Free Rad. Res. Comms., Vol. 19 Supplement, pp. S55–S61
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ON THE SUBSTITUENT EFFECT OF *tert*-BUTYLAMINOXYL GROUP FOR THE ACID-DISSOCIATION EQUILIBRIA OF α-SUBSTITUTED ACETIC ACIDS

FUMIO MORIYA*, SHOKO TANIMOTO, and KEISUKE MAKINO⁺

Department of Chemistry, Kobe University of Commerce, Nishi-ku, Kobe 651-21, Japan, and ⁺Department of Polymer Science and Engineering, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606, Japan

α-Substituted acetic acids with *tert*-butylaminoxyl groups, *t*-BuN(O·)—CHX—COOH, were produced as spin adducts in γ-irradiated aqueous solutions of amino acids and peptides with a spin trap, 2-methyl-2-nitrosopropane. The spin adducts were isolated and characterized by means of high-performance liquid chromatography and EPR spectroscopy. Their EPR spectra in acidic region changed reversibly with pH through the acid-dissociation of the carboxyl groups. The spectra at pH around the p*K* value were weighted averages of acid forms and conjugate base forms. The pK_{COOH} values for the dissociation were determined to be 3.0, 3.2, 2.0, 1.8, and 1.6 for $t\text{-BuN(O}\cdot)\text{—CH}_2\text{COOH}$, $t\text{-BuN(O}\cdot)\text{—CH(CH}_3\text{)COOH}$, $\text{NH}_3^+\text{CH}_2\text{CONHCH(COOH)N(O}\cdot)\text{—}t\text{-Bu}$, and $\text{NH}_3^+\text{—}^*\text{CH(CH}_3)\text{—CONH—}^*\text{CH(COOH)N(O}\cdot)\text{—}t\text{-Bu}$ [a pair of diastereomers], respectively. The electron-withdrawing character of the aminoxyl group is responsible for the observation that the pK_{COOH} values are lower than those of $\text{X—CH}_2\text{—COOH}$. The substituent effect of the *tert*-butylaminoxyl group on the acid-dissociation equilibria of α-substituted acetic acids was investigated in relation to the Taft equation. The aliphatic polar substituent constant (σ^*) has been evaluated to be ≈ +0.9 for the $t\text{-BuN(O}\cdot)\text{CH}_2$ group [or the inductive substituent constant $\sigma_I \approx +0.4$ for the *t*-BuN(O·) group].

KEY WORDS: Spin trapping, aminoxyl radical, acid-dissociation of free radical, p*K* of α -substituted acetic acid, aliphatic substituent constant, HPLC-EPR method.

INTRODUCTION

Proton transfer reactions and equilibria of free radicals have been studied by use of kinetic absorption spectrophotometry, EPR spectroscopy, conductometry, potentiometric titrations, and pulse polarography[1,2]. Several attempts have been made to correlate p*K* values of radicals with substituent constants[1-5]. The EPR measurement of p*K* values is the only method that relates the acid and conjugate base forms of radicals directly and leaves no doubt that the phenomenon observed in an acid-dissociation equilibrium. This method is also independent of radical yields, side reactions, or impurities, which might complicate the other methods.

Stable aminoxyl radicals (spin adducts) produced in γ-irradiated aqueous solutions of amino acids and peptides with a spin trap, 2-methyl-2-nitrosopropane (MNP), were separated and identified by means of high-performance liquid chromatography (HPLC) and EPR spectroscopy[6-10]. It was demonstrated that EPR spectra of the spin adducts changed reversibly with pH through the acid-dissociation

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* Senior author for correspondence

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equilibria of the carboxyl or amino groups. Some of the spin adducts can be regarded as α-substituted acetic acids with the *tert*-butylaminoxyl groups, *t*-Bu—N(O·)—CHX—COOH.

In this work, the pK_{COOH} values have been determined for this type of radicals. We have also investigated the substituent effect of the aminoxyl group for the dissociation equilibria of α-substituted acetic acids.

