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The Effects of pH on the Kinetics of Human Liver Ornithine–Carbamyl Phosphate Transferase*

Philip J. Snodgrass

ABSTRACT: The effects of pH on the kinetics of human liver ornithine–carbamyl phosphate transferase have been examined. As the pH of the assay increases, the concentration of ornithine which gives maximum activity decreases, and inhibition becomes apparent at each pH if an optimal ornithine concentration is exceeded. The K_m for ornithine decreases as the pH is raised from 6 to 8, but the K_m for the zwitterion form ($pK'_2 = 8.69$) remains constant over this range, indicating that the zwitterion is the actual substrate of human ornithine–carbamyl phosphate transferase.

Further, pH does not affect the K_m for carbamyl phosphate. Hence, when either carbamyl phosphate or the zwitterion of ornithine bind, apparently no groups at the active center of the enzyme or of the enzyme–substrate complex ionize. However, the effect of pH on V_{max} implies that an ionizing group in the enzyme–substrate complex with an apparent pK of 6.6 affects the rate of the breakdown of the complex into free enzyme and products.

Carbamyl phosphate–L-ornithine carbamyl transferase (EC 2.1.3.3) from rat or beef liver catalyzes the formation of citrulline by a single displacement reaction in which ornithine and carbamyl phosphate are bound simultaneously to separate sites on the enzyme (Reichard, 1957; Joseph *et al.*, 1963). Although the latter kinetic study indicates that carbamyl phosphate is bound in part through its phosphate moiety, the presence of groups on the enzyme which might play a role in substrate binding or in the catalytic process have not been sought by standard kinetic methods. As a part of a study on enzymes which participate in the urea cycle of human liver, we have therefore examined ornithine carbamyl transferase (OCT)[1] for the effects of pH on K_m and V_{max} of both substrates.

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: OCT, ornithine–carbamyl phosphate transferase.

Experimental Section

Reagents. Metal-free distilled water was used in all preparations. L-Ornithine hydrochloride (Calbiochem) and l-citrulline (Mann), both reported to be homogeneous by paper chromatography, were dissolved in appropriate buffers and stored at 4°. The dilithium salt of the carbamyl phosphate preparations used (Sigma) ranged in purity from 95 to 99%; hence, all calculations were corrected for the actual carbamyl phosphate content. To minimize spontaneous hydrolysis (Allen and Jones, 1964) carbamyl phosphate was weighed and dissolved in buffer just before use. Triethanolamine (Fisher) and maleic acid (Sigma) were employed as buffers without further purification. All buffers were adjusted to the desired pH at 37°, using a Beckman Model G pH meter and a glass electrode.

Measurement of Citrulline. Citrulline was measured by the formation of a yellow complex with diacetyl monoxime and phenazone (Kulhanek and Vojtiskova, 1964; Ceriotti and Gazzaniga, 1966). Just before use, one volume of diacetyl monoxime, 0.4 in 7.5% NaCl, was mixed with two volumes of a solution containing in 1 l. 3.7 g of phenazone (Merck) 2.5 g of ferric ammonium sulfate, 250 ml of concentrated H_2SO_4, and 250 ml of 85% H_3PO_4. The absorptivity of the citrulline–diacetyl monoxime–phenazone complex is 37,800 at its maximum at 464 mμ (P. J. Snodgrass, unpublished observations) using these reagents and conditions.

Measurement of OCT Activity. An assay for human liver OCT was employed which corrects accurately for the nonenzymatic formation of citrulline by carbamyl phosphate and ornithine (Reifer and Kleczkowski, 1960). The reaction was started by adding carbamyl phosphate to a tube containing l-ornithine, buffer, and enzyme, the total volume being 0.6 ml. The blank reaction contained the same reagents, but the enzyme had previously been inactivated by heating at 100° for 10 min. After a 10-min incubation at 37°, the enzyme and blank reactions were stopped with 0.6 ml of 0.5% phosphotungstic acid in 5% trichloracetic acid, the protein precipitates were removed by centrifugation, and 0.5 ml of the clear supernatants was added to 3.0 ml of the diacetyl monoxime–phenazone–acid color reagent. These mixtures were heated at 100° for 15 min and cooled, and the absorbance at 464 mμ was measured in 1-cm quartz cuvets using a Beckman DU spectrophotometer. The initial enzyme activity was calculated as the absorbance of the test reaction minus the absorbance of the blank reaction at 10 min. One unit of OCT activity is defined as the amount of enzyme which produces 1 μmole of citrulline/min at 37°. Specific activity is expressed as units per milligram of nitrogen, determined by a micro-Kjeldahl procedure and nesslerization.

