Biotin Basicity*

Michael Caplow

ABSTRACT: The pK_a's in aqueous solution of biotin, desthiobiotin, imidazolidone, and dimethylurea are -1.13, -0.97, -1.05, and -0.20, respectively.

From this it may be concluded that protonation of biotin occurs on the ureido group, presumably on the carbonyl oxygen atom. The absence of any measurable interaction between the ureido system and the thioether linkage of biotin suggests that the sulfur atom does not play a significant role in enzymatic transcarboxylation from *N*-carboxybiotin.

We have recently found (Caplow, 1965, 1968) that *N*-carboxybiotin is remarkably stable as compared with other carbamates and that this stability originates from the low basicity of the biotin ureido system. Our estimates of the basicity of this center required drastic revision with the report by Glasel (1966) that the basic locus of the biotin molecule is the thiophan sulfur atom. Since thioethers have been found to be extremely nonbasic (Arnett, 1963) these results suggested some special properties for the biotin molecule which may be associated with catalysis involving this cofactor. We report here a reinvestigation of this problem, the results of which indicate that biotin protonation in acidic aqueous solutions occurs on the ureido carbonyl group rather than the sulfur atom.

Experimental Section

Materials. D-Biotin, obtained from Mann, was recrystallized from water. D-Biotin (carbonyl C-14), which had been prepared by the method of Melville *et al.* (1949), was obtained from Nuclear-Chicago. The radioactive material was diluted with nonradioactive biotin and recrystallized to constant specific activity (2.21×10^4 cpm/mg, in the assay used for determination of the biotin dissociation constant). In solubility studies it was found that the amount of radioactive material that dissolved depended upon the amount of excess solid added. This indicates that the material is impure and from studies of the concentration dependence of the solubility it was found that the crystalline product was contaminated to the extent of 2.1% with a radioactive impurity. Desthiobiotin was prepared using Raney nickel as previously described (Melville *et al.*, 1943) (mp 156–158, lit. mp 152–157°). *Anal.* Calcd C, 56.06; H, 8.46; N, 13.08; S, 0. Found: C, 56.09; H, 8.55; N, 12.73; S, 0.74. The sulfur analysis suggests that the product may contain as much as 5.65% biotin, although this is not consistent with the observed carbon content which should be 55.78% for a mixture of 5.65% biotin and 94.65% desthiobiotin. Decarboxybiotin (3,4-*cis*-diamino-2-tetrahydrothiophene-*n*-valeric)acid was prepared by a published procedure (Hofmann *et al.*, 1941) and the sulfate salt was crystallized from methanol–water to give a product of mp 258–261° (lit. mp 245–255°). Imidazolidone and dimethylurea were crystallized from chloroform and ethanol–ether, respectively.

Methods. Acid dissociation constants for imidazolidone, dimethylurea, and desthiobiotin were obtained from spectrophotometric titration at 220 mμ. The results were analyzed using the logistic procedure of Reed and Berkson (Clark, 1960).

The biotin dissociation constant was determined from solubility measurements at varying sulfuric acid concentrations. A weighed quantity of crystalline biotin was added to 2 ml of acid contained in a small stoppered test tube. The tubes were periodically mixed during an approximately 6-hr incubation at 25°; saturation was generally observed after about 3 hr. The amount of dissolved biotin was determined in a 0.1-ml aliquot obtained by careful pipetting avoiding the precipitate on the bottom of the tube. The aliquot was diluted to 1 ml with a sufficient quantity of sulfuric acid and water to bring the sulfuric acid concentration to 0.58 M. This procedure was necessary since the counting efficiency is dependent upon acid concentration. After addition of 10 ml of counting fluid (dioxane–naphthalene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene, 500 ml:100 g:5 g:0.125 g) radioactivity was measured in a scintillation counter. Corrections were made for the 2.1% contaminant described above. Values of H_0 were obtained from tables provided by Paul and Long (1957).

Proton magnetic resonance spectra were recorded on a Varian A-60 spectrometer.

Results and Discussion

The pK's of biotin derivatives are summarized in Table I. As would be expected, imidazolidone and desthiobiotin have comparable basicity. Comparison of the pK's of imidazolidone and dimethylurea indicates that incorporation of the ureido system into a ring results in a decrease in basicity. Attempts to determine the acid–base properties of biotin by spectrophotometric methods were unsuccessful because of the insolubility of this compound. This factor was eventually utilized for measuring the biotin dissociation constant. Since protonation generates a polar species the solubility of biotin is increased with increasing acidity, as outlined in eq 1, where (biotin solubility) is the solubility of neutral biotin (observed solubility), is constituted from the solubilities of neutral, and

* From the Department of Biochemistry, Yale University, New Haven, Connecticut 06510. *Received February 18, 1969.* Supported by Grant No. GM 11820 from the National Institutes of Health.

TABLE I: pK_a's of Biotin Derivatives at 25°.

