Eur. J. Biochem. 53, 151–160 (1975)

# The Binding of Coenzymes and Analogues of the Substrate-Coenzyme Complex to Tyrosine Aminotransferase

Carla BORRI VOLTATTORNI, Aldo ORLACCHIO, Anna GIARTOSIO, Filippo CONTI, and Carlo TURANO

Institute of Biochemistry, Faculty of Pharmacy, University of Perugia, Institutes of Biochemistry in the Faculties of Medicine and Pharmacy, and Institute of Chemistry, University of Rome

(Received September 3/December 2, 1974)

The interaction of tyrosine apo-aminotransferase with coenzymes and coenzyme derivatives has been studied. The derivatives include analogues of substrate-coenzyme complexes formed in the course of the transamination (pyridoxyl derivatives) and coenzyme analogues (pyridoxine 5′-phosphate, 4′-deoxypyridoxine 5′-phosphate, 1-methyl-pyridoxal 5′-phosphate, 1-methyl-pyridoxamine 5′-phosphate).

From a comparison of the behaviour of the different compounds, the $\Delta G^0$ values for the binding of the coenzyme phosphate group and of the substrate (tyrosine) carboxyl and phenyl groups have been determined as equal to $-7.1$, $-3.0$ and $-2.7$ kcal/mol ($-29.7$, $-12.5$ and $-11.3$ kJ/mol) respectively. In the binding of the substrate to the enzyme a significant fraction of the intrinsic $\Delta G^0$ appears to be used up for some associated endoergonic process.

A comparison of the interaction between the enzyme and pyridoxamine 5′-phosphate and some of its derivatives has shown that a positive charge and a large substituent at position 4′ of the coenzyme have an adverse effect on the binding.

Methylation of position 1 of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate makes their $\Delta G^0$ of binding more positive by 4.0 and 1.8 kcal/mol (16.7 and 7.5 kJ/mol) respectively.

$\Delta H^0$ and $\Delta S^0$ of binding for the different pyridoxyl-derivatives showed a much greater variability than $\Delta G^0$; $\Delta H^0$ and $\Delta S^0$ appear to be very sensitive indicators of the correct alignment of the substrate-coenzyme complex at the active site.

On the basis of these results a hypothesis on the mode binding of the pyridoxyl-amino acids is presented, which is compatible with the scheme of transamination proposed earlier for aspartate transaminase by Braunstein and by Ivanov and Karpeisky.

The study of the interactions between enzymes and compounds structurally similar to intermediates formed in the catalytic process is a powerful tool in characterizing the enzyme-intermediate complexes, otherwise too labile to be isolated, and in investigating the mechanism of enzyme action. The analysis of the binding process is important for the understanding not only of the enzymic specificity, but also of the catalytic role of the enzyme, since a large fraction of the catalytic efficiency of an enzyme can be explained by the binding process itself [1].

Phosphopyridoxyl-amino acids (*P*Pxy-amino acids), which are the products of reduction of Schiff bases formed between pyridoxal-*P* and amino acids, have a structure close to that postulated for an intermediate coenzyme-substrate complex formed in the course of the reactions catalyzed by vitamin-B6-dependent enzymes. They bind efficiently to a variety of B6-dependent apoenzymes [2–4].

The *P*Pxy-amino acids are stable, and are not in general modified when bound at the enzymic active center; therefore, upon reaction with an apoenzyme, a single equilibrium will probably be established, in contrast with substrates or quasi substrates which give origin to multiple equilibria [5] and, consequently, to equilibrium constants which are more difficult to interpret.

*Abbreviations.* *P*Pxy- = pyridoxyl-5′-phosphate-; Pxy = pyridoxyl; pyridoxal-*P* = pyridoxal 5′-phosphate; pyridoxamine-*P* = pyridoxamine 5′-phosphate.

*Enzyme.* Tyrosine aminotransferase or L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5).

The *P*Pxy-amino acids even when modified in the substrate or the coenzyme moiety may still retain a measurable association constant for the related apoenzyme [4]. This allows evaluation of the relative importance of the different functional groups for the binding.

In the present paper we describe the binding of *P*Pxy-derivatives (Fig. 1) to tyrosine aminotransferase, the inducible enzyme from rat liver. This enzyme has been chosen in view of its relatively low affinity for the coenzyme [6]; when the affinity is too high, as in aspartate transaminase from pig heart, the determination of the binding constants for coenzyme or *P*Pxy-derivatives becomes extremely difficult [4].

## MATERIALS AND METHODS

Tyrosine aminotransferase was prepared from rats treated with sodium hydrocortisone hemisuccinate (Flebocortid Richter) following the procedures of Hayashi *et al.* [6] or of Valeriote *et al.* [7] to a specific activity of 100–200 units/mg.

The apoenzyme was prepared according to Hayashi *et al.* [6]. Rates of transamination were determined according to Diamondstone [8] in a reaction mixture containing coenzyme, inhibitor, L-tyrosine, 2-oxoglutarate, in the required amounts, and 0.1 M HCl-triethanolamine buffer pH 7.6; after incubation at the desired temperature for 10 min the reaction was started by addition of apoenzyme. The reaction time was 5 min; the hydroxyphenylpyruvate formed was measured by reaction with potassium hydroxide for 30 min at 37 °C (assay method I).

Different conditions of assay were also used to provide a check for steady-state conditions, as discussed in the next section. In these assays (assay method II) the apoenzyme was incubated in the usual buffer with 9 mM 2-oxoglutarate and pyridoxal-*P* at various concentrations for 5–15 min. The reaction was then started by the addition of L-tyrosine, to a final concentration of 2.8 mM, and the product formed after 5 min was determined as in assay I.

Pxy-L-tyrosine and *P*Pxy-derivatives of L-tyrosine, D-tyrosine, L-phenylalanine, L-tryptophan and L-alanine were synthesised according to Ikawa [9]. *P*Pxy-L-glutamate and Pxy-L-glutamate were synthesised according to Severin *et al.* [3]. *P*Pxy-methylamine, *P*Pxy-tyramine and *P*Pxy-L-tyrosinol were prepared following the general procedure of Ikawa [9]; purification was achieved by means of column chromatography through an Amberlite CG-50 resin ($H^+$ form) in water followed by lyophilization. The analysis of nitrogen gave the following results: *P*Pxy-methylamine, theoretical for $C_9H_{15}O_5N_2P$: 10.69%, found 10.21%; *P*Pxy-tyramine theoretical for $C_{16}H_{21}O_6N_2P$: 7.61%, found 7.65%; *P*Pxy-tyrosinol, theoretical for $C_{17}H_{23}O_7N_2P \cdot H_2O$: 6.73%, found 6.55%. 1-Methyl-pyridoxal 5′-phosphate and 1-methyl-pyridoxamine 5′-phosphate were prepared according to Pocker and Fisher [10] and further purified by column electrophoresis to eliminate contaminating traces of active coenzyme.