The enzymatic reaction remains linear until 10% or more of either substrate is consumed. The nonenzymatic formation of citrulline is also a linear reaction and accounts for approximately 5–10% of the citrulline produced. The repeatability of the method is 3%, and activity is directly proportional to the amount of enzyme added. Saturating concentrations of carbamyl phosphate are 5–10 mM, but optimal ornithine concentrations vary with pH (*vide infra*). Lineweaver–Burk plots were constructed only from assays in which the rates were known to remain zero order over the 10-min assay period. Because of substrate inhibition at high concentrations of ornithine, V_{max} was obtained by extrapolating to infinite ornithine concentrations over a substrate range where Lineweaver–Burk plots were linear. When V_{max} was measured by varying carbamyl phosphate, ornithine concentrations were used which were optimal at the various pH values being studied. All double-reciprocal plots were calculated by the method of least mean squares. Since pure human OCT is not available, comparisons of V_{max} and K_m at different pH values were made by performing all assays within 1 hr on the same enzyme preparation. The OCT activities were then expressed as units per milliliter of enzyme solution. The buffers which resulted in the highest relative activities in the human OCT assay were triethanolamine from pH 7.1 to 8.5 and maleate from pH 5.8 to 7.1. Triethanolamine was adjusted to the desired pH with HCl, and NaOH was used to adjust the pH of maleic acid. In order to control pH within 0.1 pH unit during assay, 0.20 M buffers were employed in the assays. Varying triethanolamine chloride from 0.05 to 0.30 M reduced OCT activity only by 3% and changing ionic strength with NaCl from 0.2 to 0.5 μ decreased OCT activity by only 5%.

Preparation of Liver Fractions. Human liver was obtained at biopsy or within 4-hr post mortem, drained of blood, pulped in a stainless steel tissue press, and homogenized gently in a glass Potter-Elvehjem instrument as a 10% suspension in 0.25 M sucrose. Nuclei and residue were removed by two centrifugations at 600*g* for 7 min. Mitochondria were sedimented twice at 13,000*g* for 20 min and microsomes were removed by centrifugation at 105,000*g* for 60 min and again for 30 min. The mitochondria contained 72% of the total activity in a 0.25 M sucrose homogenate. The nuclei, microsomes, and supernatant fractions contained 0, 7, and 14% of the total activity, respectively.

OCT, 20-fold-purified, was prepared from human livers obtained at post mortem, frozen within 4 hr, and stored at −70°. The pooled livers were thawed, ground in a tissue grinder, and extracted with two volumes of 0.002 M Tris (pH 8.0), the coarse debris was filtered, and the extract was centrifuged at 10,000*g* for 20 min. The pH was adjusted to 8.0 and a Sharples centrifugation at 50,000*g* was carried out. The supernatant was dialyzed against 0.004 M Tris (pH 8.0) and applied to a DEAE-cellulose column equilibrated with this buffer. OCT did not adhere to DEAE-cellulose at this pH and ionic strength, but came through at the void volume. Owing to removal of extraneous protein, the specific activity of OCT in this column effluent was 86 units/mg of N compared with an activity of 4.3 units/mg of N in the original homogenate.

Results

It became apparent early in these studies that relatively low concentrations of ornithine inhibit human OCT (Figure 1). Moreover, the optimal ornithine con-

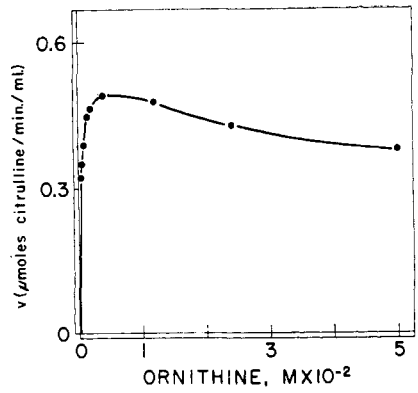

FIGURE 1: Inhibition of human OCT by ornithine. A distilled-water lysate of liver mitochondria was incubated wih various concentrations of ornithine in 0.2 M triethanolamine chloride and 5 mM carbamyl phosphate at pH 7.2 for 10 min at 37°. The optimal ornithine concentration at this pH was 5 mM.

