power of theoretical techniques such as energy minimization and molecular dynamics to investigate the structural, energetic, and dynamic properties of biomolecules. It also suggests further experiments to probe the fundamental dynamics of peptides and verify the predictions. Thus, low-temperature NMR of *cyclo*-(Ala-Pro-D-Phe)$_2$ deuteriated in the alanine methyl should be able to confirm the relative rotation rates (S. Opella, private communication) and will be attempted.

ACKNOWLEDGMENTS

We thank Dr. F. Avbelj for helpful discussions and advice.

REFERENCES

Atkins, P. W. (1978a) *Physical Chemistry*, pp 906–914, W. H. Freeman, San Francisco, CA.

Atkins, P. W. (1978b) *Physical Chemistry*, pp 864–866, W. H. Freeman, San Francisco, CA.

Brown, J. N., & Teller, R. G. (1976) *J. Am. Chem. Soc. 98*, 7565–7569.

Dauber, P., & Hagler, A. T. (1980) *Acc. Chem. Res. 13*, 105–112.

Hagler, A. T. (1985) *Peptides (N.Y.) 7*, 213–299.

Hagler, A. T., Lifson, S., & Dauber, P. (1979) *J. Am. Chem. Soc. 101*, 5122–5130.

Hall, D., & Pavitt, N. (1984) *J. Comput. Chem. 5*, 441–450.

Kitson, D. H., & Hagler, A. T. (1988) *Biochemistry 27*, 5246–5257.

Kossiakoff, A. A., & Shteyn, S. (1984) *Nature (London) 311*, 582–583.

Momany, F. A., Corruthers, L. M., McGuire, R. F., & Scheraga, H. A. (1974) *J. Phys. Chem. 78*, 1595–1620.

Williams, D. E. (1965) *J. Chem. Phys. 43*, 4424–4426.

Wittebort, R. J., Rothgeb, T. M., Szabo, A., & Gurd, F. R. N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1059–1063.

Preparation and Characterization of Singly Labeled Ruthenium Polypyridine Cytochrome *c* Derivatives†

Lian Ping Pan, Bill Durham,* Justyna Wolinska, and Francis Millett

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701

Received June 23, 1988; Revised Manuscript Received July 22, 1988

ABSTRACT: A novel two-step procedure has been developed to prepare cytochrome *c* derivatives labeled at specific lysine amino groups with ruthenium bis(bipyridine) dicarboxybipyridine [$Ru^{II}(bpy)_2(dcbpy)$]. In the first step, cytochrome *c* was treated with the mono-*N*-hydroxysuccinimide ester of 4,4′-dicarboxy-2,2′-bipyridine (dcbpy) to convert positively charged lysine amino groups to negatively charged dcbpy-lysine groups. Singly labeled dcbpy-cytochrome *c* derivatives were then separated and purified by ion-exchange chromatography. In the second step, the individual dcbpy-cytochrome *c* derivatives were treated with $Ru^{II}(bpy)_2CO_3$ to form singly labeled $Ru^{II}(bpy)_2$(dcbpy-cytochrome *c*) derivatives. The specific lysine labeled in each derivative was determined by reverse-phase chromatography of a tryptic digest. All of the derivatives had a strong luminescence emission centered at 662 nm, but the luminescence decay rates were increased relative to that of a non-heme protein control, $Ru^{II}(bpy)_2$(dcbpy-lysozyme), which was 1.8×10^6 s^{-1}. The luminescence decay rates were found to be 21, 16, 7.2, 5.7, 4.3, 4.3, and 3.5×10^6 s^{-1} for derivatives singly labeled at lysines 13, 72, 25, 7, 39, 86, and 87, respectively. There was an inverse relationship between the luminescence decay rates and the distances between the ruthenium labels and the heme group. The increased luminescence decay rates observed in the cytochrome *c* derivatives might be due to electron transfer from the excited triplet state of ruthenium to the ferric heme group. However, it is also possible that an energy-transfer mechanism might contribute to the luminescence quenching. The luminescent decay rates of the ferrocytochrome *c* derivatives were nearly as large as those of the corresponding ferricytochrome *c* derivatives, suggesting the possibility of electron transfer from ferrous heme to the excited triplet state of ruthenium.

