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Some kinetic and regulatory properties of the pea chloroplast pyruvate dehydrogenase complex *

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Results from initial rate studies of the pea chloroplast pyruvate dehydrogenase complex indicate a kinetic mechanism identical to that of bacterial and mitochondrial complexes. Interaction kinetics for all substrates resulted in parallel lines consistent with a multisite ping-pong mechanism. Product inhibition studies showed competitive inhibition between acetyl-coenzyme A and coenzyme A and between NADH and NAD. Uncompetitive inhibition was found between pyruvate and NADH or acetyl-coenzyme A, while noncompetitive inhibition was found for NAD or coenzyme A versus acetyl-coenzyme A and NADH, respectively. Activity of the chloroplast pyruvate dehydrogenase complex could be regulated by product inhibition. The plastid complex was more sensitive to the NADH/NAD ratio than the acetyl-coenzyme A/coenzyme A ratio. Additionally, there is the potential for fine regulation of chloroplast pyruvate dehydrogenase complex activity by intermediates and products of fatty acid synthesis.

Introduction

In procaryotes the pyruvate dehydrogenase complex is located in the cytosol [1], while in mammalian cells and fungi it is within the mitochondrial matrix [2–4]. Plant cells are unique in having two distinct, spatially separated PDCs, located within the plastid [5–9] and mitochondrial [10–13] compartments. The mitochondrial pyruvate dehydrogenase complex provides an entry point for carbon into the tricarboxylic acid cycle while the plastid complex provides acetyl-CoA and NADH for fatty acid biosynthesis [14,15].

A minimal pyruvate dehydrogenase complex is composed of three discrete enzymatic activities, pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoyl transacetylase (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (EC 1.6.4.3). Rather than attempt kinetic analysis of each of the components, the complex has typically been analyzed as if it was a single enzyme catalyzing the sum of the three reactions [16]. Kinetic data for the pyruvate dehydrogenase complex from various sources have been most consistent with a multi-site hexa-uni ping-pong mechanism [17].

It was previously established that pea chloroplast activity was due to an authentic pyruvate dehydrogenase complex, but with a subunit struc-

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ture and regulatory properties distinct from the mitochondrial complex [14,18]. Herein we present a partial kinetic characterization of pea chloroplast pyruvate dehydrogenase complex, examine some potential regulatory properties, and propose a mechanism for the in situ control of activity in green leaves.

Materials and Methods

Pyridine nucleotides, acetyl-CoA, and CoA were from P-L Biochemicals. Buffers and dithiothreitol were from Research Organics, Inc. All other biochemicals were purchased from the Sigma Chemical Co.

The complex was partially purified from freeze-thawed pea (*Pisum sativum* L., cv. Little Marvel) chloroplasts as previously described [14]. Unless otherwise noted, the enzyme complex was purified through the second ultracentrifugation step.

Kinetic data were plotted as reciprocal velocity versus reciprocal substrate concentration and fitted to Eqn. 1 by the least-squares method of Cleland [19]. Initial rates obtained from substrate interaction experiments were fitted to Eqns. 2 and 3 for ping-pong and ordered mechanisms, respectively. Product inhibition data were fitted to Eqn. 4 for linear competitive inhibition, Eqn. 5 for linear uncompetitive inhibition, and Eqn. 6 for linear noncompetitive inhibition. The patterns reported are those which resulted from the best fit of the data to the equations. The lines presented in the figures are from linear regression of the data, with each point the mean of at least three determinations and with a minimum of five points per line.

$$v = VA/K + A \quad (1)$$

$$v = VAB/KbA + AB \quad (2)$$

$$v = VAB/KiaKb + KbA + AB \quad (3)$$

$$v = VA/K[1 + (1/Kia)] + A \quad (4)$$

$$v = VA/K + A[1 + I/Kii] \quad (5)$$

$$v = VA/K[1 + (I/Kia)] + A[1 + (I/Ki)] \quad (6)$$

When possible, compounds evaluated as potential effectors were dissolved directly in assay buffer. Stock solutions of the more hydrophobic compounds were made up in either 95% (v/v) ethanol or dimethylsulfoxide. Assays containing these compounds also contained Triton X-100 at a final concentration of 0.125% (v/v). Control assays showed no effect of the addition of solvent alone on the enzyme assays. Free fatty acids were converted to ammonium salts before they were tested. Palmitoyl-acyl carrier protein was generously provided by Dr. J.B. Ohlrogge, and was prepared from *E. coli* acyl carrier protein and palmitate using *E. coli* acyl-acyl carrier protein synthetase [20]. In most cases pyruvate dehydrogenase complex was incubated with the effector for 1 min prior to initiation of the assay with CoA.

