Ionization Potentials of Ferricytochrome *c*, Ferrocytochrome *c*, and Ferricytochrome c_3

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Abstract: Ionization potentials of ferricytochrome *c*, ferrocytochrome *c*, and ferricytochrome c_3 have been determined from vacuum ultraviolet photoelectron spectroscopy. All samples were deposited from aqueous solution. The ionization potentials of ferricytochrome *c*, ferrocytochrome *c*, and ferricytochrome c_3 thus obtained were 6.1, 5.8, and 5.4 eV, respectively. A rather high ionization potential of cytochrome *c* has been discussed in connection with its molecular structure. The intramolecular heme-heme distance, *d*, in cytochrome c_3 has been calculated. The ionization potential of ferricytochrome *c* has been compared with that of ferricytochrome c_3 and of zinc tetraphenylporphyrin. The calculated distance, *d*, is 8.5 Å, which is almost the same as the Zn-Zn distance between the nearest neighbor in the zinc tetraphenylporphyrin crystal.

Cytochromes have important roles in biological redox processes. Cytochrome *c* having one heme in a molecule is an electron carrier in a respiratory chain of many diverse organisms in plants, animals, and bacteria. Cytochrome c_3, a member of multiheme protein, is also an electron carrier in an electron transfer chain in *D. vulgaris*. Though the two cytochromes have nearly the same molecular weight, 12 000 in cytochrome *c* and 14 000 in cytochrome c_3, their redox potentials against normal hydrogen electrode (NHE) at pH 7.0 are considerably different, +0.255 V for cytochrome *c*[2] and −0.270 V for cytochrome c_3.[3] This difference in redox potentials is due to the environment of a heme. The environment, in other words the conformation of the surrounding polypeptide side chain, may reflect the electronic states of porphyrin.

Electronic energy states of porphyrins in hemoproteins have been widely investigated by spectroscopic methods such as optical absorption, emission, magnetic resonance, and Mössbauer effect; however, they cannot give the absolute energy levels of π electrons in porphyrin.

Electronic states of several derivatives of gaseous porphyrins and phthalocyanines as a model compound of hemes have been investigated by vacuum ultraviolet photoemission spectroscopy[4] which enables direct experimental determinations of ionization potentials. It was found that ionization potentials of tetraphenylporphyrin and its metal(II) derivatives such as Mg, Mn, Fe, Ni, Cu, and Zn did not change from compound to compound. In spite of biochemical importance of metal porphyrins, however, the dependence of ionization potential on the metal oxidation state is not known well. Also it has not been established whether the electronic energy levels of hemes in actual cytochromes are the same as in these model compounds.

In this paper, we report the photoelectron spectra of cytochromes and will compare them with those of other model compounds. The influence of central metal oxidation states on the π energy level of the ligand was estimated by comparing the ionization potential of ferricytochrome *c* with that of ferrocytochrome *c*.

Experimental Section

Horse heart ferricytochrome *c* was obtained from Boehringer Mannheim as lyophilized powders. Salt free ferricytochrome *c* solution was eluted from a Sephadex G-50-fine column with distilled water. Salt free ferrocytochrome *c* solution was also eluted similarly after reduction by $Na_2S_2O_4$ in anaerobic conditions. Highly purified cytochrome c_3 was prepared from *D. vulgaris*, Miyazaki as described previously.[5] Each sample was deposited on a small copper disk emitter (12 mm in diameter) from its aqueous solution (about 10^{-4} M). The thickness of the specimen film was estimated at about 100 nm.

The photoemission measurements were carried out using a half-meter Seya-Namioka type vacuum ultraviolet monochromator. The details of the apparatus attached to the monochromator were described by Kochi et al.[6] The inside of a spherical glass photoelectron collector was coated with colloidal graphite.

Two principal pieces of information obtained from the photoemission measurements are the photoelectron energy distribution curves, EDC's, for a certain monochromatic light and the spectral dependence of the quantum yield (number of emitted electrons/number of incident photons), SDQY. EDC's were obtained by recording the derivative of the photoelectron current as a function of the retarding potential which was applied between the collector and a photoelectron emitter (sample). The details of this process have already been reported.[7] SDQY was obtained by plotting the quantum yield as a function of incident photon energy.

Results

There are several methods to obtain the ionization potentials. One method is from the EDC's. With the values of stopping voltage, V_0, and saturation voltage, V_s, the ionization potential is given as

$$I_p = h\nu - e(V_s - V_0) \quad (1)$$

where $h\nu$ is the incident photon energy and *e* is the electron charge.[7,8] When the EDC's have tails, V_0 and V_s were determined by approximating the edges of the EDC's by straight lines as shown in Figure 1.

