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Substrate Attachment in Enzymes

THE INTERACTION OF PYRIDOXAL PHOSPHATE WITH AMINO ACIDS

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A number of reactions in which amino acids take part are catalysed by enzymes which require pyridoxal phosphate. The aldehyde group of the latter compound is believed to form an imine (Schiff base) with the amino group of the substrate; the apoenzymes then bring about reactions (decarboxylation, transamination etc.) by inducing electronic rearrangements within the Schiff base. The Schiff bases can also chelate with certain metals. Many of the reactions catalysed by 'B₆ enzymes' can be simulated in chemical systems in the presence of pyridoxal and a suitable metal. The mechanisms of both the reactions *in vitro* and their enzymic counterparts have been discussed in detail by Snell (1958).

The chemical models can be related to enzyme action only if it is known: (a) how readily pyridoxal phosphate can combine with amino acids; (b) whether chelating metals influence this reaction; (c) what is the relation, if any, between the affinity of pyridoxal phosphate and an amino acid under physiological conditions, and the affinity of pyridoxal phosphate enzymes for the amino acid.

The union of both pyridoxal and pyridoxal phosphate with amino acids has been studied by Metzler (1957) and Matsuo (1957), but information is not available in all cases over the pH ranges within which the enzymes act.

Pyridoxal phosphate enzymes may operate under widely varying conditions. The pH optimum of bacterial glutamate decarboxylase is 4.5 (Gale, 1946), but alanine racemase (Wood & Gunsalus, 1951) and serine deaminase (Selim & Greenberg, 1959) show optima at pH 8.1 and 8.3 respectively. The structure of pyridoxal phosphate changes considerably over this range. The phenolic —OH and the pyridine —N= groups have pK values of 4.14 and 8.7 respectively. The zwitterion thus predominates at the pH optima of most pyridoxal phosphate enzymes, unless these pK values are drastically altered by attachment to the apoenzyme. At the acid and alkaline ends of the range, however, a substantial portion of anion or cation will be present. The secondary phosphate group dissociates with a pK of 6.2 (Williams & Neilands, 1954). The pK for dissociation of the Schiff bases

($-\text{NH}^+ \rightarrow -\text{N} + \text{H}^+$) is not known with certainty, but a value of 6.0 has been tentatively proposed (Williams & Neilands, 1954). This agrees with the value given for Schiff bases between amino acids and simple aliphatic aldehydes (Harris, 1925).

The present paper reports the affinities between pyridoxal phosphate and certain amino acids at various pH values. Comparison of these affinities with the Michaelis constants, K_m , of various pyridoxal phosphate enzymes suggests that in some cases the affinity of the enzyme for its amino acid substrate is determined mainly by the readiness with which the latter forms a Schiff base with the coenzyme. Confirmation of this was sought with leucine decarboxylase by comparing the changes in apparent K_m at different pH values with the corresponding changes in the stability of the substrate-coenzyme Schiff base.

EXPERIMENTAL

Materials

Pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts.) had molar extinction coefficients, ϵ : 1050 at λ_{min} 290 $m\mu$, and 4970 at λ_{max} 388 $m\mu$, in 0.2M-phosphate buffer, pH 7.0. The stock (mM) solution, stored at 0° and protected from light, was replaced after 7 days.

Amino acids were recrystallized where necessary to yield a single spot on a paper chromatogram, and to be free from absorption in the range 240–450 $m\mu$. L-Isomers were used except for glycine and DL-valine.

Buffers used were: pH 4.0–5.5, 0.2M-sodium acetate; pH 6.0–8.0, 0.2M-phosphate ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$). In experiments in which metal ions were added for study of chelation, precipitates were formed (except with Mg^{2+} ions) in phosphate buffers at pH 7. In these cases, and for all measurements in the presence of multivalent metals above pH 7, buffers were prepared by equilibrating $\text{N}_2 + \text{CO}_2$ (95:5) with appropriate solutions of sodium hydrogen carbonate. The pH of all buffers was checked by glass-electrode measurements.

