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The Carbonyl Reactivity of 3-Bromopyruvate and Related Compounds

GUNTER FISCHER, MANFRED SIEBER, AND ALFRED SCHELLENBERGER

Sektion Biowissenschaften, Wissenschaftsbereich Biochemie der Martin-Luther-Universität Halle/S., 402 Halle, German Democratic Republic

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The covalent hydration of β-halopyruvic acids and β-halopyruvamides has been investigated in the uv region by stopped-flow techniques. In aqueous solutions of β-bromopyruvate and related compounds, the geminal diol is the predominant form at all pH values. Kinetic investigations have also been carried out on these hydrations. General bases enhance the rate of approaching the equilibrium. Specific acid catalysis was not detected. In the water-catalyzed reaction of pyruvamides, the relationship between the equilibrium constants K and the rate constants of the forward reaction $k_f^{H_2O}$ shows that the transition state is more productlike.

α-Haloketones are among the most popular chemical classes of affinity labels in enzymology. Since the derivatives of 3-halopyruvic acid are more reactive than the ordinary α-haloketones, the former compounds have generally proved to be valuable modification reagents of the side groups of cysteine (*1*), histidine (*2*), and glutamic acid (*3*). If these substances are analogs of natural effectors of the protein, modification of a nucleophilic residue will occur within the effector-binding site with a high probability. Compounds of the 3-bromopyruvate type show a considerable acidity in 3-position and as a consequence a high enolization rate (*4*) and an increased ability of the α-carbonyl group to attack nucleophiles. For this reason the possible reaction behavior of 3-halopyruvates with proteins is rather complex, as shown in Fig. 1. Which of the reaction sequences leads to modification of the enzyme at a given binding site depends on both external reaction conditions and enzyme structure.

One of the most interesting aspects is the aptitude of 3-halopyruvates for bifunctional interactions with two different reactive groups. This property does not depend only on the alkylation potency of the halogen group but also on the increased reactivity of the carbonyl group. Thermodynamic and kinetic data describing the reactivity of the carbonyl group have been published only in the case of halogen-free 2-oxo-acids. Only with 3-fluoropyruvate was extensive hydration observed, by means of ^{13}C-nmr spectroscopy (*5*).

The experiments reported here were carried out to obtain quantitative information on the rate and equilibrium constants of the covalent addition of water to the α-carbonyl group of 3-halopyruvic acids and derivatives. There is evidence that the nucleophilic reactivity of water is linearly correlated with the affinity of some other nucleophilic groups for carbonyl addition (*6*). For a correct interpretation of

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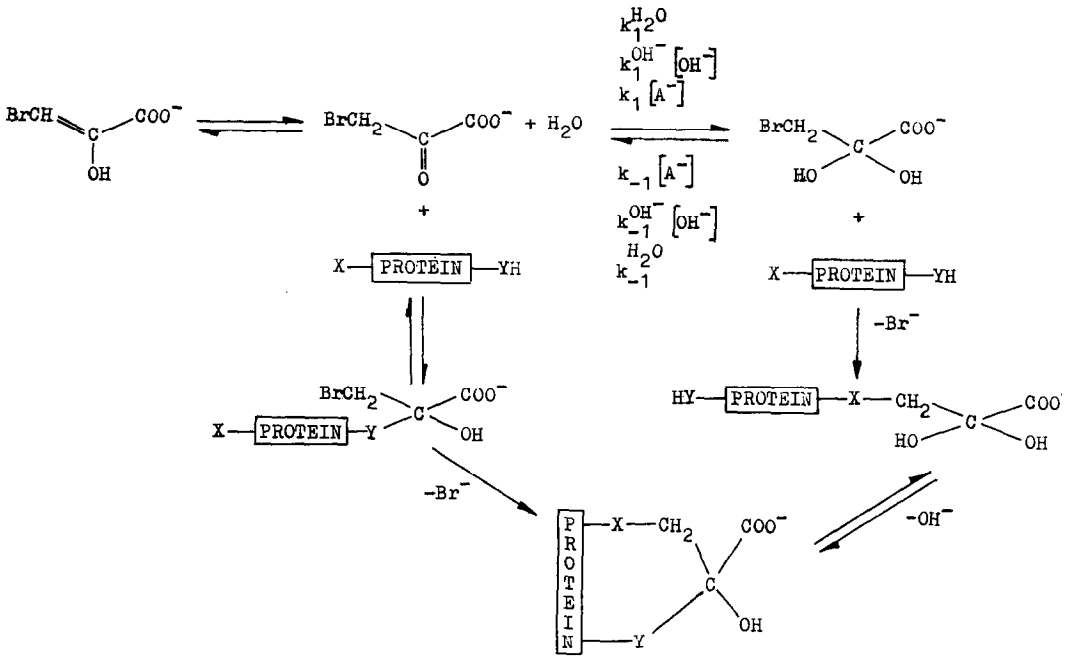