Compound	pK	Concn (M × 10^3)	H^+ Concn	ϵ_{Base}[a]	ϵ_{CA}[a,b]
Desthiobiotin	−0.97	2.36	0.42–6.65 (HCl)	118	381
2-Imidazolidone	−1.05	1.0	0.36–6.55 (HCl)	135	56.1
1,3-Dimethylurea	−0.20	10	0.11–6.73 (H_2SO_4)	47.3	27.6

[a] Molar extinction coefficient at 220 mμ. [b] CA = conjugate acid.

$$\frac{\text{(biotin solubility)}}{\text{(observed solubility)}} = \frac{K}{K + (H^+)} \quad (1)$$

protonated biotin, K = (biotin)(H^+)/(biotin-H^+), and (H^+) is the activity of the proton expressed in terms of the H_0 scale. Equation 1 predicts a sigmoidal titration curve, the midpoint of which occurs at an acidity where the solubility of biotin is doubled. The results obtained in studies of biotin solubility as a function of acidity are summarized in Table II and plotted according to a rearranged form of eq 1 in Figure 1. Although no evidence was obtained indicating that biotin undergoes hydrolysis during the course of study, the occurrence of this reaction would result in a loss of radioactivity as carbon dioxide so that no correction need be made for this process. Salts have little effect on the solubility of biotin; less than 10% variation in solubility was observed in a 0.5 M HCl solution and a 0.5 M HCl solution containing 5.25 M KCl, and in a 0.5 M H_2SO_4 solution as compared with an identical solution containing 3.0 M $KHSO_4$.

The similarity of the pK's of biotin, desthiobiotin, and imidazolidone indicates that protonation of biotin occurs on the ureido system, presumably the ureido carbonyl oxygen atom (Stewart and Muenster, 1961), rather than on the thiophan sulfur. A similar conclusion was derived from results of studies of biotin and imidazolidone basicity in anhydrous formic acid (Bowen *et al.*, 1968). The present study provides more definitive results for consideration of the properties of biotin in physiological fluids since it is conceivable that specific solvation may change the properties of the biotin molecule. The earlier conclusion that protonation occurs on sulfur was based upon an nuclear magnetic resonance study of biotin (Glasel, 1966) in which it was found that there is a change in chemical shift of the C-2 protons induced by strong acid. That this is the result of protonation of the ureido group is indicated by the fact that the change in chemical shift for the C-3 proton is *larger* than that observed for the C-2 protons (0.37 as compared with 0.21 ppm). Protonation of biotin on the sulfur atom has also been suggested from the observation that the C-2 protons of decarboxybiotin in HI:D_2O yield a single unresolved peak similar to that found by Glasel for biotin in strongly acidic solutions (Bowen *et al.*, 1968). We have recorded the nuclear magnetic resonance spectrum of decarboxybiotin in 0.043 M DCl (50 mg of the diaminocarboxylic acid sulfate in 0.75 ml of D_2O), where the thioether linkage is certainly not protonated, and have found that the C-2 protons give rise to a complex pattern with principal peaks at δ 3.14, 3.04, 2.98, 2.91, and 2.82 (relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate). In 0.26 M KOD the principal peaks associated with these protons are observed at δ 2.64, 2.54, and 2.44 (relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate). From this it may be concluded that the perturbation of the C-2 protons is associated with protonation of the amino

TABLE II: Solubility of Biotin as a Function of Acidity at 25°.

H_2SO_4 (M)	H_0	Obsd cpm[a]	Calcd cpm[b]
0.101	0.83	635	758
0.25	0.49	701	768
1.50	−0.57	960	957
2.62	−1.19	1,520	1,610
3.48	−1.62	2,798	3,070
4.08	−1.89	5,089	5,065
4.77	−2.19	9,398	9,360
5.80	−2.69	35,800	27,950

[a] Corrected for background (30 cpm) and contamination by an impurity (23 cpm/mg). [b] Calculated for a pK of −1.13 and a contribution of 750 cpm from neutral biotin (0.34 mg/ml).

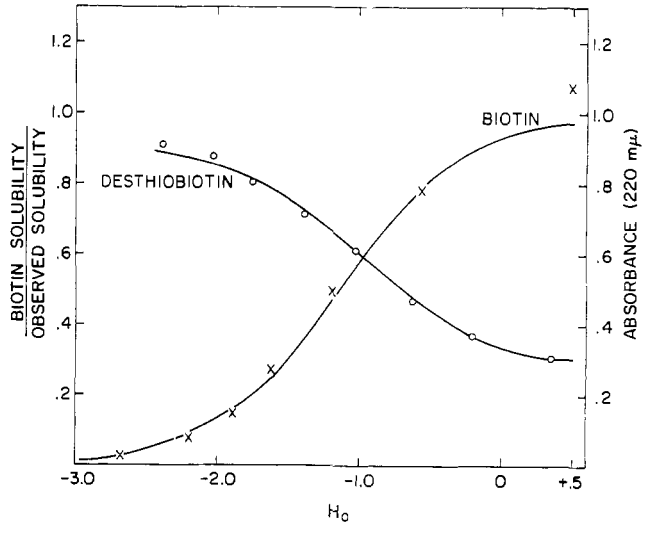

FIGURE 1: Titration of biotin and desthiobiotin. Left ordinate is for biotin, right ordinate for desthiobiotin. The solid lines are theoretical curves.

groups rather than the thioether linkage. Olah and White (1968) have observed protonation of the thioether linkage and the ureido carbonyl oxygen atom of biotin in FSO_3H-SbF_5.