Leucine decarboxylase was prepared as the supernatant obtained on centrifuging cells of *Proteus vulgaris* crushed in a Hughes press (Haughton & King, 1961). Glutamate decarboxylase was obtained from *Escherichia coli*, Wilson Type 1, laboratory stock strain no. 106.

Interaction of pyridoxal phosphate with amino acids

The amino acids were dissolved in buffer and the pH was readjusted if necessary by addition of HCl or NaOH. Stock (mM) pyridoxal phosphate was then added to give a final concentration of 0.1 mM. After equilibration for 20 min. the absorption spectrum from 240 to 420 $m\mu$ was read in the 1 cm. cell of a Unicam SP. 500 spectrophotometer. In experiments with metal chelates the chloride of the metal was added to give a final concentration of mM (unless otherwise stated) before addition of the pyridoxal phosphate.

Michaelis constant of leucine decarboxylase

Graded concentrations of leucine in buffers at pH 5, 6 or 7 were exposed to the enzyme in presence of excess (30 μmoles) of pyridoxal phosphate in nitrogen-filled manometers at 37°, and the rate of carbon dioxide output was determined as a measure of enzyme activity (Haughton & King, 1961). Values for K_m were obtained by reciprocal plots (Lineweaver & Burk, 1934).

RESULTS

Interaction of pyridoxal phosphate with amino acids

Addition of an amino acid to pyridoxal phosphate caused characteristic changes in the absorption spectrum (Matsuo, 1957; Blakley, 1955). Fig. 1 shows the effect of adding graded concentrations of arginine at pH 6. Isosbestic points at 309 and 390 $m\mu$ suggest that the spectral changes are due to a single reaction. From this family of curves we may determine the 'stability constant', K_d , of the Schiff base whose formation causes these changes:

$$K_d = \frac{[\text{pyridoxal phosphate}] \times [\text{amino acid}]}{[\text{Schiff base}]}$$

Fig. 1. Absorption spectrum of pyridoxal phosphate (0.1 mM) in phosphate buffer, pH 6: ----, alone; —, in the presence of indicated concentrations of arginine.

This expression ignores the water which enters into the reaction but which is present in large and constant excess. It is, however, more useful here than the true equilibrium constant (Matsuo, 1957), since K_d represents the concentration of amino acid at which half of the total pyridoxal phosphate will be present as Schiff base.

The value for K_d is obtained by measuring the changes in extinction (ΔE at a selected wavelength) on the addition of graded amino acid concentrations and plotting $1/\Delta E$ against $1/[\text{amino acid}]$. K_d is then the reciprocal of the intercept of the plot on the abscissa [cf. the reciprocal-plot method for determination of enzyme-substrate affinities (Lineweaver & Burk, 1934; Dixon, 1953)]. The changes in absorption on complete conversion of pyridoxal phosphate into Schiff base is given by the reciprocal of the intercept on the ordinate axis.

Fig. 2 illustrates this procedure. The reciprocal of increase in extinction at 275 m μ ($1/\Delta E_{275}$) (from Fig. 1) is plotted against $1/[\text{arginine}]$ (275 m μ gives

Table 1. *Stability constant, K_d , and molar extinction coefficient, ϵ , of Schiff base formed by pyridoxal phosphate and valine at pH 4.5–8.0*

Experimental details are given in the text.

pH	K_d (mM)	$\epsilon_{275 \text{ m}\mu}$
4.5	50	5000
5.0	44	5350
5.5	31	5650
6.6	12	5650
7.0	10	6050
8.0	7	7750

Fig. 2. Determination of affinity constant, K_d , for the arginine-pyridoxal phosphate system at pH 6. Ordinate: reciprocal of changes in extinction at 275 m μ resulting from addition of arginine to pyridoxal phosphate.

the maximum difference in E between pyridoxal phosphate and the Schiff base). From the intercept on the abscissa $-1/K_d$ is -110 M^{-1} , and K_d is 9 mM. The ordinate intercept is 2.6; to convert this into molar extinction units we multiply by 10^{-4} (since the pyridoxal phosphate concentration was 0.1 mM). The reciprocal of 2.6×10^{-4} is 3850; this is the increase in ϵ on adding arginine at infinite concentration. By adding ϵ for pyridoxal phosphate itself (1050) we obtain 4900 for ϵ of the pyridoxal phosphate-arginine Schiff base at pH 6.

