Unusual Zwitterion of D,L-β-Carboxyaspartic Acid: pK_a and X-ray Crystallographic Measurements†

Brough Richey, M. Robert Christy, R. Curtis Haltiwanger, Tad H. Koch, and Stanley J. Gill*

ABSTRACT: An investigation of the acidic properties and molecular structure of the new natural amino acid β-carboxyaspartic acid (Asa) is described. The four pK_as of Asa were determined by using a microtitration technique and are 0.8 ± 0.2, 2.5 ± 0.1, 4.7 ± 0.1, and 10.9 ± 0.1. The three pK_as of 5-hydantoinmalonic acid were similarly measured and are 1.85 ± 0.05, 4.63 ± 0.05, and 10.20 ± 0.05. 5-Hydantoinmalonic acid was used as a model for Asa with peptide bonds. Asa crystallizes in the monoclinic space group *Cc* with four molecules per unit cell of dimensions a = 13.112 (3) Å, b = 8.207 (3) Å, and c = 7.292 (2) Å and β = 108.03 (2)°. The structure was solved by direct methods and refined to final values for the discrepancy indices of R = 0.029 and wR = 0.036. The two molecules of Asa are linked by a very strong hydrogen bond between one of the β-carboxyls and the α-carboxyl group of an adjacent molecule. Analysis of the pK_a data indicates that the predominate zwitterion in solution results from ionization of a β-carboxyl group. The X-ray data indicate that in the solid state the negative charge of the zwitterion is distributed approximately equally between one of the β-carboxyls and the α-carboxyl group.

β-Carboxyaspartic acid (Asa)[1] is a naturally occurring amino acid recently identified in *Escherichia coli* ribosomal proteins (Christy et al., 1981). Asa is a homologue of γ-carboxyglutamic acid (Gla), which is formed by the vitamin K mediated posttranslational γ-carboxylation of glutamyl residues in blood coagulation proteins (Magnusson et al., 1974; Nelsestuen et al., 1974; Stenflo et al., 1974). The harsh conditions employed in conventional protein sequencing techniques cause decarboxylation of Asa to give Asp and/or elimination of ammonia from Asa to give carboxymaleic acid (Christy & Koch, 1982). These reactions have limited the detection of Asa in natural systems.

Asa has recently been synthesized by the addition of hydrazoic acid to 1,1,2-tris(carbobenzyloxy)ethylene, followed by catalytic hydrogenation, and isolated as a crystalline monohydrate hydrochloride salt (Christy et al., 1981) and recently as the zwitterion monohydrate. The availability of the pure materal enables the physical and chemical properties of Asa to be characterized and allows its molecular structure to be determined by X-ray diffraction techniques. We have performed potentiometric acid–base titration studies utilizing a specially designed small-scale apparatus (Spokane et al., 1980) in order to determine the ionization properties of Asa. We have also studied the ionic properties of 5-hydantoinmalonic acid, a derivitive of Asa that is structurally analogous to Asa in a peptide linkage. An analysis of the measured equilibrium constants of Asa shows that the major zwitterion species is different from those found in all other naturally occurring amino acids. The X-ray diffraction analysis shows that the unusual zwitterionic structure of Asa is apparent in the solid state as well.

Materials and Methods

Materials. Succinic acid, glutamic acid hydrochloride, and γ-carboxyglutamic acid were obtained from Fisher, Eastman, and Sigma, respectively, and used without further purification. β-Carboxyaspartic acid hydrochloride was prepared as described earlier (Christy et al., 1981). The purity of Asa hydrochloride was greater than 95% with less than 5% of the impurity aspartic acid hydrochloride as determined by ^1H NMR spectroscopy. 5-Hydantoinmalonic acid was prepared by saponification of dimethyl 5-hydantoinmalonate and isolated analytically pure as its monohydrate (Christy & Koch, 1982). Carbonate-free solutions of potassium hydroxide and hydrogen chloride were prepared from Dilut-It analytical concentrates (J. T. Baker) and deionized water. Certified buffer solutions for the pH meter calibration were obtained from Fisher.

