Regulation of Pyruvate Dehydrogenase Kinase and Phosphatase by Acetyl-CoA/CoA and NADH/NAD Ratios

Flora H. Pettit, John W. Pelley,* and Lester J. Reed

Clayton Foundation Biochemical Institute and the Department of Chemistry
The University of Texas at Austin, Austin, Texas 78712

Received May 29,1975

SUMMARY. The interconversion of the active, nonphosphorylated form of pyruvate dehydrogenase and its inactive, phosphorylated form is modulated by acetyl-CoA/CoA and NADH/NAD molar ratios. An increase in either ratio increases the proportion of the phosphorylated form of pyruvate dehydrogenase. The activity of pyruvate dehydrogenase kinase is stimulated by acetyl-CoA and by NADH and is inhibited by CoA and by NAD. NADH inhibits pyruvate dehydrogenase phosphatase, and this inhibition is reversed by NAD.

Activity of the mammalian pyruvate dehydrogenase complex is regulated by a phosphorylation-dephosphorylation cycle (1,2). Phosphorylation and concomitant inactivation of the pyruvate dehydrogenase component of the complex is catalyzed by a $MgATP^{2-}$-requiring kinase, and dephosphorylation and concomitant reactivation is catalyzed by a Mg^{2+}- and Ca^{2+}-requiring phosphatase (1-5). The kinase is inhibited by ADP (provided K^{+} or NH_4^{+} ions are present (6)) and by pyruvate (2,7). Results obtained with the purified pyruvate dehydrogenase system indicate that the kinase and the phosphatase maintain a steady state level of activity of the complex and that this level is modulated by the action on the kinase of the pyruvate and K^{+} (possibly NH_4^{+}) concentrations and the ATP/ADP molar ratio and by the action on the phosphatase of the concentrations of uncomplexed Mg^{2+} and Ca^{2+} ions (6,8). The present study establishes that the interconversion of the nonphosphorylated (PDH_a) and phosphorylated (PDH_b) forms of pyruvate dehydrogenase, and hence the steady state activity of the

* Present address: Department of Biochemistry, Texas Tech University School of Medicine, Lubbock, Texas 79409.

Abbreviations used: PDH_a, active, nonphosphorylated form of pyruvate dehydrogenase; PDH_b, inactive phosphorylated form; MOPS, 2-(N-morpholino)propanesulfonate.

pyruvate dehydrogenase complex, is also modulated by the acetyl-CoA/CoA and NADH/NAD molar ratios.

MATERIALS AND METHODS

Highly purified preparations of the bovine kidney and heart pyruvate dehydrogenase complexes and their component enzymes were obtained by procedures described previously (9) or by modifications thereof. The purified preparations of the complexes contained tightly bound PDH_a kinase and small amounts of PDH_b phosphatase. The assay of PDH_a kinase is based on the initial rate of incorporation of ^{32}P-labeled phosphoryl groups from [γ-^{32}P]ATP into the pyruvate dehydrogenase complex (7). In certain experiments kinase activity was determined by measuring the initial rate of inactivation of the pyruvate dehydrogenase complex in the presence of $MgATP^{2-}$ (7). The assay of PDH_b phosphatase is based on the initial rate of release of $^{32}P_i$ from preparations of the phosphorylated complex (5,7). The kidney complex was phosphorylated by incubation with 0.2 mM [γ-^{32}P]ATP for 20 min at 30°, followed by dialysis for 48 hr at 4° to remove ATP. In certain experiments phosphatase activity was determined by measuring the initial rate of reactivation of inactivated (i.e., phosphorylated) preparations of the complex in the presence of the phosphatase, Mg^{2+} and Ca^{2+} (7).

RESULTS AND DISCUSSION

In previous publications (6,8) we have shown that when both PDH_a kinase and PDH_b phosphatase are present and functional, the activity of the pyruvate dehydrogenase complex reaches a steady state within a few minutes. The steady state activity is dependent on the relative amounts of kinase and phosphatase and on the concentrations or molar ratios of substances that affect the activities of these two regulatory enzymes. Thus, the steady state activity of the complex is affected markedly by varying the concentration of Mg^{2+} or Ca^{2+} and thereby changing the activity of the phosphatase. On the other hand, at optimum Mg^{2+} and Ca^{2+} concentrations, the steady state activity is affected markedly by varying the concentration of K^+ or NH_4^+ at a fixed ATP/ADP molar ratio or by varying the ATP/ADP ratio at a fixed concentration of monovalent cation and thereby changing the activity of the kinase. Fluctuations in the steady state activity of the complex reflect changes in the distribution of pyruvate dehydrogenase between PDH_a and PDH_b. Steady state experiments with the purified pyruvate dehydrogenase system provide a relatively simple and straightforward means of determining the effects, if any, of various metabolites and cofactors on the interconversion of PDH_a and PDH_b.

