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Reactivity of Amino Acids in the Azo Coupling Reaction

I. Dependence of Their Reactivity on pH

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Azo coupling reactions of *N*-α-acetylhistidine, *N*-α-acetyltyrosine, and *N*-α-acetyllysine with *p*-methylbenzenediazonium ion were investigated as model reactions to obtain information on the relative reactivity of the histidine, tyrosine, and lysine moieties of protein, separated from structural effects. The azo coupling yields of the amino acids increased as the pH of the reaction medium was increased, indicating that the reactive species are the imidazole anion of histidine, the phenolate anion of tyrosine, and the neutral ε-amino group of lysine. It was calculated, based on percentage yields of the azo products, that the imidazole anion is more reactive than the phenolate anion and the ε-amino group, respectively.

Azo coupling reactions have been widely used for hapten synthesis (*1*), affinity labeling of antibodies (*2*), and modification of enzymes (*3*) and other proteins (*4*). Recently, Sundberg *et al.* (*5*) synthesized a bifunctional chelating agent, 1-(*p*-benzenediazonium) ethylenediaminetetraacetic acid and used the azo coupling reaction to connect this compound to albumin in an attempt to prepare a diagnostic imaging agent. Hawthorne *et al.* (*6*) prepared a ^{10}B conjugated antibody through the azo coupling reaction to rabbit anti-bovine serum albumin (BSA) with 1-(*p*-benzenediazonium)-1,2-dicarba-closo-dodecaborane.

Numerous reports on the reaction of benzenediazonium ions with proteins showed that tyrosine, histidine, and lysine are the major amino acid moieties undergoing azo couplings (*7–10*). Higgins and Harrington (*9*) reported that the imidazole group of histidine and the phenolic group of tyrosine selectively reacted when the concentration of protein was high. On the other hand, very low protein concentration compared to diazotized sulfanilic acid caused disubstitution on the imidazole and the phenolic group as well as reaction with the ε-amino group of lysine to produce the corresponding bis(benzenediazo) amino acid. The degree of azo coupling on each amino acid moiety in the protein was reported to vary, depending on the structure of the diazonium ions (*7, 11, 12*) and somewhat on the pH of the the reaction medium (*8*).

Several individual amino acids have been coupled with benzenediazonium ions (*13–15*). Spectral characteristics of azoamino acids, especially those of azotyrosine, azohistidine, and azolysine, were thoroughly studied (*16–18*). It was also reported that the functional groups undergoing azo coupling are the phenolic group of tyrosine, the imidazole group of histidine, and the ε-amino of lysine, respectively. However, the relative reactivity of these amino acids has not been reported.

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The authors would like to report in this paper the relative reactivity of the imidazole group of *N*-acetylhistidine, the phenolic group of *N*-acetyltyrosine, and the ε-amino group of *N*-acetyllysine in the azo coupling reactions. Although modification of protein side chains is in large part determined by the structure of the protein (*19*, *20*), we think that information on the relative reactivity of the amino acid residues provides better understanding of the azo coupling reaction.

MATERIALS AND METHODS

One milliliter of 0.3 *M* *p*-toluidine dissolved in 1 *M* hydrochloric acid was chilled on an ice bath. To this solution was added dropwise 1.2 ml of 0.25 *M* sodium nitrite in water. The reaction temperature was kept below 4°C and the solution was stirred for 1 hr after the addition of the sodium nitrite solution was completed. The starch–iodide test was then carried out to confirm that an exact amount of sodium nitrate was added.

The diazonium solution was diluted to 100 ml with cold water. One milliliter of the solution was added dropwise to 20 ml of 0.2 *M* resorcinol solution in 0.1 *M* sodium acetate at pH 4.5. The solution was stirred for 1 hr at room temperature and then diluted to 100 ml with methanol. The absorption spectrum of the azo solution (pH 4.5) was measured (Beckman Spectrophotometer Acta C111). The absorption maximum (378 nm) and its extinction coefficient ($e = 21\,500$) agreed with the reported value (*17*, *21*). This confirmed that the azo coupling reaction with resorcinol was quantitative and could be used for the calculation of the diazonium ion concentration.

