Mechanism of the Reaction Catalyzed by Acetoacetate Decarboxylase. Importance of Lysine 116 in Determining the pK_a of Active-Site Lysine 115[†]

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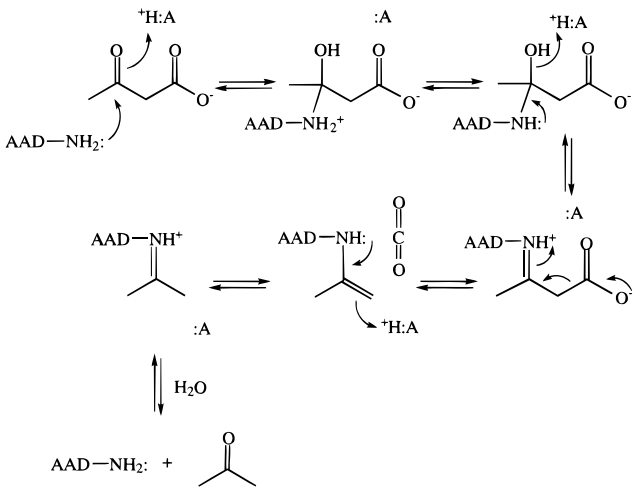
Received August 7, 1995; Revised Manuscript Received October 19, 1995[⊗]

ABSTRACT: Acetoacetate decarboxylase from *Clostridium acetobutylicum* (AAD) catalyzes the decarboxylation of acetoacetate *via* a Schiff base intermediate [Hamilton, G. A., & Westheimer, F. H. (1959) *J. Am. Chem. Soc. 81*, 6332; Fridovich, I., & Westheimer F. H. (1962) *J. Am. Chem. Soc. 84*, 3208]. The pK_a of the active-site lysine (Lys 115) is 6.0, 4.5 pK_a units less than the pK_a of lysine in solution [Kokesh, F. C., & Westheimer, F. H. (1971) *J. Am. Chem. Soc. 93***,** 7270; Frey, P. A., Kokesh, F. C., & Westheimer, F. H. (1971) *J. Am. Chem. Soc. 93*, 7266; Schmidt, D. E., Jr., & Westheimer, F. H. (1971) *Biochemistry 10*, 1249]. Westheimer and co-workers hypothesized that the pK_a of Lys 115 is decreased by its spatial proximity to the ϵ-ammonium group of Lys 116. We have investigated this proposal by studying site-directed mutants of Lys 115 and Lys 116. Two substitutions for Lys 115 (K115C and K115Q) were both catalytically inactive at pH 5.95, the pH optimum of wild type AAD, demonstrating the importance of this residue in catalysis. Activity could be restored to K115C by aminoethylation with 2-bromoethylammonium bromide (2-BEAB). Substitutions for Lys 116 (K116C, K116N, and K116R) had reduced but significant activities at pH 5.95. The effects of Lys 116 on the pK_a of Lys 115 in the mutant AADs were evaluated following imine formation with 5-nitrosalicylaldehyde and reduction with $NaBH_4$. Whereas the pK_a of Lys 115 in K116R is similar to that observed for wild type AAD, the pK_as of Lys 115 in K116C and K116N were elevated to >9.2. Alkylation of Cys 116 in K116C with 2-BEAB resulted in both significant activation and restoration of the pK_a of Lys 115 to 5.9. These data support Westheimer's hypothesis that the pK_a of the Schiff base-forming Lys 115 is decreased by its spatial proximity to the ϵ-ammonium group of Lys 116.

Acetoacetate decarboxylase (AAD, EC 4.1.1.4)[1] from *Clostridium acetobutylicum* (ATCC 824) catalyzes the decarboxylation of acetoacetate to yield acetone and carbon dioxide and displays maximal activity at pH 5.95. Westheimer and co-workers established that the mechanism of decarboxylation proceeds *via* the formation of a Schiff base intermediate with the ϵ-amino group of a lysine residue in the active site (Hamilton & Westheimer, 1959; Fridovich & Westheimer, 1962; Westheimer, 1995):



Laursen and Westheimer (1966) isolated an active-site peptide that contained the lysine proposed to participate in the mechanism; the sequence of the peptide was Glu-Leu-Ser-Ala-Tyr-Pro-Lys*-Lys-Leu, where Lys* is the lysine that participates in Schiff base formation. As deduced from the

[†] This research was supported by Grants GM-34572 (to J.A.G.) and GM-40570 (to J.A.G. and G.L.K.) from the National Institutes of Health.