EXPERIMENTAL

MNP was synthesized and purified according to the method of Stowell[11]. Glycine was purchased from Wako Pure Chemical Industries, Osaka; L-Alanine, Gly-Gly and L-Ala-Gly were from Kyowa Hakko Industrial Co., Tokyo, Protein Research Foundation, Osaka and Sigma Chemical Co, Saint Louis, respectively. All other chemicals were of reagent grade.

The amino acids (1 M) and dipeptides (0.1 M) were dissolved in 5 mg/ml of MNP aqueous solutions. Each of the sample solutions was cooled by ice and irradiated with ^{60}Co γ rays at a dose rate of ca. 100 Gy/min to a total dose of 3 kGy without deaeration. The irradiated samples were separated by HPLC with EPR detection[10]. Cation-exchange columns used were IEX-210SC (0.75 × 60 cm) of Tosoh Co., Tokyo. The EPR spectrometer was a JEOL PE-3X, which was operated at 100 kHz modulation frequency in the X band. Aminoxyl radicals, *t*-BuN(O·)—CH_2COOH [**1**], *t*-BuN(O·)—$CH(CH_3)COOH$ [**2**], *t*-BuN(O·)—$CH(COOH)NHCOCH_2NH_3^+$ [**3**] and *t*-BuN(O·)—*CH(COOH)NHCO—*$CH(CH_3)$—NH_3^+ [**4a** and **4b** (diastereomers, *S*-*S* and *S*-*R* forms)] were isolated and identified by the chromatography of the irradiated solutions of glycine[6], L-alanine[6], Gly-Gly[7] and L-Ala-Gly[8], respectively. For other experimental details on the preparations of radicals **1**–**4b** see our previous works[6-8].

The EPR spectra were recorded at ca. 25°C. The hyperfine splitting constants (hfsc's) were measured with Mn^{2+} in MgO as a reference and were accurate to ±0.003 mT. The pH values of the solutions were adjusted by addition of dilute HCl or NaOH and read to ±0.01 pH unit with a Hitachi-Horiba pH meter M-5. All experiments were carried out in the dark.

RESULTS AND DISCUSSION

Determination of Acid-Dissociation Constants of Aminoxyl Radicals

Figure 1 shows the EPR spectrum of radical **1** obtained at pH 7.0, which can be analyzed as a triple triplet. The primary triplet of 1.61 mT splitting is due to the aminoxyl nitrogen. The secondary triplet of 0.845 mT splitting with an intensity ratio of 1:2:1 is assigned to two equivalent β hydrogens. While the spectral pattern of radical **1** was pH-independent in the neutral and alkaline solutions, reversible pH-dependent shifts of the signals were observed in the acidic pH range. At pH 1.4, the hfsc's, a_N and $a_{\beta H}(2H)$, are 1.58 and 0.860 mT, respectively. Since the spectral changes occur without producing any new hyperfine splittings and the protonation of the aminoxyl group should give an additional H-hfsc of several gauss[12,13] we conclude that the aminoxyl group is not protonated in the observed pH range. Consequently, the acid-dissociation property of radical **1** was attributable to the

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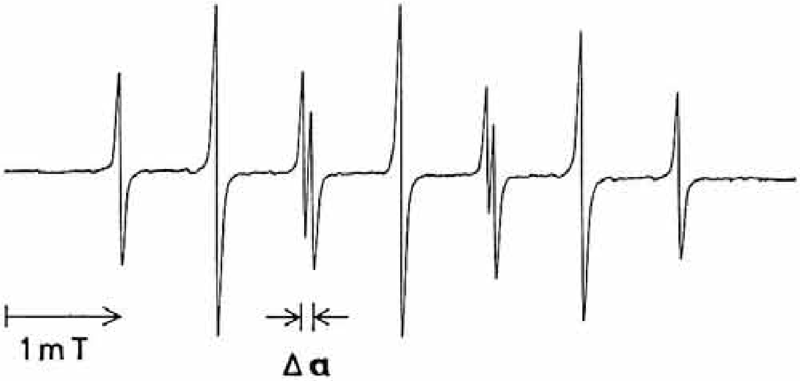

FIGURE 1 EPR spectrum of radical 1 observed at pH 7.0. The effect of pH on the EPR parameter defined by Δa was measured.

protonation of a carboxyl group near the aminoxyl center.

