KINETICS OF THE REACTION OF BLEOMYCIN-Fe(II)-O_2 COMPLEX WITH DNA

HISAO EKIMOTO, HIROSHI KURAMOCHI, KATSUTOSHI TAKAHASHI
AKIRA MATSUDA and HAMAO UMEZAWA*

Research Laboratories, Pharmaceutical Division, Nippon Kayaku Co.,
Shimo 3-31-12, Kita-ku, Tokyo, Japan
*Institute of Microbial Chemistry, Kamiosaki 3-14-23, Shinagawa-ku, Tokyo, Japan

(Received for publication January 8, 1980)

The kinetics of the reaction of BLM-Fe(II)-O_2 with DNA in the absence or presence of 2-mercaptoethanol (2-ME) were studied. The total number of bases released by BLM-Fe(II)-O_2 in the presence of 2-ME increased about 6.5 times more than that in the absence of 2-ME in the reaction of 6 hours at 37°C. The molar ratios of the released bases ware little affected by the reaction time, temperature or 2-ME. Among the four bases, thymine was preferentially released. On the basis of a reaction scheme of BLM-Fe(II)-O_2 with DNA, the equations were derived by the steady-state method. In the absence of 2-ME, the release of bases from DNA was dependent on the concentration of BLM-Fe(II)-O_2, but independent of the concentration of DNA.

In the presence of 2-ME, a biphasic reaction was observed; the first one is due to BLM-Fe(II) which originally existed and the second one is due to BLM-Fe(II) produced by the reduction of BLM-Fe(III) with 2-ME. In the second reaction, the rate of the release of bases from DNA was proportional to the concentration of BLM-Fe(II) and 2-ME, but inversely proportional to the concentration of DNA.

The rate-determining step in the reaction of BLM-Fe(II)-O_2 with DNA in the presence of 2-ME was found to be the reduction of BLM-Fe(III) to BLM-Fe(II). By these kinetic studies, the reaction of BLM-Fe(II)-O_2 with DNA in the presence of 2-ME was elucidated to proceed in a catalytic fashion. Furthermore, the maximum number of bases released by BLM from DNA was one base per twelve to thirteen bases.

Bleomycin (BLM) is a group of glycopeptide antitumor antibiotics isolated from *Streptomyces verticillus*[1]. BLM causes single- and double-strand breaks in DNA[2,3] and released free bases. Among the bases, thymine was preferentially released[4,5]. A free radical mechanism[6,7] and an enzyme-like mechanism[8] have been reported for these reactions. SAUSVILLE *et al.*[6,9] reported that an oxygen-labile complex was formed between BLM and Fe^{2+} ion, and TAKITA *et al.*[10] reported the three-dimentional structure of the ferrous complex of BLM and its implication in the reaction with DNA. TAKESHITA *et al.*[11] reported that the base sequence most readily recognized by BLM contained G-T or G-C. The formation of superoxide and hydroxy radicals in BLM-Fe(II)-O_2 system has been confirmed by ESR. (SUGIURA *et al.*[12]).

In this paper, we will report kinetic studies of the reaction of BLM-Fe(II)-O_2 with DNA in the absence or presence of 2-ME.

Materials and Methods

Bleomycin

Bleomycin A_2 (BLM) was prepared by Nippon Kayaku Co. (Japan). The Fe(II) complex of BLM was prepared by adding an equimolar amount of ferrous sulfate to the aqueous solution of BLM at 0°C immediately prior to use.

Chemicals

Chemicals were obtained as follows: Salmon sperm DNA from Sigma Chemical Co., (USA); 2-mercaptoethanol (2-ME), nucleo bases (cytosine, thymine, adenine and guanine) from Tokyo Kasei Kogyo Co., (Japan); ferrous sulfate from Hikotaro Shuzui Co., (Japan).

Determination of bases released from DNA

The incubation mixtures (0.5 ml) consist of 0.04 M Tris-HCl, pH 7.8, various concentrations of BLM-Fe(II), salmon sperm DNA and 2-ME. The mixture was prepared at 0°C and 2-ME was added last. After the incubation at 37°C for a given time, the mixture was rapidly cooled in ice bath and 10 μl of 0.5 M acetic acid was added to adjust pH to about 5.9. Immediately, to remove the unreacted DNA, the reaction mixture was passed through a DEAE-Sephadex A-25 (Pharmacia Fine Chemical Co., Sweden) column (0.8×2.5 cm) pretreated with 0.05 M phosphate buffer of pH 5.9 and the column was developed with the same buffer. The eluate was lyophilized and then dissolved in 0.5 ml of deionized water. It was subjected to the determination of nucleo bases by high-pressure liquid chromatography. The bases were completely recovered from the column of DEAE-Sephadex under this condition.

