The Binding of the Coenzyme Pyridoxal 5'-Phosphate and Analogues of the Substrate–Coenzyme Complex to Tyrosine Decarboxylase

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Phosphopyridoxyl derivatives, which are stable analogues of a substrate–coenzyme complex, are bound at the active site with great affinity. From a comparison of the interaction of a number of such compounds with the apoenzyme the ΔG° values for the binding of the substrate carboxy and phenyl groups and of the coenzyme aldehydic group were determined to be equal to (or more negative than) -3.8 , -8.4 and -12.5 kJ/mol (-0.9 , -1.9 and -3 kcal/mol) respectively; the ΔG° for the binding of the coenzyme phosphate group was shown to be more negative than -20.5 kJ/mol (-4.9 kcal/mol). Two features of the binding process of the coenzyme–substrate analogues to tyrosine decarboxylase have already been found in the case of tyrosine aminotransferase [Borri-Voltattorni, Orlacchio, Giartosio, Conti & Turano (1975) *Eur. J. Biochem.* **53**, 151–160]: (1) in the binding of the substrate to the enzyme a significant fraction of the intrinsic ΔG° appears to be used for some associated endoergonic process; (2) the ΔH° and ΔS° of binding appear to be very sensitive indicators of the correct alignment of the substrate–coenzyme analogues at the active site.

Phosphopyridoxyl-amino acids are compounds structurally similar to one of the proposed coenzyme–substrate complexes that are formed at the active site of pyridoxal 5'-phosphate-dependent enzymes. In a previous paper (Borri-Voltattorni *et al.*, 1975) we described the interaction of these compounds and of the coenzyme with apo-(tyrosine transaminase) from rat liver, showing that they bind at the active site and have a very high affinity for the enzyme; it was also possible to measure the contribution to the binding from different functional groups of the coenzyme and substrate. It was noticed that the ΔH° and ΔS° values of binding are particularly sensitive to the formation of a correct coenzyme–substrate–enzyme complex, and much more so than the ΔG° values of binding. It seemed worthwhile to study the interaction of the same analogues with another vitamin B-6-dependent enzyme with the same substrate specificity, but a different catalytic function. In the present paper we have therefore studied the binding of these compounds (Fig. 1) to tyrosine decarboxylase (L-tyrosine carboxy-lyase) from *Streptococcus faecalis* (EC 4.1.1.25).

Materials and Methods

Tyrosine decarboxylase was extracted from cells of *Streptococcus faecalis* obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); the cells were grown in a vitamin B-6-deficient medium, so that the enzyme was mostly in the apoenzyme form. To remove traces of pyridoxal 5'-phosphate from the cells, the procedure of Maruyama & Coursin (1968) was used; the only modification was to use 10 mM-sodium acetate buffer, pH 5.5, containing 5 mM-EDTA but without mercaptoethanol and tyrosine for the last washings. The cells were then suspended in 5 mM-sodium acetate buffer, pH 5.5; carborundum powder was added, and the suspension was treated in a tissue disintegrator (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) for 1 h at maximum speed. The suspension was then centrifuged at $140\,000g$ (r_{av} 5.9 cm) for 10 min at 4°C in a Beckman Spinco ultracentrifuge; the apoenzyme was present in the clear supernatant, which was used as such. The activity remained unaltered for at least a month if the solution was kept at 2 – 4°C and for at least 3 months if kept at -20°C .

The activity was measured by following the method described by Salvadori & Fasella (1970) for glutamate decarboxylase, which uses a pH-stat for measuring the amount of CO_2 liberated.

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rocal of the initial rate as a function of the reciprocal of the coenzyme concentration, in the absence and in the presence of the inhibitor. The ratio of the slopes obtained in the presence and the absence of the inhibitor is equal to $1 + ([I]/K_i)$, from which K_i can easily be calculated (Orlacchio & Borri-Voltattorni, 1979).

No difference in inhibition was found when the apoenzyme was preincubated with the inhibitor; thus the activities of the apoenzyme treated with $1.25 \mu\text{M}$ -5'-phosphopyridoxyl-L-tyrosine without preincubation or with 5 min or 15 min preincubation differed by less than 3%.

Pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate were commercial products from Merck A.G. (Darmstadt, Germany). The synthesis and the structure of the phosphopyridoxyl derivatives have been described in a previous paper (Borri-Voltattorni *et al.*, 1975). All other reagents were of analytical grade.

