A Reporter Group at the Active Site of Acetoacetate Decarboxylase. I. Ionization Constant of the Nitrophenol[1]

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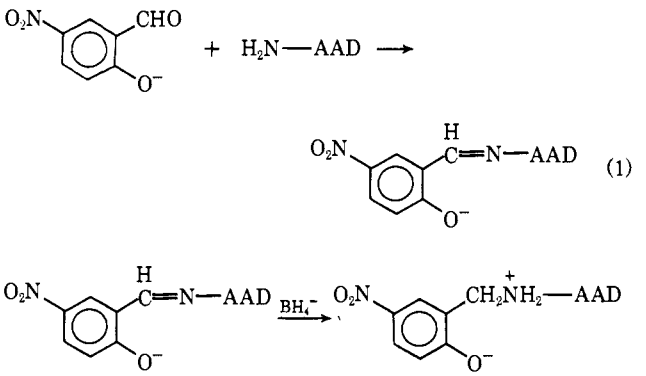
Abstract: 5-Nitrosalicylaldehyde reacts to form a Schiff base with an amino group at the active site of acetoacetate decarboxylase. Reduction of the Schiff base to an amine with borohydride ion introduces an N-substituted 2-hydroxy-5-nitrobenzyl substituent into the protein. The nitrophenol residue then provides a "reporter group" near the active site of the enzyme. The changes in absorbance at 395 nm that accompany the ionization of the nitrophenol can be correlated with a pK of 2.4; this is about 3.5 logarithmic units less than the corresponding pK of N-methyl-2-hydroxy-5-nitrobenzylamine. Since the protein is unstable at low pH, our determinations of the optical transmission of the reporter group at pH 1.8 and 2.9 were made by stopped-flow methods, so that measurements could be made in periods of time less than those that lead to denaturation. The large shift in the pK of the nitrophenol of the reporter group relative to that of the model compound is compared, in the following paper,[4] with the shift in pK of the amino group at the active site as determined by thermodynamic and kinetic methods.

Acetoacetate decarboxylase (AAD) catalyzes the decarboxylation of acetoacetic acid by way of a Schiff base (imine) as an essential intermediate.[5,6] The active ϵ-amino group of a particular lysine residue[7,8] reacts preferentially with the substrate to yield the intermediate to decarboxylation. It will also react with acetic anhydride[9] and with 2,4-dinitrophenyl propionate[10] to form inactive proteins acylated at the active site, with acetopyruvate to form a spectroscopically detectable enamine,[11] and with a few aldehydes of special structures to inhibit the enzyme and form spectroscopically detectable Schiff bases. In particular, W. Tagaki[12] found that acetoacetate decarboxylase reacts with 5-nitrosalicylaldehyde to inhibit the enzyme; the inhibition constant[13] is about 10^{-7} M. Furthermore, Tagaki showed that the resulting Schiff base can be reduced with borohydride ion so as to attach a 2-hydroxy-5-nitrobenzyl substituent irreversibly to the ϵ-nitrogen atom of the essential lysine residue, as shown in eq 1. This provides a "reporter group"[14] at the (formerly) active site of the protein. By comparing the pK's of the phenol and ammonium ions of the reporter group with those of a model compound, we can estimate the effect of the active-site environment on the ionizations.[15,16]



(1)

This paper is concerned with the spectrophotometric determination of the ionization constant of the phenolic hydroxyl group of the nitrophenol substituent. The following paper[4] treats the ionization constant of the amino group itself in 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase. These thermodynamic constants are then compared with the pK that has previously been determined by kinetic methods[10] for the essential ammonium salt group. These investigations show that the pK's of both the phenol and the ammonium ion of the reporter group at the active site of acetoacetate decarboxylase have been lowered by the enzymic environment by three to more than four logarithmic units.

Experimental Section

Materials. N-Methyl-2-hydroxy-5-nitrobenzalamine was prepared by adding 200 mg of 5-nitrosalicylaldehyde (Eastman) in 10 ml of methanol to 10 ml of a methanolic solution, 1.47 M in methylammonium chloride and 0.15 M in methylamine. A voluminous precipitate separated in a few seconds; after 5 min, 156 mg of yellow crystals was collected. An analytical sample, mp 219–220°, was prepared by crystallization from dimethoxyethane (with hot filtration to remove an insoluble impurity), followed by recrystallization from benzene.