Bases released from DNA were determined with a Hitachi 634A Liquid Chromatograph. A stainless steel column (4×250 mm) which contained Lichrosorb RP-8 (particle size 5 μm, E. Merck, West Germany) was used for the analysis. Fifty μl of the sample was charged on the column and the chromatography was developed with water at a constant flow rate of 0.6 ml/min (pressure: 95 kg/cm^2) at room temperature. The absorbance at 264 nm of the eluate was recorded and the amounts of nucleo bases were determined from their integrated peak areas by comparison with a standard curve. Cytosine, guanine, thymine and adenine were eluted in this order and their retention times were 4.4, 7.2, 11.9 and 24.0 minutes, respectively (Fig. 1).

Results

Base Release from DNA by BLM-Fe(II)-O_2

Effects of reaction time, temperature and addition of 2-ME on the base release from DNA by BLM-Fe(II)-O_2 are shown in Table 1. In all conditions tested, thymine was most readily

Table 1. Release of nucleo bases from DNA by BLM A_2-Fe(II) in the absence or presence of 2-ME.

The reaction mixture (0.5 ml) consists of 3.68×10^{-3} M salmon sperm DNA, 6.8×10^{-5} M BLM A_2, 6.8×10^{-5} M $FeSO_4$, 40 mM Tris-HCl, and pH 7.8, 2-ME when added was 1×10^{-1} M. The reactions were carried out (A) for 5 minutes at 0°C (B) for 5 minutes at 37°C and (C) for 6 hours at 37°C.

The release of nucleo bases was determined as described in Materials and Methods.

(A)

2-ME	Nucleo base (μM)				
	Thymine	Cytosine	Adenine	Guanine	Total (Ratio)
(+)	12.0 (0.56)	5.9 (0.27)	3.0 (0.14)	0.6 (0.03)	21.5 (1.00)
(−)	12.8 (0.63)	5.1 (0.25)	2.0 (0.10)	0.4 (0.02)	20.3 (1.00)

at 0°C for 5 minutes

(B)

2-ME	Nucleo base (μM)				
	Thymine	Cytosine	Adenine	Guanine	Total (Ratio)
(+)	25.1 (0.62)	10.9 (0.27)	3.7 (0.09)	0.8 (0.02)	40.5 (1.00)
(−)	20.5 (0.66)	7.3 (0.23)	3.0 (0.10)	0.4 (0.01)	31.2 (1.00)

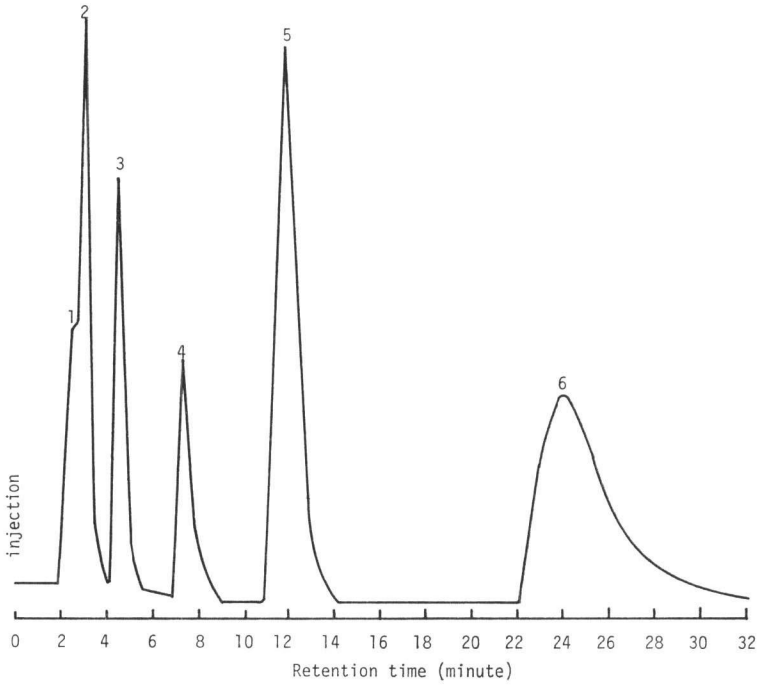
at 37°C for 5 minutes

(C)

