INTERACTION OF PYRIDOXAL PHOSPHATE ANALOGUES WITH APOENZYMES OF γ-CYSTATHIONASE AND SERINE SULPHHYDRASE

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SUMMARY: Comparative studies have been done of the interactions of some coenzyme analogues with the apoenzymes of γ-cystathionase (EC 4.2.1.15) from rat liver and serine sulphhydratase (EC 4.2.1.22) from chicken liver - pyridoxal-phosphate-dependent enzymes catalysing reactions of H_2S release from L-cystein via α,β-elimination and β-substitution, respectively. It was found that minor modifications (substitutions) in the structure of pyridoxal-5'-phosphate (pyridoxal-P; PLP) result in marked lowering of affinity of the analogues for the apoenzymes. Considerable differences were observed between the various apoenzymes in regard to the mode of their interaction with the pyridoxal-P analogues used.

INTRODUCTION

H_2S release from cysteine can result from action of several pyridoxal-P dependent enzymes catalysing different reactions (1). One of them, γ-cystathionase is able to catalyse not only cleavage of cystathionine and deamination of homoserine but likewise the degradation of L-cystine (and L-cysteine) H_2S, pyruvate and NH_3 by way of an α,β-elimination reaction (2). Serine sulphhydrase fails to catalyse elimination reactions; it is specific for β-substitution reactions of L-cysteine and its S-alkyl-derivatives, of L-serine and some β-substituted analogues of this amino acid (3,4); including the reversible reaction of L-cysteine synthesis from H_2S and serine (5) and cystathionine synthesis from serine (or cysteine) and homocysteine (4). The aim of present study was to evaluate the significance of substituent groups in different positions of the pyridoxal-P molecule for binding with an α,β-eliminating and a β-substititon specific pyridoxal-P-dependent enzyme. To this aim the interactions were studied, in comparison, between the specific proteins of these enzymes and

pyridoxal-P analogues modified at positions 2,3,4,5 and 6 of the pyridine ring.

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MATERIALS AND METHODS

The pyridoxal-P analogues were synthesized as described earlier (6). Activity of γ-cystathionase was estimated from the initial rates of α-ketobutyrate formed on incubation of the native, resp.artificial holoenzyme with L-homoserine, by a spectrophotometric assay emploing lactate dehydrogenase (7). Activity of serine sulphhydrase was assayed, as described previously (3) by determining the amount of H_2S released on incubation of the enzyme with L-cysteine and ß-mercaptoethanol; H_2S is determined spectrophotometrically in the form of colloidß PbS solution. The apoenzyme of γ-cystathionase (apoCT) was obtained on addition D-penicillamine ($5 \cdot 10^{-2}$M) to the enzyme isolated according to reference (8) and mix was kept 30 min at 37°C; to decrease residual activity, $NaBH_4$ (1 mg per mg protein) was added to the cooled solution. After 10 minutes the mixture was passed through a Sephadex G-25 column equilibrated with 0.1M K-phosphate buffer (pH 7.4) to eliminate the PLP-penicillamine complex. The apoenzyme of serine sulphhydrase (apoSSH) was obtained in a similar way from its NH_2OH complex (enzyme oxime), using purified holoenzyme as reported in reference (9). The apoenzyme solutions were stabilized with dithiothreitol ($5 \cdot 10^{-3}$M). The presence of aldimine bound in the apoenzyme-complexes with the coenzyme analogues was evidenced by reactivation of the complexes in the presence on excess of pyridoxal-P after treatment of them with $NaBH_4$ in 0.05 M tris buffer, pH 8.0, as well as by recording of the spectra of absorption and circular dichroism (CD) (6). CD spectra of complexes of apo-CT with pyridoxal-P and its analogues were read in the "Roussel-Jouan II" dichrograph in a 1 cm cuvette at a sensitivity level of 1.10^{-5} units of ectinction per 1 mm. Absorption differential spectra of the serine sulphhydrase complexes were recorded in a 1 cm cuvette "Specord" - (GDR).

RESULTS AND DISCUSSION

The dissociation constants (K_{co}) of pyridoxal-P from cystathionase and serine sulphhydrase were shown to be equal to $4.6 \cdot 10^{-6}$M and $1.0 \cdot 10^{-6}$M, respectively (Table 2). Therefore, bonding of the coenzyme in the first enzyme in somewhat more tight that in the second one. V_{max} values for the complexes of pyridoxal-P with the apoenzymes of serine sulphhydrase and γ-cystathionase are 0.8 and 46.5 M/mg/min., respectively, whereas the K_M values for substrates are fairly similar (Table 2); hence, the rate of the reaction catalysed by serine sulphhydrase is relatively low.

