pK of the Lysine Amino Group at the Active Site of Acetoacetate Decarboxylase*

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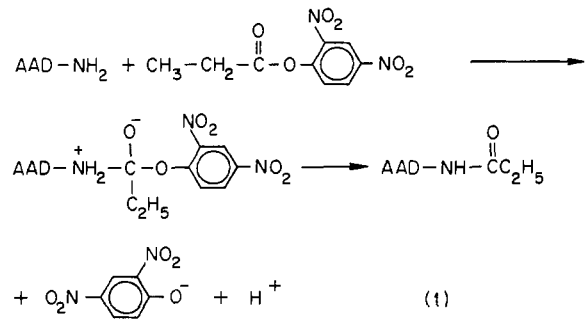
ABSTRACT: 2,4-Dinitrophenyl propionate acylates the amino group at the active site of acetoacetate decarboxylase. The pH–rate profile for this acylation can be described by a pK of 5.9. Although this value is 4 pK units less than that for an ordinary lysine amino group, it probably corresponds to the actual pK of the amino group at the active site of the enzyme.

Acetoacetate decarboxylase[1] from *Clostridium acetobutylicum* has been shown to catalyze the decarboxylation of acetoacetic acid by the mechanism shown in Scheme I (Hamilton and Westheimer, 1959; Westheimer, 1962).

The essential amino group is provided by a lysine residue. The Schiff-base salt of acetone has been trapped by borohydride reduction (Fridovich and Westheimer, 1962; Warren *et al.*, 1966) and the peptide containing this lysine isolated and identified (Laursen and Westheimer, 1966). Furthermore, the same lysine is attacked by acetic anhydride (O'Leary and Westheimer, 1968) to inactivate the enzyme.

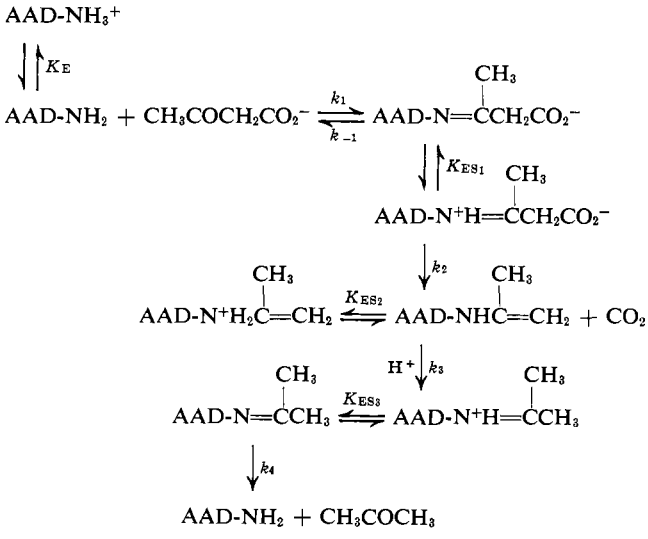
Consideration of the mechanism shown in Scheme I and the known mechanism for the formation of Schiff bases in chemical systems (Jencks, 1969) suggests that the first step in the formation of the Schiff base on the enzyme should require a free (*i.e.*, unprotonated) lysine amino group on the enzyme. This then reacts to yield a carbinolamine that loses water to yield the imine intermediate. The V_{max} *vs.* pH profile and the V_{max}/K_M *vs.* pH profile for the decarboxylation process peak around pH 6, and can be described roughly in terms of pK's in the range of 5–7 (Coutts, 1967). Perhaps one might expect that natural selection would produce an enzyme where a major fraction of the essential lysine amino group is free and therefore reactive at acidities corresponding to the rate maximum, although the pK of the lysine group must then be displaced by about 4 pK units relative to that of an ordinary lysine group. Unfortunately, one cannot draw any firm conclusions from the pH–rate profiles. In simple cases (Alberty and Massey, 1954) one can assign the pK's of essential ionizing groups on the enzyme from the V_{max}/K_M *vs.* pH profile, and the pK's of essential groups on the enzyme–substrate complex from the V_{max} *vs.* pH profile. In complicated examples, however, such as that represented by Scheme I, the pK's derived from these profiles are not simple quantities, but contain, in addition to various ionization constants, rate constant ratios of completely unknown magnitudes (*cf.* Zerner and Bender, 1961; Westheimer and Bender, 1962). In fact, the pH–rate profile cannot be used directly to determine pK's on the enzyme when different steps in the overall process depend on different levels of protonation, as in the example here under consideration. The kinetic equations derived from the mechanism of Scheme I are shown in the Appendix and illustrate this limitation. Here it suffices to say that none of the pK's that might be derived from an application of the Alberty–Massey equation has a simple interpretation.