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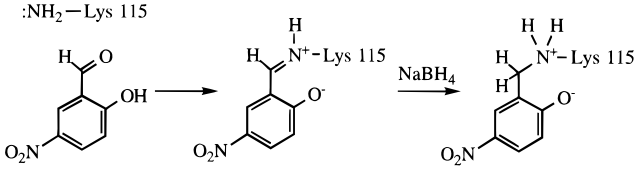
[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1995.

[1] Abbreviations: AAD, acetoacetate decarboxylase; 2-BEA, 2-bromoethylamine; 2-BEAB, 2-bromoethylammonium bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; NHNBA, *N*-methyl-2-hydroxy-5-nitrobenzylamine; 5-NSA, 5-nitrosalicylaldehyde; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

0006-2960/96/0435-41$12.00/0

protein (Samiulah, 1984) and DNA sequences (Petersen & Bennett, 1993), Lys* is Lys 115. Since the dependence of k_{cat}/K_m on pH for AAD is described by a bell-shaped curve where the maximum activity is observed at pH 5.95 (Coutts, 1967), the pK_a of the ϵ-ammonium group of Lys 115 evidently is lowered by the active-site environment such that a significant fraction of the functional group is present as the unprotonated amine at pH 6 and, therefore, reactive in formation of the Schiff base.

Westheimer and his co-workers subsequently determined that the pK_a of Lys 115 is ~6 by two independent methods. Schmidt and Westheimer (1971) studied the pH dependence of the irreversible inactivation of AAD by 2,4-dinitrophenyl propionate and determined that the rate of acylation was half-maximal at pH 5.9. Kokesh, Frey, and Westheimer used a "reporter" molecule, 5-nitrosalicylaldehyde (5-NSA), to measure the pK_a of the reactive lysine in the reduced, inactivated enzyme (Frey *et al.*, 1971; Kokesh & Westheimer, 1971). 5-NSA was suggested to form a Schiff base with the Lys 115; reduction with $NaBH_4$ afforded a covalently linked, hydrolytically stable chromophore.



The pK_as of the phenolic OH group (Frey *et al.*, 1971) and the secondary ammonium group (Kokesh & Westheimer, 1971) were decreased by 3.5 and 4.5 pK_a units (to 2.4 and 6.0), respectively, in the active site from the values determined for a model compound. This behavior was the basis of the suggestion that the perturbation in the pK_a of Lys 115 was caused by a local positive charge in the active site: electrostatic interactions involving a proximal positive charge would decrease the pK_as of both the phenolic OH group and the secondary ammonium group.

Kokesh and Westheimer (1971) hypothesized that the sequence-proximal lysine in the active-site peptide (Lys 116) could provide the positive charge that decreases the pK_a of the functional group of its sequence neighbor so that it can be unprotonated for Schiff base formation with the substrate at pH 5.95. Experimental evidence in support of Westheimer's explanation for the decreased pK_a of Lys 115 in the active site of AAD is presented in this article.

MATERIALS AND METHODS

Restriction enzymes were obtained from Boehringer-Mannheim. *Taq* polymerase was obtained from Promega. Buffer salts, 5-NSA, the lithium salt of acetoacetate, 2-bromoethylammonium bromide (2-BEAB), and DTNB were obtained from Sigma. Oligonucleotide primers were used as obtained from Oligos Inc. (Wilsonville, OR). The plasmid pDP253 containing the gene for AAD was the generous gift of Dr. George Bennett at Rice University, Houston, TX (Petersen & Bennett, 1993).

Site-Directed Mutagenesis. Prior to the construction of site-directed mutants, the *Ava*I site in the polylinker of pDP253 was removed by subcloning a 1274 bp *Eco*RI−*Hin*cII restriction fragment containing the entire gene for AAD from pDP253 into pUC18 that had been restricted with *Eco*RI and *Hin*cII. The resulting plasmid contains a unique *Eco*RI site upstream of the gene for AAD and a unique *Ava*I site in codons 118 and 119 of the gene for AAD; this plasmid was designated pLAH1.

Site-directed mutagenesis was performed by PCR methods. In each mutagenesis, the same primer containing an *Eco*RI site in the 5′-noncoding region of pLAH1 was used as the 5′-primer: 5′-GCA GT**G AAT TC**A TAA AAA C-3′ (the *Eco*RI site is noted in bold). The mutagenic 3′-primers were constructed to encode the mismatches necessary to generate the desired mutants at positions 115 and 116 as well as the unique *Ava* site that is located in codons 118 and 119 [mismatches and the *Ava*I site (**CCCATA)** are noted in bold]: K115Q: 3′-CGTATAGGA**GTC**TTCGAG**CCCAT-A**GG-5′; K115C: 3′-CGTATAGGA**ACG**TTCGAG**CCCAT-A**GG-5′; K116R and K116N: 3′-CGTATAGGATTT**T-(GATC)(GC)**GAG**CCCATA**GG-5′; K116C: 3′-CGTATA-GGATTT**A(GATC)(GC)**GAG**CCCATA**GG-5′.

