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Photoinduced Electron Transfer in Singly Labeled Thiouredopyrenetrisulfonate Cytochrome *c* Derivatives[†]

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ABSTRACT: A novel method for initiation of intramolecular electron transfer reactions in cytochrome *c* is reported. The method is based on photoexcitation of covalently attached thiouredopyrenetrisulfonate (TUPS) by the third harmonic frequency of a Nd:YAG laser (355 nm), the reaction that generates the low-potential triplet state of the dye with high quantum efficiency. TUPS derivatives of horse heart cytochrome *c* singly labeled at specific lysine residues were prepared and purified to homogeneity by ion-exchange high-pressure liquid chromatography. Eight derivatives were characterized by determination of the location of the modification, reduction potentials, and measurement of enzymatic activity with cytochrome oxidase. Transient absorption spectroscopy was used to directly measure the rate constants for the electron transfer reaction from the photoexcited triplet state of TUPS to the oxidized heme group and the back reaction from the ferrous heme to the oxidized dye. For all singly labeled derivatives, the rate constants for heme reduction were 1 or 2 orders of magnitude larger than for its reoxidation, consistent with the greater thermodynamic driving force for the oxidation reaction. Analysis of the variation of electron-transfer rates with the distance separating the dye and the heme reveals a value of coupling decay constant (β) of 0.46 \AA^{-1} . Rapid and effective photoreduction of cytochrome *c* makes it a useful tool for fast initiation of electron transfer in the reductive direction within complexes of cytochrome *c* with other redox proteins.

The mechanism of intramolecular electron transfer in redox proteins has been a subject of extensive study in recent years. A variety of experimental approaches have been employed to study electron transfer between redox sites in proteins (1–6). The progress in the field has been significantly enhanced by the use of ruthenium complexes covalently linked to metalloproteins (4–7). Utilization of Ru photochemistry provided experimentalists with a useful tool for measuring time-resolved kinetics of electron transfer in nonphotosynthetic proteins. The focus of these investigations has been on the electron transfer reactions within small soluble proteins, such as myoglobin (8, 9), azurin (10), plastocyanin (11), and cytochrome *c* (5, 6) as well as on electron transfer reactions between metalloproteins in polypeptide complexes (12–17). Cytochrome *c*, being a small stable redox-active protein with well-defined structure, served as a perfect model for these studies. The ruthenium complexes were attached via their individual histidine (5), lysine (6, 18), and cysteine (19) residues, and photoinduced electron transfer reactions from ruthenium to the oxidized heme and the back reactions from the ferrous heme group to ruthenium were measured. Up until now chemical modification with ruthenium complexes was the only approach allowing investigators to examine the dependence of the electron transfer rate in nonphotosynthetic proteins on the distance separating two, natively occurring and chemically introduced, redox centers.

We have recently introduced a new method to study electron transfer reactions in aqueous solutions (20). The method is based on the photochemistry of thiouredopyrenetrisulfonate (TUPS).¹ By means of a short duration laser pulse, it is possible to generate the triplet state of TUPS with

high yield. The triplet is a strong reductant, capable of reducing directly a variety of non-protein electron acceptors as well as ferricytochrome *c* in a bimolecular manner.

In the present paper, a novel procedure to prepare a number of singly labeled TUPS–cytochrome *c* derivatives is described. Conjugation of the dye with the protein was achieved by chemical modification of its lysines with isothiocyanatopyrenetrisulfonate, the reaction that yields the thiuredo adduct. Singly labeled TUPS–cytochrome *c* derivatives were separated and purified by ion-exchange HPLC. Transient absorption spectroscopy was employed to directly measure the rate constants of photoinduced electron transfer from the excited triplet state of TUPS to the oxidized heme group and for the back reaction from ferrous heme to the oxidized dye. Analysis of the variation of electron transfer rates with the distance separating the dye and the heme reveals a value of the coupling decay constant (β) of 0.46 \AA^{-1} .

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c* was obtained from Sigma (type VI). IPTS was purchased from Lambda Fluorescence (Graz, Austria).

Preparation of TUPS–Cytochrome *c* Derivatives. TUPS–cytochrome *c* adducts were prepared by incubation of cytochrome *c* with IPTS. Prior to the labeling, cytochrome *c* was purified from the deaminated forms of the protein as previously described (21). Chromatographically purified ferricytochrome *c* (1 mM) was mixed with freshly prepared

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¹ Abbreviations: IPTS, 1-isothiocyanatopyrene-3,6,8-trisulfonate; TUPS, thiouredopyrene-3,6,8-trisulfonate; TUPS*, excited triplet state of thiouredopyrene-3,6,8-trisulfonate.

