with a 10-nm bandwidth), the brushite-phytochrome solution was stored frozen at -20 °C in the dark.

Phytochromobilipeptides. Pepsin Digestion. This procedure was performed under green safelight[34] at 4 °C by a modification of the published procedure.[6] Brushite P_R phytochrome (614 mg, 5.1 μmol, SAR = 0.06) in 1 L of 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.8, was precipitated with 300 g of solid $(NH_4)_2SO_4$. After centrifugation (15 min, 20000*g*) the pellet was suspended in 1.3 M HCOOH (200 mL) and stirred overnight. Nine hours later this suspension was centrifuged (15 min, 20000*g*). The precipitate was resuspended in 1.3 M HCOOH (131 mL) to which a solution of pepsin (32 mL, 8.9 mg/mL in 1.3 M HCOOH) was added. The mixture was then incubated with stirring for 4.5 h at 37 °C under Ar. After digestion, the mixture was centrifuged (15 min, 20000*g*), rotary evaporated to 25 mL, and applied to a BioGel P4 column (2.5 × 33.5 cm, flow rate 45 mL/h; preequilibrated with 1.3 M HCOOH).[35] The column was then washed with 550 mL of 1.3 M HCOOH while most of the blue material remained adsorbed to the first two-thirds of the column. This blue fraction was eluted from the column with 25% aqueous HOAc and collected in a 168-mL volume, and from its absorption spectrum represents 3.1 μmol (58% yield) of phytochromobilin (with $\epsilon_{665nm} = 3.2 \times 10^4$ L mol^{-1} cm^{-1}).[6] The amino acid composition of this fraction is compiled in Table I. The ^{1}H NMR spectrum is illustrated in Figure 1 of the supplementary material.

Thermolysin Digestion. To minimize photochemical side reactions, this procedure was performed under green safelight[34] or in the dark whenever possible. The pepsin-cleaved phytochromobilin peptide fraction was lyophilized in two equal portions in 5-mL ampules. The dry, blue residues were then dissolved in 1.0 mL of 0.1 N NH_4HCO_3 to which 200 μL of thermolysin solution (0.51 mg/mL in 0.1 N NH_4HCO_3) was added. After freeze-thaw degassing twice, the ampules were sealed under vacuum. The mixtures were incubated at 37 °C for 4 h and cooled in ice; 300 μL of glacial acetic acid was introduced into each ampule.

The resulting dark blue solution was applied to a Sephadex G50 column (medium, 2.5 × 50 cm, flow rate 49 mL/h, preequilibrated with 25% aqueous HOAc) and eluted with 25% aqueous HOAc. A colorless 134-mL fraction was collected before the phytochromobilipeptide fraction eluted within 64 mL. On the basis of the absorption spectrum of this fraction, the recovery was determined as 2.4 μmol, 47% overall yield, of phytochromobilipeptides (see supplementary materials, Figure 2).

High-performance liquid chromatography of this thermolysin chromopeptide mixture was accomplished on a C_{18} reversed-phase column. As shown in Figure 3, five major phytochromobilipeptides, fractions 1-5, and three cleaved pigments, fractions 6-8, were obtained after high-performance liquid chromatography. In Tables I and II, the amino acid composition of fractions 2-5 and the sequence data for fraction 5 are tabulated. The absorption and ^{1}H NMR spectra of fraction 5, phytochromobiliundecapeptide (**2**), are illustrated in Figures 3-6 and Table III.

(35) The BioGel P4 column was prewashed with a mixture of 0.01 N EDTA and 0.1 N L-ascorbic acid to remove any trace metal or other oxidizing contaminants in the gel.

Acknowledgments. The generous assistance of Dr. Willy C. Shih in the NMR spectral analysis was invaluable. We thank Dr. Winslow Briggs and Dr. Peter Quail, Carnegie Institution of Washington, Stanford CA, for instruction and assistance in the isolation of phytochrome. The assistance of Karen Ruth, Steve Graff, and Bob Schoenleber with the isolations is gratefully acknowledged.

Supplementary Material Available: Full details of the ^{1}H NMR spectra of the peptide moiety of phytochromobiliundecapeptide (**2**), the pepsin peptide from phytochrome, and the phytochromobilioctapeptide (**3**) and the absorption spectrum of the chromopeptide fraction after thermolysin digestion (5 pages). Ordering information is given on any current masthead page.

