Protein–Protein Electron Transfer. A Marcus Theory Analysis of Reactions between *c* Type Cytochromes and Blue Copper Proteins†

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ABSTRACT: The electron-transfer rate and equilibrium constants and enthalpies of reaction for seven protein–protein reactions in 0.05 M phosphate buffer at pH 7 and 25 °C have been determined by the chemical relaxation temperature-jump method. The rate constants for the reduction by cytochrome c_{553} (*Bumilleriopsis filiformis*) of the following proteins, *Pseudomonas aeruginosa* azurin, *Alcaligenes faecalis* azurin, *Alcaligenes spp.* azurin, *Phaseolus vulgaris* plastocyanin, *Scenedesmus acutus* plastocyanin, and horse heart cytochrome *c*, and the reduction by *Pseudomonas aeruginosa* cytochrome c_{551} of *Alcaligenes spp.* azurin are 1.0×10^7, 5.2×10^5, 2.1×10^6, 4.0×10^5, 7.4×10^5, 4.0×10^4, and 3.0×10^6 M^{-1} s^{-1}, respectively. For the reaction of cytochrome c_{553} with *Ps.* azurin there is a second relaxation mode attributed to an isomerization of reduced azurin to an inactive form. The rate constant for the formation of the inactive form was 5 s^{-1} and the equilibrium constant 0.1. These data and others from the literature are analyzed with the Marcus theory formalism. For 13 protein–protein electron-transfer reactions, the data can be excellently fit by 6 apparent electron self-exchange rate constants; k_{11} (M^{-1} s^{-1}) is 2.8×10^8 for cytochrome c_{553}, 4.6×10^7 for cytochrome c_{551}, 9.9×10^5 for *Ps.* azurin, 2.6×10^5 for the other azurins, 6.6×10^2 for the plastocyanins, and 1.5×10^2 for cytochrome *c*. The constancy of the k_{11} values for each protein is taken as evidence that there is no kinetic selectivity in these reactions. Implications of this conclusion for the pathway of electron transport in photosynthesis and bacterial respiration are discussed. The k_{11} values calculated are compared to those previously calculated for some reactions of these proteins with inorganic transition metal complexes.

The extensive recent research in the field of metalloprotein electron-transfer reactivity has followed two general courses. One approach has been the study of isolated electron-transport proteins, especially the cytochromes, single blue copper proteins, and various iron–sulfur proteins, with small molecule reagents (Cummins & Gray, 1977; Goldberg & Pecht, 1976; Sutin, 1977; Wherland & Gray, 1976, 1977). The advantage of this approach is that the kinetic systems are well defined and can be studied with high precision. The goal of such research has been the elucidation of reaction pathways available to the proteins and the manner in which the polypeptide envelopes have modified the inherent reactivity of the metal centers. The second principal approach has been the classical study of photosynthetic or mitochondrial electron transport in intact or partially disrupted preparations (Bishop, 1971; Wilson et al., 1975a). The advantage here compared to the isolated systems is that the in vivo conditions are approached as close as possible.

In contrast to the case of protein–small molecule electron transfer, there are relatively few data available for protein–protein reactions, despite the proposals that electron transfer between a blue copper protein and a cytochrome *c* takes place in photosynthetic and bacterial electron transport. Notable exceptions are the work (Wilson et al., 1975b; Rosen & Pecht, 1976) on the complex *Pseudomonas aeruginosa* cytochrome c_{551} and azurin reaction, as well as the analogous reaction with *Alcaligenes faecalis* azurin (P. Rosen and I. Pecht, unpublished results), and the work of Wood (1974) on the relative reactivities of plastocyanin and azurin with mammalian and photosynthetic cytochromes.

It is the purpose of this work to extend the methods of analysis of protein electron-transfer processes, first applied to the protein–small molecule reactions, to protein–protein reaction data. The results of this analysis lead to conclusions on the kinetic selectivity and specificity of the reactions between the proteins studied, and the relationship between protein–protein and protein–small molecule electron-transfer reactivities.