All other materials and methods have been previously described [14,21].

Results

Fig. 1 shows the initial velocity patterns observed when concentrations of one of the substrates, pyruvate, CoA, or NAD was varied at a fixed concentration of the second substrate and at fixed variable concentrations of the third. For all three substrate combinations a group of parallel lines was obtained in the double reciprocal plots, consistent with an overall ping-pong mechanism. The Michaelis constants obtained from plots of reciprocal velocity versus reciprocal substrate concentration, at three different enzyme concentrations, were 36 μM, 10 μM and 120 μM for NAD, CoA and pyruvate, respectively.

Product inhibition data, obtained by varying concentrations of NADH and acetyl-CoA versus each of the substrates, are presented in Figs. 2, 3 and 4. Both NADH and acetyl-CoA were uncompetitive inhibitors with respect to pyruvate. Noncompetitive inhibition was observed with NAD as the substrate and acetyl-CoA the inhibitor (data not shown), and with CoA as the substrate and NADH the inhibitor (data not shown). Competitive inhibition patterns were observed with CoA as the substrate and acetyl-CoA the product, and with NAD as the substrate and NADH the product (Figs. 3 and 4). A summary of the product inhibition patterns is presented in Table I.

An uncompetitive pattern was observed with NAD as the variable substrate and NADPH the inhibitor (Fig. 5). A K_i value of 466 μM was

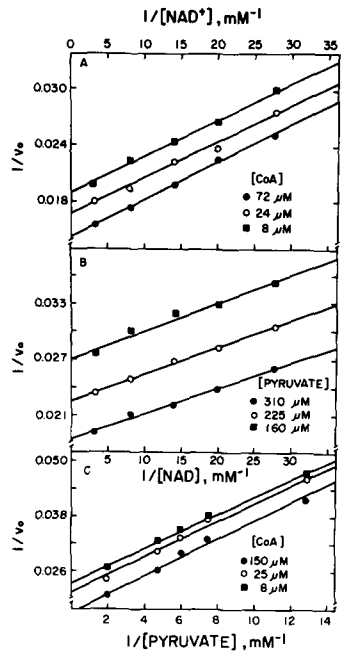

Fig. 1. Initial velocity patterns of the pyruvate dehydrogenase complex from pea chloroplasts. Panel A is a double reciprocal plot of CoA vs. varying concentrations of NAD at a fixed concentration of pyruvate (1.5 mM). The CoA concentrations were: 72 μM (●); 24 μM (○); and 8 μM (■). Panel B is a double reciprocal plot of pyruvate vs. varying concentrations of NAD at a fixed concentration of CoA (120 μM). Pyruvate concentrations were: 310 μM (●); 225 μM (○); and 160 μM (■). Panel C is a double reciprocal plot of CoA vs. varying concentrations of pyruvate at a fixed concentration of NAD (2.3 mM). The CoA concentrations were: 150 μM (●); 25 μM (○); and 8 μM (■).