The PCR reactions were performed in the buffer supplied with *Taq* polymerase (Promega) containing Mg^{2+} at a final concentration of 2.0 mM, dNTPs at final concentrations of 1.0 mM, and 1 μg of pLAH1 linearized with *Nde*I in a total volume of 100 μL. After amplification, the product fragment was isolated and restricted with *Eco*RI and *Ava*I. The resulting 686 bp fragment was ligated with the 3198 bp *Eco*RI−*Ava*I derived from pLAH1. The genes for the mutant plasmids were sequenced using a Sequenase kit (U.S.B., Cleveland, OH) both to determine the identities of the amino acids at positions 115 and 116 and to verify that no other mutations were present.

Purification of Acetoacetate Decarboxylase. Purification of wild type AAD (or mutants) from a strain of *Escherichia coli* transformed with pLAH1 (or a derivative of pLAH1 containing a mutated gene) was performed by a modification of the method for purification of the enzyme from *C. acetobutylicum* (Zerner *et al.*, 1966). Briefly, 10 L cultures of *E. coli* strain TG1 transformed with the desired plasmid were grown overnight in Luria broth at 37 °C with stirring at 300 rpm and aeration at 9 L/min in a New Brunswick fermenter. The cells were harvested by centrifugation at 14000*g* for 10 min at 4 °C. The cell pellet was weighed and resuspended in 200 mL of 50 mM potassium phosphate buffer, pH 5.9 (buffer A). The cell suspension was then disrupted by sonic treatment (Branson sonifier, Model 210). The broken cell suspension was cleared by ultracentrifugation for 60 min at 100000*g* at 4 °C. The supernatant was brought to 55% saturation with ammonium sulfate (326 mg/mL) at 4 °C. The turbid solution was cleared by centrifugation for 15 min at 20000*g*. The supernatant was then brought to 75% saturation with ammonium sulfate (an additional 127 mg/mL) at 4 °C. The precipitated enzyme was collected by centrifugation at 20000*g* for 15 min. The pellet was dissolved in 20 mL of buffer A and concentrated to 8 mL in an Amicon concentrator fitted with a PM-10 membrane. The solution was again cleared by centrifugation at 20000*g* to remove any insoluble material. The protein solution was applied to a Sephacryl S-200 column (2.6 × 95 cm) equilibrated in buffer A. The column was eluted at a flow rate of 0.75 mL/min, and 8 mL fractions were collected. Fractions were analyzed by SDS−PAGE using Coomassie staining. Fractions containing AAD were pooled and applied to a DEAE-Sephacel (Pharmacia) column (5 cm × 12 cm) equilibrated with buffer A in batches of 300 mg or less.

The DEAE-Sephacel column was washed with 1 L of buffer A or until the OD at 280 nm decreased to 0. AAD was then eluted with a linear gradient of 0.0−0.30 M ammonium sulfate in buffer A over 660 min, maintaining a flow rate of 1 mL/min (FPLC, Pharmacia). AAD elutes in the first peak well separated from the other contaminating proteins. The fractions were analyzed by SDS−PAGE. Fractions containing no visible contaminating proteins were pooled. Final purity was judged to be greater than 95% by SDS−PAGE. Typical yields of purified proteins from 10 L cultures were as follows: wild type, 80−100 mg; K115C, 20−30 mg; K115Q, 10−20 mg; K116C, 80−100 mg; K116N, 40−50 mg; and K116R, 30−50 mg.

Assay. The assay for AAD is based on the fact that 0.73% of acetoacetate is in the enol form and has an ϵ_{max} at 270 nm of 44.0 M^{-1} cm^{-1} (Pederson, 1934a,b) while the ϵ_{max} of acetone is 19.4 at the same wavelength (these values are averages of the ϵ_{max} at various pHs and were used in all studies at all pHs; Coutts, 1967). This $\Delta\epsilon_{max}$ allows a continuous, albeit rather insensitive, spectrophotometric assay to be employed. The decrease in absorbance *vs* time is proportional to the rate of decarboxylation.

