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Two Malic Enzymes in *Pseudomonas aeruginosa*

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Cell-free extract supernatant fluids of *Pseudomonas aeruginosa* were shown to lack malic dehydrogenase but possess a nicotinamide adenine dinucleotide (NAD)- or NAD phosphate (NADP)-dependent enzymatic activity, with properties suggesting a malic enzyme (malate + NAD (NADP) → pyruvate + reduced NAD (NADH) (reduced NADP [NADPH] + CO_2), in agreement with earlier findings. This was confirmed by determining the nature and stoichiometry of the reaction products. Differences in heat stability and partial purification of these activities demonstrated the existence of two malic enzymes, one specific for NAD and the other for NADP. Both enzymes require bivalent metal cations for activity, Mn^{2+} being more effective than Mg^{2+}. The NADP-dependent enzyme is activated by K^+ and low concentrations of NH_4^+. Both reactions are reversible, as shown by incubation with pyruvate, CO_2, NADH, or NADPH and Mn^{2+}. The molecular weights of the enzymes were estimated by gel filtration (270,000 for the NAD enzyme and 68,000 for the NADP enzyme) and by sucrose density gradient centrifugation (about 200,000 and 90,000, respectively).

Nicotinamide adenine dinucleotide (NAD)-specific (EC 1.1.1.39) and NAD phosphate (NADP)-specific (EC 1.1.1.40) malic enzymes have been described in different bacterial species (8, 12, 13, 16, 19, 21, 27, 31) including several pseudomonads (5, 9, 10, 14). However, only one case is known where both enzymes are present simultaneously in the same cell. This was shown in 1967 by Katsuki et al. in *Escherichia coli* (11). Further work by this group (20, 28, 34) and by Sanwal and co-workers (23-25) has provided considerable information on the properties, regulation, and possible metabolic function of these enzymes.

We have investigated the presence of malic enzyme in *Pseudomonas aeruginosa*, a bacterium with metabolic properties considerably different from those of *E. coli*. In this communication evidence is presented showing that both malic enzymes (NAD- and NADP-dependent) are present in this organism, and that they differ in several properties besides their coenzyme requirement.

MATERIALS AND METHODS

Reagents. Tris(hydroxymethyl)aminomethane (Tris), L-malate, NAD, NADP, oxaloacetate, mercaptoethanol, protamine sulfate, alumina Cγ gel, reduced NAD (NADH), reduced NADP (NADPH), alcohol dehydrogenase, catalase, cytochrome *c*, and lactate dehydrogenase were obtained from the Sigma Chemical Co., St. Louis, Mo. Sephadex G-200 and blue dextran were from Pharmacia, Uppsala, Sweden; sodium pyruvate was from Fluka, A. G. Buchs, Switzerland; Aquacide II was from Calbiochem, San Diego, Calif., and $^{54}MnCl_2$ was from New England Nuclear Corp., Boston, Mass. All other chemicals were analytical grade.

Bacterial growth. *Pseudomonas aeruginosa* (ATCC 9027) was grown for 36 h at 37 C with aeration in carboys containing 12 liters of a synthetic medium composed of 0.66% NH_4Cl, 0.2% K_2HPO_4, 0.0013% $FeSO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0013% $MnSO_4$, and 1% glucose, at pH 7.5. The inorganic components of the medium were dissolved in 90% of the final volume and any precipitate formed was removed by filtration. The filtrate was autoclaved and, after cooling, it was mixed with the glucose (dissolved in the remaining 10% of the final volume) which had been sterilized by filtration with a membrane filter (Millipore Corp.) The cells were harvested by centrifugation, washed once in 0.1 M Tris-chloride (pH 7.5) and kept frozen until used. The yield was 1 to 2 g (wet weight) per liter of culture.

The strain was kept in nutrient agar slants and was transferred to small volumes of the liquid medium before inoculating the carboys.

Preparation of cell-free extracts. The cells were resuspended in the same buffer used for the washing (1 g [wet weight] per 4 ml of buffer). The suspension was treated with a Bronwill sonic oscillator for 2 min and centrifuged for 45 min at 37,000 × g at 4 C. The precipitate was discarded and the supernatant fluid was used for the enzyme studies.

