GLYOXYLIC ACID AS AN ACTIVE SITE MARKER OF YEAST PYRUVATE DECARBOXYLASE

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Received 22 December 1975

1. Introduction

Yeast pyruvate decarboxylase (PDC, EC 4.1.1.1., mol. wt. 175 000 ± 5000 [1]) consists of two subunits [2]. Despite many attempts no evidence has been obtained so far whether the molecule contains two or four active centres, each of which with one molecule thiamine pyrophosphate (TPP). Recent experiments with glyoxylic acid as substrate have unexpectedly given new insight into this problem. If glyoxylic acid is used as substrate of PDC no CO_2 can be detected by manometric techniques. However Hübner [3] was able to estimate qualitatively the formation of $^{14}CO_2$ by taking [1,2-^{14}C]glyoxylic acid as substrate. In the same series of experiments a compound could be isolated in analogy to Holzer and Beaucamp [4] from the incubation mixture after separation of the protein fraction and chromatographic identification, which was recognized on account of its R_f value as 2-α-hydroxymethyl-TPP (HMTPP, in analogy to the product formed in the pyruvate reaction the name 'active formaldehyde' was suggested).

On the basis of these results, we supposed the glyoxylic acid to be fixed to the C_2 atom via the usual mechanism of the PDC reaction with the

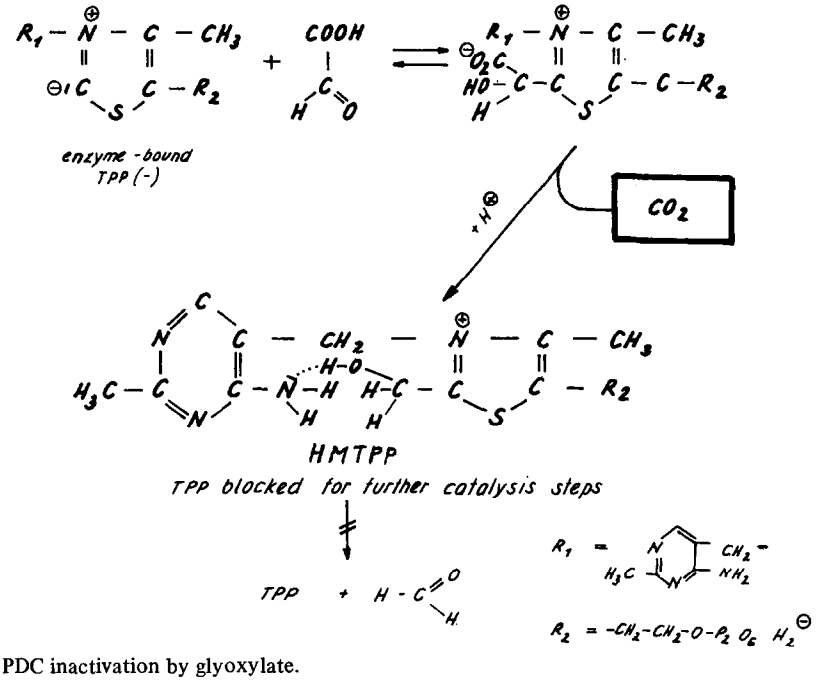

Fig.1. Mechanism of PDC inactivation by glyoxylate.

difference that the bond formed between the C_2 atom and the α-C atom of the HMTPP molecule is too strong to enable a splitting off of the formaldehyde molecule by means of subsequent intramolecular catalysis by the 4′-amino group (fig.1). This means that after incubation of the enzyme with glyoxylic acid not only any further degradation of this compound is blocked but also the catalytic function with respect to pyruvate is irreversibly suppressed.

In this paper we present a new technique which affords a quantitative estimation of the [1,2-^{14}C] glyoxylic acid degradation by adsorption of the originating $^{14}CO_2$ in potassium hydroxide solution. As this method registers only those parts of the TPP molecules which are correctly fixed within the active centre, glyoxylic acid can be regarded as a specific active centre label of the PDC molecule. As a result of experiments with enzyme fractions of different specific activities a direct correlation was registered between the content of enzyme-bound TPP molecules and the activity of the several enzyme fractions.

2. Material and methods

PDC was prepared according to Ullrich [5]. A refinement of the crude enzyme fractions was obtained by CM-Sephadex chromatography [6]. [1,2-^{14}C] glyoxylic acid was synthesized by the method of Murray et al. [7]. The quantitative $^{14}CO_2$ estimations were carried out in Warburg vessels. The incubation mixture had the following composition: Main vessel: 0.5 ml maleat buffer (0.2 M; pH 6.15); 0.5 ml bidistilled water; 0.05 ml[1,2-^{14}C]glyoxylic acid (2.5×10^{-2} M); varying amounts of enzyme solution (25 to 200 μl). Central vessel: 0.2 ml potassium hydroxide solution (0.1 N). Side vessel: 0.2 ml phosphoric acid (see below for concentration).