6,7-Dihydroxy-1-(3′-hydroxy-5′-methylphosphate-2′-methyl-4′-pyridyl)1,2,3,4-tetrahydro-3-carboxyl isoquinoline (compound IX, see Fig. 1) which is the product of reaction between L-3,4-dihydroxyphenylalanine and pyridoxal-*P* [11] was prepared according to Fellman and Roth [12]. 4′-Deoxypyridoxine 5′-phosphate was prepared according to Peterson and Sober [13].

Pyridoxine 5′-phosphate was prepared by reducing the sodium salt of pyridoxal-*P* with sodium borohydride in aqueous solution; the reaction mixture was then acidified with acetic acid to pH 3 and passed through an Amberlite CG-50 column [13].

The purity of the coenzymes and of *P*Pxy-derivatives was routinely checked by paper chromatography in the Formix mixture [13] and by paper electrophoresis in 0.05 M sodium acetate buffer, pH 5.

Occasionally it appeared from the enzymic activities that a slight contamination of the *P*Pxy-derivatives by pyridoxal-*P* was present, undetected by paper chromatography and electrophoresis and by ultraviolet spectra. In these cases the solutions of the derivatives were treated with well-washed carboxymethylcellulose hydrazide, which effectively removes every trace of coenzyme [14] leaving the *P*Pxy-derivatives in solution.

The coenzymes, the amino acids and all the other chemicals used were commercial products.

## RESULTS

### *Inhibition of Tyrosine Apo-Aminotransferase*

Litwack and Cleland [15] have shown that the steady-state kinetics of tyrosine aminotransferase are compatible with the formation of two binary substrate-enzyme complexes, taking into account a non-enzymic formation of substrate-coenzyme complexes also capable of binding to the apoenzyme.

*P*Pxy-L-tyrosine behaves as a competitive inhibitor towards the coenzyme; the other Pxy and *P*Pxy derivatives tested, as well as phosphate ions, glycerol 2-phosphate and various pyridoxal-*P* derivatives behave in the same way. Only the pyridoxyl derivatives of glutamate display an abnormal behaviour; Pxy-

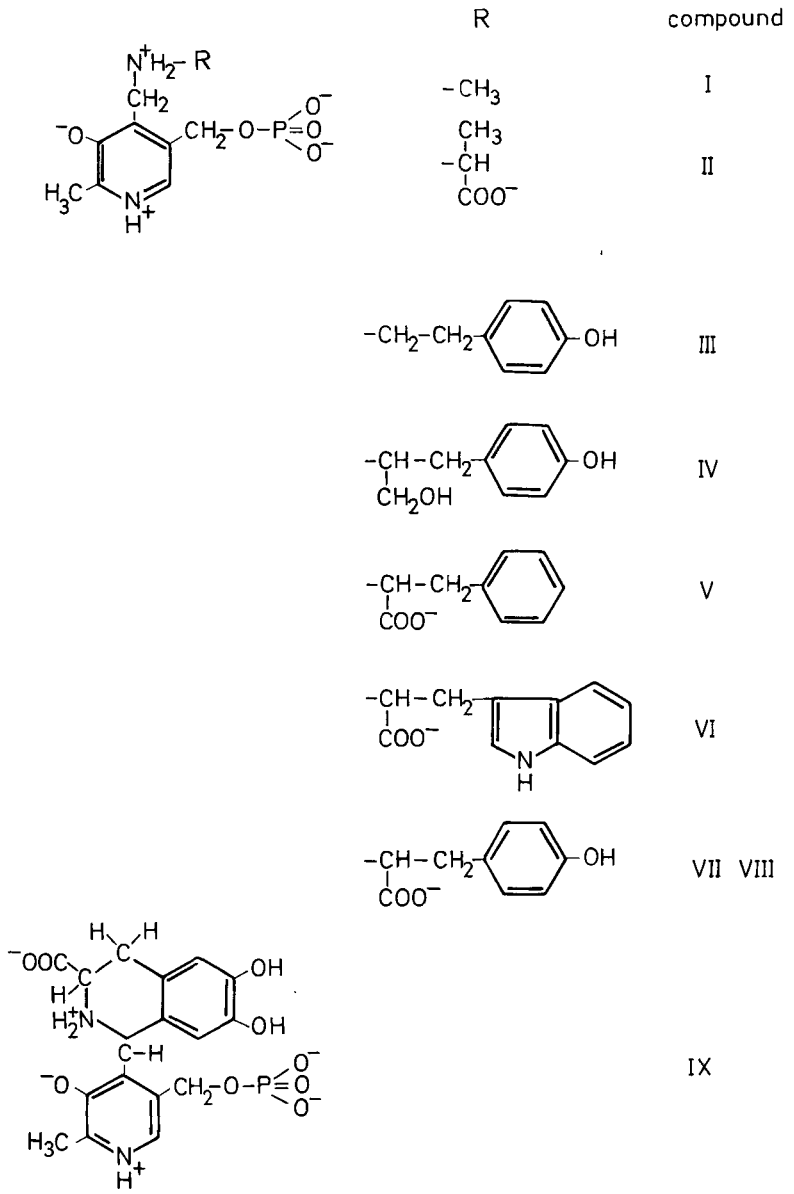


Fig. 1. *Structure of substrate-coenzyme complex analogues*

L-glutamate seems to behave normally, but gives a non-competitive inhibition if it is preincubated with the apoenzyme.

Khomutov *et al.* [16] have described an irreversible binding of *P*Pxy-L-glutamate to aspartate aminotransferase from pig heart. Since other experiments [17] seem to indicate that this may happen also in tyrosine aminotransferase, the binding of the pyridoxyl-derivatives of glutamate will not be discussed here. At present it can only be said that the glutamate site, which is present in nearly all the transaminases, must have some peculiar characteristics, which seem to be common to enzymes from different sources.

