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# The Reactivity of Imidazole Nitrogens in Histidine to Alkylation

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Copper(II)–histidine complex was allowed to react at pH 6.0–6.1 at 22°C with bromoacetic acid. The reaction was followed by means of amino acid analysis of the histidine and $N^{im}$-carboxymethylhistidine derivatives. The results of the alkylation study indicate that the nucleophilic, active histidine molecule is coordinated to the copper(II) ion through the amino nitrogen and a carboxylate oxygen with the imidazole group turned away from the copper. This model of copper-bound histidine permitted the determination of the intrinsic nucleophilic activity of the imidazole nitrogens through their respective rate constants for alkylation. The *tele*-nitrogen is three times more reactive than the *pros*-nitrogen in the histidine and in the *pros*-carboxymethylhistidine–*tele*-carboxymethylhistidine systems. The carboxymethylation of copper(II)–histidine and bovine pancreatic ribonuclease have some analogies, which suggest that in *pros*-carboxymethylhistidine-119 ribonuclease the carboxylate unit of the alkylated histidine residue points into the active site.

## INTRODUCTION

One of the more common ways to modify a protein or enzyme chemically is alkylation.[2] Of the alkylating agents used to date many are α-haloacetates or derivatives thereof. The latter reagents react with nucleophiles within proteins (*1*). The acid hydrolysis of carboxymethylated proteins yields carboxymethyl (CM) amino acids (*2, 3*). The side chain of histidine (His), bearing the imidazole ring, has two nucleophilic sites, N-1(*pros*[3]) and N-3(*tele*). The importance of histidine in catalysis by several enzymes (*4, 5*) and the relationship of imidazole ring nucleophilicity to its ability as a base catalyst prompted us to investigate the inherent nucleophilic reactivity of the two imidazole nitrogens.

Hofmann (*6*) reports that electronegative substituents on imidazole direct methylation to their adjacent nitrogen. 4(5)-Methylimidazole when treated with methyliodide yields a 1/2.2 ratio of *pros*-N alkylation and *tele*-N alkylation. Korman and Clarke (*7*) and Heinrikson et al. (*8*) do not indicate whether one of the imidazole nitrogens of histidine shows any preference for α-haloacetate. In poly-L-histidine the reactivity of

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[2] Several examples cited: R. M. S. Smellie, Ed., "Chemical Reactivity and Biological Role of Functional Groups in Enzymes," Academic Press, New York, 1970.

[3] See *Eur. J. Biochem.*, 27, 201 (1972) for full discussion on nomenclature and abbreviations of histidine and its derivatives. The abbreviations are as follows: *pros*-carboxymethylhistidine, previously known as N-1 CM-histidine, His($\pi$ CM); *tele*-carboxymethylhistine, previously known as N-3 CM-histidine, His($\tau$ CM); and dicarboxymethylhistidine, His($\pi$, $\tau$ $CM_2$).

the two nitrogens is equivalent (*9*), while in bovine pancreatic ribonuclease (EC 2.7.7.16) the *pros*-N of His-119 is much more reactive than the *tele*-N of His-12 (*10*), the relative reactivity being strongly dependent on the pH during the alkylation. Carboxymethylation of the imidazole nitrogens in $N^{\alpha}$-acetyl-L-histidine (*11*) is in agreement with the generalization of Hofmann (*6*). Crestfield et al. (*11*), however, argue that the greater reactivity of the *tele*-N over the *pros*-N is due to steric hindrance of the *pros*-N by the $N^{\alpha}$-acetyl group. The difference in reactivity also may be due to hydrogen bonding of the *pros*-N to the α-amino proton (*12–14*).