The reaction was started by adding the enzyme solution. The estimation of the blind values was carried out in experiments without enzyme solution. In another series of blind runs the enzyme solution was substituted by a TPP solution (table 2). The reaction was stopped by adding the phosphoric acid from the side arm of the vessel to the incubation mixture. The concentration of the latter was adjusted therefore to reach a final pH of 3.5 in the incubation mixture. To guarantee a quantitative diffusion of the formed CO_2 to the potassium hydroxide solution the reaction vessels were then shaken continuously for 12 h. Finally, the radioactivity which had collected within the central vessel was isolated in definite portions and the impulse rate of equal amounts was estimated by means of a measuring set of Fa. VEB Vakutronik, Dresden.

3. Results and discussion

The sensitivity and precision of the method is shown in Table 1. The reproducibility of the results (obtained from 3 single estimations) was ± 5%.

The quantitative degradation of glyoxylic acid was performed with PDC preparations of different activities. The results are summarized in fig.2.

As the enzyme charges with specific activities between 25 and 61 U probably contained different amounts of contaminating proteins, a direct relation between glyoxylic acid degradation and protein content in the incubation mixtures was not to be expected. But an exact correlation exists between the yield of $^{14}CO_2$ (TPP content of the enzyme charges) and the activity of the preparations. From fig.2 the TPP contents of the purest (most active) PDC preparations described so far in the literature (98 U [8], 93 U [6]) can be extrapolated to be 4.15 and 3.96 molecules/mol PDC respectively.

On the other hand, supposing the existence of four active centres (4 TPP molecules), it can be derived from these data that the activity of PDC will not considerably exceed the 100 U level.

As the preparations used in our experiments were

Table 1
Dependence of the yield of $^{14}CO_2$ (imp./min) on enzyme concentration after incubation with excess amounts of [1,2-^{14}C]glyoxylic acid

PDC solution (μl)	imp./min	imp./min calculated to 200 μl PDC solution
25	1632	13051
50	3327	13307
100	6405	12811
200	13312	13312

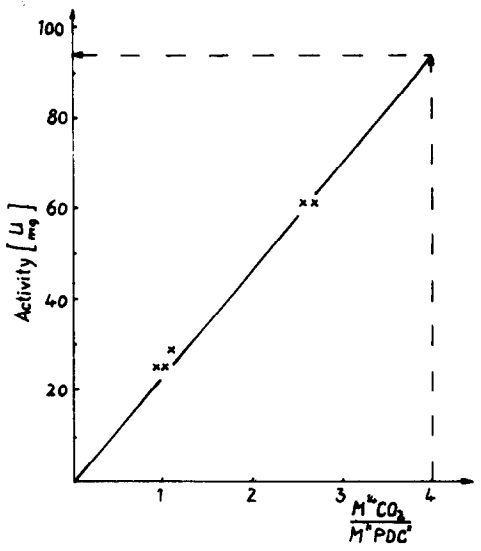

Fig.2. Relation between the specific activity (25 U, 29 U, 61 U) and TPP content of several PDC preparations, estimated from $^{14}CO_2$ production after incubation with excess [1,2-^{14}C]glyoxylate. (M "PDC" refers to the overall protein content of the solutions).

obtained at TPP saturation during the isolating steps the contaminating proteins (mainly present in preparations of lower activities) can be either foreign protein fractions with similar physical properties or PDC molecules with their ternary structure destroyed having lost the ability to bind the coenzyme molecules.

Actually with the method presented only that part of TPP molecules which is bound correctly within the active centres of the PDC molecules is registered. TPP molecules in solution or in random interaction with the proteins present cause no $^{14}CO_2$ production after the addition of [1,2-^{14}C]glyoxylic acid (table 2).

By varying the incubation periods from 1–120 min, and also by subsequent incubation of glyoxylic acid-inactivated enzyme with [1-^{14}C]pyruvate, we could finally state a quasi-irreversible blocking of the active centres of PDC in agreement with the reaction scheme shown in fig.1.

Table 2
Degradation of [1,2-^{14}C]glyoxylate (Gly) and [1-^{14}C]pyruvate (Pyr) in presence and absence of PDC

PDC (μl)	Gly (μl)	Pyr (μl)	TPP (μl)	Mg^{2+} (μl)	imp./min (total yield)
50	–	50	–	–	122031[a]
50	50	–	–	–	4595
–	50	–	50	50	331[b]
50	50	50[c]	–	–	5722[d]
–	50	50	50	50	1308[b]

Temp. 25°C. Concentrations: [1,2-^{14}C]glyoxylate 2.5 × 10^{-2} M (5 mCi/mM). [1-^{14}C]pyruvate 5.0 × 10^{-2} M (2.3 mCi/mM). TPP, Mg^{2+} 10^{-2} M.

[a] Yield of $^{14}CO_2$ after 3 min incubation period.

[b] Blind values.

[c] Addition of pyruvate after 3 min incubation of the enzyme with glyoxylate.

[d] Value from glyoxylate degradation plus pyruvate blind value (1137 imp/min).

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