Enzyme assays. Malic enzymes, malic dehydro-

genase, and alcohol dehydrogenase were measured spectrophotometrically at 30 C with an Eppendorf colorimeter at 366 nm or a Varian model 635 spectrophotometer at 340 nm. The reactions were run in cuvettes of 1-cm light path, and the volume of the mixture was 3 ml.

For malic dehydrogenase, each cuvette contained 4 μmol of L-malate, 3 μmol of NAD or NADP, 250 μmol of Tris-chloride buffer (pH 7.5), and enzyme. For the inverse reaction, 250 μmol of Tris-chloride buffer (pH 7.4), 4 μmol of oxaloacetate, 3 μmol of NADH or NADPH, and enzyme were used.

For NAD-dependent malic enzyme, each cuvette contained 5 μmol of L-malate, 3 μmol of NAD, 10 μmol of $MnCl_2$ (or $MgCl_2$ where indicated), 125 μmol of Tris-chloride buffer (pH 7.6), and enzyme. For the inverse reaction, 240 μmol of Tris-chloride buffer (pH 8.2), 150 μmol of sodium pyruvate, 150 μmol of CO_2-saturated sodium bicarbonate, 1 μmol of $MnCl_2$, 3 μmol of NADH, and enzyme were used.

For NADP-dependent malic enzyme, each cuvette contained 125 μmol of Tris-chloride buffer (pH 7.6), 50 μmol of L-malate, 3 μmol of NADP, 10 μmol of $MnCl_2$ (or $MgCl_2$ where indicated), and enzyme. To assays with the purified enzyme were added 50 μmol of KCl. For the inverse reaction, 240 μmol of Tris-chloride buffer (pH 8.2), 150 μmol of sodium pyruvate, 150 μmol of CO_2-saturated sodium bicarbonate, 1 μmol of $MnCl_2$, 3 μmol of NADH, and enzyme were used.

For alcohol dehydrogenase, each cuvette contained 48 μmol of sodium pyrophosphate buffer (pH 8.8), 1 mmol of ethanol, 25 μmol of NAD, and enzyme.

Enzyme activities are expressed in units. One unit is the amount of enzyme required to transform 1 μmol of NAD (H) or NADP (H) per minute at 30 C. Specific activity is expressed in units per milligram of protein.

Catalase was determined by measuring the breakdown of H_2O_2 at 240 nm according to the procedure of Beers and Sizer (3).

Protein determination was performed according to the method of Lowry et al. (17).

Analysis of the reaction products of the malic enzyme. Pyruvate determination: a reaction mixture for the determination of NAD-or NADP-specific malic enzyme was prepared as described above. After a predetermined change in optical density (OD), 1 ml of the reaction mixture was quickly added to 3 ml of cold 6% perchloric acid. The acid was neutralized with solid K_2CO_3 to a pH near 7, and the precipitate was discarded by centrifugation. The pyruvate content of the supernatant fluid was determined according to Bücher et al. (4) in a cuvette containing (3 ml total volume): 540 μmol of triethanolamine buffer (pH 7.6), 0.5 μmol of NADH, and 1.5 ml of supernatant fluid; the reaction was started with 0.01 ml of lactic dehydrogenase (0.5 mg/ml) in 50% glycerol.

CO_2 determination: CO_2 formation was measured manometrically at 30 C (30) by using the reaction mixtures for NAD- and NADP-specific malic enzymes described above. Pyruvate was determined at the end of the reaction.

Determination of the molecular weight of the malic enzymes. (i) Gel filtration according to the method of Andrews (1, 2) in a Sephadex G-200 column (89 by 2.5 cm), was performed. The enzymes were eluted from the column with the same procedure described for the purification of the NAD-dependent enzyme. The column was calibrated with the following compounds: $^{54}MnCl_2$ (carrier-free) (1 μCi added, determined in a well-counter); cytochrome *c* (16 mg added, followed by OD at 412 nm); yeast alcohol dehydrogenase (11 mg added, followed as described above); gamma globulin (10 mg added, followed by OD at 280 nm), and blue dextran (0.01 mg added, followed by OD at 625 nm). Malic enzymes obtained from a Sephadex G-200 column eluate as described in the purification of the NAD-dependent enzyme were concentrated in a dialysis bag by using Aquacide II for 2.5 h at 4 C. A sample (5 ml) of each enzyme was eluted separately from the column, and activity was followed as described above.

