Oxygen Isotope Effect on Activated Bleomycin Stability

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The bleomycins comprise a family of antitumor antibiotics[1−3] that act by damaging DNA[4] through formation of an activated complex of bleomycin, Fe(III), and oxygen.[5−7] When activated bleomycin[6] initiates DNA degradation, H4′ is removed from deoxyribose[8,9] and C4′ is exposed to attack by endogenous O_2.[6] How this occurs is unclear because activated bleomycin has not been adequately characterized.

Activated bleomycin can form from bleomycin, Fe(II), and O_2 (eq 1) as well as through routes involving Fe(III) and peroxides or superoxide. In each case, a dioxygen species is involved. An O−O cleavage must occur in the course of activated bleomycin formation or decay, since activated bleomycin spontaneously yields the stable compound Fe^{III}−bleomycin[5,6] and water[11] without releasing significant quantities of peroxide or superoxide.[12] It has been hypothesized that the Fe(III)-complexed oxygen in activated bleomycin is a peroxide[6,7,13] (activated bleomycin is at the redox level of a ferric peroxide,[10] and mass spectroscopy indicates that it contains both added oxygen atoms[14]). However, the absence of evidence for O−O linkage or information about the immediate products of activated bleomycin decay prevents assignment of a particular oxygen structure to the complex. Depending on this oxygen structure, activated bleomycin may be envisaged to react by breaking either an O−O or an Fe−O bond. These alternative events can be distinguished, since the distinct bond strengths of the hypothetical reactants and products (Table 1) lead to predictions of very different oxygen isotope effects on activated bleomycin decay (Table 2 and below).

Measurement of activated bleomycin decay is straightforward: in the single-turnover reaction,

$$2Fe^{II} + 2\text{bleomycin} + O_2 \rightarrow Fe^{III}\text{−bleomycin} + \text{activated bleomycin} \quad (1)$$

$$\text{activated bleomycin} + xH^+ + 2e^- \rightarrow Fe^{III}\text{−bleomycin} + yH_2O \quad (2)$$

activated bleomycin formation (eq 1) is >10-fold faster than its decay (eq 2), which is monitored as a first-order disappearance of DNA-cleaving activity.[6] The cleavage reaction releases stoichiometric quantities[15] of base propenal,[16] which provides a precise and convenient colorimetric assay[6] when subsequently reacted with 2-thiobarbituric acid.[17] DNA-cleaving activity is proportional to the activated bleomcyin quantitated by stop-freeze EPR spectrometry.[6] We emphasize that decay of activated bleomycin occurs prior to the addition of DNA, which is present only for assay of residual DNA-cleaving activity.

Table 1. Vibrational Frequencies (cm^{-1})

molecule	mode	ν_{16}	ν_{18}	ν_{54}	ν_{56}	ν_{58}
O_2[a]		1556	1467			
H_2O[b]	H−O	3824	3824			
		3939	3922			
	H−O−H	1654	1644			
Fe^{IV}=O[a]		828	791	831	828	824
Fe^{III}−OOH[a]	Fe^{III}−O	500	478	502	500	498
Fe^{III}−O_2[a]	Fe^{III}−O	567	542	569	567	565

[a] The ν_{16} and ν_{56} values are from Chase et al.[23] (O_2), Hashimoto et al.[24] (Fe^{IV}=O), and Dunn et al.[25] (Fe^{III}−O). Values of ν_{18}, ν_{54}, and ν_{58} were calculated by using $\nu_{18} = \nu_{16}(G_{18}/G_{16})^{1/2}$, and $\nu_{58} = \nu_{56}(G_{58}/G_{56})^{1/2}$, respectively, where G is the corresponding diagonal G element in the **G** matrix of Wilson et al.[26] [b] These values were calculated by using known force constants[24] and the **GF** matrix method.[26]

To measure the $^{18}O_2$ isotope effect, we formed activated bleomycin from either natural abundance O_2 or 97 atom % $^{18}O_2$ to make the overwhelmingly abundant species isotopically homogeneous. The observed isotope effect on the rate of activated bleomycin decay was 1.054 ± 0.008 for 19 pairs of determinations (Figure 1).

