INFLUENCE OF PHENYLPYRUVATE ON THE INTERCONVERSION OF PYRUVATE DEHYDROGENASE COMPLEX FROM MAMMALIAN BRAIN AND KIDNEY

Barbara T. HOFFMANN and Ferdinand HUCHO
Fachbereich Biologie der Universität Konstanz, 775 Konstanz, Germany

Received 21 March 1974

Introduction

Conflicting results have been published on the influence of metabolites occurring during phenylketonuria on the activity of the pyruvate dehydrogenase multienzyme complex. Inhibition of the brain enzyme by phenylpyruvate has been reported [1] but could not be confirmed by others [2,11]. Neither investigation included the effect of the respective metabolites on the rate of the interconversion of the pyruvate dehydrogenase from its active nonphosphorylated to its inactive phosphorylated form [3,4]. This interconversion appears to be under control by metabolites of low molecular weight, e.g. pyruvate, ATP, ADP [5,6]. Therefore we investigated the effect of a variety of metabolites involved in phenylketonuria on the pyruvate dehydrogenase kinase and phosphatase activity. We show in the present paper that phenylpyruvate influences the rate of interconversion of the pyruvate dehydrogenase complex from its active to the non-active form, i.e. it inhibits phosphorylation of the complex by the pyruvate dehydrogenase kinase. None of the compounds tested had any effect on the enzyme directly, i.e. by competitive inhibition or activation. With respect to phenylpyruvate, no significant qualitative difference was found for the kidney and the brain enzymes. At this stage of the investigation, the bearing of these findings on the interpretation of the symptoms of the disease is not clear, but they are pertinent to our understanding of the mechanism of the interconversion of the pyruvate dehydrogenase complex. Together with earlier results [4–6], they are indicative of a regulatory α-ketoacid binding site on the protein separate from the active center substrate binding site.

2. Materials

NAD^+, thiamine pyrophosphate and sodium pyruvate were obtained from Boehringer und Soehne, Germany; coenzyme A and dithiothreitol were from Serva, Germany. Substances used as effectors were as follows: sodium salts of phenylpyruvic acid (Fluka, Switzerland), L-phenylalanine and indole-3-acetic acid (Merck, Germany), L-3-phenyllactic acid and *o*-hydroxy-phenylacetic acid (EGA-Chemie, Germany), and DL-3-indolelactic acid (Aldrich, USA).

3. Methods

3.1. *Purification procedures*

The preparation of the enzyme from bovine kidney mitochondria was basically the same as described by Linn et al. [7], but without the ultimate steps of ultracentrifugation and acid precipitation. Bovine brain mitochondria were prepared by the method of Burgett [8]. Here too, the ultracentrifugation and acid precipitation steps were omitted. The mitochondrial preparations were subjected to 4 × 30 sec sonification instead of being homogenized in a Manton–Gaulin-homogenizer. The specific activity of the kidney pyruvate dehydrogenase complex was 1.4 moles NADH/min/mg protein at 25°C, while the specific activity of the brain pyruvate dehydroge-

 North-Holland Publishing Company – Amsterdam

nase complex was 0.4 moles NADH/min/mg protein at 25°C.

3.2. *Assay of pyruvate dehydrogenase complex*

The standard assay procedure was the same as described elsewhere [7]. The temperature was 30°C. The reaction was initiated by addition of enzyme.

3.3. *Assay of pyruvate dehydrogenase kinase*

The pyruvate dehydrogenase kinase activity of the pyruvate dehydrogenase complex was determined by measuring the decrease of activity of the enzyme complex in the presence of ATP.

3.4. *Assay of pyruvate dehydrogenase phosphatase*

The assay is based on the reactivation of an inactivated (i.e. phosphorylated) preparation of the pyruvate dehydrogenase complex by the phosphatase.

4. Results

4.1. *Effect of metabolites on the activity of the pyruvate dehydrogenase complex, pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase*

Kinetic studies were undertaken to investigate the effect of metabolites on the pyruvate dehydrogenase activity of bovine kidney. The method of Lineweaver and Burk [9] was used to determine the influence of the following substances on the activity of the kidney pyruvate dehydrogenase complex: phenylpyruvic acid, L-phenylalanine, indole-3-acetic acid, L-3-phenyllactic acid, Dl-3-indole-lactic acid and *o*-hydroxy-phenylacetic acid. Of the six metabolites tested, none was significantly inhibitory. However, increased concentrations of phenylpyruvate (10 mM) showed the possibility of an uncompetitive inhibition (fig. 1A).

Brain pyruvate dehydrogenase complex activity was, therefore, investigated to determine if there was any influence due to phenylpyruvate and, here too, a 10 mM concentration of the effector showed an uncompetitive inhibition (fig. 1B). Yet, since the highest recorded phenylpyruvate concentration in tissue [10] is very much below this magnitude, it is unlikely that, in phenylketonuria, phenylpyruvate has any direct effect on the pyruvate dehydrogenase activity.