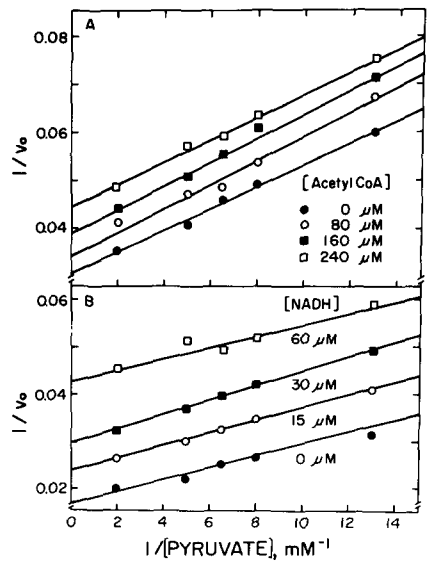

Fig. 2. Product inhibition of pea chloroplast pyruvate dehydrogenase complex. Panel A is a double reciprocal plot of acetyl-CoA vs. pyruvate at fixed concentrations of CoA (40 μM) and NAD (1.4 mM). Panel B is a double reciprocal plot of NADH vs. pyruvate at fixed concentrations of CoA (20 μM) and NAD (72 μM).

calculated from a replot of the inhibition data. Uncompetitive inhibition was also observed when ATP was tested at varying concentrations of NAD or CoA (Figs. 6 and 7). In both instances, relatively high concentrations of ATP were necessary to inhibit chloroplast PDC activity.

The percent inhibition when the ratios of NADH to NAD, and of acetyl-CoA to CoA were varied from 0 to 4.4 at a total pyridine nucleotide and coenzyme A concentrations of 100 μM and 20 μM, respectively, is presented in Fig. 8. A curvilinear response was observed upon varying the proportion of NADH to NAD, with ratio of 0.2 resulting in 50% inhibition. There was a linear increase in inhibition of PDC activity with an increasing proportion of acetyl-CoA in the assays. An acetyl-CoA-to-CoA ratio of 2.2 was required for 50% inhibition.

When tested at concentrations of 0.25 to 1 mM, intermediates of the photosynthetic carbon reduction cycle and the photorespiratory carbon cycle had no major effect upon pyruvate dehydrogenase

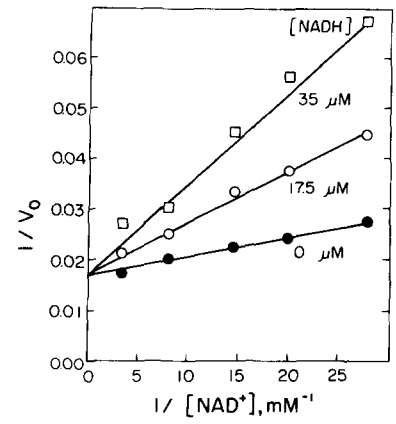

Fig. 3. Inhibition of pea chloroplast pyruvate dehydrogenase complex by fixed variable concentrations of NADH at varying concentrations of NAD. Concentrations of CoA and pyruvate were fixed at 40 μM and 1.5 mM, respectively.

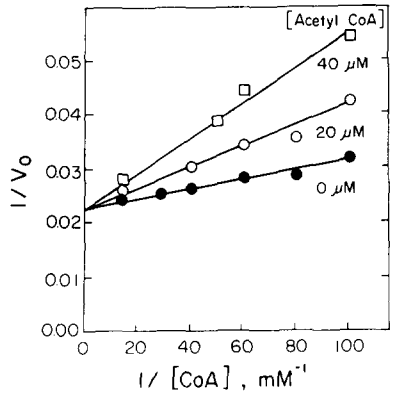

Fig. 4. Inhibition of pea chloroplast pyruvate dehydrogenase complex by fixed variable concentrations of acetyl-CoA at varying concentrations of CoA. Concentrations of NAD and pyruvate were fixed at 2.3 mM and 1.5 mM, respectively.

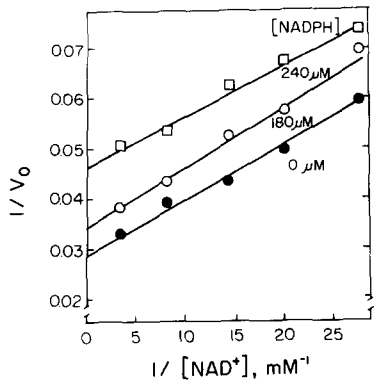

Fig. 5. Inhibition of pea chloroplast pyruvate dehydrogenase complex by fixed variable concentrations of NADPH at varying concentrations of NAD. Concentrations of pyruvate and CoA were fixed at 1.5 mM and 40 μM, respectively.