Titrations. The pH meter was calibrated with three buffer solutions of pH 4.00, 7.00, and 10.00 (ionic strength ≈ 0.03) to within ±0.02 pH unit. The acids to be titrated had typical concentrations of 0.01 M and were titrated with 0.1 M potassium hydroxide. If the acid had a group with a pK_a very much less than 2, titrations were also performed with 0.1 M acid and 1 M potassium hydroxide.

The titrations were performed on 100-μL acid samples under a nitrogen atmosphere. The potassium hydroxide solutions were injected into the sample by means of a calibrated 100-μL Hamilton syringe. A precision stepping motor syringe drive controlled by a Cybernetics Microsystems programmer was used to set the potassium hydroxide injection rate slow enough to ensure near equilibrium conditions throughout the titration. Typically it took 15 min to inject each equivalent of potassium hydroxide.

Data Analysis. The thermodynamic acid dissociation constants were determined by an analysis of the titration curve data by a method similar to that described by Anderegg (1961) and Willis (1981). Ionic strength effects were accounted for by calculating activity coefficients for all charged species. The value of the activity coefficient γ was approximated by use of the Davies equation as given by Butler (1964):

$$-\log \gamma = AZ^2[I^{1/2}/(1 + I^{1/2}) - 0.2I] \quad (1)$$

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[1] Abbreviations: Asa, β-carboxyaspartic acid; Gla, γ-carboxyglutamic acid; Asp, aspartic acid; Glu, glutamic acid; NMR, nuclear magnetic resonance.

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Table I: Positional and Thermal Parameters[a]

atom	x	y	z	B_{11}[b]	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
O(1)	0.127	−0.3317 (2)	0.6602	3.48 (10)	2.76 (9)	1.52 (7)	−1.03 (8)	1.22 (7)	−0.34 (6)
O(2)	0.2660 (2)	−0.1621 (2)	0.7029 (4)	2.31 (8)	2.71 (8)	1.44 (7)	−0.38 (7)	0.22 (6)	−0.52 (6)
O(3)	−0.0679 (2)	−0.0857 (3)	0.1216 (4)	2.11 (9)	2.32 (8)	3.36 (9)	0.44 (7)	0.20 (7)	−0.15 (7)
O(4)	0.0750 (3)	0.0012 (2)	0.3537 (4)	2.69 (9)	2.13 (8)	3.59 (10)	0.48 (7)	0.18 (8)	−0.85 (7)
O(5)	0.1512 (2)	−0.3203 (2)	0.0060 (3)	1.91 (7)	2.61 (8)	1.31 (7)	−0.25 (7)	0.55 (5)	−0.05 (6)
O(6)	−0.0200 (2)	−0.3845 (3)	−0.0576 (4)	1.56 (8)	3.09 (8)	2.22 (8)	0.04 (6)	0.20 (6)	−0.94 (7)
O(7)	0.3847 (3)	−0.2722 (3)	1.0691 (4)	2.49 (9)	3.05 (9)	2.37 (8)	0.15 (7)	−0.21 (7)	0.03 (7)
N(1)	0.2618 (2)	−0.1696 (3)	0.3390 (4)	1.63 (9)	2.26 (9)	1.40 (8)	−0.31 (7)	0.45 (7)	−0.14 (7)
C(1)	0.1950 (3)	−0.2519 (3)	0.6055 (4)	2.1 (1)	1.69 (9)	1.22 (8)	0.33 (9)	0.39 (7)	0.04 (7)
C(2)	0.1874 (3)	−0.2823 (3)	0.3936 (4)	1.60 (10)	1.51 (9)	1.22 (9)	−0.08 (7)	0.46 (7)	−0.06 (7)
C(3)	0.0736 (3)	−0.2757 (3)	0.2545 (4)	1.62 (9)	1.56 (8)	1.50 (9)	−0.33 (8)	0.67 (8)	−0.07 (7)
C(4)	0.0269 (3)	−0.1049 (3)	0.2488 (4)	1.73 (10)	1.81 (9)	1.65 (9)	−0.07 (8)	0.72 (8)	0.00 (8)
C(5)	0.0637 (3)	−0.3335 (3)	0.0498 (4)	1.67 (9)	1.49 (8)	1.35 (8)	0.08 (8)	0.29 (7)	−0.02 (7)