Table 1 summarizes the results thus obtained for the pyridoxal phosphate-valine system. The stability constant of the Schiff base, K_d , progressively falls (i.e. stability rises) as pH is raised. There is a sharp rise in the extinction of the Schiff base at the higher pH values, possibly due to loss of a proton from the $-\text{NH}^+$ group.

The relation between stability of the Schiff bases and pH is shown in Figs. 3 (a)–(c). In Fig. 3 (a), pyridoxal phosphate is shown to combine more readily with valine than with glycine or alanine. Alanine forms very little Schiff base at the lower pH values. Glutamic acid and arginine [Fig. 3 (b)], however, show a rise in affinity at low pH; both are decarboxylated by bacterial enzymes with acid pH optima. The guanidino group of arginine does not appear to interact with pyridoxal phosphate under our conditions since: (a) the changes in the absorption spectrum of pyridoxal phosphate on adding arginine are very similar to those obtained with valine; (b) no spectral changes are seen when creatine is added to pyridoxal phosphate. Serine and ornithine [Fig. 3 (c)] both show weaker Schiff-base formation; the former shows little variation in affinity over the pH range studied.

Pyridoxal forms much less stable Schiff bases than pyridoxal phosphate. Below pH 7 there was little change in the absorption spectrum of pyridoxal on adding valine, even at 0.1 M concentration. This is in agreement with the findings of Metzler (1957): from his results it can be calculated that conversion of half the pyridoxal into Schiff base would require about 0.2 M-valine at pH 7 and 2 M at pH 5. Combination occurred more readily at higher pH values, however (K_d is 1 mM at pH 9). The requirement in enzymic systems for the phosphate ester rather than pyridoxal itself is probably due to some extent to the relatively weak affinity of the latter for amino acids, though the role of the phosphate group in binding the coenzyme to the apoenzyme is perhaps the more important factor (Snell, 1958).

Chelation with metals

Matsuo (1957; cf. Fasella, Lis, Silliprandi & Baglioni, 1957) described chelation complexes between pyridoxal phosphate and certain metals.

Most of Matsuo's experiments were performed with glycine; the metal ions were added at concentrations equimolar with the pyridoxal phosphate. From a wide range of bi- and ter-valent metals he obtained strongest chelation with Co^{2+} ions; we therefore used this ion for most of our experiments. We confirmed complete chelation of the metal with

the glycine-pyridoxal phosphate Schiff base, when added in equimolar amount. When glycine was replaced by certain other amino acids, however, an excess of Co^{2+} ions was required to drive the reaction to completion. We used mm-Co^{2+} ion and 0.1 mm -pyridoxal phosphate; this gave complete chelation of all the Schiff bases studied.

Added Co^{2+} ions do not alter the absorption spectra of either pyridoxal phosphate or the amino acids under our experimental conditions. Addition of Co^{2+} ions to a solution of pyridoxal phosphate with any of the amino acids reported in Figs. 3 (a)–(c) yielded spectral changes similar to those reported by Matsuo (1957). The $275 \text{ m}\mu$ maximum, characteristic of the Schiff base, is no longer clearly defined; the $410 \text{ m}\mu$ peak moves to $375 \text{ m}\mu$ with some increase in extinction.

To investigate whether chelating metals assist the binding of the amino acids as Schiff bases, pyridoxal phosphate (0.1 mm) and cobaltous chloride (mm) in buffer were placed in the blank cell of the spectrophotometer; the reading cell contained the same reagents plus the amino acid in graded concentrations. The differential extinction, ΔE , at $375 \text{ m}\mu$ was then measured and K_d obtained by plotting $1/\Delta E_{375}$ against $1/[\text{amino acid}]$. The K_d values obtained are shown by the open circles in Figs. 3 (a)–(c). In most cases Co^{2+} ions significantly alter the stability of the Schiff bases only at the higher pH values. With serine, ornithine, arginine and glutamic acid, however, a complication arose, the spectra failing to form isobestic points at the higher pH values. This suggests that changes more complex than the formation of a single amino acid-pyridoxal phosphate-cobalt compound were taking place; the nature of the complexes formed has not been investigated. Results obtained under conditions giving this phenomenon have been excluded from Figs. 3 (a)–(c).

