The Oxidation–Reduction Potential of Copper–Bleomycin

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Abstract

The oxidation–reduction potential of Cu(II)–bleomycin was measured at 25 °C, pH 7.0. With dithionite as reductant, the potential was −105 ± 9 mV with an *n* value near unity. With cysteine as reductant, the potential was −49 ± 10 mV, with an *n* value substantially greater than unity. The potentials are within a range that would allow the reduction of Cu(II)–bleomycin to take place in a cell.

Introduction

The bleomycins constitute a family of glycopeptide antibiotics with antineoplastic activity [1, 2]. They form complexes with a number of transition metal ions, some of which have been reported to cleave DNA [3–5]. Of these, Fe(II)–bleomycin has been shown to react with O_2, forming a ternary complex which, by single electron reduction, can be activated to cleave DNA [6]. Cu(II), which also forms a complex with the drug, is an inhibitor of the Fe(II)-dependent reaction, presumably by replacing Fe(II) [7]. Recently, it has been reported [5] that Cu(II)–bleomycin can be activated to cleave DNA with NADPH and microsomal NADPH–cytochrome P-450 reductase ($E^{\circ\prime}$ = −371, −274 and −109 mV [8]), presumably by reducing the bound metal ion to Cu(I) [5]. Yet, these findings are difficult to reconcile with the inability to anaerobically reduce Cu(II)–bleomycin with microsomes in the presence of a two-fold molar excess of NADPH [9] ($E^{\circ\prime}$ = −324 mV) and with the reported oxidation–reduction potential for the copper complex ($E^{\circ\prime}$ = −310 mV, measured polarographically in phosphate buffer, pH 8 [10]). For this reason, we have reexamined the oxidation–reduction potential of Cu(II)–bleomycin and found that it can vary with the method of measurement. Using reduced pyridine nucleotide as reductant, it can be as low as −390 mV *versus* the SHE**, while with dithionite as reductant, the $E^{\circ\prime}$ was found to be −105 ± 9 mV. With cysteine, the potential is −49 ± 12 mV and with an *n* value near 2. For the reaction with cysteine, no evidence for dimer formation or free radical generation was obtained.

Experimental

Materials and Methods

Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories and was used without further purification. This drug was a metal-free, lyophilized preparation containing approximately 60% bleomycin A_2, 30% bleomycin B_2 and 10% various other bleomycins [11]. Solutions were prepared in metal-free, deionized, distilled water. A molecular weight of 1550 was assumed with an $\epsilon_{292} = 1.45 \times 10^{-4}$ M^{-1} cm^{-1}. All solutions of bleomycin were used immediately after preparation.

Hepes, phenazine methosulfate, cysteine, NADH and NADPH were purchased from Sigma. Ascorbic acid and sodium dithionite were obtained from Nahaisi Chemicals and Fisher Chemical Co., respectively. Reagent grade hydrated ferrous ammonium sulfate (J. T. Baker Chemical Co.) and cupric acetate (Mallinkrodt) were dissolved in distilled water before use.

A Cary 14 R spectrometer was used for optical studies and a Varian E-12 spectrometer equipped with a Varian E-213 multipurpose cavity, a Varian NMR gaussmeter and a System-Donner frequency counter were used for EPR studies.

Coulometric–Potentiostatic Measurements

These were performed as previously described in 0.05 M Hepes buffer, pH 7.0 containing 50 μM mediator [12]. Cu(II)–bleomycin samples were

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**Abreviations used: Hepes = *N*-(2-hydroxyethyl) piperazine *N'*-2-ethanesulfonic acid; PMS = phenazine methosulfate; SHE = standard hydrogen electrode; TLC = thin layer chromatography.

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injected in 5 to 100 μl aliquots into the sample cell with a Hamilton gas-tight syringe. Residual currents were a function of the applied potential and ranged from 0.5 to 10 nA.