Anal. Calcd for $C_8H_8N_2O_3$: C, 53.33; H, 4.48; N, 15.55. Found: C, 53.47; H, 4.45; N, 15.60.

N-Methyl-2-hydroxy-5-nitrobenzylamine was prepared by adding 12 ml of an aqueous solution, 0.26 M in sodium borohydride and 0.005 M in sodium hydroxide, to a suspension of the corresponding benzalamine in methanol, prepared (as above) from 600 mg of

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(16) F. H. Westheimer, *Search*, 1, 33 (1970).

5-nitrosalicylaldehyde. The mixture was stirred for 5 min at room temperature and the solid product (400 mg) was recovered by filtration. One recrystallization from 120 ml of hot water yielded 236 mg of fluffy yellow crystals, mp 218° dec. The nmr spectrum showed singlets at δ 4.2 and 2.7 in the ratio of 2:3 for the methylene group and the *N*-methyl group of the benzylamine, respectively, and a multiplet at low field for the aromatic protons.

Anal. Calcd for $C_8H_{10}N_2O_3$: C, 52.45; H, 5.50; N, 15.30. Found: C, 52.74; H, 5.59; N, 15.69.

Acetoacetate decarboxylase was prepared from *Clostridium acetobutylicum*.[17]

Substituted Protein. One milliliter of a solution of acetoacetate decarboxylase (20 mg/ml) in 0.05 *M* potassium phosphate buffer, pH 6, was mixed with 0.1–0.3 ml of a 3×10^{-3}–2×10^{-2} *M* aqueous solution of the nitrosalicylaldehyde, and chilled in an ice bath for 5 min. (Such solutions, when subjected to spectrophotometric examination, show formation of imine.) The solution was then treated with 0.01 ml of a cold solution of 0.1 *M* sodium borohydride. After 5–10 min, the protein was precipitated by bringing the solution to 75% saturation with ammonium sulfate and refrigerating for at least 30 min. The reporter-labeled AAD was isolated by centrifugation at 20,000 × *g* for 20 min. The resulting pellet was dissolved in 1.0 ml of 0.01 or 0.005 *M* potassium phosphate buffer, pH 5.9, and desalted by passing this solution through a 1.5 × 30 cm column of Sephadex G25 that had been equilibrated with the same buffers. The labeled protein is yellow, and can be followed visually on the column. It can be crystallized by the same procedure as that used for native enzyme; when the crystallization procedure was used, solutions of the protein were desalted just prior to spectroscopic studies.

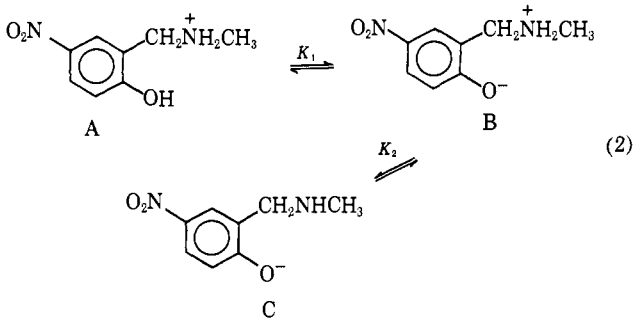
Methods. Spectra were determined with a Cary-15 spectrophotometer, in which the sample cuvet was thermostated in a special cell holder at 25.0 or 30.0 ± 0.1°. The stopped-flow measurements were made with a Durrum Model 13701 apparatus equipped with a Harrison 6515A dc power supply for the photomultiplier tube, and connected to a Zeiss PM QII spectrophotometer and M4 QII monochrometer. The photomultiplier output was monitored with a Tektronix Model 564 storage oscilloscope, and recorded with a Tektronix C30 Polaroid camera.

pH measurements were made with a Radiometer TTTlc pH meter and a GK2321C combination electrode. When necessary, the nomograph furnished with the electrode was used to make corrections for sodium ion response. The electrode was standardized using pH 4.0 and pH 7.4 Beckman buffers.

Least-squares analysis of data was conducted with an IBM 1620 computer. The program is outlined in the Appendix of the accompanying paper.[4]

Results

Model Compound. The spectra for *N*-methyl-2-hydroxy-5-nitrobenzylamine at pH 3.5, 8.0, and 13.4 are presented in Figure 1. These spectra correspond roughly to the absorptions of the species A, B, and C in eq 2.