Some experiments were also performed with Mg^{2+} , Ca^{2+} and Mn^{2+} ions in the system valine-pyridoxal phosphate at pH 5 and 7. In no case were the spectral changes observed distinct enough to justify the conclusion that appreciable amounts of the metal ion were being chelated even when present at a concentration of 10 mm (100-fold excess over the pyridoxal phosphate).

*Relation between pH and Michaelis constant
in leucine decarboxylase*

The K_m values for leucine decarboxylase were determined for pH 5, 6 and 7; the results (Fig. 4) are compared in Table 2 with the corresponding K_d values for the Schiff base formed by pyridoxal phosphate and leucine. The two sets of values are of the same order of magnitude and show similar variations with pH.

Fig. 3. Pyridoxal phosphate and amino acid affinities in relation to pH. (a) $\circ$, $\bullet$, Valine; $\square$, $\blacksquare$, alanine; $\blacktriangle$, glycine. (b) $\circ$, $\bullet$, Glutamic acid; $\square$, $\blacksquare$, arginine. (c) $\circ$, $\bullet$, Serine; $\square$, $\blacksquare$, ornithine; $\blacktriangle$, isobutylamine. Open symbols, in presence of mm-Co^{2+} ion; closed symbols, no metals present (except Na^+ and K^+ ions in buffer).

Fig. 4. Michaelis constant of leucine decarboxylase at different pH values. Ordinate: reciprocal of rate of enzymic decarboxylation of leucine. $\circ$, pH 5; $\bullet$, pH 6; $\square$, pH 7.

Table 2. *Stability constant, K_d , of leucine-pyridoxal phosphate Schiff base and Michaelis constant, K_m , of leucine decarboxylase*

Experimental details are given in the text. K_d was determined from the results of Fig. 5 by the method of least squares.

pH	K_d (mm)	K_m (mm)
5.0	85	45
6.0	52	27
7.0	27	16

DISCUSSION

The aldehyde group of the coenzyme is generally assumed to be one point of substrate attachment in pyridoxal phosphate enzymes, even though the aldehyde group may itself be bound to the enzyme when no substrate is present (cf. Shukuya & Schwert, 1960; Sutton & King, 1962). We must assume that there are other sites of attachment to explain the specificity of the enzymes, and such phenomena as the inhibition of leucine decarboxylase by isovaleric acid, which cannot react with pyridoxal phosphate. How important then is the coenzyme site of attachment? One approach to this problem can be made by comparing the Michaelis affinity constant of the enzyme system for an amino acid substrate with the affinity of the isolated coenzyme for the same amino acid, measured under comparable conditions. Two assumptions are made here: (a) The coenzyme attaches itself much more firmly to the apoenzyme than to the amino acid substrate. This is certainly true; in most pyridoxal phosphate enzymes the coenzyme is very firmly anchored [K is about 10^{-5} to 10^{-8} M (Snell, 1958)], and the substrate

Fig. 5. Relation between Michaelis constants, K_m , of enzymes and affinities, K_d , of substrates for pyridoxal phosphate. $\bullet$, No metal ions present (except Na^+ and K^+ ions in buffer); $+$, mm- Co^{2+} ion present. 1, Serine deaminase, K_m at 8.3 (Selim & Greenberg, 1959); 2, leucine decarboxylase, K_m at 5.0 (Table 2); 3, glutamate decarboxylase (*Escherichia coli*), K_m at 4.5 (N. Lucas, H. K. King & S. J. Brown, unpublished work); 4, leucine decarboxylase, K_m at 6.0 (Table 2); 5, conversion of glycine into serine, K_m for glycine at 7.2 (Blakley, 1957); 6, transaminase, K_m for alanine at 7.0 (Darling, 1951); 7, leucine decarboxylase, K_m at 7.0 (Table 2); 8, transaminase K_m for glutamate at 7.0 (Darling, 1951); 9, leucine decarboxylase, K_d for isobutylamine at 6.0 (T. Whalley, personal communication); 10, alanine racemase, K_m at 8.0 (Wood & Gunsalus, 1951); 11, glutamate decarboxylase (brain), K_m at 6.3 (Roberts & Frankel, 1951); 12, ornithine decarboxylase, K_m at 5.2 (Gale, 1946); 13, arginine decarboxylase, K_m at 5.2 (Gale, 1946).