(ii) For sucrose density gradient centrifugation, gradients were prepared at 4 C with 2.5 ml each of 5 and 20% sucrose in 0.05 M Tris-chloride, pH 7.6. On top of the gradients was placed 0.1 ml of a solution prepared by mixing 0.1 ml of horse liver alcohol dehydrogenase (30 mg/ml), 0.005 ml of beef liver catalase (12.5 mg/ml), and 0.2 ml of malic enzyme (NAD-dependent, NADP-dependent, or crude extract). Centrifugation was performed at 4 C for 16 h at 90,000 × *g* in a Spinco model L-2-65B with an SW-65 rotor. After centrifugation, the tubes were punctured and 15 fractions of equal volume were collected. Alcohol dehydrogenase, catalase, and malic enzyme activities were determined as described above. The linearity of the gradient was controlled by preparing a gradient with the heavy sucrose solution containing 0.005% methylene blue. This gradient was centrifuged and fractionated as described, and the methylene blue concentration was determined spectrophotometrically at 670 nm.

RESULTS

Malate oxidizing activity in the crude extract. Malic enzyme and malic dehydrogenase activities were measured in the crude extract supernatant fluid (Table 1). Both reactions, using NAD or NADP, required Mg^{2+} as expected for malic enzyme. The NADH oxidizing activity was not stimulated by oxaloacetate or Mg^{2+}, and it was probably due to an NADH oxidase present in this bacterium (32).

Nature and stoichiometry of the reaction products. Table 2 shows the results obtained when the amount of pyruvate produced was compared to that of CO_2 evolved or pyridine nucleotide reduced. A stoichiometry in agreement with the reaction catalyzed by malic enzyme was observed with either NAD or NADP as cofactor.

Heat stability of the malic enzyme. The results (Fig. 1) show that the NADP-dependent activity was considerably more stable, suggest-

TABLE 1. *Malic dehydrogenase and malic enzyme activity in the cell-free extract supernatant fluid*[a]

Reaction mixture	Activity (mU)
Malate, NADP, Mg^{2+}	90.0
Malate, NADP	45.3
Malate, NADP, EDTA	0
Malate, NAD, Mg^{2+}	54.3
Malate, NAD	0
Oxalacetate, NADH, Mg^{2+}	13.5
Oxalacetate, NADH, EDTA	9.0
NADH	18.0
Oxalacetate, NADPH, Mg^{2+}	0

[a] Activity was followed by measuring oxidation of NADH or reduction of NAD or NADP. Concentrations of substrates and cofactors are as described for malic dehydrogenase in Materials and Methods. Mg^{2+} (10 μmol) and ethylenediaminetetraacetic acid (EDTA) (3 μmol) were added. A sample (0.1 ml) of enzyme was used in each assay (total volume, 3 ml).

TABLE 2. *Products of malate oxidation with supernatant fluids of cell-free extracts*[a]

Expt no.	NAD-dependent activity (μmol)			NADP-dependent activity (μmol)		
	NADH	CO_2	Pyruvate	NADPH	CO_2	Pyruvate
1	0.076		0.076	0.076		0.078
2	0.151		0.147	0.151		0.177
3		2.4	2.12		4.8	4.4
4		4.49	4.1		7.5	7.23

[a] Assay conditions were as described in Materials and Methods.