Optical Potentiometric Measurements

These studies were performed as previously reported, utilizing a redox cell (4 ml operating volume) modified by the addition of a saturated KCl–agar salt bridge attached to the Calomel electrode [12]. Cu(II)–bleomycin samples (3–4 mM) were prepared using 10% molar excess drug, concentrations that were adequate for optical monitoring. Reduction of solutions, previously made anaerobic by purging with highly purified, water-saturated argon, was carried out by the addition of small volume increments of anaerobic cysteine or other reducing agents (1 equivalent per 50 μl). With NADH and NADPH, higher concentrations (1 equivalent per 20 μl) were used when it became apparent that reduction was not proceeding to completion. For studies with Fe(III)–bleomycin, 0.06–1.2 mM samples were prepared in 20 mM sodium phosphate buffer or 50 mM Hepes buffer, pH 7.0. When used, PMS was added at a concentration equal to 5% of the metal bleomycin present. Potentiometric data were fitted to the Nernst equation with the aid of a computer, as described previously [12].

Thin Layer Chromatography

The bleomycins were separated on GF-precoated silica gell glass plates (Uniplate, Analtech) in methanol + 10% ammonium acetate (*w/v*) + 10% ammonium hydroxide (*v/v*) (10:9:1). Two μg of the drug can be visualized with an ultraviolet lamp. At high concentrations, Cu(II)–bleomycin is visually detectable as a blue spot on the plate. At low concentrations, sodium bathocuproine sulfonate (2 mM) with either 10 mM $Na_2S_2O_4$ or 10% hydroxylammonium chloride plus 10% ammonium acetate, pH 7.0, was used to stain for copper. For anaerobic handling and preparations of TLC plates, a glove box purged 1 h with N_2 followed by a 1 h argon flush was used. Running buffer and samples were made anaerobic by argon purging.

Results

Potentiostatic Reduction of Cu(II)–Bleomycin

The reduction proved difficult and non-reproducible in the absence of mediator. Even with PMS, the current–time integrals showed a large degree of variation. The integrals became reproducible only at potentials below −160 mV *versus* the SHE. Although computer aided analysis resulted in a poor fit to the Nernst equation (not shown), the data suggested that the redox potential was probably more negative than −20 mV but more positive than −200 mV. The number of electrons involved in the reduction reaction, *n*, was difficult to estimate due to the scatter of data points. Because of the limited success of this method, the remaining investigation focused on a potentiometric method utilizing chemical reductants.

Optical Potentiometry of Cu(II)–Bleomycin

The reductive titration of Cu(II)–bleomycin was studied optically at 603 nm, which is the absorption maximum for the complex ($\epsilon = 118^{-1}$ M^{-1} cm^{-1}) [7], and was monitored throughout the range +330 mV to −330 mV *versus* the SHE with several reductants. The loss of absorbance at 603 nm is due to the reduction of Cu(II) as determined by EPR spectroscopy [7]. Reaction with cysteine ($E^{\circ\prime} = -340$ mV) or sodium dithionite ($E^{\circ\prime} = -527$ mV) led to complete reduction of Cu(II) at pH 7.0 in 0.05 M Hepes buffer, although with cysteine, approximately 15 min was required for this to occur. Spectral stability, *i.e.* constant absorbance, was obtained before the potential completely stabilized. This may have resulted either from a slow response of the electrode to the solution potential or from complex formation of Cu(II)–bleomycin with cysteine [7].

A typical Nernst plot for cysteine reduction of Cu(II)–bleomycin assayed optically against the measured potential was fitted by a computer-generated least-squares procedure to the Nernst equation (Fig. 1). The average value of the redox potential