(2)

The large spectral change that accompanies the ionization of a *p*-nitrophenol (λ_{max} 310 nm) to a *p*-nitrophenolate ion (λ_{max} 392 nm) is the basis of many assay methods. In our model compound, a second small spectral shift accompanies the ionization of the ammonium salt group; presumably the change in ionic environment (perhaps enhanced, in this example, by hydrogen bonding between ammonium salt group and phenolate anion) causes the change in extinction coefficients. Whatever the cause, the shift in the absorption maximum for the nitrophenolate chromophore from 395 nm at pH 8 to 410 nm at pH 13.4 and the concomitant increase in extinction coefficient from 18,100 to 20,000 M^{-1} cm^{-1} provide an adequate tool for the determination of the second p*K*. The data needed for the calculation of the two p*K*'s are presented in Tables I and II, and shown graphically in Figures 2 and 3. The two p*K*'s are so widely separated that they can be individually determined without mutual interference.

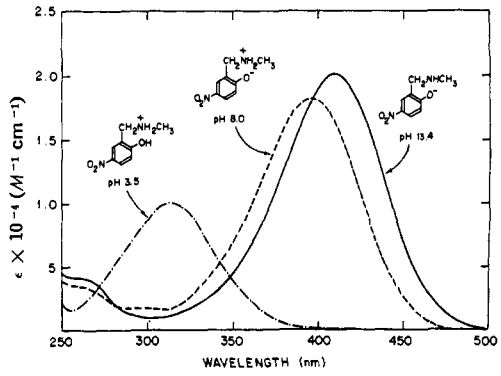


Figure 1. Absorption spectra of *N*-methyl-2-hydroxy-5-nitrobenzylamine at pH 3.5, 8.0, and 13.4. At these pH's, the spectra are nearly completely those of the cation, the zwitterion, and the anion, respectively.

Table I. Extinction Coefficients (M^{-1} cm^{-1}) at 400 nm of *N*-Methyl-2-hydroxy-5-nitrobenzylamine at pH 4.1–9.3, and Calculated Values of the pK_1, ϵ_{acid}, and ϵ_{base} at 25°

pH	$\epsilon \times 10^{-3}$
4.08[a]	0.39
4.49[a]	0.89
4.84[a]	1.65
5.02[a]	2.30
5.25[a]	3.37
5.57[a]	5.85
5.83[b]	8.50
6.20[b]	11.7_2
6.42[b]	13.4_1
6.51[b]	14.5_0
6.78[b]	15.1_7
6.95[b]	15.8_5
7.37[b]	16.9_8
7.69[b]	17.24
8.62[c]	17.4_5
9.29[c]	17.6_3
pK_1[d]	5.88
ϵ_{acid}[d]	0.19 × 10³
ϵ_{base}[d]	17.4_4 × 10³
Rms dev[e]	0.17 × 10³

[a] 0.1 *M* acetate buffer. [b] 0.1 *M* phosphate buffer. [c] 0.1 *M* glycine buffer. [d] Least-squares values. [e] Root-mean-square deviation of observed and calculated ϵ's.

Labeled Protein. The uv–visible spectra of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase at pH 4.97 and 9.68 are shown in Figure 4. The large absorption around 280 nm is that typical of all proteins; the small absorption at 320 nm is that previously

(17) F. H. Westheimer, *Methods Enzymol.*, **14**, 231 (1969).

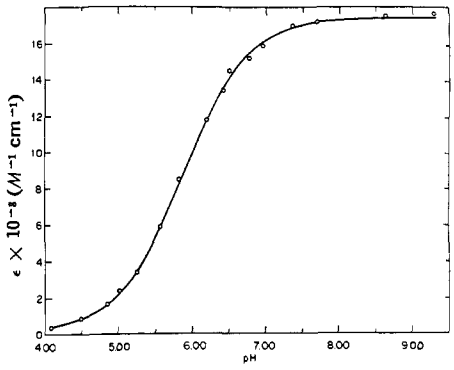


Figure 2. Titration data for pK_1 (the ionization of the nitrophenol group) for *N*-methyl-2-hydroxy-5-nitrobenzylamine at 25°. The solid line was calculated using an extinction coefficient for the acid form of 190 M^{-1} cm^{-1} and for the zwitterion of 17,400 M^{-1} cm^{-1} and a pK of 5.88; these are the values obtained from least-squares treatment of the experimental data.