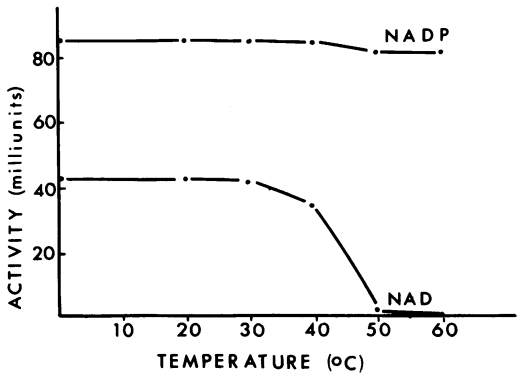

FIG. 1. *Effect of temperature on the NAD-and NADP-dependent malic enzyme activities. Crude extract supernatant fluid was incubated for 3 min at the temperatures indicated. Assays were performed as described in Materials and Methods.*

ing that the activities are due to separate enzymes.

Fractionation of the NADP-dependent activity. All steps were performed at 4 C. Volumes (0.05) of 1 M $MnCl_2$ were added to the crude extract to precipitate nucleic acids. The mixture was centrifuged for 15 min at 27,000 × *g*, and the pellet was discarded. To the supernatant fluid was added ammonium sulfate up to 80% saturation. After a 15-min incubation, the suspension was centrifuged and the precipitate was resuspended in one-sixth of the original volume of 0.1 M Tris-chloride buffer pH 7.6. The pH was lowered to 5.6 with 1 N acetic acid, and aged calcium phosphate gel (prepared according to reference 26) was added (14 mg/ml of enzyme). After stirring for 30 min at 4 C, the gel was centrifuged for 15 min at 15,000 × *g*. The pH of the supernatant fluid was lowered to 5.3 and the mixture was heated for 3 min at 50 C. The suspension was centrifuged and the supernatant fluid was adjusted to pH 7.5 with 0.1 N NaOH.

A summary of the purification is presented in Table 3. Attempts at further purification by using ion-exchange chromatography with Sephadex or cellulose derivatives have not been successful due to instability of the enzyme. The NADP-dependent enzyme can be obtained practically free of the NAD-dependent activity.

Fractionation of the NAD-dependent activity. To the supernatant fluid (placed in a 36 C water bath) was added, over a 5-min period, 2% protamine sulfate in 0.05 M Tris-Chloride, pH 7.5 (1 ml/20 ml of extract). After 5 min at 0 C the cloudy mixture was centrifuged for 10 min at 12,000 × *g* and the precipitate was discarded.

To the supernatant fluid, 12 mg (dry weight) of alumina Cγ was added per ml and the mixture was gently stirred for 20 min at 0 C. The slurry was centrifuged for 10 min at 3,000 × *g* and the supernatant fluid was discarded. The precipitate was resuspended in 10% of the original crude extract supernatant fluid volume of 0.5 M potassium phosphate buffer, pH 7.6, and stirred for 15 min at 0 C. This extraction

TABLE 3. *Fractionation of the NADP-dependent malic enzyme*

Fraction	Volume (ml)	Total activity (U)	Specific activity (U/mg of protein)	
			NADP	NAD
Cell-free supernatant fluid	90	106.5	0.044	0.048
$MnCl_2$-treated	89	103.8	0.048	0.036
$(NH_4)_2SO_4$ (0–80%)	15	147.5	0.133	0.075
Calcium phosphate-heat treated	13.5	110.4	0.209	0.024

was repeated with a similar volume of buffer after centrifugation at 3,000 × *g* for 10 min, and the supernatant fluids were pooled.

The supernatant fluid was put in a Sephadex G-200 column (89 by 2.5 cm) equilibrated with 0.05 M Tris-chloride buffer (pH 7.6) containing 1 mM mercaptoethanol. Elution was performed with upward flow controlled by a peristaltic pump at a rate of 28 ml/h. Figure 2 shows an example of the elution pattern obtained.

A summary of the results is presented in Table 4. The Sephadex G-200 step yields fractions completely free of the NADP-dependent activity. NADP-dependent enzyme free of NAD-dependent activity is also obtained in this step, but total purification from the crude extract does not exceed threefold.

Metal requirements. Both malic enzymes had an absolute requirement for divalent metal cations. When the purified enzymes were dialyzed against 100 vol of 0.05 M Tris-chloride (pH 8.0) containing 1 mM ethyenediaminetetraacetic acid for 4 h at 4 C, followed by 12 h against the Tris buffer alone, no enzymatic activity was observed without the addition of cations. Both enzymes seemed to have considerably more affinity for Mn^{2+} than Mg^{2+} as shown by the difference in apparent K_m values (see Table 5).

