in part by the donors of the Petroleum Research Fund, administered by the American Chemical Society.

Registry No. 1, 36749-09-4; **2**, 78-09-1; **3**, 105-58-8; **4**, 115-80-0; **5**, 23786-93-8; **6**, 80866-27-9; **7**, 53143-91-2; **8**, 1900-58-9; **9**, 80866-28-0; **10**, 80866-29-1; **11**, 80866-30-4; **12**, 4362-57-6; **13**, 80866-31-5; **14**, 111-70-6; **15**, 50635-65-9; **16**, 80866-32-6; **17**, 23418-82-8.

Chemical Reactivity of the New Amino Acid β-Carboxyaspartic Acid (Asa)

M. Robert Christy and Tad H. Koch*

Department of Chemistry, University of Colorado Boulder, Colorado 80303

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We recently reported the laboratory synthesis of the new naturally occurring amino acid β-carboxyaspartic acid (Asa) and its identification in the ribosomal proteins of *E. coli*.[1] Asa is a homologue of γ-carboxyglutamic acid (Gla), which is formed by the vitamin-K-mediated post-translational γ carboxylation of glutamyl residues in blood coagulation proteins.[2] We have reported that Asa from base hydrolysis of the ribosomal proteins of *E. coli* is acid labile with respect to decarboxylation to aspartic acid (Asp).[1] We as well as Hauschka and co-workers have noted similar reactivity for synthetic Asa.[1,3]

Recently, we have observed that Asa is also base labile with respect to elimination of the amine functional group and now report in detail on the decarboxylation and elimination reactions of Asa and of a model system for Asa residues in a protein. The elimination reaction is particularly significant because it limits the detectability of Asa in proteins by alkaline hydrolysis followed by amino acid analysis.

Failure to detect Asa in any protein subjected to acid hydrolysis is due to its quantitative decarboxylation to Asp. The reaction follows clean first-order kinetics in 1 M hydrochloric acid. Plots of $-\ln (A_t - A_\infty)$ vs. time are linear for the entire reaction, where A_t and A_∞ are the absorbances at 207 nm at time t and time ∞. The rate constants as a function of temperature are as follows: 321 K, $(2.06 \pm 0.01) \times 10^{-5}$ s^{-1}; 338 K, $(2.13 \pm 0.02) \times 10^{-4}$ s^{-1}; 343 K, $(3.72 \pm 0.02) \times 10^{-4}$ s^{-1}; 348 K, $(7.56 \pm 0.09) \times 10^{-4}$ s^{-1}. The data give a linear Arrhenius plot with an activation energy of 29.8 ± 0.04 kcal/mol and a preexponential factor of 3.7×10^{15} s^{-1}. Hauschka and co-workers independently determined the free energy of activation to be 26.8 kcal/mol.[3]

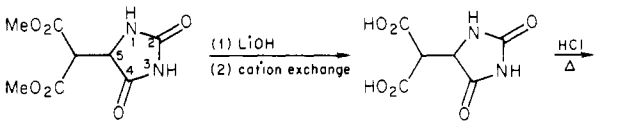
We have investigated the stability of Asa incorporated in a protein with respect to decarboxylation using 5-hydantoinmalonic acid (**1**) as a model system. 5-Hydantoinmalonic acid was synthesized from dimethyl 5-hydantoinmalonate (**2**) by saponification in aqueous tetrahydrofuran solvent (1:2 v/v) with a 2-fold excess of lithium hydroxide at 25 °C for 8 h and was isolated, after cation-exchange chromatography on a column of Hamilton HC-X4.00 resin eluting with 0.10 M hydrochloric acid, as the monohydrate in 73% yield.[4] Dimethyl 5-hydantoinmalonate was prepared in 26% yield by reaction of dimethyl sodiomalonate with 5-chlorohydantoin using a modified version of the procedure reported for the preparation of diethyl 5-hydantoinmalonate.[5]

Table I. Reactivity of β-Carboxyaspartic Acid as a Function of pH

NaOH,[a] equiv	pH	reaction composition, %[b] unreacted Asa	Asp	tricarboxyethylene
1.0	1.8	0	100	0
2.0	4.6	35	50	15
3.0	9.8	74	0	26
3.5	10.0	76	0	24
4.0	12.2	99	0	1

[a] The appropriate quantity of a 2.0 M solution of sodium hydroxide was added to a solution of β-carboxyaspartic acid hydrochloride (~0.1-0.3 mM) in deionized water. [b] Samples were heated to 60 ± 0.2 °C for 64 h in sealed, Teflon-lined vessels and analyzed by ^{1}H NMR spectroscopy in deuterium oxide and hexadeuteriodimethyl sulfoxide solvents.