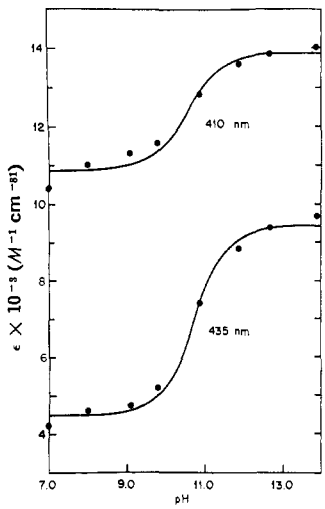


Figure 3. Titration data for pK_2 (the ionization of the benzylammonium group) for *N*-methyl-2-hydroxy-5-nitrobenzylamine. The data were obtained at 410 and 435 nm and 25°. The solid line was calculated using extinction coefficients for the zwitterion and anion of 10,880 and 13,850 M^{-1} cm^{-1} at 410 nm and of 4500 and 9410 M^{-1} cm^{-1} at 435 nm, and a pK of 10.65; these are the values obtained by least-squares treatment of the data.

found[18] for acetoacetate decarboxylase, but still unexplained; the changes in this absorption band with pH are treated in the accompanying paper. The peak at 400 nm is typical of nitrophenolate anions; its extinction coefficient of 18,300 M^{-1} cm^{-1} (based on one reporter group for each 60,000 mol wt units; *cf.* Tagaki and Westheimer[19]) is comparable to that for species B of *N*-methyl-2-hydroxy-5-nitrobenzylamine. The small but measurable changes in absorption of this peak at neutral to basic pH's are treated in the accompanying paper.

The absorption peak with a maximum at 400 nm is nearly invariant over the pH range from 6 to 3 and provides evidence that the nitrophenol is fully ionized over this entire range of acidity. In order to measure the pK of the phenolic hydroxyl group, the pH had to be lowered below 3, so that an appreciable fraction of the phenolate ion could be protonated. However,

(18) F. Lederer, S. Coutts, R. A. Laursen, and F. H. Westheimer, *Biochemistry*, 5, 823 (1966).
(19) W. Tagaki and F. H. Westheimer, *ibid.*, 7, 895 (1968).

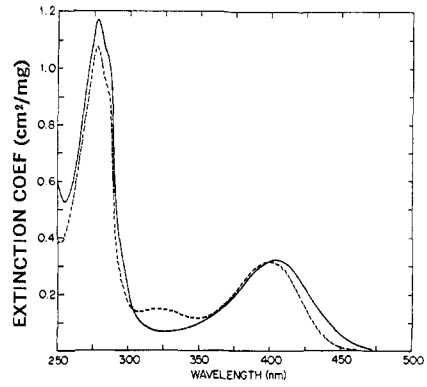


Figure 4. Absorption spectra of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase, determined at 30° at pH 4.97 (dashed line) and pH 9.68 (solid line).

Lederer, *et al.*,[18] have already shown that the enzyme is rapidly and irreversibly denatured at pH's below 3.

Measurements of the optical density were nevertheless made successfully at pH 2.9 and at 1.8 by using a stopped-flow spectrophotometer. Typical photographs of the oscilloscope output are shown in Figures 5 and

Table II. Extinction Coefficient (M^{-1} cm^{-1}) of *N*-Methyl-2-hydroxy-5-nitrobenzylamine at pH 7–13.4, and Calculated Values of the pK_2, ϵ_{acid}, ϵ_{base} at 25°

	ε × 10⁻³			
	λ, nm			
pH	360	460	435	460
7.00[a]	6.54	10.38	4.20	0.62
8.00[b]	6.58	11.00	4.58	0.70
9.08[b]	6.56	11.26	4.72	0.70
9.80[c]	6.60	11.52	5.18	0.94
10.90[d]	5.50	12.78	7.40	1.80
11.90[d]	4.76	13.58	8.82	2.38
12.70[d]	4.64	13.86	9.36	2.60
13.40[d]	4.48	14.00	9.66	2.70
pK_2[e]	10.87	10.56	10.73	10.76
10^{-3} ϵ_{acid}[e,f]	6.60	10.88	4.50	0.67
10^{-3} ϵ_{base}[e,f]	4.54	13.85	9.41	2.61
10^{-3} rms dev[g]	0.08	0.25	0.20	0.07

[a] 0.1 *M* phosphate buffer. [b] 0.1 *M* Tris buffer. [c] 0.1 *M* glycine buffer. [d] Sodium hydroxide solution. [e] Least-squares value. [f] ϵ_{acid} and ϵ_{base} are apparent extinction coefficients, obtained by dividing observed absorbance values by the approximate amine concentration. [g] Root-mean-square deviation of observed and calculated ϵ's.