Our understanding of the factors involved in biological electron transfer has been significantly enhanced by the use of metalloproteins specifically labeled with ruthenium complexes. In the pioneering work of Winkler et al. (1982) and Isied et al. (1982), intramolecular electron transfer was found to take place between a $Ru^{II}(NH_3)_5$ group attached to histidine 33 of cytochrome *c* and the ferric heme group with a rate constant of about 30 s^{-1}. This long-range electron-transfer reaction has a driving force of 0.11 eV and a separation of about 12 Å between the ruthenium and the heme group. Paradoxically, Bectold et al. (1986) found that the rate of electron transfer from the ferrous heme group of cytochrome *c* to $Ru^{III}(NH_3)_4$(isonicotinamide)(histidine 33) was over 10^5 slower than the above rate, even though the driving force was larger (0.18 eV). Elias et al. (1988) have recently prepared a zinc-substituted derivative of cytochrome *c* containing $Ru^{III}(NH_3)_5$(histidine 33) and found that the rate of electron transfer from the triplet excited zinc porphyrin to ruthenium was 7.7×10^5 s^{-1}, while the rate of the thermal back-reaction was 1.6×10^6. The much larger rate constants for these reactions relative to those involving the native iron are presumably due to the large driving force of the reactions (0.88 V for the reaction involving triplet zinc). Intramolecular electron-transfer studies have also been carried out on ru-

† This work was supported by NIH Grants GM20488 and RR07101.

0006-2960/88/0427-7180$01.50/0

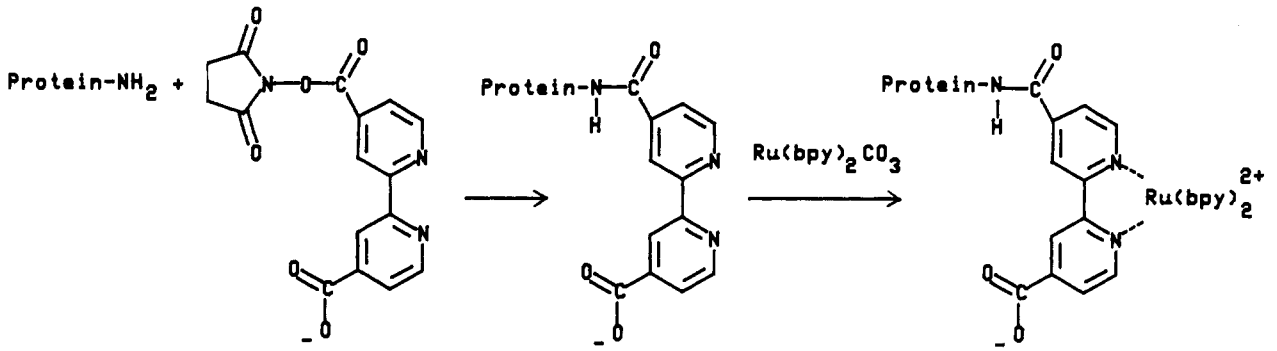

FIGURE 1: Scheme for the preparation of $Ru^{II}(bpy)_2$(dcbpy-cytochrome *c*) derivatives.

thenium-labeled azurin and myoglobin derivatives (Kostić et al., 1983; Crutchley et al., 1986; Axup et al., 1988), mixed-metal hybrid hemoglobins (McGourty et al., 1987), and organic complexes (Closs et al., 1986). The goal of these studies has been to determine the dependence of electron transfer on the driving force of the reaction, the distance between donor and acceptor, and the protein medium through which electron transfer occurs.

Up until now, all of the specifically labeled ruthenium derivatives have involved substitution reactions at surface histidine residues. In the present paper we describe a novel two-step procedure to prepare a number of different cytochrome *c* derivatives that are singly labeled at individual lysine amino groups with ruthenium bis(bipyridine) dicarboxybipyridine [$Ru^{II}(bpy)_2$(dcbpy)].[1] The first step involves treatment of cytochrome *c* with the mono-*N*-hydroxysuccinimide ester of 4,4′-dicarboxy-2,2′-bipyridine to convert positively charged lysine amino groups to negatively chaged dicarboxybipyridine (dcbpy) lysine groups (Figure 1). Singly labeled dcbpy-cytochrome *c* derivatives are then separated and purified by ion-exchange chromatography. The second step involves treatment of the individual dcbpy-cytochrome *c* derivatives with $Ru^{II}(bpy)_2CO_3$ to form singly labeled Ru(bpy)$_2$(dcbpy-lysine cytochrome *c*) derivatives. The method is quite general in that the high affinity of the dcbpy group for metal atoms should allow a wide variety of different ruthenium complexes to be attached to each of the dcbpy-cytochrome *c* derivatives. Preliminary luminescence lifetime studies of the Ru(bpy)$_2$(dcbpy-cytochrome *c*) derivatives have also been carried out, which suggest the possibility of electron transfer from the excited state of ruthenium to the heme group.