AZO COUPLING REACTIONS

N-α-Acetylhistidine, *N*-α-acetyltryrosine, *N*-α-acetyllysine, histidine, tyrosine, or lysine, respectively, was dissolved in 0.1 *M* sodium bicarbonate–sodium carbonate buffer solutions at different pH values. Fifty microliters of 3×10^{-3} *M* *p*-methylbenzenediazonium ion was then added to 1 ml of 2.4×10^{-3} *M* each of the amino acid solutions in a 1.5-ml vial at 5°C. The vial was closed and the solution was occasionally stirred for 10 hr at the same temperature. The completion of the reaction was confirmed by the fact that the reaction mixture did not have coupling power with α-naphthol dissolved in methanol–0.1 *M* sodium carbonate. There was no change in the pH of the buffer solution throughout the reaction. The azohistidine and the azotyrosine solutions were stable at least for 4 days at 5°C. Each of the azo solutions was titrated to pH 13.4 with 10 *N* NaOH (Fisher Accument Model 320). The azo-*N*-α-acetylhistidine and the azohistidine solutions showed an absorption maximum at 410 nm while the azotyrosine solutions gave an absorption maximum at 472 nm. The azo solutions from *N*-α-acetyllysine and lysine did not show an absorption at 378 nm for the corresponding bisazolysines. The amino acids did not show absorptions at the above wavelengths. Weak absorption due to the hydrolysis products of the diazonium ion was substracted from the total absorbance of the azo coupling solution to get the net absorbance of the azo compound. The concentration of *p*-tolueneazotyrosine was calculated using the molar absorptivity ($\varepsilon = 10\,400$ at 472 nm) of *p*-tolueneazo-*N*-acetyltyrosine ethyl ester.

The concentration of *p*-tolueneazohistidine was calculated based on the molar absorptivity ($\varepsilon = 21\ 700$ at 410 nm) (*17*).

PREPARATION OF MONO-(*p*-METHYLBENZENEAZO)-*N*-ACETYLTYROSINE ETHYL ESTER

N-Acetyltyrosine ethyl ester, 0.54 g (2×10^{-3} mol), was dissolved in 50 ml of ethanol–0.1 *M* aqueous sodium carbonate (1:1). The solution was cooled on an ice bath and 10 ml of 0.1 *M* *p*-methylbenzenediazonium ion was added dropwise to the solution keeping the reaction temperature at 5°C. The reaction solution was stirred overnight at that temperature. A yellowish product was collected and washed with 0.1 *M* sodium carbonate solution. The product was then purified by a preparative tlc method. The R_f of the yellowish product was 0.43 on silica gel plate developed with a mixture of *n*-butanol (30 ml), glacial acetic acid (6 ml), and water (10 ml). Melting point was 130 to 132°C.

Anal. Calcd for $C_{20}H_{23}N_3O_4$: C, 65.02%; H, 6.28%; N, 11.38%. Found: C, 64.96%; H, 6.36%; N, 11.30%.

RESULTS

p-Methylbenzenediazonium ion was allowed to react, respectively, with *N*-α-acetyltyrosine, *N*-α-acetylhistidine, *N*-α-acetyllysine, L-tyrosine, L-histidine, or L-lysine dissolved in 0.1 *M* sodium bicarbonate–sodium carbonate buffer solutions at different pH values. The concentration of the amino acid was about 15-fold in excess of the diazonium ion in order to obtain a pseudo-first-order rate expression and also to avoid disubstitution on the phenolic group of the tyrosine and the imidazole group of the histidine.