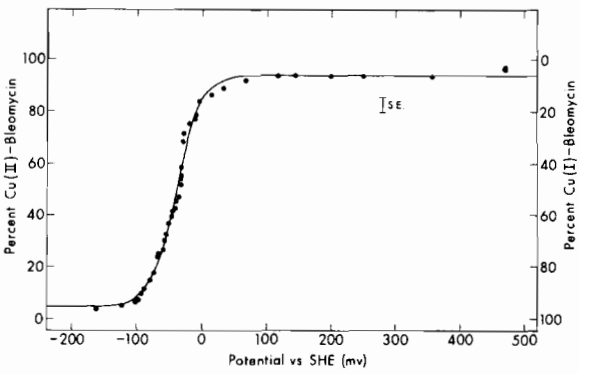

Fig. 1. A typical oxidation–reduction potential analysis of Cu(II)–bleomycin determined from a spectrophotometric titration. Cysteine reduction of an anaerobic solution of Cu(II)–bleomycin (0.8 mM) in 50 mM Hepes buffer, pH 7.0, was followed both spectrophotometrically and potentiometrically as described. Data were plotted as percentage of Cu(II)–bleomycin, determined optically, *vs.* potential compared to the SHE. The line is a computer-generated least-squares fit of the data to the Nernst equation, yielding $E^{\circ\prime} = -40$ mV and $n = 1.85$. The computer fit of the data to the Nernst equation with fixed values of either 1 or 2 for *n* showed lower mean weight errors and higher correlation coefficients for an $n = 2$ process. S.E., the standard error, is an expression of the deviation of the data from the iterated least-squares fit to the equation.

TABLE I. Oxidation–Reduction Potential of Cu(II)–Bleomycin at pH 7.0, 25 °C

Reductant	$E^{\circ\prime}$ (mV)	n
Cysteine	−40	1.85
Cysteine	−62	1.39
Cysteine	−37	1.41
Cysteine	−62	2.19
Cysteine	−46	2.01
	av: −49 ± 10	1.77 ± 0.30
Dithionite	−114	1.15
Dithionite	−96	0.96
	av: −105 ± 9	1.06 ± 0.10

determined from five independent measurements with cysteine as reductant was −49 ± 12 mV *versus* the SHE with an n value of 1.77 ± 0.30 (Table I).

With dithionite as the reducing agent, a significant difference was observed. An average $E^{\circ\prime}$ of −105 ± 9 mV *versus* the SHE and an n value near unity was obtained (Table I). The deviation in n value is small, and more significantly, the value is that expected for a one-electron process.

With other reductants that might be physiologically relevant, *i.e.*, NADH ($E^{\circ\prime}$ = −320 mV) and NADPH ($E^{\circ\prime}$ = −324 mV), full reduction of Cu(II)–bleomycin was not observed at pH 7.0. Addition of a 12-fold molar excess of NADH only resulted in a 30% reduction of the Cu(II) within 30 min. Analysis of the partial reduction data suggested an apparent potential of −390 mV. The presence of PMS did not facilitate the rate or extent of reduction.

Attempts at utilizing less than stoichiometric amounts of cysteine as a mediator, but with a large excess of a second reducing agent such as NADH, resulted in reduction of that amount of Cu(II)–bleomycin that was stoichiometrically equivalent to half the amount of cysteine added. Thus, cysteine not only acts as an electron transfer reagent for Cu(II)–bleomycin reduction, but also has a secondary role in the redox system.

No Evidence for a Bleomycin Dimer

To further investigate the unusual stoichiometry of the reduction of Cu(II)–bleomycin by cysteine, two approaches were taken. Were the n value to represent a two-electron process, then one electron equivalent is accounted for by the reduction of Cu(II) to Cu(I), and the other electron equivalent could either generate a free radical or lead to the formation of a dimeric species. EPR spectral studies could not detect any long-lived radical (data not shown), so a search for dimer formation was undertaken by thin layer chromatography.

When Cu(II)–bleomycin was run on TLC, three major blue spots were seen, each of which corresponded to that for the metal-free drug, but with a slightly higher R_f value [11]. These spots are of the Cu(II)-bound forms of bleomycin A_2, B_2 and demethyl A_2. When Cu(II)–bleomycin was anaerobically reduced with a 5-fold excess of cysteine, and subsequently run on TLC in an argon-filled glove box, four spots were observed, one of which was due to free copper and the remaining three to the bleomycin isomers. When a similar, cysteine-reduced Cu(II)–bleomycin sample was allowed to air oxidize before running on TLC, the resulting pattern consisted of 6 major spots grouped as 3 sets of 2. Each set was of a single bleomycin isomer, with one spot for the metal-free form and the other set for the copper-bound form. The six R_f values were in close agreement with those observed for individually purified metal-free and Cu(II)-bound drug isomers [11]. There was no indication of bleomycin dimer formation or any other species.