Experimental Procedures

Materials. Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (type VI), and 4,4′-dimethyl-2,2′-bipyridine was obtained from GFS chemicals. *N*-Hydroxysuccinimide and dicyclohexylcarbodiimide were obtained from Pierce Chemical Co. 4,4′-Dicarboxy-2,2′-bipyridine (dcbpy) was prepared from 4,4′-dimethyl-2,2′-bipyridine by the method of Sprintschnik et al. (1978). $Ru^{II}(bpy)_2CO_3$ was prepared as described by Johnson et al. (1978). The mono-*N*-hydroxysuccinimide ester of dcbpy was prepared as follows: dcbpy (0.32 mmol in 3 mL of water) was titrated with 0.32 mmol of KOH (molar ratio = 1) to convert one of the two protonated carboxyl groups to the K^+ salt. After all of the water was removed under vacuum, the compound (0.32 mmol) was dissolved in 1.2 mL of dry DMF, dicyclohexylcarbodiimide (0.32 mmol) and *N*-hydroxysuccinimide (0.32 mmol) were each added in a molar ratio of 1, and the solution was stirred at 25 °C for 15 h as described by Anderson et al. (1964). The precipitated dicyclohexylurea was removed, and the DMF was evaporated under a stream of nitrogen. The mono-*N*-hydroxysuccinimide ester of dcbpy was dissolved in 1 mL of 50 mM Tris-HCl, pH 8.0. The small amount of the di-*N*-hydroxysuccinimide ester of dcbpy present in the original reaction mixutre has a low solubility in the Tris buffer and was removed by centrifugation.

Preparation of dcbpy-cytochrome c Derivatives. Horse heart cytochrome *c* (10 mM in 4 mL of 50 mM Tris-HCl, pH 8.0) was treated with the freshly prepared mono-*N*-hydroxysuccinimide ester of dcbpy (40 mM final concentration; molar ratio of 4 relative to cytochrome *c*) for 2 h at 25 °C. The sample was diluted with 7 mL of water and applied to a 2.5 × 70 cm column of Bio-Rex 70. The column was eluted with an exponential gradient from 50 mM ammonium phosphate, pH 7.2, to 160 mM ammonium phosphate, pH 7.2. Each of fractions 1–7 was concentrated on a small Bio-Rex 70 column and rechromatographed on a 1.5 × 25 cm column of Whatman sulfopropyl SE-53 using an exponential gradient from 20 to 250 mM sodium phosphate, pH 6.0.

Preparation of $Ru^{II}(bpy)_2$(dcbpy-cytochrome c) Derivatives. Each of the purified dcbpy-cytochrome *c* fractions was concentrated to 1 mM and treated with 10 mM $Ru^{II}(bpy)_2CO_3$ in 100 mM sodium acetate, pH 4.0, for 24 h at 25 °C in the dark. The sample was passed through a small Bio-Gel P-2 column to remove excess reagent and then chromatographed on a 0.6 × 45 cm Whatman CM-32 column using a gradient from 20 to 400 mM sodium phosphate, pH 6.0. UV/visible spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. The redox potential of the heme group in the cytochrome *c* derivatives was determined as described by Smith et al. (1977). Ru(bpy)$_2$(dcbpy-lysozyme) was prepared in the same manner as described above, except that the derivative was not purified by ion-exchange chromatography.

Identification of the Lysine Modified in the Ru(bpy)$_2$(dcbpy-cytochrome c) Derivatives. Each derivative was dialyzed into 0.1 M Bicine, pH 8.0, at a concentration of 1 μg/μL and digested with 50 ng/μL TPCK-treated trypsin for 15 h at 37 °C. The tryptic digests were separated on a Dynamax 300-Å reverse-phase HPLC column using a linear gradient from 0.01% trifluoroacetic acid to 100% methanol. The gradient was generated on a Spectra Physics SP 8700 solvent delivery system, and the eluent was monitored at 210 and 290 nm on Kratos 757 and Tracor 980A detectors in series. The amino acid composition of each purified peptide was determined by amino acid analysis acccording to the procedures of Smith et al. (1977).