There is also some evidence that under certain conditions, such as a long preincubation with the apo-enzyme and high inhibitor concentration, also *P*Pxy-L-tyrosine, which is the derivative with the highest affinity for the enzyme, may become irreversibly bound. However these conditions are far more extreme than the ones we have used to measure the inhibition constant of this compound. The reliability of the value found by us for this constant is also supported by a measurement performed with assay method II, in which the inhibitor is preincubated for 5 min with the apoenzyme before the reaction takes place. An inhibition constant of $3.1 \times 10^{-9}$ M has

been found, in good agreement with the value found with the assay method I ($6.5 \times 10^{-9}$ M). Also the inhibition constant of Pxy-L-tyrosine was measured with both methods, and again similar values were found ($6.6 \times 10^{-4}$ M with assay I, and $2.4 \times 10^{-4}$ M with assay II).

We can assume that for all the inhibitors tested the following reaction takes place:

$$I + E \rightleftharpoons IE$$

(where I is the inhibitor and E is the apoenzyme) characterised by the dissociation constant $K_i$ where

$$K_i = \frac{[I]\,[E]}{[IE]} \qquad (1)$$

Introducing this equilibrium in the mechanism proposed by Litwack and Cleland [15], and using their terminology, we can write the following expression for the initial reaction rate $v$:

$$v = \frac{V}{1 + \frac{1}{C_t}\left(\frac{k_3}{k_1 + k_3}\,\frac{K_{ic}K_a}{A} + \frac{k_3 K_{ac}}{k_1 + k_3} + \frac{k_1}{k_1 + k_3}\,\frac{K_b K_{id}}{B} + \frac{k_1 K_{bd}}{k_1 + k_3}\right)\left(1 + \frac{[I]}{K_i}\right) + \frac{K_a k_3}{A(k_1 + k_3)} + \frac{K_b k_1}{B(k_1 + k_3)}} \qquad (2)$$

where $V$ is the maximal reaction rate, $k_1$ and $k_3$ are the rate constants of the rate-limiting steps, $A$, $B$ and $C_t$ are respectively the concentrations of tyrosine, 2-oxoglutarate and total coenzyme (pyridoxal-*P* + pyridoxamine-*P*), $K_{ic}$ and $K_{id}$ are the dissociation constants of the two holoenzyme forms, *i.e.* pyridoxal-*P* enzyme and pyridoxamine-*P* enzyme, $K_a$ and $K_b$ are the dissociation constants for the holoenzyme-substrate complexes and $K_{ac}$ and $K_{bd}$ are the dissociation constants of the apoenzyme · Schiff-base complexes.

The steady-state kinetics for all the inhibitors tested is compatible with the proposed mechanism; as expected, the inhibitors show non-competitive behaviour against the substrates.

From Eqn (2) it can be seen that the inhibition constants can be determined by plotting $1/v$ against $1/C_t$ in the presence and in the absence of the inhibitor; $K_i$ is easily calculated from the ratio of the slopes of the two lines.

The $K_i$ obtained with the different *P*Pxy-amino acids and the other inhibitors tested are shown in Table 1. 1-Methyl-pyridoxal-*P* and 1-methyl pyridoxamine-*P* bind well to the apoenzyme; the resulting complexes show no appreciable activity (less than 2% of that with the real coenzymes); therefore their dissociation constants have been measured as inhibition constants.

Inspection of Eqn (2) further reveals that the dissociation constants for the coenzymes ($K_{ic}$ and $K_{id}$) can be measured by plotting $1/v$ *versus* $1/A$ (or $1/B$), at two concentrations, $c'$ and $c''$, of pyridoxal-*P* (or pyridoxamine-*P*), in the absence of inhibitor.

From Eqn (2) the following equation can be derived:

$$K_{ic}\ (\text{or } K_{id}) = \frac{c'\,c''\,(r - 1)}{c'' - r\,c'} \qquad (3)$$

where $r$ is the ratio of the slopes of the two straight lines obtained respectively with $c'$ and $c''$.

$K_{ic}$ and $K_{id}$ are true equilibrium constants only if the rate constants for coenzyme binding and dissociation from the protein are large compared to those for the other steps of the enzymic reaction, *i.e.* if the assumptions made by Litwack and Cleland [15] are valid. No proof of this has been offered by these authors; furthermore the validity of the entire steady-state treatment can be questioned, considering that the reaction is started, in their measurements as well as assay method I, by the addition of the apoenzyme to a solution of substrates and coenzyme: a slow formation of holoenzyme could give origin to an apparent saturation curve of the coenzyme even if $K_{ic}$ and $K_{id}$ were close to zero.

If this hypothesis is correct, a preincubation with the coenzyme should saturate the apoenzyme. That this is not the case was shown by experiments performed with the assay method II. The apoenzyme was preincubated with pyridoxal-*P*, in the concentration range 1.5–20 nM, and 2-oxoglutarate, and then the reaction was started by the addition of L-tyrosine. Also in these conditions saturation curves with pyridoxal-*P* were obtained; an increase of the time of preincubation of the apoenzyme with the coenzyme did not affect the results. Thus a preincubation with 2 nM pyridoxal-*P* for 5 and 15 min gave respectively transamination rates of 12.0 and 12.5 nmol of product formed/min; a preincubation with 5 nM pyridoxal-*P* for 5 and 15 min gave transamination rates of 15.3 and 15.0 nmol of product formed/min respectively. A $K_{ic}$ in the $10^{-8}$ M range has been found.

These data show that by addition of pyridoxal-*P* to the apoenzyme a finite, measurable equilibrium is established between the newly formed holoenzyme and the apoenzyme plus the coenzyme.

Therefore the use of the steady-state treatment originally proposed by Litwack and Cleland [15] appears to be legitimate, at least in the experimental conditions of the present work.