The relative sizes of equilibrium and kinetic isotope effects provide information about the transition state structure.[18,19] A kinetic isotope effect may exceed the equilibrium isotope effect, but it cannot exceed a maximum set by the equilibrium value for total loss of the bond defining the reaction coordinate without compensating bond formation.[20] Thus, the values of $^{18}K_e$ for reactions 4, 5, and 6 (Table 2), in which no new bonds form, are theoretical upper limits for kinetic isotope effects on the dissociations of Fe^{III}−$O_2^{\bullet-}$ (reaction 1), Fe^{III}−OOH (reaction 2), and Fe^{IV}=O (reaction 3), respectively. The observed kinetic effect of 1.054 ± 0.008 clearly exceeds the upper limit for dissociation of Fe^{III}−$O_2^{\bullet-}$ (1.0417) and Fe^{III}−OOH (1.0451), thereby excluding reactions 1 and 2 from bleomycin decay. This conclusion is consistent with the observations[12] that no superoxide is generated during the decay of activated bleomycin and that peroxide release accounts for no more than 7% of activated bleomycin decay. With respect to reaction 3, activated bleomycin could in principle be an oxo−iron complex that decays with release of the oxo oxygen as water (reaction 3). Here the observed kinetic isotope effect (1.054 ± 0.008) does not

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Table 2. Summary of Equilibrium Isotope Effects on Various Reactions of Fully Enriched Iron and Oxygen Species

no.	reaction	$^{18}K_e$	$^{58/54}K_e$
1	$Fe^{III}-O_2^{\bullet-} \overset{H^+}{\rightleftharpoons} Fe^{III} + HO_2^{\bullet}$	0.9997[a]	1.0039[a]
2	$Fe^{III}-OOH \overset{H^+}{\rightleftharpoons} Fe^{III} + H_2O_2$	0.9951[a]	1.0036[a]
3	$Fe^{IV}=O \overset{e^-,2H^+}{\rightleftharpoons} Fe^{III} + H_2O$	0.9926[b]	1.0091[b]
4	$Fe^{III}-O_2^{\bullet-} \rightleftharpoons Fe^{III} + O_2^{\bullet-}$	1.0429[c]	
5	$Fe^{III}-OOH \rightleftharpoons Fe^{III} + {}^{-}OOH$	1.0453[d]	
6	$Fe^{IV}=O \overset{e^-}{\rightleftharpoons} Fe^{III} + O^{2-}$	1.0484[b]	
7	$Fe^{III}-OOH \overset{e^-,H^+}{\rightleftharpoons} Fe^{IV}=O + H_2O$	1.0322[e]	0.9946[a]
8	$Fe^{III}-O-OH \rightleftharpoons Fe^{III}-O^{\bullet} + {}^{\bullet}OH$	1.0831[f]	
9	$HO_2^{\bullet} \overset{-H^+}{\rightleftharpoons} O_2^{\bullet-}$	1.0432[g]	
10	$O_2 \overset{e^-}{\rightleftharpoons} O_2^{\bullet-}$	1.0673[h]	
11	$O_2 \overset{e^-,H^+}{\rightleftharpoons} HO_2^{\bullet}$	1.0231[h]	
12	$H_2O_2 \overset{-H^+}{\rightleftharpoons} HO_2^{-}$	1.0504[i]	
13	$O_2 \overset{2e^-,H^+}{\rightleftharpoons} HO_2^{-}$	1.0692[h]	
14	$O_2 \overset{2e^-,2H^+}{\rightleftharpoons} H_2O_2$	1.0179[h]	
15	$Fe^{II} + O_2 \overset{2e^-,2H^+}{\rightleftharpoons} Fe^{IV}=O + H_2O$	1.0556[b]	
16	$Fe^{II} + O_2 \overset{e^-,H^+}{\rightleftharpoons} Fe^{III}-OOH$	1.0227[h]	
17	$Fe^{II} + O_2 \rightleftharpoons Fe^{III}-O_2^{\bullet-}$	1.0108[j]	

	$^{18}K_e$			$^{58/54}K_e$		
no.	ZPE[k]	EXC	MMI	ZPE	EXC	MMI
1	1.0329	1.0090	0.9592	1.0097	1.0013	0.9930
2	1.0262	1.0111	0.9590	1.0097	1.0019	0.9920
3	1.0244	1.0038	0.9653	1.0170	1.0006	0.9916
6	1.0934	1.0037	0.9553			
7				0.9928	1.0013	1.0005
8	1.0735	1.0025	0.9670			
15	1.0622	0.9966	1.0556			