The yields of the azo-*N*-α-acetyltyrosine (7.6%) and the azo-*N*-α-acetylhistidine (7.2%) are very small at pH 8.5. However, the yields increase steadily as the pH of the medium is increased (Table 1). The production of the azo-*N*-α-acetylhistidine becomes more favorable than that of the azo-*N*-α-acetyltyrosine at pH values greater than 9.1. The yield of the azo-*N*-α-acetylhistidine is 76.5% at pH 11.4 as compared to 29.8% for the azo-*N*-α-acetyltyrosine. The same dependence of the yields on pH was also observed for the azo coupling reactions of histidine and tyrosine. The yield of the azohistidine resulting from the substitution of the diazonium ion on the imidazole group of histidine increases from 1.7% at pH 8.5 to 13.6% at pH 10.2 and 60.9% at pH 11.4. The azo coupling on the phenolic group of tyrosine also increases from 2.4% at pH 8.5 to 4.2% at pH 10.2% and to 8.8% at pH 11.4, but the increase is not as drastic as for histidine (Table 2). A pentazene, the product from the reaction of the ε-amino group on *N*-α-acetyllysine or L-lysine with two molar equivalents of the diazonium ion, was not detected. However, the presence of the equal molar concentration (2.29×10^{-3} *M*) of *N*-α-acetyllysine reduced the production of the azo-*N*-α-acetylhistidine significantly at all pH values (Table 3). Likewise the presence of the equal molar concentration of lysine reduced the production of the azo-*N*-α-acetylhistidine from 76.5 to 51.5% at pH 11.40

TABLE 1

THE AZO COUPLING YIELD OF *N*-α-ACETYLHISTIDINE (2.29×10^{-3} *M*) AND *N*-α-ACETYLTYROSINE (2.29×10^{-3} *M*) WITH *p*-METHYLBENZENEDIAZONIUM ION (1.50×10^{-4} *M*) IN BUFFER SOLUTIONS AT VARIOUS pH VALUES AT 5°C

Amino acid	pH	Yield based on diazonium ion[a] (%)
N-α-Acetylhistidine	8.50	7.2 ± 0.3
N-α-Acetylhistidine	9.10	13.4 ± 0.1
N-α-Acetylhistidine	9.50	20.6 ± 0.3
N-α-Acetylhistidine	9.80	25.7 ± 0.2
N-α-Acetylhistidine	10.20	41.3 ± 0.2
N-α-Acetylhistidine	10.75	58.1 ± 0.5
N-α-Acetylhistidine	11.40	76.5 ± 0.5
N-α-Acetyltyrosine	8.50	7.6 ± 0.0
N-α-Acetyltyrosine	9.10	12.6 ± 0.1
N-α-Acetyltyrosine	9.50	16.6 ± 0.1
N-α-Acetyltyrosine	9.80	19.6 ± 0.1
N-α-Acetyltyrosine	10.20	23.8 ± 0.2
N-α-Acetyltyrosine	10.75	28.5 ± 0.2
N-α-Acetyltyrosine	11.40	29.8 ± 0.4

[a] Average values ±SD of three experiments.

TABLE 2

THE YIELD OF THE AZO COUPLING REACTION OF THE PHENOLIC GROUP OF TYROSINE (2.29×10^{-3} *M*) AND THE IMIDAZOLE GROUP OF HISTIDINE (2.29×10^{-3} *M*) WITH *p*-METHYLBENZENEDIAZONIUM ION (1.43×10^{-4} *M*) IN BUFFER SOLUTIONS AT VARIOUS pH VALUES AT 5°C

Amino acid	pH	Yield based on the diazonium ion[a] (%)	Yield based on the amino acid[a] (%)
Tyrosine	8.5	2.4 ± 0.7	0.12 ± 0.04
Tyrosine	9.80	4.4 ± 0.4	0.28 ± 0.02
Tyrosine	10.20	4.2 ± 0.5	0.21 ± 0.04
Tyrosine	10.75	5.1 ± 0.0	0.32 ± 0.00
Tyrosine	11.40	8.8 ± 1.1	0.39 ± 0.01
Histidine	8.50	1.7 ± 0.3	0.09 ± 0.01
Histidine	9.80	5.0 ± 0.1	0.32 ± 0.01
Histidine	10.20	13.6 ± 0.9	0.67 ± 0.09
Histidine	10.75	27.8 ± 0.5	1.75 ± 0.01
Histidine	11.40	60.9 ± 0.5	3.64 ± 0.42

[a] Average values ±SD of three to six experiments.