FIG. 1. Possible reactions of 3-halopyruvates with proteins.

kinetic results about interactions of 3-halopyruvates with enzymes utilizing pyruvate as a substrate or an effector, it is necessary to take into account the fact that both species, hydrate and carbonyl form, exist in solution. It is an advantage of 3-halopyruvamides that pyruvate-dependent enzymes will bind these compounds like pyruvate, but are not able to accept them as substrates during the catalytic process. This has been proved for pyruvate decarboxylase (EC 4.1.1.1) (*7*) and should be true at least for all thiamindiphosphate-dependent pyruvate decarboxylating enzymes, e.g., pyruvate dehydrogenase complexes or pyruvate oxidase. Investigations of the reaction between substrate analogous halomethyl ketones with subtilisin BPN′ (*8*), or with anhydro chymotrypsin (*9*) indicate that the formation of hemiacetals occurs before the covalent modification of a nucleophilic side chain at the active center, as indicated in Fig. 1.

RESULTS AND DISCUSSION

Ultraviolet spectroscopy is one of the main methods for determining the equilibrium constants and rates of hydration of carbonyl compounds in aqueous and mixed aqueous solutions. The decrease of concentration of the carbonyl group is monitored by using the n-π^*-band, a spectral region in which the geminal diol is transparent.

The initial absorbancy A_0, necessary for the calculation of the equilibrium constant K, was deduced by extrapolating the linear semilogarithmic first-order plots

to $t = 0$. In the presence of phosphate buffer there exists a catalytic component for each acidic and basic species present in the reaction solution:

$$k_{obs} = k_{obs}^{H_2O} + k_{obs}^{OH^-}[OH^-] + k_{obs}^{H_3O^+}[H_3O^+] + k_{obs}^{H_2PO_4^-}[H_2PO_4^-] + k_{obs}^{HPO_4^{2-}}[HPO_4^{2-}].$$

In the present studies, several series of kinetic investigations were carried out in phosphate buffers, each at constant buffer ratio r, with simultaneously varying concentrations of HPO_4^{2-}. For each of these series, the observed rate constants of the reversible reaction k_{obs} were plotted against the concentration of buffer to evaluate

$$k_0 = k_{obs}^{H_2O} + k_{obs}^{H_3O^+}[H_3O^+] + k_{obs}^{OH^-}[OH^-]$$

as the intercept on the ordinate.

Figure 2 is a graph of the extrapolated rate constants of the hydration reaction (k_0) as a function of pH for 3-bromopyruvamide and 3,3-dibromopyruvate. A catalytic contribution by the term $k_{obs}^{H_3O^+}$ is negligible for all the compounds of Table 1 at pH values >3.5. These results are in qualitative agreement with many other carbonyl analogous reactions of the 2-oxo acid derivatives (*4*, *10*).

The hydration reactions of 3-bromopyruvic acid are more complex than the corresponding processes with the pyruvamides and 1,3-dibromoacetone, because of ionization of the keto acid ($pK_a < 1.5$ (*11*)) and its hydrate ($pK_a < 3.0$ (*11*)). In the pH range studied, only hydration of the anionic forms is involved. It should be noted that the nucleophilic attack of H_2O and OH^- on the 3-halogen position and the hydrolysis of the amide group are slow in all cases in comparison with hydration. Table 1 shows equilibrium constants and catalytic contributions obtained by

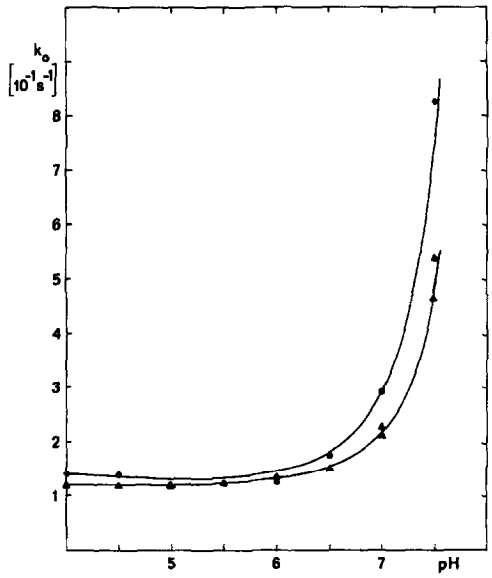

FIG. 2. Plot of k_0 as a function of pH for bromopyruvamide (▲) and 3,3-dibromopyruvate (●) at 25°C (I = 1, 0).