TABLE I

PRODUCT INHIBITION PATTERNS OBSERVED FROM PEA CHLOROPLAST PYRUVATE DEHYDROGENASE COMPLEX

Substrate	Inhibitor	Inhibition pattern
Pyruvate	NADH	uncompetitive
Pyruvate	acetyl CoA	uncompetitive
NAD	acetyl CoA	noncompetitive
NAD	NADH	competitive
CoA	acetyl CoA	competitive
CoA	NADH	noncompetitive

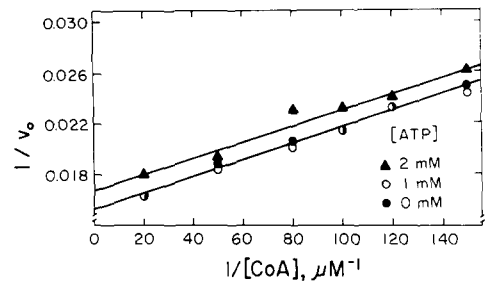

Fig. 6. Inhibition of pea chloroplast pyruvate dehydrogenase complex by fixed variable concentrations of ATP at varying concentrations of CoA. Concentrations of NAD and pyruvate were fixed at 100 μM and 1.5 mM, respectively. The ATP concentrations were 2 mM (△), 1 mM (○), and 0 mM (●).

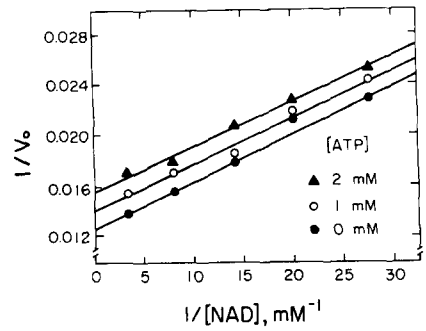

Fig. 7. Inhibition of pea chloroplast pyruvate dehydrogenase complex by fixed variable concentrations of ATP at varying concentrations of NAD. Concentrations of CoA and pyruvate were fixed at 40 μM and 1.5 mM, respectively. The ATP concentrations were 2 mM (△), 1 mM (○), and 0 mM (●).

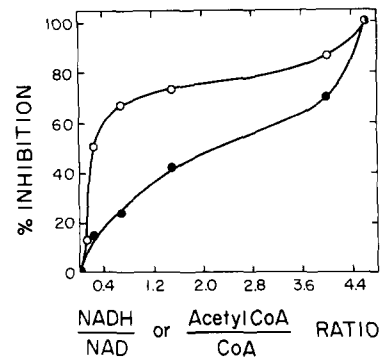

Fig. 8. Regulation of pea chloroplast pyruvate dehydrogenase complex activity by substrate-product ratios. The total pyridine nucleotide concentration was 100 μM and the total CoA + acetyl-CoA concentration was 20 μM. NADH/NAD (○); acetyl-CoA/CoA (●).

TABLE II

ACETYL-CoA DERIVED PLASTID METABOLITES AS POTENTIAL EFFECTORS OF THE PEA CHLOROPLAST PYRUVATE DEHYDROGENASE COMPLEX

The following compounds (at the indicated mM concentration) had no discernable in vitro effect: β-sitosterol (0.10), campesterol (0.10), carotene (0.10), squalene (0.10), HMG-CoA (1.00), abscisic acid (1.00), zeatin (1.00), zeatin riboside (1.00). Control rates were 0.11 μmol/min per assay.

Effector	Concentration (mM)	Percent of control
Palmitic acid	0.05	136 ± 3
Palmitoyl-CoA	0.05	86 ± 2
Palmitoyl-ACP	0.05	99 ± 3
Stearic acid	0.05	100 ± 2
Steroyl-CoA	0.05	86 ± 1
Oleic-acid	0.05	43 ± 5
Oleoyl-CoA	0.05	79 ± 1
Phytol	0.50	93 ± 2
Gibberellic acid A_3	1.00	114 ± 4

complex activity in vitro (data not presented). Similarly, fructose 2,6-bisphosphate, malonyl-CoA, acetate, and the amino acids alanine, leucine, isoleucine, and valine were without effect. At a final concentration of 10 mM, inorganic phosphate inhibited in vitro complex activity by 24 ± 9%.