However, the acylation of the enzyme offers, at least in principle, a possible way of determining the pK of the essential group. Here, in a stoichiometric reaction, presumably only one step is involved, and the pH–rate profile may then reflect the



(1)

SCHEME I



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[1] Abbreviations used are: AAA-NH$_2$, acetoacetate decarboxylase; DNPP, dinitrophenyl propionate; DNP, dinitrophenol (or its anion); AAD-NH-P, propionylacetoacetate decarboxylase; TMDA, *N,N',N'',N'''*-tetramethylethylenediamine; DAM, 4-(2-dimethylaminoethyl)-morpholine.

pK of the ionizable group. Although prior work had been carried out on acetylation (O'Leary and Westheimer, 1968), the present research utilized 2,4-dinitrophenyl propionate (eq 1) as the acylating agent.

The present paper presents the pH–rate profile for the acylation, and the evidence that the acylating agent does indeed attack the active site of the enzyme, and offers the argument that the pK determined by the kinetic method is, in this case, significant.

Experimental Section

Materials. Crystalline acetoacetate decarboxylase was prepared by the method developed in these laboratories (Westheimer, 1969) including the heat-activation step discovered by Neece and Fridovich (1967). The material used in this research had a specfic activity of 45 units or more.

2,4-Dinitrophenyl propionate was synthesized by adding propionyl chloride (11.0 g, 0.12 mole) in 10 ml of dry ether to a suspension of 2,4-dinitrophenol (22.0 g, 0.12 mole) and pyridine (9.8 g, 0.12 mole) in 20 ml of ether. The resulting mixture, protected from moisture, was stirred overnight. The solid (pyridinium chloride) was removed by filtration, and the product, mp 67–68°, obtained by evaporation of the solvent and two recrystallizations from chloroform–hexane. *Anal.* Calcd for $C_9H_8N_2O_6$: C, 45.02; H, 3.55; N, 11.66. Found, C, 45.09; H, 3.30; N, 11.41. Other values are ϵ (400 nm), calculated for dinitrophenylate ion, 11,500 cm^2/mole; found (after saponification of the ester in 1 N sodium hydroxide), 11,400 ± 100.

4-(2-Dimethylaminoethyl)morpholine was prepared by the method of Godfrey (1966), and boiled at 54–55° (2 mm). Titration gave an equivalent weight of 79; calcd 79. The nuclear magnetic resonance spectrum showed 6 protons (singlet) at δ 2.2, 8 protons (multiplet) at δ 2.4, and 4 protons (multiplet) at δ 3.7. The pK's of the conjugate acid of the amine were determined as 4.16 and 8.95 by electrometric titration of 0.1 M amine in the presence of 0.1 M potassium chloride with 1 M hydrochloric acid.

Other chemicals were reagent grade.

Methods. Reactions kinetics were followed by ultraviolet spectrophotometry in thermostatted 3-ml cuvets of a Cary 15 spectrophotometer equipped with a 0.0–0.1 optical density slide-wire. After a base line was established, 10–30 μl of a solution of 2,4-dinitrophenyl propionate of known concentration (*e.g.*, 1.09 mg/ml) in Spectrograde quality acetonitrile was added in a buffer solution to the cuvets, and the "spontaneous" hydrolysis followed for several hundred seconds. Next a portion of a stock solution of acetoacetate decarboxylase (10–35 mg/ml in 0.05 M potassium phosphate buffer, pH 5.91) was placed on the tip of a flattened glass rod. The rod was introduced into the cuvet through a hole in the top of the cell holder, and moved rapidly up and down to mix the solution. The increase in optical density at 400 nm that accompanies the liberation of 2,4-dinitrophenol was then measured; the rate eventually diminished to one only slightly greater (*vide infra*) than the original, "spontaneous" rate.

pH values were measured with a Radiometer automatic titrator, equipped with a scale expander. Nuclear magnetic resonance spectra were determined with a Varian A60 spectrometer.

Catalysis of the Hydrolysis of 2,4-Dinitrophenyl Propionate by Proteins. As noted above, the rate of hydrolysis of 2,4-dinitrophenyl propionate with acetoacetate decarboxylase after the initial burst is slightly greater than the buffer rate alone. A small (20–30%) increase in rate is caused also by bovine serum albumin or by acetoacetate decarboxylase that has been inhibited by acetopyruvate.