Another method is from the quantum yield measurement. According to Lyons and Morris,[9] the threshold energy of ionization, E_{th}, is given by the photon energy at which the quantum yield is 10^{-9} electron/photon.

The other method is from the relation between the quantum yield *Y* and the incident photon energy $h\nu$. Near the threshold energy, the quantum yield is given as[6,10]

$$Y^{1/3} \propto (h\nu - E_{th}) \quad (2)$$

The first method has some ambiguities when it is difficult to determine V_0 and V_s precisely. The determination of V_0 in the high-photon-energy region is also difficult, because high-energy electrons lose energy by scattering due to nonuniformity in sample and/or intrinsic secondary effects. Therefore V_0 and V_s values at 7.75 eV photon energy are more reliable than others. The ionization potential thus determined is noted by EDC_{th}. The ionization potential obtained from the V_0 and V_s averaged over the whole measured range of photon energies is expressed by EDC_{av}. The second method is not applicable to the present study because the lowest detectable quantum yield was 10^{-5} electron/photon, far from 10^{-9}.

The EDC's for various samples are shown in Figures 2-4. Especially in Figure 2 the energy structure has been found in the large retarding potential, which is thought to be the appearance of the conduction band structure.[11] In Figure 5 the data appearing in Figures 2-4 are plotted to estimate I_p values

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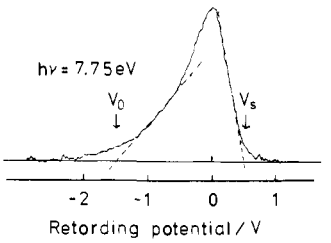

Figure 1. Determination of stopping voltage V_0 and saturation voltage V_s from EDC. Primary electrons are scattered near the edge of the EDC. Scattered secondary electrons are shown as an envelope of EDC.

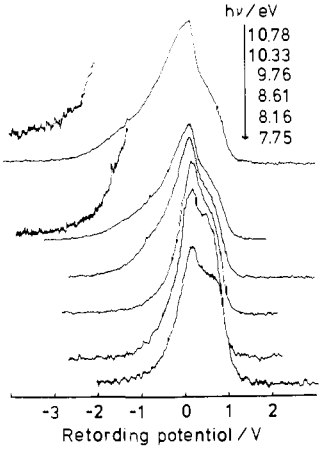

Figure 2. EDC's of ferricytochrome *c*. The arrow indicates the photon energy of the incident light for the respective EDC. An energy structure other than scattered electrons appeared in the low-energy region resulting from the conduction band.

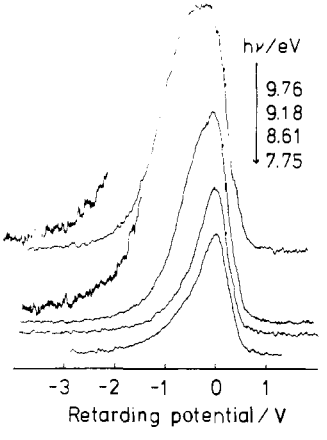

Figure 3. EDC's of ferrocytochrome *c*.

by means of eq 1. SDQY's and cube root plots of the quantum yield as a function of incident photon energy are plotted in Figures 6 and 7.

The values of I_p calculated by various methods as described above are listed in Table I. The average value obtained from the EDC's method, EDC_{av}, is not so accurate because of the ambiguity of stopping voltage, while EDC at the threshold photon energy is the most reliable one. Taking into account the advantage and disadvantage of each method as mentioned above, the most probable values of I_p are shown in Table I.

Discussion

Oxidation States of Cytochrome *c*. The ionization potential

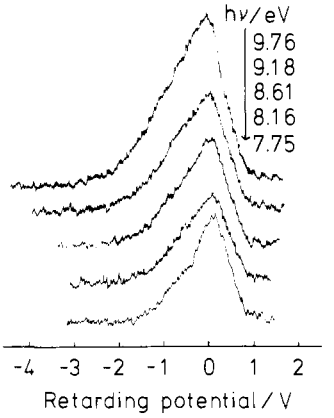

Figure 4. EDC's of ferricytochrome c_3.