In the acidic pH range the largest changing EPR parameter of radical **1** is Δa visualized as the distance between the two signals shown in Figure 1. The parameter, $\Delta a = 2a_{\beta H} - a_N$, was plotted as a function of pH in Figure 2a. In the dissociation equilibrium of the carboxyl group of radical **1**, if the interchange between acid form (A) and base form (B) is sufficiently rapid as compared with the time scale of EPR, which is the case here, the observed parameter (Δa) represents the weighted average of the two forms (Eqn. 1)[5].

$$\Delta a = (c_A \Delta a_A + c_B \Delta a_B)/(c_A + c_B) \qquad (1)$$

where Δa_A and Δa_B are the Δa values for the acid and base forms, respectively, and c_A and c_B refer to the concentration of each form. The equilibrium is characterized by its thermodynamic dissociation constant, K_a, which is usually expressed in terms of $pK_a = -\log K_a$. The pK_a value can be given by Eqn. 2,

$$pK_a = pH + \log(c_A/c_B) + \log(f_A/f_B) \qquad (2)$$

where f_A and f_B refer to the activity coefficient of each form. In analyses of acid-base properties of radicals, the last term in Eqn. 2 is usually neglected and dissociation constants are measured as pK values[2]. For a given solution the pK value can be calculated by Eqn. 3.

$$\begin{aligned} pK &= pH + \log(c_A/c_B) \\ &= pH + \log[(\Delta a - \Delta a_B)/(\Delta a_A - \Delta a)] \qquad (3) \end{aligned}$$

On this basis Δa is expected to describe a sigmoid curve as a function of pH according to

$$\begin{aligned} \Delta a &= \Delta a_A/[1 + 10^{(pH - pK)}] + \Delta a_B/[1 + 10^{(pK - pH)}] \\ &= (1/2)(\Delta a_A + \Delta a_B) \\ &\quad + (1/2)(\Delta a_A - \Delta a_B)[1 - 10^{(pH - pK)}]/[1 + 10^{(pH - pK)}] \qquad (4) \end{aligned}$$

For radical **1**, Δa_A and Δa_B were 0.14 and 0.08 mT, respectively. For every 0.1 unit

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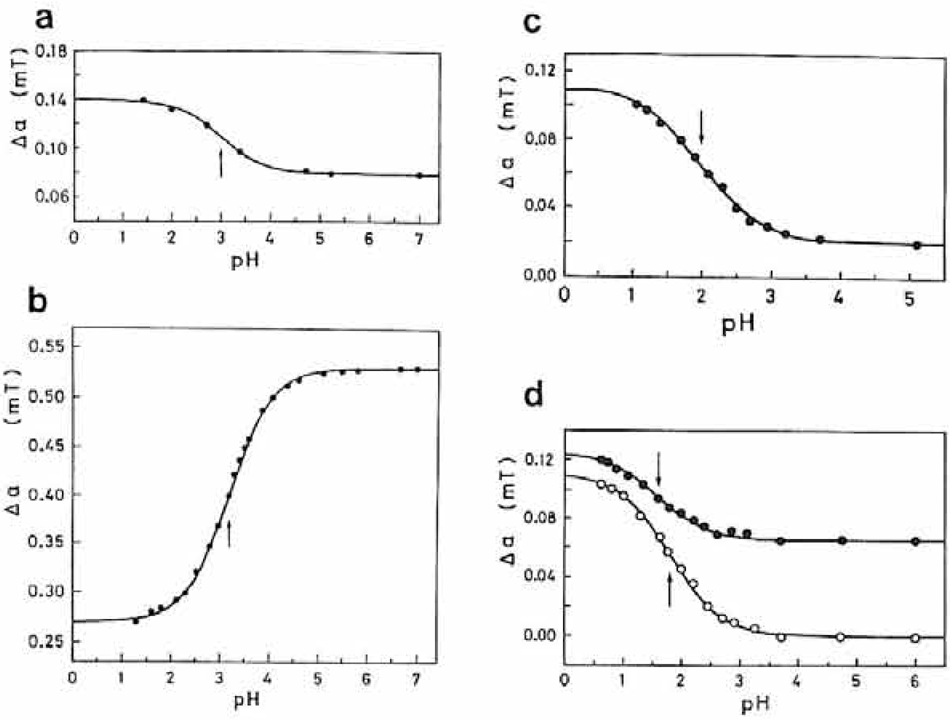