L-Phenylalanine, indole-3-acetic acid, L-3-phenyllactic acid, and phenylpyruvate were tested as to their effects on the phosphorylation and dephosphorylation of the kidney pyruvate dehydrogenase complex. Only phenylpyruvate inhibited the phosphorylation of the pyruvate dehydrogenase complex with increased concentrations, as shown in fig. 2A, whereas the dephosphorylation of the pyruvate dehydrogenase complex was not significantly influenced by any of the effectors.

In view of the above results, the protective effect of phenylpyruvate on the activity of the brain pyruvate dehydrogenase complex was investigated. Here too, the investigation showed (fig. 2B) that the phosphorylation of the enzyme complex was inhibited to about the

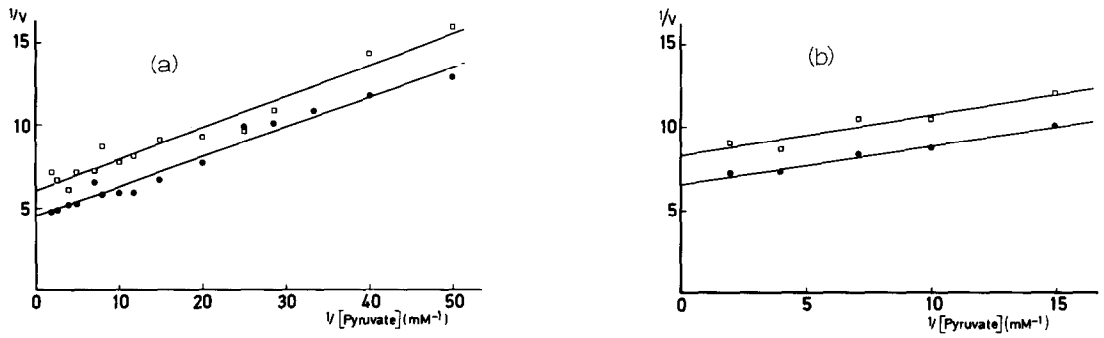

Fig. 1a and 1b. Pyruvate dehydrogenase complex activity in the presence of phenylpyruvate. The initial rate of activity was assayed in the presence of (□) 10 mM and (•) without phenylpyruvate. The standard assay at 30°C was as described in 'Methods'. The reaction mixture contained a protein concentration of 25 μg/ml (kidney) and 39 μg/ml (brain). a: kidney pyruvate dehydrogenase complex. b: brain pyruvate dehydrogenase complex.

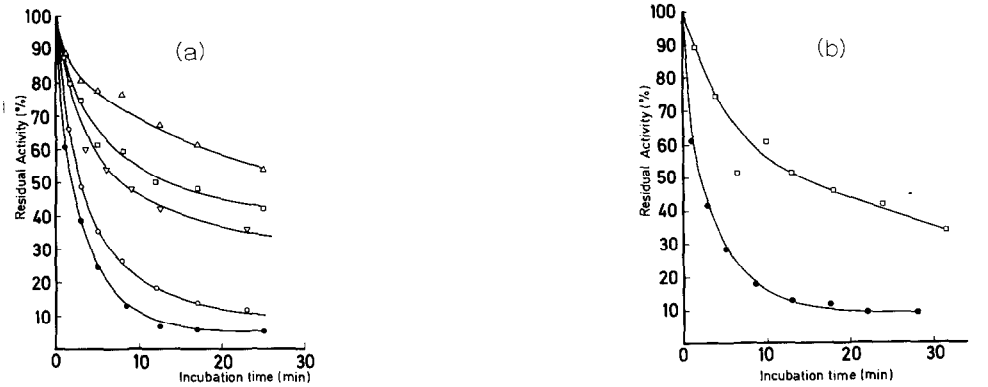

Fig. 2a and 2b. Protective effect of phenylpyruvate on the inactivation of pyruvate dehydrogenase complex by pyruvate dehydrogenase kinase. Pyruvate dehydrogenase complex was incubated with 0.01 mM ATP (kidney) and 0.1 mM ATP (brain) at room temperature. ATP was omitted from the control. The reaction mixture contained 20 mM phosphate buffer, pH 7.0; 2 mM dithiothreitol; 0.2 mM $MgCl_2$; and phenylpyruvate with the following concentrations: (○) 2 mM, (▽) 5 mM, (□) 10 mM, and (△) 20 mM; (●) contains no phenylpyruvate. At the time indicated 50 μl aliquots were removed and assayed under standard conditions. a: kidney pyruvate dehydrogenase complex; b: brain pyruvate dehydrogenase complex.

same extent as the kidney enzyme, while the dephosporylation of the brain pyruvate dehydrogenase complex was not significantly affected (fig. 3).