Several acetyl-CoA-derived plant products were tested as possible effectors of chloroplast pyruvate dehydrogenase complex activity. Sterols, carotene, squalene, 3-hydroxy-3-methyl-glutaryl-CoA, and the terpenoid plant hormones, zeatin and abscissic acid, were without effect on plastid complex activity in vitro (Table II). Palmitic acid stimulated activity while oleic acid was inhibitory and stearic acid was without effect. Acyl-CoAs elicited less pronounced effects than did the corresponding free fatty acids. At the concentrations tested, palmitoyl-acyl carrier protein had no effect upon pea chloroplast pyruvate dehydrogenase complex activity.

Discussion

While higher plant cells contain a mitochondrial pyruvate dehydrogenase complex, as do other eucaryotes, they differ in that they also have a second type of complex, located within the plastids. This is probably true for all young, developing or expanding plant tissues, both green and nongreen [7–9,22]. Based upon substrates required and products formed it was shown that the plastid activity was due to an authentic pyruvate dehydrogenase complex [14]. It was reported, however, that the subunit composition and regulatory properties of plastid complexes were distinct from those of their mitochondrial counterparts [14,18,23,24]. It was particularly notable that plastid pyruvate dehydrogenase complex is not regulated by reversible phosphorylation [14,18,23]. Additionally, there has only recently been published the first direct comparison of the kinetic mechanism of plastid pyruvate dehydrogenase complex with that of the mitochondrial complex from the same tissue, *Ricinus* endosperm [24,25]. This is a tissue, however, which is not photosynthetic, and the plastids are specialized for the conversion of glycolytic intermediates into fatty acids [37]. As such, the regulatory properties of the complex could be distinct from those required in photosynthetic tissues.

Cleland has developed the rate equations for a three-site ping-pong mechanism [17]. From these rate equations the initial veloicty patterns predicted are parallel lines, regardless of the substrate varied. The substrate interaction patterns observed for the pea chloroplast pyruvate dehydrogenase complex are consistent with the predicted patterns. Product inhibition patterns predicted by Clelands model require that each product will be competitive with the substrate combining at the same site and uncompetitive against other substrates. However, noncompetitive inhibition is predicted if the product acts as a dead-end inhibitor or if it in some way hinders binding of the substrate [17]. With the pea chloroplast complex, acetyl-CoA and NADH were both uncompetitive versus pyruvate. Noncompetitive inhibition was observed with acetyl-CoA versus NAD and with NADH versus CoA. Similar results have been obtained with bacterial [26,27] and mitochondrial pyruvate dehydrogenase complexes from a variety of animal [28–30] and plant [11,31,32] sources, and with the plastid complex isolated from developing castor oil seeds [25]. Tsai et al. [16] presented evidence indicating that the observed noncompetitive inhibition patterns were the result of protein interactions, whereby acetyl-CoA binding

hindered the binding of NAD, and NADH binding hindered that of CoA. It seems likely then that all pyruvate dehydrogenase complexes have, at least, a very similar kinetic mechanism, regardless of the tissue or subcellular location.

One primary component of regulation of eucaryotic pyruvate dehydrogenase complexes is reversible phosphorylation. As previously reported, however, the plastid enzyme complexes more closely resemble those of bacteria in that they are not regulated by covalent modification [18]. Bacterial pyruvate dehydrogenase complexes are regulated in part by metabolites [1]. Thus, a number of plastid metabolites were tested as potential regulators of the plastid enzyme. There was no evidence for metabolite effects in vitro from intermediates of the photosynthetic carbon reduction cycle, amino acid metabolism, or the photorespiratory carbon cycle.