TABLE 1

RATE AND EQUILIBRIUM CONSTANTS FOR THE REVERSIBLE HYDRATION OF SUBSTITUTED PYRUVIC ACIDS AT 25°C IN 10% DIOXANE/WATER

	$k_{obs}^{H_2O}$ (sec^{-1})	$k_{obs}^{OH^-}$ (M^{-1} sec^{-1})	$k_{obs}^{H_2PO_4^-}$ (M^{-1} sec^{-1})	$k_{obs}^{HPO_4^{2-}}$ (M^{-1} sec^{-1})	K	ϵ_0[a]
$BrCH_2COCONH_2$	1.20×10^{-1}	1.1×10^6	6.4×10^{-1}	1.09×10^1	5.49	14.6
$ClCH_2COCONH_2$	7.40×10^{-2}	7.3×10^5	3.3×10^{-1}	4.17	3.88	5.56
$CH_3COCONH_2$ (*12*)	4.60×10^{-2}	1.4×10^5	—	—	0.75[b]	24.9
FCH_2COCOO^-	—	—	—	—	5.6[c]	—
$BrCH_2COCOO^-$	1.24×10^{-2}	7.3×10^4	7.8×10^{-2}	2.4×10^{-1}	1.84	34.4
$ClCH_2COCOO^-$	1.24×10^{-2}	7.3×10^4	4.8×10^{-2}	2.1×10^{-1}	1.21	22.1
$Br_2CHCOCOO^-$	1.35×10^{-1}	1.8×10^6	1.5×10^{-1}	4.65	14.5	46.6
$CH_3COCOOH$	7.7×10^{-1} (*15*)	—	—	—	1.45 (*14*)	—
CH_3COCOO^-	3×10^{-3}[d]	—	—	—	5.3×10^{-2} (*18*)	—
$CH_3COCOOCH_3$ (*10*)	3.3×10^{-2}	—	—	—	2.2	—
$BrCH_2COCH_2Br$	4.1×10^{-2}	1.8×10^5	1.8×10^{-1}	2.0	1.70[e]	59.4

[a] Extinction coefficient of the carbonyl form at the wavelength given under Experimental.
[b] $K = 0.8 \pm 0.04$ (*13*).
[c] Kokesh (*5*).
[d] Extrapolated from the values at 0°C (*17, 14*).
[e] Wolfenden *et al.* (*16*) obtained 1.85 in D_2O at 34°C.

us together with values taken from literature for some other pyruvic acid derivatives.

The values of K demonstrate that all 3-halopyruvic acids and their amides exist in solution mainly as hydrates. The effect of substitutions at the alkyl and carboxyl part of the α-carbonyl compounds is examined. In the series of pyruvic acid derivatives a relationship between $\Sigma\sigma_r^*$ (*19*) and log K is observed. The linear regression yields $\log K = 5.52\,\Sigma\sigma_r^* - 1.01$, $r^2 = 0.847$, $n = 10$ (1,3-dibromoacetone was omitted). Greenzaid *et al.* (*18*) found similar linear correlations for various aldehydes and ketones using an equation containing two parameters, $\Sigma\sigma^*$ and Δ, the number of aldehyde protons. The different amides listed in Table 1 show that the tendency for electrophilic reactivity ($k_1^{H_2O}$) increases with increasing affinity (K) to the nucleophile. From this finding we may conclude that specific bonding effects and solvent configurations characteristic of the product of the reaction are present in the transition state, possibly reflecting an sp^3-analogous transition state for the uncatalyzed addition of water. The same conclusion was drawn from estimations of secondary β-deuterium isotope effects in the case of this reaction (*12*). The substitution of the amide group by the carboxylate group causes a decrease both in $k_1^{H_2O}$ and also in $k_{-1}^{H_2O}$.

The catalysis of approaching the equilibrium of hydration by means of $H_2PO_4^-$ and HPO_4^{2-} is a general base-catalyzed reaction typical of hydration/dehydration. In the case of methyl pyruvate no general acid catalysis has been observed for monobasic acids (*10*).