Spectrophotometric investigation showed γ-cystathionase exhibits clearly pronounced optical features: it has a positive CD band with λ_{max} at 427 Hm. Serine sulphhydrase is devoid of a coenzyme-linked CD extrema. Absorption spectra were recorded for serine sulphydrase; a maximum at 430 Hm was found, characteristic for the aldimine complexes of PLP with proteins. Spectrophotometric analysis showed that the positions of absorption maxima of both enzymes were pH independent.

The formyl group of PLP is known to play an essential role in aldimine formation with enzymes and protein bound amino substrates. Modification of the aldehyde group at position 4 of the cofactors results in drastic lowering of the affinity of analogues for apo-CT and apo-SSH. Pyridoxine-P and pyridoxamine-P do not reactive these apoenzymes, neither do they exhibit any inhibitory action in the case of apo-CT (Table I). As shown by the results of experiments with $NaBH_4$, reduction the slight inhibitory effect of pyridoxamine-P on apoSSH cannot be considered as specific.

All the cofactor analogues modified at position 2 are completely devoid of reactivating activity for γ-cystathionase apoenzyme but considerably inhibit binding of pyridoxal-P to the apoenzyme (Table 1); the K_i value for 2-nor-PLP with apo-CT is $4.7 \cdot 10^{-5}$ M. The complex of 2-nor-PLP with apo-CT shows positive CD with λ_{max} = 427 Hm, typical of aldimines of the natural cofactor (fig. 1). In the case of serine sulphhydrase 2-nor-PLP is the only analogue found capable to reactivate the apoenzyme (to 83%) by formation of a complex with properties similar to those of the natural holoenzyme (Table 1, fig.2). The experimental data showed that absence of the 2-CH_3 group in analogue results in approx. 10-fold increase of the K_{co} value, i.e. in a considerable lowering of affinity for the apoenzyme. K_M for substrate is augmented in this case and V_{max} is somewhat lower than with natural holoenzyme (Table 2).

2'-Isopropyl- and 2'-Phenyl-PLP inhibit interaction of the cofactor with both apoenzymes. Weak inhibitory activity of 2'-Phenyl-PLP for PLP binding to apo-CT is apparently caused by the bulky volume of the substituent, limiting its interaction with the protein. The data presented show that the 2'-Methyl group of the cofactor is of importance both for binding to the enzymes proteins and for catalytic activity in γ-cystathionase and serine sulphhydrase.

Table 1

Cofactor	Degree of activation (% of activation by PLP)		Degree of inhibition on natural holoenzyme (%)		K_i (M)	
	CT	SSH	CT	SSH	CT	SSH
Pyridoxine-P	0	0	9	0	-	-
Pyridoxamine-P	0	0	0	13	-	-
3-O-methylpyridoxal-P	0	2	8	15	-	-
3-Deoxypyridoxal-P	0	0	0	33	-	$4,5 \cdot 10^{-4}$
2-nor-Pyridoxal-P	0	83	80	-	$2,8 \cdot 10^{-5}$	-
2'-Isopropylpyridoxal-P	0	0	53	65	$4,7 \cdot 10^{-5}$	$1,0 \cdot 10^{-4}$
2'-Phenylpyridoxal-P	0	0	8	71	-	$0,8 \cdot 10^{-4}$
2-Methylpyridoxal-P	100	0	-	60	-	$1,2 \cdot 10^{-4}$
2-nor-6-Methyl-pyridoxal-P	47	0	-	56	-	$1,5 \cdot 10^{-4}$
Pyridoxal	0	0	8	0	-	-
5'-Methyl-pyridoxal-P	26	70	-	-	-	-
5'-Deoxypyridoxal	0	0	24	30	$9,1 \cdot 10^{-5}$	$1,2 \cdot 10^{-3}$
5-nor-5-β-Carboxy-ethyl-pyridoxal	0	0	100	27	$2,7 \cdot 10^{-6}$	$1,25 \cdot 10^{-3}$
5-nor-5-β-Carboxy-vinil-pyridoxal	0	0	57	40	$4,8 \cdot 10^{-5}$	$4,3 \cdot 10^{-4}$

In the reactivation experiments the apoenzyme of γ-cystathionase (5.10^{-8} - 10^{-7}M) was preincubated 20 min at 25° with an excess of PLP or analogue (100-300 equivalents) in 0.1M K-phosphate buffer (pH 8.1). The apoenzyme of serine sulphhydrase (10^{-6}M) was preincubated 20 min with an excess of analogue (50-200 equivalents) in 0.1 M K-phosphate buffer (pH 7.2) at 37° and tested for enzymatic activity. The degree of inhibition of γ-cystathionase by coenzyme analogues was determined by keeping the apoenzyme (5.10^{-8} - 2.10^{-7}M) at 4° an analogue ($2.5 \cdot 10^{-5}$M) and with PLP (10^{-6}M) in 0.1 M K-phosphate buffer pH 7.2 for 18 hours. For serine sulphhydrase, inhibition assay, mixtures containing the apoenzyme (10^{-6} M) were preincubated at 4° both with an analogue (10^{-4}M) and PLP (2.10^{-5}M) in 0.1 M K-phosphate buffer (pH 7.2) for 18 hours. Thereafter to the samples with either apoenzymes their respective substrates were added and the activity was assayed. K_i values for analogues exhibiting inhibitory action were estimated by the graphic method of Dixon.