Titration of Model Compound (NHNBA). The synthesis of the compound *N*-methyl-2-hydroxy-5-nitrobenzylamine (NHNBA) that models the adduct between Lys 115 and 5-NSA was accomplished as described by Frey *et al.* (1971). UV/vis spectra were obtained at pH values ranging from 2.0 to 13 so that the pK_as of the phenolic OH and secondary ammonium groups could be confirmed (Kokesh & Westheimer, 1971). Titration curves describing the data were generated using Cleland's WAVL program (Cleland, 1979).

Labeling of AADs with 5-NSA. AAD (wild type or mutant) was concentrated to 20 mg/mL (360 μM active sites assuming half-of-sites reactivity; O'Leary & Westheimer, 1968; Tagaki *et al.*, 1968) using a Centricon spin concentrator (Amicon). The labeling with 5-NSA was performed according to the procedure described by Frey *et al.* (1971). After gel filtration, the protein-containing fractions were concentrated using a Centricon PM-10 concentrator (Amicon). [Wild type recombinant AAD was judged to display half-of-sites reactivity based upon the amount of 2,4-dinitrophenolate released from 2,4-dinitrophenyl propionate (Schmidt & Westheimer, 1971).] The inactivated proteins so produced were designated WT-NSA, K116C-NSA, and K116N-NSA.

Alkylation of Wild Type AAD, K115C, and K116C with 2-Bromoethylammonium Bromide. Wild type AAD, K116C, and K115C were each concentrated to ∼10 mg/mL in 50 mM Tris, pH 8.2, using a Centricon PM-10 concentrator (Amicon). The solutions were brought to 50 mM 2-bromoethylammonium bromide (2-BEAB) by the addition of freshly prepared 2 M 2-BEAB in the same buffer. After 16−24 h, unreacted 2-BEAB was removed by repeated washing in a Centricon PM-10 concentrator (Amicon). The aminoethylated proteins so produced were designated WT-EA, K115C-EA, and K116C-EA.

Titration of 5-NSA Inactivated AADs. UV/vis spectra were obtained as a function of pH for the 5-NSA modified and reduced samples of wild type AAD and its Lys 116 mutants. The absorbance data at 320 and 400 nm were analyzed using WAVL (Cleland, 1979) to determine the pK_as that described the observed titration.

5-NSA Labeling of K116C-EA. The labeling of K116C-EA with 5-NSA (to produce K116C-EA-NSA) was performed according to the procedure used for the wild type and mutant proteins. The titration of the resulting K116C-EA-NSA was performed as described for the NSA derivatives of wild type AAD and the site-directed substitutions for Lys 116.

Quantitation of Sulfhydryl Groups. Sulfhydryl groups were quantitated using the method of Ellman (1959). The number of surface accessible sulfhydryl groups was quantitated using enzyme solutions containing approximately 8.0 μM subunits (27.54 kDa/subunit) in 50 mM Tris, pH 8.2, at 25 °C. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was added to a final concentration of 500 μM, and the absorbance at 412 nm was monitored as a function of time. The number of total sulfhydryl groups was analogously determined following incubation of protein for 5 min in the same buffer containing 8 M urea prior to the addition of DTNB.

Tryptic Digestion of 5-NSA-Inactivated AADs. Tryptic digests of the 5-NSA-modified proteins were performed using the procedure described by Laursen and Westheimer (1966). The progress of the reaction was analyzed by HPLC as described in the next section. When the reaction was judged to be completed as assessed by no change in the HPLC chromatogram monitored at 214 nm, the digested protein was lyophilized, resuspended in 0.5 mL of 0.1 M NH_4HCO_3, and stored at −20 °C.

Isolation of 5-NSA-Labeled Peptides by HPLC. The trypsin-digested samples were chromatographed on a Beckman System Gold HPLC equipped with a C_{18} column (Beckman); the eluent was monitored with a diode array spectrophotometer (System Gold Model 168). Buffer A was water containing 0.1% TFA. Buffer B was water/2-propanol/acetonitrile, 3:2:2, containing 0.1% TFA and 0.1% nonylamine. The digested protein was applied to the C_{18} column and eluted by a linear gradient from 0% to 50% buffer B over 60 min at 1 mL/min. The column was then washed with 100% buffer B for 5 min and re-equilibrated in buffer A.

The isolation of the peptide containing Lys 115 was facilitated by the observation that fully protonated NHNBA (and, therefore, presumably the 5-NSA-labeled peptide) has an extinction coefficient of ∼13 000 M^{-1} cm^{-1} at 320 nm in the solvent system used to separate tryptic peptides. Accordingly, the eluent was monitored both at 214 nm (peptide bond) and at 320 nm. The eluent containing impure 5-NSA-labeled peptide was collected, lyophilized to dryness, and resuspended in 0.5 mL of 100 mM NH_4HCO_3, pH 7.7.