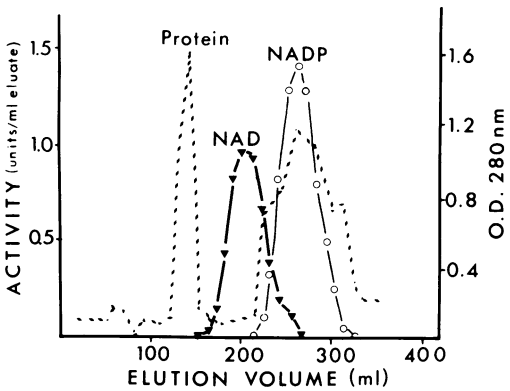

FIG. 2. *Elution pattern of the malic enzymes from a Sephadex G-200 column. A sample (14.3 ml) of the alumina Cγ eluate (see text) was applied to the column and 10.6-ml fractions were collected. Protein concentration was estimated by measuring the OD at 280 nm.*

TABLE 4. *Purification of the NAD-dependent malic enzyme*

Fraction	Volume (ml)	Total activity (U)	Specific activity (U/mg of protein)	
			NAD	NADP
Cell-free supernatant fluid	47	50.7	0.027	0.026
Protamine sulfate	46	46.5	0.033	0.035
Alumina gel eluate	14.3	34.2	0.061	0.033
Sephadex G-200 eluate pool	42.4	15.9	0.371	0

Monovalent metal cations had no effect on the NAD-dependent enzyme; K^+ (up to 65 mM), NH_4^+ (up to 18 mM), and Na^+ (up to 20 mM) did not activate or inhibit the enzyme. The NADP-dependent enzyme was affected by K^+ and NH_4 (Fig. 3). Na^+ had no effect on this enzyme up to a concentration of 25 mM. All of these experiments were performed with chloride salts.

Reversibility of the reaction. No reversibility of the malic enzyme reaction could be observed when the crude extract was used. However, the inverse reaction could be clearly demonstrated for both malic enzymes by using the partially purified preparations (Table 6). The activity observed for the NADP-specific malic enzyme in the absence of $MnCl_2$ was probably due to the presence of sufficient $MnCl_2$ in the enzyme preparation, because this salt was added in the first step of the purification procedure.

Molecular weight determination. Figure 4 shows the results of the molecular weight determinations using Sephadex G-200 gel filtration. The molecular weights of the malic enzymes interpolated in the graph are 270,000 for the NAD-dependent and 68,000 for the NADP-dependent enzyme. The values for the molecular weights of the standards are those given by Andrews (2).

The results of the sucrose density gradient centrifugation with a sample of the crude extract are shown in Fig. 5. The positions of the peaks of the malic enzymes were identical when the purified preparations were used. The molecular weights were calculated according to Martin and Ames (18), and the values used for the standard enzymes were: horse liver alcohol dehydrogenase, mol wt 84,000 and $s_{20,w}$ 5.11 (7), and catalase, mol wt 244,000 and $s_{20,w}$ 11.4 (22). The following results were obtained: NAD-dependent malic enzyme mol wt 180,000 (standard, alcohol dehydrogenase) and 204,000 (standard, catalase); NADP-malic enzyme mol wt 84,000 (standard, alcohol dehydrogenase) and 95,200 (Standard, catalase).

DISCUSSION

The evidence presented shows that the soluble fraction of a *Pseudomonas aeruginosa* cell-free extract lacks malic dehydrogenase and

TABLE 5. *Apparent Michaelis constants for substrates and cofactors of the malic enzymes*[a]

Substrate	K_m	
	NAD enzyme (M)	NADP enzyme (M)
Malate	5.2×10^{-4}	2.7×10^{-3}
NAD	1.3×10^{-4}	
NADP		7×10^{-5}
Mg^{2+}	1.1×10^{-2}	3.4×10^{-4}
Mn^{2+}	2.7×10^{-4}	4.0×10^{-6}
K^+		7×10^{-4}

[a] Values for K_m were obtained from Lineweaver-Burk plots (19). Constant substrates were used in concentrations as indicated in Materials and Methods. The values for the NADP-dependent enzyme were determined in the absence of K^+.