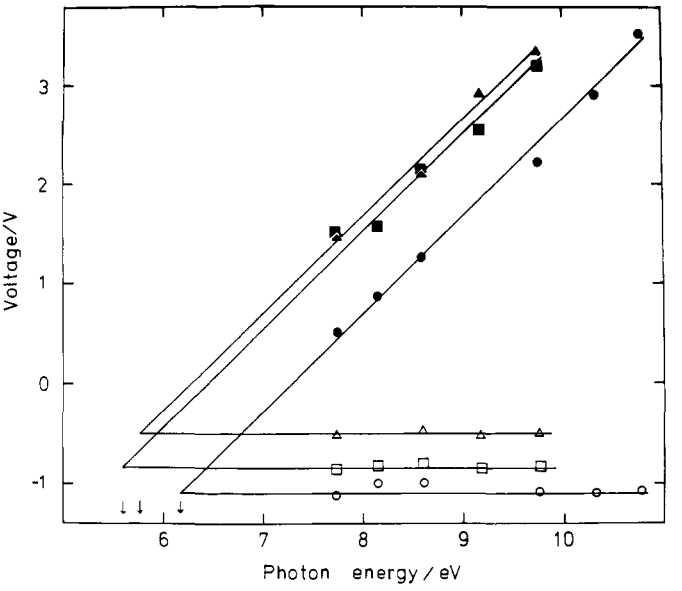

Figure 5. Graphical determination of ionization potential using eq 1 with V_0 and V_s. Open marks are V_s and black marks are V_0: ○, ferricytochrome *c*; △, ferrocytochrome *c*; □, ferricytochrome c_3.

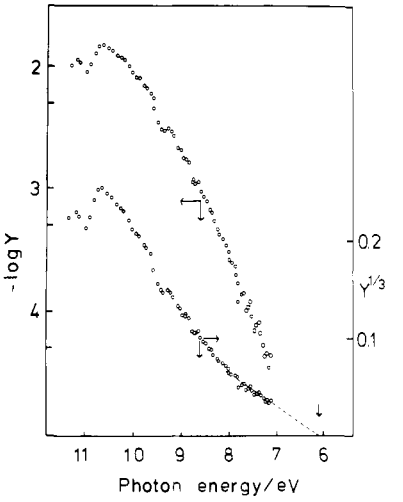

Figure 6. The quantum yield of photoemission and cube root plot of the quantum yield as a function of photon energy. Sample: ferricytochrome *c*.

of ferricytochrome *c*, 6.1 eV (1 eV ≈ 1.6×10^{-19} J), is larger than that of the ferro form, 5.8 eV. It is apparent that the oxidation states of the central metal ion affect the energy level

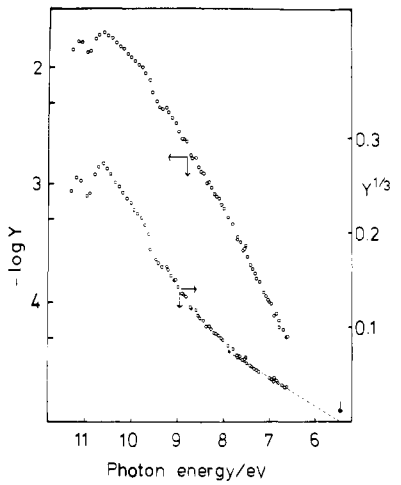

Figure 7. The quantum yield of photoemission and cube root plot of the quantum yield as a function of photon energy. Sample: ferricytochrome c_3.

Table I. Ionization Potentials of Cytochromes Obtained from Several Methods

	cyt c (Fe^{3+})	cyt c (Fe^{2+})	cyt c_3 (Fe^{3+})
$Y^{1/3}$ [a]	6.0_6		5.4_2
EDC_{av} [b]	6.1_5	5.7_5	5.5_8
EDC_{th} [c]	6.1_2	5.7_6	5.3_7
I_p [d]	6.1	5.8	5.4

[a] $Y^{1/3}$, cube root plot of quantum yield. [b] EDC_{av}, average value obtained from EDC's method. [c] EDC_{th}, value obtained from EDC's at threshold energy. [d] I_p, most probable value of ionization potential.

of a ligand. It was suggested[12] from X-ray photoelectron emission of the Fe $2p_{3/2}$ orbital that there is a charge difference of 0.44 e on the central iron between the ferro and ferri forms of porphyrin. According to the ab initio MO calculations by Dedieu et al.,[13] the net electron population of iron(II) porphyrin is +1.22 e. The net charge density of ligands is hence −1.22 e. The negative value of net charge on ligands enhances the energy levels of π orbitals, that is, the ionization potential of ligand π orbital is lowered. Though the charge distribution of iron(III) porphyrin has not yet been calculated, the net charge of the central iron is expected to be about +1.66 e from the above figures cited. If the charge of axial ligand is −1.00 e, the charge density of porphyrin ligands in this case is then −0.66 e which may lower the energy level of the highest occupied MO. It is reasonable to consider that the different amounts of net charge of ligands result in the difference of energy levels of the ligand π orbitals by as much as 0.3 eV.