Table 2

KINETIC PARAMETERS OF COMPLEXES OF THE APOENZYMES OF γ-CYSTATHIONASE (CT) AND SERINE SULPHHYDRASE (SSH) WITH PYRIDOXAL-P AND ITS ANALOGUES MANIFESTING REACTIVATION ABILITY

Compounds	K_{Co} (M)		K_M for substrate		V_{max} M/mg/min	
	CT	SSH	CT	SSH	CT	SSH
Pyridoxal-P	$4{,}6 \cdot 10^{-6}$	$1{,}0 \cdot 10^{-6}$	$2{,}2 \cdot 10^{-2}$	$1{,}8 \cdot 10^{-2}$	46,5	0,80
6-Methyl-pyridoxal-P	$4{,}2 \cdot 10^{-6}$	-	$2{,}4 \cdot 10^{-2}$	-	50,0	-
2-nor-6-Methyl-pyridoxal-P	$6{,}6 \cdot 10^{-6}$	-	$2{,}3 \cdot 10^{-2}$	-	19,6	-
5'-Methyl-pyridoxal-P	-	$1{,}5 \cdot 10^{-5}$	$1{,}5 \cdot 10^{-2}$	$4{,}0 \cdot 10^{-2}$	9,3	0,44
2-nor-Pyridoxal-P	-	$0{,}9 \cdot 10^{-5}$	-	$3{,}0 \cdot 10^{-2}$	-	0,56

To estimate dissociation constants (K_{Co}) of PLP and its analogues, the apoenzyme solution was kept with varying concentrations of the coenzyme or its analogues at 4° until the equilibrium under the experimental conditions used was attained (9). The concentrations of apo- and holoenzymes in the equilibrium mixture were estimated on the basis of measured enzyme activity. K_{Co} values was calculated as follows:

$$K_{Co} = [\text{apoenzyme}] \times [\text{cofactor}] \; / [\text{holoenzyme}]$$

Values of V_{max} and K_m (for substrate) were estimated from graphs of double reciprocal plots of initial reaction rates at 37° and substrate concentrations randing between $2 \cdot 10^{-3}$ and 10^{-2}M. For calculation of dissociation constants, molecular weights of CT and SSG assumed to be 43.000 (2) and 45.000 (10) per 1 active site, respectively.

6-substituted analogues - 6-Methyl-PLP and 2-nor-6-Methyl-PLP manifest cofactor properties in regard to γ-cystathionase; they reactivate apo-CT to 100 and 47%, respectivery (Table I). Comparison of K_{co}, V_{max} and K_M values with CD extrema obtained for the complexes of apo-CT with PLP.

6-Methyl- or 2-nor-6-Methyl-PLP shows that the introduction of a CH_3 group at position 6 of the analogue does not influence its cofactor activity (Table 2, fig.1). Simultaneous removal of the CH_3 group at position 2 leads to a decrease in affinity of the analogue for apo-CT. 6-Methyl- and 2-nor-6-Methyl-PLP are completely devoid of ability to reactivate the apoenzyme of SSH but act as rather powerful competitive inhibitors of PLP binding by corresponding competition for internal aldimine formation (λ 430 Hm) with the enzyme protein (fig.2).

Experiments with 3-O-methyl- and 3-Deoxy-PLP showed that these compounds neither reactivate apo-CT nor influence interaction between PLP and the protein. These analogues are also practically devoid of coenzyme activity for the apoenzyme of SSH. Although 3-O-methyl and 3-Deoxy-PLP inhibit interaction of apo-SSH with PLP to the extent of 15 and 33%, respectively (Table 1); experiments with NaB^{H}_{4} treatment have indicated that the action of these compounds is not associated with formation of internal aldimine bond. This indicates that the hydroxyl group at position 3 of the cofactor is absolutely essential both for manifestation of catalytic activity and for specific binding to apo-CT and apo-SSH. The 5'-Phosphate group of PLP is known to play the main role in maintaining tight binding of the cofactor to the protein. One can see (Table 1) that all analogues lacking a 5'-phosphate group do not display any ability to reactivate the apoenzymes of -cystathionase and serine sulphhydrase. Only 5'-methyl-PLP possesses cofactor properties (Table 1). Comparison of the binding of 5'-Methyl-PLP with apo-CT and apo-SSH clearly shows that the affinity of this analogue for the enzyme proteins of PLP (Table 2). It was found that 5'-Deoxypyridoxal and analogues containing 5-substituents spatially similar to the acidic moiety of PLP (viz., 5-nor-5-ß-carboxyethyl- and 5-nor-5-ß-carboxyvinylpyridoxal) are powerful competitive inhibitors in case of cystathionase (Table 1). In the case of serine sulphydrase all three 5'-modified analogues only moderately inhibit binding of the coenzyme to the protein. Free pyrido-

