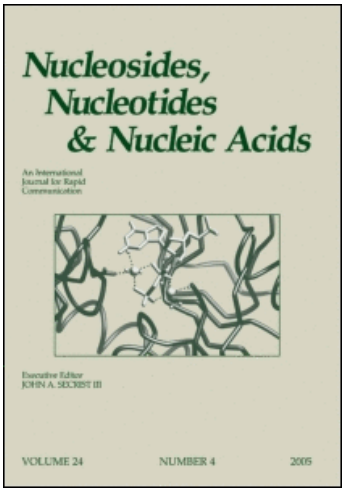

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EFFECTS ON THE ACTIVITY OF THE ENZYME PHOSPHORIBOSYL-AMINOIMIDAZOLE CARBOXYLASE, INVOLVED IN THE BIOSYNTHESIS OF PURINE NUCLEOTIDES DE NOVO BY BI-VALENT METAL COMPLEXES OF THE NATURAL SUBSTRATE 5-AMINO-1-β-D-RIBOFURANOSYLIMIDAZOLE-4-CARBOXYLIC ACID 5'-PHOSPHATE

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Abstract: A series of bi-valent metal complexes of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylic acid and its 5'-phosphate derivative (CAIR), a central intermediate in de novo biosynthesis of purine nucleotides have been synthesised. The nucleotide complexes were found to affect the activity of the enzyme phosphoribosylaminoimidazole carboxylase (EC.4.1.1.21).

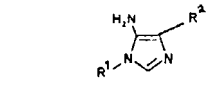
In earlier publications in this series[1], and elsewhere[2], we have reported that transition metal ions inhibit the enzyme (AIR-carboxylase; EC.4.1.1.21) catalysed decarboxylation of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylic acid 5'-phosphate (1a) (CAIR) to 5-amino-1-β-D-ribofuranosylimidazole 5'-phosphate (1b) (AIR) presumably by complex formation. We have been interested in the synthesis of metal complexes of CAIR with a view to better understanding their properties and inhibition of the enzyme AIR-carboxylase. In preliminary studies we isolated[3] the model CAIR copper(II) complex of (1c) in crystalline form and analysis of this compound suggests the stoichiometry $CuL_2,2H_2O$. The structure (2) was assigned to this substance, which is in agreement with the earlier assignments[4,5] of pK_a values to the carboxylate anion (pK_a3.2) and the doubly bonded nitrogen atom N-3(pK_a2.2).

In contrast the nucleoside 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylic acid (1d) prepared as a sodium salt by hydrolysis of the corresponding ethyl ester[6] (1e) with aqueous ethanolic sodium hydroxide (0.5 mol dm^{-3}) for 2h, with copper(II) nitrate gave a green solution, t.l.c. examination of which showed the presence of three green coloured u.v. absorbing compounds, each of which, in addition gave positive tests for ribose and for diazotisable aromatic amine[7]. Two of the salts were isolated by precipitation or by chromatography on silica gel and elution with water : ethanol (1:1). Analysis of the compounds indicated that they had structures of stoichiometry $CuL_2,nCu(OH)_2$ n = 1 and 2 respectively. The third compound (probably from the t.l.c. behaviour n = 0) was not isolated. In a similar manner complexes $ML_2nM(OH)_2$ n = 2 were obtained from the sodium salt with cobalt(II) and nickel(II) nitrates with t.l.c. evidence in each case for compounds corresponding to n = 0 and 1.

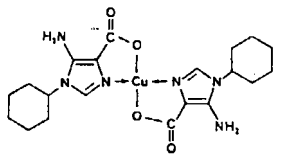
Similar results have been obtained using the nucleotide CAIR (1a). In these experiments we have used a solution of CAIR at pH 7.45 to which was added solutions of copper(II), cobalt(II) and nickel(II) salts (nitrates or chlorides) respectively. The choice of this pH was made on the basis that the non-enzymic decarboxylation is not significantly catalysed by hydrogen ions above pH 7.4. With excess CAIR over

cation concentration the colour changes in the resulting solutions were identical with those produced from the nucleoside and loss of the visible absorption maximum characteristic of each of the free cations was complete suggesting that the complexes are essentially undissociated in aqueous solution.