2-ME	Nucleo base (μM)				
	Thymine	Cytosine	Adenine	Guanine	Total (Ratio)
(+)	142.0 (0.64)	39.0 (0.18)	29.0 (0.13)	12.3 (0.05)	222.3 (1.00)
(−)	21.0 (0.61)	10.5 (0.31)	2.8 (0.08)	0.1 (0.003)	34.4 (1.003)

at 37°C for 6 hours

Fig. 1. Separation of nucleo bases by high-pressure liquid chromatography.
Details were described in Materials and Methods.
1: Unknown 2: Unknown 3: Cytosine 4: Guanine 5: Thymine 6: Adenine



released (56~66% of the total of the released base) and the molar ratio of the released thymine to the total of the bases released was little affected by varing the reaction conditions. Cytosine was 18~31% of the total, adenine was 8~14% and guanine was most slowly released (0.3~5%). The reaction-time dependency of the base release was observed only when 2-ME was added to the reaction mixture. It will be discussed in the following section. The total amount of base released was increased about 6.5 times by 2-ME in the reaction at 37°C for 6 hours. The temperature effect on the base release (37°C *versus* 0°C) was an increase of 1.5 times in the absence of 2-ME and 1.9 times in the presence of 2-ME at 5-minute reaction time.

Time-dependency of the Base Release from DNA by BLM-Fe(II)-O_2 in the Presence of 2-ME

The results are shown in Fig. 2. The bases were released biphasically. Within the initial 5

Fig. 2. Time-dependency of the base release from DNA by BLM A_2-Fe(II)-O_2 in the presence of 2-ME.

Experimental conditions are the same as described in Table 1. The reaction time is 5, 10, 20, 30 and 60 minutes respectively.

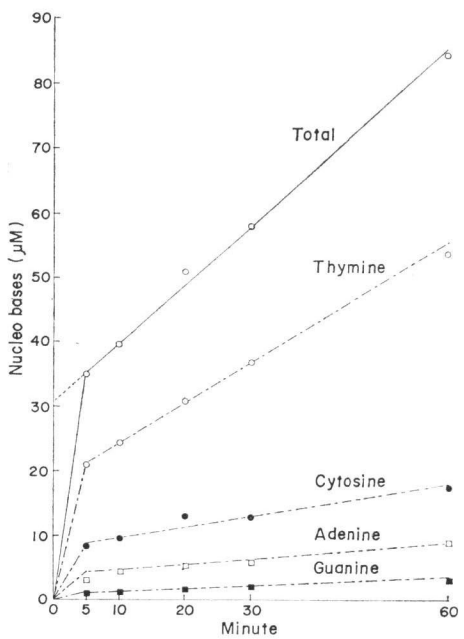
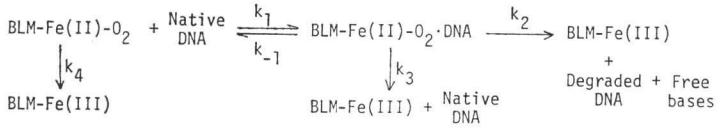


Fig. 3. The reaction scheme of BLM-Fe(II)-O_2 with DNA in the absence or presence of reducing agents.

(A) in the absence of reducing agents.



(B) in the presence of reducing agents

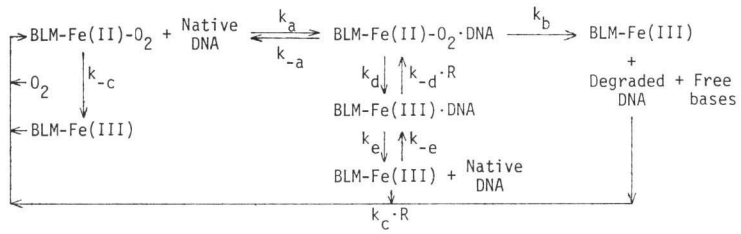
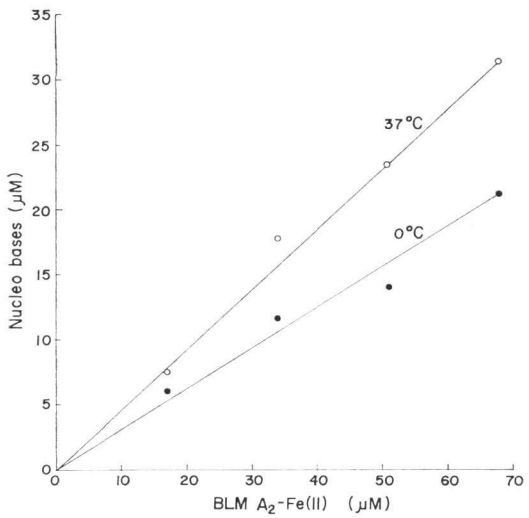


Fig. 4. The release of nucleo bases from DNA at various concentrations of BLM A_2-Fe(II) in the absence of 2-ME.