The plastid pyruvate dehydrogenase complex is thought to provide acetyl-CoA and NADH needed for fatty acid synthesis [14,35], a process that occurs in the light [33]. The effect of NADPH and ATP on the plastid complex thus becomes important, since these metabolites increase at a time when high enzyme activity is necessary. An inhibitory effect of these metabolites might be predicted since both have structural similarities to CoA and NAD and because of a previous report of inhibition of *Ricinus* leucoplast pyruvate dehydrogenase complex by ATP. The latter, however, was most likely the result of chelation of Mg^{2+} from the reaction mixture. In the present study the K_i for NADPH was high (466 μM) and ATP was only marginally inhibitory at a concentration as high as 2 mM. The relative insensitivity to inhibition by NADPH and ATP, plus the previously described alkaline pH optimum and requirement for high concentrations of $MgCl_2$ [7,14,18], are all consistent with an active chloroplast pyruvate dehydrogenase complex during illumination when the demand for acetyl-CoA and NADH for fatty acid biosynthesis is high.

The pea chloroplast pyruvate dehydrogenase complex was sensitive to product inhibition, as are all pyruvate dehydrogenase complexes examined to date. The plastid complex appears to be more sensitive to changes in the NADH/NAD ratio than the acetyl-CoA/CoA ratio. As shown in Fig. 8, small increases in the NADH/NAD ratio resulted in a substantial inhibition of the enzyme, whereas large increases in acetyl-CoA are needed to effectively inhibit the complex. The NADH/NAD ratio required for 50% inhibition of pea chloroplast pyruvate dehydrogenase complex activity in vitro is approx. 4-fold lower than the ratio required for equal inhibition of the mitochondrial complex [11,12,23]. Thus, control of the plastid complex through changes in the NADH/NAD ratio is suggested.

The plastids of plant cells contain several enzyme systems capable of synthesizing acetyl-CoA [34,35]. The most direct route includes a complete set of glycolytic isozymes plus the pyruvate dehydrogenase complex [36–38]. Acetyl-CoA produced within the plastids can be used directly for synthesis of fatty acids. Although there has been some controversy concerning the localization of some early enzymes involved in isopentenyl-pyrophosphate production (e.g., Refs. 39 and 40), plastids are the site of synthesis of complex terpenoids, quinones, hydrocarbons, and most of the plant hormones (reviewed in Ref. 41). Selected intermediates and end products of terpene synthesis were tested as potential effectors of the plastid pyruvate dehydrogenase complex, but none had any significant effect in vitro. There was, however, a stimulation of pea chloroplast enzyme activity by palmitate and an inhibition by oleate, the two primary end products of plastid fatty acid biosynthesis [42]. The specificity would seem to indicate that these are not simple detergent effects. These fatty acid effects could be an additional component of the fine regulation of plastid pyruvate dehydrogenase complex activity.

The substrate requirements and kinetic mechanism of plastid pyruvate dehydrogenase complexes are the same as those of other organisms and organelles. The occurrence of varied biosynthetic pathways within this compartment has, however, resulted in the evolution of a unique set of regulatory properties. As our knowledge of plastid metabolism continues to increase, additional regulatory properties of the plastid pyruvate dehydrogenase complex will certainly come to light.