Results

All pyridoxal derivatives tested behave as competitive inhibitors towards the coenzyme.

The ΔG^0 values of binding of the inhibitors were calculated from the K_i values at 37°C , and are shown in Table 1.

Effect of temperature on the formation of the complexes

The variation of K_i as a function of temperature between 10 and 37°C was also examined. Van't Hoff plots of the results obtained in this temperature range are linear, within experimental error, indicating that the heat capacity change, ΔC_p , is very low. From these plots the ΔH^0 values for the binding of the inhibitor were calculated (Fig. 2). The estimated error for the ΔG^0 values is $\pm 5\%$ and that for ΔH^0 $\pm 15\%$. The values of ΔG^0 , ΔH^0 and ΔS^0 (calculated from ΔG^0 and ΔH^0) of binding are shown in Table 1.

Discussion

In interpreting the kinetics of tyrosine decarboxylase, the non-enzymic formation of the Schiff base between tyrosine and pyridoxal 5'-phosphate must be taken into account (Orlacchio & Borri-Voltattorni, 1979). A rate equation was therefore proposed, which fitted well the experimental data. This rate equation is also compatible with a direct binding of the non-enzymically formed Schiff base with the enzyme active site. However, it must be pointed out that the occurrence of this binding is not yet proved; the same conclusion was reached by Litwack & Cleland (1968), when a similar binding of the Schiff bases was proposed for tyrosine aminotransferase.

The pyridoxal derivatives that are the subject of the present paper are structurally similar to the

Fig. 2. Van't Hoff plots of 5'-phosphopyridoxyl derivatives ■, Pyridoxal 5'-phosphate-dopa compound; ▲, 5'-phosphopyridoxyl-methylamine; ●, 5'-phosphopyridoxyl-L-phenylalanine; □, 5'-phosphopyridoxyl-L-alanine; △, 5'-phosphopyridoxyl-L-tryptophan; ▼, 5'-phosphopyridoxyl-L-tyrosinol. The values of $\log K_i$ for 5'-phosphopyridoxyl-L-tyrosine (○) refer to the right-hand scale, all the others to the left-hand scale.

postulated substrate-coenzyme intermediate complex, and have been shown to have a high affinity for the apoenzyme. Being stable compounds, they are not transformed when bound at the enzymic active centre and the equilibrium constant of their binding can be measured with precision.

Table 1. *Apparent thermodynamic parameters of the binding of coenzyme and coenzyme derivatives to tyrosine decarboxylase*
Details are given in the text.

Coenzyme and derivatives	ΔG^0 [kJ/mol (kcal/mol)]	ΔH^0 [kJ/mol (kcal/mol)]	ΔS^0 [J/mol per degree (cal/mol per degree)]
5'-Phosphopyridoxyl-methylamine (I)	-29.3 (-7.0)	-50.2 (-12.0)	-67 (-16)
5'-Phosphopyridoxyl-L-alanine (II)	-33.1 (-7.9)	-36.0 (-8.6)	-8 (-2)
5'-Phosphopyridoxyl-tyramine (III)	-37.2 (-8.9)	-54.1 (-12.9)	-54 (-13)
5'-Phosphopyridoxyl-L-tyrosinol (IV)	-38.1 (-9.1)	-58.6 (-14.0)	-67 (-16)
5'-Phosphopyridoxyl-L-phenylalanine (V)	-32.2 (-7.7)	-94.6 (-22.6)	-201 (-48)
5'-Phosphopyridoxyl-L-tryptophan (VI)	-35.1 (-8.4)	-23.8 (-5.7)	+38 (+9)
5'-Phosphopyridoxyl-L-tyrosine (VII)	-37.2 (-8.9)	-123.5 (-29.5)	-276 (-66)
Pyridoxal 5'-phosphate-dopa compound* (VIII)	-28.5 (-6.8)	-11.7 (-2.8)	+54 (+13)
Pyridoxal 5'-phosphate	-46.9 (-11.2)	-31.4 (-7.5)	+50 (+12)
Pyridoxamine 5'-phosphate	-23.0 (-5.5)	-64.0 (-15.3)	-134 (-32)
Pyridoxine 5'-phosphate	-38.1 (-9.1)	-61.1 (-14.6)	-75 (-18)
4'-Deoxypyridoxine 5'-phosphate	-33.9 (-8.1)	-71.1 (-17.0)	-121 (-29)
Pyridoxyl-L-tyrosine	>-17 (>-4)		
Phosphate	>-4 (>-1)		

* Product of reaction between L-3,4-dihydroxyphenylalanine and pyridoxal 5'-phosphate: 3-carboxy-1,2,3,4-tetrahydro-6,7-dihydroxy-1-(3'-hydroxy-5'-methylphosphato-2'-pyrid-4'-yl)isoquinoline.

