Redox chemistry of carbon-centered α-amino acid radicals †

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In this work we present the one-electron oxidation potentials of the α-amino carbon-centered radicals of glutathione, glutathione diethylester hydrochloride, cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride in aqueous solution and *N*-dimethylglycine ethylester, *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine in acetonitrile measured by photomodulation voltammetry. The potentials were found to be −0.30, −0.27, −0.06, 0.05, −0.23, −0.40, −0.48 and −0.38 V *vs.* NHE, respectively, under the present experimental conditions. On the basis of these results and previously published results, the nature of α-amino acid and peptide radicals, glutathione in particular, is discussed.

Introduction

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) is a well known biologically active peptide, participating in various redox systems.[1] Its redox behavior has been studied in detail by various groups, and is outlined in Scheme 1.

$$GSH/GS^- + RO^\bullet \longrightarrow GS^\bullet + ROH/RO^-$$
$$GS^\bullet + GS^- \rightleftharpoons GSSG^{\bullet -}$$
$$GS^\bullet \rightleftharpoons {}^\bullet CR$$

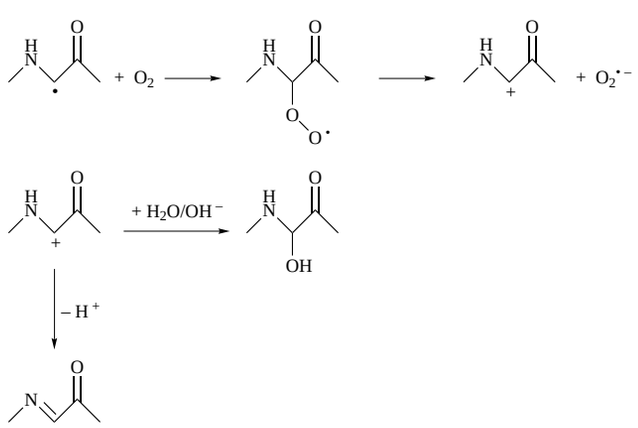
Scheme 1

Reaction of GSH with various one-electron oxidants initially leads to the formation of the sulfur-centered radical $GS^\bullet$. This thiyl radical can undergo intramolecular hydrogen transfer to form an α-amino carbon-centered radical, $^\bullet CR$, as described by Grierson *et al.*[2] and later proposed by Zhao *et al.*[3] The rate constant for this process was measured to be $1.2 \times 10^5\ s^{-1}$ at pH 10.5.[3] As would be expected for the formation of α-amino carbon-centered radicals, this reaction exhibits a significant pH dependence since the effects of NH_3^+-substitution and NH_2-substitution on the C–H bond dissociation energy differ significantly. Intramolecular hydrogen transfer has also been reported for homocysteine, cysteine[3] and 2-mercaptoethanol.[4] Akhlaq *et al.* have also observed intermolecular hydrogen transfer to thiyl radicals.[5] These results are surprising since it was assumed that thiyl radicals are unreactive with respect to hydrogen abstraction to form carbon-centered radicals. A recent theoretical study by Armstrong *et al.* has evaluated the C–H bond dissociation energy of a series of glycyl peptides.[6] It has been estimated that D_{C-H} is *ca.* 348 kJ mol^{-1} for a glycyl peptide[6] and 331 kJ mol^{-1} for neutral glycine,[7] while that of aliphatic S–H bonds is *ca.* 370 kJ mol^{-1}.[8] Zhao *et al.* have recently estimated an upper limit of 331 kJ mol^{-1} for the glycine α-C–H bond dissociation energy based on thermochemical cycles.[9] The very low α-C–H bond dissociation energies for neutral amino acids have been suggested to be the result of the capto-dative effect of the $-NH_2$ and $-CO_2H$ substitution. This means that the combined effect of the two substituents is larger than the sum of the separate effects of the two substituents. Thus, there seems to be a thermodynamic driving force for the formation of α-amino acid carbon-centered radicals from sulfur-centered amino acid radicals based on the gas-phase bond dissociation energies. An interesting question here is: why is the hydrogen atom not abstracted directly from the α-carbon? This can be explained on kinetic grounds since, in general, hydrogen abstraction from more polar bonds, *e.g.* S–H bonds, is more rapid than hydrogen abstraction from less polar bonds, *e.g.* C–H bonds.[10] Another example of this is hydrogen abstraction from phenol, aniline and toluene, which have more or less the same R–H bond dissociation energies, where the rate of hydrogen abstraction displays a strong R–H polarity dependence.[11]