5-Hydantoinmalonic acid was dissolved in 1.0 M hydrochloric acid and heated in a Teflon-lined, sealed vessel at 70 ± 1 °C for 30 h. The progress of the reaction was monitored by ^{1}H NMR spectroscopy, which indicated that 5-hydantoinmalonic acid cleanly decarboxylated to 5-hydantoinacetic acid (**3**)[6] with first-order



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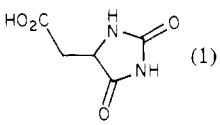

3 (1)

kinetics. After 30 h the reaction was 82% complete. A plot of −ln (% 5-hydantoinmalonic acid) vs. time gave a first-order rate constant of $(1.41 \pm 0.03) \times 10^{-5}$ s^{-1} and a $\tau_{1/2}$ of 13.7 h. Hence, Asa decarboxylates 25 times faster than 5-hydantoinmalonic acid at 70 °C. Gla is similarly stabilized by peptide bonds.[3]

As shown in Table I, β-carboxyaspartic acid reacts at higher pH by elimination of the amine functional group to give tricarboxyethylene, characterized as its trisodium salt **4** by a singlet at δ 6.28 in its ^{1}H NMR spectrum in deuterium oxide solvent. The identification of **4** was achieved by conversion to 1-hydroxy-1,2,2-tricarboxyethane (**5**)[6] by cation-exchange chromatography and by conversion to the known tricarboxyethane (**6**)[7] with catalytic hydrogenation followed by cation-exchange chromatography. Both **5** and **6** were independently prepared in 95% and 94% yields, respectively, by saponification of tris(carbomethoxyethylene)[8] (**7**) followed by cation-exchange chromatography or followed by catalytic hydrogenation and cation-exchange chromatography. Tricarboxyethane was also prepared in 62% yield by saponification of tris(carbomethoxy)ethane[9] (**8**) followed by cation-exchange chromatography. These reactions are summarized in Scheme I. A control experiment showed that Asa was stable to the condition of catalytic hydrogenation and cation-exchange chromatography.

The stability of Asa under the basic conditions commonly employed for protein hydrolysis was also examined. Heating a

(1) Christy, M. R.; Barkley, R. M.; Koch, T. H.; Van Buskirk, J. J.; Kirsch, W. M. *J. Am. Chem. Soc.* **1981**, *103*, 3935.

(2) Stenflo, J.; Fernlund, P.; Egan, W.; Roepstorff, P. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2730. Nelsestuen, G. L.; Zythovicz, T. H.; Howard, J. B. *J. Biol. Chem.* **1974**, *249*, 6347. Magnusson, S.; Sottrup-Jensen, L.; Petersen, T. E.; Morris, H. R.; Dell, A. *FEBS Lett.* **1974**, *44*, 189.

(3) Hauschka, P. V.; Henson, E.; Gallop, P. M. *Anal. Biochem.* **1980**, *108*, 57.

(4) 5-Hydantoinmalonic acid hydrate has the following spectroscopic and analytical properties: IR (KBr) 2.75-3.7 (b) and 5.5-6.2 μm (b); ^{1}H NMR (Me_2SO-d_6) δ 3.62 (d, J = 4.5 Hz, 1 H), 4.37 (d, J = 4.5 Hz, 1 H), 7.80 (s, 1 H), 9.30-10.50 (b, 4 H), 10.65 (s, 1 H); ^{13}C NMR (D_2O) δ 52.4 (t, 1:1:1), 57.5, 160.3, 169.8, 170.6, 176.5. Anal. Calcd for $C_6H_8N_2O_7$: C, 32.73; H, 3.66; N, 12.73. Found: C, 32.73; H, 3.67; N, 12.69.