The reaction mixture (0.5 ml) consists of 3.68×10^{-3} M salmon sperm DNA, 40 mM Tris-HCl of pH 7.8 and 1.7, 3.4, 5.1 and 6.8×10^{-5} M BLM A_2-Fe (II). Reaction time, 5 minutes; reaction temperature, 0°C and 37°C.



minutes, they were released rapidly and thereafter gradually and linearly with the time of the reaction. In the absence of 2-ME, the reaction proceeded as rapidly in the initial phase as in the presence of 2-ME, but it reached a plateau within 5 minutes (data are not shown). The total amount of nucleo bases released in the absence of 2-ME was almost the same as that indicated by extrapolation to 0 time of the line of the total amount of nucleo bases released in the presence of 2-ME, that is, in the absence of 2-ME, 34.4 μM nucleo bases were released from DNA by 68 μM BLM-Fe(II) for 6 hours (Table 1) and the extrapolated value at 0 time in Fig. 2 was 31 μM.

BLM-Fe(III) does not release bases from DNA in the absence of 2-ME. Therefore, it can be concluded that in the biphasic reaction of BLM-Fe(II) with DNA in the presence of 2-ME, the first rapid release is due to BLM-Fe(II) initially added and the second slow release is due to BLM-Fe(II) produced by reduction of BLM-Fe(III) by 2-ME.

Kinetics in the Reaction of BLM-Fe(II)-O_2 with DNA in the Absence of 2-ME

The reaction scheme of BLM-Fe(II)-O_2 with DNA in the absence of 2-ME can be postulated as shown in Fig. 3-A. BLM binds to Fe(II) and oxygen immediately. A labile BLM-Fe(II)-O_2 binds and reacts with DNA. BLM-Fe(II)-O_2 partly degrades itself and loses the reaction activity, but in the presence of DNA, BLM remains without degradation (data are not shown). Therefore, the decom-

position of BLM-Fe(II)-O_2 itself can be excluded from the reaction scheme. Kinetical analysis of the reaction shown in the scheme was performed by the steady-state method. The amounts of bases released from DNA at a certain time are given by the following equation (1).

$$[\text{base}]_t = \frac{k_2[D]_0[B]_0}{(k_2+k_3)[D]_0+k_4/k_1(k_{-1}+k_2+k_3)}(1-e^{-kt}) \quad (1)$$

$$k = \frac{k_1(k_2+k_3)}{k_{-1}+k_2+k_3}[D]_0+k_4$$

The k_1, k_{-1}, k_2, k_3 and k_4 are the rate constants shown in Fig. 3-A. $[D]_0$ and $[B]_0$ represent the concentrations of DNA and BLM-Fe(II) at 0 time, respectively. If k_3 or k_4 is assumed to be negligible, the equation (2) or (3) can be derived, respectively, at $t=\infty$.

$$[\text{base}]_\infty = \frac{k_2[D]_0[B]_0}{k_2[D]_0+k_4/k_1(k_{-1}+k_2)} \quad (2)$$

$$[\text{base}]_\infty = \frac{k_2}{k_2+k_3}[B]_0 \quad (3)$$

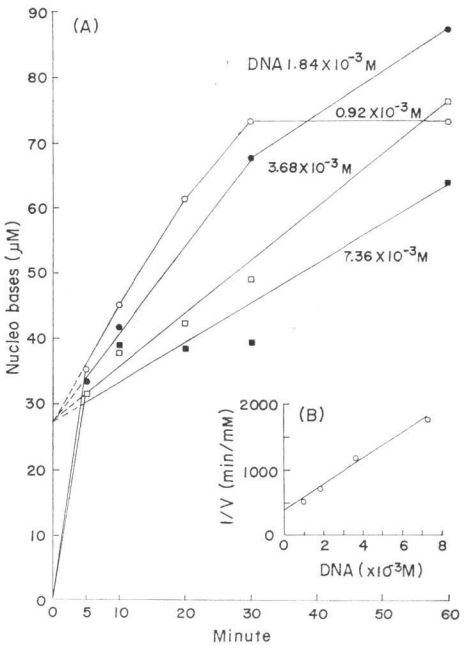
If the equation (3) is satisfied, the reaction should be dependent on the concentration of BLM-Fe(II)-O_2, but independent of the concentration of DNA. If the equation (2) is satisfied, the reaction should be dependent on the concentrations of BLM-Fe(II)-O_2 and DNA.