In order to determine the intrinsic reactivity of the imidazole nitrogens, it was necessary to use histidine in a form where the risk of imidazole side-chain interaction with the α-amino or the α-carboxyl (*15*) was absent. Copper (II)–histidine chelate was selected, although its structure is not clear. The proposed structures include the following: (a) α-amino and α-carboxylate ion as the ligands and the imidazole ring free in solution (*16–18*), (b) imidazole *pros*-N and α-carboxylate ion as the ligands and the α-amino group pointing away from the metal ion (*19–21*), and (c) imidazole *pros*-N and α-amino as the ligands and the α-carboxylate ion free (*22*, *23*). Recent studies of Sarkar and Kruck (*23–26*) indicate that several structures may exist simultaneously in solution. These include complex (a), complex (b), and a complex where the α-amino group, the α-carboxyl, and the *pros*-N occupy three adjacent corners of the octahedral coordination sites of copper (*27*). The relative amount of the different species of histidine–copper(II) complex varies with the pH of the solution and the histidine–copper ratio (*23–26*, *28*). The carboxymethylation results we report below are consistent with the model where the α-amino and the α-carboxylate ion are bound to the metal ion, and this model was used for the calculation of the relative reactivity of the two imidazole nitrogens.

## EXPERIMENTAL

### *Bulk Preparation of Copper(II)–Histidine*

L-Histidine hydrochloride monohydrate (Aldrich), 10.5 g (50 mmoles), was dissolved in 80 ml of water. Sodium hydroxide, 0.1 *M*, 40 ml, and an equal volume of distilled–deionized water containing 11.2 g of $CuSO_4 \cdot 5H_2O$ (45 mmoles) were added. After stirring the solution for 2 hr at room temperature and concentrating, a dark blue cupric histidine complex was isolated by filtration, washed with methanol, and dried *in vacuo* over phosphorous pentoxide and sodium hydroxide pellets (8.7 g). The blue product is believed to be [Cu(His)($H_2O$)(OH)] (*29*). If the preparation of the complex is carried out with 2 equiv histidine to 1 equiv copper salt, a dark blue-green precipitate is obtained. This product is probably a mixture of the monohistidine–copper(II) complex and the bishistidine–copper(II) complex (*26*, *28*, *29*).

### *Bulk Preparation of CM-Histidines*

Copper(II)–histidine (8.7 g, 34.3 mmoles) was dissolved in 400 ml 0.2 *M* monobasic sodium phosphate ($NaH_2PO_4$). Bromoacetic acid (Eastman Chemicals), 27.8 g (0.2 moles), was added and the pH of the solution was adjusted to pH 6 with 0.2 *M* dibasic

sodium phosphate. After 8 days at room temperature the blue solution turned brown, and some brown material precipitated out. The precipitate is believed to be copper bromide because of the color (*30*), the melting point (above 300°C), and its being ninhydrin and iodine negative on thin-layer chromatography. The mother liquor was taken down to dryness, and the residue was redissolved in 2 *N* HCl. After the acid solution was shaken with Chelex 100 (Bio-Rad, sodium form 200–400 mesh), the chelating polymer was removed and washed with 0.1 *N* HCl. The CM-histidine preparation was concentrated and then desalted on a Bio-Gel P-2 column (2 × 33 cm). The column was monitored by spotting aliquots of the collected fractions on thin-layer