Experimental Section

Pseudomonas aeruginosa (*Ps.*) azurin and cytochrome c_{551} were prepared by the method of Ambler & Wynn (1973). *Alcaligenes faecalis* and *Alcaligenes spp.* (Iwasaki-II) were prepared by the same procedure except that the azurin elution buffer for the CM-cellulose column was pH 4.9 to 5.1 instead of pH 4.6. Repeated attempts to purify the *Alcaligenes spp.* azurin gave a constant ratio of A_{280}/A_{625} of 2.9. Extra steps included DE- and CM-cellulose chromatography, G-75 gel filtration, and ammonium sulfate precipitation. Stable apoazurin may be the impurity, but Cu(I) in the presence of ascorbate did not improve the ratios of the oxidized protein. Gel electrophoresis showed no significant impurities. The other two azurins had the more typical ratio of 2.

Bean plastocyanin (*Phaseolus vulgaris*) was prepared by the method of Milne et al. (1974), except that the first step was juicing of the leaves. The A_{278}/A_{597} ratio was 1.15. Leaves were collected fresh from mature plants.

Cytochrome c_{553} from the algae *Bumilleriopsis filiformis* and plastocyanin from *Scenedesmus acutus* were gifts of Peter Böger, Peter Kroneck, and coworkers, and were prepared as previously described (Lach et al., 1973; Siegelman et al., 1976). The reduced cytochrome c_{553} had an A_{278}/A_{597} ratio of 2.8.

Horse heart cytochrome *c* was Sigma type VI and was used as supplied, after oxidation with $Fe(CN)_6^{3-}$.

Concentrations were determined from extinction coefficients as given in Table I. All values are from the literature except

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TABLE I: Properties of the Redox Proteins Studied.

Protein	Mol wt (10^{-3})	Lys	Arg	His[a]	Glu[b]	Asp[b]	Metal[c] site	Other[d]	Charge[e]	pI	λ (nm)	ε (mM^{-1} cm^{-1})	E (mV)	Ref
Cytochrome c_{553} (*Bumilleriopsis filiformis*)	7.36	3	2	0 (1)	(7)	(10)	1/0	−2	>−13/−14	<6	553	23.2	335	*f*
Cytochrome c_{551} (*Ps. aeruginosa*)	8.1	8	1	0 (1)	5	5	1/0	−2	−2/−3	4.7	551	30	286	*g, h*
Cytochrome *c* (horse heart)	12.5	19	2	1 (3)	9	3	1/0	−3	7.5/6.5	10.5	550	27.7	260	*g, i*
Plastocyanin (*Scenedesmus* monomer)	9.9	5	0	2 (4)	(8)	(10)	1/0	0	>−10/−11	3	598	4.9	350	*j*
Plastocyanin (bean)	10.7	5	0	0 (2)	9	5	1/0	0	−10/−9	<6	598	4.5	350	*k, l*
Azurin (*Ps. aeruginosa*)	13.9	11	1	2 (4)	4	11	1/0	0	−1/−2	5.2	625	5.7	304	*o, p,fr q*
Azurin (*Alcaligenes spp.*)	14.0	14	1	2 (4)	4	11	1/0	0	2/1	<6	625	5.4	260	*r*
Azurin (*Alcaligenes faecalis*)	13.9	13	1	2 (4)	7	10	1/0	0	−1/−2	<6	625	4.6	266	*r, s*

[a] Total lysines are in parentheses, but those with shifted p*K*s are subtracted. [b] Numbers in parentheses are the total of asparagines and aspartates or glutamines and glutamates. [c] For cytochromes, the deprotonated heme nitrogens contribute −2; for the blue copper proteins a thiolate is assumed. [d] Two propionate side chains for all cytochromes and an additional correction for the acetylated N terminus of the horse heart protein. [e] Calculated as the sum of lysines, arginines, half of the histidines, metal site, and "other", minus the glutamates and aspartates. [f] Lach et al. (1973). [g] Dickerson & Timkovich (1976). [h] Morton et al. (1970). [i] Margalit & Shejter (1973). [j] Siegelman et al. (1976). [k] Milne et al. (1974). [l] Wood (1974). [m] Ramshaw, et al. (1973). [n] Sailasuta, N., Anson, F. C., & Gray, H. B., unpublished results. [o] Yamanaka (1966). [p] Ambler & Brown (1967). [q] Rosen & Pecht (1976). [r] Ambler (1971). [s] Rosen, P., & Pecht, I., unpublished results.

for that for *Alcaligenes spp.* azurin, for which the extinction coefficient and potential were determined from a ferricyanide titration of the reduced protein.