Luminescence. Steady-state luminescence specta were obtained on aa Perkin-Elmer 650-40 fluorescence spectrometer using excitation at 450 nm. Luminescence lifetimes were measured by using the third harmonic of a Nd:YAG laser to

[1] Abbreviations: bpy, 2,2′-bipyridine; dcbpy, 4,4′-dicarboxy-2,2′-bipyridine.

Table I: Luminescence Decay Rates of $Ru^{II}(bpy)_2$(dcbpy-cytochrome *c*) Derivatives[a]

fraction	lysine modified	Fe^{III}(cyt *c*) *k* (10^6 s^{-1})	Fe^{II}(cyt *c*) *k* (10^6 s^{-1})	distance (Å)[b]
1A	86	4.3 ± 0.2	4.6 ± 0.2	10–22
1B	87	3.5 ± 0.2	3.3 ± 0.2	10–24
2B	13	21 ± 2	18 ± 2	3–10
3A	72	16 ± 2	14 ± 3	6–16
4A	25	7.2 ± 0.2	6.0 ± 0.2	9–16
4B[c]	7, 25 (64%, 36%)	5.7 ± 0.2	5.0 ± 0.2	12–16, 12–16
4C[c]	27, 7 (82%, 18%)	>40, 5.7 ± 0.2	>40, 5.2 ± 0.2	3–10, 12–16
5A[c]	39, 60 (64%, 36%)	4.3 ± 0.2	3.7 ± 0.2	15–18, 17–19
5B[c]	39, 60 (51%, 49%)	4.5 ± 0.2	3.8 ± 0.2	15–18, 17–19

[a] The luminescence decay of each derivative (1 μM) was measured in 100 mM sodium phosphate, pH 7.0, at 25 °C and fitted to a first-order exponential decay curve with a rate constant *k*. [b] The distance between the edge of the ruthenium group and the closest part of the heme group was estimated from a model of the crystal structure of cytochrome *c* (Swanson et al., 1977), taking into account the uncertainty in the orientation of the lysine side chain. [c] The luminescence decay of fraction 4C was biphasic, with a fast phase that was too rapid to measure with our instrumentation and a slow phase with the rate constant shown. The luminescence decays of fractions 4B, 5A, and 5B were monophasic, indicating that the individual components in these mixtures had nearly the same decay rates.

excite at 365 nm with a 20-ns pulse. The luminescence passed through a 600-nm long-pass filter and was detected by using an R928 photomultiplier tube. The signal was recorded on a Biomation 8100 digitizer and transferred to an IBM microcomputer for kinetic analysis using a weighted linear least-squares program. All luminescence experiments were carried out in 100 mM sodium phosphate, pH 7.0, at 25 °C.

Results

Preparation of $Ru(bpy)_2$(dcbpy-cytochrome c) Derivatives. *N*-Hydroxysuccinimide has been widely used as a carboxyl activating group in peptide synthesis (Anderson et al., 1964), and the mono-*N*-hydroxysuccinimide ester of dcbpy was found to react efficiently with the lysine amino groups on cytochrome *c*. The dcbpy-cytochrome *c* derivatives were separated into seven different fractions on a Bio-Rex 70 column as shown in Figure 2A, and then each of the fractions was rechromatographed on a Whatman SE-53 column. Fractions 1–3 were resolved into subfractions as shown in Figure 2B, while fractions 4–7 were not further resolved. Spectral analysis indicated that all the fractions contained a single equivalent of dcbpy. Each of the purified dcbpy-cytochrome *c* fractions was then incubated with $Ru^{II}(bpy)_2CO_3$ at pH 4 to form the $Ru^{II}(bpy)_2$(dcbpy-cytochrome *c*) derivatives. A control experiment indicated that no residues on native cytochrome *c* reacted with $Ru(bpy)_2CO_3$ under these conditions, although histidine 33 did react at pH values above 6.0. Each of the derivatives was purified a final time on a Whatman CM-52 column, which resolved a small band due to the unmodified dcbpy-cytochrome *c* from a major band that contained 1 equiv of $Ru(bpy)_2$(dcbpy) per cytochrome *c* (Figure 2C). Fractions from the leading and trailing edges of the final chromatograms of fractions 4 and 5 were analyzed separately. The lysine residue modified in each derivative was determined by reverse-phase HPLC of a tryptic digest of the derivative. In the HPLC chromatogram of fraction 4A shown in Figure 3, the only changes relative to the chromatogram of native cytochrome *c* were that a new ruthenium-labeled peptide appeared, which was shown by amino acid analysis to contain the sequence 23–27. Fraction 4A therefore contains a single Ru-$(bpy)_2$(dcbpy) group on lysine 25. The identification of the other singly labeled derivatives was carried out in a similar fashion and is summarized in Table I. Fractions 4B, 4C, 5A, and 5B were found to contain mixtures of singly labeled derivatives, while all the other fractions shown in Table I contained one singly labeled derivative. All of the derivatives had a UV/visible spectrum that was equal to the sum of the spectra of 1 equiv of $Ru^{II}(bpy)_2$(dcbpy) and 1 equiv of native cytochrome *c* (Figure 4). The 695-nm band was unmodified in all of the derivatives, indicating that the methionine 80 heme