Kinetics of Thiamine Cleavage by Bisulfite Ion

Daniel R. Doerge and Lloyd L. Ingraham*

Contribution from the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received August 13, 1979*

Abstract: The rate of cleavage of thiamine by bisulfite ion was found to be dependent on the square of the concentration of bisulfite ion at low concentrations of bisulfite. These results support the Zoltewicz-Kaufmann mechanism for thiamine cleavage in which the second step is an S_N2 displacement by bisulfite ion. Support is also given to the Zoltewicz-Kauffman suggestion that the enzyme thiaminase utilizes a similar mechanism in thiamine cleavage by amines.

Introduction

The bisulfite cleavage of thiamine has been known since 1935.[1] The products of the reaction are 2-methyl-4-aminopyrimidyl-5-methanesulfonate (V) and 4-methyl-5-(2-hydroxyethyl)thiazole. This reaction has been considered to be a simple displacement reaction by bisulfite ion for many years in spite of the fact that the reaction is unique for bisulfite ion and that the Japanese literature contained several reports that bisulfite ion catalyzed the cleavage by other nucleophiles, predominately amines.

Zoltewicz and Kauffman[2] have proposed a novel mechanism for the cleavage as a result of their studies of the bisulfite-catalyzed azide cleavage of thiamine. These authors proposed that the first step is a bisulfite addition to the 6-position of the pyrimidine ring (cf. Scheme I) followed by either an S_N1 or S_N2 displacement by azide ion or the formation of a sultone (III) followed again by reaction with azide ion. The Zoltewicz-Kauffman mechanism for bisulfite cleavage would replace the azide ion with bisulfite ion and thus require two bisulfite ions for the reaction. Zoltewicz and Kauffman found a unimolecular dependence of the rate on the concentration of bisulfite ion as was found earlier by Leichter and Joslyn.[3] This result could be found for the sultone mechanism, the S_N1 mechanism, or an S_N2 mechanism in which the rate of reaction of II with bisulfite ion is faster than the dissociation of II to I and bisulfite ion.

In order to investigate the latter possibility, we have studied the kinetics at low concentrations of bisulfite ion where the reaction of II with bisulfite ion would be slower than the dissociation.

Experimental Section

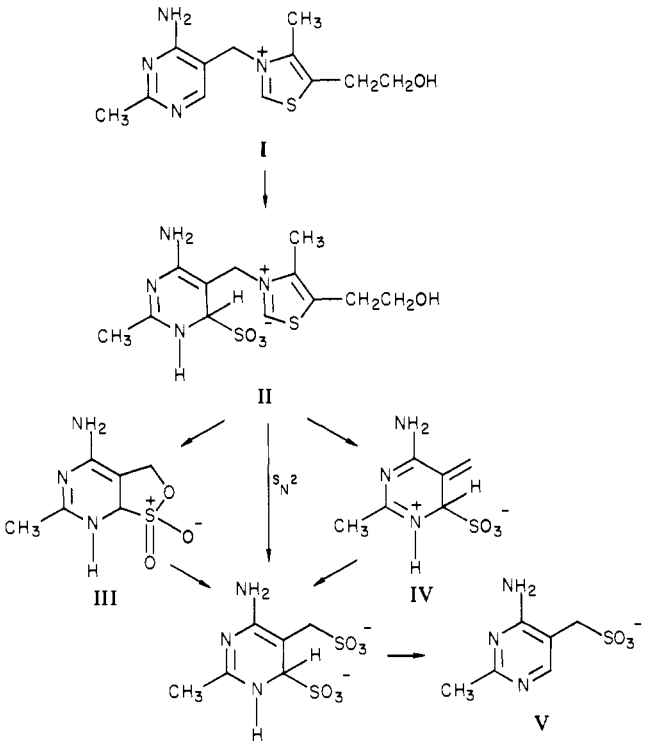
Materials. All reagents were of analytical reagent purity.

Kinetic Procedure. Reactions were run in a sealed glass vessel maintained at 25.0 ± 0.2 °C by a Lauda circulating water bath. All reactions were carried out in 1 mM buffer (citrate for pH 3.5, 4.0, 4.5; acetate for pH 5.0; tris for pH 6.8) and pH values checked before and after each reaction. The pH values did not change within experimental error during the reaction. Sodium bisulfite (Mallinkrodt) solutions were prepared

(1) Williams, R. R. *J. Am. Chem. Soc.* **1935**, *57*, 229.
(2) Zoltewicz, J. A., Kauffman, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 3134.
(3) Leichter, J.; Joslyn, M. A. *Biochem. J.* **1969**, *113*, 611.