(5) Henson, E. B.; Gallop, P. M.; Hauschka, P. V. *Tetrahedron* **1981**, *37*, 2561.

(6) 5-Hydantoinacetic acid was characterized by comparison of its ^{1}H NMR spectral properties with those of an authentic sample.

(7) 1-Hydroxy-1,2,2-tricarboxyethane polymerized upon attempted further purification and was characterized by an ^{1}H NMR singlet at δ 4.88 and as its trisodium salt by an AB pattern at δ 3.53 and 4.31 (J = 4.5 Hz), both in deuterium oxide solvent.

(8) Hall, H. K. Jr.; Ykman, P. *J. Am. Chem. Soc.* **1975**, *97*, 800.

(9) Bischoff, C. A. *Justus Liebigs Ann. Chem.* **1882**, *214*, 38.

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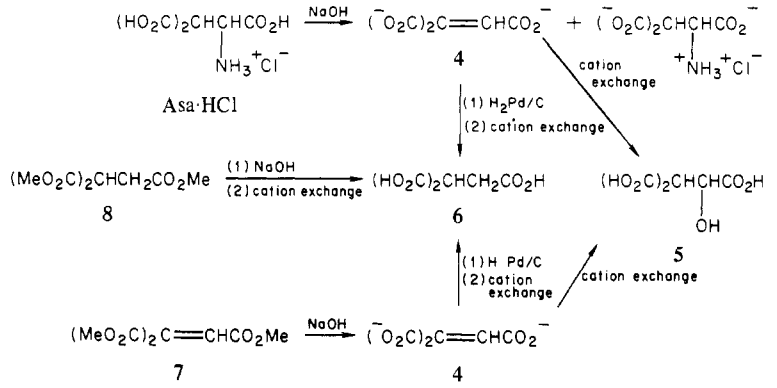
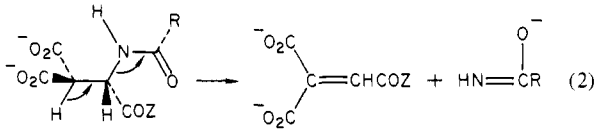
Scheme I



sample of Asa at 110 °C for 24 h in 2 M potassium hydroxide in a Teflon-lined, sealed vessel led to only 5% deamination as determined by ^{1}H NMR analysis.

The reactivity of Asa incorporated in a protein in basic medium was approximated by using dimethyl 5-hydantoinmalonate (**2**) as a model. The dimethyl ester is an acceptable model because ester saponification occurs prior to amide hydrolysis (vide supra). Heating **2** at 100 °C in a sealed, Teflon-lined vessel for 24 h in 2 M potassium hydroxide gave 55% Asa and 45% tricarboxyethylene as determined by ^{1}H NMR spectroscopy.

The extent of elimination as a function of pH can be explained in terms of the leaving group. Even with 3.5 equiv of sodium hyroxide at pH 10 some of the Asa is present with the amine functional group protonated, and the leaving group is ammonia. At higher pH Asa is completely deprotonated and elimination is much slower because the leaving group is amide. 5-Hydantoinmalonic acid is more reactive in 2 M potassium hydroxide with respect to elimination than is Asa because until the 1-2 amide bond of **1** is hydrolyzed, the leaving group is carboxamide. Carboxamide is resonance stabilized and leaves more rapidly than does amide. However, to achieve the geometry necessary for facile E2 or E1cB elimination mechanisms with resonance stabilization of the incipient carboxamide, initial hydrolysis of the 3-4 amide bond of the hydantoin is required. This hydrolysis allows for the necessary bond rotation to occur. The 3-4 amide bond of the hydantoin ring system is known to hydrolyze in basic medium before the 1-2 amide bond.[10] In a protein or peptide containing Asa residues an initial amide hydrolysis is not required, and the elimination reaction should be at least as facile as in the model system. Hence, at least 45% of the Asa was probably lost during the hydrolysis of *E. coli* ribosomal proteins in 2 M potassium hydroxide[1] (see eq 2).