Table 1. *Dissociation constants for coenzymes and analogues at 37 °C*
$K_{ic}$ and $K_{id}$ are apparent dissociation constants according to the terminology of Litwack and Cleland [15]

| Coenzymes and analogues | $K_{ic}$ and $K_{id}$ | $K_i$ |
|---|---|---|
| | M | |
| Pyridoxal 5'-phosphate | $5.6 \times 10^{-9}$ | |
| Pyridoxamine 5'-phosphate | $5.3 \times 10^{-7}$ | |
| Pyridoxine 5'-phosphate | | $2.0 \times 10^{-7}$ |
| 4-Deoxypyridoxine 5'-phosphate | | $1.0 \times 10^{-7}$ |
| 1-Methyl-pyridoxal-*P* | | $4.4 \times 10^{-6}$ |
| 1-Methyl-pyridoxamine-*P* | | $9.8 \times 10^{-6}$ |
| *P*Pxy-L-tyrosine (VII) | | $6.5 \times 10^{-9}$ |
| *P*Pxy-D-tyrosine (VIII) | | $1.7 \times 10^{-7}$ |
| *P*Pxy-L-tryptophan (VI) | | $1.2 \times 10^{-7}$ |
| *P*Pxy-L-phenylalanine (V) | | $1.0 \times 10^{-7}$ |
| *P*Pxy-tyramine (III) | | $8.5 \times 10^{-7}$ |
| *P*Pxy-L-alanine (II) | | $5.2 \times 10^{-7}$ |
| *P*Pxy-L-tyrosinol (IV) | | $7.2 \times 10^{-7}$ |
| *P*Pxy-methylamine (I) | | $1.9 \times 10^{-6}$ |
| Compound IX | | $2.7 \times 10^{-6}$ |
| Pxy-L-tyrosine | | $6.6 \times 10^{-4}$ |
| Phosphate | | $4.0 \times 10^{-3}$ |
| Glycerol 2-phosphate | | $1.2 \times 10^{-2}$ |

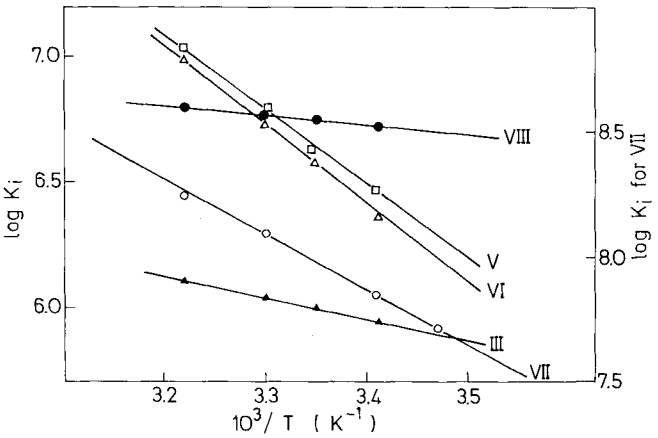


Fig. 2. *Van't Hoff plots of* P*Pxy-derivatives.* (○——○) *P*Pxy-L-tyrosine; (●——●) *P*Pxy-D-tyrosine; (△——△) *P*Pxy-L-tryptophan; (□——□) *P*Pxy-L-phenylalanine; (▲——▲) *P*Pxy-tyramine. The values of log $K_i$ for *P*Pxy-L-tyrosine are shown at the right side of the graph, all the others at the left side

This conclusion is strengthened by the identity of the $K_i$ values found with the two assay methods both for *P*-Pxy-L-tyrosine and Pxy-L-tyrosine, as mentioned above.

### *The Effect of Temperature on the Formation of the Complexes*

In order to determine the enthalpy and entropy changes for the formation of the complexes between the apoenzymes and the inhibitors or the coenzymes, the temperature dependence of the binding reaction has been measured in the temperature range from 10° to 37 °C.

Van't Hoff plots (Fig. 2) have been used to calculate $\Delta H^0$; from this value, and the $\Delta G^0$ derived from the values of $K_i$, $K_{ic}$ or $K_{id}$, $\Delta S^0$ has been calculated. In all cases, in the temperature range examined, the van't Hoff plots show no appreciable curvature, so that heat capacity changes must be negligible.

The estimated error for the $\Delta G^0$ values is ± 5%, and for $\Delta H^0$ ± 10%.

The values found, shown in Tables 1 and 2, are given as the apparent $\Delta H^0$ and $\Delta S^0$ since they may contain heat and entropy of protonation or dissociation accompanying the inhibitor or coenzyme binding to the protein. Despite this limitation, information may be gathered by the $\Delta(\Delta H^0)$ and $\Delta(\Delta S^0)$ values, as they are derived from a homologous series of compounds.

## DISCUSSION

The assumption that the *P*Pxy-amino acids are good models of coenzyme-substrate complexes is supported by a number of data [3,4,18] on the great affinity of these compounds for the active center of pyridoxal-*P*-dependent apoenzymes and also by experiments in which aspartate aminotransferase in the presence of excess substrate has been subjected to mild reduction with sodium borohydride forming *P*Pxy-derivatives of the substrate [19].

The data reported in the present paper confirm this assumption. Some of these compounds show a very high affinity for the enzyme, and the affinity is clearly related to the similarity between the *P*Pxy-derivative and the real complex (*i.e.* the affinity is greatest when the amino acid moiety of the compound is the real amino acid substrate).

Under the conditions used in the present research the complexes formed appear to be readily dissociable, and the relative equilibrium constants are easily determined.

As shown in Table 1 the use of Pxy-derivatives makes it possible to introduce poor substrates or amino acid derivatives which are not substrates or even coenzyme derivatives (*e.g.* non-phosphorylated Pxy-tyrosine) at the enzymic active center, so that the contributions of the different functional groups of the coenzyme-substrate analogue to the binding can be evaluated.

Table 2. *Apparent thermodynamic parameters for the binding of coenzymes and coenzyme derivatives to tyrosine aminotransferase*
$\Delta G^0$ values are those obtained at 37 °C