Static spectra were recorded on a Cary 118 spectrophotometer under deoxygenated argon, when necessary.

Kinetic Measurements. Temperature-jump measurements were made with a double beam spectrophotometer as previously described (Rosen & Pecht, 1976). After the protein samples were mixed, they were maintained in the temperature-jump cell under a flow of nitrogen or argon scrubbed by passage through vanadium(II) or reduced methyl viologen solutions. Reduced azurin and reduced cytochromes other than cytochrome c_{553} were also maintained in an oxygen-free environment. Cytochromes were reduced by ascorbate and then passed through a G-15 column to remove the excess ascorbate and its oxidation products. Azurins were reduced by H_2 in the presence of catalytic amounts of platinum black which was subsequently filtered off.

All measurements were made in 0.05 M phosphate buffer (pH 7.0) containing 10 μM EDTA (ionic strength 0.1 M). Temperature jumps of 3 °C to a final temperature of 25 °C were used.

Data were collected at the absorption maximum of the reduced cytochrome near 550 nm, except for the cytochrome c_{553}–cytochrome *c* reaction which was monitored at 557 nm, an isosbestic point of the latter cytochrome. The average of 3 to 12 digitized traces, typically 9, was examined for its fit to a single exponential by a least-squares procedure (Strehlow & Jen, 1971; cf. Rosen & Pecht (1976) and Goldberg & Pecht (1976) for further experimental details). At least 500 of the digitized data points for each averaged trace, covering a minimum of 3 half-lives, were included in the fitting. The amplitudes and relaxation times were fit to the appropriate equations in a least-squares sense by varying the equilibrium constant and fitting the linear variables by an unweighted linear least-squares procedure until a minimum was reached on the error surface. The final equilibrium constant was a compromise between the values obtained from the amplitude and the reciprocal relaxation time analysis. This compromise was taken as the geometric mean $(KK')^{1/2}$, thus basing the average on the free-energy changes.

The relaxation curves for all reactions except that between *Ps.* azurin and cytochrome c_{553} were a single exponential and were analyzed according to the bimolecular equilibrium mechanism:

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C + D \qquad K = \frac{k_{12}}{k_{21}} = \frac{[C][D]}{[A][B]} \tag{1}$$

where A and C are the two oxidation states of one protein, and B and D represent the second protein. The reciprocal relaxation time for this mechanism is:

$$1/\tau = k_{12}([A] + [B]) + k_{21}([C] + [D]) \tag{2}$$

Under the constraint that initial solutions contain only A or B, the reciprocal relaxation time may be expressed:

$$1/\tau = k_{12}\left([S] + [Q] + [X]\left(\frac{2}{K} - 2\right)\right) \tag{3}$$

where [S] = [A] + [C], [Q] = [B] + [D], [X] = [C] = [D], and [X] is a quadratic equation[1] in terms of [S], [Q], and *K*. The expression for the amplitude is (Jovin, 1975):

$$-\frac{\Delta I}{2.3 d I_0} = \Gamma \frac{\Delta T}{RT^2} \Delta H \Delta\theta \tag{4}$$

where I_0 is the total light intensity in mV, ΔI is the amplitude in mV, *d* is the path length, 0.7 cm, ΔT is the temperature rise,

[1] $$[X] = \frac{-K([S] + [Q]) + \sqrt{K^2([S] + [Q])^2 + 4(1 - K)K[S][Q]}}{2(1 - K)}$$

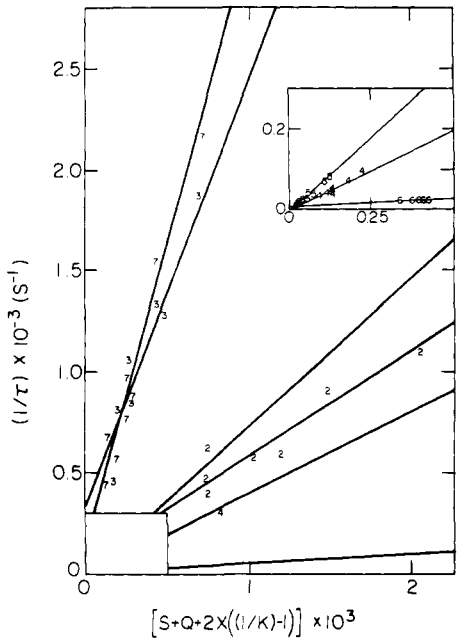

FIGURE 1: Dependence of $1/\tau$ on concentrations according to eq 3. Reactions are numbered as in Table II.

