These analyses show that this cyclic AMP phosphodiesterase contains at least 15 ng of zinc per activity unit. The sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fraction 18 suggests that the specific activity of pure enzyme is at least 104 units/mg. Thus 1 mol (65000 g; Fujimoto et al., 1974; Londesborough, 1974) contains about 1.6 g-atoms of zinc. We are now trying to make large enough amounts of pure enzyme to permit accurate zinc analyses and a reliable estimate of the $A_{15\%}^\text{E}$.

When 1,10-phenanthroline was added to reaction mixtures it inhibited the enzyme in a simple competitive fashion ($K_i = 480 \mu M$). Attempts to prevent this inhibition by addition of metals, such as Zn, Ni and Co, which are strongly chelated by 1,10-phenanthroline, were complicated by the strong inhibition exerted by these metals themselves (Fujimoto et al., 1974). The inhibition by 1-3 mM-1,10-phenanthroline was decreased by small amounts of NiSO$_4$, but the protection was never complete. Maximum protection (an approximate doubling of the reaction rate) was reached when the concentration of Ni(OH)$_3$ was a third of that of the 1,10-phenanthroline, corresponding to formation of Ni(OP)$_3$. The enzyme was not inactivated by preincubation with 5 mM-1,10-phenanthroline for 2 h at room temperature before assay of 50-fold diluted samples. By contrast, the enzyme was inactivated by preincubation with 8-hydroxyquinoline. The extent of inactivation depended on the concentration of 8-hydroxyquinoline, and the temperature and duration of the incubation. At 30°C, 40% of the activity was lost in 1 min, and the remainder in 90 min at 5 mM-8-hydroxyquinoline, and 50% was lost in 160 min at 0.5 mM-8-hydroxyquinoline. This irreversible inactivation was prevented by addition of 1 mg of bovine albumin/ml, so that an instantaneous inhibition by 8-hydroxyquinoline could be studied in reaction mixtures containing 1 mg of bovine albumin/ml. Under these conditions, 8-hydroxyquinoline was a simple competitive inhibitor ($K_i = 1.1 \text{mM}$). In the absence of albumin, mixed inhibition was observed, and results indicated that significant irreversible inactivation occurred during the time (1 min) required to measure the reaction rate.

Inhibition by mercaptoethanol was studied by the spectrophotometric assay, and was mixed ($K_i^{\text{E}} = 0.4 \text{mM}$, $K_i^{\text{PS}} = 1.6 \text{mM}$). Inhibition by mercaptoethylamine was similar, but non-linear. At 0.5 mM-cyclic AMP, 50 mM-KCN caused 70% inhibition (t.l.c. assay), but NaN$_3$ had no effect. This is consistent with the much smaller stability constants of the complexes of Zn$^{2+}$ with N$_3^-$ than with C$_2^-$. Inhibition by mercaptoethylamine and CN$^-$ is not likely to be caused by binding to a hydrophobic site (rather than a metal), as might be the case for 1,10-phenanthroline and 8-hydroxyquinoline. Further, the small size of the CN$^-$ ligand argues that if its binding site is not actually a part of the enzyme's catalytic site, it must in any case be very close.


Regulatory Properties of Pyruvate Carboxylase from *Aspergillus nidulans*: Evidence for the Presence of a Masked Activator Site

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Studies on pyruvate carboxylases obtained from various organisms have resulted in the identification of two major species of this enzyme. Pyruvate carboxylases obtained from mammals, birds, yeasts and various bacteria are typically found to be activated by an
acyl derivative of coenzyme A and inhibited by a dicarboxylic acid. L-Glutamate, and in some cases α-oxoglutarate, is the effective inhibitor for the mammalian and avian enzymes, whereas those obtained from bacterial and fungal sources are typically inhibited by L-aspartate and less effectively by α-oxoglutarate. In contrast, in pseudomonads and related bacteria the catalytic activity of pyruvate carboxylase is insensitive to any effectors tested thus far (cf. Scrutton, 1978, and references therein). Pyruvate carboxylase in Aspergillus niger appears, however, as a major exception to the classification since it has previously been reported that the activity of this enzyme is unaffected by addition of acetyl-CoA (Bloom & Johnson, 1961), but is inhibited by L-aspartate (Feir & Suzuki, 1969). We have therefore partially purified and examined in greater detail the properties of pyruvate carboxylase in the related organism Aspergillus nidulans.

A. nidulans R21 (paba A1, y A2) mycelia were grown in liquid shake cultures with glucose as the carbon source (Skinner & Armit, 1972; Roberts, 1970) and were harvested by filtration towards the end of the rapid growth phase (18–20h of growth at 36°C). After washing, the mycelial mat was stored at −60°C for up to 2 months. For preparation of the enzyme the mycelia were suspended in 50mM-Tris/HCl, pH7.8, containing 2 mM-MgCl₂, 1 mM-EDTA, 0.1 mM-dithioerythritol and 10 μM-phenylmethylsulphonyl fluoride, and broken by sonication. Pyruvate carboxylase was partially purified from the cell-free extract according to a modification of the method of Feir & Suzuki (1969) to a specific activity of 1.8 μmol/min per mg of protein (25°C). The enzyme activity was stable for 6 weeks at 0°C when stored in the above buffer supplemented with 20% (v/v) glycerol. Pyruvate carboxylase activity was determined in the presence of malate dehydrogenase and NADH as previously described (Young et al., 1969).