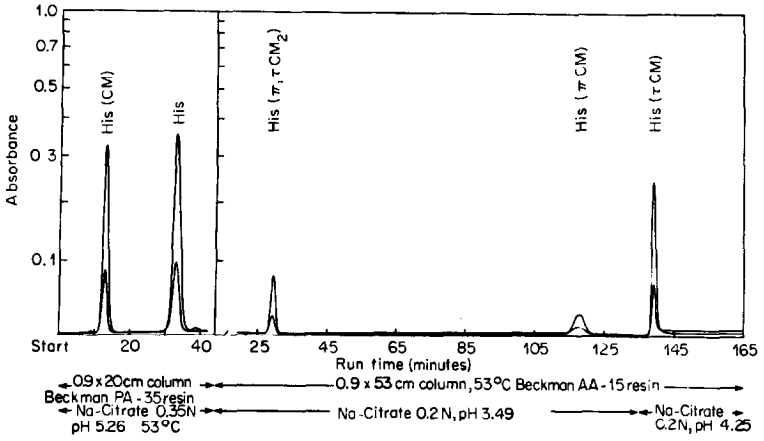


FIG. 1. Amino acid analysis of carboxymethylhistidine, where His(CM) refers to all three carboxymethylhistidines, His($\pi$, $\tau$ $CM_2$) refers to dicarboxymethylhistidine, His($\pi$ CM) refers to *pros*-carboxymethylhistidine or N-1 CM-His(2, 3, 11), and His($\tau$ CM) refers to *tele*-carboxymethylhistidine or N-3 CM-His(2, 3, 11).

plates (Eastman Chromagram Sheet 6061) and staining with ninhydrin, reading the conductivity (Radiometer, CDM2e) and optical density at 220 nm (Beckman DU2 Spectrophotometer). The His(CM) composition of the Bio-Gel P-2 peptide peak was essentially independent of the elution position. The front position of the void peak had approximately a 10% enrichment of dicarboxymethylhistidine over the tail position of that peak. Lyophilization of the peptide peak yielded 0.07 equiv *pros*-carboxymethylhistidine (His($\pi$ CM)), 0.23 equiv *tele*-carboxymethylhistidine (His($\tau$ CM)), 0.09 equiv dicarboxymethylhistidine (His($\pi$, $\tau$ $CM_2$)), and 0.61 equiv histidine. These values are based on amino acid analyses (Beckman Amino Acid Analyzer Model 121C, Fig. 1) of the freeze-dried samples, assuming that the color factors for histidine and its CM derivatives are equivalent. Isolation of the individual His(CM) derivatives may be achieved by the ion-exchange method described by Crestfield et al. (*11*).

### *Kinetic Analysis of the Carboxymethylation Reaction*

Cupric histidine (151.7 mg, 600 $\mu$moles) was dissolved in 15 ml 0.2 *M* $NaH_2PO_4$. Bromoacetic acid, 1.39 g (10 mmoles), in 1 ml of water was added, and the pH was adjusted to 6.1 with 8.5 ml 1 *M* NaOH. A 1-ml aliquot was removed immediately, and

subsequently at 8- and 16-hr intervals for 1 week. These aliquots were diluted with 1 ml 1 *N* HCl, shaken with Chelex 100, and passed through a fine sintered-glass funnel. The filtrate was lyophilized, redissolved in the pH 2.2 amino acid analyzer sample buffer (*31*), and analyzed on the Amino Acid Analyzer (Beckman, 121C).

Figure 2 illustrates the simplest kinetic model which accounts for the products obtained during the alkylation studies. Compound 1 is histidine, 2 is His($\pi$ CM), 3 is His($\tau$ CM), and 4 is His($\pi$, $\tau$ $CM_2$). If $X_i$ is the concentration of compound $i$ at

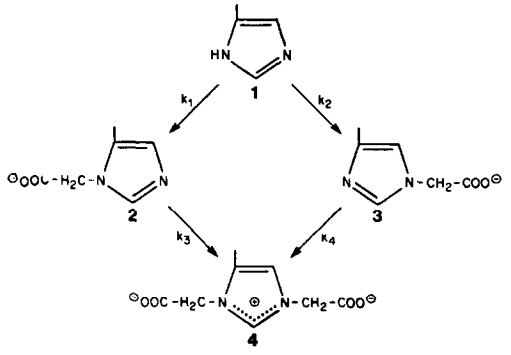


FIG. 2. Carboxymethylation of (1) histidine, and (2 and 3) *pros*-carboxymethylhistidine– and *tele*-carboxymethylhistidine–copper(II) complex, respectively. Only the imidazole portion of the complexes is shown.