TABLE 3

EFFECT OF *N*-α-ACETYLLYSINE (2.29×10^{-3} *M*) ON THE REACTION OF *N*-α-ACETYLHISTIDINE (2.29×10^{-3} *M*) WITH *p*-METHYLBENZENEDIAZONIUM ION (1.50×10^{-4} *M*) IN 0.1 *M* SODIUM CARBONATE–SODIUM BICARBONATE BUFFER AT DIFFERENT pH VALUES AT 5°C

pH	Amino acid	Concentration[a] (*M*)	Yield[b] (%)	k_H/k_L
9.80	*N*-α-Acetylhistidine	4.56×10^{-6}	25.7 ± 0.2	
9.80	*N*-α-Acetylhistidine	4.56×10^{-6}	13.3 ± 0.2	33.2
	+			
	N-α-Acetyllysine	5.50×10^{-4}		
10.20	*N*-α-Acetylhistidine	1.14×10^{-5}	40.4 ± 0.5	
10.20	*N*-α-Acetylhistidine	1.14×10^{-5}	22.7 ± 0.2	45.9
	+			
	N-α-Acetyllysine	1.01×10^{-3}		
10.75	*N*-α-Acetylhistidine	4.00×10^{-5}	58.1 ± 0.5	
10.75	*N*-α-Acetylhistidine	4.00×10^{-5}	40.3 ± 0.3	56.2
	+			
	N-α-Acetyllysine	1.69×10^{-3}		
11.40	*N*-α-Acetylhistidine	1.69×10^{-4}	76.5 ± 0.5	
11.40	*N*-α-Acetylhistidine	1.69×10^{-4}	61.9 ± 0.1	40.7
	+			
	N-α-Acetyllysine	2.12×10^{-3}		
Average				44.0

[a] The molar concentrations of imidazole anion of the histidine and neutral ε-amino group of the lysine were calculated based on pK_a 12.5 for the imidazole and pK_a 10.30 for the ε-amino group.

[b] Average values ±SD of three experiments.

(Table 4). This yield reduction is very similar to that (54.5%) caused by the combination of the equal molar concentration of 4-aminobutyric acid and alanine.

The effect of sodium acetate on the production of the azo-*N*-α-acetylhistidine and the azo-*N*-α-acetyltyrosine was also investigated. However, the presence of 2.29×10^{-3} *M* sodium acetate in the reaction of the diazonium ion with *N*-α-acetylhistidine or *N*-α-acetyltyrosine did not affect the azo coupling yields at all pH values studied.

The concentrations of the anions and the neutral amino groups were calculated based on pK_a values of 10.07 for the phenolic group of *N*-α-acetyltyrosine, 12.50 for the imidazole group of *N*-α-acetylhistidine (*22*, *23*), 10.30 for the ε-amino group of *N*-α-acetyllysine, 10.40 for the 4-amino group of 4-aminobutyric acid, and 9.87 for the α-amino group of alanine. The second-order rate constant for the reaction of the imidazole anion of *N*-α-acetylhistidine was compared with that for the reaction of the phenolate of *N*-α-acetyltyrosine. The relative rate constant for both reactions was calculated based on the following scheme.

