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Comparative Behavior in the Kinetics of Reduction by Superoxide and Dithionite Ions

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Abstract: Rate constants for reaction of superoxide and dithionite ions with a number of oxidants are reported. Potassium superoxide dissolved at a pH ≥11.5 represents a source of O_2^- in aqueous solution which can be used for kinetic studies of the radical. Disproportionation rate data for superoxide ion at pH 1-13 are in good agreement with values obtained by pulse radiolysis. It is found that the relative rate constants for reduction by SO_2^- and O_2^- ions are generally approximately constant and the ratio of $\sim 10^3$ has predictive value.

Ever increasing attention is being paid to the chemistry of the superoxide ion (O_2^-) and to the related enzyme superoxide dismutase.[1,2] Methods of generation[2,3] of O_2^- in aprotic solvents, in which is it stable, include electrochemical reduction of dioxygen,[4] as well as by direct use of salts including soluble $Me_4N^+O_2^-$[5] and KO_2 "solubilized" by crown ethers.[6] The very rapid disproportionation of superoxide ion in aqueous solution, except at high pH, presents problems however in the study of its kinetic reactivity in that medium. One has therefore to resort to specialized methods, of which the most used for kinetic studies[7] involves pulse radiolysis.[8] Superoxide ion is produced within microsecs by e_{aq}^- reduction of O_2. The reaction of O_2^- with substrate is examined in situ[9] or after mixing in a stopped-flow apparatus.[10] Reaction of O_2 with reduced flavins,[11] dyes, and biochemical reductants,[12,13] differential mixing of O_2^- in Me_2SO with a large volume of water,[14] and photochemical methods[15] have also been employed to generate superoxide ion in aqueous solution.[15d] A largely overlooked[16] source of O_2^- ions in aqueous solution is by dissolution of KO_2 at pH ≥11.5, where disproportionation is slow. We have found that such a solution can be mixed with buffer, with or without substrate, and the rate of disproportionation of O_2^- or of its reduction reactions measured. In this way, the rate constants for the reaction of O_2^- with a number of oxidants have been determined (or remeasured) and compared with those of SO_2^- (or $S_2O_4^{2-}$) ion. In reductions by dithionite, $S_2O_4^{2-}$ or SO_2^- or sometimes both are kinetically important species.[17-25] Both O_2^- and SO_2^- are small, singly charged ions, and they should generally have a constant comparative reactivity on the basis of the Marcus relationship for outer-sphere reactions.[26]

Experimental Section

Chemicals used were the purest commercial products. Horse heart cytochrome *c* was Type III (Sigma) and potassium superoxide was 96.5% pure (Alfa). The complexes $[Co(terpy)_2]Br_3$ and $K[Mn(CyDTA)]\cdot 2H_2O$ were prepared by literature methods.[27] Doubly distilled water was further purified by extraction with dithizone dissolved in CCl_4 and redistilled in the presence of 0.1 mM Na_2EDTA.

Kinetics. In a typical procedure, freshly powdered potassium superoxide ($\sim$5 mg) was dissolved in swirling water (100 mL) containing 0.1 mM EDTA at pH $\sim$11.5. The concentration of O_2^- in the fresh solutions was $\sim$0.2 mM by using absorbance at 250 nm[9] (ϵ 2188 M^{-1} cm^{-1}) and represented $\sim$30% of the theoretical amount. The solution was transferred quickly to one of the syringes of a stopped-flow apparatus (solution A). For a study of disproportionation, solution A was mixed with buffer (10 mM citrate, acetate, phosphate, or borate) at the appropiate pH in the other syringe. The spectral change was monitored at 250 nm, and the second-order rate constant for loss of O_2^- was computed by using molar absorbance coefficients at various pH listed by Bielski.[9] For the study of other O_2^- reactions, solution A was mixed with substrate in the second syringe (usually ≥0.5 mM reactant at pH 9.2 by using 15-30 mM $Na_2B_4O_7$ solution containing 0.1 mM EDTA). The reaction was monitored at a wavelength usually determined by the spectral characteristics of the substrate and of the reduced product. Good first-order kinetics were obtained, and the first-order rate constant was linearly dependent on substrate concentration. The latter was determined by weight or by using known absorbance coefficients. That used for azurin was 5.7×10^3 M^{-1} cm^{-1} at 625 nm.[28] The final reaction pH was quite close to that of the contents of the second syringe. Reactions of dithionite were studied anerobically by using reductant in excess[19] and with the same conditions as used in the superoxide experiments. A Gibson-Dionex stopped-flow apparatus linked to an OLIS data collecting system was used to measure