In the study by Armstrong *et al.*,[6] the reduction potential, E^0, in aqueous solution for the reaction (1) has been calculated to

$$R^\bullet + H^+ + e^- \rightleftharpoons RH, \text{ where } R^\bullet \text{ is } -HN–CH{\cdot}C(O)– \quad (1)$$

be 0.8 V *vs.* NHE, suggesting that α-C–H bonds are susceptible to oxidation by $RS^\bullet$, whose reduction potential is higher under analogous conditions. These findings have initiated an intense discussion on the biological relevance of carbon-centered radicals formed by oxidative damage. Once formed, these carbon-centered radicals react with molecular oxygen to form peroxyl radicals, which can expel superoxide anion radicals to give carbocations which then hydrolyse to the corresponding alcohol or deprotonate to give an imine (Scheme 2).[12,13]



Scheme 2

The α-amino acid radicals can also be oxidized by other relatively mild oxidizing agents, *e.g.* $IrCl_6^{2-}$ and $Fe(CN)_6^{3-}$.[12,13] The life-time of the alkylperoxyl radical depends on the oxidation potential of the corresponding alkyl radical.

Clearly, direct measurements of the α-C–H bond dissociation energy of amino acids and the redox behavior of their respective carbon-centered radicals are needed to provide an answer to

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this ongoing controversy. Clark *et al.* have reported the α-C–H bond dissociation energy of $(CH_3)_2NCH_2CO_2C_2H_5$ to be 331–347 kJ mol^{-1} measured by photoacoustic calorimetry [14] which is well in line with the previously mentioned numbers. In our present work, we wish to present our experimental results on the oxidation potential of the glutathione, glutathione diethylester hydrochloride, cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride α-carbon-centered radicals in aqueous solution and the *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine α-carbon-centered radicals in acetonitrile measured by photomodulation voltammetry. Under our experimental conditions, glutathione diethylester hydrochloride, cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride possess the electron-withdrawing NH_3^+-group. Glutathione is in the zwitterionic form and the *N*-Boc protected amino acids possess the slightly electron-donating Bu^{t}OC-(O)NH-group [judging from the Hammett constant for the MeOC(O)NH-group] [15] and a free acid functionality. For comparison, we have chosen the *N*-dimethylglycine ethylester for our studies. Furthermore, the α-C–H bond dissociation energies of the α-amino C–H bonds are discussed.

Experimental

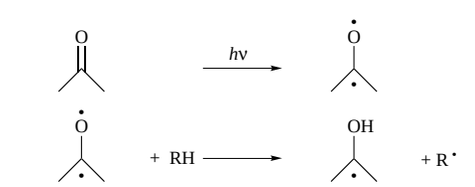
General

Glutathione diethylester hydrochloride was prepared according to the published procedure from glutathione (Aldrich) and ethyl alcohol under acidic conditions.[16] *N*-Boc-Gly and *N*-Boc-Leu were prepared according to Itoh *et al.* [17] and characterized by ^{1}H NMR (*N*-Boc-Gly): ^{1}H NMR (δ in ppm $CDCl_3$): 11.02 [1H, s br, C(O)OH], 5.12 (1H, s br, −N*H*), 3.97 (2H, d, $J_{N-H} = 5.0$, $-CH_2-$), 1.46 [9H, s, $(CH_3)_3C-$]; *N*-Boc-Leu: ^{1}H NMR (δ in ppm $CDCl_3$): 5.00 (1H, d, $J_{N-H} = 7.9$, NH), 4.31 (1H, m, CH), 1.77–1.48 (3H, m, overlapping signals due to CH_2 and CH groups), 1.45 [9H, s, $(CH_3)_3C-$], 0.96 (1H, d, $J_{HH} = 6.1$).

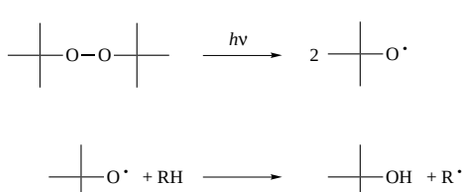
N-Boc-Pro, cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride were used as received (Aldrich). Ethyl alcohol was dried using Na and distilled from NaOEt. All other solvents were used as received without further treatment. ^{1}H NMR spectra were recorded at 200.132 MHz on a Bruker AC 200 NMR spectrometer. All chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The ^{1}H NMR chemical shifts are relative to tetramethylsilane ($\delta = 0$ ppm), which was added as an internal standard. Spectral assignments were made using 2D-COSY. All measurements were carried out at 293 K unless otherwise specified.

Photomodulation voltammetry

The instrument has been described in detail previously.[18] The carbon-centered α-amino acid radicals were generated in the electrochemical cell by photolysis, through an optically transparent gold electrode, of acetone (10% v/v) in water (unbuffered solutions) to form a triplet [reaction (2)] or di-*tert*-butyl-



(2)



(3)

peroxide (10% v/v) in acetonitrile to form *tert*-butoxyl radical [reaction (3)] which abstract the α-hydrogen from the amino acid or peptide (concentration 1 mmol dm^{-3}).