6. At pH 2.9 the optical transmission is constant, as shown in the first trace, for almost 1 min before the precipitation of denatured protein causes it to decrease (second and third traces). At pH 1.8 the transmission is constant for only about 0.2–0.5 sec before it falls. But even this brief time is more than sufficient to allow estimation of the optical transmission without interference by precipitation.

The transmission after mixing but before significant denaturation has occurred is 81% (average of three determinations) and 50% (average of three determinations) at pH 1.80 and 2.88, respectively. Since the optical cell of the stopped-flow apparatus is 2.0 cm in length, these transmittances correspond to absorbance values for a 1-cm path length of 0.043 and 0.150. If we use $A^{395} = 0.430$ for a 1-cm path length for the

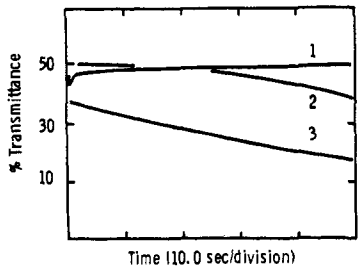


Figure 5. Transmittance *vs.* time at 395 nm of a solution (0.7 mg/ml) of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase, determined at 30° and pH 2.9 with a Durrum stopped-flow apparatus. The first oscilloscope trace shows an almost constant transmittance at about 50% over a 50-sec scan. The second and third traces show a diminution of transmittance as denatured protein precipitated in the cell.

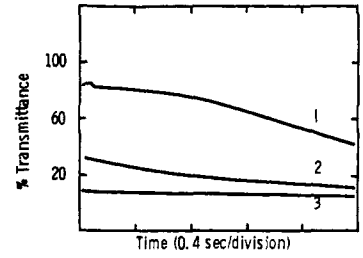


Figure 6. Transmittance *vs.* time at 395 nm of a solution (0.7 mg/ml) of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase, determined at 30° and pH 1.8. The first sweep shows that the transmittance is constant, at about 80%, for several tenths of a second before the transmittance drops as denatured protein precipitated in the cell.

original protein solution (prior to a twofold dilution in the stopped-flow apparatus), then we calculate pK_1 as 2.40 and 2.42.

Discussion

The measured pK of the nitrophenol group in 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase is 2.4; this is 3.5 logarithmic units less than the corresponding pK (5.9) for the model compound, *N*-methyl-2-hydroxy-5-nitrobenzylamine. The measured value of the pK for the nitrophenol of the reporter group is probably accurate. Even though the protein undergoes denaturation in the pH range where the measurements were made, the denaturation requires much more time than that needed to make the optical observations for the determination of the pK. Of course, the enzyme may undergo a rapid conformational change at low pH, prior to the irreversible changes observed after 0.2–30 sec. In fact, some unfolding of the protein probably occurs during the induction period, before precipitation of protein occurs. This, however, is unlikely to have had any effect upon the measured pK, since the value observed at pH 1.8 is the same as that at pH 2.9, where denaturation is much slower. Furthermore, if a rapid conformational change has nevertheless affected the ionization constant of the phenolic group, then in all probability the change has been such as to increase the measured, as compared to the true, pK. This follows because the very low pK of the nitrophenolic group on the labeled protein must be caused by a special environment at the active site; partial denaturation of the protein would most probably partially disrupt this special environment. It follows then that the pK as measured is either correct or else represents an upper limit to the true values. The difference between the pK of the reporter group and that of the model is therefore at least 3.5 logarithmic units.

The thermodynamic determination of the ionization constant of the special amino group of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase is reported in the accompanying paper.[4] The significance of the low pK found in this investigation for the phenolic group is there considered as part of the broader discussion of the properties and mechanism of action of the enzyme.