Each impure peptide was reapplied to the C_{18} column in 75 μL fractions and eluted by a shallower gradient of buffers A and B. Each pure peptide was collected and lyophilized. The peptide was resuspended in 50 mM NH_4HCO_3, pH 8.0, and the concentration was quantitated assuming that the extinction coefficient for the *p*-nitrophenyl substituent was 18 000 M^{-1} cm^{-1} at 400 nm (the same for NHNBA at the same pH). Aliquots of ∼500 pmol were sequenced with an Applied Biosystems 477A protein sequencer (Protein Nucleic Acid Laboratory, University of Maryland, College Park).

RESULTS AND DISCUSSION

The Importance of Lys 115. The K115Q and K115C mutants possessed no detectable activity (Table 1), demon-

Table 1: Kinetic Constants for Wild Type (WT), Mutant, and Aminoethylated Samples of AAD at pH 5.95 and 25 °C[a]

enzyme	k_{cat} (s^{-1})	K_m (mM)
WT	1560 ± 25	8.2 ± 0.8
K115C	0	NA[b]
K115Q	0	NA[b]
K115C-EA	119 ± 20	11.6 ± 1.2
WT-EA	740 ± 25	7.2 ± 0.8
K116C	38 ± 6	8.4 ± 1.4
K116N	30 ± 6	10.0 ± 2.4
K116R	302 ± 15	14.7 ± 1.6
K116C-EA	410 ± 20	8.0 ± 0.4

[a] Rates were determined from three independent trials using three substrate concentrations higher and three lower than the K_m. The rates for each substrate concentration were determined in duplicate. Kinetic constants were calculated from three independent determinations of k_{cat} and K_m. [b] Not applicable.

strating that Lys 115 is absolutely essential for catalytic activity. This result supports Westheimer's mechanistic studies of AAD, including the identification of Lys 115 as the Schiff base-forming residue (Laursen & Westheimer, 1966).

Partial activity could be restored to the inactive K115C mutant by incubating the protein with 2-BEAB to generate a protein containing 4-thialysine at residue 115. The generation of this analog of Lys 115 (in K115C-EA) restored ~8% of wild type activity to the mutant enzyme (Table 1). Wild type AAD analogously modified with 2-BEAB has ~50% of type activity, presumably the result of additional alkylation reactions elsewhere on the surface of the protein: sulfhydryl titrations of the alkylated enzymes revealed that one sulfhydryl group (out of a total of three) was modified when wild type AAD was alkylated with 2-BEAB; two sulfhydryl groups (out of a total of four) were modified when K115C was alkylated with 2-BEAB. That the activity of K115C-EA is 16% that of the modified wild type enzyme suggests either that (1) the catalytic activity of the AAD molecule is dependent upon the exact position of the amino group of residue 115 in the active site, since the side chains of lysine and thialysine cannot be expected to be strictly isosteric (Ammon *et al.*, 1990); or (2) the lower pK_a of the ammonium group of thialysine relative to lysine reduces the nucleophilicity of the amino group of thialysine (Hermann & Lemke, 1968; Gloss & Kirsch, 1995), with a possible consequence that the rate of Schiff base formation with acetoacetate is reduced. Irrespective of the exact explanation for the lower activity of K115C-EA, the restoration of substantial catalytic activity to K115C by alkylation with 2-BEAB provides additional evidence that an amino group at residue 115 is essential for catalysis.

Substitutions for Lys 116. The activities of the mutant AADs were determined at pH 5.95, the pH optimum for wild type AAD, and found to be decreased from that of wild type (Table 1). In addition, each of the mutants could be irreversibly inactivated by (1) incubation with substrate or product in the presence of $NaBH_4$ or (2) incubation with 5-NSA followed by reduction with $NaBH_4$. These observations suggest that the mechanisms of the reactions catalyzed by the mutants also involve formation of a Schiff base with the ϵ-amino group of Lys 115. [5-NSA does, in fact, react with Lys 115 in wild type AAD and in the Lys 116 mutants (*vide infra*).]

The K116R mutant showed only modestly decreased activity, i.e., ~20% that of wild type at pH 5.95 (Table 1).