FIGURE 2 The effects of pH on the EPR parameters: (a) $\Delta a = 2a_{\beta H} - a_N$ for radical **1**; (b) $\Delta a = a_{\beta H}$ for **2**; (c) $\Delta a = a_{\beta N} - a_{\beta H}$ for **3**; (d) $\Delta a = a_{\beta N} - a_{\beta H}$ for **4a** (○) and **4b** (●). Best-fitted solid curves were calculated by use of Eqn. 4. Vertical arrows indicate the pK_{COOH} values determined for the radicals.

of pK value the calculated curves were compared with the experimental points. Good agreement was obtained by substituting $pK = 3.0$ to Eqn. 4 as shown by the solid curve in Figure 2a.

Whereas in neutral and alkaline solutions the EPR pattern of radical **2** was independent of pH, in the acidic pH range the EPR signals exhibited reversible pH-dependent shifts. The hfsc's of radical **2**, a_N, $a_{\beta H}$ and $a_{\gamma H}(3H)$ are 1.61, 0.53 and 0.042 mT at pH 7.0, and 1.59, 0.27 and 0.035 mT at pH 1.3, respectively. In Figure 2b, the β-H hfsc was plotted against the pH of the solution. By substituting $\Delta a = a_{\beta H}$, $\Delta a_A = 0.27$ mT and $\Delta a_B = 0.53$ mT to Eqn. 4, the variation of $a_{\beta H}$ with pH can be calculated. The pK_{COOH} value of radical **2** was determined to be 3.2 by fitting Eqn. 4 to the experimental points as shown by the solid curve in Figure 2b.

Radical **3** showed reversible pH-dependent shifts of the EPR signals in the acidic pH range. (At pH 6.0 $a_N = 1.56$ mT, $a_{\beta N} = 0.24$ mT and $a_{\beta H} = 0.22$ mT, and at pH 1.0 $a_N = 1.52$ mT, $a_{\beta N} = 0.26$ mT and $a_{\beta H} = 0.16$ mT.). The differences of the β hfsc's, $\Delta a = a_{\beta N} - a_{\beta H}$, were plotted as a function of pH in Figure 2c. The pK_{COOH} value of radical **3** was determined to be 2.0 by substituting $\Delta a_A = 0.11$ mT and $\Delta a_B = 0.02$ mT to Eqn. 4.

A pair of diastereomeric radicals **4a** and **4b** showed pH-dependent spectral behavior similar to radical **3**. The hfsc's of radical **4a** are $a_N = 1.55$ mT,

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TABLE I
Dissociation constants pK_{COOH} for radicals t-BuN(O$\cdot$)$-$CHX$-$COOH and Taft's σ^* substituent constants of t-BuN(O$\cdot$)CHX groups

radical	structure	pK_{COOH}	$\Sigma\sigma^*$
1	t-BuN(O$\cdot$)$-$CH$_2-$COOH	3.0	0.9
2	t-BuN(O$\cdot$)$-$CH(CH$_3$)$-$COOH	3.2	0.8
3	t-BuN(O$\cdot$)$-$CH(NHCOCH$_2$NH$_3{}^+$)$-$COOH	2.0	1.8
4a	t-BuN(O$\cdot$)$-$CH[NHCOCH(CH$_3$)NH$_3{}^+$]$-$COOH	1.8	1.8
4b	(a pair of diastereomers)	1.6	1.8