IPTS (1 mM) and incubated for 42 h at 38 °C in 5 mM Hepes (pH 7.5) and 0.5 M KCl. The labeled protein was separated from nonbound dye and salt on a 200 × 10 mm G-25 Sephadex "coarse" column, equilibrated with 5 mM Hepes (pH 8.0). The protein eluted in the void volume, containing a mixture of labeled derivatives, was subjected to further HPLC purification.

Purification of Individual TUPS–Cytochrome *c* Derivatives. The TUPS–monocytochrome *c* derivatives were resolved by ion-exchange HPLC. For initial separation, 50 mg of protein was incubated in 5 mL of 5 mM Hepes (pH 8.0) with 1 mM ferricyanide for 1 min and then loaded onto a 250 × 4.6 mm Phenomenex W-Porex 5CM HPLC cation exchange column equilibrated with 5 mM Hepes (pH 8.0). Cytochrome *c* and its derivatives were eluted at room temperature in 5 mM Hepes (pH 8.0) at a flow rate of 0.6 mL/min by a linear KCl gradient from 0 to 0.5 M over a period of 1 h. The gradient was generated by a dual-pump system (Model 626), and the elution of cytochrome *c* fractions was monitored at 590 nm ($\epsilon_{590} \sim 2500 \text{ M}^{-1} \text{ cm}^{-1}$) on a UV/visible detector (Model PDA 996) both from Waters (Waters Chromatography Division of Millipore, Milford, MA). Fractions corresponding to individual peaks were collected, and the label content in the fractions was determined by absorption spectroscopy. The spectrum of the native cytochrome was subtracted from that of the fraction to obtain TUPS absorbance. The TUPS content was quantitated on the basis of the absorbance coefficient of $\epsilon_{372} = 30\,000 \text{ M}^{-1} \text{ cm}^{-1}$ (20) and cytochrome *c* content on the basis of absorbance coefficient of $\epsilon_{550} = 28\,000 \text{ M}^{-1} \text{ cm}^{-1}$. The main peaks, eluted from the column in 150–450 mM KCl, contained 1/1 labeled cytochrome *c* and subjected to another purification step. The secondary purifications of fractions were carried out on the same column, using a linear KCl gradient from 0 to 0.5 M. Examples of the secondary purification are given in Figure 1B,C. The purified derivatives were collected and passed through 200 × 10 mm G-25 Sephadex "coarse" column in 5 mM Hepes (pH 7.0) to separate them from KCl.

Identification of the Lysine Modified in the TUPS–Cytochrome Derivatives. The derivatives (100 $\mu\text{g/mL}$) were incubated with trypsin in 50 mM ammonium bicarbonate (pH 8.3) for 30 min at 37 °C with end-over-end mixing. As digestion of derivatives proceeds with different efficiencies, three concentrations of trypsin were used: 25 $\mu\text{g/mL}$ for hydrolysis of Lys-86, Lys-87, Lys-72, Lys-27, and Lys-7; 5 $\mu\text{g/mL}$ for hydrolysis of Lys-13 and Lys-8; and 1 $\mu\text{g/mL}$ for hydrolysis of Lys-39 derivatives. The tryptic digests were separated at room temperature on a C18 reversed-phase HPLC column, 250 × 4.6 mm (Vydac, Hesperia, CA). The peaks were eluted at a flow rate of 0.7 mL/min by a linear gradient of acetonitrile from 4 to 50% (0.1% TFA). The elution was followed, monitoring the absorbance of the peptide bond, the dye, and the heme at 214, 372, and 405 nm, respectively, on a UV/visible detector (Model PDA 996) from Waters. The TUPS-containing fraction differs from the heme-containing one by a relatively low absorbance ratio of 405 to 372 nm. The peak containing TUPS label was collected and analyzed by mass spectroscopy in order to confirm the peptide purity. The fractions containing more than one peptide were rechromatographed in the same conditions as described above. The purified labeled peptides, originating from all TUPS–cytochrome *c* derivatives were sequenced on a PROCISE-HT protein sequencer at the