| Coenzymes and derivatives | $\Delta G^0$ | $\Delta H^0$ | $\Delta S^0$ |
|---|---|---|---|
| | kcal/mol (kJ/mol) | | cal × mol$^{-1}$ × K$^{-1}$ (J × mol$^{-1}$ × K$^{-1}$) |
| Pyridoxal 5′-phosphate | − 11.7 (− 48.9) | + 11.6 (+ 48.5) | + 75 (+ 314) |
| Pyridoxamine 5′-phosphate | − 8.9 (− 37.2) | + 7.5 (+ 31.4) | + 53 (+ 222) |
| Pyridoxine 5′-phosphate | − 9.5 (− 39.7) | + 13.6 (+ 56.9) | + 74 (+ 310) |
| 4′-deoxypyridoxine 5′-phosphate | − 9.9 (− 41.4) | | |
| Phosphopyridoxyl-L-tyrosine (VII) | − 11.6 (− 48.5) | + 9.9 (+ 41.4) | + 69 (+ 289) |
| Phosphopyridoxyl-D-tyrosine (VIII) | − 9.6 (− 40.2) | + 1.8 (+ 7.5) | + 37 (+ 155) |
| Phosphopyridoxyl-L-tryptophan (VI) | − 9.8 (− 41.0) | + 14.9 (+ 62.3) | + 80 (+ 335) |
| Phosphopyridoxyl-L-phenylalanine (V) | − 9.9 (− 41.4) | + 14.8 (+ 61.9) | + 80 (+ 335) |
| Phosphopyridoxyl-tyramine (III) | − 8.6 (− 36.0) | + 3.9 (+ 16.3) | + 40 (+ 167) |
| Phosphopyridoxyl-L-alanine (II) | − 8.9 (− 37.2) | + 5.1 (+ 21.3) | + 45 (+ 188) |
| Phosphopyridoxyl-L-tyrosinol (IV) | − 8.7 (− 36.4) | | |
| Phosphopyridoxyl-methylamine (I) | − 8.1 (− 33.9) | + 7.6 (+ 32.0) | + 51 (+ 213) |
| Compound IX | − 7.9 (− 33.0) | | |
| Pyridoxyl-L-tyrosine | − 4.5 (− 18.8) | + 1.5 (+ 6.3) | + 19 (+ 79) |
| Phosphate | − 3.4 (− 14.2) | + 9.1 (+ 38.1) | + 40 (+ 167) |

It is important to note that in order to get the intrinsic free energy of binding of a single group of a protein ligand (*e.g.* the phosphate group of *P*Pxy-derivatives) it is more convenient to deduce it from a comparison of the binding of the entire ligand and a derivative lacking that group (*e.g.* a comparison between a *P*Pxy-amino acid and a Pxy-amino acid) rather than measuring the binding of the isolated group (phosphate ion alone in our example). In the latter case the observed $\Delta G^0$ of binding will usually appear less negative than the intrinsic $\Delta G^0$ because of the entropy loss necessary to freeze the group at the enzymic site [1] while in the first case this entropy loss will not appear. However, even in the first case, the observed $\Delta G^0$ of binding may not coincide with the intrinsic $\Delta G^0$ since a fraction of the latter can be used to distort the ligand, alter the protein conformation, *etc.* [1]. For this reason the values which will be given in the following discussion as representative of the intrinsic $\Delta G^0$ values are to be considered as higher limits, while the real intrinsic $\Delta G^0$ might well be more negative.

Some recent papers on aminoacyl-tRNA synthetases are good examples of the usefulness of this approach for the study of the interactions at the enzyme active site [20, 21].

### *The Binding of the Phosphate Group*

The free energy contribution to the binding of the phosphate group can be evaluated by the $\Delta(\Delta G^0)$ of binding of *P*Pxy-L-tyrosine and Pxy-L-tyrosine (Table 2) which is −7.1 kcal/mol (−29.7 kJ/mol).

The phosphate ion by itself has a $\Delta G^0$ of binding of −3.4 kcal/mol (−14.2 kJ/mol). The difference between these two values is equal to −3.7 kcal/mol (−15.5 kJ/mol).

It is easily seen from Table 2 that this difference derives mostly from the entropy term, *i.e.* +40 cal × mol$^{-1}$ × K$^{-1}$ (+167 J × mol$^{-1}$ × K$^{-1}$) is the $\Delta S^0$ for phosphate and +50 cal × mol$^{-1}$ × K$^{-1}$ (+209 J × mol$^{-1}$ × K$^{-1}$) is the $\Delta S^0$ difference between *P*Pxy-L-tyrosine and Pxy-L-tyrosine.

Therefore this −3.7 kcal/mol (−15.5 kJ/mol) is probably due to the fact that, whilst the binding of the phosphate ion requires a loss of entropy, the binding of the phosphate already linked to the Pxy-L-tyrosine moiety avoids this loss.

It is interesting that Mulivor and Rappaport [20] in an analysis of the binding of phenylalanine to phenylalanyl-tRNA synthetase, have calculated as an upper limit for this entropy advantage the value of −3.7 kcal/mol (−15.5 kJ/mol), as $T\Delta S^0$ or $\Delta G^0_c$, and were able to use successfully this value throughout their analysis.

The value of −7.1 kcal/mol (−29.7 kJ/mol) should be close to the real intrinsic $\Delta G^0$ of binding of the phosphate group. It is a greater energy contribution than that previously estimated for other transaminases [22] and emphasizes the importance of this group for the binding of pyridoxal 5′-phosphate and its derivatives.

In the discussion up to this point a possible energetic contribution from the displacement of a counter ion from the phosphate-binding site has not been considered. Extrapolation of measurements of *P*Pxy-

L-tyrosine binding at three different ionic strengths gives a $\Delta G^0$ of binding more negative than that indicated in Table 2 by 0.35 kcal/mol (1.5 kJ/mol). Although significant, this small difference in $\Delta G^0$ (an upper limit value) does not alter the general conclusions reached about the binding of the phosphate group.

An attempt to confirm the present results by measuring the binding of other non-phosphorylated Pxy-amino acids failed, owing to their relatively high $K_i$ and their poor solubility.

*The Binding of the Groups in the 4′ Position*

It is well known that pyridoxamine-*P* binds to transaminases less tightly than pyridoxal-*P*, owing to the fact that the latter form of the coenzyme has a covalent bond between the carbon in the 4′ position of the pyridine ring and an amino group of the protein. The data presented in Table 2 give −2.8 kcal/mol (−11.7 kJ/mol) as the difference in $\Delta G^0$ of binding between the two coenzyme forms.

Concerning the binding of pyridoxamine-*P* and three of its derivatives the order of increasing affinity is: *P*Pxy-methylamine, pyridoxamine-*P*, pyridoxine 5′-phosphate, 4′-deoxypyridoxine 5′-phosphate, with $\Delta G^0$ respectively −8.1, −8.9, −9.5 and −9.9 kcal/mol (−33.9, −37.2, −39.7 and 41.4 kJ/mol). It seems therefore that two factors, bulky substituents and a positive charge in the 4′ position, decrease the affinity for the active site.

Unfavourable electrostatic interaction may arise from the presence at the active site of a lysine amino group, which seems a general feature of B 6-dependent enzymes [23].

The effect of bulky substituents might indicate that the group in 4′ position is not freely exposed on the protein surface, but instead has its own site in the interior of the protein molecule. This situation is similar to the one which has been found in aspartate transaminase from pig heart, where the amino group of pyridoxamine-*P* appears to be partially masked towards acylating reagents [24].

