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REGULATORY PROPERTIES OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *PSEUDOMONAS AERUGINOSA*

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Summary

The pyruvate dehydrogenase multienzyme complex of *Pseudomonas aeruginosa* was subjected to a steady-state kinetic analysis using the exponential model for a regulatory enzyme and a sensitive statistical fitting procedure. This showed that all the substrates, pyruvate, CoA and NAD^+, exhibit cooperative kinetics towards the native multienzyme complex.

Introduction

The pyruvate dehydrogenase multienzyme complexe catalyses the oxidative decarboxylation of pyruvate thus·

pyruvate + NAD^+ + CoASH → acetyl-CoA + CO_2 + NADH + H^+

and has elaborate quaternary structures containing multiple copies of three enzymic components. pyruvate dehydrogenase (E1) (pyruvate : lipoamide oxidoreductase (decarboxylating and acceptor-acetylating, EC 1.2.4.1), lipoyl transacetylase (E2) (acetyl-CoA dihydrolipoamide *S*-acetyltransferase, EC 2.3.1.12) and lipoamide dehydrogenase (E3) (NADH : lipoamide oxidoreductase, EC 1.6.4.3) [1,2]. The rationale for such structures is probably a physiological one since the pyruvate dehydrogenase complex lies at the crossroads between mainstream carbohydrate matabolism (glycolysis and gluconeogenesis)

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and energy generation (the tricarboxylic acid cycle). The regulatory properties of these enzymes are therefore important but in procaryotic organisms (*Escherichia coli* in particular) only the cooperative behaviour of pyruvate has been examined in detail [3—6].

In the present paper we describe an analysis of the steady-state kinetics of the pyruvate dehydrogenase multienzyme complex of *Pseudomonas aeruginosa* [7,8]. In addition to the cooperative behaviour of pyruvate, we also demonstrate for the first time, using a sensitive statistical fitting procedure [9], that both CoA and NAD^+ can exhibit cooperative kinetics towards the native multienzyme complex.

Materials and Methods

Enzymes and reagents *P aeruginosa* strain PAO1 was kindly provided by Professor B.W. Holloway. The pyruvate dehydrogenase multienzyme complex of glucose-grown *P aeruginosa* was isolated in pure form by ethanol-Sepharose 2B chromatography, followed by sucrose-gradient centrifugation [7,8]. It contained no contaminating activities. Citrate (*si*)-synthase (EC 4.1.3.7) was obtained from Boehringer and all other reagents were of the A.R. grade.

Determination of enzyme activity. The pyruvate dehydrogenase complex was assayed with (μmol in a final volume of 1 ml): Tris-HCl (100, pH 7.8), pyruvate (5), $MgCl_2$ (2), dithiothreitol (2.5), thiamine pyrophosphate (0.4), CoA (0.13) and NAD^+ (0.6). The reaction was started with the varied substrate and the production of NADH followed by the increase in absorbance at 340 nm. Enzyme activity is expressed as μmol NADH produced/min. All assays were performed at 25°C using a double-beam recording spectrophotometer (Unicam SP1800). When CoA was the varied substrate the assay was modified by the inclusion of a CoA-regenerating system· citrate (*si*)-synthase (2.5 units) plus oxaloacetate (0.2 μmol). Protein was determined by the method of Lowry et al. [10] with bovine serum albumin as the reference protein.

Analysis of the steady-state kinetics Kinetic results were analyzed using the exponential model for a regulatory enzyme [9] This model allows rate data, containing not more than a single inflexion, to be expressed in terms of three parameters only: the maximal velocity (V_A) obtained at saturating concentrations of the varied substrate (A); the association constant (α_A) of the unbound enzyme for ligand A; and the cooperativity (k_A). Thus, the rate function may be expressed as:

$$v = \frac{V_A \;\; A \;\; \alpha_A \cdot \exp(k_A v/V_A)}{1 + A \;\; \alpha_A \cdot \exp(k_A v/V_A)} \qquad (1)$$

where v is the initial velocity of reaction. The cooperativity (k_A) may be measured much more accurately than the Hill coefficient (h) but bears a simple relation to it

$$h = \frac{4}{4 - k_A} \qquad (2)$$

and Eqn. 1 shows that when $k_A = 0$, the reaction follows Michaelis-Menten kinetics.