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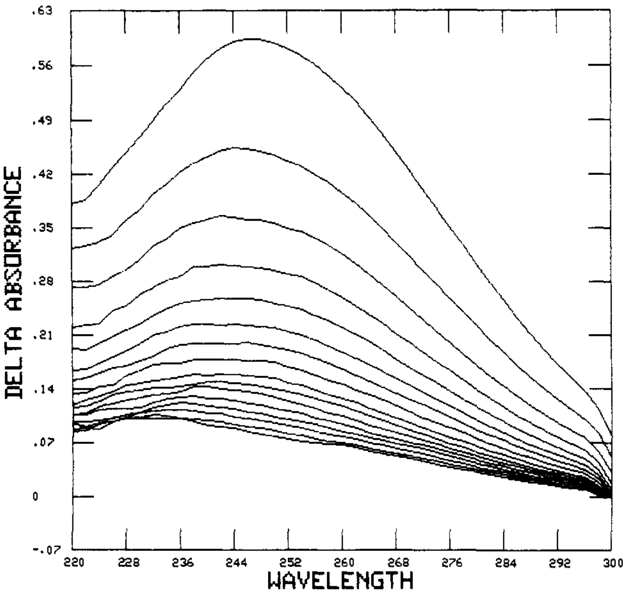

Figure 1. Rapid scan of O_2^- after rapid mixing of O_2^- at pH 11.5 with buffer at pH 9.2 (16 spectra in 5 s, 7.0-ms sweep time, 220–300 nm). Final reference spectrum after 35 s. Data at 25 °C.

the rates of all reactions studied. The rapid scan spectral stopped-flow apparatus used a Harrick Rapid Scan Monochromator and was designed by Dr. DeSa (OLIS Jefferson, GA).

Results

Disproportionation of O_2^- at pH 11.5 and above is quite slow, requiring many minutes for substantial completion of the reaction. It was found that increasing the ionic strength with NaCl had a small effect on the disproportionation rate constant at pH 11.5 (20 mM NaCl, $k = 64$ M^{-1} s^{-1}; 400 mM NaCl, $k = 85$ M^{-1} s^{-1}; 1.0 M NaCl, $k = 91$ M^{-1} s^{-1}). However, Na_2SO_4 markedly accelerated the rate constant (10mM Na_2SO_4, $k = 101$ M^{-1} s^{-1}; 330 mM Na_2SO_4, $k = 652$ M^{-1} s^{-1}). In any ionic strength adjustments, NaCl was therefore used. A rapid scan of the spectrum of O_2^- when a solution at pH 11.5 was rapidly mixed with a borate buffer at pH 9.1 (final pH 9.2) is shown in Figure 1. A maximum at 248 nm was observed. Use of the appropriate absorbance coefficient (2.25×10^3 M^{-1} cm^{-1}) leads to $k = 4.6 \times 10^3$ M^{-1} s^{-1}. Kinetic data were always obtained by continual observation at 250 nm. The variation with pH of the second-order rate constant for disproportionation of superoxide ion is shown in Figure 2 and is interpreted in terms of reactions 1–4.[9] The computed constant