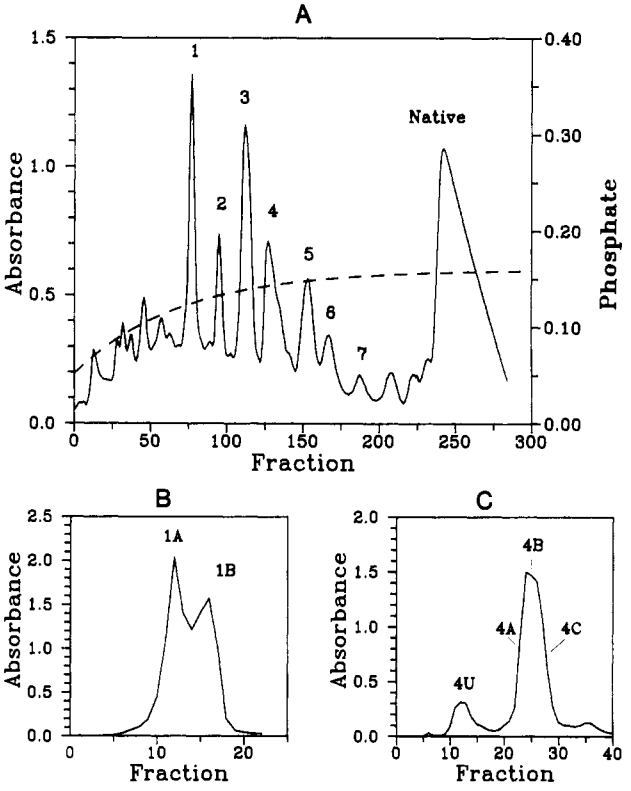

FIGURE 2: Purification of dcbpy-cytochrome *c* and $Ru(bpy)_2$-(dcbpy-cytochrome *c*) derivatives. (A) The crude reaction mixture of dcbpy-cytochrome *c* (500 mg) was chromatographed on a 2.5 × 70 cm Bio-Rex 70 column using an exponential gradient from 50 mM ammonium phosphate, pH 7.2, to 160 mM ammonium phosphate, pH 7.2. The flow rate was 25 mL/h and the fraction size was 3.8 mL. The absorbance was measured at 542 nm. (B) Fraction 1 from (A) was rechromatographed on a 1.5 × 25 cm column of Whatman sulfopropyl SE-53 using an exponential gradient from 20 to 250 mM sodium phosphate, pH 6.0. The fraction size was 1 mL and the absorbance was measured at 542 nm. (C) Repurified fraction 4 was treated with $Ru^{II}(bpy)_2CO_3$ and chromatographed on a 0.6 × 45 cm Whatman CM-32 column using a gradient from 20 to 400 mM sodium phosphate, pH 6.0. The fraction size was 1 mL and the absorbance was measured at 542 nm. The fraction marked 4U contained unmodified dcbpy-cytochrome *c*.

ligand was not disturbed. The heme group redox potentials of the derivatives were all in the range 250–260 mV, essentially the same as that of native cytochrome *c*, 260 ± 10 mV.

Luminescence of $Ru^{II}(bpy)_2$(dcbpy-cytochrome c) Derivatives. The steady-state luminescence emission spectra of each of the derivatives had the same line shape as that of Ru-$(bpy)_2$(dcbpy), with a maximum at 662 nm (Figure 4). However, both the intensity of the steady-state luminescence and the lifetime of the luminescence were decreased relative