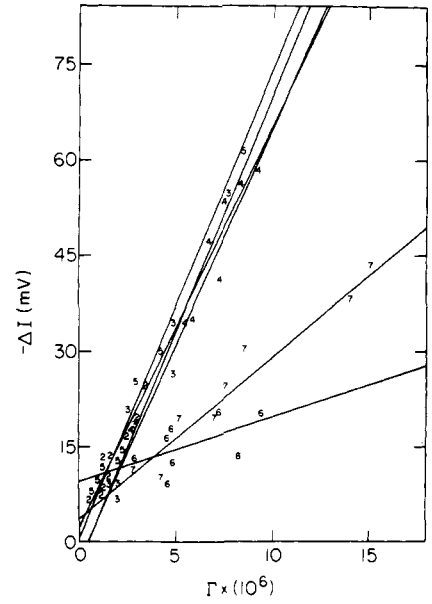

FIGURE 2: Dependence of relaxation amplitudes on concentrations according to eq 4-6. Reactions are numbered as in Table II.

3 °C, T is the final temperature, 298 K, ΔH is the enthalpy change of the reaction, $\Delta\theta$ is the extinction change of the reaction ($\epsilon_C + \epsilon_D - \epsilon_A - \epsilon_B$), and Γ is a function of the concentrations of the various species. For a given system under the fixed conditions given:

$$-\Delta I = F\Gamma \tag{5}$$

where F incorporates all of the constants and for the bimolecular mechanism given above:

$$\Gamma^{-1} = \left(\frac{1}{[A]} + \frac{1}{[B]} + \frac{1}{[C]} + \frac{1}{[D]}\right) = \frac{2[X](1-K) + ([S]+[Q])K}{[X]^2} \tag{6}$$

With an estimate of θ, the F value can be related to ΔH by eq 4. The spectral bandwidth of the temperature-jump spectrometer (10 nm in this work) is large compared to the spectrophotometer used to measure the spectra and determine extinction values; thus the magnitude of $\Delta\theta$ is overestimated and that of ΔH is underestimated. This effect is not larger than 20%.

Equilibrium constants of all the reactions studied kinetically were calculated using reported redox potentials of the proteins. The reaction of *Ps.* azurin with cytochrome c_{553} was studied both statically and dynamically, as this system was found to exhibit a multistep equilibrium. Duplicate static titrations were performed at four different temperatures and the data were evaluated at three wavelengths (553, 520, and 625 nm). For both titrations and all four temperatures, the equilibrium constant was evaluated at three points in the titration. For the reaction cytochrome c_{553}(II) + azurin(II) = cytochrome c_{553}(III) + azurin(I) at 6, 16, 25, and 30 °C the equilibrium constants are 0.07, 0.08, 0.10, and 0.11, each ± 0.01, respectively. These values lead to a ΔH of 3.4 kcal/mol and a ΔS of 7 cal/(mol deg). The temperature-jump experiments yielded two well-separated relaxations with amplitudes of opposite sign, analogous to the result with *Ps.* azurin and cytochrome c_{551} (Rosen & Pecht, 1976). Thus, the following mechanism is proposed:

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C + k_{32} \underset{E}{\overset{D}{\rightleftharpoons}} k_{23} \tag{7}$$

The following symbols are used: A, cytochrome c_{553}(II); B, azurin(II); C, cytochrome c_{553}(III); D, azurin(I); E, azurin(I) isomer; [S] = [A] + [C]; [Q] = [B] + [D]; [X] = (reducing equivalents); K_1 = [C][D]/[A][B]; K_2 = [E]/[D]; K_0 = [C]([D] + [E])/[A][B] = $K_1(1 + K_2)$.