As shown in Fig. 4, the release of bases from DNA was linearly dependent on the concentration of BLM-Fe(II)-O_2 and independent of the concentration of DNA (data are not shown).

Fig. 5. DNA concentration dependency of the reaction of BLM-Fe(II)-O_2 in the presence of 2-ME.

(A) The reaction mixture (0.5 ml) consists of 6.8×10^{-5} M BLM A_2, 6.8×10^{-5} M $FeSO_4$, 0.1 M 2-ME, 40 mM Tris-HCl of pH 7.8 and 0.92, 1.84, 3.68 and 7.36×10^{-3} M salmon sperm DNA. The base-release was measured at 5, 10, 20, 30 and 60 minutes, respectively.

(B) Kinetics of the reaction of BLM A_2-Fe(II) with various concentrations of DNA in the presence of 2-ME. From Fig. 5-A, the initial reaction velocities of each reactions are obtained.



The result indicates that the reaction of BLM-Fe(II) with DNA in the absence of 2-ME satisfies equation (3), that is, k_4 is negligibly small but k_3 is not.

Kinetics on the Reaction of BLM-Fe(II)-O_2 with DNA in the Presence of 2-ME

Fig. 3-B shows the reaction scheme of BLM-Fe(II)-O_2 with DNA in the presence of 2-ME. Application of the steady-state method gives equation (4), when $k_{-d}\cdot R$ is assumed as negligible.

$$v = \frac{\{f/(b+e[R]_0)\}[R]_0[B]_0}{1+\{(a+c[R]_0)/(b+e[R]_0)\}/[D]_0+\{d/(b+e[R]_0)\}[D]_0} \quad (4)$$

a, b, c, d and e in the equation represent the following constants consisting of the rate constants of each reaction shown in Fig. 3-B:

$a = k_bk_ck_e+k_{-c}k_dk_e+k_bk_{-c}k_e+k_{-a}k_{-c}k_e,$
$b = k_ak_ck_d+k_{-a}k_{-c}k_{-e}+k_ak_dk_e+k_ak_bk_e,$
$c = k_ck_dk_e+k_bk_ck_e+k_bk_{-c}k_d+k_{-a}k_{-c}k_d+k_{-a}k_ck_e,$

Fig. 6. BLM-Fe(II) concentration dependency of the base-release from DNA in the presence of 2-ME.

(A) The reaction mixture (0.5 ml) consists of 3.68×10^{-3} M salmon sperm DNA, 1.7, 3.4, 5.1 and 6.8×10^{-5} M BLM A_2-Fe(II), 0.1 M 2-ME and 40 mM Tris-HCl of pH 7.8.

(B) Kinetics of the reaction of various concentrations of BLM A_2-Fe(II) with DNA in the presence of 2-ME. From Fig. 6-A, the initial reaction velocities of each reactions are obtained.

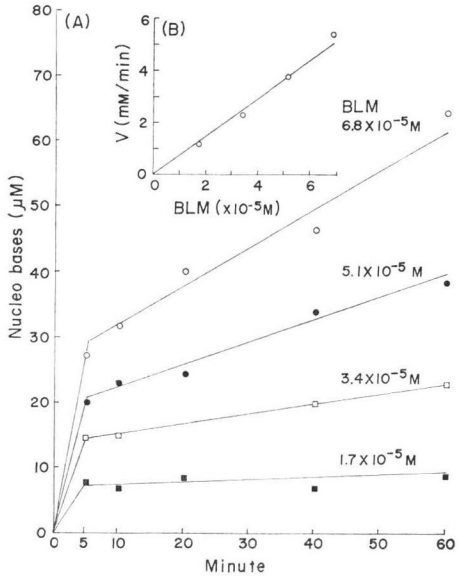
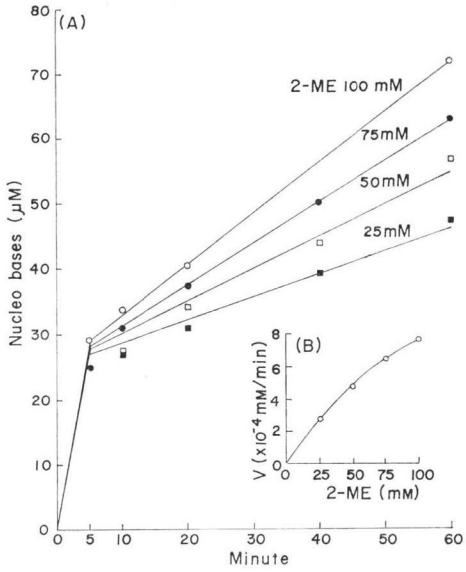


Fig. 7. 2-ME concentration dependency of the reaction of BLM-Fe(II)-O_2 with DNA.