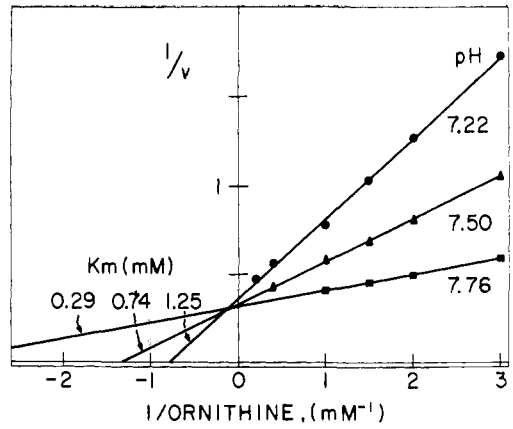

FIGURE 2: The effect of pH on the K_m for ornithine. Human liver OCT was partially purified on DEAE-cellulose columns (see Methods section). Carbamyl phosphate was 5 mM and 0.2 M triethanolamine–chloride was the buffer at 37°. OCT activity is expressed as units per milliliter of enzyme solution. The K_m's calculated for the zwitterion form of ornithine are 0.040 mM at pH 7.22, 0.044 mM at pH 7.50, and 0.030 mM at pH 7.76.

centration varies with pH. At pH 7.2 and 37°, 8 mM ornithine results in maximal activity, but 4 mM is optimal at pH 7.5 and 1.5 mM at pH 8.0. This effect of pH suggests that the zwitterion of ornithine serves as the actual substrate, *i.e.*, that species which has a pK'_2 of 8.69 and a net charge of zero. To test this hypothesis, the effects of pH on both the K_m and V_{max} for ornithine were studied over a pH range of 6–9.

When carbamyl phosphate concentrations are held constant and ornithine concentrations are varied over a range where inhibition is not encountered, Lineweaver–Burk plots are linear at pH 7.22, 7.50, and 7.76 (Figure 2). The K_m values calculated from the slopes and intercepts of these lines decrease progressively as the pH increases. This is true both for OCT purified on DEAE-cellulose columns (Figure 2) and for distilled-water lysates of human mitochondria (Table I) over a pH range of 6–8.

Potentiometric titrations of ornithine in the assay system for OCT indicated a pK'_2 of 8.70 at 37°, almost identical with that of 8.69 determined by Batchelder and Schmidt (1940). The pK'_1 for ornithine was determined by these authors as 1.705 and the pK'_3 as 10.755. Using the measured pK'_2 of 8.70, the K_m's for ornithine (Figure 2) were recalculated from a generalized titration curve to determine the Michaelis constants of the zwitterion species, $K_m(z)$. The variation of K_m with pH is thus abolished, resulting in a $K_m(z)$ at pH 7.22 of 0.040 mM, at pH 7.50 of 0.044 mM, and at pH 7.76 of 0.030 mM. Similarly, with OCT derived from four mitochondrial lysates (Table I), the $K_m(z)$ averages 0.039 ± 0.017 mM, whereas the K_m's calculated on the basis of total ornithine vary from 17.7 to 0.36 mM over the same pH range of 6–8. Twenty successive determinations of K_m between pH 6 and 8 revealed that Lineweaver–Burk plots become nonlinear and exhibit an upward curvature whenever the calculated concentration of the zwitterion form of ornithine exceeds 0.25 mM. Thus the optimal total ornithine concentration is 14 mM at pH 7.0 and 1.5 mM at pH 8.0.

As expected, the K_m for total ornithine continues to decrease from pH 8 to 9 such that in mitochondrial lysate E, the K_m is 0.33 mM at pH 7.95, 0.15 mM at pH 8.55, and 0.13 mM at pH 9.00. These K_m's reduce to $K_m(z)$'s of 0.050, 0.062, and 0.086 mM, respectively. The maximal velocity, however, reaches its peak at pH 8,

TABLE I: K_m's for Total Ornithine and for the Zwitterion of Ornithine at Various pH Values.[a]

OCT Preparation	pH	K_m, Total Ornithine (mM)	K_m, Ornithine Zwitterion (mM)
Mitochondrial lysate A	7.00	2.24	0.044
	7.50	0.50	0.030
	8.00	0.39	0.064
Mitochondrial lysate B	7.10	1.56	0.038
	7.55	0.47	0.031
	8.00	0.36	0.060
Mitochondrial lysate C	6.00	12.2	0.024
	6.50	3.66	0.023
	7.07	1.14	0.026
	7.37	0.78	0.035
	7.95	0.40	0.061
Mitochondrial lysate D	6.00	17.7	0.035
	6.45	3.98	0.023
	7.10	1.06	0.026
	8.00	0.46	0.076

[a] Lineweaver–Burk plots were constructed by varying ornithine at each pH while holding carbamyl constant at 5 mM. K_m's were calculated by the method of least mean squares. The concentrations of the zwitterion form of ornithine at each pH were used to calculate the K_m's for this neutral species.