atom	x	y	z	B	atom	x	y	z	B
H(1)	0.142 (4)	−0.320 (6)	0.847 (8)	3.2	H(6)	0.330 (4)	−0.168 (6)	0.437 (7)	2.8
H(2)	0.213 (4)	−0.387 (6)	0.400 (7)	2.3	H(7)	0.231 (3)	−0.054 (6)	0.330 (6)	2.8
H(3)	0.031 (4)	−0.349 (6)	0.292 (6)	2.4	H(8)	0.361 (5)	−0.238 (7)	0.955 (8)	3.8
H(4)	−0.082 (5)	0.001 (7)	0.124 (8)	3.7	H(9)	0.446 (4)	−0.217 (6)	1.157 (8)	3.8
H(5)	0.257 (4)	−0.205 (6)	0.219 (8)	2.8					

[a] The quantities given in the table are in units of angstroms squared. [b] The form of the anisotropic thermal ellipsoid is $\exp[-0.25(B_{11}h^2a^{*2} + B_{22}k^2b^{*2} + B_{33}l^2c^{*2} + 2B_{12}hka^*b^* + 2B_{13}hla^*c^* + 2B_{23}klb^*c^*)]$.

The value of A is taken to be 0.509, Z is the net charge on the species, and I is the ionic strength of the solution. Activity coefficients were evaluated for each species at every titration point by an iterative nonlinear least-squares fitting program that calculated the values of the thermodynamic acid dissociation constants.

X-ray Structure Analysis. Crystals of Asa monohydrate suitable for X-ray analysis (furnished courtesy of Synthetech Corp., Boulder, CO) were grown by crystallization of racemic β-carboxyaspartic acid hydrochloride in pH 2 hydrochloric acid and had the following crystal data: $C_5H_7NO_6 \cdot H_2O$, fw = 195.13 amu, monoclinic, *Cc*, a = 13.112 (3) Å, b = 8.207 (3) Å, c = 7.292 (2) Å, β = 108.03 (2)°, V = 746.2 (3) Å^3, Z = 4, D_m = 1.73 g/mL (floatation, $CHCl_3$ + $CHBr_3$), D_c = 1.74 g/mL. Three-dimensional X-ray data were collected on a Syntex P$\bar{1}$ diffractometer by using graphite monochromated Mo Kα radiation and θ–2θ scans. Cell dimensions and space group were determined on the diffractometer. The 1648 reflections measured to 2θ = 55.0° were corrected for Lorentz and polarization factors and were averaged to 856 independent reflections. Of these, 765 were observed, $F_o^2 \geq 3.0\sigma F_o^2$, and used in subsequent calculations. The structure was determined by using MULTAN 78 (Main et al., 1978). Hydrogen atoms were located from three-dimensional difference maps after anisotropic refinement on the heavy atoms. The proton H(1) was found centered between O(1) of the α-carboxyl, O(1)–H(1) 1.21 Å, and O(5) of the β-carboxyl, O(5)–H(1) 1.24 Å. Subsequent refinement of the model including positional parameters for the hydrogen atoms resulted in a shift of this hydrogen toward O(5) of 0.07 Å. The function minimized in the least squares was $w(|F_o| - |F_c|)^2$. At convergence the residuals $R = \sum||F_o| - |F_c||/\sum|F_o| = 0.029$ and $wR = [\sum w(|F_o| - |F_c|)^2/(\sum wF_o^2)]^{1/2} = 0.036$. Final positional and thermal parameters are given in Table I. A table of observed and calculated structure factors may be obtained from the authors. A more detailed description of the X-ray procedures has been reported previously (Haltiwanger et al., 1978).