time $t$, and $X_0$ is the initial concentration of histidine in the reaction mixture, then the following four equations describe $X_i$ in terms of $X_0$ and the pseudo-first-order rate constants, $k_j$, as defined in Fig. 2 (first-order conditions were assumed since bromoacetate is in 17-fold excess over histidine):

$$X_1 = X_0 e^{-(k_1+k_2)t}, \tag{1}$$

$$X_2 = X_0 \frac{k_1}{k_3-(k_1+k_2)}(e^{-(k_1+k_2)t} - e^{-k_3 t}), \tag{2}$$

$$X_3 = X_0 \frac{k_2}{k_4-(k_1+k_2)}(e^{-(k_1+k_2)t} - e^{-k_4 t}), \tag{3}$$

$$X_4 = X_0\left\{1 + \frac{k_1}{k_3-(k_1+k_2)}e^{-k_3 t} + \frac{k_2}{k_4-(k_1+k_2)}e^{-k\ t} + \frac{k_1k_3+k_2k_4-k_3k_4}{[k_3-(k_1+k_2)][k_4-(k_1+k_2)]}e^{-(k_1+k_2)t}\right\}. \tag{4}$$

Clearly,

$$\sum_1^4 X_i = X_0. \tag{5}$$

This allows the amino acid analyses to be used for the determination of the rate constants since,

$$\frac{X_i}{X_0} = X_i\left(\sum_1^4 X_i\right)^{-1}, \tag{6}$$

assuming the ratio of the integral of a His(CM) peak over the sum of the integrals of all the His(CM) peaks and the His peak represents the composition of the reaction mixture. Thus, the plot of Eq. (7) (obtained from Eqs. 1 and 6),

$$\log[X_1(\sum X_i)^{-1}] = \frac{-1}{2.303}(k_1 + k_2)t \tag{7}$$

would yield the sum of $k_1$ and $k_2$. After Taylor expansion of Eqs. (2) and (3) (assuming that the exponential terms are less than 1), these equations become

$$X_2(\sum X_i)^{-1} = k_1 t, \tag{8}$$

$$X_3(\sum X_i)^{-1} = k_2 t, \tag{9}$$

respectively. The initial slope of the plots of $X_2/\sum X_i$ and $X_3/\sum X_i$ versus time yields $k_1$ and $k_2$, respectively. Alternatively, Eqs. (2) and (3) may be rewritten as

$$\frac{X_2}{X_0} = \frac{k_1}{k_3 - (k_1 + k_2)} e^{-k_3 t}\{e^{[k_3 - (k_1 + k_2)]t} - 1\}, \tag{10}$$

$$\frac{X_3}{X_0} = \frac{k_2}{k_4 - (k_1 + k_2)} e^{-k_4 t}\{e^{[k_4 - (k_1 + k_2)]t} - 1\}, \tag{11}$$

respectively. If the terms $[k_3 - (k_1 + k_2)]t$ and $[k_4 - (k_1 + k_2)]t$ respectively are very much less than 1, then the Taylor expansion of that portion of Eqs. (10) and (11) carrying these exponentials and incorporating Eq. (6) yields

$$X_2(\sum X_i)^{-1}\frac{1}{t} = k_1 e^{-k_3 t}, \tag{12}$$

$$X_3(\sum X_i)^{-1}\frac{1}{t} = k_2 e^{-k_4 t}, \tag{13}$$

respectively. A plot of the logarithm of the left side of Eqs. (12) and (13) versus time would yield $k_3$ and $k_4$ from the slope and $k_1$ and $k_2$ from the intercept of the ordinate.

The equations with single exponentials (Eqs. 1, 12, and 13) using the amino acid analysis data were also solved by a least-squares fit program on a Wang Calculator (600 Series).

## RESULTS

### *Bulk Preparation of His(CM)*

The preparation of the various His(CM) compounds described above, as markers for the analysis of CM-proteins, has some advantages over those previously described (*9*, *11*). The latter require acid hydrolysis, an unnecessary step in the present preparation. The recovery of the copper(II)–His(CM) represents 75% of the original histidine used. A disadvantage is the time necessary to obtain the products.