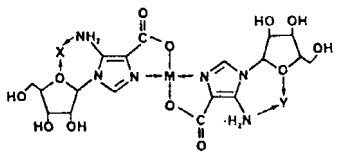
Possible structures for the nucleoside complexes would be (3a), (3b) and (3c) corresponding to n - 0, 1 and 2 respectively. The structures are based on pK_a measurements. Thus, the carboxylate anion has a pK_a 3.2 and the doubly bonded nitrogen 2.2 in contrast to the singly bonded nitrogen pK_a 11.72[5].



(1) a; R^1 = 1-β-D-ribofuranosyl 5'-phosphate, R^2 = CO_2H
b; R^1 = 1-β-D-ribofuranosyl 5'-phosphate, R^2 = H
c; R^1 = cyclohexyl, R^2 = CO_2H
d; R^1 = 1-β-D-ribofuranosyl, R^2 = CO_2H
e; R^1 = 1-β-D-ribofuranosyl, R^2 = CO_2Et



(2)



(3) a; X & Y, absent ; M = Cu, Co, Ni
b; X = $M(OH)_2$, Y, absent
c; X = Y = $M(OH)_2$

The carboxylate group and imidazole nitrogen atom, especially N3, of the nucleotide CAIR (1a) can act as electron pair donors. At the pH where enzyme activity was measured (pH 7.45) neither of these groups will be protonated as their pK_a values are both less than 3.5[5]. Thus under the conditions of the assay, CAIR can act as a chelating ligand. Although no complex formation constants have been measured for CAIR it may be reasonably compared with pyridine-2-carboxylic acid, for which complex formation constants have been measured with many metal ions[8,9]. Measurements show that complexes with up to three pyridine-2-carboxylate ligands may be formed. Accordingly we can write equations (1) - (3) for complex formation by CAIR with a divalent metal ion:

$$M + CAIR \overset{K_1}{\rightleftharpoons} M(CAIR) \qquad (1)$$

$$M(CAIR) + CAIR \overset{K'_2}{\rightleftharpoons} M(CAIR)_2 \qquad (2)$$

$$M(CAIR)_2 + CAIR \overset{K'_3}{\rightleftharpoons} M(CAIR)_3 \qquad (3)$$

Formation constants are quoted for formation according to equations (4) and (5).

$$M + 2\ CAIR \overset{K_2}{\rightleftharpoons} M(CAIR)_2 \qquad (4)$$

$$M + 3\ CAIR \overset{K_3}{\rightleftharpoons} M(CAIR)_3 \qquad (5)$$

The values of K_1, K_2 and K_3 used for the calculations were based on values for pyridine-2-carboxylic acid. As enzyme assays were carried out in a 0.1 mol dm^{-3}

phosphate buffer, complex formation by HPO_4^{2-} and $H_2PO_4^-$ with metal ions must be taken into account, equations (6) and (7).

$$M^{2+} + HPO_4^{2-} \underset{}{\overset{K_P}{\rightleftharpoons}} M.HPO_4 \qquad (6)$$

$$M^{2+} + H_2PO_4^- \overset{K'_P}{\rightleftharpoons} M.H_2PO_4 + \qquad (7)$$

Inspection of values of Kp and K'p in tabulated data[8,9] shows that the formation by $H_2PO_4^-$ can be neglected in comparison to that of HPO_4^{2-}. With Cu^{2+}, HPO_4^{2-} forms a complex with formation constant 100 times greater than that of the complex with $H_2PO_4^-$. At pH 7.45 hydrolysis of metal ions must be considered, equations (8) and (9).

$$M^{2+} + OH^- \overset{K_{OH}}{\rightleftharpoons} [M\,OH]^+ \qquad (8)$$

$$M + 2OH^- \overset{K'_{OH}}{\rightleftharpoons} M(OH)_2 \qquad (9)$$

At the concentrations of metal ions employed, and in the presence of phosphate and CAIR, only Fe^{3+} will precipitate as insoluble $Fe(OH)_3$.

Equilibrium concentrations may be calculated as follows.