The parameters V_A, α_A and k_A, were obtained by fitting the raw data (A, v) to Eqn. 1 by Gauss-Newton non-linear regression [11]. In the data presented below the term 'saturating' always refers to an extrapolated value obtained by computer calculation Thus, the saturation value of α_A is $\alpha_A \cdot \exp(k_A)$ obtained when $v = V_A$, see Eqn. 1.

Results

Cooperative kinetic behaviour of pyruvate and CoA Variations in initial velocity of the reaction of the pyruvate dehydrogenase complex with pyruvate (P) and CoA (C) as the varied substrates are shown in Fig. 1a and b together with the linear replots for Eqn. 1 [9]. The fitted values of the cooperativities of these substrates k_P and k_C, suggest that both display positive cooperative kinetics towards the native complex. With pyruvate the calculated Hill coefficient ($h = 1.34$) is similar to the value (1.1) obtained for the pyruvate dehydrogenase complex of *E coli* [4,5] and is somewhat lower than that (2 5) observed for the enzyme complex from *Azotobacter vinelandii* [6]. The interpretation of positive cooperativity for pyruvate can be given with confidence despite the low value of k_P, because the fitting was performed using untransformed data in the mid-range of fractional saturations and the residual sum of squares for the fitted curve was acceptably small approx. ($1 \cdot 10^{-4}$). The results in Table I show that the fitted values for the maximal velocities attained with pyruvate and CoA as the varied substrates are the same, within the limits of experimental error.

Regulatory effects of NADH upon the variation of the initial velocity with NAD$^+$. In the absence of NADH, the kinetics with respect to NAD$^+$ (N) approached the limiting Michaelis-Menten form because the value of k_N is not sufficiently different from zero to justify the interpretation of positive kinetic cooperativity (Fig. 1c and Table I). However, upon the addition of 30 μM

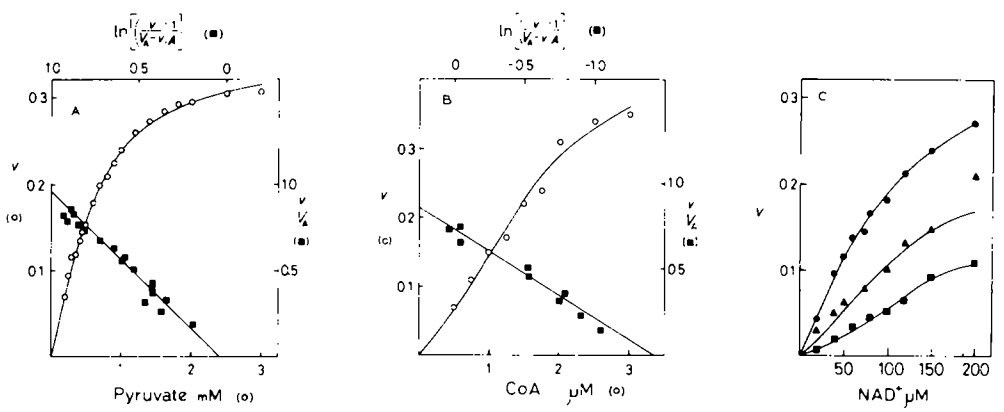

Fig 1 Variation of the initial velocity when (a) pyruvate, (b) CoA and (c) NAD$^+$ are the varied substrates The values for v are expressed in μmol NADH produced/min In (a) and (b) the solid lines were calculated by substituting the fitted parameters into Eqn 1 (○———○), or its linear transform (■———■) [8] In (c) the lines were calculated by substituting the fitted parameters into Eqn 1 only and the concentrations of NADH were 0 μM (●———●), 30 μM (▲———▲), 120 μM (■———■)

TABLE I

KINETIC PARAMETERS OF THE EXPONENTIAL MODEL FOR PYRUVATE, CoA AND NAD^+ AS THE VARIED SUBSTRATES

The parameters were obtained by fitting the raw (A, v) data to Eqn 1 by Gauss-Newton non-linear regression [11] V_A is expressed as μmol NADH formed/min