Results

pK_a Measurements. The values determined in this study for the acid dissociation constants of succinic acid and glutamic acid are presented in Table II along with literature values obtained under similar solution conditions. The agreement between these sets of results indicates that ionic strength effects are adequately treated by eq 1 and that the errors caused by the liquid junction effects of the glass electrode are small. The results of the titration of γ-carboxyglutamic acid are also presented in Table II and compare reasonably well with those

Table II: Acid Dissociation Constants Corrected to Zero Ionic Strength at 25 °C

acid	pK_1	pK_2	pK_3	pK_4
succinic acid				
obsd	4.12 ± 0.03	5.59 ± 0.03		
lit.[a]	4.18	5.61		
glutamic acid				
obsd	2.10 ± 0.05	4.25 ± 0.05	9.92 ± 0.05	
lit.[a]	2.16	4.27	9.6	
γ-carboxy-glutamic acid				
obsd	1.8 ± 0.1	2.91 ± 0.05	5.01 ± 0.05	10.3 ± 0.1
lit.[b]	1.7 ± 0.2	3.2 ± 0.1	4.75 ± 0.1	9.9 ± 0.1

[a] Obtained from Christensen et al. (1976). [b] Obtained from Märki et al. (1977).

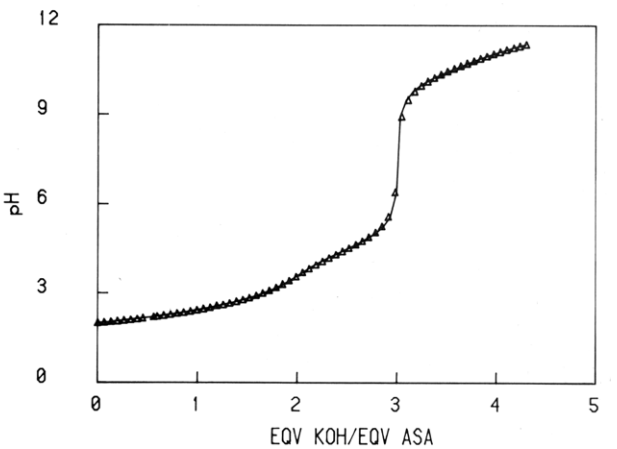

FIGURE 1: Typical titration of Asa. A volume of 100 μL of 9.90 × 10^{-3} M Asa was titrated with 0.1 M KOH. The triangles are the experimental points, and the solid line represents the best fit titration curve generated with values of 1.2, 2.5, 4.7, and 10.9 for pK_1–pK_4, respectively. Note that our best estimate of pK_1 given in Table III was determined by analogous experiments at higher concentrations of Asa.