Results

Magnitude of the Burst. The extinction coefficient of 2,4-dinitrophenolate ion at 400 nm is 11,500 cm^2/mole, and that of 2,4-dinitrophenol at this wavelength is negligible (Carstens and Eisen, 1953). A series of experiments was conducted at various pH's with 0.72 mg/ml of enzyme, where the molecular weight of an active site (apparently two subunits) is 60,000 (O'Leary and Westheimer, 1968; Tagaki *et al.*, 1968). From these facts, one calculates that the optical density anticipated is 1.37×10^{-2}. The optical density found at low pH must be corrected for the ionization of 2,4-dinitrophenol, with pK of 4.00 (Ali *et al.*, 1965). The data found are listed in Table I.

TABLE I: Calculated and Observed Values of the Burst.

pH	Optical Density × 10^2 (Cor for Ionization)[a]
4.99	1.35
5.93	1.31
6.43	1.36
6.91	1.31
7.81	1.40
Calculated	1.37

[a] Average of two or more measurements.

Inactivation of the Enzyme. The rate of inactivation of the enzyme by 2,4-dinitrophenyl propionate is the same as the rate of liberation of dinitrophenol as predicted by eq 1. The reaction, carried out at 30° in pH 6.00 lutidine–lutidine sulfate buffer, employed 3×10^{-6} M enzyme (based on the concentration of active sites of mol wt 60,000) and 6×10^{-6} M 2,4-dinitrophenyl propionate. At intervals, 0.05 ml of solution was withdrawn for assay, which was conducted at 285 nm rather than at 270 nm in order to minimize interference from dinitrophenol.

The dilution that accompanied the assay procedure effectively quenched the reaction between enzyme and 2,4-dinitrophenyl propionate, and the overall reaction was sufficiently slow that the brief interval (a couple of seconds) involved in making the transfer did not interfere with the kinetics. The results are presented in Figure 1, and show that the inactivation is essentially quantitative and occurs at the same rate as does the liberation of dinitrophenol.

Active Site. In order to demonstrate that 2,4-dinitrophenyl propionate reacts at the active site of the enzyme, the reaction was attempted with enzyme inhibited with (a) acetopyruvate and (b) acetic anhydride. (a) A solution of decarboxylase (8.1×10^{-5} M) in 0.05 M phosphate buffer (pH 5.91) was inhibited by bringing the solution to a concentration of 5.0×10^{-4} M in acetopyruvate. The resulting enzyme solution had only about 2% of initial activity. The buffer (3 ml) was treated with 0.015 ml of a solution of 2,4-dinitrophenyl propionate in acetonitrile (1.09 mg/ml) to achieve a final concentration of 2.27×10^{-5} M. Then 0.06 ml of enzyme solution (active or

inhibited) was added and the burst monitored. The increase in optical density for the enzyme solution was 1.24×10^{-2}, whereas that for the inhibited solution was 0.14×10^{-2}, actually slightly less than the increase in optical density calculated for the absorption of the enzyme–acetopyruvate complex. Prior work had shown that acetopyruvate reacts with the enzyme at the lysine of the active site to yield an enamine (Tagaki and Westheimer, 1968). (b) A portion of the decarboxylase (4.1×10^{-4} M with respect to active sites, mol wt 60,000) was inactivated with acetic anhydride (final concentrations 5.0×10^{-4} M). Assay showed that less than 1% of the enzymic activity remained; prior work had shown that the acetic anhydride attacks at the essential lysine residue (O'Leary and Westheimer, 1968). The solutions were tested as described for the enzyme that had been inactivated with acetopyruvate. The burst for 0.03 ml of enzyme solution was 3.67×10^{-2} optical density unit, whereas that for the same quantity of inactivated enzyme was only 0.23×10^{-2} optical density unit, or slightly less than the increase caused by the acetylated enzyme added. The final rate of liberation of 2,4-dinitrophenol from the solution of inactivated enzyme was indistinguishable from the spontaneous rate of hydrolysis of the ester.

Kinetics of the Reaction of the Enzyme with 2,4-Dinitrophenyl Propionate. The reaction between excess 2,4-dinitrophenyl propionate and acetoacetate decarboxylase, after correction for the small amount of "spontaneous" reaction, is strictly first order (pseudounimolecular). Numerous experiments gave constants valid to a few per cent.