Redox Potential of Fe(III)–Bleomycin in the Presence of Cu(II)

The redox potential of a system containing both Fe(III)– and Cu(II)–bleomycin was investigated. The relative extinction coefficients in the visible region determined the range of concentrations of metal–drug complexes feasible for this investigation. The 4 ml sample used in this study consisted of 0.9 mM bleomycin, 40 μM Fe(III) and 0.825 mM Cu(II) in 0.05 mM Hepes buffer, pH 7.0. The Fe(III)– and Cu(II)–bleomycin chromophores were clearly resolved in the optical spectrum, permitting independent monitoring of the degree of reduction for each. Cysteine was used as the reductant since both the Cu(II)– and Fe(III)–bleomycin reactions with cysteine have been characterized [7]. The Nernst plot of the reduction titration yielded a characteristic $E^{\circ\prime}$ value for Fe(III)–bleomycin of +135 mV and a corresponding n value of 1.23, well within the range observed in a redox study of Fe(III)–bleomycin alone (+129 mV) [12]. Similarly, the $E^{\circ\prime}$ value for Cu(II)–bleomycin was −40 mV with an n value of 1.41, again within the range for Cu(II)–bleomycin alone (Table I). This result indicates the distinct and non-interacting nature of the two metal forms of the drug which are capable of undergoing reduction concurrently. This further suggests that both the iron-bound and copper-bound forms are capable of undergoing reduction independently when the potential is poised between the $E^{\circ\prime}$ values for the iron and for the copper complex of the drug.

Discussion

The values obtained for the oxidation–reduction potential of Cu(II)–bleomycin are significantly different from those reported by Dabrowiak and Santillo [10]. Using a polarographic and voltametric approach, these authors report a half-wave potential in phosphate buffer, pH 8, of –310 mV, a value considerably lower than the redox potential obtained by us using either cysteine or dithionite. We have shown that chemical reduction was extremely slow, and it is possible, therefore, that the measured half-wave potential did not reflect the equilibrium of the system. With dithionite as reductant, the measured redox potential of –105 mV is considerably higher than that obtained by the polarographic method, and would support the idea that equilibrium was not obtained by the latter method. And yet, reduced pyridine nucleotides, with potentials of –320 mV, are incapable of completely reducing Cu(II)–bleomycin. One must conclude, then, that the measured potential is not that of a simple Cu(I)–bleomycin/Cu(II)–bleomycin couple, and that more complex equilibria are operative when reduction takes place.

Results from initial experiments conducted with the potentiostat suggested that the reduction of Cu(II)–bleomycin might occur only at low potentials, possibly outside the realm available in living cells. NADH or NADPH are unable to fully reduce Cu(II)–bleomycin [7] due to a low $E^{\circ\prime}$ value for the complex. In contrast, our studies with cysteine as reductant demonstrate an $E^{\circ\prime}$ of –49 mV, a potential easily obtainable within the cell. This potential is the basis for the chemical reduction demonstrated previously *in vitro* [12].

The formation of a cysteine Cu(II)–bleomycin complex may account for the elevation in the redox potential. An n value greater than unity for the reduction is compatible with this idea. The ternary complex thus formed could undergo reduction and eventually release Cu(I). This scheme explains why the presence of cysteine in catalytic amounts, together with other reducing agents in excess, does not lead to complete reduction of Cu(II).

Another possible reason for finding an n value greater than unity is that two reducing equivalents are involved in the reduction of Cu(II)–bleomycin by cysteine. If this were so, then a radical species or dimer should result. Static EPR experiments did not detect the presence of a radical species. Thin layer chromatography yielded no evidence for the existence of dimer and thus gave additional support to the idea that reduction of Cu(II)–bleomycin with cysteine also leads to a loss of copper from the complex.

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