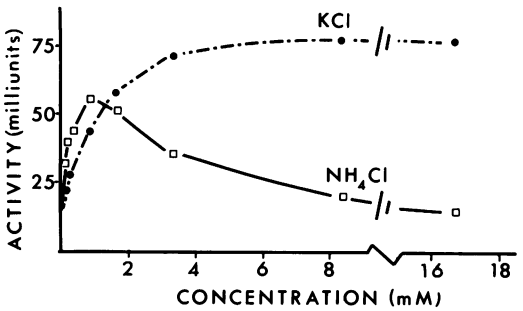

FIG. 3. *Effect of K^+ and NH_4^+ on the activity of the NADP-specific malic enzyme. The experimental conditions were as indicated in the enzyme assay.*

TABLE 6. *Carboxylation of pyruvate by the malic enzymes*[a]

Reaction system	Carboxylation (mU)	
	NAD-specific enzyme	NADP-specific enzyme
Complete system	18	57
$-MnCl_2$	3	45
– Pyruvate	0	0

[a] Assays were performed as described in Materials and Methods by following the oxidation of NADH or NADPH. The reactions were started by the addition of bicarbonate; in its absence no change in OD was observed. A 0.1-ml sample of the NADP- and 0.2 ml of the NAD-specific purified malic enzymes were used in the assays; their specific activities for the forward reaction were 0.209 and 0.157, respectively.

possesses two distinct malic enzymes, thus confirming and extending the observations of Tiwari and Campbell (29). These conclusions are based on the following evidence. (i) The activity observed in the cell-free supernatant fluid requires divalent metal cations; (ii) the reaction products are pyruvate, reduced pyridine nucleotides, and CO_2 in stoichiometric amounts; (iii) the inverse reaction requires bicarbonate, pyruvate, and a reduced pyridine nucleotide; (iv) the NAD- and NADP-requiring activities show different heat stabilities and can be resolved from each other by purification.

Several differences are found when comparing the properties of the two enzymes. Although both require Mg^{2+} or Mn^{2+} for activity,

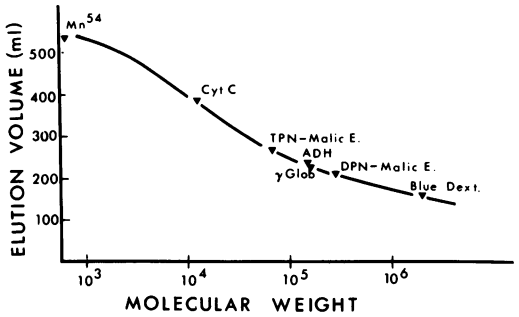

FIG. 4. *Relationship between elution volume and log of the molecular weight of several substances on a Sephadex G-200 column. Details of the procedure used are described in Materials and Methods. Abbreviations: Cyt C, cytochrome c; γGlob, gamma globulin; Blue Dext., blue dextran.*

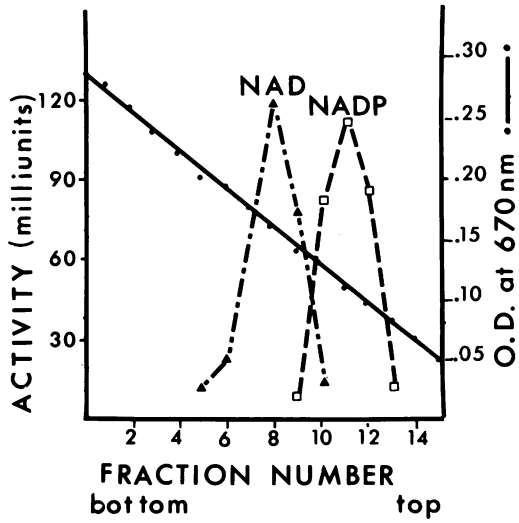

FIG. 5. *Profiles of the NAD- and NADP-dependent malic enzymes in a sucrose density gradient. Experimental conditions as described in the text. Activity is expressed in units per milliliter of gradient mixture. Catalase and alcohol dehydrogenase are not included for the sake of clarity; their activity peaks were in fractions 6 and 11, respectively. The continuous line indicates the concentration of methylene blue, obtained from a parallel experiment.*