In the experiments to be described, the desired steady state activity

of the pyruvate dehydrogenase complex was achieved by adjusting the amount of added phosphatase and by selecting an appropriate ATP/ADP molar ratio and K^+ concentration. Optimum concentrations of Mg^{2+} and Ca^{2+} were used. The data presented in Fig. 1 and Fig. 2 show that the steady state activity of the bovine kidney pyruvate dehydrogenase complex is sensitive to changes in the acetyl-CoA/CoA and NADH/NAD molar ratios. An increase in either ratio decreased the steady state activity. Similar results (not shown) were obtained with the bovine heart pyruvate dehydrogenase complex. From the data presented in these figures, the PDH_a/PDH_b ratios can be calculated (6). For example, a steady state activity of 50% corresponds to a PDH_a/PDH_b ratio of 1.0. Varying the acetyl-CoA/CoA ratio between 0.1 and 10 changed the steady state activity

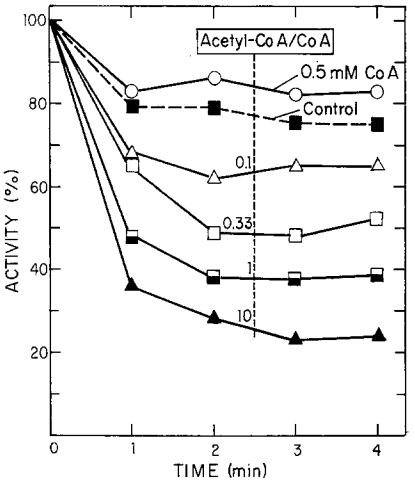

Fig. 1. Effect of changes in acetyl-CoA/CoA ratio on the steady state activity of the bovine kidney pyruvate dehydrogenase complex. The reaction mixtures contained 0.17 mg of enzyme complex (contains tightly bound PDH_a kinase), 50 mM MOPS (adjusted to pH 7.0 with KOH), 60 mM potassium acetate (pH 7.0), 0.4 mM ATP, 0.1 mM ADP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, and 52 units of PDH_b phosphatase (9) in a final volume of 0.2 ml. The total concentration of acetyl-CoA + CoA was 0.5 mM. ATP and ADP were added after the mixtures had been preincubated at 30° for 3 min (zero time). After 2.5 min of preincubation with the effectors and at the indicated time intervals after addition of ATP and ADP, a 0.01-ml aliquot was added to 0.99 ml of assay mixture for measurement of NAD-reduction activity (9). Acetyl-CoA and CoA were omitted from the control mixture.

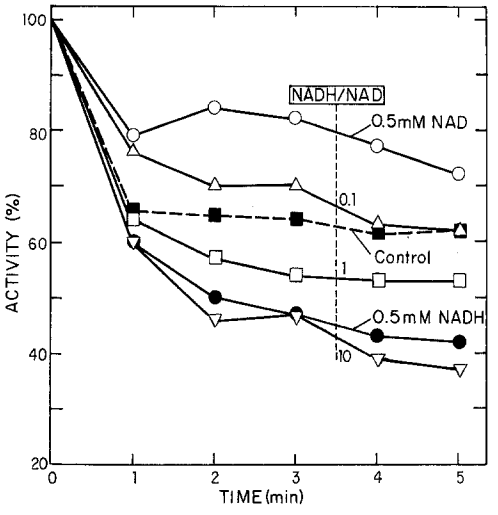

Fig. 2. Effect of changes in NADH/NAD ratio on the steady state activity of the bovine kidney pyruvate dehydrogenase complex. The components and conditions were as described in Fig. 1, except that 24 units of PDH_b phosphatase were used and acetyl-CoA and CoA were omitted. The total concentration of NADH + NAD was 0.5 mM. NADH and NAD were omitted from the control mixture.

---

of the complex from 65% to 27% (Fig. 1). These latter activities correspond to PDH_a/PDH_b ratios of 1.86 and 0.37, respectively, a fivefold change. Varying the NADH/NAD ratio between 0.1 and 10 changed the steady state activity from 62% to 37% (Fig. 2), corresponding to PDH_a/PDH_b ratios of 1.63 and 0.59, respectively, a threefold change.

