite.

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