The relaxation times are:

$$1/\tau_1 = k_{12}([A] + [B]) + k_{21}([C] + [D]) \tag{8}$$

$$1/\tau_2 = k_{23} + k_{32} - \frac{k_{23}}{[D]}\left\{1 \Big/ \left(\frac{1}{[A]} + \frac{1}{[B]} + \frac{1}{[C]} + \frac{1}{[D]}\right)\right\} \tag{9}$$

and the various species are re-expressed in terms of the equilibrium constants K_0 and K_2. The data were fit as before by varying the nonlinear parameters and solving for the linear ones.

A further complication applies to all measurements on cytochrome c_{551}. For this protein, an isomerization to an inactive form has been indicated in the reactions with *Ps.* azurin and *Alc. faecalis* azurin (P. Rosen and I. Pecht, unpublished results). This isomerization is fast on the time scale of the measurements and does not involve a change in the cytochrome spectrum. Its influence was included by the authors of the work mentioned above, but the other data in Table II have not been adjusted to include this finding. The influence on the reciprocal relaxation time is to change k_{12} (eq 1 and 7) to $k_{12}/(1 + K_c)$, where K_c is the equilibrium constant for the formation of the active form of the reduced cytochrome. The typical value for K_c is 1.9 (Rosen & Pecht, 1976). This difference does not significantly alter any of the later discussion.

The results for all systems are summarized in Table II and displayed in Figures 1 and 2. For reactions 2 through 7, eight or nine different concentration conditions were used. All relaxation times and amplitudes and solution compositions, along with the best fit values, are listed in the Supplementary Material (see paragraph at end of paper). The additional results for the *Ps.* azurin cytochrome c_{553} are values of K_2 = 0.04 and

TABLE II: Kinetic and Thermodynamic Data for Protein Electron-Transfer Reactions.

Oxidant	Reductant	k (M^{-1} s^{-1})	K^a	F^k (10^{-6})	$\Delta\theta$ (mM^{-1} cm^{-1})	ΔH (kcal/mol)	ΔS (cal/(mol deg))	K (predicted)^b	log (k_{12}^2/K)^c Obsd	Calcd	k (M^{-1} s^{-1}) calcd	k(calcd)/k(obsd)	Ref
1. Azurin (*Ps.*)	Cytochrome c_{553}	1.43 (10^7)	0.1 (0.1)					(0.3)	15.31	14.44	5.3 (10^6)	0.4	*d*
2. Azurin (*Alc. f.*)	Cytochrome c_{553}	5.23 (10^5)	0.01	−6.15	−17.3	−1.6	−15	0.07	13.44	13.86	8.5 (10^5)	1.6	*d*
3. Azurin (*Alc. spp.*)	Cytochrome c_{553}	2.11 (10^6)	0.07	−7.33	−17.9	−1.9	−12	0.05	13.80	13.86	2.3 (10^6)	1.1	*d*
4. Plastocyanin (bean)	Cytochrome c_{553}	4.00 (10^5)	1.4	−6.55	−18.6	−1.5	−4	1.8	11.06	11.26	5.0 (10^5)	1.3	*d*
5. Plastocyanin (*Sc.*)	Cytochrome c_{553}	7.36 (10^5)	1.7	−7.25	−19.7	−1.7	−5	1.8	11.50	11.26	5.6 (10^5)	0.8	*d*
6. Cytochrome *c*	Cytochrome c_{553}	3.96 (10^4)	0.08	−1.00	−8.0	−0.6	−7	0.05	10.29	10.61	5.7 (10^4)	1.4	*d*
7. Azurin (*Alc. spp.*)	Cytochrome c_{553}	2.96 (10^6)	0.5	−2.54	−22.7	−0.5	−3	0.4	13.24	13.08	2.5 (10^6)	0.8	*d*
									13.67	13.66	6.0 (10^6)	1.0	*e*
8. Azurin (*Ps.*)	Cytochrome c_{551}	6.10 (10^6)	0.8 (3.9)			−5.9	−20	(2)	13.15	13.08	1.9 (10^6)	0.9	*f*
9. Azurin (*Alc. f.*)	Cytochrome c_{551}	2.10 (10^6)	0.31			−5.3	−20	0.5	9.65	9.83	8.2 (10^4)	1.2	*g*
10. Cytochrome *c*	Cytochrome c_{551}	6.7 (10^4)	1.0					0.4	7.17	8.17	5.2 (10^3)	3.2	*h*
11. Cytochrome *c*	Azurin (*Ps.*)	1.6 (10^3)						0.18	10.44	10.48	7.8 (10^5)	1.0	*i*
12. Plastocyanin (parsley)	Cytochrome c_{551}	7.5 (10^5)						20.3	5.09	4.34	1.5 (10^2)	0.4	*j*
13. Cytochrome *c*	Cytochrome *c*	3.5 (10^2)						1	10.30	14.90	2.2 (10^3)	2 (10^{-3})	*i*
14. Plastocyanin (parsley)	Cytochrome *c*	1.0 (10^6)						50					*i*
15. Plastocyanin (parsley)	Cytochrome *f*	3.6 (10^7)						2					*i*
16. Cytochrome *f*	Azurin (*Ps.*)	6.0 (10^6)						6					