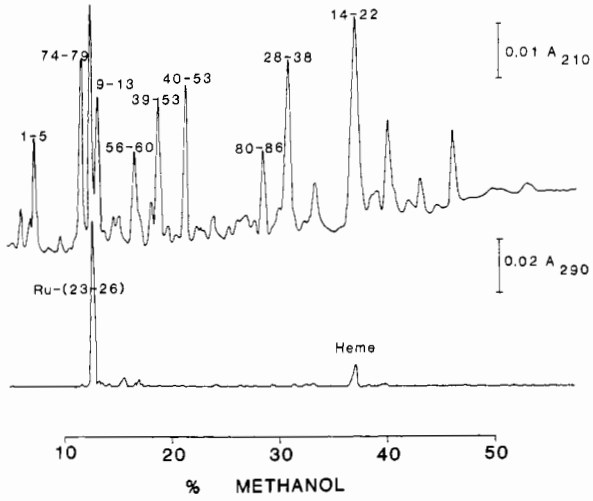

FIGURE 3: HPLC chromatogram of the tryptic digest of $Ru(bpy)_2$-(dcbpy-cytochrome *c*) fraction 4A. The tryptic digest (50 μg) was eluted on a Dynamax 300-Å column using a linear gradient from 0.01% trifluoroacetic acid to 100% methanol. The native and ruthenium-modified peptides were identified by amino acid analysis and indicated on the figure. Small peptides such as 23–25 and 26–27 eluted in the void volume of the column.

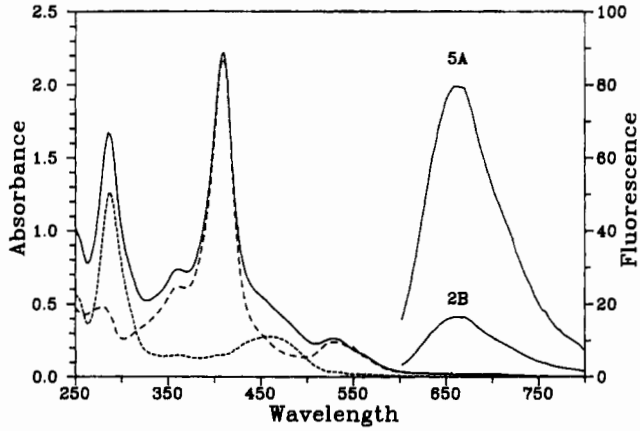

FIGURE 4: Absorbance and luminescence spectra of $Ru(bpy)_2$-(dcbpy-cytochrome *c*). Absorbance spectra are shown for 22 μM fraction 5A (—) and 22 μM native cytochrome *c* (--), and the difference spectrum of fraction 5A minus native is also shown (---). Uncorrected luminescence spectra are shown on the right of the figure for 1 μM fraction 5A and 1 μM fraction 2B with excitation at 450 nm.

to that of a non-heme protein control, $Ru^{II}(bpy)_2$(dcbpy-lysozyme) (Table I). The most extreme case was for the lysine 13 derivative where the luminescence decay rate was 21×10^6 s^{-1} relative to 1.8×10^6 s^{-1} for $Ru^{II}(bpy)_2$(dcbpy-lysozyme). In both cases the luminescence decay was accurately fit by a single first-order exponential. The ruthenium label at lysine 13 is about 3–10 Å from the closest edge of the heme group, estimated from a model of the X-ray crystal structure of cytochrome *c* by taking into account the uncertainty in the orientation of the lysine side chain (Figure 5). In contrast, the ruthenium derivative of lysine 39, which is on the back of cytochrome *c* 15–18 Å from the heme group, had a luminescence decay rate of 4.3×10^6 s^{-1}. The luminescence decay rates of the ferrocytochrome *c* derivatives were nearly as large as those of the corresponding ferricytochrome *c* derivatives (Table I).

Discussion

The two-step procedure for labeling cytochrome *c* lysines with ruthenium compplexes has several advantages over other possible methods. The mono-*N*-hydroxysuccinimide ester of

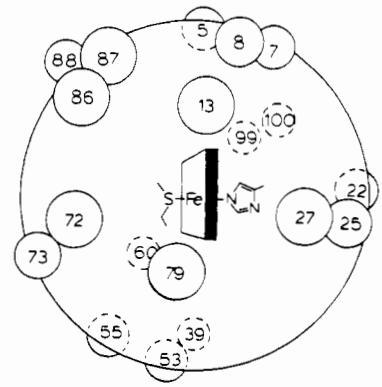

FIGURE 5: Schematic diagram of horse heart cytochrome *c* viewed from the front of the heme crevice. The approximate positions of the β-carbon atoms at the lysine residues are indicated by closed and dashed circles for residues located toward the front and back of cytochrome *c*, respectively.

dcbpy reacts very selectively with lysine amino groups, with only a slight excess of reagent. The resulting dcbpy-lysine group has a negative charge, which allows singly labeled derivatives to be purified by ion-exchange chromatography techniques that have been developed in this laboratory over a number of years (Smith et al., 1978). Treatment of the dcbpy-cytochrome *c* derivatives with $Ru(bpy)_2CO_3$ at pH 4 was found to efficiently lead to the formation of $Ru(bpy)_2$-(dcbpy-cytochrome *c*) derivatives without modification of any other residues. The $Ru(bpy)_2$(dcbpy-cytochrome *c*) derivatives have the same net charge as native cytochrome *c*, which might be advantageous for studies of their electron-transfer reactions with other proteins.