$a_{\beta N} = a_{\beta H} = 0.233$ mT at pH 6.0, and $a_N = 1.52$ mT, $a_{\beta N} = 0.26$ mT, $a_{\beta H} = 0.16$ mT at pH 0.8. For radical **4b**, $a_N = 1.56$ mT, $a_{\beta N} = 0.258$ mT, $a_{\beta H} = 0.193$ mT at pH 6.0, and $a_N = 1.53$ mT, $a_{\beta N} = 0.27$ mT, $a_{\beta H} = 0.15$ mT at pH 0.8. In Eqn. 4 ($\Delta a = a_{\beta N} - a_{\beta H}$), $\Delta a_A = 0.11$ mT, $\Delta a_B = 0$ for radical **4a** and $\Delta a_A = 0.125$ mT, $\Delta a_B = 0.065$ mT for **4b** were substituted. The pK_{COOH} values were determined to be 1.8 and 1.6 for radicals **4a** and **4b**, respectively, as shown in Figure 2d.

Substituent Effect of Aminoxyl Group for the Dissociation Equilibria of α-Substituted Acetic Acids

The dissociation contants (pK_{COOH}) of radicals **1–4b** summarized in Table I seem to be dependent strongly on their structure. That is to say, the pK_{COOH} values are lower than those for the corresponding compounds (acetic acid, propionic acid, Gly-Gly and L-Ala-Gly) in which the *tert*-butylaminoxyl groups of the radicals are substituted by hydrogens. The electron-withdrawing character of the aminoxyl group is responsible for this difference.

The substituent effects for the dissociation constants of aliphatic carboxylic acids (X$-$CH$_2-$COOH) can be generally expressed by a linear free energy relationship, called the Taft equation[14-17],

$$\log(K/K_0) = \rho^*\sigma^* (\text{or } \rho_I\sigma_I) \tag{5}$$

where σ^* is a polar substituent constant of the X$-$CH$_2$ group (σ_I is an inductive substituent constant of the X group.) and ρ^* (or ρ_I) is a reaction constant. K and K_0 are the acid-dissociation constants of substituted and unsubstituted [X = H, $\sigma^* = 0$ (or $\sigma_I = 0$)] acetic acids, respectively. The additive nature of the σ^* values permit that $\Sigma\sigma^*$ is used to replace σ^* in Eqn. 5. For the dissociation of α-substituted acetic acids (water, 25°C), Eqn. 6 was derived by taking $\rho^* = +1.721$ and $\log K_0 = -4.65$.

$$pK = +4.65 - 1.721\,\Sigma\sigma^* \tag{6}$$

More detailed treatments were demonstrated on the correlation analysis of substituent effects[18].

In order to make a rough estimate of the Taft's σ^* constant of the *tert*-butylaminoxylmethylene group [t-BuN(O$\cdot$)CH$_2$] for the dissociation equilibria of α-substituted acetic acids, the pK_{COOH} values of radicals **1–4b** and their corresponding compounds [X$-$CH$_2-$COOH] have been correlated with Eqn. 6. The adopted σ^* values were -0.10, $+0.9$ and $+0.9$ for the CH$_3$CH$_2$, NH$_3{}^+$CH$_2$CONHCH$_2$ and NH$_3{}^+$CH(CH$_3$)CONHCH$_2$ groups, respectively. The $\Sigma\sigma^*$ values of radicals **1–4b** were considered with Eqns. 7 and 8, etc.

$$\sigma^*[t\text{-BuN(O}\cdot\text{)CH(CH}_3\text{)}] = \sigma^*[t\text{-BuN(O}\cdot\text{)CH}_2] + \sigma^*[\text{CH}_3\text{CH}_2] \quad (7)$$

$$\sigma^*[t\text{-BuN(O}\cdot\text{)CH(NHCOCH}_2\text{NH}_3^+\text{)}] = \sigma^*[t\text{-BuN(O}\cdot\text{)CH}_2] + \sigma^*[\text{NH}_3{}^+\text{CH}_2\text{CONHCH}_2] \quad (8)$$