$$HO_2 \rightleftarrows H^+ + O_2^- \quad K_1 \qquad (1)$$

$$HO_2 + HO_2 \rightarrow O_2 + H_2O_2 \quad k_2 \qquad (2)$$

$$HO_2 + O_2^- \rightarrow O_2 + HO_2^- \quad k_3 \qquad (3)$$

$$O_2^- + O_2^- \rightarrow O_2 + O_2^{2-} \quad k_4 \qquad (4)$$

for reactions 1–4 compared with literature values at 23 °C[9] (in parentheses) are as follows: $K_1 = 2.23 \times 10^{-5}$ (2.1×10^{-5}) M; $k_2 = 1.1 \times 10^6$ (8.6×10^5) M^{-1} s^{-1}; $k_3 = 1.8 \times 10^8$ (1.0×10^8) M^{-1} s^{-1}; $k_4 = 5.0$ (<0.35) M^{-1} s^{-1}. The larger values of k_4 probably arises from catalysis by impurities in the potassium superoxide.[2,3,9]

The studies of reduction of substrate by O_2^- were mostly carried out at pH 9.2. Here disproportionation of O_2^- was sufficiently slow, especially at the low concentrations examined (<50 μM), not to interfere in the study of the pseudo-first-order rates of reaction of O_2^- with substrate in excess (>100 μM). The plots of k_{obsd} vs. the concentration of substrate were linear (Figure 3), and from these the second-order rate constants were computed (Table I). As disproportionation of O_2^- occurred in the solution standing at pH 11.5, the absorbance change of the examined reaction decreased, but the second-order (for disproportionation) or first-order rate constants remained invariant. This allowed for easy recognition of the O_2^- reaction. The reactions of O_2^- with

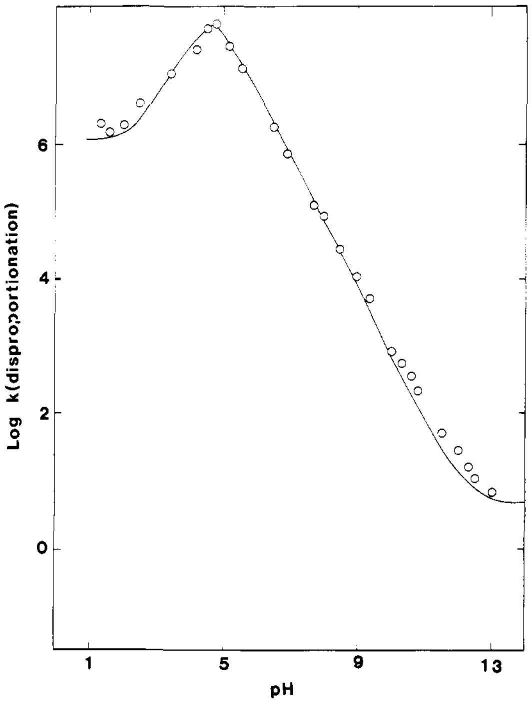

Figure 2. Experimental values of second-order disproportionation constant (k_{obsd}) vs. pH at 25 °C. Total superoxide = 0.4–1.5 mM. Buffers in 0.1 mM EDTA were 10 mM sodium citrate, acetate, phosphate, and borate. λ 250 nm (also 240 and 270 nm). The full line fits the equation $k_{obsd} = (k_2 + k_3K_1[H^+]^{-1})(1 + K_1[H^+]^{-1})^{-1} + k_4$ by using values in the text.