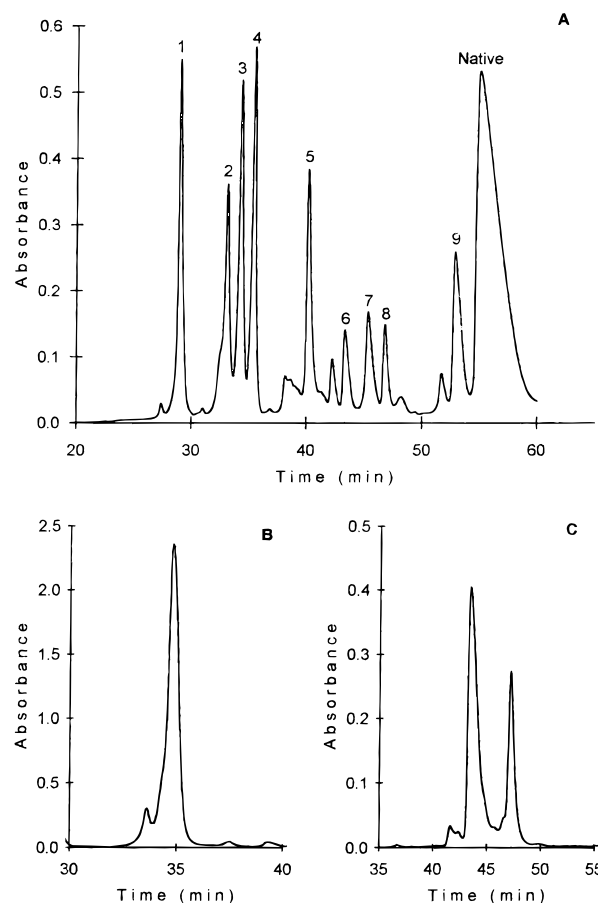


FIGURE 1: HPLC separation of TUPS–cytochrome *c* derivatives. (A) The crude reaction mixture of TUPS–cytochrome *c* derivatives (50 mg) was chromatographed on a 250 × 4.6 mm HPLC W-Porex 5CM column in 5 mM Hepes (pH 8.0) with a linear gradient from 0 to 500 mM KCl at a flow rate of 0.6 mL/min. Cytochrome *c* elution was followed by the absorbance at 590 nm ($\epsilon_{590} \sim 2500 \text{ M}^{-1} \text{ cm}^{-1}$). The main fractions eluted are indicated by the numbers in the figure. Fractions 3 (B) and 6 (C) from (A) were rechromatographed on the same column under the same gradient as in (A).

Protein Center of the Israel Institute of Technology. The TUPS-modified lysine, obtained by Edman degradation, elutes earlier than any of the unlabeled amino acids when separated by HPLC. This property enabled the identification of the position of the label in the protein sequence.

Reduction Potentials of TUPS–Cytochrome *c* Derivatives. The standard redox potentials of all derivatives were measured in 10 mM potassium phosphate (pH 7.0) by spectrophotometric titration of the oxidized labeled cytochromes with potassium ferrocyanide as described by Margalit and Schejter (22).

Reactivity with Cytochrome Oxidase. Cytochrome oxidase was isolated from a Keilin–Hartree heart muscle preparation (23) essentially as described by Yonetani (24). Cytochrome *c* oxidase activity was followed spectrophotometrically at 550 nm by monitoring the oxidation of ferrocytochrome *c*. The reaction solution contained 20 mM potassium phosphate (pH 7.0), 0.5% Tween 80, and ferrocytochrome *c*. The later was prepared by incubation of ferricytochrome *c* with ascorbate and subsequent removal of the reductant by gel filtration. The enzymatic reaction was initiated by addition of an appropriate amount of cytochrome oxidase preparation, and the initial rates were measured at different ferrocytochrome *c* concentrations, ranging from 0.5 to 20 μM . The dependence of the initial rate on the substrate concentration was analyzed in a Lineweaver–Burk plot to obtain the K_m and

V_{\max} values. The enzyme exhibited a maximum activity of $9 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$, a value compatible with the rates reported in the literature.

Transient Absorption Kinetics. The sample (1.5 mL), containing 5 mM Hepes (pH 7.0), TUPS–cytochrome *c* derivative (15 μM), and cytochrome oxidase was placed in a four-face 1 cm quartz cuvette. Cytochrome oxidase was added in substoichiometric concentration to oxidize the ferrocyanochrome *c* always present in cytochrome *c* preparations. The oxygen dissolved in the solution was removed by continuous bubbling of Ar at 15 mL/min through the cuvette for 15 min and throughout the experiment. Excitation of the dyes was initiated by the third harmonic frequency of a Nd:YAG laser (355 nm, 2 ns full-width at half-maximum, 3 mJ/pulse), which was focused on the side of cuvette over a spot having a surface area of 0.3 cm². Continuous monitoring of the redox state of the dye in TUPS adducts was carried out at 487 nm. The redox state of the cytochrome heme group was monitored at 550–556 nm (isosbestic point of cytochrome *c*). The probing beam, either the 458 and 487 nm bands of a CW argon laser or the output of a “Coherent” CR-599 dye laser with rhodamine-110 dye at 550 and 556 nm, crossed the pulse-irradiated face of the cuvette perpendicular to the excitation beam. The probing beam was directed to a monochromator photomultiplier assembly, and transients were stored and averaged by a Tektronix TDS 520A digital oscilloscope as previously described (25). The response time of the detection system is equal to 20 ns. The transients are the average of 20 pulses collected at a frequency of 0.02 Hz. Reduction of cytochrome *c* was quantitated by the absorbance coefficient difference of $\epsilon_{550-556} = 19\,000 \text{ M}^{-1} \text{ cm}^{-1}$.