We have investigated the molecular basis of these effects of the acetyl-CoA/CoA and NADH/NAD ratios on the interconversion of PDH_a and PDH_b. The data presented in Table I demonstrate that acetyl-CoA and NADH stimulate the activity of bovine kidney and heart PDH_a kinase, whereas CoA and NAD inhibit kinase activity. These data and additional experiments (data not shown) indicate that the stimulatory effects of acetyl-CoA and NADH are additive. Potassium ions are required for these allosteric effects on kinase activity. Thus, similar results were obtained with 20 mM potassium phosphate buffer or with 50 mM MOPS-imidazole buffer plus 60 mM potassium chloride, but little effect was observed with MOPS-imidazole buffer in the absence of K^+ (data not

Table I

Effects of CoA, Acetyl-CoA, NAD, and NADH on PDH_a Kinase Activity

Additions	Kinase activity (%)	
	Heart	Kidney
None	100	100
CoA	78	85
Acetyl-CoA	152	148
NAD	88	73
NADH	119	117
CoA + NAD	74	53
Acetyl-CoA + NADH	182	161

The incubation mixtures contained 50 mM MOPS-imidazole buffer, pH 7.0, 60 mM potassium acetate, 0.1 mM EDTA, 1 mM $MgCl_2$, 10 mM NaF, 2 mM dithiothreitol, 0.4 mM [γ-^{32}P]ATP (25,000 cpm/nmol), 0.5 mM each allosteric effector, and 0.51 mg of heart pyruvate dehydrogenase complex or 0.24 mg of kidney pyruvate dehydrogenase complex and 0.31 mg of kidney PDH_a in a final volume of 0.25 ml. The mixtures were preincubated for 1 min at 30° prior to addition of ATP, and 30 sec (kidney) or 1 min (heart) after addition of ATP a 0.05-ml aliquot was assayed for protein-bound radioactivity. Since the activity of endogenous kinase in the kidney complex was tenfold higher than that of endogenous kinase in the heart complex, excess PDH_a was added to the former system to permit measurement of meaningful rates. In the absence of allosteric effectors, 5.3 nmol of ^{32}P were incorporated/30 sec/mg of kidney complex and 1.3 nmol/min/mg of heart complex.

---

shown). Table II shows that NADH inhibits kidney PDH_b phosphatase activity and that this inhibition is reversed by NAD. Potassium ions are not required for this allosteric effect on phosphatase activity. In spectrophotometric phosphatase assays (Methods) acetyl-CoA appeared to inhibit slightly kidney PDH_b phosphatase activity by increasing the apparent K_m for PDH_b about twofold (data not shown). CoA had no effect on the apparent K_m for PDH_b, but appeared to decrease V_{max} about 30%. However, the dominant effects of acetyl-CoA and CoA in the steady state experiments (Fig. 1) are apparently exerted on the kinase.

Although fatty acids are known to inhibit pyruvate oxidation in various mammalian tissues, as well as in isolated mitochondria, and to increase the

Table II

Effects of NADH and NAD on PDH_b Phosphatase Activity

Additions	Phosphatase activity	
	nmol $^{32}P_i$ released/min	Percent of control
None	0.87	100
NADH	0.36	41
NADH:NAD (10:1)	0.54	62
NADH:NAD (3:1)	0.69	79
NADH:NAD (1:1)	0.71	82
NADH:NAD (1:3)	0.76	87
NADH:NAD (1:10)	0.83	95
NAD	0.89	102

The incubation mixtures contained 50 mM MOPS (adjusted to pH 7.0 with KOH), 60 mM potassium acetate, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.39 mg of kidney ^{32}P-labeled pyruvate dehydrogenase complex (5.1 nmol ^{32}P/mg protein), 28 units of kidney PDH_b phosphatase, and a total concentration of 0.5 mM of allosteric effector(s) in a total volume of 0.2 ml. The reaction was started after 1 min preincubation at 30° by addition of $MgCl_2$. Aliquots (0.05 ml) were withdrawn at 30-sec intervals after addition of $MgCl_2$ and were assayed for protein-bound radioactivity.

---

proportion of PDH_b, the molecular basis of this effect of fatty acids has not been established (10). This inhibitory effect of fatty acids has been attributed to (a) inhibition of the activity of the pyruvate dehydrogenase complex by acetyl-CoA and NADH (11), (b) an increase in the intramitochondrial ATP/ADP ratio resulting from inhibition of the adenine nucleotide translocase by fatty acyl-CoA (12), or (c) an increase in the NADH/NAD ratio or the fatty acyl-CoA concentration which somehow affects the activity of PDH_a kinase (10). It should be noted in this connection that Taylor *et al.* have reported recently (10) that octanoate increased the proportion of PDH_b in isolated rat liver mitochondria without altering appreciably the intramitochondrial ATP/ADP ratio.