0002-7863/80/1502-4828$01.00/0

Scheme I



daily and bisulfite concentrations standardized iodometrically. Small but measurable rates of air oxidation were corrected for (<1% of total reaction). In a typical experiment, 40 mM thiamine hydrochloride at the indicated pH was mixed with 500 μM sodium bisulfite (final ionic strength 0.05 M). Minimum ionic strength conditions were employed to maximize rates. At various times, an aliquot was removed and the remaining concentration of bisulfite determined colorimetrically by the method of Nury et al.[4]

Kinetic Analysis. Initial rates were obtained from the linear portion of the disappearance of [bisulfite] vs. time plot (<10% of total reaction) at various pHs. Slopes and intercepts (initial [bisulfite]) were obtained by linear least-squares analysis. Initial rates were replotted vs. initial [bisulfite] as shown in an example at pH 4.0 (Figure 2). k_1 was determined by dividing out the [thiamine] from the slope of the straight-line portion of the curve determined by least-squares analysis. The intercept of the straight-line portion of the curve on the [bisulfite] axis, k_2/k_3, was determined and associated error limits were indicated by bars in Figure 2.

Kinetics

The reaction of thiamine (I) with bisulfite ion (S) to give a complex (II) followed by a rate-determining bisulfite cleavage leads to rate eq 1. Expansion of eq 1 by algebraic division gives

$$\mathrm{I} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{II}$$

$$\mathrm{II} + \mathrm{S} \xrightarrow{k_3} \mathrm{P}$$

$$\frac{-\mathrm{dI}}{\mathrm{d}t} = \frac{k_1 k_3 (\mathrm{I})(\mathrm{S})^2}{k_2 + k_3(\mathrm{S})} \qquad (1)$$

eq 2 and retention of only the first two terms gives eq 3 which

$$\frac{-\mathrm{dI}}{\mathrm{d}t} = k_1(\mathrm{I})(\mathrm{S}) - \frac{k_1 k_2}{k_3}(\mathrm{I}) + \frac{k_1 k_2^2(\mathrm{I})}{k_3^2(\mathrm{S})} - \frac{k_1 k_2^3(\mathrm{I})}{k_3^3(\mathrm{S})^2} + \ldots \qquad (2)$$

$$\frac{-\mathrm{dI}}{\mathrm{d}t} = k_1(\mathrm{I})(\mathrm{S}) - \frac{k_1 k_2}{k_3}(\mathrm{I}) \qquad (3)$$

shows that $-\mathrm{dI}/\mathrm{d}t$ is linear in (S) at large values of (S) in agreement with previous work. Extrapolation of the linear portion of the curve to $-\mathrm{dI}/\mathrm{d}t = 0$ gives an intercept on the S-axis of (S) $= k_2/k_3$.

(4) Nury, F. S.; Taylor, D. H.; Brekke, J. E. *J. Agric. Food Chem.* **1959**, *1*, 351.

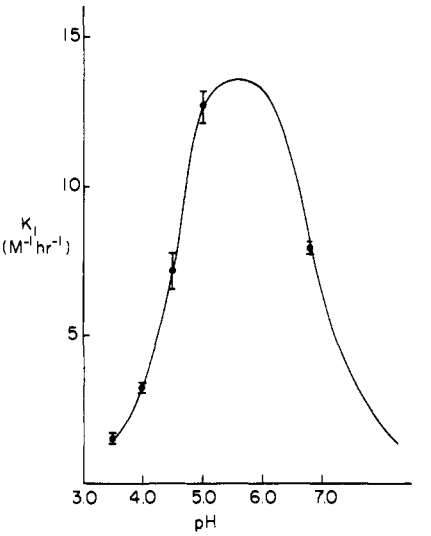


Figure 1. Second-order rate constant for disappearance of bisulfite in the presence of thiamine as a function of pH.