RESULTS

Preparation of TUPS–Cytochrome *c* Derivatives. Isothiocyanates are widely used in protein modification studies as a specific modifier of lysine residues. Incubation of the protein with stoichiometric concentrations of IPTS results in a mixture of singly and poly-labeled TUPS–cytochrome *c* derivatives. The individual singly labeled derivatives were separated one from another on a CM-Porex HPLC column (Figure 1A). The poly-labeled derivatives, carrying a negative charge, were eluted in the void volume. Nine major and several minor peaks were obtained in the primary separation of singly labeled TUPS–cytochromes *c*. Spectral analysis proved that all these fractions contained a single equivalent of TUPS. Most of the fractions collected from the primary separation were already more than 95% pure; however, several fractions were contaminated with neighboring ones. The latter fractions were further purified by HPLC. Examples of secondary purification are given in Figure 1B,C. The second chromatography of the fractions yielded complete separation between peaks.

Localization of the Modified Lysines. The lysine residue modified in each derivative was determined by sequencing of the labeled peptide obtained by tryptic digestion of the derivative. Figure 2 shows separation of the tryptic digest of TUPS–cytochrome *c* fraction 6 by reversed-phase HPLC. The peptides absorbing at 372 nm (absorbance maximum of TUPS (20)) contain either the label or the heme. The TUPS-containing fraction, with relatively low absorbance at 405 nm, is indicated by the arrow in the figure. The fraction was rechromatographed and sequenced on a protein se-

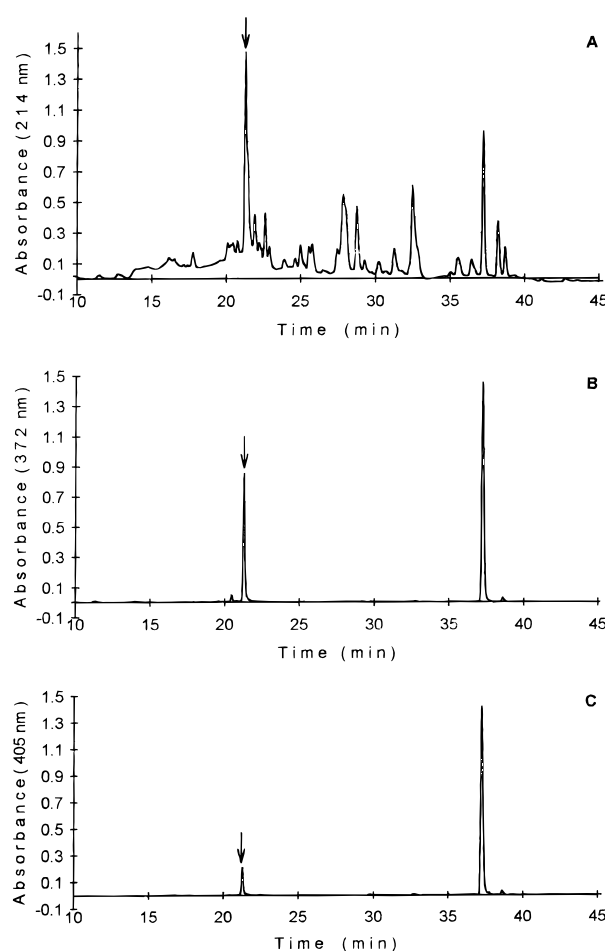


FIGURE 2: HPLC separation of tryptic digest of TUPS–cytochrome *c* fraction 6. The peptides obtained by tryptic digestion of fraction 6 (20 μg) were eluted from a C18 reversed-phase HPLC column with a linear gradient of acetonitrile from 4 to 50% in 0.1% TFA at 0.7 mL/min flow rate. The elution was monitored at 214 (A), 372 (B) and 405 nm (C). The TUPS-containing fraction is indicated by the arrows in the figure.