The supporting electrolytes used were 0.1 mol dm^{-3} TBAP (tetrabutylammoniumperchlorate) and 1 mol dm^{-3} KCl in acetonitrile and water, respectively. For the electrochemical experiments water was deionized and distilled and acetonitrile (Omnisolv) was distilled from CaH_2 under 1 atm of argon prior to use. The supporting electrolyte TBAP was recrystallized three times from 10% hexane in ethyl acetate and dried in a vacuum oven (40 °C, 10 Torr).

The output from the lamp was modulated with a light chopper so the light intensity (and therefore the radical concentration) rose and fell as a sine wave. Samples flowed slowly through the cell so as to avoid problems associated with sample depletion and/or product formation. The photolysis source was a 1000 W mercury–xenon lamp which was only capable of generating average radical concentrations of 10^{-7}–10^{-8} mol dm^{-3}, *i.e.* well below the normal level of detection for conventional electrochemical apparatus. The voltage at the working electrode was scanned slowly (20 mV s^{-1}) until the oxidation potentials of the radicals were reached, at which small currents oscillating at the modulation frequency (100 Hz) were obtained due to the formation of ions. A phase-sensitive detector (Stanford Research Systems Model SR530 lock-in amplifier) gave the amplitude of the oscillating signals, which was output onto an *x*–*y* recorder (HP 7045 B). The resulting trace is a polarogram of the free radical. The electrochemical cell was fully *iR* compensated and was controlled with a PAR Model 174 polarographic analyser. Potential calibration in acetonitrile and water was accomplished using ferrocene ($E^0 = 0.44$ V *vs.* SCE) [19] and ferricyanide ($E^0 = 0.37$ V *vs.* NHE),[20] respectively. The polarograms are reversible which indicates that the half-wave potentials ($E_{1/2}$) are true thermodynamic potentials. The limitations of the method have been thoroughly discussed by Nagaoka *et al.* [21]

Table 1 Oxidation potentials of α-amino acid carbon-centered radicals

Radical	$E^{ox}_{1/2}$ (V *vs.* NHE)	Solvent
1	−0.23	MeCN
2	−0.40	MeCN
3	−0.48	MeCN
4	−0.38	MeCN
5	0.05	H_2O
6	−0.27	H_2O
7	−0.30	H_2O
8	−0.06	H_2O

Results and discussion

The oxidation potentials of the α-carbon-centered radicals measured by photomodulation voltammetry are given in Table 1. (1: *N*-dimethylglycine ethylester, 2: *N*-Boc-leucine, 3: *N*-Boc-proline, 4: *N*-Boc-glycine, 5: cystine dimethylester dihydrochloride, 6: glutathione diethylester hydrochloride, 7: glutathione, 8: cysteine ethylester hydrochloride.)

The reduction of the sulfur-centered thiyl radical of cysteine ethylester hydrochloride can also be seen in the polarogram (0.88 V *vs.* NHE), however, this potential and the reduction potentials of the other thiyl radicals are very close to the upper practical limit of the method and are therefore rather uncertain. For comparison, the oxidation potentials of some alkyl and amino alkyl radicals are given in Table 2.

As can be seen in Table 2, α-amino alkyl radicals have very low oxidation potentials compared to other alkyl radicals. For instance, a primary α-amino alkyl radical has an oxidation potential more than 1 V lower than that of a tertiary alkyl radical. The oxidation potential of a secondary amino alkyl

Table 2 Oxidation potentials of alkyl radicals in acetonitrile

Radical	$E^{ox}_{1/2}$ (V *vs.* NHE)	Reference
$(CH_3)_2NCH_2^{\bullet}$	−1.27	22
$(CH_3CH_2)_2NCH^{\bullet}CH_3$	−1.36	22
$(CH_3)_3C^{\bullet}$	−0.15	23
$CH_3OCH_2^{\bullet}$	−0.48	23
$CH_3CH_2OCH^{\bullet}CH_3$	−0.69	23