The first-order rate constant depends on the concentration of 2,4-dinitrophenyl propionate, as anticipated for a second-order reaction between enzyme and acylating agent; the data for pH 5.35 in TMDA sulfate buffer are shown in Figure 2. The deviation of the last point, at a concentration of 2.2×10^{-4} M of dinitrophenyl propionate, is considered in the discussion section.

pH–Rate Profile. The pH–rate profile for the reaction of the enzyme with 2,4-dinitrophenyl propionate is shown in Figure 3. The error limits suggested in the figure are obtained from the agreement between duplicate runs, and correspond to ±5%. The solid line has been calculated for eq 2, with $k_0 = 2.18 \times 10^3\ M^{-1}\ sec^{-1}$ M and $pK_1 = 5.88$.

$$k_{obsd} = \frac{k_0}{1 + (H^+)/K_1} \qquad (2)$$

Pyrophosphate Buffers. The reaction of 2,4-dinitrophenyl propionate with the enzyme was measured in buffers of lutidine–lutidine sulfate, TMDA sulfate, and DAM sulfate. Phosphate buffers can also be used. But, except at pH values below 5.5, rates of liberation of DNP and of inactivation of the enzyme are considerably higher in pyrophosphate buffers. The spontaneous, *i.e.*, buffer, rate is also higher with pyrophosphate, and in fact at pH 5.86 with 0.2 M pyrophosphate the rate is 7.6 times as great as in a lutidine buffer of the same pH. The rate of reaction between enzyme and 2,4-dinitrophenyl propionate in pyrophosphate buffers depends on the concentration of pyrophosphate, and extrapolates, at zero buffer concentration, to the same rate as that obtained with other buffers. Apparently the enzyme catalyzes a reaction between itself, 2,4-dinitrophenyl propionate, and pyrophosphate. This special behavior has not been further investigated, but perhaps is related to the general binding of anions by the enzyme (Fridovich, 1963). The choice of sulfate as the anion in cationic buffers was made because this anion does not inhibit the enzyme, and although the decarboxylation rate is more or less normal in the presence of pyrophosphate (Coutts, 1967), the interaction of enzyme and anions is probably related to the enzymic catalysis of a reaction between the substrate, pyrophosphate, and the enzyme itself.

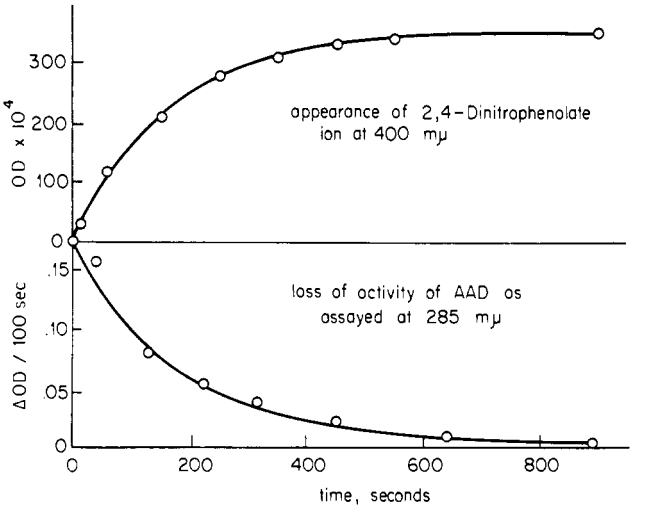

FIGURE 1: The release of 2,4-dinitrophenol (upper curve) from 2,4-dinitrophenyl propionate as measured at 400 nm, and the loss of the activity of the enzyme (lower curve), assayed on aliquots removed from the reaction mixture, both as a function of time in a lutidine–lutidine sulfate buffer, pH 5.90 at 30°.

Discussion

The reaction between 2,4-dinitrophenyl propionate and acetoacetate decarboxylase occurs at the active site, as demonstrated (a) by the complete and stoichiometric inhibition of the enzyme by the acylating agent and (b) by the demonstration that enzyme inhibited with acetopyruvate or by acetylation does not cause the liberation of dinitrophenol from DNPP. The reaction does not show Michaelis–Menton kinetics, but is essentially second order, at least up to a concentration of substrate of about 10^{-4} M. At a concentration of 2×10^{-4} M, the data shown in Figure 2 deviate from linearity in the direction expected for formation of an enzyme–sub-