The rate of the formation of the azohistidine is:

$$\text{Rate}_H = k_H \text{[imidazole anion] [diazonium ion]}. \quad (1)$$

Likewise, the rate of the hydrolysis reaction of the diazonium ion can be expressed:

$$\text{Rate}_{other} = k_{other} \text{[other reactive entities] [diazonium ion]}, \quad (2)$$

TABLE 4

EFFECT OF AMINO ACIDS (2.29×10^{-3} *M*) ON THE REACTION OF *N*-α-ACETYLHISTIDINE (2.29×10^{-3} *M*) WITH *p*-METHYLBENZENEDIAZONIUM ION (1.50×10^{-4} *M*) IN 0.1 *M* SODIUM CARBONATE (pH 11.4) AT 5°C

Amino acid added	Concentration of neutral amine[a] ($M \times 10^{-3}$)	Yield based on the diazonium ion[b] (%)
—	—	76.5 ± 0.5
Lysine	2.12,[c] 2.28[d]	51.5 ± 0.2
N-α-Acetyllysine	2.12	61.9 ± 0.1
4-Aminobutyric acid	2.08	61.9 ± 0.4
Alanine	2.22	64.0 ± 0.4
4-Aminobutyric acid and alanine	2.08 2.22	54.4 ± 0.1

[a] The concentration of the neutral amino group was calculated based on pK_a 10.30 for the ε-amino group of lysine or *N*-α-acetyllysine, pK_a 8.90 for the α-amino group of lysine, pK_a 10.40 for the amino group of 4-aminobutyric acid, and pK_a 9.87 for the α-amino group of alanine.

[b] Average values ±SD.

[c] Concentration of the ε-amino group.

[d] Concentration of the α-amino group.

where k_{other} and [other reactive entities] are the rate constant for the hydrolysis reaction of the diazonium ion and the concentration of hydroxide, respectively. The percentage yield ratio of the azohistidine to the other products from the diazonium decomposition is determined by the rate ratio of Eq. (1) to Eq. (2).

$$\frac{k_H\,[\text{imidazole anion}]}{k_{other}\,[\text{other reactive entities}]} = \frac{(\%\ \text{azohistidine})}{(100 - \%\ \text{azohistidine})}. \tag{3}$$

Similarly, the percentage yield ratio of the azotyrosine to the other products is:

$$\frac{k_T\,[\text{phenolate}]}{k_{other}\,[\text{other reactive entities}]} = \frac{(\%\ \text{azotyrosine})}{(100 - \%\ \text{azotyrosine})}. \tag{4}$$

The rate of hydrolysis accompanying the azo coupling of *N*-α-acetylhistidine is the same as that accompanying the azo coupling of *N*-α-acetyltyrosine when the reactions at the same pH are compared. Division of Eq. (3) by Eq. (4) gives:

$$\frac{k_H\,[\text{imidazole anion}]}{k_T\,[\text{phenolate}]} = \frac{(\%\ \text{azohistidine})\,(100 - \%\ \text{azotyrosine})}{(\%\ \text{azotyrosine})\,(100 - \%\ \text{azohistidine})}. \tag{5}$$

The concentrations of the anions are nearly constant throughout the reaction because the concentration of the amino acid was about 15 times larger than that of the diazonium and the reaction was run in a buffer solution. The relative rate constant, k_H/k_T, was calculated according to Eq. (5). The average k_H/k_T obtained at six different pH values is 243 (range, 164 to 277) (Table 5). The relative reactivity of the imidazole anion of *N*-α-acetylhisititidine and the ε-amino group of *N*-α-acetyllysine was also

TABLE 5

THE RELATIVE SECOND-ORDER RATE CONSTANT OF THE REACTION OF *p*-METHYLBENZENEDIAZONIUM ION WITH THE IMIDAZOLE ANION OF *N*-α-ACETYLHISTIDINE TO THAT WITH THE PHENOLATE ANION OF *N*-α-ACETYLTYROSINE AT VARIOUS pH VALUES