There is an inverse relationship between the luminescence decay rates of the derivatives and the distances between the $Ru(bpy)_2$(dcbpy) groups and the heme group of cytochrome *c* (Table I). Two mechanisms for the quenching of ruthenium luminescence by the heme group have been suggested, energy transfer and electron transfer (Sutin & Creutz, 1978; Nocera et al., 1984). There is almost no overlap between the luminescence emission of $Ru^{II}*(bpy)_2$(dcbpy) and the absorption of ferri- or ferrocytochrome *c*, but some type of energy-transfer mechanism involving spectrally forbidden triplet states might be possible. Winkler et al. (1982) demonstrated that $Ru^{II}*$-$(bpy)_3$ was able to transiently reduce ferricytochrome *c* but did not rule out the possibility that energy transfer made some contribution to the luminescence quenching. The following mechanisms have been suggested for oxidative and reductive electron-transfer quenching (Sutin & Creutz, 1978):

$$Ru^{II}\text{–}Fe^{III} \xrightarrow{h\nu} Ru^{II}*\text{–}Fe^{III} \xrightarrow[\Delta E^0 = 0.88\ V]{k_1} Ru^{III}\text{–}Fe^{II} \xrightarrow[\Delta E^0 = 1.12\ V]{k_2} Ru^{II}\text{–}Fe^{III}$$

$$Ru^{II}\text{–}Fe^{II} \xrightarrow{h\nu} Ru^{II}*\text{–}Fe^{II} \xrightarrow[\Delta E^0 = 0.81\ V]{k_1} Ru^{I}\text{–}Fe^{III} \xrightarrow[\Delta E^0 = 1.19\ V]{k_2} Ru^{II}\text{–}Fe^{II}$$

where the redox potential differences have been estimated as described by Sutin and Creutz (1978) using the spectral and electrochemical data of Cherry and Henderson (1984) and Elliott and Hershenhart (1982). An upper limit to the electron-transfer rate constant k_1 can be estimated by subtracting the luminescence decay rate in the absence of quenching from the observed decay rate of the derivatives. The luminescence decay of the non-heme protein $Ru^{II}(bpy)_2$(dcbpy-lysozyme) was accurately fit by a single exponential with a rate constant of 1.8×10^6 s^{-1}, which was identical with that of $Ru^{II}*$-

$(bpy)_2(dcbpy)$ itself. Since this derivative is undoubtedly a mixture of many derivatives labeled at different lysine groups on lysozyme, it appears that the enviornment on the surface of the protein does not significantly affect the luminescence decay rate. Therefore, an upper limit to the true rate of electron transfer can be obtained by subtractng 1.8×10^6 s^{-1} from the luminescence decay rates shown in Table I. These values are of comparable magnitude to the rates of other electron-transfer reactions with similar distances and driving forces (Axup et al., 1988; Elias et al., 1988). The similarity in the luminescence decay rates of the corresponding ferro- and ferricytochrome *c* derivatives is consistent with the similarity in the ΔE^0 values for the oxidative and reductive electron-transfer reactions. Additional experiments are planned to provide direct evidence on whether the quenching mechanism for the ferro- and ferricytochrome *c* derivatives does indeed involve electron transfer. The new class of derivatives introduced here should allow great flexibility in the design of experiments to determine the effect of driving force, distance, and protein medium on electron-transfer reactions.

ACKNOWLEDGMENTS

We thank Dr. Joan Hall for assistance with the luminescence lifetime measurements.