(A) The reaction mixture (0.5 ml) consists of 3.68×10^{-3} M salmon sperm DNA, 6.8×10^{-5} M BLM A_2-Fe-(II), 25, 50, 75 and 100 mM 2-ME and 40 mM Tris-HCl of pH 7.8.

(B) Kinetics of the reaction of BLM A_2-Fe(II) with DNA in the various concentrations of 2-ME. From Fig. 7-A, the initial reaction velocities of each reactions are obtained.



$d=k_ak_dk_{-e}+k_ak_bk_{-e}$,
$e=k_ck_{-d}k_{-e}+k_ak_ck_e+k_ak_ck_d$,
$f=k_ak_bk_ck_e$.

$[D]_0$, $[B]_0$ and $[R]_0$ represent the concentrations of DNA, BLM-Fe(II) and 2-ME at 0 time, respectively. The assumption that k_{-c} is negligible and $k_c\cdot R$ is small leads to equation (5).

$$v=\frac{\{k_bk_c/(k_b+k_d)\}[R]_0[B]_0}{1+[D]_0/K_e} \tag{5}$$

Where K_e is the dissociation constant of BLM-Fe(III)·DNA complex. Equation (5) can be converted to equation (6).

$$1/v=\{(k_b+k_d)/k_bk_c\}/[R]_0[B]_0+\{(k_b+k_d)/k_bk_c\}[D]_0/K_e[R]_0[B]_0 \tag{6}$$

If the assumptions described above are satisfied, a plot of 1/v *versus* $[D]_0$ should be linear with the intercept of $\{(k_b+k_d)/k_bk_c\}/[R]_0[B]_0$ and the slope of $\{(k_b+k_d)/k_bk_c\}/K_e[R]_0[B]_0$. From the intercept, k_c is calculated since $k_b=k_d$ was led from Fig. 4 and equation (3). Furthermore, from the slope, K_e is calculated. Then, a plot of v *versus* $[B]_0$ or $[R]_0$ is linear with the slope of $\{k_bk_c/(k_b+k_d)\}[R]_0/(1+[D]_0/K_e)$ or $\{k_bk_c/(k_b+k_d)\}[B]_0/(1+[D]_0/K_e)$, respectively.

DNA Concentration Dependency of the Base-release in the Reaction of BLM-Fe(II)-O_2 with DNA

In order to obtain a plot of 1/v *versus* $[D]_0$, the concentration of DNA was varied from 0.92 to

7.36×10^{-3} M. From equation (5), it can be presumed that v is reduced by an increase of DNA concentration. This presumption was certified by the time course study of the base-release at various concentrations of DNA (Fig. 5-A). With 0.92×10^{-3} M DNA, the nucleo bases were most rapidly released from DNA and the reaction reached a plateau at 30 minutes. From this result, it was calculated that one nucleo base per twelve to thirteen bases of DNA was released maximally.

Fig. 5-B shows the relationship between the reciprocal of v and the concentration of DNA. The velocity was obtained from the linear slope of the released bases after 5 minutes. The plot of 1/v *versus* $[D]_0$ was linear.

Accordingly, the results shown in Fig. 5 satisfy equation (6). From the intercept, k_e was calculated to be 1.2×10^{-5} ($mM^{-1} \cdot second^{-1}$) and the half life period of BLM-Fe(III) was calculated to be 578 seconds in the presence of 0.1 M 2-ME. From the slope, K_e was calculated to be 2.0×10^{-3} M.

BLM-Fe(II) Concentration Dependency of the Base-release in the Reaction of BLM-Fe(II)-O_2 with DNA

In order to obtain the plot of v *versus* $[B]_0$, the concentration of BLM-Fe(II) was varied from 1.7 to 6.8×10^{-5} M. Fig. 6-A shows the time course of the reaction to release bases from DNA at various concentrations of BLM-Fe(II). As shown in Fig. 6-B, v was a linear function of $[B]_0$.