and Mn^{2+} appears more effective in both cases, the apparent K_m's for both metal ions are about two orders of magnitude lower for the NADP enzyme (see Table 5). The NADP enzyme is affected by K^+ and NH_4^+ (Fig. 3), whereas the NAD enzyme is not. The molecular weights also show considerable differences, with the NAD enzyme being two to three times larger than the NADP enzyme.

Fractionation of the NAD-specific malic enzyme and study of its properties have been attempted in extracts from several bacterial species. Some of the results obtained are summarized in Table 7 and are mostly in good agreement with those reported here for the *Pseudomonas* enzyme.

Table 8 shows some properties of bacterial NADP-dependent malic enzymes; most of them are similar to the properties of the *Pseudomonas* enzyme. A striking difference is found, however, when the molecular weights of the *E. coli* and *Pseudomonas* enzymes are compared. The molecular weight values for the *Pseudomonas* enzyme (68,000 to 95,000, depending on the method used) resembles more that of a subunit of the *E. coli* enzyme, 67,000 (27), obtained when the enzyme is wholly dissociated in the presence of 5 M guanidinium-hydrochloride.

The existence of two or more enzymes catalizing a similar reaction in the same cell has been described a number of times in bacteria. Some of the best known examples are the threonine deaminases (33) and the aspartokinases (6) of *E. coli*. These enzymes usually perform a different physiological function in the cell as can be shown by study of their regulatory properties. Two malic enzymes, one requiring NADP and the other NAD, have been found in *E. coli* (11), and many of their regulatory properties have been studied (20, 23–25, 28). Murai et al. (20) have suggested that the NAD enzyme plays a role in the catabolism of malate, controlling the concentration of dicarboxylic acids in the Krebs cycle, whereas the NADP enzyme participates in supplying pyruvate from malate for the biosynthesis of several amino acids and supplying acetyl-coenzyme A via pyruvate for the biosynthesis of lipids and other compounds as well as for the operation of the Krebs cycle; they suggest that it would also provide NADPH required for biosynthetic purposes.

No adequate explanation is available for the metabolic need of two malic enzymes by *P. aeruginosa*. A study of their regulatory properties could shed some light on this question and

TABLE 7. *Some properties of bacterial NAD malic enzymes*[a]

Organism	Mol wt	Divalent metal cation requirement	Effect of monovalent metal cation	Reversibility of reaction
Escherichia coli	198,000[b] 203,000[c]	Absolute; Mn^{2+} more effective than Mg^{2+} (34)	K^+, inhibitory over 100 mM; Na^+ and NH_4^+, no effect (28)	Reversible (28)
Streptococcus faecalis		Absolute; Mn^{2+} more effective than Mg^{2+} (16)	Na^+ and K^+, no effect; NH_4^+, 1 mM activates but higher concn inhibit (16)	Reversible (16)
Lactobacillus arabinosus		Absolute (13)	K^+ activates (13)	Reversible (13)

[a] Numbers in parentheses indicated references.
[b] By sedimentation equilibrium centrifugation (34).
[c] By sucrose density gradient centrifugation (34).

TABLE 8. *Some properties of bacterial NADP malic enzymes*[a]

Organism	Mol wt	Divalent metal cation requirement	Effect of monovalent metal cation	Reversibility of reaction
Escherichia coli	546,000[b](27) 345,200[c](24)	Absolute (27)	K^+ activates; NH_4^+ activates at low concn, inhibits at high concn; Na^+, no effect (28)	
Mycobacterium 607		Absolute; Mn^{2+} more effective than Mg^{2+} (21)	K^+ activates; NH_4 and Na^+ inhibit (21)	Reversible (21)

[a] Numbers in parentheses indicate references.
[b] By sedimentation equilibrium centrifugation.
[c] By sucrose density gradient centrifugation.

on the specific physiological function of each of the enzymes.

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