(2)

Henson and co-workers have also reported a synthesis of Asa.[5] These authors imply that their final step, the saponification and hydrolysis of diethyl 5-hydantoinmalonate in 2 M potassium hydroxide at 100 °C for 24 h, was quantitative, even though the resulting Asa was never isolated. The results described above suggest that the yield of Asa from this step is actually less than 55% because of the facile elimination.

Quantitative measurement of peptide-bound Asa is unlikely from protein hydrolysis in basic medium. A more accurate measure of Asa may now require an initial chemical transformation of the malonic acid moiety prior to hydrolysis. Zytkovic and Nelsestuen have shown that the malonic acid moiety of peptide-bound Gla can be reduced to the tritiated 1,3-diol with tritiated diborane and the Gla detected as tritiated δ,δ'-dihydroxyleucine by amino acid analysis after acidic hydrolysis.[11] This methodology is presently under investigation for analysis of peptide-bound Asa.

Acknowledgment. We thank the National Cancer Institute (Grant CA-24665) and the General Medical Institute (Grant GM-24965) of the NIH for financial assistance. We also thank John Van Buskirk and Wolff Kirsch for stimulating discussions.

Registry No. 1, 80754-77-4; **2**, 80754-78-5; **3**, 5427-26-9; **4** trisodium salt, 80754-79-6; **5**, 80754-80-9; **6**, 922-84-9; **7**, 51175-48-5; **8**, 40967-67-7; β-carboxyaspartic acid, 75898-26-9; dimethyl sodiomalonate, 18424-76-5; 5-chlorohydantoin, 32282-43-2.

(10) Ware, E. *Chem. Rev.* **1950**, *46*, 403 and references therein.

(11) Zytkovicz, T. H.; Nelsestuen, G. L. *J. Biol. Chem.* **1974**, *250*, 2968.

Preparation and Crystal Structure of Bis[bis(pentamethylcyclopentadienyl)ytterbium(III)] Undecacarbonyltriferrate, $[(C_5Me_5)_2Yb]_2[Fe_3(CO)_{11}]$: A Compound with Four Isocarbonyl (Fe-CO-Yb) Interactions

T. Don Tilley and Richard A. Andersen*

Chemistry Department and Materials and Molecular Research Division of Lawrence Berkeley Laboratory, University of California Berkeley, California 94720

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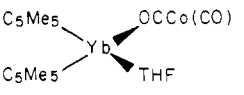
It has been shown recently that the divalent lanthanide complex $(C_5Me_5)_2Yb(OEt_2)$ reduces $Co_2(CO)_8$, cleaving the metal-metal bond to give I.[1a] The tetracarbonylcobaltate anion is bonded to



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the trivalent Lewis acid fragment $[Yb(C_5Me_5)_2(THF)]^+$ by donation of a lone pair of electrons on one of the carbon monoxide groups. Infrared and X-ray data suggest that the carbon-oxygen bond of the bridging carbonyl ligand was significantly weakened, resulting in a charge disparity in the sense Co(δ-)-C(δ+)-O(δ-)-Yb(δ+). This should enhance the reactivity of the bridging carbon monoxide group toward nucleophilic and/or electrophilic reagents. Such carbon- and oxygen-bonded carbonyl ligands have

* To whom correspondence should be addressed at the Chemistry Department, University of California, Berkely, CA.

(1) (a) Tilley, T. D.; Andersen, R. A. *J. Chem. Soc., Chem. Commun.* **1981**, 985-986. (b) Infrared spectra have been used to suggest isocarbonyl interactions with f-block metal atoms: Marianelli, R. S.; Durney, M. T. *J. Organomet. Chem.* **1971**, *32*, C41-C43. Bennet, R. L.; Bruce, M. I.; Stone, F. G. A. *Ibid.* **1971**, *26*, 355-356. Crease, A. E.; Legzdins, P. *J. Chem. Soc., Dalton Trans.* **1973**, 1501-1507. Onaka, S.; Furuichi, *J. Organomet. Chem.* **1979**, *173*, 77-88. *Inorg. Chem.* **1980**, *19*, 2132-2136.

0002-7863/82/1504-1772$01.25/0