Goren and Barnard (*9*, *10*) obtained a radioactive peak, peak 12 (*3*), at the position of isoleucine from the acid hydrolysis of $C^{14}$-CM-ribonuclease or $C^{14}$-CM-poly-L-histidine. The evidence offered suggested that this peak was derived from His($\pi$, $\tau$ $CM_2$). The present preparation of His(CM) did not result in appearance of a ninhydrin-positive

peak in this position. Peak 12 (*3*), which was not normally seen in the acid hydrolysate of CM-poly-L-histidine, could be detected when this polymer was hydrolyzed with other protein (*9*). The identification of peak 12 is still under investigation.

*Carboxymethylation of Copper(II)–Histidine Complex*

Table 1 shows the data of the amino acid analysis expressed in terms of the rate equations. These data, when plotted in the manner described in the Experimental

TABLE 1

RELATIVE AMOUNTS OF HISTIDINE AND CM-HISTIDINE DERIVATIVES OBTAINED WITH INCREASING TIME OF REACTION[a]

| Time (hr) | $X_1(\sum X_i)^{-1}$ | $X_2(\sum X_i)^{-1}$ | $X_3(\sum X_i)^{-1}$ | $X_4(\sum X_i)^{-1}$ |
|---|---|---|---|---|
| 0 | 100.0 | 0 | 0 | 0 |
| 8 | 99.3 | 0 | 0.7 | 0 |
| 24 | 95.0 | 1.23 | 3.78 | 0 |
| 32 | 93.5 | 1.67 | 4.85 | 0 |
| 48 | 88.6 | 2.43 | 7.97 | 1.01 |
| 56[b] | 93.54 | 1.23 | 4.48 | 0.75 |
| 72 | 84.9 | 2.81 | 10.37 | 1.91 |
| 80 | 83.2 | 2.84 | 11.38 | 2.54 |
| 104 | 78.9 | 3.05 | 14.11 | 3.92 |
| 120 | 78.6 | 2.86 | 14.23 | 4.34 |
| 128 | 77.6 | 2.79 | 14.91 | 4.67 |
| 144 | 79.3 | 2.28 | 13.50 | 4.90 |
| 152 | 73.9 | 2.46 | 15.65 | 8.02 |
| 168 | 73.8 | 2.83 | 18.00 | 5.42 |

[a] The relative amounts of histidine and its analogues are expressed in percentile units. $X_1$, $X_2$, $X_3$, $X_4$ are the concentrations of histidine, *pros*-carboxymethylhistidine, *tele*-carboxymethylhistidine, and dicarboxymethylhistidine respectively. $\sum X_i$ is the sum of the concentrations of histidine and its carboxymethyl derivatives.

[b] Data of this analysis were not used in the least-squares fit program or in Fig. 3.

section (Fig. 3), yield the respective pseudo-first-order rate constants (Table 2). The following points add to the credibility of the calculated rate constants: (i) the agreement of the rate constants determined by several independent methods; (ii) the coefficient of correlation of the data in fitting the least-squares fit program; and (iii) the ability to calculate $X_4/X_0$ (Table 3), using the rate constants in Eq. (4). The second-order rate constants are $k_1' = 0.34 \times 10^{-6}\ M^{-1}\ \text{sec}^{-1}$, $k_2' = 1.02 \times 10^{-6}\ M^{-1}\ \text{sec}^{-1}$, $k_3' = 6.36 \times 10^{-6}\ M^{-1}\ \text{sec}^{-1}$, and $k_4' = 2.40 \times 10^{-6}\ M^{-1}\ \text{sec}^{-1}$. By means of these second-order rate constants, the composition of the bulk preparation of His(CM) after 16 hr of reaction and after 192 hr of reaction (reported in the Experimental section) could be calculated within the error of reproducibility of the amino acid analyzer.