pH	Amino acid	[Anion][a] (*M*)	Yield (%)	k_H/k_T
8.50	*N*-α-Acetyltyrosine	6.00×10^{-5}	7.6	247
8.50	*N*-α-Acetylhistidine	2.29×10^{-7}	7.2	
9.10	*N*-α-Acetyltyrosine	2.22×10^{-4}	12.6	262
9.10	*N*-α-Acetylhistidine	9.11×10^{-7}	13.4	
9.50	*N*-α-Acetyltyrosine	4.86×10^{-4}	16.6	277
9.50	*N*-α-Acetylhistidine	2.29×10^{-6}	20.6	
9.80	*N*-α-Acetyltyrosine	8.00×10^{-4}	19.6	249
9.80	*N*-α-Acetylhistidine	4.56×10^{-6}	25.7	
10.20	*N*-α-Acetyltyrosine	1.32×10^{-3}	23.8	260
10.20	*N*-α-Acetylhistidine	1.14×10^{-5}	41.3	
10.75	*N*-α-Acetyltyrosine	1.89×10^{-3}	28.5	164
10.75	*N*-α-Acetylhistidine	4.00×10^{-5}	58.1	
Average				243

[a] The molar concentrations of the imidazole anion of the histidine and the phenolate anion of the tyrosine were calculated based on pK_a 12.5 for the imidazole and pK_a 10.07 for the phenolic group.

calculated based on Eq. (5). The results are shown in Table 3. The average k_H/k_L obtained at four different pH values is 44 (range, 33 to 56).

DISCUSSION

Benzenediazonium ions are known to be mild electrophiles which undergo electrophilic substitution reactions with aromatic compounds activated by electron-donating substituents such as hydroxy and amino groups (*24*). They were reported to react with amino compounds (*24*) and heterocyclic compounds containing an imidazole moiety (*25*, *26*). However, the relative reactivity of isolated amino acids, tyrosine, histidine, and lysine has not been reported.

The azo coupling yields of tyrosine and histidine increase with *p*-methylbenzenediazonium ion steadily as the pH of the medium is increased (Tables 1 and 2). The plots of the logarithm of the azo coupling yields of histidine and tyrosine against pH values of the medium show a steady increase in the yields up to pH 11.4 (Fig. 1), indicating that the reactive species for the azo coupling reaction are the imidazole anion of histidine and the phenolate of tyrosine, respectively.

Mechanisms of azo coupling of phenol derivatives (naphthols), imidazoles, and amines have been studied quite extensively. Brown *et al.* (*25*, *26*) found that the rate of azo coupling of diazotized sulfanilic acid with imidazole is faster at higher pH. They concluded that the rate data could be explained best by considering the imidazole anion as the reactive species. Wolff and Covelli (*23*) estimated that the reactivity of the

imidazole anion of histidine to be about 10^{10} times faster than imidazole in the iodination of histidine. Wittwer and Zollinger (*27*) reported that the reactive entities for azo coupling reaction of benzenediazonium ions with naphthol derivatives are the diazonium ions and the corresponding naphtholates. The reactivity of the phenolates (*28*) to the diazonium ion was reported to be 10^{10} times greater than that of the phenol, and consequently the formation of the azo product must be favored at higher pH value. However, the concentration of the diazonium ion decreases by 10^2 when the pH is increased by 1 unit at higher pH values due to the diazonium–diazotate equilibrium reported by Wittwer and Zollinger (*27*) and Lewis and Suhr (*29*). The dependence of the production of the azo-*N*-α-acetylhistidine and the azo-*N*-α-acetyltyrosine on pH (Table 1) can be explained by the mechanism with parallel competing reactions proposed by Wittwer and Zollinger (*27*). However, unlike the azo coupling of diazotized *m*-

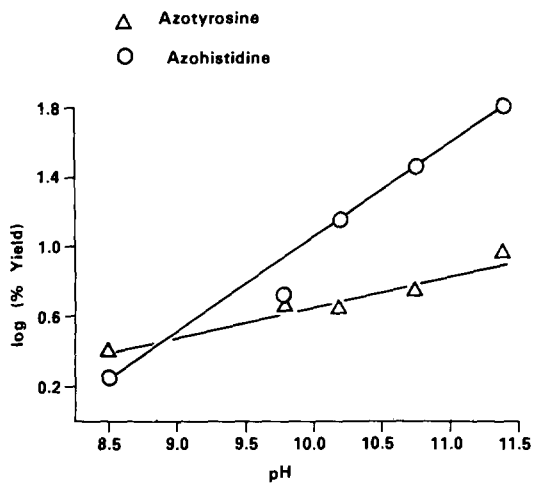

FIG. 1. Plots of pH versus logarithm of azo coupling yields from the reaction of histidine (2.29×10^{-3} *M*) and tyrosine (2.29×10^{-3} *M*) with *p*-methylbenzenediazonium ion (1.43×10^{-4} *M*) at 5°C.