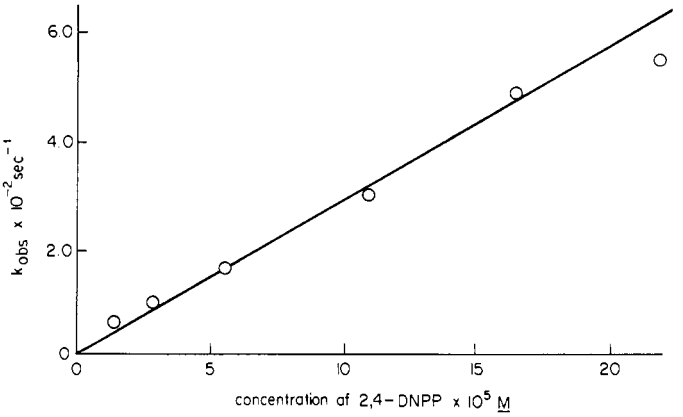

FIGURE 2: First-order (pseudounimolecular) rate constants for the reaction of 2,4-dinitrophenyl propionate with acetoacetate decarboxylase, plotted as a function of the concentration of 2,4-dinitrophenyl propionate. All the reactions were carried out in lutidine–lutidine sulfate buffers, pH 5.90 at 30°, in the presence of a large excess of 2,4-dinitrophenyl propionate relative to enzyme.

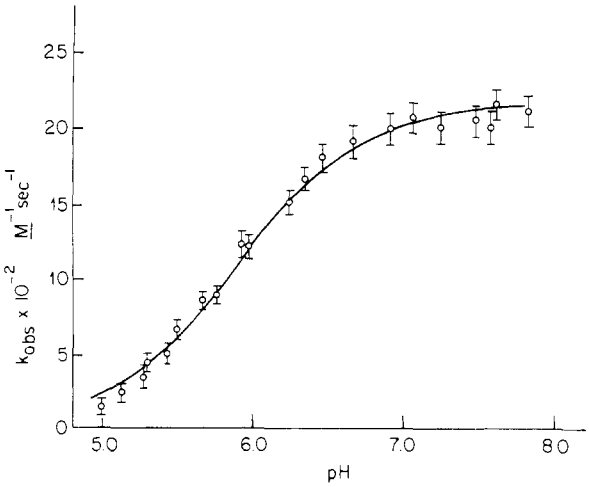

FIGURE 3: pH–rate profile for the reaction of 2,4-dinitrophenyl propionate with acetoacetate decarboxylase.

strate complex. Nevertheless, within the lower concentration range, the second-order rate constant is properly employed, as in Figure 3, in the pH–rate profile. That profile can be described by a simple titration curve, with a pK of 5.9.

Can this pK be ascribed to the lysine amino group at the active site of the enzyme?

If the acylation scheme is the simplest one, as shown in eq 3, then this is certainly the case. For

$$\begin{array}{l} \text{DNPP} + \text{AAD-NH}_2 \xrightarrow{k_0} \text{AAD-NH-P} + \text{DNP} \\ \quad\downarrow\uparrow K_1 \\ \text{AAD-NH}_3^+ \end{array} \qquad (3)$$

$$k_{obsd} = \frac{k_0(\text{DNPP})_0}{1 + (\text{H}^+)/K_1}$$

i.e., eq 2. More complicated kinetic schemes lead to more complicated equations for the pH–rate profile. Thus if

$$\begin{array}{lll} \text{DNP} + \text{AAD-NH}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{AAD-NH}_2\cdot\text{DNPP} & & \\ \quad\downarrow\uparrow K_1 & & \downarrow k_2 \\ \text{AAD-NH}_3^+ & & \text{AAD-NH-P} + \text{DNP} \end{array}$$

then

$$k_{obsd} = \frac{k_2(\text{DNPP})_0}{K_M(1 + (\text{H}^+)/K_1) + (\text{DNPP})_0} \qquad (4)$$

where $K_M = (k_1 + k_3)/k_1$, and if

$$\begin{array}{l} \text{DNPP} + \text{AADNH}_2 \overset{K}{\rightleftharpoons} \\ \quad\downarrow\uparrow K_1 \\ \text{AAD-NH}_3^+ \end{array} \qquad \begin{array}{l} \\ \\ \end{array}$$

$$\begin{array}{l} \text{AADNH}_2\cdot\text{DNPP} \xrightarrow{k_2} \text{AAD-NH-P} + \text{DNP} \\ \quad\downarrow\uparrow K_2 \\ \text{AAD-NH}_3^+\cdot\text{DNPP} \end{array}$$

then

$$k_{obsd} = \frac{k_2(\text{DNPP})_0/(1 + (\text{H}^+)/K_2)}{\dfrac{K(1 + (\text{H}^+))/K_1}{(1 + (\text{H}^+)/K_2)} + (\text{DNPP})_0} \qquad (5)$$