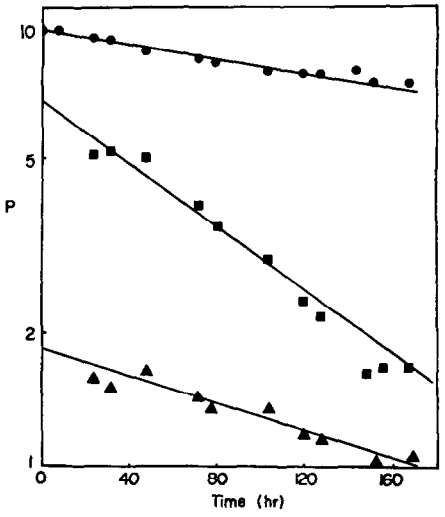


FIG. 3. Plot of log $P$ versus time, where (●—●) $P = [X_1(\sum X_t)^{-1}] \times 10^1$. (■—■) $P = [X_2(t \sum X_t)^{-1}] \times 10^4$ hr$^{-1}$, and (▲—▲) $P = [X_3(t \sum X_t)^{-1}] \times 10^3$ hr$^{-1}$. $X_1$, $X_2$, and $X_3$ are the concentrations of histidine, *pros*-carboxymethylhistidine, and *tele*-carboxymethylhistidine, respectively. $\sum X_t$ is the sum of the concentrations of histidine and its carboxymethyl derivatives.

TABLE 2

FIRST-ORDER RATE CONSTANTS FOR THE CARBOXYMETHYLATION OF HISTIDINE AND CARBOXYMETHYLHISTIDINE[a]

| Equation: | (7) | (8) | (9) | (12) | (13) |
|---|---|---|---|---|---|
| ($\times 10^3$ hr$^{-1}$) | | | | | |
| $k_1$ | 1.98(1.83)[b] | 0.50 | — | 0.72(0.72)[c] | — |
| $k_2$ | | — | 1.50 | — | 1.85(1.81)[d] |
| $k_3$ | — | — | — | 9.56(9.34)[c] | — |
| $k_4$ | — | — | — | — | 3.52(3.53)[d] |

[a] The rate constants $k_1$ and $k_2$ are for the carboxymethylation of the *pros*-nitrogen and the *tele*-nitrogen, respectively. The rate of dicarboxymethylhistidine formation from *pros*-carboxymethylhistidine- and *tele*-carboxymethylhistidine–copper(II) complex is $k_3$ and $k_4$, respectively. Figures in parentheses were determined from the respective equation when the data were submitted to a least-squares fit program.

[b] Least-squares fit of Eq. (1). Coefficient of correlation was 0.98.

[c] Coefficient of correlation was 0.98.

[d] Coefficient of correlation was 0.93.

## DISCUSSION

### *Structure of Copper(II)–Histidine*

Prolonged treatment of histidine with bromoacetate yields $N^{\alpha}$-carboxymethyl histidine ($N^{\alpha}$-CM-His), histidine glycollic acid ester (His-*O*-CM), and the imidazole carboxymethylated products (*1–3*). The amino group of $N^{\alpha}$-CM-His is secondary,

TABLE 3

RELATIVE AMOUNT OF DICARBOXYMETHYLHISTIDINE FORMED WITH INCREASING TIME OF REACTION

| Time (hr) | $X_4/X_0$ calculated[a] | $X_4/X_0$ found[b] |
|---|---|---|
| 0 | 0 | 0 |
| 8 | −0.002 | 0 |
| 24 | 0.002 | 0 |
| 32 | −0.001 | 0 |
| 48 | 0.004 | 0.0101 |
| 56 | 0.008 | 0.0075 |
| 72 | 0.021 | 0.0191 |
| 80 | 0.025 | 0.0254 |
| 104 | 0.042 | 0.0392 |
| 120 | 0.05 | 0.0434 |
| 128 | 0.054 | 0.0467 |
| 144 | 0.07 | 0.049 |
| 152 | 0.074 | 0.0802 |
| 168 | 0.099 | 0.0542 |

[a] Using Eq. (4), where $k_2 = 1.50 \times 10^{-3}$ hr$^{-1}$, $k_1 = 0.50 \times 10^{-3}$ hr$^{-1}$, $k_4 = 3.53 \times 10^{-3}$ hr$^{-1}$, and $k_3 = 9.34 \times 10^{-3}$ hr$^{-1}$.