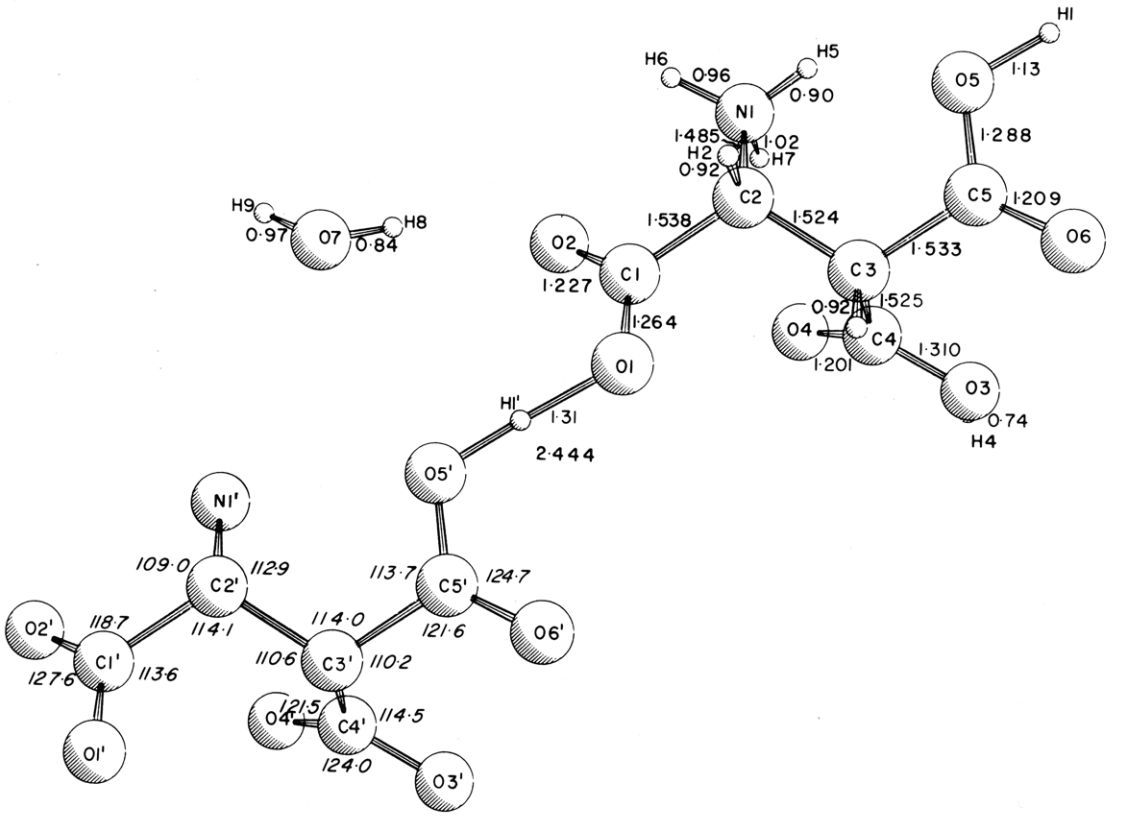

FIGURE 2: PLUTO (Motherwell & Clegg, 1978) drawing of Asa showing the very short O(1)–H(1)–O(5) hydrogen bond, the numbering scheme, the bond lengths (Å), and the bond angles (degrees). Angles involving hydrogen atoms are omitted for clarity. Atoms shown with prime marks are generated by the symmetry operation $x, y, 1.0 + z$. The average standard deviation of the bond lengths is 0.003 for heavy atoms and 0.05 for bonds involving hydrogen, and that of the bond angles is 0.2°.

Table III: Acid Dissociation Constants of Asa and Dimethyl 5-Hydantoinmalonate Corrected to Zero Ionic Strength at 25 °C

	pK_1	pK_2	pK_3	pK_4
Asa[a]	0.8 ± 0.2	2.5 ± 0.1	4.7 ± 0.1	10.9 ± 0.1
5-hydantoin-malonic acid[b]	1.85 ± 0.05	4.63 ± 0.05	10.20 ± 0.05	

[a] Amino acid concentration was 0.01 M except for runs used to determine pK_1 for Asa where 0.1 M Asa was used. [b] Hydantoin concentration was 0.01 M.

Table IV: Hydrogen Bond Distances and Angles[a]

A···H–B	A···B	A···H	<AHB	<HBA
O(1)–H(1)–O(5)[b]	2.444 (2)	1.31 (6)	180	0
O(2)···H(8)–O(7)	2.793 (3)	1.98 (6)	163	12
O(7)[c]···H(4)–O(3)	2.647 (4)	1.93 (6)	163	12
O(6)···H(9)[d]–O(7)[d]	2.919 (3)	2.16 (5)	134	32
O(5)···H(5)–N(1)	2.711 (3)	1.97 (5)	139	29
O(7)[e]···H(5)–N(1)	3.024 (3)	2.33 (5)	134	34
O(6)[f]···H(6)–N(1)	2.760 (3)	2.00 (5)	134	31
O(2)[g]···H(7)–N(1)	2.903 (3)	2.12 (4)	138	29

[a] Symmetry operations are as denoted by the remaining superscripts. [b] $x, y, 1.0 + z$. [c] $-0.5 + x, -0.5 + y, 1.0 + z$. [d] $-0.5 + x, -0.5 - y, -1.5 + z$. [e] $x, y, -1.0 + z$. [f] $0.5 + x, -0.5 - y, 0.5 + z$. [g] $x, y, -1.0 + z$.

previously obtained under different solution conditions (Märki et al., 1977).