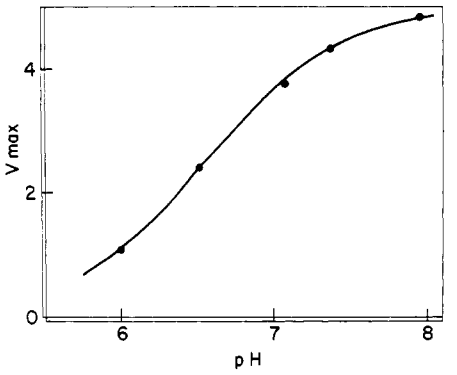

FIGURE 3: The effect of pH on V_{max}. OCT was added as a distilled-water lysate of human liver mitochondria C, the activities being expressed as micromoles of citrulline per minute per milliliter of mitochondrial lysate at 37°. Carbamyl phosphate was 5 mM, and the buffer was 0.2 M triethanolamine chloride–sodium maleate. The solid line is a theoretical curve resulting from the titration of an ionizing group with a pK' of 6.55. The experimental points are V_{max} results from Lineweaver–Burk plots obtained by varying ornithine concentrations at each pH.

and falls off above this level. Although human OCT in buffer is unstable at pH values above 8 (Table II), this decrease in V_{max} is probably intrinsic since the enzyme between pH 8 and 9 is sufficiently stable in the presence of its substrates to allow assays which are linear for 5 min. The Lineweaver–Burk plots for the K_m's at these pH values were linear over ornithine concentrations of 0.2 to 1.0 mM. The human enzyme also becomes unstable below pH 6.0 (Table II).

OCT derived from human mitochondrial lysates B and C was employed to measure the K_m values for carbamyl phosphate over the pH range of 5.8–8.0. Ornithine concentrations were chosen which are optimal at each pH. Over this pH range K_m values vary between 0.29 and 0.98 mM in a random manner (Table III). Using the purified OCT preparation the K_m for carbamyl phosphate is 0.29 mM at pH 7.6–9.0. In four different mitochondrial lysates, the average K_m in 19 experiments at different pH values between 6 and 8 was 0.48 ± 0.16 mM. Substrate inhibition is not encountered when highly purified carbamyl phosphate is employed.

TABLE II: Effect of pH on Stability of Human OCT.[a]

Preincubation Time (min)	Preincubation pH 5.8	6.5	7.6	8.5
	% Control Activity			
0	98	98	100	95
5	90	98	99	38
10	77	92	95	14
15	57	92	95	7
20	30	92	100	4

[a] Liver mitochondria were preincubated in 0.02 M maleate–triethanolamine buffers for the times indicated, and then an aliquot was removed to 0.2 M buffer at pH 7.6 for a 10-min assay of OCT activity at pH 7.6. Control assays were preincubated at pH 7.6.

TABLE III: Effect of pH on the K_m for Carbamyl Phosphate.[a]

OCT Preparation	pH	[Ornithine] (mM)	K_m, Carbamyl Phosphate (mM)
Mitochondrial lysate B	6.43	25	0.82
	6.90	10	0.62
	7.23	5	0.98
	7.65	2.5	0.48
	8.07	1.25	0.73
Mitochondrial lysate C	6.50	25	0.29
	7.07	8	0.52
	7.40	3	0.31
	7.95	1	0.33

[a] Lineweaver–Burk plots were constructed by varying carbamyl phosphate concentrations from 0.1 to 5 mM at each pH. The ornithine concentrations are optimal at each pH.

In contrast to this lack of pH dependence of the K_m (z) for ornithine or of the K_m for carbamyl phosphate, V_{max} is clearly pH dependent. Using mitochondrial lysate C and varying ornithine, the graph of V_{max} *vs.* pH is a sigmoid curve (Figure 3) and closely follows the theoretical curve for the titration of a single group ionizing with a pK' of 6.55. The variation of V_{max} with pH also follows closely to this curve in the cases of mitochondrial lysates B and D (Table I); the pK' values are 6.6 and 6.5. When optimal concentrations of ornithine are employed and V_{max} is obtained by varying carbamyl phosphate concentrations (Table III), sigmoid-shaped curves are also obtained which closely approximate the titration of a single group but with a pK' of 6.8.