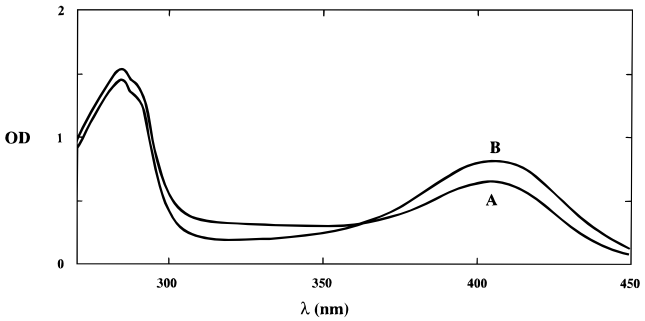


FIGURE 1: UV/vis spectra of WT-NSA at pHs 5.13 (spectrum A) and 6.98 (spectrum B).

This level of activity may result from either distortion of the active site by the greater size of the arginine side chain relative to that of lysine or the differing influence of the more diffuse positive charge of the guanidinium group on the reactivity of the ammonium group of Lys 115. In contrast, the activities of the K116C and K116N mutants are more significantly reduced, i.e., ~2% that of wild type at pH 5.95 (Table 1). The simplest explanation for the latter decreases in activity would be that in the absence of a positive charge at residue 116 the pK_a of Lys 115 is "normal", with the consequence that a significantly lower percentage of the functional group would be unprotonated at pH 5.95.

We also determined the dependencies of both k_{cat} and k_{cat}/K_m on pH for the substitutions for Lys 116 (data not shown). These studies revealed that the pH optima for K116C and K116N were not shifted to higher values of pH [the pH optimum for wild type AAD is 5.95 (Coutts, 1967)], as might have been expected if the dependencies of the kinetic parameters on pH reflected the pK_a of Lys 115 (*vide infra*). The identities of the rate-determining steps in k_{cat} and k_{cat}/K_m for wild type AAD are unknown, so the observed decreases in activity cannot be interpreted in terms of the mechanism.

Titration of Wild Type AAD Modified with 5-NSA. We attempted to measure directly the pK_a of Lys 115 in the mutant enzymes by inactivation with 2,4-dinitrophenyl propionate (Schmidt & Westheimer, 1971), but the rates of production of 2,4-dinitrophenol for K116C and K116N could not be distinguished from background. While this behavior suggested that the pK_as of Lys 115 are elevated in these mutants, we concluded that this method could not be used to accurately measure the pK_as of Lys 115 in the mutant enzymes. Accordingly, we used the "reporter" group method described by Westheimer's laboratory (Frey *et al.*, 1971; Kokesh & Westheimer, 1971) to assess the effect of substitutions for Lys 116 on the ionization behavior of Lys 115 in the covalently modified enzymes. Kokesh and Westheimer (1971) observed that the pK_a of the secondary amino group in the 5-NSA-modified enzyme (derived from the ϵ-amino group of Lys 115) is indistinguishable from that determined from the pH dependence of the reaction with 2,4-dinitrophenyl propionate, indicating that the pK_as of Lys 115 in the modified enzymes can be assessed properly by the "reporter" group method.

At pH values below 6.0, a significant amount of phenolate anion is present in the modified wild type AAD as judged by significant absorbance at 400 nm and insignificant absorbance at 320 nm (Figure 1). This observation confirms that reported by Frey *et al.* (1971) and the conclusion that

Table 2: pK_a Values for the OH and $R_2NH_2^+$ Groups of the NHNBA Model Compound and the Reporter Groups in the 5-NSA-Modified and Reduced Proteins As Assessed by the Dependence of the Increase in Absorbance at 400 nm on pH

enzyme	pK_a of OH	pK_a of $R_2NH_2^+$
NHNBA	6.36 ± 0.02	10.66 ± 0.22
WT	<4	6.40 ± 0.02
K116C	6.24 ± 0.06	>9.2
K116N	6.36 ± 0.06	>9.2
K116R	<4	6.27 ± 0.11
K116C-EA	<4	5.91 ± 0.10

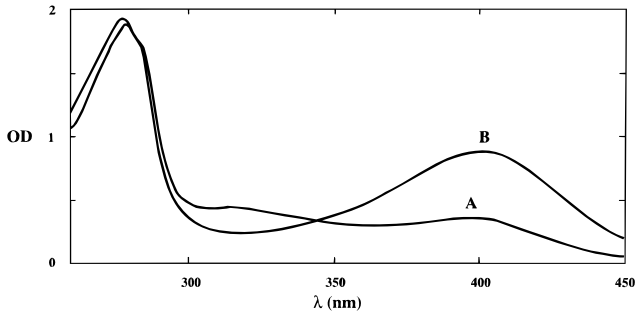


FIGURE 2: UV/vis spectra of K116C-NSA at pHs 4.87 (spectrum A) and 9.15 (spectrum B).