Figure 1 shows a typical experimental titration curve of Asa. The solid line is the best fit titration curve generated by using the values determined for the four acid dissociation constants. The values of the acid dissociation constants determined for Asa and 5-hydantoinmalonic acid are presented in Table III. The first pK_a of Asa is lower than that observed for any other amino acid. The large error associated with this value results from the experimental difficulties encountered in determining such a low pK_a. High concentrations of Asa (0.1 M) must be used in order to ensure that a reasonable fraction is fully protonated. The resulting high ionic strength of such solutions cannot be treated correctly by eq 1, and thus the results obtained under such conditions have larger errors. The other three acid dissociation constants of Asa were evaluated by titrating 0.01 M Asa solutions.

X-ray Measurements. Bond distances and angles for Asa monohydrate derived from the positional parameters given in Table I are presented in Figure 2. The numbering scheme used is indicated in the figure. Table IV gives a summary of hydrogen bonding distances and relevant angles. The additional contacts H(6)···O(2), 2.34 (5) Å, N(1)···O(2), 2.638 (3) Å, H(7)···O(4), 2.15 (4) Å, and N(1)···O(4), 2.853 (3) Å, are less than the sum of the van der Waals radii but do not appear to be H-bonding interactions. Similar ionization of one of the β- and the α-carboxyl groups is evident from the O–H and C–O bond lengths.

Discussion

pK_a Data. The α-carboxyl groups of natural amino acids typically have pK_as between 1.8 and 2.4. This narrow range is maintained even though the various amino acids have different substituent groups with large differences in charge and inductive strengths. Because the value of pK_1 (0.8) for Asa is so far out of this range, one must consider the possibility that one of the β-carboxyl groups gives rise to the very low pK_a.

Edsall (Cohn & Edsall, 1943) illustrates a semiempirical method that predicts the influence of various charge and dipolar substituent groups on the pK_as of organic acids. We use this method to determine whether the α-carboxyl or one of

the β-carboxyl groups of Asa ionizes first as the pH is raised, thereby determining the structure of the predominant zwitterion species. The ionization constant of the α-carboxyl group on fully protonated Asa is estimated as follows. If one takes the nominal pK_a for a carboxyl group to be 4.8, the effect of the α-NH_3^+ group one carbon away will reduce this value to 2.3 (Greenstein, 1932). The two β-carboxyl groups two carbons away reduce the pK_a by 0.25 unit each (Cohn & Edsall, 1943) to yield a final value of 1.8 for the pK_a of the Asa α-carboxyl group. This value is within the range normally observed for α-carboxyls of amino acids. The pK_a of an Asa β-carboxyl group is estimated by starting with the pK_1 of malonic acid, which is 2.8. The influence of the charged α-NH_3^+ group at a position two carbons away reduces this value to 1.5 (Cohn & Edsall, 1943). The α-carboxyl group two carbons away further lowers this value by 0.3 unit to yield a final value of 1.2 for the estimated pK_a of an Asa β-carboxyl group. Taking the ionization of both the α-carboxyl and the β-carboxyl groups into account gives the estimated macroscopic dissociation constant as 1.1. This estimate is within experimental error of the experimentally determined pK_1 value for Asa. The experimental Asa titration results are thus consistent with the ionization of a β-carboxyl group to yield the predominant zwitterion species. On the basis of the above estimates the β-carboxyl zwitterion is approximately 4 times as prevalent as the α-carboxyl zwitterion.

The conclusions reached with Asa concerning the nature of the zwitterion species are further supported when the same analysis is applied to Gla. The semiempirical method predicts the macroscopic pK_1 of Gla to be 1.6 with the zwitterion species resulting from ionization of one of the γ-carboxyl groups to be slightly favored over the species resulting from the ionization of the α-carboxyl group. The predicted value of pK_1 agrees well with the measured values given in Table II. The prediction concerning the relative populations of the two zwitterion species is consistent with that determined directly by the ^{13}C NMR studies of Sperling et al. (1978).