Discussion

For human OCT the concentration of ornithine which results in optimal activity decreases as the pH increases, suggesting that the zwitterion form is bound to the enzyme and accepts the carbamyl group to form citrulline. In beef liver OCT, Cohen and Marshall (1962) noted that the K_m for ornithine decreased from 9.8×10^{-4} M at pH 7.58 to a value of 3.7×10^{-4} M at pH 8.25, a change which they suggested was consistent with the increasing concentrations of the form of ornithine which has a pK'_2 of 8.69. They assigned this pK'_2 to the δ-amino group and the pK'_3 of 10.75 to the α-amino group of ornithine. Considering the pK's of lysine or ε-aminocaproic acid (Edsall and Blanchard, 1933) it would be reasonable that the α-amino of ornithine has a pK' of 8.69 while the δ-amino group would have a pK' of 10.75. Since the assignment of these pK's in ornithine has not

been settled, we have chosen to call the species of substrate which ionizes with a pK'_2 of 8.69 the zwitterion.

The hypothesis that the zwitterion is the actual substrate of human OCT would seem to be supported by the Lineweaver–Burk plots for ornithine over the pH range from 6 to 8 (Figure 2; Table I). The K_m's for total ornithine show a 50-fold decrease whereas those for the zwitterion vary only from 0.023 to 0.076 mM. Apparently, the neutral rather than the charged species is the substrate of human OCT, and pH determines the fraction of the total substrate present in the form which is bound by the enzyme. The same pH dependence of the K_m for ornithine was also observed using mitochondrial lysates from rat liver (P. J. Snodgrass, unpublished observations). However, Joseph *et al.* (1963) did not observe inhibition of beef liver OCT up to 50 mM ornithine at pH 8.

The decreased activity which results when a certain total ornithine concentration is exceeded at any pH is apparently due to inhibition by the zwitterion form, since when the zwitterion concentration exceeded 0.25 mM, the rate of the reaction declined. The positively charged species of ornithine does not seem to inhibit, since as the pH decreases and its relative concentration increases, the inhibition diminishes. This phenomenon, wherein one ionic species of a compound serves as the substrate for an enzyme, while serving as an inhibitor when in excess, is reported infrequently in the literature. Frieden and Alberty (1955) considered the dianions of fumarate and malate to be the substrates of fumarase, and the dianion of fumarate was shown to inhibit at high concentrations. They calculated pH-independent K's for the dianion forms, much as has been done here to establish the pH-independent K_m for the ornithine zwitterion.

Since variation of pH from 6 to 8 does not affect the K_m for carbamyl phosphate (Table III) or that of the zwitterion form of ornithine significantly (Table I), apparently no groups at the active center of the enzyme or the enzyme–substrate complex ionize when either substrate binds to human OCT (Dixon, 1953). These conclusions have been drawn from the effect of pH on K_m, not on K_s. The use of K_m is also valid when Briggs–Haldane conditions apply, *i.e.*, when K_m is not a true equilibrium constant but is equal to $(k_{-1} + k_{+2})/k_{+1}$. Both Dixon and Webb (1964) and Laidler (1955) derive equations which indicate that, with rare exceptions and with small errors in the apparent ionization constants, this pH analysis using K_m applies to the Briggs–Haldane case as well as to the equilibrium approach.

The maximal velocity of OCT is, however, clearly affected by pH. The sigmoid-shaped curve of Figure 3 suggests a titration curve for one ionizing group near the active center with a pK' of 6.55. The curves relating V_{max} to pH were also compared with the theoretical curve obtained when two groups ionize with the same intrinsic affinity for hydrogen ions, resulting in a statistical effect such that $k_1 = 4k_2$ (Klotz, 1953). The best fit was still obtained, however, with the titration curve for a single group, yielding a range of pK' values of 6.5–6.8 in five experiments, varying either substrate.

An apparent pK value of 6.5–6.8 has been interpreted as evidence that the imidazolium nitrogen of a histidine residue is being titrated at the active center (Gutfreund, 1955). Since α-amino groups may ionize in this range in proteins (Hill and Davis, 1967), and other amino acid side chains show marked shifts in pK', presumably due to the effects of neighboring groups, the identity of this group in human OCT must await studies of the pure enzyme with site-specific reagents. The present results do serve, however, as the first evidence that an ionizing group at the active center of OCT with a pK' of 6.6 is involved in the catalytic mechanism.

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