Table 1: Electron Transfer Rate Constants of TUPS–Cytochrome *c* Derivatives

fraction ^a	modified lysine	rate constant, ^b 10^3 s^{-1}		distance, ^c Å
		heme reduction	heme reoxidation	
1	13	4400	24	13.1
2	86	467	8.1	18.3
3	87	120	1.3	20.0
4	72	6800	150	8.9
5	8	138	1.6	21.2
6	27	11000	66	10.9
7	7	210	2.9	18.7
9	39	120	1.7	19.5

^a From Figure 1. ^b Measured as in Figure 3. ^c Estimated between a nitrogen atom of the ϵ -amino group of the correspondent lysine and the heme's iron using a model of the X-ray crystal structure of cytochrome *c* (31). Error limits estimated to be $\pm 10\%$.

quencer. Prior to the sequencing procedure, the sample was analyzed by mass spectroscopy in order to confirm its purity. The characteristic HPLC elution time of TUPS-modified lysine, obtained by Edman degradation, is different from that of the unlabeled amino acids. This enables recognition of the modified lysine residue in the sequencer records. The same TUPS identification strategy was applied for eight TUPS–cytochrome *c* derivatives, and the results are summarized in Table 1.

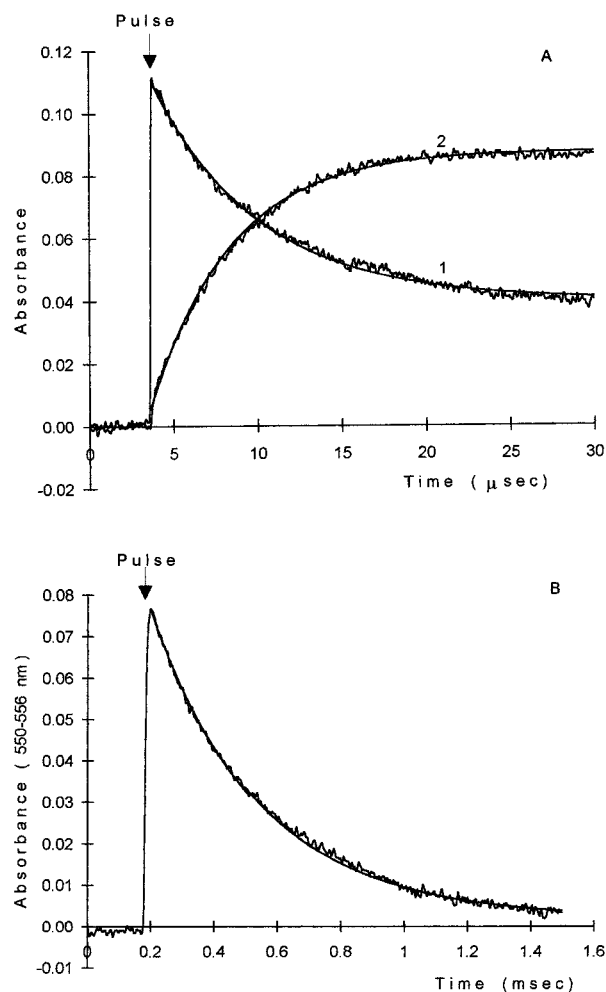


FIGURE 3: Transient kinetics of the TUPS Lys-7 cytochrome *c* derivative. TUPS–cytochrome *c* (15 μ M) in solution containing 5 mM Hepes (pH 7.0) and 0.1 μ M cytochrome oxidase was bubbled with Ar for 15 min and irradiated by a 3 mJ pulse of a Nd:YAG third harmonic frequency as described in Materials and Methods. (A) The absorbance transients were monitored at 487 (curve 1) and 550 – 556 nm (curve 2). The transient at 487 nm (curve 1) indicates the formation and decay of TUPS excited state. The transient at 550 – 556 nm accounts for reduction of the protein's heme group. The transients are fit by a single-exponential decay having the rate constants $k_{\text{obs}} = (1.8 \pm 0.3) \times 10^5 \text{ s}^{-1}$ and $k_{\text{obs}} = (2.1 \pm 0.3) \times 10^5 \text{ s}^{-1}$ for curves 1 and 2, respectively. (B) The transient at 550 – 556 nm accounts for reoxidation of the protein's heme group and fit by a single-exponential decay having a rate constant $k_{\text{obs}} = (2.9 \pm 0.3) \times 10^3 \text{ s}^{-1}$.