affinities are much lower. (b) The classical K_m value is a measure of enzyme-substrate affinity. This is precisely correct only when the rate of formation of the enzyme-substrate complex greatly exceeds its rate of breakdown. Otherwise K_m will be greater than the true dissociation constant, determination of which is technically difficult in most cases; K_m is therefore commonly taken as a first approximation of the true affinity constant.

In Fig. 5 the K_m values of a number of pyridoxal phosphate enzymes are plotted against the K_d values of the corresponding Schiff bases determined at the same pH—in most cases the pH optimum of the enzyme concerned. The drawn line represents: $K_m = K_d$. Determinations made in the presence or absence of a chelating metal are shown by crosses and circles respectively. Circles 1–10 fall reasonably close to the line (four of them refer to the same enzyme under different conditions). In

these cases there is close correlation between K_m and K_d and we suggest that: (a) the coenzyme may be the binding site which largely determines substrate affinity; (b) the apoenzyme does not greatly alter the stability of the coenzyme-substrate link.

Points 11, 12 and 13 lie well below the line, i.e. K_m is lower (affinity is greater) than binding of the coenzyme alone could account for. All three refer to amino acid decarboxylases; some values in this region have been reported for microbial glutamate decarboxylases (cf. Shukuya & Schwert, 1960). In view of the discrepancies in the literature, we determined K_m for this decarboxylase in cell-free extracts of *Escherichia coli* (in 20 mM-phthalate, pH 4.5) and this value is used for point 4. The anomalous points 11-13 indicate one of the following situations (the data available do not permit decision between them): (a) other binding sites anchor the substrate more firmly than does the coenzyme; (b) the stability of the imine is increased considerably by union with the apoenzyme; (c) K_m diverges widely from the true affinity constant in these cases.

The seven K_d determinations made in the presence of Co^{2+} ions show no correlation with the corresponding K_m values. Metal ions play an important part in the chemical models of pyridoxal catalysis, though Co^{2+} ion—the metal ion most readily chelating with the Schiff bases (Matsuo, 1957)—shows a relatively poor performance (Longenecker & Snell, 1957). The role of metals in the enzyme systems, however, is very uncertain; in few, if any, cases has a clear-cut requirement been demonstrated or even suggested [for discussion, see Snell (1958)].

Leucine decarboxylase

The changes in K_m of this enzyme as the pH is altered closely follow the corresponding changes in K_d . Likewise, the K_i of the inhibitor isobutylamine is close to its K_d value (point 9 of Fig. 5). On the other hand, isovalerate, which cannot be bound through the coenzyme, has an affinity of a different order [K_i 1.8 mM (Haughton & King, 1961)]. It is not easy to explain why the loss of one binding group (amino) should cause an increase in affinity. The side chain clearly plays an important role; otherwise one would expect alanine and glycine to be either substrates or inhibitors for this enzyme.

The reciprocal plots of decarboxylation rate against substrate concentration (Fig. 4) for pH 5, 6 and 7 intercept the ordinate axis at approximately the same point. Rate of enzyme action at substrate saturation is thus almost independent of pH over this range; the decline in rate on the acid side of the pH optimum may thus be attributed to decreased affinity of the coenzyme for the substrate

rather than to lower activity of the apoenzyme itself.

The present findings give some support for the suggestion that the coenzyme site for binding of substrate may play an important part in determining substrate affinity in some of the enzyme systems considered. However, other factors must come into play, otherwise there would be no specificity of binding of substrate. The substrate must 'fit' the enzyme surface; but, once this condition is met, the readiness with which it forms an imine with the coenzyme would seem to be a major factor in determining enzyme-substrate affinity.