By taking +0.9 as $\sigma^*[t\text{-BuN(O}\cdot\text{)CH}_2]$, the $\Sigma\sigma^*$ values of the radicals were calculated and summarized in Table 1. Figure 3 shows a linear correlation of the pK_{COOH} values for the radicals and their corresponding compounds with the polar substituent constants. Since the σ^* and σ_I constants are related by $\sigma_I(X) = 0.45\,\sigma^*(CH_2X)$[17], $\sigma_I[t\text{-BuN(O}\cdot\text{)}] \approx +0.4$ has been derived. The pK_{COOH} values for the radicals could also be correlated with Eqn. 9[15,16].

$$pK = +4.712 - 3.95\,\Sigma\sigma_I \quad (9)$$

Consequently, the strength of the electron-withdrawing substituent effect of *tert*-butylaminoxyl group for the acid-dissociation equilibria of α-substituted acetic acids is nearly equal to that of the $NH_3{}^+CHRCONH$ groups and the substituent constant σ^* (or σ_I) has been suggested to be $\approx +0.9$ for the $t\text{-BuN(O}\cdot\text{)CH}_2$ group [or $\approx +0.4$ for the $t\text{-BuN(O}\cdot\text{)}$ group].

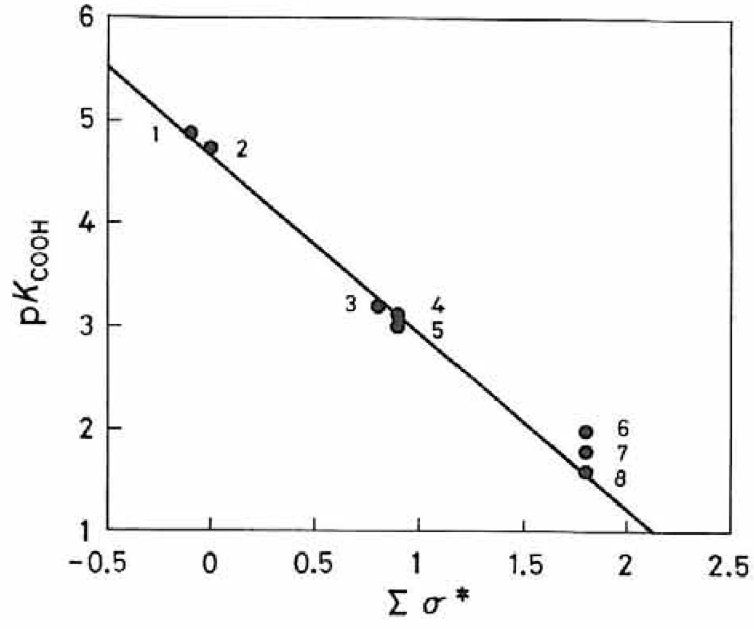

FIGURE 3 Correlation of the pK_{COOH} values for R—COOH with Taft's σ^* substituent effect. Solid linear line was described by Eqn. 6. 1, CH_3CH_2COOH; 2, CH_3COOH; 3, $t\text{-BuN(O}\cdot\text{)CH(CH}_3\text{)COOH}$ [radical **2**]; 4, $NH_3^+CH_2CONHCH_2COOH$ and $NH_3^+CH(CH_3)CONHCH_2COOH$; 5, $t\text{-BuN(O}\cdot\text{)—CH}_2\text{COOH}$ [radical **1**]; 6, $t\text{-BuN(O}\cdot\text{)CH(NHCOCH}_2\text{NH}_3^+\text{)COOH}$ [radical **3**]; 7 and 8, $t\text{-BuN(O}\cdot\text{)—CH[NHCOCH(CH}_3\text{)NH}_3^+\text{]COOH}$ [a pair of diastereomeric radicals **4a** and **4b**, respectively].

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Acknowledgements

The authors express gratitude to Prof. Hiroyuki Hatano, International Institute of Technological Analysis, Health Research Foundation, Dr. Souji Rokushika and Dr. Fumiko M. Yamamoto, Kyoto University for their contributions.

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