Photoinduced Electron Transfer Kinetics. In the previous paper (20) we have shown that photoexcitation of TUPS generates the triplet state of the dye (TUPS*) with high quantum efficiency. The triplet, being a strong reductant ($E \sim -0.9 \text{ eV}$), is capable of reducing the heme group of cytochrome *c* in a diffusion-controlled reaction. When covalently linked to a cytochrome *c* molecule, TUPS* initiates the sequence of intramolecular redox reactions. Figure 3 depicts the transient adsorbance measurement with the Lys-7 derivative. Kinetic transients obtained at 487 (curve 1) and 550 – 556 nm (curve 2) correspond to the excited state of the dye (20) and the redox state of cytochrome *c*, respectively. In curve 1, the initial rise in absorbance at 487 nm is due to the formation of the triplet state (see ref 20), and the subsequent absorbance decay corresponds to triplet disappearance. The decay does not proceed completely to the preflash baseline, as a result of

Table 2: Activity of TUPS–Cytochrome *c* Derivatives with Cytochrome Oxidase

cytochrome <i>c</i>	K_m , ^a μ M	V_{max} , ^a μ M/(min mg)	elution time, ^b min
native	2.2	9.0	55.0
TUPS Lys-39	2.5	9.0	52.8
TUPS Lys-7	3.2	9.0	45.4
TUPS Lys-27	3.6	9.0	43.4
TUPS Lys-8	6.3	9.0	40.2
TUPS Lys-72	16.7	9.0	35.5
TUPS Lys-87	22.2	9.0	34.3
TUPS Lys-86	40.0	9.0	33.1
TUPS Lys-13	220.0	9.0	29.0

^a Obtained by analysis of the dependence of the enzymatic rate on cytochrome *c* concentration in a Lineweaver–Burk plot. The reaction was assayed in 20 mM potassium phosphate (pH 7.0) as described in Materials and Methods. Error limits estimated to be $\pm 10\%$. ^b Estimated from Figure 1.

the heme reduction. The later is associated with a positive absorbance change at 487 nm ($\Delta\epsilon = 9000 \text{ M}^{-1} \text{ cm}^{-1}$). In curve 2, an absorbance increase at 550 – 556 nm is due to the cytochrome heme reduction by TUPS*. The rate constant for the 550 – 556 nm transient, $(2.1 \pm 0.3) \times 10^5 \text{ s}^{-1}$, is identical, within the experimental error, to the rate constant for the 487 nm transient. The rate constant was independent of the concentration of the TUPS–cytochrome *c* derivative from 10 to 100 μ M, which is indicative of an intramolecular electron transfer mechanism. This reductive branch of the photochemical process generates two redox-active species: the reduced heme and the oxidized form of TUPS. Recombination, namely, reoxidation of the heme by the oxidized dye, brings the system to its initial prepulsed state. Figure 3B shows the kinetic transient at 550 – 556 nm associated with heme reoxidation. The transient is monophasic with a rate constant of $(2.9 \pm 0.3) \times 10^3 \text{ s}^{-1}$. As in the case of heme reduction, the rate constant was independent of concentration of the TUPS–cytochrome *c* derivative. The redox cycle initiated by photoexcitation of TUPS can be repeated hundreds of times without decrease of the signal amplitude. The rate constants for heme reduction and reoxidation were measured in a similar fashion for seven other singly labeled derivatives. The results are summarized in Table 1. The rates of electron transfer in both directions are dependent on the position of the label in the protein molecule. The amplitudes of the transients, however, are equal for all derivatives tested. The efficiency of the perturbation is high and about 30% of the cytochrome *c* molecules undergo intramolecular reduction in a single pulse.

Reduction Potential of TUPS–Cytochrome *c* Derivatives. The reduction potentials of the TUPS–cytochrome *c* derivatives were determined. They ranged from 0.26 to 0.28 eV for TUPS Lys-13, -7, -72, -27, -39, -87, -86, and -8. The values are essentially the same, within the experimental error, as that of native cytochrome *c*, 0.26 eV (22). This indicates that the heme properties are unaffected by the modification of any given lysine.