The decreased affinity of *P*Pxy-methylamine with respect to pyridoxamine-*P* may also originate from the fact that, while in pyridoxamine-*P* the 4′-amino group is free to rotate, in *P*Pxy-methylamine, if the additional methyl group is in a fixed position after the binding, this rotational freedom will be lost.

In the other analogues examined, the 4′ substituents resemble more closely the true substrate and allow the evaluation of the energy contribution to the binding of the carboxyl group and the *p*-hydroxyphenyl side chain of the amino acid substrate.

The contribution for the binding of the carboxyl group of the substrate can be evaluated from the difference in $\Delta G^0$ between *P*Pxy-L-tyrosine and *P*Pxy-tyramine (−3.0 kcal/mol, −12.5 kJ/mol) or from the difference in $\Delta G^0$ between *P*Pxy-L-alanine and *P*Pxy-methylamine (−0.8 kcal/mol, −3.3 kJ/mol).

The $\Delta G^0$ of binding for the carboxyl 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 phenolic group. Its $\Delta G^0$ of binding can be calculated from the difference in $\Delta G^0$ between *P*Pxy-L-tyrosine and *P*Pxy-alanine (−2.7 kcal/mol, −11.3 kJ/mol) or from difference in $\Delta G^0$ between *P*Pxy-tyramine and *P*Pxy-methylamine (−0.5 kcal/mol, −2.1 kJ/mol).

These data indicate that in the binding of the carboxyl group, or of the hydroxyphenyl group, in the absence of the second group (*i.e.* in *P*Pxy-L-alanine or *P*Pxy-tyramine) the observed $\Delta G^0$ of binding is much less negative than the intrinsic $\Delta G^0$. This can be explained by a loss in entropy (which is indeed present as seen in Table 2 in going from *P*Pxy-methylamine to *P*Pxy-L-alanine or *P*Pxy-tyramine) due to a freezing of the substituent in position 4′ of the coenzyme at the active site: however a $T\Delta S^0$ factor alone is not likely to explain entrirely the low values (−0.8 and −0.5 kcal/mol, −3.3 and −2.0 kJ/mol) of the observed $\Delta(\Delta G^0)$. Additional factors might be the following: (a) a protein conformational change, which could be a local change requiring an energy expenditure for an induced-fit effect, thus explaining the ease of binding of the second group of the substrate; (b) a distortion of the bound *P*Pxy-derivative or an imperfect fit at the active center, which could mean that the enzyme favours the binding of compounds having sp2 carbon atoms at position 4′ of the coenzyme and α of the substrate, as in a hypothetical transition state; (c) a displacement of the coenzyme part of the analogue from the position occupied when the substrate is absent, requiring an energy expenditure. The last hypothesis will be discussed below.

Our data do not permit distinction among the different possibilities; however it appears clearly that in the binding of the substrate part of the analogue a part of the free energy is used to overcome some energetically unfavourable events, which are probably important for the catalysis.

The $\Delta G^0$ values of −3.0 and −2.7 kcal/mol (−12.5 and −11.3 kJ/mol) should be close to the intrinsic $\Delta G^0$ values of binding of respectively the carboxyl and the phenolic group.

In particular −2.7 kcal/mol (−12.5 kJ/mol) is a much more likely value than −0.5 kcal/mol (−2.0 kJ/

mol) for a group which is responsible for specificity as is the hydroxyphenyl group of the substrate.

It is also interesting to compare the binding of *P*Pxy-L-tyrosine with that of compound IX and to relate the binding to their tridimensional structures.

According to an X-ray crystallographic study, the molecule of *P*Pxy-L-tyrosine has an extended conformation, with the planes of the two rings nearly parallel [A. Mangia, M. Nardelli, G. Pelizzi, C. Borri Voltattorni, A. Orlacchio and C. Turano (1975) *J. Chem. Soc. (Lond.)* in press].

Evidence has recently been presented [25] that *P*Pxy-L-3-aminotyrosine, which is similar to our compound, has indeed an extended conformation. It has also been shown that a cyclization product formed from a Schiff base between 3-aminotyrosine and pyridoxal-*P*, which is very similar to our compound IX, has the plane of the tyrosine ring perpendicular to that of the coenzyme ring.

From our data, it appears that *P*Pxy-L-tyrosine binds much better than compound IX (Table 1); the latter binds as well as *P*Pxy-methylamine. This means that neither the carboxyl nor the tetrahydroisoquinoline ring of compound IX can interact with a specific binding site, being oriented in a plane approximately perpendicular to that of the coenzyme [25].

It could be suggested that the real substrate-coenzyme complex bound to tyrosine aminotransferase has an extended conformation, like that of *P*Pxy-L-tyrosine: however this conclusion needs further experimental support, since the energy differences for different conformations of *P*Pxy-L-tyrosine in solution are not likely to be large.

### *The Binding of* N-*Methyl-pyridoxal*-P *and* N-*Methyl-pyridoxamine*-P

The interaction with transaminases of vitamin B6 coenzymes methylated on the pyridine nitrogen has been already investigated: 1-methyl-pyridoxal was found to be unable to bind to pyridoxamine pyruvate aminotransferase [26], while 1-methyl-pyridoxal-*P* and 1-methyl-pyridoxamine-*P* showed a good affinity for aspartate apo-aminotransferase [27,28].

Tyrosine aminotransferase offers the opportunity to measure the dissociation constants for these modified coenzymes and to compare them with those of the real coenzymes (Table 1).

The introduction of a methyl group at nitrogen 1 decreases strongly the affinity of pyridoxal-*P* for the enzyme but affects that of pyridoxamine-*P* much less, so that the two coenzymes, methylated in position 1, bind to the apoenzyme with nearly the same affinity constant. This can be explained by assuming that pyridoxamine-*P* is more free at its binding site than pyridoxal-*P*, which has an additional bond with the protein (*i.e.* the aldimine bond with a protein amino group); Churchich [29] in fact found that pyridoxamine-*P* has a rather high mobility when bound to aspartate apo-transaminase.

Therefore the introduction of a bulky substituent in position 1 which is likely to displace the coenzyme from its normal position at the active site, is bound to affect pyridoxal-*P* more than pyridoxamine-*P*.