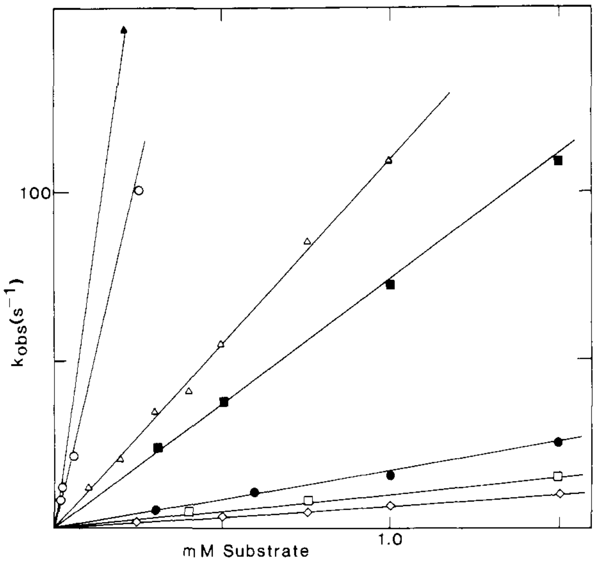

Figure 3. Variation of k_{obsd} (s^{-1}) with substrate concentration for reactions of O_2^- at pH 9.2 using 15 mM sodium borate, 0.1 mM EDTA, and 25 °C. Substrates: azurin (□); ferricytochrome *c* (•); $Fe(CN)_6^{3-}$ (◇); $Mn(CyDTA)^-$ (▲); $Co(terpy)_2^{3+}$ (■); nitro blue tetrazolium (△); DCIP (○). For $Mn(CyDTA)^-$ there is an additional point at 0.3 mM substrate, $k = 202$ s^{-1}. For ferricytochrome *c*, the concentration ordinate is 0.15 mM not 1.5 mM. For azurin, concentration ordinate is 0.15 mM and rate constant ordinate is 10 s^{-1} not 100 s^{-1}.

$Co(terpy)_2^{3+}$ and nitro blue tetrazolium were also studied at pH 6.0 and 7.0, respectively, and no difference from the rate constants at pH 9.2 was observed. Reduction by dithionite ion of the substrates examined (azurin, nitro blue tetrazolium, and 2,6-dichlorophenol indophenol) all conformed to the two-term rate law (5). The linear plots of $k_{obsd}[S_2O_4^{2-}]^{-1/2}$ vs. $[S_2O_4^{2-}]^{1/2}$ (see Figure

$$\text{rate} = k_1[\text{substrate}][S_2O_4^{2-}] + k_2[\text{substrate}][S_2O_4^{2-}]^{1/2} = k_{obsd}[\text{substrate}] \qquad (5)$$

4) yield values from the intercept of k_2 and from the slope, k_1.

Table I. Rate Constants ($M^{-1}\ s^{-1}$) for Reactions of O_2^-, SO_2^-, and $S_2O_4^{2-}$ Ions at 25 °C

oxidant	$k_{O_2^-}$	$k_{SO_2^-}$	$2k_{S_2O_4^{2-}}$	log ($k_{SO_2^-}$/ $k_{O_2^-}$)	log ($2k_{S_2O_4^{2-}}$/ $k_{O_2^-}$)
Pseudomonos aeruginosa azurin	9.3×10^3 [a,b]	4.6×10^6, [a,b] 2.5×10^6 [c]	6.6×10^2, [a,b] 3.7×10^3 [c]	2.7	−1.1
horseheart ferricytochrome *c*	1.8×10^5, [a,d] 1.0×10^5 [f]	3.9×10^7 [e]	1.5×10^4 [e]	2.3	−1.1
horseradish ferriperoxidase	$>10^7$, [a,b] $\sim 10^8$ [h]	5.0×10^5 [g]		−1.3	
horseheart metmyoglobin	6×10^3 [i]	4.5×10^6, [j] 2.7×10^6 [k]		2.9	
$Fe(CN)_6^{3-}$	6.9×10^3, [a,d] 2.7×10^2 [m]	$\sim 2 \times 10^8$ [l]	1.0×10^5 [l]	4.5	1.2
$Fe(EDTA)^-$	1.3×10^6 [n]		3.6×10^4 [o]		−1.6
ferrioxamine	$<2 \times 10^5$ [n]	3.5×10^3 [p]			
$Mn(CyDTA)^-$	7.2×10^5, [a,b] $\sim 10^6$ [q]		2.1×10^6 [o]		0.3
$Co(terpy)_2^{3+}$	7.0×10^4 [a,b,r]	5.0×10^7 [s]	8.7×10^4 [s]	2.9	0.1
H_2O_2	0.13, [t] <0.3 [u]	2.6×10^2 [v]		≥3.3	
nitro blue tetrazolium	1.1×10^5, [a,b,r] 5.9×10^4 [w]	1.4×10^8 [a,b]	9.4×10^4 [a,b]	3.1	−0.1
2,6-dichlorophenol indophenol	4.3×10^5 [a,b]	3.3×10^8 [a,b]	3.6×10^5 [a,b]	2.9	−0.1
methyl viologen	1.0×10^4 [x]	9.0×10^6 [x]		3.0	