Activity of TUPS–Cytochromes *c* with Cytochrome *c* Oxidase. The kinetic parameters of cytochrome *c* oxidase with TUPS–cytochromes are shown in Table 2. The kinetic data for the reactions of the TUPS–cytochromes *c* with cytochrome oxidase were measured spectrally by monitoring the initial rates of oxidation of fully reduced samples of cytochrome *c*. It has been demonstrated earlier (26) that K_m values obtained from spectroscopic measurements at low ionic strength reflect the binding affinity of cytochrome *c*

to the low-affinity binding site of cytochrome oxidase. Modification of cytochrome *c* lysines results in an increase of apparent K_m . The maximum rate of cytochrome oxidation, obtained by extrapolation of the enzymatic rate to the infinite substrate concentration, was shown to be the same for all derivatives tested. The order of K_m values for the TUPS-modified cytochromes *c* is as follows: native cytochrome *c* < Lys-39 < Lys-7 < Lys-27 < Lys-8 < Lys-72 < Lys-87 < Lys-86 < Lys-13 (see Table 2). Almost the same order has been previously reported for the affinities of monocarboxydinitrophenyl- and thiopropionyl-cytochrome *c* derivatives to the high-affinity binding site of cytochrome oxidase (26, 27). The effect of chemical modification by negatively charged labels has been attributed to the changes in the electrostatic field of the protein, the factor that governed the interaction of cytochrome *c* with cytochrome oxidase (27). The latter is proved by direct correlation of the TUPS-cytochrome *c* derivative's affinity toward cytochrome oxidase with the HPLC elution times (Table 2), the parameter characterizing the affinity of the protein for the column's resin. Binding of cytochrome *c* to the polyanion CM matrix can thus serve as a model for interaction of the protein with cytochrome oxidase.

DISCUSSION

The electrostatic field of the cytochrome *c* molecule is strongly altered by introducing the polyanionic TUPS label. This allows separation of the labeled derivatives by ion-exchange chromatography. The primary separation of singly labeled TUPS-cytochrome *c* derivatives by HPLC yields nine major and a number of minor individual peaks. Separation of the same mixture by low-pressure chromatography is less efficient and in optimal conditions yields five overlapping peaks (not shown), each containing a mixture of several individual derivatives.

In general, binding of proteins to the ion-exchange resin is determined by the charge of the protein molecule. The difference in the binding affinity of singly labeled TUPS derivatives is most likely attributed to the changes in the vectorial electrostatic field of the protein molecule, as the sum charge of the derivatives is independent of the position of the label, at least at neutral pH. The key role of the electrostatic field of cytochrome *c* in governing its interaction with physiological reaction partners has been proposed earlier (28). The results of the present work show that the same electrostatic factors control the interaction of cytochrome *c* with either cytochrome oxidase or the polyanionic matrix (see Table 2).

TUPS-cytochrome *c* adducts have a number of favorable properties for studying the intra- and intermolecular electron transfer reactions. By means of a short-duration laser pulse, it is possible to generate the triplet state of TUPS with high yield (20). The triplet has a long natural life time of 0.5 ms and is a strong ($E^\circ \sim -0.9$ eV) reducing agent. Intramolecular reduction of the heme group of cytochrome *c* by the covalently attached TUPS was shown to proceed with high efficiency. The rather low excitation energy (3 mJ/pulse) needed to generate the triplet makes the approach a gentle one. The protein can be exposed to laser excitation hundreds of times with no reduction of the signal amplitude. The redox perturbations, initiated by photoexcitation, are reversible. The reduction of the heme is followed by its reoxidation by the oxidized form of the dye. This oxidative stage of

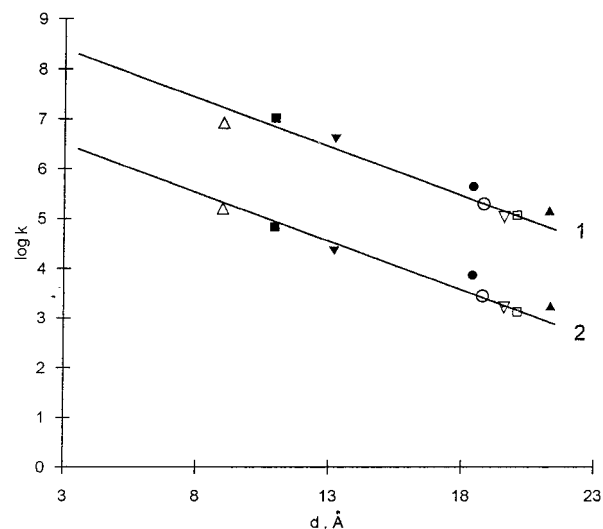


FIGURE 4: Dependence of the logarithm of the rate constants for heme reduction (curve 1) and reoxidation (curve 2) in TUPS-cytochrome *c* derivatives on the separation distance (d). The rate constants were measured as shown in Figure 3. Distances between the nitrogen atom of the ϵ -amino group of Lys-7 (○), Lys-8 (▲), Lys-13 (▼), Lys-27 (■), Lys-39 (▽), Lys-72 (△), Lys-86 (●), and Lys-87 (□) and the heme iron were estimated as in Table 1. Best-fit linear approximations: $\log k = 8.4 - 0.2(d - 3)$ and $\log k = 6.5 - 0.2(d - 3)$ for curves 1 and 2, respectively.

the process proceeds much slower than the reductive one, the property that enables one to separate the reactions in time.