Results of studies of nonenzymatic unimolecular decarboxylation of carbamates, and acyl transfer from esters of *N*-carboxyimidazolidone (Caplow, 1965, 1968) suggest that the principal determinants underlying the reactivity of *N*-carboxybiotin in enzymatic carbon dioxide transfer are the basicity of the cofactor, electrophilic activation of the carboxylate group, and the catalysis by the enzyme directed to activation of the nucleophilic acceptor. The absence of any measurable interaction between the ureido system and the thioether linkage in neutral or protonated biotin suggests that the sulfur atom does not play a significant role in enzymatic transcarboxylation, as suggested previously (Mildvan *et al.*, 1966).

References

Arnett, E. M. (1963), *Progr. Phys. Org. Chem. 1*, 223.
Bowen, C. E., Rauscher, E., and Ingraham, L. L. (1968), *Arch. Biochem. Biophys. 125*, 865.
Caplow, M. (1965), *J. Am. Chem. Soc. 87*, 5774.
Caplow, M. (1968), *J. Am. Chem. Soc. 90*, 6795.
Clark, W. M. (1960), Oxidation–Reduction Potentials of Organic Systems, Baltimore, Md., Williams & Wilkins Co., p 149.
Glasel, J. A. (1966), *Biochemistry 5*, 1851.
Hofmann, K., Melville, D. B., and DuVigneaud, V. (1941), *J. Biol. Chem. 141*, 207.
Melville, D. B., Dittmer, K., Brown, G. B., and DuVigneaud, V. (1943), *Science 98*, 497.
Melville, D. B., Pierce, J. G., and Partridge, C. W. H. (1949), *J. Biol. Chem. 180*, 299.
Mildvan, A. S., Scrutton, M. C., and Utter, M. F. (1966), *J. Biol. Chem. 241*, 3488.
Olah, G. A., and White, A. M. (1968), *J. Am. Chem. Soc. 90*, 6087.
Paul, M. A., and Long, F. A. (1957), *Chem. Rev. 57*, 1.
Stewart, R., and Muenster, L. J. (1961), *Can. J. Chem. 39*, 401.

On the Reported Presence of Biotin in Carbamyl Phosphate Synthetase*

R. Bruce Huston† and Philip P. Cohen

ABSTRACT: Purified carbamyl phosphate synthetase from frog and beef liver mitochondria and from *Escherichia coli* was tested for the presence of biotin as a coenzyme. Avidin preparations which were proved effective in inhibiting pyruvate carboxylase had no effect on either the beef liver or the *E. coli* carbamyl phosphate synthetase. Also, a direct assay for biotin using *Neurospora crassa* as a test organism showed no significant amount of biotin in any of the three enzymes Thus biotin is not a coenzyme in the carbamyl phosphate synthetase reaction.

The results on the *E. coli* enzyme are in direct conflict with the conclusion of the report (Wellner, V. P., Santos, J. I., and Meister, A. (1968), *Biochemistry 7*, 2848) that biotin was present and functional.

The formation of carbamyl phosphate from bicarbonate, ammonia, and 2 moles of ATP by carbamyl phosphate synthetase includes the ATP-dependent activation of CO_2 as a postulated partial reaction (Anderson and Meister, 1965; Metzenberg *et al.*, 1958). This has led in the past to a number of negative, and therefore unpublished, experiments concerning the possible inhibition by avidin, and possible presence of biotin, in the frog liver mitochondrial enzyme (M. Marshall, R. L. Metzenberg, and P. P. Cohen, unpublished results).

The recent report of the presence of biotin in the glutamine-utilizing *Escherichia coli* carbamyl phosphate synthetase (Wellner *et al.*, 1968) and the accompanying suggestion that both the ammonia- and glutamine-utilizing animal enzymes might also contain biotin has led to a reinvestigation of this question in the frog liver mitochondrial carbamyl phosphate synthetase and the extension of the study to include the mitochondrial enzyme from beef liver. As a control, *E. coli* carbamyl phosphate synthetase was also prepared and tested.

Two recent papers have stated the absence of biotin in the rat liver mitochondrial carbamyl phosphate synthetase (Guthöhrlein and Knappe, 1968) and the lack of inhibition by avidin of the frog liver mitochondrial enzyme and the pigeon liver glutamine-utilizing enzyme (Peng and Jones, 1969).

Experimental Section

Materials. Two samples of avidin were used, both products

* From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received March 17, 1969.* This study was supported in part by Grant C-3571 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and Grant GB 7105 from the National Science Foundation.

† National Institutes of Health postdoctoral fellow.