SUMMARY

1. The changes which occur in the absorption spectrum of pyridoxal phosphate on addition of graded concentrations of glycine, alanine, valine, leucine, glutamic acid, arginine, ornithine, serine and isobutylamine are reported. Measurements were made over the pH range 4-8.

2. From these results the affinity constants for the formation of Schiff bases between pyridoxal phosphate and the amino acids have been calculated.

3. Comparison is made between these affinity constants and the classical Michaelis constants of pyridoxal phosphate enzymes reacting with the amino acids under investigation.

4. The variation in Michaelis constant with pH had been investigated in the leucine decarboxylase of *Proteus vulgaris*; the values run parallel with those obtained for the leucine-pyridoxal phosphate affinity constant. Maximum rate of enzyme action at substrate saturation remains constant from pH 5 to 7, the apparent pH optimum at pH 6.5-7 being due to the fall in substrate affinity at lower pH values.

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The Effect of Dietary Changes on the Fatty Acid Composition of Normal Human Depot Fat

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Reports of similar diets but dissimilar depot-fat composition of the herbivora (Hilditch, 1956; Dole, James, Webb, Rizack & Sturman, 1959) and of the different diets but similar composition of human and chimpanzee depot fats (Gunstone, 1955; Kingsbury, Paul, Crossley & Morgan, 1961*b*) emphasize the apparent stability of the depot-fat composition and the overriding importance of phylogenetic rather than dietary factors in determining its fatty acid pattern. However, the fatty acid composition of mammalian depot fat can be varied by large and prolonged dietary alterations (Hilditch, 1956), though such extensive temporary changes in the diets of normal people are unusual so that, if the fatty acid composition of human depot fat does vary markedly in relatively short periods of time, this could provide evidence for the importance of endogenous rather than exogenous determinants of fatty acid composition.

In order to examine the effect of dietary changes on the fatty acid composition of the depot fat, various oils and fats rich in polyunsaturated fatty acids were fed to normal volunteers who were on a controlled basal diet. Serial depot-fat samples were taken and their fatty acid patterns examined. In addition, as the plasma fatty acids are readily influenced by diet (Ahrens *et al.* 1959; Kingsbury, 1961), a simultaneous study of the total plasma fatty acids and of the ester class containing the highest percentage of the polyunsaturated fatty acids (the plasma cholesteryl esters) was carried out both as a check on the absorption of the dietary fatty acids and as an interesting metabolic comparison.

EXPERIMENTAL

Subjects. These were eleven male students, aged 19–23 years, with no history of any lipid abnormality, or of liver, pancreatic, kidney or thyroid disease. Their plasma lipids and proteins were normal and their fasting plasmas clear. All were of normal weight and activity, with no previous idiosyncracies of diet, and their diets over the previous year had been similar. Volunteers with low, medium and high normal plasma cholesterol concentrations were chosen.

Diets. The subjects were given the same basal diet for the experimental period of 6 weeks; the various fats were added to this. The experiment was divided into three phases, each of 2 weeks, consisting of an initial control period and then two consecutive supplemental periods.

The control and basal diets represented the student's normal quantity and type of food, assessed from knowledge of the student's usual diet and by questioning. The menus were planned to avoid dislikes and the concentration of any type of food on particular days. For this reason fried foods were not given. The fat was a mixture of animal and dairy fats; no fish foods were included.

The experiment began with the control diet shown in Table 1 but, as the volunteers considered that the fat content was less than they normally consumed and their plasma cholesterol concentrations fell slightly (Kingsbury, Morgan, Aylott & Emmerson, 1961*a*), the diet was changed after the first 6 days, as shown in Table 1. On this diet the plasma cholesterol concentrations rose to their starting values and the students considered the amount of fat to be similar to that normally eaten.

After the second week on the control diet there followed the two consecutive supplemental periods, each of 2 weeks. In the first fortnight group 1 (2 students) was given 10 g. of ethyl arachidonate/day for 11 days, followed by 10 g. of Ethiv 315 (the 'pentaene' fraction from cod-liver oil) daily