Binding of the groups on the 4'-position

By comparison of the ΔG^0 values of binding for pyridoxamine 5'-phosphate, pyridoxine 5'-phosphate, 4'-deoxypyridoxine 5'-phosphate and 5'-phosphopyridoxyl-methylamine it can be inferred that a positive charge in the 4'-position has a strong unfavourable effect on the binding to the apoenzyme. Although this effect is also present in tyrosine aminotransferase (Borri-Voltattorni *et al.*, 1975), it is much more pronounced for tyrosine decarboxylase, the value for which, by comparing pyridoxamine 5'-phosphate and 4'-deoxypyridoxine 5'-phosphate, is 10.9 kJ/mol (2.6 kcal/mol). This effect also explains the large difference in binding between pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate, which amounts to 23.9 kJ/mol (5.7 kcal/mol). In tyrosine aminotransferase this difference is only 11.7 kJ/mol (2.8 kcal/mol), but it should be noticed that, whereas in the latter enzyme pyridoxamine 5'-phosphate has a role as a coenzyme, in the decarboxylase it has no role at all. This unfavourable effect of the positive charge may be due to the proximity of a lysine residue, i.e. the one that forms an aldimine bond with pyridoxal 5'-phosphate; it can be argued that in the transaminase, but not in the decarboxylase, the electrostatic repulsion between the positive charges of pyridoxamine 5'-phosphate and lysine is partly compensated by some unknown factor, in order to allow the aminic form of the coenzyme to be bound efficiently.

The energetic contribution of the aldimine bound in the decarboxylase can be estimated by comparison of the binding of pyridoxal 5'-phosphate with that of

pyridoxamine 5'-phosphate or 4'-deoxypyridoxine 5'-phosphate. It amounts to -8.4 to -12.6 kJ/mol (-2 to -3 kcal/mol), and is very similar to that in tyrosine aminotransferase (Borri-Voltattorni *et al.*, 1975).

The higher affinity of 5'-phosphopyridoxyl-methylamine compared with that of pyridoxamine 5'-phosphate is not easily explained; one possibility is that the 4'-methyl group contributes to the binding by interacting with a hydrophobic area of the substrate binding site.

Examination of the 5'-phosphopyridoxyl derivatives with larger substituents in the 4'-position revealed that the compounds with the highest affinity for the enzyme are, besides 5'-phosphopyridoxyl-L-tyrosine, which resembles more closely the real substrate-coenzyme intermediate complex, those that lack the carboxyl function (i.e. 5'-phosphopyridoxyl-tyramine and 5'-phosphopyridoxyl-L-tyrosinol). This is another feature that distinguishes tyrosine decarboxylase from tyrosine aminotransferase, and is likely to be correlated with the different type of reaction catalysed by the former enzyme. Before discussing this point, it is worthwhile to examine the contribution to the binding of the two functional groups that compose the substrate moiety of the 5'-phosphopyridoxyl derivatives, i.e. the carboxy group and the phenyl group. The contribution for the binding of the carboxy group of the substrate can be evaluated from the difference in ΔG^0 between 5'-phosphopyridoxyl-L-tyrosine and 5'-phosphopyridoxyl-tyramine (0 kJ/mol) or from the difference in ΔG^0 between 5'-phosphopyridoxyl-

L-alanine and 5'-phosphopyridoxyl-methylamine [-3.8 kJ/mol (-0.9 kcal/mol)].

The ΔG° of binding of the carboxy group therefore depends on whether the *p*-hydroxyphenyl side chain of the amino acid is present or absent.

A similar situation is found with the hydroxyphenyl group. Its ΔG° of binding can be calculated from the difference in ΔG° between 5'-phosphopyridoxyl-L-tyrosine and 5'-phosphopyridoxyl-L-alanine [-4.4 kJ/mol (-1.0 kcal/mol)] or from the difference in ΔG° between 5'-phosphopyridoxyl-tyramine and 5'-phosphopyridoxyl-methylamine [-7.9 kJ/mol (-1.9 kcal/mol)].