sulfanilic acid ($pK_1 + pK_2/2 = 10.84$) with 2-naphthol-6-sulfonic acid ($pK_a = 8.94$), for which the maximum azo coupling rate was obtained at pH 10, high pK_a values of the phenolic group (10.07) of tyrosine and the imidazole group (12.5) of histidine as well as the high $(pK_1 + pK_2)/2$ value of *p*-methylbenzenediazonium ion (12.50) make the percentage yields increase with respect to pH up to 11.4.

Comparing *N*-α-acetylated histidine and tyrosine with histidine and tyrosine, one sees that the azo coupling yields of *N*-α-acetylated compounds are much higher than those of unacetylated compounds, in agreement with the previous report (*17*) that the α-amino group reduced the azo coupling at the phenolic group and the imidazole group of the corresponding amino acids.

The azo coupling of *N*-α-acetyllysine or lysine did not produce a detectable amount of a uv-absorbing bis(benzenediazo) amino acid at all pH values studied. However, competition studies showed that the azo coupling yield of *N*-α-acetylhistidine was reduced in the presence of 2.29×10^{-3} *M* *N*-α-acetyllysine and lysine, respectively, at

all pH values studied. This finding indicates that lysine or *N*-α-acetyllysine can compete with *N*-α-acetylhistidine for the diazonium ion, although they do not form products with an absorption maximum above 320 nm. This result is different from that previously reported (*8*, *9*), namely, that azo coupling of the ε-amino group of lysine produced the corresponding pentazene with an absorption maximum at 378 nm. The discrepancy seems to be caused by the differences in the relative concentration of the amino acid to the diazonium ion. Under our conditions the concentration of the amino acid was 15 times higher than that of diazonium ion in order to get a pseudo-first-order rate expression. Consequently the chance of disubstitution on the ε-amino group was much reduced. *N*-α-acetyllysine has two reactive functional groups, ε-amino and carboxylate groups, while lysine has an α-amino group in addition to the above functional groups. The contribution of each functional group to the yield reductions was studied by allowing *N*-α-acetylhistidine to react with the diazonium ion in the presence of sodium acetate, alanine, and 4-aminobutyric acid. It was found that the equal molar concentration of sodium acetate did not reduce the yield of the azo-*N*-α-acetylhistidine at all pH values studied, while 4-aminobutyric acid reduced the yield to the same extent as *N*-α-acetyllysine did at pH 11.4. Alanine is slightly less effective than 4-aminobutyric acid in reducing the azo coupling yield of *N*-α-acetylhistidine, and the combination of the equal molar concentration of alanine and 4-aminobutyric acid can account for the reduced yield of the azo-*N*-α-acetylhistidine due to lysine.

In summary, the reactive entities undergoing the azo coupling reaction are the imidazole anion of *N*-α-acetylhistidine, the phenolate anion of *N*-α-acetylhistidine, and the ε-amino group of *N*-α-acetyllysine. The phenolate anion and the ε-amino group are less reactive than the imidazole anion. The azo coupling yield of the amino acids is determined by competition between the reaction of the reactive functional groups of the amino acids with the diazonium ion and the hydrolysis reaction of the diazonium ion. A maximum coupling yield is, therefore, obtained when the pH of the medium is at the vicinity of the pK_a of the reactive group. The studies (Tables 1 and 3) on the model amino acids suggest that the selective modification of protein at tyrosine, histidine, or lysine sites cannot be achieved by the azo coupling reaction if the amino acid residues are in the same concentration.

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