However, experimentally k_{obsd} is linear in the concentration of dinitrophenyl propionate (Figure 2) at least until the concentration of the acylating agent approaches 2×10^{-4} M. Therefore, at lower concentrations of DNPP, the terms in the denominators of eq 4 and 5 for the concentration of DNPP must be small relative to the other terms of these denominators, so that eq 4 and 5 reduce to equations of the form of eq 2. It follows that, for any of the three mechanisms here advanced, the value of K_1 (the ionization constant of the essential lysine residue in the enzyme) corresponds to the value obtained from the pH–rate profile shown in Figure 3, *i.e.*, pK_1 = 5.9.

Of course, the three mechanisms shown above are not the only possibilities. Among others, perhaps the least unlikely is a transfer mechanism, where the acylating agent reacts first with an imidazole residue, in a rate limiting step, and then, in a fast subsequent step, the acyl group is transferred from the imidazole to the lysine residue of the active site.

$$\begin{array}{l} \text{DNPP} + \begin{matrix} \text{B} \\ \text{H}_2\text{N} \end{matrix} \longrightarrow \begin{matrix} \text{CH}_3\text{CH}_2\text{CO-B} \\ \text{H}_2\text{N} \end{matrix} + \text{DNP}^- \\ \qquad\qquad\qquad\qquad\qquad\qquad\downarrow \text{fast} \\ \qquad\qquad\qquad\qquad\qquad\qquad \begin{matrix} \text{B} \\ \text{CH}_3\text{CH}_2\text{CO-NH} \end{matrix} + \text{H}^+ \end{array}$$

However, such a mechanism would be completely irrelevant to the function of the enzyme, which requires a nucleophilic lysine residue. Furthermore, acetoacetate decarboxylase does not readily react with, and is not inactivated by, iodoacetate (except in a photochemical reaction), and this also makes it unlikely that an imidazole residue is at the active site (Lee and Westheimer, 1966). Other possibilities than those here considered might be envisioned, and perhaps lead to a different conclusion. Nevertheless, the conclusion here reached seems the most probable one, namely that the reaction follows one of the mechanisms (3, 4, or 5) outlined, and that therefore the pK of the essential lysine residue is about 5.9.

A sharp decrease in the pK of an amino group in proteins has been observed before, although the magnitude of the shift here is perhaps somewhat greater than usual. Lysine-41 in ribonuclease has a pK of 7 (Murdock *et al.*, 1966) and pK's of basic groups in other proteins are similarly lowered (Carty and Hirs, 1968; Hill and Davis, 1967; Breslow and Gurd, 1962). Similarly, large displacements have been observed for the pK's of buried tyrosine residues (Timasheef and Gorbunoff, 1967). The introduction of a reporter group (Burr and Koshland, 1964) into acetoacetate decarboxylase (Frey and Westheimer, 1970; F. Kokesh, unpublished) shows that the environment at the active site is indeed such as to lower the pK of the essential lysine amino group to a value close to that here reported. However, the present observation on the native enzyme provides, in our view, the best estimate of the true pK of the amino group at the active site of acetoacetate decarboxylase so far available.

Appendix

The kinetic equations that describe the system of equations in Scheme I are given below.

$$V_{max} = \frac{V_1}{\frac{K_1}{(H^+)} + 1 + \frac{(H^+)}{K_2}}$$

$$\frac{V_{max}}{K_M} = \frac{V_2}{\frac{K_3}{(H^+)} + 1 + \frac{(H^+)}{K_4}}$$

where

$$V_1 = \frac{k_2(E_0)}{1 + \frac{k_2}{k_4} + \frac{k_2}{k_3 K_{ES_2}}}$$

$$V_2 = \frac{k_1(E_0)}{1 + k_{-1}K_{ES_1}/k_2K_E}$$

$$K_1 = \frac{K_{ES} + k_2/k_3}{1 + \frac{k_2}{k_4} + \frac{k_2}{k_3 K_{ES_2}}}$$

$$K_2 = \frac{1 + \frac{k_2}{k_4} + \frac{k_2}{k_3 K_{ES_2}}}{k_2/k_4 K_{ES_3}}$$

$$K_3 = \frac{k_{-1}K_{ES_1}/k_2}{1 + k_{-1}K_{ES_1}/k_2K_E}$$

$$K_4 = K_E + k_{-1}K_{ES_1}/k_2$$

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