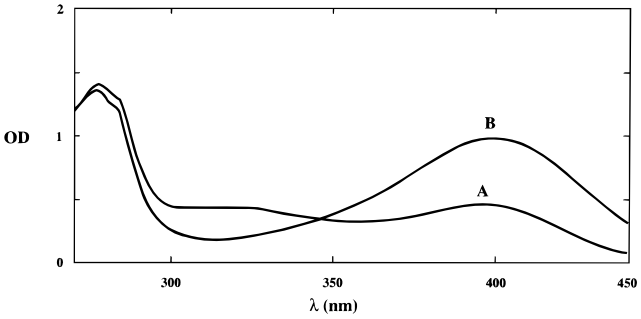


FIGURE 3: UV/vis spectra of K116N-NSA at pHs 5.26 (spectrum A) and 8.92 (spectrum B).

the pK_a of the phenolic OH group is significantly decreased in the active site of wild type AAD such that it is deprotonated at pH 6. As the pH is increased from 5 to 8, the absorbance at 400 nm increases by about 50% and shifts to slightly longer wavelength; the change in absorbance at 400 nm is described by a pK_a of 6.40 ± 0.02 (Table 2), in good agreement with the value reported by Kokesh and Westheimer (1971). This pK_a describes the titration of the secondary ammonium group derived from the amino group of Lys 115 and confirms that the pK_a of the ammonium group is decreased by >4 pK_a units by the active-site environment.

pK_a of Lys 115 in K116C and K116N. UV/vis spectra of K116C-NSA and K116N-NSA were recorded at pH values ranging from 4.8 to 9.35. Spectral data could not be recorded below pH 4.7 for these modified proteins, since, like wild type, they both rapidly denature at low pH. The two proteins showed similar spectrophotometric characteristics (K116C, Figure 2; K116N, Figure 3). Below pH 6.0, the absorbance at 320 nm is significant but the absorbance at 400 nm is small. When the pH values were raised from 5 to 8, a marked (>2-fold) increase in absorbance with a λ_{max} at 395 nm was observed for K116C-NSA and at 394 nm for K116N-NSA; these were formed concomitant with disappearance of the absorbance at 320 nm. In contrast to the adduct formed between wild type AAD and 5-NSA, both the insensitivity of the λ_{max}s (394−395 nm) to increased pH and

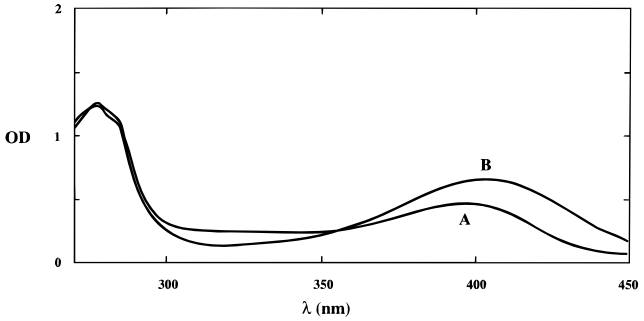


FIGURE 4: UV/vis spectra of K116R-NSA at pHs 5.05 (spectrum A) and 8.26 (spectrum B).

the magnitudes of the increases in absorbance as the pH values are increased from 5 to 8 demonstrate that the phenolic OH groups of the 5-NSA adducts with both K116C and K116N are protonated at pH 5 and deprotonated at pH 8. The absorbance data at 400 nm for K116C-NSA are described by a titration curve with a pK_a of 6.24 ± 0.06 (Table 2). The absorbance data for K116N-NSA are described by a titration curve with a pK_a of 6.36 ± 0.06 (Table 2). These pK_as describe the ionization of the phenolic OH group in the adducts.

As the pHs were raised to >9, the λ_{max} values for both K116C-NSA and K116N-NSA shifted toward 410 nm, the λ_{max} expected for the anionic chromophore produced by titration of the ammonium group (Kokesh & Westheimer, 1971). Titration of the ammonium group is also supported by a small increase in the absorbance at 410 nm. The pK_as describing these changes in spectral properties were too high to be quantitated accurately, although they necessarily are >9.2 (Table 2).