The fact that the binding of either the carboxy or the hydroxyphenyl group is made less favourable as a consequence of the presence of the second group strongly suggests that some distortion or strain is present in the substrate-coenzyme-apoenzyme complex (Jencks & Page, 1972).

The ΔG° of binding of the carboxy group [-3.8 kJ/mol (-0.9 kcal/mol)] or the phenyl group [-7.9 kJ/mol (-1.9 kcal/mol)] when the second group is absent (as in 5'-phosphopyridoxyl-L-alanine or in 5'-phosphopyridoxyl-tyramine) might represent the apparent intrinsic ΔG° of interaction for these groups. As pointed out elsewhere (Jencks & Page, 1972; Borri-Voltattorni *et al.*, 1975), the apparent intrinsic ΔG° of interaction can be more positive than the real intrinsic value owing to the possible occurrence of at least two unfavourable contributions: a decrease in entropy due to freezing of the compounds at the active site on binding of the carboxy or the phenyl group, and some distortion that could be introduced in the compounds and that would increase the apparent ΔG° of interaction.

As shown above, the existence of some distortion is strongly suggested for 5'-phosphopyridoxyl-L-tyrosine, which is the derivative that more closely resembles the real substrate-coenzyme complex.

The carboxy group is mainly responsible for this distortion, or anyway for this decrease in binding, as shown by the fact that the 5'-phosphopyridoxyl derivatives lacking the carboxy group (5'-phosphopyridoxyl-tyramine and 5'-phosphopyridoxyl-tyrosinol) bind just as well as or even better than does 5'-phosphopyridoxyl-L-tyrosine. Since the carboxy group is the one eliminated during the catalytic process, this 'distortion' caused by the carboxy group is likely to be relevant for the efficiency of the catalysis.

The compound formed by condensation of dopa (3,4-dihydroxyphenylalanine) and pyridoxal 5'-phosphate binds very poorly, as is the case with tyrosine aminotransferase (Borri-Voltattorni *et al.*, 1975); this seems to rule out the possibility that in these enzymes a situation in which the aromatic ring of the substrate is perpendicular to that of the coenzyme is favoured for the formation of an inter-

mediate complex (Borri-Voltattorni *et al.*, 1975; Raso & Stollar, 1973).

Binding of the phosphate group

Whereas the energetic contribution for the binding of the phosphate group can be evaluated in the case of tyrosine aminotransferase (Borri-Voltattorni *et al.*, 1975), where it is found to be equal to about -29 kJ/mol (-7 kcal/mol), it cannot be evaluated with tyrosine decarboxylase, owing to the poor binding of the phosphate ion itself and of the non-phosphorylated pyridoxyl derivative of L-tyrosine. As shown in Table 1, the lower limits of the ΔG° of binding of phosphate and pyridoxyl-L-tyrosine are respectively -4.2 and -16.7 kJ/mol (-1 and -4 kcal/mol).

From the difference in the ΔG° of binding of 5'-phosphopyridoxyl-L-tyrosine and pyridoxyl-L-tyrosine, it follows that the intrinsic ΔG° of binding of the phosphate group should be equal to or more negative than about -20.5 kJ/mol (-4.9 kcal/mol).

The fact that the binding of phosphate alone is not detected and that the corresponding ΔG° is higher than -4.2 kJ/mol (-1 kcal/mol) is not surprising, since in the latter case a large loss of entropy is expected, and in the case of the phosphate group already linked to pyridoxyl-L-tyrosine this loss is avoided; this fact has been illustrated for tyrosine aminotransferase (Borri-Voltattorni *et al.*, 1975).

Although no quantitative value can be given, it is clear that for tyrosine decarboxylase also the phosphate group is essential for the binding of the coenzyme and its derivatives.

Influence of temperature on binding

The ΔH° values for the binding for coenzyme derivatives to the apoenzyme are usually large and negative, and overcome the unfavourable ΔS° values, which are also negative (with two exceptions), as shown in Table 1.

These values, being calculated from Van't Hoff plots, are subject to rather large errors; moreover they are composed from various contributions, which may derive from protonations or deprotonations, changes in conformation of the protein and/or the inhibitor and so on. For these reasons they cannot be used as such for a detailed description of the binding process; however, some useful considerations emerge from a comparison of the $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ for different analogues, which in some cases are certainly larger than the experimental error.