### *The Influence of Temperature on Binding*

Temperature affects the dissociation constants of the various inhibitors to a very different extent, as shown in Table 2, where the apparent $\Delta H^0$ and $\Delta S^0$ values are recorded. In view of the fact that these values may in part reflect protonations or deprotonations accompanying the formation of the complexes, no attempt can be made to correlate their sign and magnitude to the different kinds of weak interactions which take place between the protein active site and the coenzyme-substrate analogues.

Also the fact that $\Delta H^0$ is not determined directly, but by means of van't Hoff plots adds some uncertainty to these parameters. However it may worthwhile to examine the $\Delta(\Delta H^0)$ and the $\Delta(\Delta S^0)$ for different analogues. These differences are in some cases quite large, certainly larger that the experimental error, and since there is a certain degree of enthalpy entropy compensation, the corresponding $\Delta(\Delta G^0)$ is much smaller. This seems to indicate that solvation effects are important in these binding processes [30,31] and partly obscure the possible variations in $\Delta H^0$ and $\Delta S^0$ due to strain effects, conformational changes or freezing of the analogues at the active site. A similar situation. *i.e.* small $\Delta(\Delta G^0)$ and large $\Delta(\Delta H^0)$ and $\Delta(\Delta S^0)$, has been noticed in a calorimetric study on aldolase [32].

Considering the *P*Pxy-derivatives, the $\Delta H^0$ of binding decreases in the following order: *P*Pxy-L-tryptophan, *P*Pxy-L-phenylalanine, *P*Pxy-L-tyrosine, *P*Pxy-methylamine, *P*Pxy-L-alanine, *P*Pxy-tyramine, *P*Pxy-D-tyrosine, with a difference of more than 13 kcal/mol (54.4 kJ/mol) between the first and the last term of the series.

The derivatives with the highest $\Delta H^0$ of binding are the ones more closely resembling the true substrate-coenzyme complexes, *i.e.* the ones in which the amino acid part is true substrate (tyrosine, tryptophan, phenylalanine).

A good example of what has been said before about $\Delta(\Delta H^0)$ and $\Delta(\Delta G^0)$ is provided by the comparison of *P*Pxy-L-tyrosine and *P*Pxy-D-tyrosine (D-tyrosine is inactive as a substrate for tyrosine amino-

transferase) which differ only 2 kcal/mol (8.4 kJ/mol) in $\Delta G^0$, but differ by 8.1 kcal/mol (33.9 kJ/mol) in $\Delta H^0$. The relatively small difference in $\Delta G^0$ might be explained by assuming that *P*Pxy-D-tyrosine does indeed bind at the active site, being able to interact by means of its carboxyl and phenol groups with the specific enzymic sites; in fact it binds better than *P*Pxy-tyramine and *P*Pxy-L-alanine, and nearly as well as *P*Pxy-L-phenylalanine. However, as shown by the $\Delta(\Delta H^0)$ values, the binding must have some peculiar characteristics, which results in an imperfect fit at the active center.

It is not possible at present to say what an "imperfect fit" means in terms of molecular structure, and what is the origin of this difference in $\Delta H^0$; it might even reside outside the active site if the protein molecule as a whole is influenced by the events taking place at the active site.

Whatever may be the nature of the event that is reflected in the large variations of $\Delta H^0$ and $\Delta S^0$, this event is not important energetically for the formation of the substrate-coenzyme complex, but somehow must be important for its correct formation, so as to allow catalysis to take place. $\Delta H^0$ and $\Delta S^0$ therefore appear to be very sensitive indicators of this correct formation of the complex, being much more sensitive, for this purpose, than the $\Delta G^0$ values.

Although the interpretation of the $\Delta H^0$ and $\Delta S^0$ values is at present much harder than that of $\Delta G^0$, their sensitivity to the structure of intermediate complexes indicates they should not be overlooked, and that a search for their origin is highly desirable. More data are necessary for this purpose, firstly the pH dependence of the thermodynamic parameters. These measurements, and a more direct measurement of $\Delta H^0$ by calorimetry are at present under way.

### *The Formation of the Substrate-Coenzyme-Apoenzyme Complex*

The results discussed so far may suggest an hypothesis on the mode of formation of the substrate-coenzyme-enzyme complex.

It has been shown that a large substituent in position 4 of the pyridine ring of the coenzyme hinders the binding at the active site of the enzyme. One may wonder how the substrate, which in the real transamination process binds in this position, could fit in the active site at all. *P*Pxy-derivatives having only one part of the substrate moiety, *i.e.* *P*Pxy-L-alanine and *P*Pxy-tyramine, show indeed a very small increase in affinity over *P*Pxy-methylamine. On the other hand a further increase of the size of the substituent, as in *P*Pxy-L-tyrosine, where the entire substrate is present,

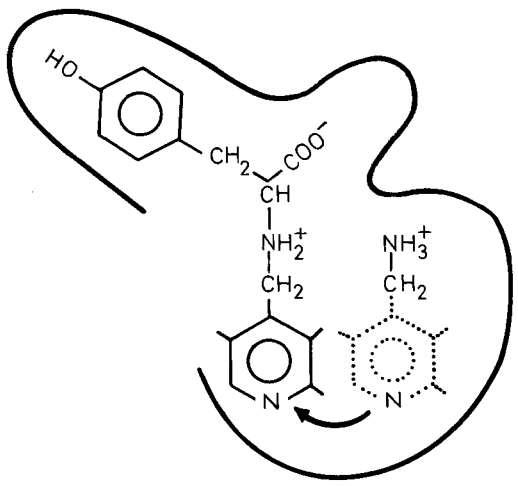


Fig. 3. *Schematic presentation of pyridoxamine-*P *and* P*Pxy-L-tyrosine bound to the apoenzyme*

produces a strong increase in affinity for the active site.

To reconcile these observations we propose that the coenzyme can occupy two different positions at the apoenzyme binding site, as shown schematically in Fig. 3. In the position occupied by the coenzyme in the absence of substrate there is no room for bulky substituents in position 4′ of the pyridine ring. Therefore when *P*Pxy-L-tyrosine, is bound, the coenzyme occupies the second position, as indicated in Fig. 3. If we assume that this second coenzyme position is thermodynamically unfavourable and if we further assume that even the presence of only one group of the substrate (as in *P*Pxy-L-alanine or *P*Pxy-tyramine) causes the coenzyme to bind in the second position, we can explain the small $\Delta(\Delta G^0)$ between *P*Pxy-L-alanine (or *P*Pxy-tyramine) and *P*Pxy-methylamine.