2-ME Concentration Dependency of the Base-release in the Reaction of BLM-Fe(II)-O_2 with DNA

In order to obtain the plot of v *versus* $[R]_0$, the concentration of 2-ME was varied from 6.25×10^{-3} to 1.0×10^{-1} M. Fig. 7-A indicates the time course of the base-release from DNA at various concentrations of 2-ME. The plot of v *versus* $[R]_0$ was linear at lower concentrations of 2-ME, but curved down at the higher concentrations (Fig. 7-B).

This suggests that 2-ME may coordinate to BLM-Fe(III). In fact, the visible spectrum of BLM-Fe(III) was changed by the addition of cysteine and showed a new absorption maximum at 570 nm (data are not shown).

As described above, the reaction of BLM-Fe(II) in the presence of 2-ME satisfied equations (5) and (6).

Discussion

BLM reacts with DNA in two steps: Binding to DNA through the bithiazole and terminal amine moieties of BLM[13~16], generation of reactive oxygen radicals at the ferrous-binding site of BLM[6,9,10,12]. TAKESHITA *et al.*[11] reported the recognition of base sequence and the base specificity liberated by the reaction of BLM with DNA using the sequence-technique. We confirmed that pyrimidines, especially thymine, were more easily released than purines in salmon sperm DNA (Table 1) and one nucleo base per twelve to thirteen bases of DNA was found to be maximally released by BLM A_2 (Fig. 5-A).

Concerning the mechanism of action of BLM to release bases, the reaction scheme of BLM-Fe(II)-O_2 with DNA in the absence or presence of reducing agents can be proposed as shown in Fig. 3-A and -B.

In the absence of 2-ME, the release of bases from DNA was dependent on the concentrations of BLM-Fe(II) but independent of the concentrations of DNA (Figs. 4, 5-A and Eq. 3). Namely, the reactivity of BLM-Fe(II)-O_2 with DNA is determined by k_2 and k_3 in equation (3) and k_4 can be neglected.

BLM-Fe(II)-O_2 bound to DNA effectively released bases because $k_2/(k_2+k_3)$ value was about 0.5 at 37°C. This means that one half of the BLM-Fe(II)-O_2 molecules bound to DNA release bases (Table 1 and Fig. 4); whereas the reactivity of BLM-Fe(II)-O_2 with DNA decreased at 0°C and the value of $k_2/(k_2+k_3)$ was 0.3 (Table 1 and Fig. 4). Since BLM-Fe(II) remained without degradation for 30 minutes at 0°C (data are not shown), k_4 is negligible.

These results suggest that the difference of reaction efficiency between 37°C and 0°C is due to the temperature dependency of k_2 and/or k_3.

In the presence of 2-ME, the base-release reaction was biphasic. The initial rapid release is due to BLM-Fe(II) added and the second slow one is due to BLM-Fe(II) continually produced by the reduction of BLM-Fe(III) by 2-ME. In the second reaction, the rate of the release of bases was proportional to the concentration of BLM-Fe(II) and of 2-ME, but inversely proportional to the concentration of DNA (Figs. 5, 6 and 7).

The reaction rate was determined at the step of the production of BLM-Fe(II) from BLM-Fe(III) by 2-ME, namely, $k_c \cdot R$ (Fig. 3-B). Fe(III) in BLM-Fe(III)·DNA complex is hardly reduced by 2-ME because the reaction rate decreased with the increase of DNA concentration (Fig. 5-A). It is a problem to be solved whether the reduction of Fe(III) by 2-ME occurs in its free form or in its complex with BLM. If BLM-Fe(III) is reduced, the rate of base-release should be proportional to the concentration of added BLM-Fe(II), and if free Fe(III) ion is reduced, the rate of base-release will not be proportional to the concentration of added BLM-Fe(II). Our data indicated that the rate of the base-release is proportional to the concentration of added BLM-Fe(II) (Fig. 6-B). This result indicates that Fe(III) in BLM-Fe(III) is reduced (Fig. 3-B), although SAUSVILLE *et al.*[9] reported that free Fe(III) generated by the oxidation of BLM-Fe(II) may undergo reduction by a reducing agent and become available for the further reaction with DNA.

At higher concentrations of 2-ME, the plot of v *versus* $[R]_0$ curved down (Fig. 7-B). This suggests that 2-ME may coordinate to BLM-Fe (III). In fact, the visible spectrum of BLM-Fe(III) showed a new absorption maximum at 570 nm by the addition of cysteine (data are not shown). BLM binds strongly with Cu^{2+} and forms an equimolar complex BLM-Cu(II). When metal-free BLM is injected, BLM binds partially or completely with Cu^{2+} in the blood[17]. BLM-Cu(II) does not cleave DNA *in vitro*, but does *in vivo*. The copper in the BLM-Cu(II) is reductively removed[18] and replaced by Fe^{2+} in cells.