EXPERIMENTAL

3-Bromopyruvamide

The amide was prepared by oxidation of 3-bromo-2-hydroxypropionamide (mp 107°C), obtained from glycidamide (*20*) and HBr in analogy to the method described by Stubbe *et al.* (*21*). In a typical run, a mixture of 2.67 g CrO_3 in 10 ml 23% H_2SO_4 is added with stirring to a solution of 2.0 g 3-bromo-2-hydroxy propionamide in 300 ml acetone. After 30 min at 0°C the mixture is evaporated and the residue is extracted with 150 ml hot chloroforme. After drying with $MgSO_4$ the solvent is removed by distillation to give 0.9 g (30–50%) white crystals. The material was purified by recrystallization from isopropanol and by sublimation under reduced pressure over P_4O_{10} immediately before use, mp 100–102°C (lit. (*21*) 80–83°C), pure by thin-layer chromatography. ^{1}H-nmr spectrum in CD_3OD showed signals at $\delta = 3.51$ (2H, hemiacetale, quadruplet, $J = 10.5$ Hz), $\delta = 4.23$ (2H, s, carbonyl form).

Anal. Calcd for $C_3H_4BrNO_2 \cdot H_2O$: C, 19.55; H, 3.26; N, 7.60; Br, 43.40. Found: C, 19.36; H, 3.10; N, 7.28; Br, 43.25.

UV-n-π^*-band: λ_{max} = 372 nm (chloroforme), 366 nm (dioxane), 349 nm (water).

3-Chloropyruvamide

In analogy to 3-bromopyruvamide from 3-chloro-2-hydroxypropionamide (mp 114°C). The total yield was 30–40%, mp 85–88°C. ^{1}H-nmr spectrum in CD_3OD: δ = 3.61 (2H, hemiacetale, quadruplet, $J = 12.0$ Hz).

Anal. Calcd for $C_3H_4ClNO_2 \cdot H_2O$: C, 25.82; H, 4.33; N, 10.04. Found: C, 25.98; H, 4.45; N, 9.96.

3-Bromopyruvic acid (*22*) (mp 76–77°C; lit. (*22*) mp 77–79°C), 3,3-dibromopyruvic acid (*23*) (mp 87–89°C; lit. (*23*) mp 89–91°C), and 1,3-dibromoacetone (*24*) (mp 27°C; lit. (*24*) mp 27°C) were synthesized according to published procedures.

3-Chloropyruvic acid was prepared by the method of Cracoe and Robb (*25*) with some modifications. After purification we obtained a mp 73–75°C (carbonyl form) and a yield of 75%. Lit. (*26*) mp 57–58°C (monohydrate); lit. (*26*) mp 45°C (carbonyl form); lit. (*27*) mp 83–84°C (carbonyl form); ^{1}H-nmr-spectrum in CD_3OD: δ = 3.60 (2H, hemiacetale, quadruplet, $J = 12.0$ Hz), $\delta = 4.62$ (s, carbonyl form); in $CDCl_3$: $\delta = 4.62$ (s, 2H).

Anal. Calcd for $C_3H_3ClO_3$: C, 29.40; H, 2.45; Cl, 28.91. Found: C, 29.56; H, 2.73; Cl, 28.68.

Kinetic Studies

Hydration was initiated by injecting the solution of the haloketone in dry dioxane (0.1–0.01 *M*) by means of the 1 : 10 ratio system of a Dionex D-110 stopped-flow spectrometer into the aqueous buffer solution. A rapid diminution of the absorbancy at 338 nm (332 nm for 3-chloropyruvamide and 289 nm for 1,3-dibro-

moacetone) was recorded and digitally stored on a biomation transient recorder. The data were afterwards copied on a conventional strip card recorder for analysis and storage. Excellent pseudo-first-order kinetics were observed. Because of the reversible nature of hydration, the observed rate constant k_{obs} is actually the sum of the first-order rate constants of forward (k_1) and reverse reaction (k_{-1}). For mechanistic considerations the rate constant k_1 was evaluated by multiplying k_{obs} with the value of $K/(1 + K)$. Estimations of the equilibrium constant K (K = hydrate/carbonyl form) need the absorbancy A_0 for the pure carbonyl form of the keto compounds in 10% dioxane/water. This value was obtained from extrapolated pseudo-first-order plots to $t = 0$; $K = [A_0 - A_\infty]/A_\infty$. The A_∞ can be measured directly. The final solution of the reactants contains 10% dioxane. The ionic strength was held constant ($I = 1.0$) by addition of KCl. Cell compartment and mixing syringes of the stopped-flow instrument were thermostated to 25 ± 0.2°C. In the case of 3-halogen-2-oxo acids the stock solution of the buffer components contained equivalent amounts of KOH to neutralize the acid. Measurements of pH after the reaction were carried out on a radiometer pHM 26. Hydroxide ion concentrations were calculated from the pH values, using a $pK_w^{25°}$ (10% dioxane) of 14.17. Great care was taken to avoid contamination of the carbonyl compounds and the dioxane by moisture. Dioxane (uvasol, Merck) was boiled and distilled over sodium, stored over molecular sieves (4 Å) in the dark, and used within 3 days after preparation.