The addition of the second group of the substrate (as in *P*Pxy-L-tyrosine) greatly increases the affinity, since the endoergonic displacement of the coenzyme has already taken place. This change of coenzyme position would of course also occur in the formation of the real substrate-coenzyme complex in the course of the catalytic process. In this case the substrate, located at its specific site, would bind to the coenzyme through the usual aldimine or ketimine bond, while shifting it from its first to its second position, as indicated by the arrow in Fig. 3. This endoergonic shift is compensated by the free energy of substrate-apoenzyme and substrate-coenzyme interactions.

Nothing can be said from our data about the spatial relationship between the two hypothetical coenzyme positions. In this respect it must be pointed out that Fig. 3, in which the third dimension is ignored, can

serve only as a rough scheme of the process; the real displacement of the coenzyme is likely to be rather small.

The possibility must be also considered that the endoergonic process is actually a protein conformational change. The existence of an endoergonic process is in every case an important phenomenon, which can be relevant for the mechanism of catalysis.

It should be noticed that Ivanov and Karpeisky [33] and Braunstein [34] have proposed a mechanism for aspartate transaminase, which requires a movement of the coenzyme upon binding to the substrate; this movement is supposed to be a rotation around the bonds connecting the pyridine ring with substituents in positions 2 and 5. Considerable experimental evidence has been presented by these authors in support of their mechanism, so that the identification of the endoergonic process with this rotation of the coenzyme can be proposed as a working hypothesis.

The technical assistance of Luigi Della Penna and Marco Codini is gratefully acknowledged. The authors are indebted to Dr Gianni Perfetti for preparing the 1-methyl derivatives of the coenzymes. This work was supported by C.N.R.

## REFERENCES

1. Jencks, W. P. & Page, M. I. (1972) *Abstr. 8th Meet. Fed. Eur. Biochem. Soc.* no. 216.
2. Morino, Y. & Snell, E. E. (1967) *J. Biol. Chem. 242*, 5591–5601.
3. Severin, E. S., Gulyaev, N. N., Khurs, E. N. & Khomutov, R. M. (1969) *Biochem. Biophys. Res. Commun. 35*, 318–323.
4. Turano, C., Borri Voltattorni, C., Orlacchio, A. & Bossa, F. (1970) in *Enzymes and Isoenzymes* (D. Shugar, ed.) pp. 123–131, Academic Press, London.
5. Fasella, P. & Turano, C. (1970) *Vitam. Horm. 28*, 157–194.
6. Hayashi, S., Granner, D. K. & Tomkins, G. M. (1967) *J. Biol. Chem. 242*, 3998–4006.
7. Valeriote, F. A., Auricchio, F. & Tomkins, G. M. (1969) *J. Biol. Chem. 244*, 3618–3624.
8. Diamondstone, T. I. (1966) *Anal. Biochem. 16*, 395–401.
9. Ikawa, M. (1967) *Arch. Biochem. Biophys. 118*, 497–500.
10. Pocker, A. & Fischer, E. H. (1969) *Biochemistry, 8*, 5181–5188.
11. Schott, H. F. & Clark, W. G. (1952) *J. Biol. Chem. 196*, 449–462.
12. Fellman, J. H. & Roth, E. S. (1971) *Biochemistry, 10*, 408–414.
13. Peterson, E. A. & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169–175.
14. Mezzasoma, I., Borri Voltattorni, C., Giartosio, A., Orlacchio, A. & Turano, C. (1970) *Boll. Soc. Ital. Biol. Sper. 46*, 913–914.
15. Litwack, G. & Cleland, W. W. (1968) *Biochemistry, 7*, 2072–2073.
16. Khomutov, R. M., Severin, E. S., Khurs, E. N. & Gulyaev, N. N. (1969) *Biochim. Biophys. Acta, 171*, 201–202.
17. Turano, C., Borri Voltattorni, C., Giartosio, A. & Orlacchio, A. (1973) *Abstr. 9th Int. Congr. Biochem.* p. 75, no. 2j22.
18. Snell, E. E. (1969) *Proc. 4th Int. Congr. Pharmacol.*, vol. 5, p. 210, Schwabe & Co Publ., Basel/Stuttgart.
19. Riva, F., Vecchini, P., Turano, C. & Fasella, P. (1964) *Abstr. 6th Int. Congr. Biochem.* sect. IV, no. 140.
20. Mulivor, R. & Rappaport, H. P. (1973) *J. Mol. Biol. 76*, 123–134.
21. Holler, E., Rainey, P., Orme, A., Bennet, E. L. & Calvin, M. (1973) *Biochemistry, 12*, 1150–1159.
22. Braunstein, A. E. (1964) *Vitam. Horm. 22*, 451–484.
23. Fasella, P. (1967) *Annu. Biochem. Rev. 36*, 185–210.
24. Turano, C., Giartosio, A., Riva, F. & Baroncelli, V. (1967) *Biochem. J. 104*, 970–977.
25. Raso, V. & Stollar, B. D. (1973) *J. Am. Chem. Soc. 95*, 1621–1628.
26. Snell, E. E. (1970) *Vitam. Horm. 28*, 265–290.
27. Furbish, F. S., Fonda, M. L. & Metzler, D. E. (1969) *Biochemistry, 8*, 5169–5180.
28. Fonda, M. L. (1971) *J. Biol. Chem. 246*, 2230–2240.
29. Churchich, J. E. (1970) *J. Biol. Chem. 245*, 448–449.
30. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill Book Co.
31. Lumry, R. & Rajender, S. (1970) *Biopolymers, 9*, 1125–1227.
32. Crowder, A. L., Swenson, C. A. & Barker, R. (1973) *Biochemistry, 12*, 2852–2855.
33. Ivanov, V. I. & Karpeisky, M. Ya. (1969) *Adv. Enzymol. 32*, 21–53.
34. Braunstein, A. E. (1970) in *Enzymes and Isoenzymes* (D. Shugar, ed.) pp. 101–122, Academic Press, London.

C. Borri Voltattorni and A. Orlacchio
Istituto di Chimica Biologica, Facoltà di Farmacia, Via del Liceo, I-06100 Perugia, Italy

A. Giartosio
Istituto di Chimica Biologica dell'Università, Città Universitaria, I-00185 Roma, Italy

F. Conti
Istituto Chimico dell'Università, Città Universitaria, I-00185 Roma, Italy

C. Turano
Istituto di Chimica Biologica dell'Università, Facoltà di Farmacia, Città Universitaria, I-00185 Roma, Italy