The model compounds of hemoprotein such as various derivatives of porphyrins and phthalocyanines have been well studied. The ionization potential of gaseous porphyrin was reported by Khandelwal and Roebber.[4] The minor variation of photoelectron spectra upon changing the central metal ion supports that the emitted electron comes from porphyrin π orbitals, not from localized d orbitals. The first peak appeared between 6.4 and 6.5 eV from compound to compound. Therefore, we may accept a value of I_p of zinc(II) mesotetraphenylporphyrin (ZnTPP) as the average I_p value of the porphyrin molecule. The ionization potential of the ZnTPP film was found to be 5.0 eV by means of EDC's.[14] The energy diagrams of isolated porphyrin, aggregated porphyrin, and also porphyrin surrounded by protein (cytochrome) are given in Figure 8.

It should be noticed that there is only a difference of 0.7 eV in I_p between the $Zn^{II}TPP$ molecule and ferrocytochrome c

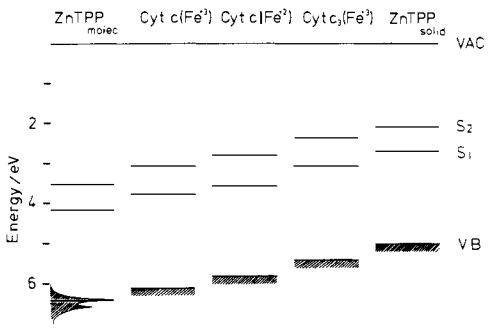

Figure 8. Energy diagram of ZnTPP, cytochrome c, and cytochrome c_3. The position of the valence band (VB) of all cytochromes has been obtained from I_p of the deposited film of the respective sample. The VB position of ZnTPP molecule is taken from ref 2 and that of the aggregated sample was obtained from I_p of the evaporated film: S_1, Q band of π-π transition of porphyrin: S_2, B band of π-π transition of porphyrin.

(Fe^{2+}). This finding supports that the heme in cytochrome c has the character of an isolated state in part.

The ionization potential of the solid is related to that of the molecule as follows

$$I_p(\text{solid}) = I_p(\text{gas}) - P_+ \quad (3)$$

where P_+ stands for polarization energy by the surrounding molecules in the solid. In the case of ZnTPP, P_+ is estimated at 1.5 eV. On the other hand, P_+ of ferrocytochrome c is estimated at 0.7 eV corresponding to the difference in the $Zn^{II}TPP$ molecule and ferrocytochrome c, because I_p of gaseous ferrocytochrome c is thought to be lower than that of the $Zn^{II}TPP$ molecule. The small P_+ may be explained from the small polarization energy of a polypeptide chain and from the cavity around a heme, since each heme is surrounded by a polypeptide chain. X-ray structural analyses of cytochrome c by Dickerson et al.[15] show that a heme in cytochrome c is not kept in contact with the polypeptide chain, but it is suspended in the central cavity with two cysteines, methionine, and histidine residues. That is, the heme in cytochrome c is isolated in a space. This unique conformation is the reason for a small difference in the ionization potential between the $Zn^{II}TPP$ molecule and ferrocytochrome c.

Oxidation Potential in Solution. The oxidation potential of $Fe^{III}TPP$ in solution which corresponds to the ionization potential in solution was determined electrochemically.[16] The three steps of one-electron oxidation have been reported in which potential is given in V vs. saturated calomel electrode, SCE.

$$Fe^{II}TPP \xrightarrow[-0.32V]{} Fe^{III}TPP \xrightarrow[1.18V]{} Fe^{III}TPP \xrightarrow[1.50V]{} Fe^{III}TPP^{2+}$$

The first step is due to metal oxidation and second and third steps are due to ligand oxidation. If we neglect the reorganization energy by solvent, the oxidation potential can be converted to the ionization potential under vacuum (absolute energy scale) using the equation[17]

$$U_{ox} = U_H - eE^\circ_{ox} - eE^\circ_{SCE} \quad (4)$$

where U_{ox} is energy level of porphyrin; U_H is the energy level of NHE,[18] −4.5 eV; E°_{ox} is the oxidation potential of porphyrin in solution; and E°_{SCE} is the standard potential of SCE vs. NHE, +0.241 V. The ionization process of ferricytochrome c corresponds to the oxidation of $Fe^{III}TPP$ to $Fe^{III}TPP^+$. The oxidation potential (1.18 V) is then converted to −5.92 eV with the use of eq 4. This calculated value is in good agreement with the ionization potential, 6.1 eV, of ferricytochrome c.