[a] This work. [b] pH 9.2, 15 mM borate. [c] pH 7.0, 20 °C, ref 24. [d] pH 9.2, 10 mM borate, $I = 0.2$ M. [e] pH 8.0, $I = 0.41$ M, ref 17. [f] pH 9.7, ref 29. [g] pH 6.3, $I = 0.15$ M, ref 25. [h] pH 7.0, ref 30. [i] pH 7.0, 66 mM phosphate, ref 31. [j] pH 8.2, $I = 0.50$ M, ref 19. [k] pH 6.4, $I = 0.3–0.8$ M, ref 20. [l] pH 6.8, $I = 0.1$ M, insensitive to pH from pH 7 to 10, ref 21. [m] pH 9.5–9.7, $I = 0$, ref 32. [n] pH 7.0, $I = 0.1$ M, ref 33; for effect of pH see ref 34. [o] pH 7.0, ref 22. [p] pH 6.8, $I = 0.5$ M, ref 23. [q] 20 °C, ref 35. [r] Similar value at pH 6.0 and 10.0. [s] pH 8.0, $I = 0.5$ M; extended data of ref 22 shows contribution from SO_2^- as well as $S_2O_4^{2-}$ (Bemtgen, J. M., Wilkins, R. G., unpublished data). [t] pH 7, 23.5 °C, ref 36. [u] pH 9.6, ref 37. [v] pH 6.5, $I = 0.15$ M, ref 18. [w] pH 7–11, $I = 0.1$ M, ref 38. [x] Calculated from the rate constant for the reduced methyl viologen–O_2 reaction [$7.7 \times 10^8\ M^{-1}\ s^{-1}$, pH 6-8 (Farrington, J. A.; Ebert, M.; Land, E. J.; Fletcher, K. *Biochim. Biophys. Acta* 1973, *314*, 372)] and the redox potential estimated equilibrium constant (8×10^4). [y] pH independent, 7.0–9.0, $I = 0.5$ M (Tsukahara, K.; Wilkins, R. G., unpublished data).

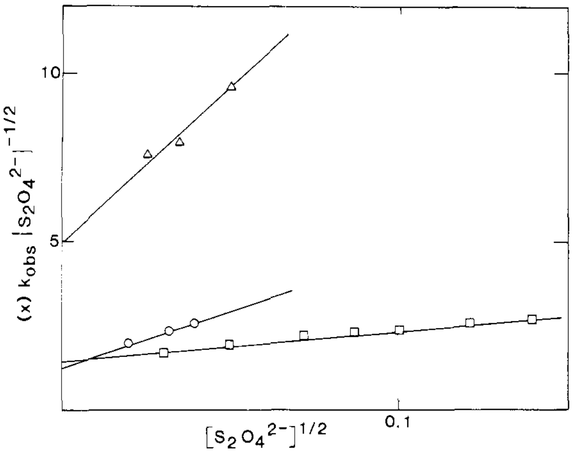

Figure 4. $(x)k_{obsd}[S_2O_4^{2-}]^{-1/2}$ vs. $[S_2O_4^{2-}]^{1/2}$ for reaction of dithionite at pH 9.2 using 15 mM sodium borate, 0.1 mM EDTA, and 25 °C. Substrates: azurin (□), $x = 10^{-2}$; nitro blue tetrazolium (△), $x = 10^{-3}$; DCIP (○), $x = 10^{-4}$.