The largest (negative) values of ΔH° of binding are those of 5'-phosphopyridoxyl-L-phenylalanine and 5'-phosphopyridoxyl-L-tyrosine [-94.6 to -123.5 kJ/mol (-22.6 to -29.5 kcal/mol)]. A second group of inhibitors, including pyridoxamine 5'-phosphate, 5'-phosphopyridoxyl-L-tyrosinol, 5'-phosphopyridoxyl-tyramine and 5'-phosphopyridoxyl-methyl-

amine, has ΔH° values ranging from -50 to -63 kJ/mol (-12 to -15 kcal/mol). Even smaller (negative) ΔH° values are shown by other inhibitors, such as 5'-phosphopyridoxyl-L-alanine, 5'-phosphopyridoxyl-L-tryptophan and pyridoxal 5'-phosphate-dopa compound [-12 to -38 kJ/mol (-3 to -9 kcal/mol)]. The first group of inhibitors, i.e. the one with the largest negative values, includes the 5'-phosphopyridoxyl derivatives of L-tyrosine and L-phenylalanine, which are the physiological substrates of the enzyme (Mitoma & Udenfriend, 1960).

The other groups include the 5'-phosphopyridoxyl derivative of tryptophan, which is a very poor substrate (Mitoma & Udenfriend, 1960), and other compounds that are not substrates at all. It should be remembered that some of these 5'-phosphopyridoxyl derivatives (i.e. those of tyramine and tyrosinol) bind just as well as does 5'-phosphopyridoxyl-L-tyrosine itself. Considering ΔS° , the 5'-phosphopyridoxyl derivatives of L-tyrosine and L-phenylalanine have the largest (negative) values, -276 and -201 J/mol per degree (-66 and -48 cal/mol per degree) respectively.

This situation resembles the one found with tyrosine aminotransferase, where the 5'-phosphopyridoxyl derivatives of substrates have the largest (positive in this case) enthalpy variations.

In both enzymes the difference among the ΔH° values for the various inhibitors greatly exceed those among the ΔG° values.

Thus, for example, on comparing the binding of two analogues differing only for the carboxy group (5'-phosphopyridoxyl-L-tyrosine and 5'-phosphopyridoxyl-tyramine) there is no difference in ΔG° , but the difference in ΔH° amounts to 69.4 kJ/mol (16.6 kcal/mol).

Therefore the ΔH° and ΔS° of binding of coenzyme analogues to apo-(tyrosine decarboxylase) appear to be very sensitive indicators of the formation of the correct substrate-coenzyme-apoenzyme complex; although the origin of these large ΔH° and ΔS° values is not known, the magnitude of these differences indicate some rather extensive solvation or conformational change of the protein, perhaps not limited to the active site. The fact that this change is so specific for the analogues structurally more similar to the physiological intermediate complex suggests that this event is important for the catalytic process.

Comparison of tyrosine aminotransferase and tyrosine decarboxylase

A comparison of the binding process of coenzyme analogues to two enzymes with different catalytic functions, namely tyrosine aminotransferase and tyrosine decarboxylase, reveals the existence of at

least two common features: (1) an endoergonic process in the binding of the substrate part of the analogues; (2) a large difference in enthalpy and entropy in the formation of the complexes more closely resembling the natural ones.

As discussed above, both these features may be important for the catalytic process, and their occurrence in both enzymes reinforces this view.

A difference in behaviour between the two enzymes, however, can be detected in a more detailed analysis of the binding in the case of decarboxylase. A strain on binding of the substrate moiety can be detected only when both the hydroxyphenyl group and the carboxy group are present; this is shown, for example, by the fact that 5'-phosphopyridoxyl-L-tyrosine does not bind better than 5'-phosphopyridoxyl-tyramine.

With the aminotransferase, instead, a strain appears to be present when either the hydroxyphenyl group alone or the carboxy group alone is present; this is shown by the fact that 5'-phosphopyridoxyl-L-tyramine and 5'-phosphopyridoxyl-L-alanine do not bind better than pyridoxamine 5'-phosphate (Borri-Voltattorni *et al.*, 1975).

This difference, which seems to indicate a different architecture of the substrate-coenzyme-apoenzyme complexes for the two enzymes, is probably related to the different specificity; this is compatible with Dunathan's (1966) hypothesis, according to which the type of reaction catalysed by a vitamin B-6-dependent enzyme depends in the first place on the mutual orientation of the substrate and of the coenzyme aromatic ring.

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