# THE INHIBITION OF BOVINE PANCREATIC CARBOXYPEPTIDASE A BY DIHYDROXYACETONE PHOSPHATE

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In a recent study of the effects of various ligands on the rate of proteolytic digestion of rabbit muscle aldolase, it was observed that dihydroxyacetone phosphate (DHAP) acts as a powerful inhibitor of bovine pancreatic carboxypeptidase A (CPA) (Adelman et al., 1968). The present work demonstrates that this inhibition is competitive for both peptidase and esterase activities. Because this represents the first observation of competitive inhibition of CPA by a compound lacking an aromatic group, it was of interest to characterize further the structural requirements for this interaction.

## MATERIALS AND METHODS

CPA was a DFP-treated preparation, recrystallized three times, purchased from the Worthington Biochemical Corporation, and $CPA_{\alpha}$ was a generous gift of Dr. B. L. Vallee. Aliquots of the crystalline suspensions were dissolved in 5 mM Tris-HCl-500 mM lithium chloride buffer, pH 7.5, prior to use.

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Peptidase activity toward hippuryl-L-phenylalanine (HPA) and esterase activity toward hippuryl-L-phenyllactic acid (HPLA) were assayed according to previously described spectrophotometric procedures (McClure et al., 1964; Whitaker et al., 1966).

DHAP was purchased from the Boehringer Mannheim Corporation as the dicyclohexylammonium salt of the dimethyl ketal, and the free compound was generated by treatment with Dowex-50 ($H^+$), followed by hydrolysis for 4 hours at 39°. This preparation was free of glyceraldehyde 3-phosphate. Acetol phosphate (the DHAP analogue in which $CH_3$ is substituted for $CH_2OH$) was a generous gift of Dr. W. J. Rutter in the form of the dicyclohexylammonium salt of the dimethyl ketal. The free compound was generated as above followed by hydrolysis for 24 hours at 39°, and assayed with α-glycerophosphate dehydrogenase and DPNH (Rose and O'Connell, 1968). D,L-Glyceraldehyde 3-phosphate was purchased from the Boehringer Mannheim Corporation as the barium salt of the diethyl acetal, and the free aldehyde prepared as above followed by hydrolysis for 4 hours at 40°. This compound was free of DHAP.

All other compounds were obtained from commercial sources in the highest purity available.

## RESULTS AND DISCUSSION

The inhibition of both peptidase activity and esterase activity by DHAP was competitive with substrate (Fig. 1). In order to determine the specific structural requirements for inhibition, the effects of α-glycerophosphate, D,L-glyceraldehyde 3-phosphate and acetol phosphate were also examined. DHAP and acetol phosphate were equally potent competitive inhibitors of both peptidase and esterase activity of CPA (Table I). Since the hydroxyl group at carbon-3 represents the only structural difference between these

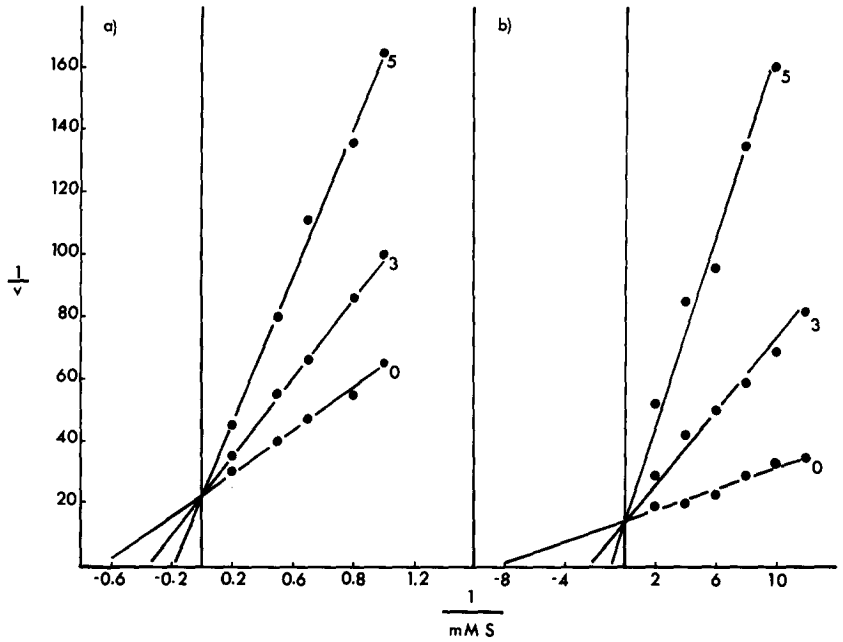


Fig. 1. Competitive inhibition of CPA by DHAP. The mM concentrations of inhibitor are indicated on the figure (v represents μmoles substrate hydrolyzed per minute). a) Peptidase activity; the HPA concentrations ranged from 1.0 to 5.0 mM (CPA concentration = $2.8 \times 10^{-8}$ M). b) Esterase activity; the HPLA concentrations ranged from 0.08 to 0.50 mM (CPA concentration = $1.4 \times 10^{-9}$ M).

two inhibitors, any contribution of this functional group to the binding of the inhibitors appears to be negligible.