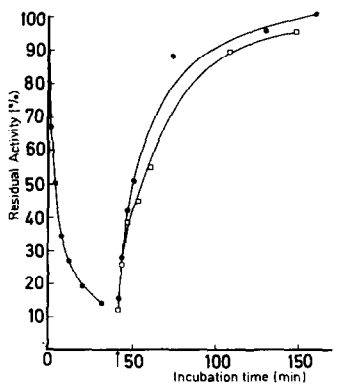

Fig. 3. Reactivation of brain pyruvate dehydrogenase complex by pyruvate dehydrogenase phosphatase. Brain pyruvate dehydrogenase complex was incubated at room temperature with 0.1 mM ATP and the reaction mixture as described in the legend of fig. 2. At 85% inactivation by pyruvate dehydrogenase kinase the incubation mixture was divided into two parts. To one part phenylpyruvate was added, while the other half was assayed without phenylpyruvate. Reactivation by pyruvate dehydrogenase phosphatase was started by adding $MgCl_2$ (arrow) to a final concentration of 10 mM. At the time indicated, 50 μl aliquots were removed and assayed under standard conditions. (●) without phenylpyruvate; (□) 10 mM phenylpyruvate.

5. Discussion

The experiments presented here lead to the following conclusions:

1. Phenylpyruvate inhibits the interconversion of the pyruvate dehydrogenase complex from the active non-phosphorylated to the inactive phosporylated form. Other metabolites occurring during phenylketonuria (L-phenylalanine, indole-3-actic acid, L-3-phenylactic acid. DL-3-indole-lactic acid and *o*-hydroxy-phenyl-acetic acid) had no significant effect.
2. The reverse reaction, the interconversion from the inactive to the active form, is not significantly affected by phenylpyruvate.
3. Phenylpyruvate does not influence the activity of the component enzymes of the multienzyme complex directly, i.e. it does not compete with the substrate for the active site.
4. The results with the pyruvate dehydrogenase complex isolated from mammalian brain and kidney are qualitatively the same.

With these results it remains very doubtful if changes in the pyruvate dehydrogenase complex activity caused by elevated concentrations of

certain metabolites during phenylketonuria can be responsible for the observed symptoms. At present it is not clear how a shift of the enzyme to its active non-phosporylated state in vivo can cause for example the deficiency in myelin deposition in the nervous tissue which is characteristic for the disease.

The effect of phenylpyruvate reported here, may have, on the other hand, important implications for the enzymology of the pyruvate dehydrogenase complex and the mechanism of its interconversion. We reported elsewhere a strong inhibition of the interconversion by pyruvate (for a discussion of possible physiological implications of this effect |see ref. [6]). From various lines of evidence, we postulated that the binding site of the pyruvate which protects the multienzyme complex against phosphorylation and concomitant inactivation, is not identical with the binding site of the substrate pyruvate, i.e. we proposed that there is a regulatory pyruvate binding site. The data presented here now indicate that phenylpyruvate does not compete for the substrate binding site, because it is neither substrate nor competitive inhibitor, but, like pyruvate, it still protects the complex against inactivation.

The binding site for this protective molecule could be located on the pyruvate dehydrogenase kinase, the enzyme which catalyses the phosphorylation and inactivation of the complex. But for the protective pyruvate we have shown no competition with ATP for the active site of the kinase [5]. Together with the results reported in this paper, these experiments may indicate that the pyruvate dehydrogenase contains a separate regulatory site which is involved in the inactivation of the enzyme by phosphorylation and which binds protective molecules as pyruvate or phenylpyruvate. Further experiments are in progress to substantiate this conclusion.

Acknowledgements

We wish to thank Dr. Horst Sund, Konstanz, for his encouragement and for reviewing the manuscript. We are grateful to the Gesellschaft für Molekularbiologische Forschung, Stöckheim, for making available its facilities for the preparation of the kidney mitochondria and Mr. Michael Janda for technical assistance. This work was supported by research grants from the Deutsche Forschungsgemeinschaft.

References

[1] Bowden, J. A. and McArthur, C. L. (1972) Nature 235, 230.
[2] Blass, J. P. and Lewis, C. (1973) Biochem. J. 131, 31.
[3] Linn, T. C., Pettit, F. H., and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U.S. 62, 234.
[4] Linn, T. C., Pettit, F. H., Hucho, F. and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U.S. 64, 227.
[5] Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. and Reed, L. J. (1972) Arch. Biochem. Biophys. 151, 328.
[6] Hucho, F. (1974) Angew. Chemie in press.
[7] Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., and Reed, L. J. (1972) Arch. Biochem. Biophys. 148, 327.
[8] Burgett, M. W. (1972) PhD-Thesis, University of Texas, Austin, Texas.
[9] Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 158.
[10] Patel, M. S. (1972) Biochem. J. 128, 677.
[11] Land, J. M. and Clark, J. B. (1973) Biochem. J. 134, 539.