The acidic properties of 5-hydantoinmalonic acid were examined because the compound is a close analogue of Asa with peptide bonds. The pK values determined for the two β-carboxyl groups of this compound differ by approximately 1 pK unit from malonic acid (pK_1 = 2.82, pK_2 = 5.66), showing that the peptide bonds exert a strong effect on the ionization properties of the β-carboxyl groups. Under physiological conditions these groups will be fully ionized and free to participate in chelate formation.

X-ray Data. The unusual zwitterionic structure for Asa suggested by the pK_a data analysis prompted the X-ray structural study. We hoped that the site of negative charge would be apparent in the solid state from C–O and O–H bond lengths.

The X-ray data do in fact show an unionized β-carboxyl group, a partially ionized β-carboxyl group, and a partially ionized α-carboxyl group. The proton that determines the ionization, H(1), is located between O(1) and O(5) of the α- and β-carboxyl groups in adjacent molecules in what, according to the distance criterion of Emsley (1980), is a very strong hydrogen bond (Speakman, 1972). Careful examination of difference maps for Asa calculated without H(1) included reveals a broad peak significantly elongated along the vector between O(1) and O(5) with a maximum approximately halfway between O(1) and O(5) (Table IV). Although it is not possible without a neutron diffraction study to determine accurately the position of the hydrogen atoms, this peak suggests that the negative charge is distributed approximately equally between the β- and α-carboxyl groups.

Examination of the C–O bond lengths also supports this assignment of the sites of negative charge. C–O bond lengths in completely ionized carboxyl groups are approximately equal and average 1.25 Å (Sutton, 1965). Examples include the C–O bonds in the α-carboxyl of Asp (Derissen et al., 1968; Rao, 1973) and Glu (Lehmann et al., 1972). C–O bond lengths in unionized carboxyl groups differ by approximately 0.1 Å from each other as observed in the β- and γ-carboxyls of Asp and Glu, respectively, and in the unionized β-carboxyl of Asa (Figure 2). The four C–O bonds of Asa, C(1)–O(1), C(1)–O(2), C(5)–O(5), and C(5)–O(6), are intermediate between these extremes, which again suggests that the negative charge is distributed between the two sites. The α-carboxyl is probably slightly more ionized than the β-carboxyl since the C(1)–O(1) bond is slightly shorter than the C(5)–O(5) bond.

Analogous hydrogen bonding and C–O bond lengths were observed in the X-ray study of Gla. In this case the negative charge is distributed between one of the γ-carboxyl groups and the α-carboxyl group. The hydrogen equivalent to H(1) of Asa was found in two partially occupied positions between the α- and γ-carboxyl groups of adjacent molecules (Satyshur & Rao, 1979).

The X-ray data also indicate that the hydrogens of the protonated amino group of Asa interact with adjacent oxygens and that these interactions determine the conformation about the C(2)–C(3) bond. H(5) apparently participates in a bifurcated hydrogen bond to O(5) and O(7), while H(6) and H(7) participate in normal hydrogen bonds to O(6) and O(2). H(5) lies in the plane formed by O(5), O(7), and N(1) (deviation 0.11 Å). This geometry is typical of bifurcated hydrogen bonds (Parthasarathy, 1969; Koetzle et al., 1972). As mentioned previously, H(6) and H(7) are in close contact with O(2) and O(4). Presumably these contacts result from a Coulombic attraction and along with the intramolecular hydrogen bond O(5)···H(5)–N(1) accounts for the amino group being situated between the two β-carboxyls. As a consequence the hydrogens H(2) and H(3) are gauche. Examination of Corey–Pauling–Koltun models suggests that a conformation with these two hydrogens anti would be sterically less hindered.

Summary. We report the pK_as of Asa and 5-hydantoinmalonic acid and the X-ray structure of Asa. Analysis of the pK_a data indicates that Asa is the most acidic natural amino acid and that the predominant zwitterion in solution is that resulting from ionization of a β-carboxyl group. The X-ray data show approximately equal ionization of the α-carboxyl group and one of the β-carboxyl groups in the solid state.