REFERENCES

1. J. E. G. Barnett and F. Koliss, *Biochem. J.* **143,** 487 (1974); M. L. Fonda, *J. Biol. Chem.* **251,** 229 (1976); G. Chang and R. Y. Hsu, *Biochem. Biophys. Res. Commun.* **55,** 580 (1973); P. J. Hudson, D. B. Keech, and J. C. Wallace, *ibid.* **65,** 213 (1975).
2. P. O. Göthe and P. O. Nyman, *FEBS Lett.* **21,** 159 (1972); H. Yoshida and G. Wood, *J. Biol. Chem.* **253,** 7650 (1978).
3. H. P. Meloche, C. T. Monti, and R. A. Hogue-Angeletti, *Biochem. Biophys. Res. Commun.* **84,** 589 (1978).
4. G. Fischer and A. Schellenberger, *Tetrahedron* **28,** 4113 (1972); G. Fischer, G. Küllertz, and A. Schellenberger, *ibid.* **29,** 4313 (1973).
5. T. J. Hurley, H. L. Carrell, R. K. Gupta, J. Schwartz, and J. P. Glusker, *Arch. Biochem. Biophys.* **193,** 478 (1979); F. C. Kokesh, *J. Org. Chem.* **41,** 3593 (1976).
6. E. G. Sander and W. P. Jenks, *J. Amer. Chem. Soc.* **90,** 6154 (1968); C. D. Ritchie, *ibid.* **97,** 1170 (1975).
7. M. Sieber, Dissertation, University Halle, German Democratic Republic, 1981.
8. T. L. Poulos, R. A. Alden, S. T. Freer, J. J. Birktoft, and J. Kraut, *J. Biol. Chem.* **251,** 1097 (1976).
9. H. Weiner, W. N. White, D. G. Hoare, and D. E. Koshland, Jr., *J. Amer. Chem. Soc.* **88,** 3851 (1966).
10. Y. Pocker, J. E. Meany, and C. Zadorojny, *J. Phys. Chem.* **75,** 792 (1971).
11. N. Hellström and S.-O. Almquist, *J. Chem. Soc. B* 1396 (1970).
12. G. Fischer, G. Küllertz, and A. Schellenberger, *Tetrahedron* **32,** 1503 (1976).
13. P. R. Young, L. G. Howell, and T. C. Owen, *J. Amer. Chem. Soc.* **97,** 6544 (1975).
14. Y. Pocker, J. E. Meany, and B. J. Nist, *J. Phys. Chem.* **73,** 2879 (1969).
15. H. Patting and H. Strehlow, *Ber. Bunsenges. Phys. Chem.* **73,** 534 (1969).
16. C. A. Lewis and R. Wolfenden, *Biochemistry* **16,** 4886 (1977).

17. Y. POCKER AND Y. E. MEANY, *J. Phys. Chem.* **74,** 1486 (1970).
18. P. GREENZAID, Z. LUZ, AND D. SAMUEL, *J. Amer. Chem. Soc.* **89,** 749 (1967).
19. D. F. DE TAR, *J. Org. Chem.* **45,** 5166 (1980); A. PALM, "Quantitative Theorie organischer Reaktionen," p. 126, Akademieverlag, Berlin, 1971.
20. G. B. PAYNE AND P. H. WILLIAMS, *J. Org. Chem.* **26,** 651 (1961).
21. J. A. STUBBE AND G. L. KENYON, *Biochemistry* **11,** 333 (1972).
22. N. F. BLAU AND C. G. STUCKWISCH, *J. Org. Chem.* **22,** 83 (1957).
23. H. WICHELHAUS, *Liebig's Ann. Chem.* **152,** 265 (1869).
24. J. U. NEF, *Liebig's Ann. Chem.* **335,** 259 (1904).
25. E. J. CRACOE AND C. M. ROBB, *Org. Synth.* **40,** 54 (1960).
26. M. GARINO AND J. MUZIO, *Gazz. Chim. Ital.* **52,** 226 (1926).
27. P. V. NAIR AND H. BUSCH, *J. Org. Chem.* **23,** 137 (1958).