radical is *ca.* 100 mV lower than that of a primary amino alkyl radical. Interestingly, the difference in potential between a primary and secondary alkoxy substituted alkyl radical is *ca.* 200 mV. A similar trend has been observed for the reduction potential of substituted benzene radical cations, *i.e.* the effects of two substituents are not purely additive.[24] The very strong lowering effect of the amino substituent is almost cancelled out by the effect of the $-CO_2CH_2CH_3$ substituent for the radical of *N*-dimethylglycine ethylester. This, in combination with the low α-C–H bond dissociation energy of *N*-dimethylglycine ethylester (331–347 kJ mol^{-1}),[14] is well in line with the theory of the capto-dative stabilization of the C-centered α-amino acid radical. The low bond dissociation energy indicates that the radical is stabilized relative to the amino acid and the rather high oxidation potential indicates that the radical is stabilized relative to the oxidized state. The somewhat lower oxidation potentials of the C-centered radicals of *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine in combination with the somewhat higher C–H bond dissociation energies, *i.e.* the calculated bond dissociation energy for a glycyl peptide, indicate that the capto-dative effect is less pronounced in peptides than in neutral amino acids, assuming that the effect of a Bu^{t}OC(O)NH-substituent is comparable to the effect of a peptide bond.

For *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine we can also see the effects of alkyl substitution on the α-carbon. The oxidation potential of the radical of *N*-Boc-leucine is slightly lower than that of *N*-Boc-glycine and the oxidation potential of the radical of *N*-Boc-proline is significantly lower. In the latter case, the decrease in potential can be due to substitution on both the carbon and the nitrogen and also the ring strain. More detailed studies will be necessary to delineate between these two effects. Alkyl substitution on the α-carbon also seem to affect the C–H bond dissociation energy of peptides judging from photoacoustic calorimetry measurements on glycine anhydride and alanine anhydride. The oxidation potential of the α-carbon-centered radical of glycine anhydride is 90 mV higher than the potential of the corresponding radical of alanine anhydride and the α-C–H bond dissociation energy of alanine anhydride is 15 kJ mol^{-1} lower than that of glycine anhydride.[25] Also, semi-empirical quantum chemical calculations on the α-C–H bond dissociation energy of a number of neutral amino acids indicate that α-carbon alkyl substitution lowers the bond dissociation energy.[26]

The potentials determined in aqueous solution are not as straightforward to interpret. In unbuffered solutions amino acids exist in their zwitterionic form, *i.e.* as NH_3^+–CH(R)–CO_2^-. It would thus be interesting to measure the oxidation potential of amino acid radicals at a pH where the amino group is not protonated. Unfortunately, measurements in aqueous solution using photomodulation voltammetry are difficult to perform at higher pH where the potential range decreases dramatically. As can be seen in Table 1, the oxidation potentials of the C-centered radicals of cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride are considerably higher than the potentials of radicals 1–4. The main reason for this difference is that the NH_3^+-group is more electron-withdrawing than the Bu^{t}OC(O)NH-group but differences in the solvation properties of water and acetonitrile may also play an important role. More detailed studies are under way.

The oxidation potentials of the glutathione and glutathione diethylester hydrochloride C-centered radicals are significantly lower than the potentials of the cysteine ethylester hydrochloride and cystine dimethylester dihydrochloride C-centered radicals. This indicates that the radical center of the glutathione based radicals is not adjacent to a NH_3^+-group under the present conditions, *i.e.* the hydrogen is primarily abstracted from the cysteine α-carbon. It has been suggested that the thiyl radical of glutathione abstracts a hydrogen atom in an intramolecular fashion from the glutamyl group under physiological conditions.[3] Furthermore, the carbon-centered radical thus formed reduces Methyl Viologen rapidly at pH 10.5 which indicates that its oxidation potential is lower than −0.45 V *vs.* NHE, the reduction potential of Methyl Viologen.[27] The radical formed at pH 10.5, where the amino groups are not protonated, is consequently not identical to the radical formed under the conditions used in this work. However, under physiological conditions, only a small fraction of the amino groups are not protonated. It is therefore reasonable to believe that two different C-centered radicals with different redox properties can be formed.

It was also our intention to measure the α-C–H bond dissociation energies of *N*-dimethylglycine ethylester, *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine using photoacoustic calorimetry. We managed to reproduce the value by Clark *et al.*[14] for the *N*-dimethylglycine ethylester (*ca.* 331 kJ mol^{-1}) but we were not able to measure the bond dissociation energies for *N*-Boc-proline, *N*-Boc-leucine and *N*-Boc-glycine since the rates of hydrogen abstraction from these compounds are too slow for the time window of the method. This observation in itself could indicate that the bond dissociation energies of the latter three compounds are somewhat higher than the bond dissociation energy of the *N*-dimethylglycine ethylester as predicted by the calculations by Armstrong *et al.*[6] [assuming that the effect of a Bu^{t}OC(O)NH-substituent is comparable to the effect of a peptide bond]. An example of a situation where the observed kinetics of a hydrogen abstraction correlate with the C–H bond dissociation energy is hydrogen abstraction from structurally similar alkanes by bromine.[28]

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