Let C_M and C_L be the total concentration of M^{2+} and CAIR added to the system respectively and let [M] and [L] be the concentration of 'free' uncomplexed metal and CAIR at equilibrium. Equations (10) and (11) describe the mass balance.

$$C_M = [M(HPO_4)] + [M(OH)] + [M(OH)_2] + [M(CAIR)] + [M(CAIR)_2] + [M(CAIR)_3] + [M] \qquad (10)$$

$$C_L = [M(CAIR)] + 2[M(CAIR)_2] + 3[M(CAIR)_3] + [L] \qquad (11)$$

Substitution of the appropriate equilibrium constants gives equations (12) and (13).

$$C_M = K_P[M][HPO_4^{2}] + K_{OH}[M][OH^-] + K'_{OH}[M][OH^-]^2 + K_1[M][L] + K_2[M][L]^2 + K_3[M][L]^3 + [M] \qquad (12)$$

$$C_L = K_1[M][L] + 2K_2[M][L]^2 + 3K_3[M][L]^3 + [L] \qquad (13)$$

These may be rearranged to give equations (14) and (15).

$$[M] = \frac{C_M}{K_P[HPO_4^{2-}] + K_{OH}[OH^-] + K'_{OH}[OH]^2 + K_1[L] + K_2L^2 + K_3[L]^3 + 1} \qquad (14)$$

$$\frac{C_L - [L]}{K_1[L] + 2K_2[L]^2 + 3K_3[L]^3} \qquad (15)$$

This gives a 4th order equation in [L], equation (16).

$$K_3[L]^4 + \{K_3(3C_M - C_L) + K_2\}[L]^3 + \{K_2(2C_M - C_L) + K_1\}[L]^2 + \{K_1(C_M - C_L) + 1 + K_P[HPO_4^{2-}] + K_{OH}[OH^-] + K'_{OH}[OH^-]^2\}[L] - \{1 + K_P[HPO_4^{2-}] + K_{OH}[OH^-] + K'_{OH}[OH^-]^2\}C_L = 0. \qquad (16)$$

Table Calculated concentrations of species formed when CAIR is mixed with Copper(II) ions (5×10^{-5} mol dm^{-3}) at pH 7.45.

10^4[CAIR]$_{total}$ /mol dm^{-3}	10^5[CAIR]$_{free}$ /mol dm^{-3}	10^{10}[Cu^{2+}]$_{free}$ /mol dm^{-3}	10^7[Cu(CAIR)] /mol dm^{-3}	10^5[Cu(CAIR)$_2$] /mol dm^{-3}	10^8[Cu(HPO_4)] /mol dm^{-3}
1.00	0.32	75.00	18.00	4.70	71.00
1.10	1.07	7.20	5.60	4.90	6.80
1.20	2.03	2.00	3.00	5.00	1.90
1.30	3.02	0.91	2.00	5.00	0.86
1.40	4.02	0.51	1.50	5.00	0.49
1.50	5.01	0.33	1.20	5.00	0.31
1.60	6.01	0.23	1.00	5.00	0.22
1.70	7.01	0.17	0.88	5.00	0.16
1.80	8.01	0.13	0.77	5.00	0.12
1.90	9.01	0.10	0.68	5.00	0.10
2.00	10.00	0.08	0.61	5.00	0.08

For any known mixture of initial concentrations of CAIR and M of C_L and C_M respectively all concentrations apart from [L] are known, and equation (16) can be solved by Newton's method to give [L]. The concentration of M can then be calculated from equation (15) and consequently this leads to values for concentrations of all other complexes present.

Data for copper(II), nickel(II), zinc(II), manganese(II) and cobalt(II) complexes were obtained, (e.g. for copper(II) in Table). Iron(III) cannot be used since $Fe(OH)_3$ precipitates at pH 7.45. When calcium(II) and magnesium(II) ions are added no CAIR is complexed. Four fifths of the added calcium(II) or magnesium(II) complex with HPO_4^{2-}, the remaining fifth is uncomplexed. Iron(II) is readily oxidised to iron(III) at pH 7.45 by traces of oxygen so no work was carried out with this metal.

The calculations show that the concentration of complexes varied with CAIR concentration ([CAIR]). Also, with magnesium(II), manganese(II) and zinc(II) the metal phosphates, $M(HPO_4)$ were the predominant species. However, in the formation of the copper(II) and cobalt(II) complexes, a constant concentration of $Cu(CAIR)_2$ and $Co(CAIR)_2$ was achieved on addition of excess CAIR. Thus, taking copper as our example (TABLE), the calculations showed that on adding excess CAIR ($[CAIR]_{free}$), over that required for $Cu(CAIR)_2$ complex formation, a direct proportional relationship resulted with increase in non-complexed CAIR ($[CAIR]_{free}$). These results enabled enzyme kinetic studies [for copper(II), cobalt(II) and nickel(II)] to be established in which the concentration of metal complex ([inhibitor]) was kept constant whilst the substrate concentration ($[CAIR]_{free}$) was varied, against initial velocity (Vo). The results for the enzyme kinetics involving $Cu(CAIR)_2$ are given in Fig.1. The concentration on non-complexed copper(II), cobalt(II) or nickel(II) (given as Cu^{2+}_{free} in Table) were considerably (some 10,000 times) less than the respective $M(CAIR)_2$ complexes and were therefore considered to be insignificant with respect to enzyme inhibition.