Acknowledgements

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References

1 Reed, L.J. (1974) Acc. Chem. Res. 7, 40–46.
2 Pettit, F.J. and Reed, L.J. (1982) Methods Enzymol. 89, 408–414.
3 DeLattre, P., Mareck, A. and Foucher, B. (1985) Biochimie 67, 633–636.
4 Yeaman, S.J. (1986) Trends Biochem. Sci. 11, 293–296.
5 Reid, E.E., Lyttle, C.R., Canvin, D.T. and Dennis, D.T. (1975) Biochem. Biophys. Res. Commun. 62, 42–47.
6 Elias, B.A. and Givan, C.V. (1979) Plant Sci. Lett. 17, 115–122.
7 Williams, M. and Randall, D.D. (1979) Plant Physiol. 64, 1099–1103.
8 Liedvogel, B. (1984) Z. Naturforsch. 40c, 182–188.
9 Treede, H.-J. and Heise, K.-P. (1986) Z. Naturforsch. 41c, 1011–1017.
10 Randall, D.D. and Rubin, P.M. (1977) Plant Physiol. 59, 1–3.
11 Randall, D.D., Rubin, P.M. and Fenko, M. (1977) Biochim. Biophys. Acta 485, 336–349.
12 Rubin, P.M. and Randall, D.D. (1977) Arch. Biochem. Biophys. 178, 342–349.
13 Rao, K.P. and Randall, D.D. (1980) Arch. Biochem. Biophys. 200, 461–466.
14 Camp, P.J. and Randall, D.D. (1985) Plant Physiol. 77, 571–577.
15 Reid, E.E., Thompson, P., Lyttle, C.R. and Dennis, D.T. (1977) Plant Physiol. 59, 842–848.
16 Tsai, C.S., Burgett, M.W. and Reed, L.J. (1973) J. Biol. Chem. 248, 8348–8352.
17 Cleland, W.W. (1973) J. Biol. Chem. 248, 8353–8355.
18 Miernyk, J.A., Camp, P.J. and Randall, D.D. (1985) Curr. Top. Plant Biochem. Physiol. 4, 175–190.
19 Cleland, W.W. (1967) Adv. Enzymol. 29, 1–17.
20 Kuo, T.M. and Ohlrogge, J.B. (1984) Arch. Biochem. Biophys. 230, 110–116.
21 Miernyk, J.A. and Randall, D.D. (1987) Plant Physiol. 83, 306–310.
22 Journet, E.-P. and Douce, R. (1984) C.R. Acad. Sci. Paris Serie III, 298, 365–370.
23 Thompson, P., Reid, E.E., Lyttle, C.R. and Dennis, D.T. (1977) Plant Physiol. 59, 854–858.
24 Rapp, B.J., Miernyk, J.A. and Randall, D.D. (1987) J. Plant Physiol. 127, 293–306.
25 Thomson, P., Reid, E.E., Lyttle, C.R. and Dennis, D.T. (1977) Plant Physiol. 59, 849–853.
26 Bosman, H.J., De Graaf-Hess, A.C., De Kok, A., Veeger, C., Visser, A.J.W.G. and Voordouw, G. (1982) Ann. N.Y. Acad. Sci. USA 378, 265–286.
27 Carlsson, J., Kujala, U. and Edlund, M.-B.K. (1985) Infect. Immun. 49, 674–678.
28 Carlsson, J., Kujala, U. and Edlund, M.-B.K. (1985) Infect. Immun. 49, 674–678.
28 Denton, R.M., Randle, P.J., Bridges, B.J., Cooper, R.H., Kerbey, A.L., Pask, H.T., Severson, D.L., Stansbie, D. and Whitehouse, S. (1975) Mol. Cell. Biochem. 9, 27–53.
29 Hansford, R.G. (1976) J. Biol. Chem. 251, 5483–5489.
30 Randle, P.J. (1981) Curr. Top. Cell. Reg. 18, 107–129.
31 Rubin, P.M., Zahler, W.L. and Randall, D.D. (1978) Arch. Biochem. Biophys. 188, 70–77.
32 Crompton, M. and Laties, G.G. (1971) Arch. Biochem. Biophys. 143, 143–150.
33 Roughan, P.G., Kagawa, T. and Beevers, H. (1980) Plant Sci. Lett. 18, 221–228.
34 Murphy, D.J. and Walker, D.A. (1982) Planta 156, 84–88.
35 Givan, C.V. (1983) Physiol. Plantarum 57, 311–316.
36 Simcox, P.D., Reid, E.E., Canvin, D.T. and Dennis, D.T. (1977) Plant Physiol. 59, 1128–1132.
37 Dennis, D.T. and Miernyk, J.A. (1982) Annu. Rev. Plant Physiol. 33, 27–50.
38 Liedvogel, B. and Bauerle, R. (1986) Planta 169, 481–489.
39 Kreuz, K. and Kleinig, H. (1981) Planta 153, 578–581.
40 Schultz, G., Soll, J., Fiedler, E. and Schultz-Siebert, D. (1985) Physiol. Plantarum 64, 123–129.
41 Givan, C.V. and Harwood, J.L. (1976) Biol. Rev. 51, 365–406.
42 Stumpf, P.K. (1980) in: The Biochemistry of Plants, Vol. 4, pp. 177–204, Academic Press, New York.