Accordingly, the following two roles of reducing agents in the action of BLM to DNA in cells can be proposed: First, the reductive removal of the copper from the BLM-Cu(II); second, the production of BLM-Fe(II) from BLM-Fe(III). Since the rate-determining step in DNA breakage by BLM-iron complex in the presence of reducing agents is $k_c \cdot R$, BLM action should be dependent on the kinds and concentrations of reducing agents in cells.

References

1) UMEZAWA, H.; Y. SUHARA, T. TAKITA & K. MAEDA: Purification of bleomycins. J. Antibiotics, Ser A 19: 210~215, 1966
2) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA: On the mechanism of action of bleomycin: Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22: 446~448, 1969
3) HAIDLE, C. W.: Fragmentation of deoxyribonucleic acid by bleomycin. Mol. Pharmacol. 7: 645~652, 1971
4) HAIDLE, C. W.; K. K. WEISS & M. T. KUO: Release of free bases from deoxyribonucleic acid after the reaction with bleomycin. Mol. Pharmacol. 8: 531~537, 1972
5) MÜLLER, W. E. G.; Z. YAMAZAKI, H. BRETER & R. K. ZAHN: Action of bleomycin on DNA and RNA. Eur. J. Biochem. 31: 518~525, 1972
6) SAUSVILLE, E. A.; J. PEISACH & S. B. HORWITZ: A role for ferrous ion and oxygen in the degradation of DNA by bleomycin. Biochem. Biophys. Res. Commun. 73: 814~822, 1976
7) LOWN, J. W. & S. K. SIM: The mechanism of the bleomycin-induced cleavage of DNA. Biochem. Biophys. Res. Commun. 77: 1150~1157, 1977
8) MÜLLER, W. E. G. & R. K. ZAHN: Bleomycin in antibiotic that removes thymine from double-stranded

DNA. Progr. Nucleic Acids Res. Mol. Biol. 20: 21~57, 1977
9) SAUSVILLE, E. A.; J. PEISACH & S. B. HORWITZ: Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. Biochemistry 17: 2740~2746, 1978
10) TAKITA, T.; Y. MURAOKA, T. NAKATANI, A. FUJII, Y. IITAKA & H. UMEZAWA: Chemistry of bleomycin. XXI. Metal-complex of bleomycin and its implication for the mechanism of bleomycin action. J. Antibiotics 31: 1073~1077, 1978
11) TAKESHITA, M.; A. P. GROLLMAN, E. OHTSUBO & H. OHTSUBO: Interaction of bleomycin with DNA. Proc. Natl. Acad. Sci., U.S.A. 75: 5983~5987, 1978
12) SUGIURA, Y. & T. KIKUCHI: Formation of superoxide and hydroxy radicals in iron(II)-bleomycin-oxygen system: Electron spin resonance detection by spin trapping. J. Antibiotics 31: 1310~1312, 1978
13) CHIEN, M.; A. P. GROLLMAN & S. B. HORWITZ: Bleomycin-DNA interaction: Fluorescence and proton magnetic resonance studies. Biochemistry 16: 3641~3647, 1977
14) STRONG, J. E. & S. T. CROOKE: DNA breakage by tallysomycin. Cancer Res. 38: 3322~3326, 1978
15) POVIRK, L. F.; M. HOGAN & N. DATTAGUPTA: Binding of bleomycin to DNA: Intercalation of the bithiazole rings. Biochemistry 18: 96~101, 1979
16) KASAI, H.; H. NAGANAWA, T. TAKITA & H. UMEZAWA: Chemistry of bleomycin. XXII. Interaction of bleomycin with nucleic acids, preferential binding to guanine base and electrostatic effect of the terminal amine. J. Antibiotics 31: 1316~1320, 1978
17) KANAO, M.; S. TOMITA, S. ISHIDA, A. MURAKAMI & H. OKADA: Chelation of bleomycin with copper *in vivo*. Chemotherapy (Tokyo) 21: 1305~1310, 1973
18) TAKAHASHI, K.; O. YOSHIOKA, A. MATSUDA & H. UMEZAWA: Intracellular reduction of the cupric ion of bleomycin copper complex and transfer of the cuprous ion to a cellular protein. J. Antibiotics 30: 861~869, 1977