Both heme reduction and reoxidation are characterized by high driving forces. The ΔG° values for the forward and reverse electron transfer reactions can be estimated to be 1.16 and 0.62 eV, respectively. The estimation is based on $E^\circ = -0.9$ eV for TUPS*/TUPS_{ox} (20), $E^\circ = 0.88$ eV for TUPS/TUPS_{ox} (25), and $E^\circ = 0.26$ eV for cytochrome *c* (22). The combination of the above mentioned properties of the dye, together with the high time resolution attained by our method ($\tau < 100$ ns), provided us with high-quality measurements of intraprotein electron transfer rates.

The rates of electron transfer depend on the position of the label on the protein molecule (see Table 1). It is informative to compare the observed rate constants with those predicted by the widely used treatment introduced by Marcus. According to Marcus theory (29, 30), the rate of a nonadiabatic outer-shell electron transfer between adjacent reactants is given by the following equation:

$$k_{el} = 10^{13} \exp(-\beta(d-3)) \exp(-(\lambda + \Delta G^\circ)^2/4\lambda RT) \quad (1)$$

where d is the distance between the redox-active centers, β is a scalar that correlates the rate with separation distance (d), ΔG° is the standard free energy of the reaction, and λ is the reorganization energy. Equation 1 quantitates the dependence of the rate on two molecular parameters. The first exponent expresses the effect of the distance (d) factorized by β on the reaction's rate, while the second exponent is the Boltzmann expression for the activation energy. At present, the study of electron transfer within cytochrome *c* is based on experiments where d is variable while ΔG° is constant.

Equation 1 was used to predict the distance dependence of the rate constants for both heme reduction and reoxidation (see Figure 4). Electron transfer rates in both cases can be described by a 0.46 \AA^{-1} distance decay constant, with no

significant deviations from the simple exponential distance dependence. The d values given on the figure are the distances between the nitrogen atom of the ϵ -amino group of the corresponding lysine and the heme iron estimated by using a model of the X-ray crystal structure of cytochrome *c* (31). Evaluation of the real distance between redox centers is a rather complicated problem. In hemes, the redox center might be just the central Fe atom or include the conjugated macrocycles and amino acid ligands. In the TUPS label, the redox center may be delocalized or associated with certain atoms. The preliminary quantum mechanical calculations (M. Shokhen, Tel Aviv University, unpublished) show that the electron-donating orbital of the label's triplet is localized on the double-bonded carbon and sulfur atoms of TUPS. Measurement of the real distances between the label and the heme is underway in our laboratory and includes estimation of the energetically optimal orientation of TUPS in each derivative, using molecular modeling programs and ab initio calculations of the electronic wave function of segments of the system.

The value of β determined in the present study is much smaller than those reported earlier (6, 32, 33). This discrepancy is not attributed to erroneous estimation of the distance between the reactants in the present work. When the dependence of the electron transfer rate on the edge-to-edge distances was analyzed in the same fashion as in Figure 4, almost the same β value was obtained. The very low value of the distance decay constant may also not be attributed to the reduction of cytochrome *c* amino acids by TUPS, as none of the 20 standard amino acids examined was capable of redox interaction with TUPS*.

The treatment of distance dependence data, obtained from eq 1, suggests that the only term varying with distance is electronic coupling. However, the outer-sphere reorganization energy also increases with distance, contributing to the distance decay constant (34). The size of this contribution can be quite large depending on the balance between the inner- and the outer-sphere reorganization energies, the driving force of the reaction, and the polarity of the intervening media. The difference in the reorganization energies for the reactions of the ruthenium and TUPS species with the heme group may be the reason for the difference of the apparent β values in the previous (6) and the present work.

The employment of TUPS—cytochrome *c* derivatives in which reduction of the heme is completed within less than 1 μ s, provides us with a direct tool for fast reduction of cytochrome *c* bound to other redox proteins and for measuring the resulting electron redistribution in polypeptide complexes with the same level of time resolution. High enzymatic activity and specific interaction of several TUPS—cytochrome *c* derivatives with cytochrome oxidase (see Table 2) make the approach promising for investigation of electron transfer in this enzyme substrate complex.

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