The ionization of the porphyrin ligand in solution is relaxed by polarization and/or reorganization of surrounding solvent molecules. Thus we have to subtract the relaxation energy

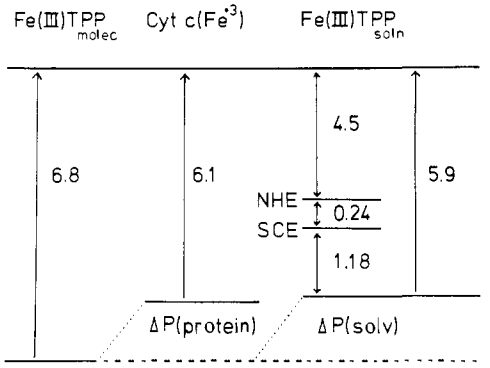

Figure 9. Ionization potential of iron(III) porphyrin in solution and under vacuum. All the figures are given in electron volts.

ΔP(solv) from the calculated value, −5.92 eV, in order to obtain the true ionization potential of the $Fe^{III}TPP$ molecule under vacuum. On the other hand, the porphyrin ligand in cytochrome *c* is already surrounded by protein. We cannot obtain the real ionization potential of the porphyrin ligand in cytochrome *c* experimentally. However, the polarization energy by the protein, ΔP(protein), can be estimated likewise as in the case of ferrocytochrome *c*. Assuming the same polarization energy in both cytochromes, ΔP(protein) = 0.7 eV, the ionization potential of ligand in cytochrome *c* becomes 6.8 eV. The energy diagrams of absolute scale and of electrochemical scale are shown in Figure 9.

The straightforward consideration on oxidation potential of the central metal in solution leads to the estimate of the ionization potential of iron or porphyrin under vacuum. We have obtained −4.42 eV for the process, $Fe^{II}TPP \rightarrow Fe^{III}TPP$. The value roughly coincides with the redox potential of cytochrome *c*, +0.25 V vs. NHE (=−4.75 eV under vacuum).

Multihemoprotein. Cytochrome c_3 was reported to have four hemes in a molecule.[5] The heme-heme interaction among these four hemes was suggested by EPR[19] and Mössbauer[20] studies. It is naturally considered that this interaction may be reflected in the ionization potential. It is known that the ionized molecule in the solid state is energetically stabilized by the polarization effect of surrounding molecules and the ionization potential of the solid may be lowered (the difference is termed polarization energy). Therefore, four hemes in cytochrome c_3 may stabilize its ionized state and may decrease its ionization potential. This is the reason for the difference in ionization potential, 0.7 eV, between cytochrome *c* and cytochrome c_3. The number of surrounding hemes and their distance, hence, determine the magnitude of the polarization energy of cytochrome c_3. The polarization energy for several organic molecular crystals was given by Lyons as follows[21]

$$P_+ = -\sum_{k=1}^{N-1} e^2\alpha/2r_k^4 \text{ erg} \qquad (5)$$

where α is molecular polarizability; r_k is the distance between the charged molecule and the uncharged molecule; and N is the total number of molecules in the crystal. Though the molecular polarizability of porphyrin is not known, we can estimate it from P_+ of ZnTPP. In summing up the right terms of eq 5, we used the crystal data of orthorhombic ZnTPP.[22] Using the 12 nearest-neighbor molecules, 18 next-neighbor molecules, and P_+ of ZnTPP (1.5 eV), α is obtained as follows,[23] α = 170.8 Å^3.

The heme-heme distance in cytochrome c_3 can be estimated using eq 5 and the polarization energy of cytochrome c_3, P_+ = 0.7 eV, if we assume that all the distances are equal. The distance, 8.5 Å (1 Å = 0.1 nm), thus obtained corresponds with the nearest-neighbor Zn-Zn distance in ZnTPP, 8.3 Å. This result is very striking in the point that four hemes are located so close to each other in cytochrome c_3. The vicinity of hemes in cytochrome c_3 was also suggested by Zeeman splitting of Mössbauer spectrum[20] and NMR paramagnetic shift.[24]

Cytochrome c_3 exhibits some unusual properties different from other *c*-type cytochromes. (1) The apparent number of electrons transferred to an electrode (*n* in Nernst equation) is less than unity,[3] (2) the band width of optical spectrum in the Soret region is significantly narrower than for other *c* cytochromes, and (3) redox potential (E_0') is the lowest of all *c* cytochromes.[3,5] These unusual properties can be attributed to this unique structure which made intramolecular heme-heme interaction possible.

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