[a] Estimated using ν for Fe−O and O−H stretches (Table 1) according to Tian and Klinman.[27] Fe−O−O bending modes are unavailable, and bending mode contributions are generally less important than stretches. [b] Calculated using ν from Table 1 and Tian and Klinman.[27] [c−e] $^{18}K_e$ and $^{58/54}K_e$ calculated using (*c*) no. 1 × no. 9, (*d*) no. 2 × no. 12, and (*e*) no. 15 ÷ no. 16. (Although reactions 3 and 7 are shown as one-electron events, the reaction 7 electron for Fe−bleomycin may originate internally and thereby preserve its net redox state. Regenerating ferric bleomycin by reaction 3 would then be a 2-electron reaction.) [f] Obtained by squaring the singly labeled species' value obtained from H_2O_2 vibrational frequencies,[27] assuming that the HO• stretch and the O−H stretch in H_2O_2 are the same. Substituting Fe for H in H_2O_2 does not appreciably affect the isotope effect.[27] [g] No. 10 ÷ no. 11. [h] Obtained by squaring the singly labeled species' value.[27] [i] No. 13 ÷ no. 14. [j] The square of $^{18}K_e$ for oxymyoglobin formation from singly labeled dioxygen.[27] [k] Contributed from zero point energies (ZPE), excited vibrational states (EXC), and mass and moments of inertia (MMI).

significantly exceed the calculated upper limit (1.0484). However, if activated bleomycin decay occurred by reaction 3, the similarity of the observed and theoretically limiting kinetic isotope effects would require an extremely late transition state and an unlikely O^{2-} for product, making this mechanism implausible. The remaining possibility, an O−O cleavage mechanism (reaction 7), is consistent with the experimental data. An upper bound to the kinetic isotope effect for this reaction is the equilibrium effect calculated for reaction 8, wherein no new bond forms, unlike reaction 7. The observed kinetic effect (1.054 ± 0.008) is well within the kinetic upper limit defined by reaction 8 ($^{18}K_e$ = 1.0837).

Complementary predictions for $^{58/54}$Fe kinetic isotope effects were also made, and their measurements,[21] although less robust, gave results consistent with the inferences from the oxygen experiment. The Fe-isotope effect, measured in 20 pairs of reactions, was 1.0006 ± 0.003. This value is closer to the equilibrium effect predicted for O−O cleavage (Table 2, reaction 7: $^{58/54}K_e$ = 0.9946) than to that predicted for Fe−O cleavage (reaction 3: $^{58/54}K_e$ = 1.0091).

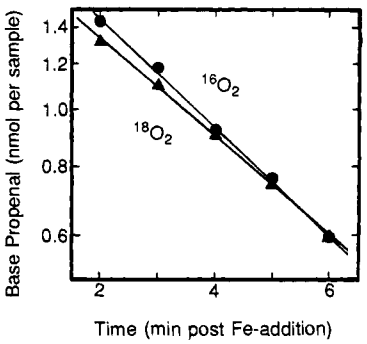

Figure 1. Kinetics of activated bleomycin decay. Incubation[28] samples removed when shown were assayed with DNA and 2-thiobarbituric acid for residual activity in forming base propenal.[6] This typical incubation pair displays an $^{18/16}$O isotope effect of 1.07.

Identifying activated bleomycin as a peroxide complex is consistent with its sloth in decaying spontaneously and in reacting with DNA ($t_{1/2}$ = 2 min at 4 °C), since monooxygenated complexes are typically more active. The presence of peroxide in activated bleomycin establishes that O−O cleavage occurs after formation of activated bleomycin. The peroxide configuration also raises the possibility that activated bleomycin reacts to form an unstable, monooxygenated product that could be the proximate DNA-attacking species, a hypothesis consistent with the inability of DNA to affect the rate of activated bleomycin decay.[6] The possibility that such a species exists would be strengthened by the existence of a repository for oxidizing equivalents; a suitable delocalized π-electron system, analogous to that in iron porphyrins, has been proposed for iron−bleomycin on the basis of resonance Raman spectra.[22] Although such a feature could support the possibility of mononuclear oxygens in activated bleomycin, the isotope effects observed above rule this out.

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(21) Done as for oxygen, but initiated by adding matched Fe(II) solutions, of either ^{58}Fe (Isotec, 93 atom %) or ^{54}Fe (U.S. Services, 99.8 atom %), prepared[6] in dilute H_2SO_4, to otherwise complete, air-equilibrated reaction mixtures.

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