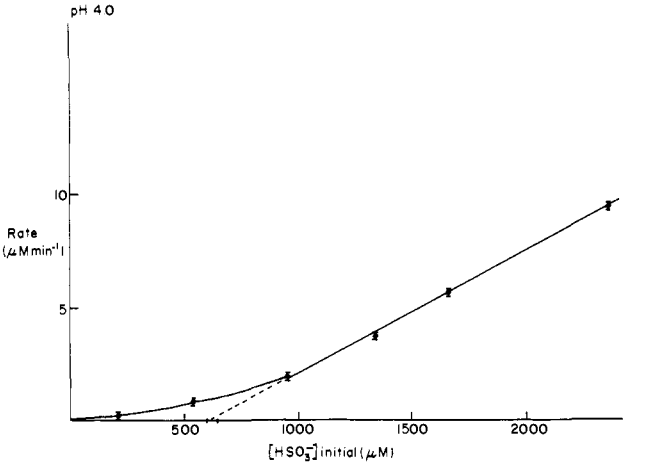


Figure 2. Dependence of the initial rates for disappearance of bisulfite ion in the presence of thiamine at pH 4.0 on the initial concentration of bisulfite ion.

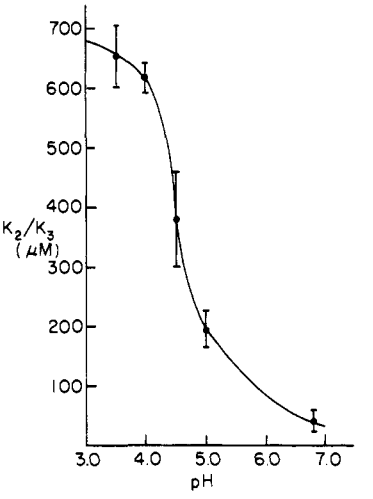


Figure 3. Dependence of the intercept as in Figure 2 on pH of the solution.

Results

The rates of disappearance of bisulfite ion were measured as a function of the concentration of bisulfite ion. The bimolecular

rate constant k_1, determined from the linear portion of the rate curves at high [bisulfite], showed the same bell-shaped pH dependence as previously reported (cf. Figure 1).[2,3] Extrapolation of these rates to low concentrations of bisulfite ion showed an intercept (Figure 2). The intercept has a strong dependence on pH as shown in Figure 3. The inflection point at pH = 4.7 corresponds to the pK of the pyrimidine portion of thiamine (4.7). A more complete explanation of the pH dependence of k_2 and k_3 is not possible since the acid-base properties of the intermediate(s) are not known.

Thus the pH dependence of k_1 is in agreement with the mechanism proposed and the pH dependence of the intercept is at least consistent.

These results show clearly that two bisulfite ions are involved in thiamine cleavage as proposed by Zoltewicz and Kauffman.

Our results at low concentrations of bisulfite clearly show a rate-determining attack of bisulfite ion on the bisulfite-thiamine adduct. S_N2 reactivity at the methylene carbon could be expected to increase the bisulfite-thiamine adduct due to the increased electron-donating properties of the bisulfite-thiamine adduct relative to thiamine.

These results certainly rule out both the S_N1 mechanism and the sultone mechanism. Zoltewicz and Kauffman did not support the sultone mechanism but preferred the S_N1 mechanism over the S_N2 mechanism. They point out that the S_N2 mechanism encounters more steric hindrance in the adduct II over I even though electronically S_N2 would be favored in II over I. The electronic effects may compensate for the steric hindrance in compound II.

Zoltewicz and Kauffman have proposed that this mechanism may be functioning in certain enzyme-catalyzed reactions such as the thiaminase reaction. We concur and add that a two-step reaction was considered for the thiamine reaction in 1963.[5] The thiaminase-catalyzed rate of cleavage of thiamine by substituted anilines increased with the value of Hammett's σ constant for negative values of σ constants but decreases with positive values of σ constants. A change in rate-determining step was proposed between negative and positive values of σ constants which requires a two-step mechanism.

(5) Mazrimas, J. A.; Song, P. S.; Ingraham, L. L.; Draper, R. D. *Arch. Biochem. Biophys.* **1963**, *100*, 409.