Our findings with the purified pyruvate dehydrogenase system suggest that the inhibitory effect of fatty acids is due, at least in part, to modulation of the activities of PDH_a kinase and PDH_b phosphatase by the acetyl-

CoA/CoA and NADH/NAD ratios. Increases in these ratios would stimulate kinase activity and inhibit phosphatase activity, thereby increasing the steady state level of PDH_b.

Taylor et al. also reported (10) that rotenone, which is known to inhibit oxidation of intramitochondrial NADH, decreased the proportion of PDH_a in isolated rat liver mitochondria without altering appreciably the intramitochondrial ATP/ADP ratio. In addition, glutamate or 3-hydroxybutyrate decreased and ammonium chloride or acetoacetate increased the proportion of PDH_a in liver mitochondria. Glutamate, ammonium chloride, or 3-hydroxybutyrate had effects on the proportion of PDH_a in rat adipose tissue segments similar to those observed with liver mitochondria. However, acetoacetate decreased the proportion of PDH_a in adipose tissue segments. Our findings with the purified pyruvate dehydrogenase system suggest that decreases in the proportion of PDH_a produced by rotenone, glutamate, or 3-hydroxybutyrate may be due, at least in part, to inhibition of PDH_b phosphatase activity and possibly to stimulation of PDH_a kinase activity resulting from an increase in the intramitochondrial NADH/NAD ratio. Conversely, ammonium chloride or acetoacetate decrease the intramitochondrial NADH/NAD ratio which, in turn, stimulates PDH_b phosphatase activity and apparently inhibits PDH_a kinase activity. In adipose tissue, which contains a low level of 3-hydroxybutyrate dehydrogenase, the inhibitory effect of acetoacetate may be due to an increase in the acetyl-CoA/CoA ratio, which stimulates PDH_a kinase activity.

Our latest findings, together with previous results, indicate that PDH_a kinase and PDH_b phosphatase can sense fluctuations in the concentrations of pyruvate, K^+, Mg^{2+}, and Ca^{2+} and fluctuations in the ATP/ADP, acetyl-CoA/CoA, and NADH/NAD molar ratios, and then integrate these changes to produce appropriate modulation of the pyruvate dehydrogenase activity.

ACKNOWLEDGMENTS. This work was supported in part by USPHS Grant GM06590. We thank Cheryl Hyslop and Cynthia Hook for skilled technical assistance and Dr. Merle Olson and Dr. Earl Stadtman for valuable discussions.

REFERENCES

1. Linn, T.C., Pettit, F.H., and Reed, L.J. (1969) Proc. Nat. Acad. Sci. U.S.A. 62, 234-241.
2. Linn, T.C., Pettit, F.H., Hucho, F., and Reed, L.J. (1969) Proc. Nat. Acad. Sci. U.S.A. 64, 227-234.
3. Denton, R.M., Randle, P.J., and Martin, B.R. (1972) Biochem. J. 128, 161-163.
4. Siess, E.A., and Wieland, O.H. (1972) Eur. J. Biochem. 26, 96-105.
5. Pettit, F.H., Roche, T.E., and Reed, L.J. (1972) Biochem. Biophys. Res. Commun. 49, 563-571.
6. Roche, T.E., and Reed, L.J. (1974) Biochem. Biophys. Res. Commun. 59, 1341-1348.
7. Hucho, F., Randall, D.D., Roche, T.E., Burgett, M.W., Pelley, J.W., and Reed, L.J. (1972) Arch. Biochem. Biophys. 151, 328-340.
8. Reed, L.J., Pettit, F.H., Roche, T.E., and Butterworth, P.J. (1973) in Protein Phosphorylation in Control Mechanisms (F.Huijing and E.Y.C. Lee, eds.) pp. 83-95, Academic Press, New York.
9. Linn, T.C., Pelley, J.W., Pettit, F.H., Hucho, F., Randall, D.D., and Reed, L.J. (1972) Arch. Biochem. Biophys. 148, 327-342.
10. Taylor, S.I., Mukherjee, C., and Jungas, R.L. (1975) J. Biol. Chem. 250, 2028-2035.
11. Nicholls, D.G., Shepherd, D., and Garland, P.B. (1967) Biochem. J. 103, 677-691.
12. Wieland, O.H., and Portenhauser, R. (1974) Eur. J. Biochem. 45, 577-588.