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Structure of Eukaryotic 5S Ribonucleic Acid: A Study of *Saccharomyces cerevisiae* 5S Ribonucleic Acid with Ribonucleases†

Roger A. Garrett* and Svend Ole Olesen

ABSTRACT: The structure of 5S RNA from *Saccharomyces cerevisiae* was examined by using ribonucleases A, S_1, T_1, and T_2 and a double helix specific cobra venom ribonuclease as probes. The 5′- and 3′-^{32}P-end labeled RNAs were examined, and this enabled a clear distinction to be made between primary and secondary cuts; a selection was also made for nicked, but intact, 5S RNA molecules. The relative degree of cutting was estimated, and the data were tested against secondary structural models. Support is provided for the minimal secondary structural model containing five helical regions; cobra venom ribonuclease cuts were detected in three of the five putative helical regions (helices I, II, and IV), and no single-strand-specific enzyme cuts were observed in the others (helices III and V). Of the putative non-double-helical regions, C_{10}–A_{11}, U_{33}, G_{37}–C_{39}, Ψ_{50}, G_{75}, and U_{90}–G_{91} were very accessible to the single-strand-specific ribonucleases; less accessible sites were G_{25}, G_{52}, U_{54}–A_{55}, C_{73}–C_{74}, and U_{83},G_{85}. Other putative non-double-helical regions A_{22}–A_{24}, C_{26}–C_{28}, C_{34}–C_{36}, C_{40}–C_{44}, U_{53}, A_{56}–G_{57}, A_{76}–A_{79}, and G_{101}–C_{105} were not cut by any ribonucleases and were assumed to be involved in the tertiary structure. Evidence implicating helices III and V in the RNA tertiary structure is also presented. An exceptional degree of flexibility in the sequence A_{22}–G_{57} was induced by primary cuts at U_{38} and C_{39}. The bulged nucleotides A_{63}/A_{64} in helix II and A_{84}/G_{85} in helix IV that have recently been proposed as protein recognition sites became selectively more accessible to the single-strand-specific ribonucleases as magnesium was removed from the RNA. Comparisons between the present results on a eukaryotic 5S RNA and those obtained earlier with *Escherichia coli* and *Bacillus stearothermophilus* 5S RNAs [Douthwaite, S., & Garrett, R. A. (1981) *Biochemistry 20*, 7301–7307] reveal a high level of structural homology and a few marked differences.

5S RNA is the smallest ribosomal RNA and is an integral part of the large ribosomal subunit; it has been localized, together with its bound proteins, in the neighborhood of the peptidyltransferase center in eubacterial ribosomes (Garrett et al., 1981). Although the RNA was thought to form an essential attachment site for elongator tRNAs, this has recently been refuted (Pace et al., 1982) and the precise role of the RNA remains unknown. Nevertheless, the relative structural simplicity of 5S RNA, and its native protein complexes, renders it an obvious choice for detailed studies on both ribosomal RNA secondary structure and the chemical specificity of protein–RNA interactions.

Phylogenetic sequence comparisons have proved to be a powerful aid in determining the secondary structures of both eubacterial (Fox & Woese, 1975) and eukaryotic 5S RNAs (Nishikawa & Takemura, 1974; Fox & Woese, 1975) and, more recently, of the large ribosomal RNAs (Noller & Woese, 1981). On the basis of such studies three double helices, I–III, which are also found in eubacterial 5S RNAs, were proposed at an early stage (Nishikawa & Takemura, 1974; Fox & Woese, 1975). More recently, there has been general agreement about two further helices, one that is analogous to helix IV in eubacterial 5S RNA and an additional one, V, that has no clear equivalent in eubacteria (Nishikawa & Takemura, 1974; Garrett et al., 1981; Luehrsen & Fox, 1981). Never-

† From the Division of Biostructural Chemistry, Kemisk Institut, Aarhus Universitet, DK-8000 Aarhus C, Denmark. *Received March 25, 1982.* The Danish Research Council provided a licentiate stipendium to S.O.O. and a temporary laboratory grant to R.A.G.

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