Varied substrate (A)	V_A 10	α_A (mM^{-1})	k_A
Pyruvate (P)	3 57 ± 0 06	1 03 ± 0 04	1 02 ± 0 12
CoA (C)	4 71 ± 0 45	239 ± 19 20	1 95 ± 0 30
NAD^+ (no NADH) * (N)	3 76 ± 0 41	6 51 ± 0 44	0 89 ± 0 38
NAD^+ (plus 30 μM NADH) *	2 20 ± 0 68	4 22 ± 0 90	1 69 ± 1 09
NAD^+ (plus 120 μM NADH) *	1 53 ± 0 22	2 64 ± 0 23	2 15 ± 0 31

* Refers to the initial concentration of NADH

NADH, V_N and α_N decreased, whereas k_N increased, and these trends were exaggerated by increasing the initial concentration of NADH (Table I).

The effect of a 'pure' competitive inhibitor upon Eqn. 1 is to decrease the value of α only, leaving the value of k_A unaffected [13—15]. Table I shows that this condition is not satisfied for the NADH inhibition. This fact, and the observation that saturating concentrations of NAD^+ are not sufficient to overcome the net inhibition by NADH (the operational definition of a competition [16]), suggest that the inhibition is 'mixed'. The changes of α_N and k_N induced by NADH have a complementary effect such that at low and intermediate concentrations of NAD^+, the degree of inhibition produced by NADH is greater than would be observed if the rate function remained Michaelis-Menten. A similar phenomenon has been observed for a number of other unrelated enzymes [12,15].

It is interesting that the final affinity for NAD^+, α_N exp (k_N), of the enzyme saturated with this substrate appears the same for both sets of data obtained in the presence of NADH and different to that obtained in its absence. This may indicate that NADH induces a gross conformational change in the multienzyme complex.

Discussion

The pyruvate dehydrogenase multienzyme complex of *P aeruginosa* appears to be similar but smaller, than that of *E. coli* [7,8]. So, the cooperative behaviour observed for each of the three substrates of the overall reaction (i.e., pyruvate, CoA and NAD^+) could arise by the binding of these ligands to distinct sites on the individual components (E1, E2 and E3, respectively). For the enzymes from *E coli* and *A vinelandii*, several studies have implicated the pyruvate dehydrogenase component as the primary locus of pyruvate cooperativity and modulation by physiological effectors [3—6].

The observed positive cooperative steady-state kinetics for CoA are novel, although Danson et al. [17] recently suggested from transient kinetic evidence, that CoA may allosterically stimulate the 'posting' of acetyl groups between identical subunits of the E2 complex of *E coli*. Interestingly, Bresters et al. [6] suggested that the apparent steady-state kinetic cooperativity of CoA, observed

for the pyruvate dehydrogenase from *A vinelandii*, was due to the inhibitory effects of accumulating acetyl-CoA that could be abolished with a CoA-regenerating system. However, this does not apply to the present study. Thus, it is concluded that the regulatory influence of CoA upon the native complex is real and may serve a physiological function.

Cooperative kinetics for NAD^+ have not been observed for other pyruvate dehydrogenase complexes. Hansen and Henning [18] suggested on the basis of preliminary qualitative evidence that the binding of NADH was competitive with respect to NAD^+ for the enzyme from *E coli*. However, they also observed that at saturating concentrations of NAD^+, 50% inhibition was still observed at a NADH/NAD^+ ratio of as low as 0.02. This last result contradicts the operational definition of a competition [16]. Several studies [19,20] have shown that for the *E coli* enzyme NADH reacts with the flavoprotein component (E3) of the complex and that excess NADH causes this component to become doubly reduced, thereby causing inactivation of the native complex. Although this may account for the effects on V_N observed for the *P aeruginosa* enzyme, it cannot explain the enhancement of NAD^+ cooperativity by NADH, which is probably conformational in origin. Indeed, for the *A vinelandii* enzyme, inhibitor studies have implicated an NADH-promoted conformational change [21].

In this study, reliable and sensitive statistical fitting procedures have been used for estimating the kinetic parameters and cooperative effects. This avoids any reliance on visual inspection or linear regression analysis which are notoriously misleading, particularly for rate functions that are not of the Michaelis-Menten type [22].

It is not clear to what extent the results obtained with *P. aeruginosa* can be generalized, but the observed cooperative effects have obvious physiological and structural importance and highlight the need for a general re-examination of the kinetics of the bacterial pyruvate dehydrogenase complexes.

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