[b] $X_4/X_0 = X_4(\sum X_i)^{-1}$. This ratio was found on amino acid analysis of the carboxymethylated product. $X_4$ is the concentration of dicarboxymethylhistidine, $X_0$ is the concentration of histidine at the start of the reaction, and $\sum X_i$ is the sum of the concentrations of histidine and its carboxymethyl derivatives.

and its reaction with ninhydrin would yield the proline type of product. On amino acid analysis proline is recorded with the 440-nm detection printout. The basicity of $N^{\alpha}$-CM-His should be equivalent to or less basic than one of the mono-$N^{im}$-CM-histidines ($N^{\alpha}$-CM-lysine is less basic than $N^{\varepsilon}$-CM-lys (*3*)). It follows, therefore, that if $N^{\alpha}$-CM-His was present in the alkylation product either a 440-nm peak would be recorded in the vicinity of the $N^{im}$-CM-histidines or the printout of one of the latter analogues would have the wrong 570 nm/440 nm printout ratio.

His-*O*-CM would be expected to have the same basicity as histidine. The additional bulk of this CM-His analogue would, however, retard the elution of the compound off the pH 5.26 column (Fig. 1).

The amino acid analysis of the alkylation reaction did not show a 440 nm peak, an anomolous 570 nm/440 nm printout ratio, a ninhydrin peak after histidine (except for a small ammonia peak), or tailing of the histidine peak. This inability to demonstrate $N^{\alpha}$-CM-His and His-*O*-CM in the bromoacetate–Cu(II)–histidine reaction at pH 6.0 and 22°C leads one to conclude that the complex being alkylated has the α-amino group and the α-carboxyl group of histidine bound to the copper ion (a nucleophile bound to a metal ion loses its nucleophilicity).

If the imidazole ring of histidine was also bound to the copper ion—that is, if histidine

were acting as a tridentate ligand—then the nitrogen of the imidazole ring not participating in the complex would act as a poor nucleophile. The nucleophilicity of the latter nitrogen would be very small since the free electrons of this nitrogen would have been withdrawn into the copper imidazole bonding system. Indirect evidence for this is available from the $pK_a$ of imidazole, 14.52 (*32*), and of imidazole bound to copper(II), 11–12 (*20*). The poor nucleophilicity of imidazole nitrogen when the second nitrogen is bound to copper ion, and the fact that both His($\pi$ CM) and His($\tau$ CM), and His($\pi$, $\tau$ $CM_2$) (Fig. 1, Table 1) are obtained during the alkylation reaction leads to the conclusion that histidine does not act as a tridentate ligand when being alkylated at pH 6.0 and 22°C.

It may be proposed that the histidine–Cu(II) complex exists in an equilibrium between the bidentate form described above and other forms where the imidazole ring is unavailable for alkylation (*20, 23, 27, 33*). Defining the equilibrium constant between the bidentate and the unreactive forms of copper(II)–histidine as

$$K = C_u/C_b, \tag{14}$$

where $C_b$ and $C_u$ are the concentrations of the respective forms, the kinetic equations (1–4) are modified by substitution of $X_0/(1 + K)$ for $X_0$. Thus Eq. (1) becomes Eq. (15) in the form used for the least-squares fit program:

$$X_1\left(\sum_1^4 X_i\right)^{-1} = \left(\frac{1}{1+K}\right) e^{-(k_1+k_2)t}. \tag{15}$$

The value of $1/(1 + K)$ is 0.976. This value was assumed to be unity since it is within the error of measurement of reaction products. However, the experimental error does not exclude the possibility that the relative amount of unreactive forms of copper(II)–histidine is greater than the 2.5% calculated from $K = 0.024$.