REFERENCES

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839-1842.
Axup, A. W., Albin, M., Mayo, S. L., Crutchley, R. J., & Gray, H. B. (1988) *J. Am. Chem. Soc. 110*, 435-439.
Cherry, W. R., & Henderson, L. J., Jr. (1984) *Inorg. Chem. 23*, 983-986.
Closs, G. L., Calcaterra, L. T., Green, N. J., Penfield, K. W., & Miller, J. R. (1986) *J. Phys. Chem. 90*, 3673-3683.
Crutchley, R. J., Ellis, W. R., Jr., & Gray, H. B. (1986) *Frontiers in Bioinorganic Chemistry* (Xavier, A. V., Ed.) Verlag Chemie, Weinheim, FRG.
Bechtold, R., Kuehn, C. L., Lepre, C., & Isied, S. S. (1986) *Nature (London) 322*, 286-289.
Elias, H., Chou, M. H., & Winkler, J. R. (1988) *J. Am. Chem. Soc. 110*, 429-434.
Elliott, C. M., & Hershenhart, E. J. (1982) *J. Am. Chem. Soc. 104*, 7519-7526.
Isied, S. S., Worosila, G., & Atherton, S. J. (1982) *J. Am. Chem. Soc. 104*, 7659-7661.
Johnson, E. C., Sullivan, B. P., Salmon, D. J., Adeyemi, S. A., & Meyer, T. J. (1978) *Inorg. Chem. 17*, 2211-2215.
Kostić, N. M., Margalit, R., Che, C.-M., & Gray, H. B. (1983) *J. Am. Chem. Soc. 105*, 7765-7767.
McGourty, J. L., Peterson-Kennedy, S. E., Ruo, W. Y., & Hoffman, B. M. (1987) *Biochemistry 26*, 8302-8312.
Nocera, D. G., Winkler, J. R., Yocom, K. M., Bordignon, E., & Gray, H. B. (1984) *J. Am. Chem. Soc. 106*, 5145-5150.
Smith, H. T., Staudenmayer, N., & Millett, F. (1977) *Biochemistry 16*, 4971-4975.
Sprintschnik, G., Sprintschnik, H. W., Kirsh, P. P., & Whitten, D. G. (1977) *J. Am. Chem. Soc. 99*, 4947.
Sutin, N., & Creutz, C. (1978) *Adv. Chem. Ser. No. 168*, 1-27.
Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 759-770.
Winkler, J. R., Nocera, D. G., Yocom, K. M., Bordignon, E., & Gray, H. B. (1982) *J. Am. Chem. Soc. 104*, 5798-5800.

ABC Excinuclease Incises both 5′ and 3′ to the CC-1065–DNA Adduct and Its Incision Activity Is Stimulated by DNA Helicase II and DNA Polymerase I†

Christopher P. Selby* and Aziz Sancar

Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7260

Received June 7, 1988; Revised Manuscript Received July 18, 1988

ABSTRACT: CC-1065 is a large molecule that binds covalently to adenine residues of DNA in a sequence-specific manner and lies in the minor groove about four bases to the 5′ side of the adducted residue. Using a reconstituted *Escherichia coli* nucleotide excision repair system, we have obtained data showing that the ABC excinuclease makes incisions both 5′ and 3′ to the CC-1065 adduct and that the incision activity is stimulated by the addition of helicase II and DNA polymerase I (and dNTPs). Our results with the CC-1065 adduct are consistent with the reported in vitro processing of other adducts (e.g., cisplatin, UV photoproducts) but do not agree with a recent study that reported anomalous processing of the CC-1065 adduct by ABC excinuclease and helicase II. Our results also imply that, in binding to damaged DNA, ABC excinuclease does not make important contacts in the minor groove four bases to the 5′ side of the damaged residue.

ABC excinuclease was first described as an enzyme that incises the phosphodiester backbone of DNA on the 3′ and 5′ sides of pyrimidine dimers in vitro (Sancar & Rupp, 1983). The same dual incision pattern was observed later with (acetylamino)fluorene, psoralen (Sancar et al., 1985), and cisplatin (Beck et al., 1985) adducts. However, it was also recognized that occasionally the enzyme incises only on one side of the adduct, i.e., in an "uncoupled" manner. Uncoupled incisions, observed with pyrimidine dimers (Yeung et al., 1983) and psoralen adducts (Van Houten et al., 1986), were usually seen at low frequencies. In contrast, a recent report claimed that the enzyme incised only on the 5′ side of CC-1065 adducts (Tang et al., 1988). Also, as opposed to UV photoproducts and cisplatin adducts (Caron et al., 1985; Husain et al., 1985), with the CC-1065 adduct, helicase II did not stimulate ABC

† This work was supported by grants from the National Institutes of Health (GM32833) and the National Science Foundation (PCM8351212) and partly by a grant from The Council for Tobacco Research (1872R2).

0006-2960/88/0427-7184$01.50/0