Plots of initial velocity versus substrate concentration for effects of each of the metal copper(II), cobalt(II) and nickel(II) complexes (M-CAIR) on AIR-carboxylase activity are shown in Figs. 1, 2 and 3: repetition of the experiments revealed consistency in the results. It was also found that the increased substrate concentration produced an enhancement in activity in the experiments involving copper(II) and nickel(II) complexes respectively but not with the cobalt(II) complex.

It appears from our results, therefore, that the metal complexes are modulating changes in enzyme activity, at least in the backwards reaction (CAIR ⟶ AIR) of enzyme EC.4.1.1.21., and that our observations are not merely heavy metal inhibition,

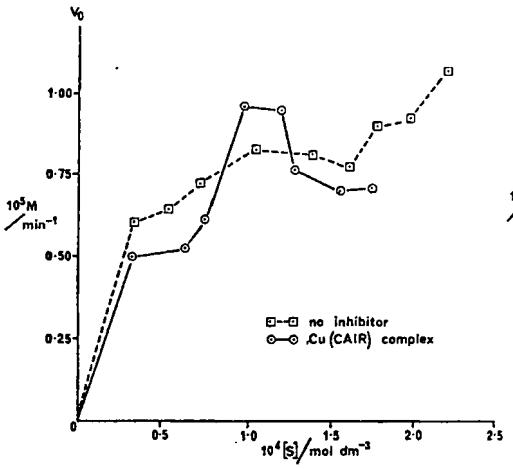


Fig 1 Effect of Cu(CAIR) complexes on AIR-carboxylase activity

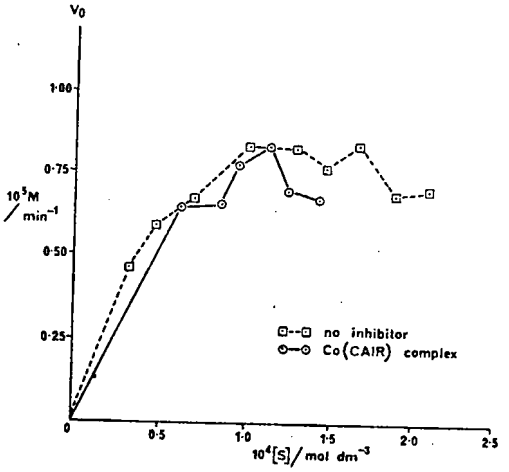


Fig 2 Effect of Co(CAIR) complexes on AIR-carboxylase activity

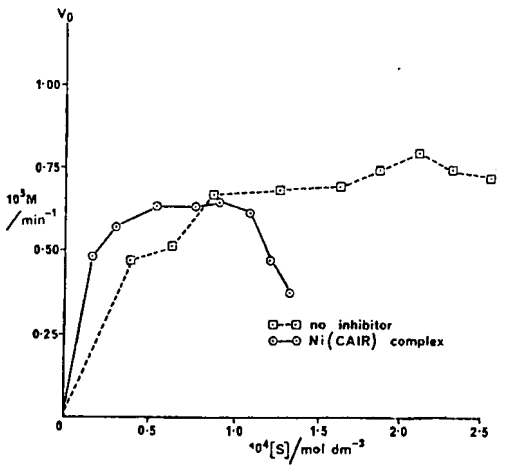


Fig 3 Effect of Ni(CAIR) complexes on AIR carboxylase activity

or simply changes in equilibrium of the enzyme catalyzed reaction brought about by complex formation with the substrate ligand (CAIR).

We have previously[10] reported evidence to suggest that AIR-carboxylase is a branch point allosteric enzyme which involves both the de novo pathway to purine nucleotides and thiamine biosynthesis. Our results could indicate therefore that metal CAIR complexes may modify the enzyme's allosteric properties. Such modifications could have implications in explaining some of the less well understood biological effects of transition metals[11].

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