### *Intrinsic Activity of Imidazole Nitrogens of Histidine*

The rate of carboxymethylation of the *tele*-N is about three times the rate of carboxymethylation of the *pros*-N, regardless of whether the opposite nitrogen is already carboxymethylated or not (compare $k_2$ to $k_1$ and $k_3$ to $k_4$). Examining compound 1 behavior (Fig. 2), one might suspect that if the *tele*-N were the secondary amine, that is the major tautomer form, then the ratio of $k_2$ to $k_1$ would approximate the one obtained. This view is based on the observation that the nitrogen of diallylamine, $pK_a = 9.29$ (*32*), is more nucleophilic than the nitrogen of pyridine, $pK_a = 5.14$ (*32*). In the mono-CM histidine derivatives only the pyridine nitrogen is available for alkylation. The intrinsic reactivity of the *tele*-N, however, remains three times that of the *pros*-N. The distribution of electrons about the imidazole ring cannot, therefore, be the basis for the difference in the alkylation rates of the two nitrogens.

One feature common to both compound 1 and compound 3 (Fig. 2) is the adjacence of the alkyl side chain to the *pros*-N. The assumption is made that, in order for the *pros*-N to attack the $\alpha$-carbon of the bromoacetate, more energy is required than for the same attack by the *tele*-N. This assumption is based on possible interference by the alkyl side chain. The increased transition energy would be manifested by a lower rate constant: that is, $k_1$ versus $k_2$ and $k_4$ versus $k_3$. The ratio of the reactivity of the

two nitrogens would therefore be independent of what other substituents are on the imidazole ring.

The rate of formation of His($\pi$, $\tau$ $CM_2$) from either of the mono-CM forms (compound 2 and 3, Fig. 2) is six to seven times greater than that of the same nitrogen in the first carboxymethylation ($k_4$ versus $k_1$ and $k_3$ versus $k_2$). The increased nucleophilicity of the nitrogens may be due to the electron inductive effect that the carboxymethyl group has on the imidazole ring.

*Carboxymethylation of Bovine Pancreatic Ribonuclease*

In bovine pancreatic ribonuclease, bromoacetate reacts with histidine-119 at the *pros*-N position (*10*) and with histidine-12 and histidine-105 at the *tele*-N position (*4*, *34*). In addition, Goren and Barnard (*4*, *10*) and Bello and Nowoswiat (*34*) have shown that the *tele*-N of His($\pi$ CM)-119 is also carboxymethylated, the rate of carboxymethylation of the *tele*-N of histidine-105 being approximately equal to that of the *tele*-N of His($\pi$ CM)-119 (*34*). Based on the relative reactivity of the *tele*-N of histidine when the *pros*-N is carboxymethylated or not ($k_3 > k_2$), it may be assumed that the relative rate of formation of His($\pi$, $\tau$ $CM_2$)-119 should be greater than the rate of His($\tau$ CM)-105 formation. Since the $pK_a$ of the imidazole ring of His-105 is normal (6.7 (*35*)), its nucleophilicity should be normal. It follows that the carboxymethylation of the *tele*-N of His($\pi$ CM)-119 is inhibited.

Only that imidazole-nitrogen of histidine-119 which points into the active site is available for reaction with bromoacetate. Normally, this is the *pros*-N. When this nitrogen is carboxymethylated, histidine-119 has the option of leaving the carboxymethyl group in the active site or flipping over and thus allowing the *tele*-N to enter the active site. The apparently reduced tendency for alkylation of the *tele*-N indicates that His($\pi$ CM)-119-ribonuclease has the carboxylate pointing into, and the *tele*-N away from, the active site (*36*).

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