Although α-glycerophosphate was also found to inhibit peptidase activity competitively, the inhibition of esterase activity was not competitive (Table I). Furthermore, DHAP was a somewhat better inhibitor than α-glycerophosphate, especially for esterase activity (Table I). D,L-Glyceraldehyde 3-phosphate (10 mM) was not an inhibitor, which may be attributed to the change in position of the carbonyl function.

The competitive inhibition of both activities, seen only in the presence of DHAP and acetol phosphate, suggested that the keto group contributes substantially to the binding of inhibitor to the enzyme. Schiff base formation

Table I

Inhibition of CPA by Triose Phosphates

| Substrate | Inhibitor[c] | Type of Inhibition | $K_i$[e] (mM) |
|---|---|---|---|
| HPA[a] | DHAP | Competitive | 2.2 |
| " | Acetol phosphate | " | 2.0 |
| " | α-Glycerophosphate | " | 3.5 |
| HPLA[b] | DHAP | " | 0.6 |
| " | Acetol phosphate | " | 0.5 |
| " | α-Glycerophosphate | Not competitive[d] | 2.3[f] |

Inhibition of CPA by Keto Acids

| Substrate | Inhibitor[c] | Type of Inhibition | $K_i$[e] (mM) |
|---|---|---|---|
| HPA[a] | Acetoacetic | Competitive | 73 |
| | Levulinic | —[g] | |
| | α-Ketobutyric | Competitive | 10 |
| | α-Ketoisocaproic | " | 14 |
| | α-Ketoisovalenic | " | 43 |
| | Hydroxypyruvic | " | 4.1 |
| HPLA[b] | Acetoacetic | " | 9.4 |
| | Levulinic | Not competitive[d] | 12[f] |
| | α-Ketobutyric | " | 100[f] |
| | α-Ketoisocaproic | " | 6.9[f] |
| | α-Ketoisovalenic | " | 45[f] |
| | Hydroxypyruvate | None | |

[a] This substrate was studied over a concentration range of 1.0-5.0 mM, from which a $K_m$ of 1.5 mM was determined.

[b] This substrate was studied over a concentration range of 0.08-0.50 mM, from which a $K_m$ of 0.08 mM was determined.

[c] Inhibitor concentrations ranged from 1-100 mM.

[d] Although the plots of $\frac{1}{v}$ versus $\frac{1}{s}$ converge at a point in the upper

left quadrant, no steps were taken to differentiate between mixed and non-competitive inhibition.

[e] These values were calculated according to Spolter et al. (1965).

[f] This value was calculated on the assumption that the inhibition was non-competitive.

[g] This value could not be determined due to an increase in absorbance resulting from an interaction between levulinic acid and a reaction product.

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at the active center could not be demonstrated, since treating mixtures of CPA and DHAP with sodium borohydride (Lai et al., 1967) in the presence and absence of 1.0 mM o-phenanthroline failed to inactivate the enzyme[3], although 0.3 equivalents per mole of $^{14}$C-DHAP was incorporated.

In order to determine the effect of the distance between the keto group and the negative charge, several keto acid analogues of DHAP were studied. The inhibition of CPA by α-keto acids already has been reported (Geratz, 1965), although its nature was not indicated. The inhibition by α-keto-isocaproic acid (as reported by Geratz) indicated that an aromatic group was not necessary to inhibit CPA activity (see also Smith et al., 1951), and also suggested that a structural relationship between keto group and negative charge may be important with respect to binding of the inhibitor to the enzyme (see also Smith, 1949). Although CPA is inhibited by several keto acids (Table I), only acetoacetate behaved in a manner similar to DHAP and acetol phosphate; the inhibition was competitive toward both peptidase and esterase activities. Construction of molecular models confirmed that of the keto acids tested, acetoacetic acid most closely resembled the structure of DHAP (or acetol phosphate). Presumably, the inhibition results from

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[3] In these experiments $CPA_{\alpha}$ (Bargetzi et al, 1963) was employed, since the commercially obtained preparation is irreversibly inactivated by o-phenanthroline.

interaction between the active center of the enzyme and the keto and negatively-charged phosphate groups of DHAP. Size of the inhibitor molecule also is of importance, since fructose 1-phosphate[4/] does not inhibit at all.

The differences in type of inhibition observed with esterase and peptidase activity by the same inhibitor are consistent with the model of Vallee (1968), which postulates the existence of overlapping sites for ester and peptide substrates at the active center of CPA.

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4/ It is essential to convert the dicyclohexylammonium salt to the sodium salt, since the former (10 mM) inhibits esterase activity and enhances peptidase activity (see also Davies et al., 1968).