Taken together, these observations demonstrate that the pK_as of the phenolic OH groups and the secondary ammonium groups in K116C-NSA and K116N-NSA are not significantly decreased from those observed for the NHNBA model (6.36 ± 0.02 and 10.66 ± 0.22, respectively; Table 2).

pK_a of Lys 115 in K116R. UV/vis spectra of the reduced adduct of K116R with 5-NSA were recorded at pH values ranging from 5.05 to 9.65. Even at pH values <6, a significant absorbance was present at 401 nm, indicating that the phenolic OH group is deprotonated (Figure 4). As the pH was increased from 5 to 8, the λ_{max} shifted to 408 nm, indicating that the secondary ammonium group was being titrated. The increase in absorbance at 400 nm is described by a titration curve with a pK_a of 6.27 ± 0.11 (Table 2). Thus, the behavior of K116R-NSA is similar to that observed for WT-NSA, i.e., the pK_a of the ammonium group is decreased by >4 pK_a units by the active-site environment.

The spectral properties of the adducts of 5-NSA with the three substitutions for Lys 116 are consistent with Westheimer's proposal that the cationic group of Lys 116 decreases the pK_a of Lys 115 in wild type AAD.

Partial Restoration of Wild Type Activity to K116C by Aminoethylation. As described previously, the catalytically inactive K115C mutant could be "rescued" by reaction with 2-BEAB. We, therefore, attempted to "rescue" the K116C mutant with 2-BEAB. After reaction with 2-BEAB, the activity of the K116C mutant at pH 5.95 increased 11-fold to a value 55% that of WT-EA (Table 1).

K116C-EA was modified with 5-NSA and reduced with $NaBH_4$. UV/vis spectra of the adduct were recorded at pH

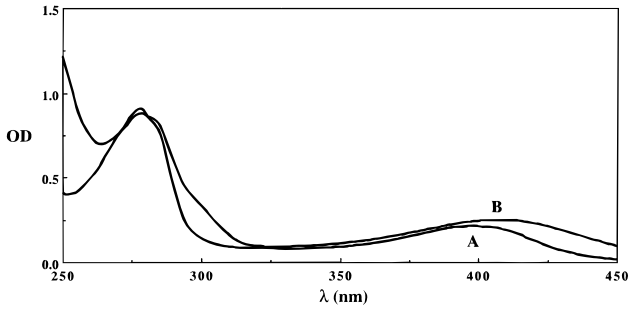


FIGURE 5: UV/vis spectra of K116C-EA-NSA at pHs 4.82 (spectrum A) and 10.15 (spectrum B).

values ranging from 4.82 to 10.15. Even at pH values <6, significant absorbance was present at 400, indicating that the phenolic OH group is deprotonated at low pH. As the pH was increased from 5 to 8, the λ_{max} shifted to 408 nm, indicating the ammonium group was being deprotonated (Figure 5). The absorbance data at 400 nm are described by a titration curve with a pK_a of 5.91 ± 0.10 (Table 2).

The pK_a of the secondary ammonium group in K116C-EA is decreased by >4 pK_a units by the active site environment. The spectral properties of aminoethylated K116C are similar to those of wild type AAD and K116R; i.e., the pK_a of the ammonium group is decreased by >4 pK_a units by the active-site environment.

Isolation and Sequence Analysis of Active-Site Peptides. The implicit assumption in these spectral analyses is that 5-NSA reacts with Lys 115 and not some other amino group on the surface of the protein. We have verified this assumption for the adducts with wild type AAD and the K116C, K116N, and K116R mutants by isolating and sequencing the peptide that contains the chromophore.

For each protein, the HPLC chromatogram of a tryptic digest demonstrated that the major product of reaction with 5-NSA and reduction is a single peptide. Also, in each case, the sequence of the labeled peptide was Glu-Leu-Ser-Ala-Tyr-Pro-X, where X is an unidentified amino acid. The sequence of the first six residues of this peptide is that expected for residues 109−114 in AAD; X is assumed to be the reduced adduct of Lys 115 with 5-NSA. Thus, the spectral titrations reported in this article do allow assessment of the ionization properties of Lys 115.

Conclusions. Westheimer's proposal that the pK_a of Lys 115 is significantly decreased by its proximity to the ammonium group of Lys 116 has withstood the test of experimental scrutiny. That large shifts in pK_a can be produced by spatially proximal cationic residues is undoubtedly relevant to other enzymes, including mandelate racemase where a pK_a of ∼6 has been assigned to the ϵ-ammonium group of Lys 166 (Kallarakal *et al*., 1995*)*; this functional group is in close proximity to both the ϵ-ammonium group of Lys 164 and an essential Mg^{2+} (Landro *et al.*, 1994).

ACKNOWLEDGMENT

We thank Professor F. H. Westheimer for his helpful comments.

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BI9518306