The value of k_2 is composite since it equals the product $K^{1/2}k_{SO_2^-}$. Using the

$$S_2O_4^{2-} \rightleftarrows 2SO_2^- \qquad K \tag{6}$$

$$SO_2^- + \text{substrate} \rightarrow \text{product} \qquad k_{SO_2^-} \tag{7}$$

value of $K = 1.4 \times 10^{-9}$ M,[17] that of $k_{SO_2^-}$ can be assessed. These together with k_1 ($=2k_{S_2O_4^{2-}}$) are contained in Table I. $k_{S_2O_4^{2-}}$ is the bimolecular rate constant for reaction of substrate with $S_2O_4^{2-}$ to give $S_2O_4^-$.

Discussion

The results obtained indicate that under certain conditions commercial KO_2 is a good source of O_2^- ions for kinetic studies. The disproportionation data (Figure 2) are generally in very good agreement with those obtained by pulse radiolysis.[9] The relative stability of O_2^- in high pH, allows its manipulation and rapid mixing with substrate at lower pH. Provided the reaction is faster than the disproportionation (a necessary condition regardless of the method of generation of O_2^-) the kinetics of reduction of the substrate can be measured. The superoxide solutions contain appreciably larger concentrations of O_2 and H_2O_2 than of O_2^-. Any reactions of these can be usually distinguished from that of O_2^- since the latter is attended by continually decreasing absorbance changes as disproportionation occurs in the stock solution at pH 11.5. Reactions of H_2O_2 or O_2 are often slower or nonexistent in the systems studied and can be discounted. Data for the reaction of O_2^- with a number of oxidants are shown in Table I.[29-38] Good agreement with rate data which had been previously obtained for ferricytochrome *c*, $Fe(CN)_6^{3-}$, $Mn(CyDTA)^-$, and nitro blue tetrazolium is noted. The reactions of O_2^- with azurin, $Co(terpy)_2^{3-}$, and 2,6-dichlorophenol indophenol have not previously been reported.

The kinetics of reaction of *both* superoxide and dithionite with only a few oxidants had been previously studied. A now expanded list is shown in Table I. Examination of the table shows that in general SO_2^- is more reactive than O_2^- toward a common substrate by a factor of $\sim 10^3$ and that $S_2O_4^{2-}$ has a similar or a slightly less reactivity than that of O_2^-.[39] It is easily shown from the

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(39) The conditions for the study of the O_2^- and SO_2^- reactions are usually different. However, effects of pH and small ionic strength changes are minimal when these have been examined for these reactions, and the relative rate constants are unlikely to be much different with identical reaction conditions.

Marcus relationship for outer-sphere reactions[26] (see, for example, ref 40) that the reactivity ratio R toward a common substrate is expected to be a constant, for example (eq 8), where $k(SO_2/SO_2^-)$

$$R = \frac{k_{SO_2}}{k_{O_2^-}} = \left[\frac{k(SO_2/SO_2^-)}{k(O_2/O_2^-)} 10^{16.9\Delta E} \right]^{1/2} \qquad (8)$$