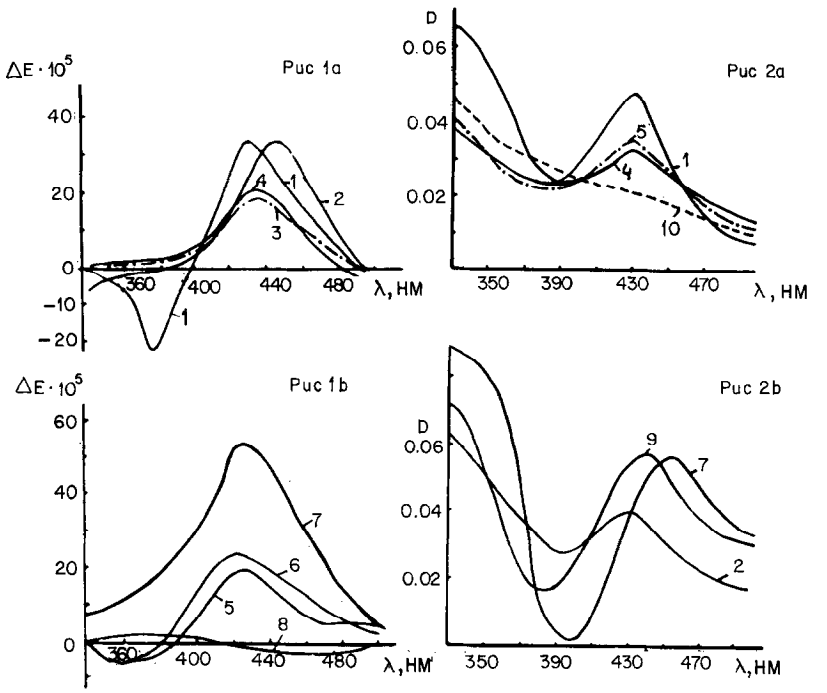

Fig.1 (a and b). CD spectra of apo-CT with PLP analogues protein concentration 4 mg/ml, that of PLP and analogues 30 equivalents. Preincubation was carried out in 0.1 M K-phosphate buffer (pH 7.2) at 4° for 18 hrs.
1 - PLP; 2 - 6-Methyl-PLP; 3 - 2-nor-6-Methyl-PLP; 4-5'-Methyl--PLP; 5 - 2-nor-PLP; 6 - 5-nor-5-β-carboxyethyl-pyridoxal; 7- 5--nor-β-carboxyvinil-pyridoxal; 8-pyridoxal.

Fig.2 (a and b). Absorbance spectra of the complexes between apo-SSH and PLP or its analogues (different spectrum: apoenzyme+ +analogues against analogue). Protein concentration 0.53 mg/ml, that of PLP and analogues 20 equivalents. Preincubation was carried out in 0.15 K-phosphate buffer pH 7.0 at 4° for 18 hrs. Curves 1-8 see legend to fig.1; 9 - 2-Phenyl-PLP; 10 - apoSSH.

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xal fails to reactivate either apoenzymes and practically does not inhibit their interaction with PLP. Spectrophotometric studies showed (fig.1 and 2) that all PLP-analogues modified at position 5 (excepting pyridoxal) display considerable affinity for the active sites of CT and SSH forming aldimine bonds as evidenced by absorption maxima at 415-440 nm.

Comparison of the data concerning interaction of PLP analogues with the apoenzymes of CT and SSH suggest that these enzymes which catalyse H_2S release from L-cysteine <u>via</u> dissimilar reaction mecha-

nism, exhibit different requirements regard to structure of the cofactor. 6-Methyl-, 2-nor-6-Methyl-PLP and 5'-Methyl-PLP display coenzyme activity for CT whereas only 2-nor- and 5'-Methyl-PLP are active in the case of SSH. In the case of γ-cystathionase all analogues alkyl-substituted of position 2 completely lose cofactor activity, but retain high affinity for the protein . In this respect γ-cystathionase is clearly distinct from serine sulphhydrase and other PLP-containing enzymes, such as aspartate transaminase, glutamate decarboxylase, D-serine hydratase etc. (11-13). According to our data (9), serine sulphydrase is similar to the rat liver cystathionine-β-synthetase (EC 4.2.1.21) in the nature of the interactions of the apoenzyme with different PLP analogues. Some other properties of these enzymes are likewise fairly similar (4,); the data presented here strongly suggest that the enzymes mentioned are functionally closely related.

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