The kinetic properties of the interaction of A. nidulans pyruvate carboxylase with its substrates resemble those described for the enzyme from A. niger (Feir & Suzuki, 1969) and for other pyruvate carboxylases studied (cf. Scrutton & Young, 1972). Saturation of the enzyme by the substrates ATP and pyruvate follows first-order kinetics, and the presence of K⁺ ions and free Mg²⁺ ions is required for maximal catalytic activity. L-Aspartate, at millimolar concentrations, almost completely inhibits the A. nidulans enzyme, but a significant residual extent of activity (about 5%) is observed in the presence of saturating concentrations of this inhibitor. Inhibition by L-aspartate follows second-order kinetics and the affinity is independent of the concentrations of ATP or pyruvate. α-Oxoglutarate serves as a somewhat less effective inhibitor, whereas glutamate and various other tricarboxylic acid cycle intermediates have no effect on the enzyme activity over a similar concentration range. Acetyl-CoA has no effect on the activity of A. nidulans pyruvate carboxylase in the presence of saturating or non-saturating concentrations of the substrates. However, acetyl-CoA completely reverses inhibition by L-aspartate. The reversal of inhibition follows first-order kinetics, and the affinity of the enzyme for acetyl-CoA depends on the concentration of L-aspartate. As the aspartate concentration increases, the affinity for acetyl-CoA decreases.

Conversely, as the acetyl-CoA concentration increases, the affinity of the enzyme for aspartate decreases, but the second-order character of the inhibition is retained. Long-chain acyl-CoA derivatives, such as palmitoyl-CoA, also reverse the inhibition of A. nidulans pyruvate carboxylase by L-aspartate and are effective at lower concentrations than acetyl-CoA.

These data therefore establish the relationship of pyruvate carboxylase from A. nidulans to the bacterial and fungal pyruvate carboxylases which are activated by acetyl-CoA and inhibited by L-aspartate (Scrutton & Young, 1972; Scrutton, 1978). They also provide an unusual example of a regulated enzyme in which the activator site is expressed only in the presence of the regulatory inhibitor.

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The Inactivation of Penicillinase by Methyl acetimidate

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The exoenzyme penicillinase (penicillin amido-β-lactam hydrolase, EC 3.5.2.6) from Staphylococcus aureus PC1 (mol.wt. 28823) contains 43 lysine residues (Ambler, 1975). Methyl acetimidate amidinates enzyme amino groups with a minimal change in bulk and charge: the product may retain a large part of normal catalytic activity (e.g. Lambert et al., 1977). Solutions of the reagent were prepared with the precautions suggested by Bates et al. (1975). Penicillinase activity was measured as described by Bristow & Virden (1978). Sodium acetate buffer, pH 5.0, contained 0.5 mM-EDTA (disodium salt) and 50 mM-sodium acetate/27 mM-acetic acid.

Penicillinase (10 mg/ml) in 0.43 M-K₂SO₄/sodium acetate buffer, pH 5.0, was titrated to pH 9.4 with N-ethylmorpholine. After addition of 0.1 M-methyl acetimidate (added as a 1 M-solution in the same buffer, adjusted to pH 9.4 and diluted with an equal volume of 2 M-NaOH) and incubation at 22°C there was a decrease in enzyme activity (t₁/₂ ≈ 30 min) measured in samples diluted 10-fold with 0.43 M-K₂SO₄/sodium acetate buffer, pH 5. A further equal portion of reagent was added after 4 h; enzyme activity decreased to below the limit of detection (<1%) during the next 2 h. There was a parallel decrease in content of free amino groups measured by the method of Habeeb (1966), with the loss of at least 85–90% of total amino groups after 4 h.

A sample (12 mg) of enzyme was treated with methyl acetimidate as described above and applied to the top of a column (2 cm x 60 cm) of Sephadex-G75 equilibrated and eluted at room temperature with 0.43 M-(NH₄)₂SO₄/sodium acetate buffer, pH 5.0, at a flow rate of 18 ml/h. A major peak of protein was eluted at the same elution volume (99 ml) as untreated enzyme: a minor peak preceding the major peak contained 14% of the total A₂₇₆.5r compared with 8% in the untreated enzyme. Thus there was little unexpected intermolecular cross-linking in the amidinated enzyme.

Cephaloridine is a cephalosporin that, during hydrolysis by penicillinase, induces partial, reversible inactivation of the enzyme with kinetics similar to those with the penicillin quinacillin (Virden et al., 1978). Incubation of penicillinase (1.0 mg/ml) with 10 mM-cephaloridine in 0.43 M-K₂SO₄ and 19.5 mM-NaH₂PO₄/30.5 mM-Na₂HPO₄, pH 7.0, led to partial inactivation of benzylpenicillinase activity to 13% of normal: low activity persisted for 30–40 min until cephaloridine hydrolysis was complete. Addition, after 5 min of incubation of enzyme with cephaloridine, of 0.1 M-N-ethylmorpholine and titration to pH 9.4 with 1 M-NaOH extended the lifetime of the low activity state for at least 240 min; after 18 h enzyme activity had increased to 75% of normal. Fig. 1 shows the results of adding 0.1 M-methyl acetimidate (as a 1 M-solution in the same buffer adjusted to pH 9.4 and diluted with an equal volume of 2 M-NaOH) after preincubation of enzyme with cephaloridine and adjustment to pH 9.4 as described above. Samples were added to an equal volume of a buffer containing 5 mM-EDTA (disodium salt) and 0.5 mM-sodium acetate/0.27 M-acetic acid, pH 5; the diluted samples for measurement of penicillinase activity (0.1 ml) were incubated at 4°C for 18 h to allow recovery of the enzyme from the low activity state induced by cephaloridine. The diluted samples for assay of amino groups (1 ml) were dialysed against 400 vol. of sodium acetate buffer, pH 5.0, at 4°C for 18 h. The enzyme, in the state induced by the presence of cephaloridine, was substantially protected against inactivation by methyl acetimidate, although the extent of modification of amino groups was similar whether cephaloridine