Communications to the Editor

$C_3H_5^+$ Isomers: Evidence for the Existence of Long-Lived Allyl and 2-Propenyl Cations in the Gas Phase

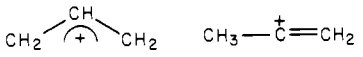
Sir:

Several years ago Aue, Davidson, and Bowers[1] suggested that reactions 1 and 2 formed, at least in part, the 2-propenyl cation

$$BH^+ + CH_2{=}C{=}CH_2 \rightleftarrows C_3H_5^+ + B \quad (1)$$

$$BH^+ + CH_3C{\equiv}CH \rightleftarrows C_3H_5^+ + B \quad (2)$$

II rather than the more stable[2] allyl cation I. The suggestion



I II

was based on the measured proton affinities of $CH_2{=}C{=}CH_2$ and $CH_3C{\equiv}CH$ which yield[1-3] $\Delta H_f°(C_3H_5^+) = 230 \pm 2$ kcal/mol, a value 4 kcal/mol greater than the well-known[3,4] heat of formation of allyl cation I and on the fact that $C_3H_5^+$ ions formed from protonation of allene and propyne protonate CH_3OH while those formed from electron-impact-induced fragmentation of allyl chloride do not.

In order to further investigate this problem we have formed $C_3H_5^+$ ions in the source of a high-performance ZAB-2F mass spectrometer[5] either by electron-impact-induced fragmentation of a molecular ion or by selected ion-molecule reactions. The ions are analyzed by collision-induced dissociation spectroscopy (MIKES-CID).[6] A large number of product ion peaks result from collisional excitation of $C_3H_5^+$.[7] The ion peaks that show most clearly differences in MIKES-CID spectra are those containing two carbon atoms. A summary is given in Table I.

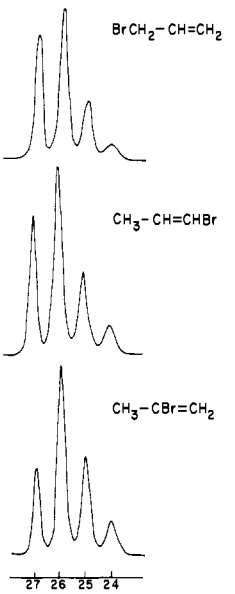


Figure 1. MIKES-CID spectra of $C_3H_5^+$ ions in the region C_2^+ to $C_2H_3^+$. The $C_3H_5^+$ ions were generated by electron impact on the three isomeric bromopropenes shown.

(1) D. H. Aue, W. R. Davidson, and M. T. Bowers, *J. Am. Chem. Soc.*, **98**, 6700 (1976).

(2) Revisions in the proton affinity scale and double-resonance experiments with allene and propyne yield proton affinities (PA) of these species of 182 kcal/mol on a scale where $PA(NH_3) = 205$ kcal/mol (ref 3). The uncertainty in the heat of formation of 2-propenyl ion could be as great as 3-5 kcal/mol, however; see ref 3 and F. Houle and J. L. Beauchamp, *J. Am. Chem. Soc.*, **101**, 4071 (1979).

(3) D. H. Aue and M. T. Bowers, Basicity Measurements in "Gas Phase Ion Chemistry", Vol. II, M. T. Bowers, Ed., Academic Press, New York, 1979, pp 1-52.

(4) F. P. Lossing, *Can. J. Chem.*, **50**, 3973 (1972); **49**, 356 (1971). See also D. W. Berman, V. G. Anicich, and J. L. Beauchamp, *J. Am. Chem. Soc.*, **101**, 1239 (1979).

(5) Manufactured by V. G. Micromass. For a working description, see R. P. Morgan, J. H. Beynon, R. H. Bateman, and B. N. Green, *Int. J. Mass Spectrom. Ion Phys.*, **28**, 171 (1978).

(6) R. G. Cooks in "Collision Spectroscopy", R. G. Cooks, Ed.; Plenum Press, New York, 1978. For a more general reference, see R. G. Cooks, J. H. Beynon, R. M. Caprioli, and G. R. Lester, "Metastable Ions", Elsevier, Amsterdam, 1973. See also N. M. M. Nibbering, T. Nishishita, C. C. van de Sande, and F. W. McLafferty, *J. Am. Chem. Soc.*, **96**, 5668 (1974); F. W. McLafferty, *ACS Symp Ser.*, **No. 70**, 45-47 (1978); K. Levsen, "Fundamental Aspects of Organic Mass Spectrometry", Verlag Chemie, Weinheim, West Germany, 1978.

(7) A. Maquestiou, Y. van Hoverbeke, R. Flamming, C. de Meyer, and A. Menu, *Org. Mass Spectrom.*, **12**, 707 (1979).

0002-7863/80/1502-4830$01.00/0