and $k(O_2/O_2^-)$ are the self-exchange rate constants and ΔE is the difference between the reduction potentials of O_2 and SO_2. The reduction potential of the O_2/O_2^- system is −0.16V [2] and that estimated for SO_2/SO_2^- is −0.26 V.[41] An R value in (8) of 10^3 requires that $k(SO_2/SO_2^-)/k(O_2/O_2^-)$ be $\sim 10^4$. Since the rate constant for the SO_2^-–O_2 reaction is 1.3×10^6 M^{-1} s^{-1} (pH 8.0),[18] that for O_2^-/O_2 self-exchange can be estimated as $\sim 10^3$ M^{-1} s^{-1}. This is slightly larger than that assessed by application of Marcus theory to the $Ru(NH_3)_6^{2+}$–O_2 reaction.[42] This means that the SO_2^-/SO_2 self-exchange is predicted to be $\sim 10^7$ M^{-1} s^{-1}. The reduced reactivity of O_2^- toward H_2O_2[36,37] and ferrioxamine[33] is understandable since reaction of SO_2^- with these substrates is relatively slow. One glaring exception to the enhanced reactivity of SO_2^- is toward horseradish peroxidase. The higher rate constant for O_2^- may reflect an innersphere mechanism not perhaps shown by SO_2^-, since oxyferroperoxidase is the immediate product of the reaction of O_2^- ion.[30]

In summary, we have used a simple source of superoxide ions and demonstrated its value for studying a number of reduction reactions of O_2^- in aqueous solution. Interference from reactions of oxygen and hydrogen peroxide must always be considered and this will be particularly the case with the study of oxidation reactions by O_2^-. Each system will require separate scrutiny. We have shown for seven different types of oxidant a relation between the reactivity of O_2^- and SO_2^- and suggested that this has predictive value.

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Registry No. $Fe(CN)_6^{3-}$, 13408-62-3; $Mn(CyDTA)^-$, 73360-48-2; $Co(terpy)_2^{3+}$, 19137-07-6; O_2^-, 11062-77-4; $S_2O_4^{2-}$, 14844-07-6; Na_2SO_4, 7757-82-6; SO_2^-, 12143-17-8; nitroblue tetrazolium, 298-83-9; 2,6-dichlorophenol, 87-65-0; ferriperoxidase, 9003-99-0.

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The Relaxational Behavior of Self-Associated 6-Methylpurine

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Abstract: Information about the geometry and the thermodynamic parameters of molecular stacks in aqueous solution was obtained from the concentration and temperature dependence of the ^1H chemical shift and the relaxation behavior of ^{13}C and ^1H. All diverging association models and assumptions about the stack shift increments which have been proposed in the literature gave an equally good simulation of the chemical shift data. The obtained range of thermodynamic parameter sets was then analyzed by using a description of the relaxation behavior based on the Woessner formalism yielding the selection of an association model and intermolecular proton–proton distances. The predominantly intermolecular character of the proton relaxation within each stack as well as the existence of two distinct association geometries were proved by the change of the relaxation after partial deuteration. The model compounds used for these investigations are 6-methylpurine and 8-deuterio-6-methylpurine.

While numerous reports exist on the measurement of the stacking behavior of molecules on the basis of chemical shifts,[1-8] only a few investigators have used relaxation behavior for the study of the stacking phenomenon.[9-11] The scope of this study was a careful and critical examination of the application of the relaxation data to obtain information about the geometry and the thermodynamics of molecular stacking systems.

Measurements

^1H and ^{13}C T_1 relaxation times were measured at various field strengths and molar concentrations. The data obtained together with some T_2 and NOE enhancement values are listed in Table I.

Discussion

A three-step approach was used to obtain information about the stacking phenomenon of 6-methylpurine and its deuterated analogue from the relaxation behavior. First, a model was proposed with use of assumptions about the stack structure and the relaxation channels. Next, the ^1H chemical shifts and the ^{13}C relaxation times were fit by a computer program to a theoretical description of the stacking molecular system. This, in turn, gave theoretical values for the relaxation times of the protons. A comparison of these theoretical values with the observed proton relaxation times at different field strengths allowed the determination of a thermodynamic description of the stacks. Finally, the relaxation times of the undeuterated and deuterated 6-methylpurine were compared to gain information about the molecular structure of the stacks.

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