[a] Experimental value for electron-transfer equilibrium constant; numbers in parentheses are for the overall equilibrium if this is different. [b] Value calculated from reduction potentials of Table I. [c] Using experimental values for K where available. [d] This work. [e] Rosen & Pecht (1976). [f] Rosen, P. & Pecht, I., unpublished results. [g] Morton et al. (1970). [h] Greenwood et al. (1971). [i] Wood (1974). [j] Gupta (1973). [k] Amplitude coefficient (eq 5).

$k_{23} = 5\ s^{-1}$. In this latter system, the two relaxation times at 16 different concentrations were used to evaluate the constants. Attempts were also made to measure the cross-reactions between cytochrome *c* and bean plastocyanin, cytochrome *c* and *Alc.* azurin, and cytochrome c_{551} and bean plastocyanin. Only an estimate of the rate constants could be obtained in the first two cases because the reaction was slow on the temperature-jump time scale. The third case gave no relaxation.

Discussion

Prior to further analysis of the data, several points may be made. First, the rate constants for the cytochrome c_{551} and c_{553} reactions with the azurins are among the fastest measured for electron-transfer reactions involving proteins. Thus, from the viewpoint of the mere magnitude of the rate constant, these reactions are efficient and reasonable candidates to be natural partners. Of course, the cytochrome c_{553} reaction could not be considered a natural partner of an azurin since they do not occur together in the same species. The reactions of the plastocyanins and cytochrome *c* are not particularly fast in any of the cases considered here. Second, the equilibrium constants calculated from the kinetic data and listed in Table II may be compared with those calculated from the potentials given in Table I. There is good agreement, as is shown in Table II, with the possible exception of the *Alc. faecalis* azurin–cytochrome c_{553} reaction, which was rather difficult to measure as it was furthest from the ideal equilibrium constant of 1, and shared a low enthalpy with the other cytochrome c_{553} reactions. Thus, there is no clear evidence for additional steps other than those already proposed. For the *Ps.* azurin–cytochrome c_{553} reaction, the parameters for the step which has been assigned to an isomerization of reduced azurin are somewhat different than those previously derived from the *Ps.* azurin–cytochrome c_{551} reaction, K_2 of 0.7 and k_{23} of 12 s^{-1} (Rosen & Pecht, 1976). The origin of this difference is being pursued through further experiments on reduced azurin alone.

The enthalpy changes given in Table II, reactions 2 to 7, are significantly different from those found in the two previous studies, reactions 8 and 9, and remarkably constant. The cytochrome c_{553} reactions with the four blue copper proteins have virtually identical enthalpies. The *overall* equilibrium for the *Ps.* azurin–cytochrome c_{553} reaction gives a somewhat higher enthalpy, 3.4 kcal/mol, than the *Ps.* azurin–cytochrome c_{551} reaction, ~0 kcal/mol (Rosen & Pecht, 1976). The inability to observe a temperature-jump signal for the cytochrome c_{551}–bean plastocyanin reaction indicates that the enthalpy for this reaction is close to zero, assuming the mechanism of eq 1. An interpretation of these results must await data on the temperature dependences of the rate constants and the reduction potentials.

In order to further analyze the data for evidence of specificity, the Marcus theory formalism (Marcus & Sutin, 1974) for describing outer-sphere electron transfer will be used. The reactions are assumed to proceed by an outer-sphere mechanism, and the Marcus theory formalism is used to dissect, out of the rate constant data, the contribution from the activation process each protein must undergo in order to transfer an electron. This analysis exposes any changes in this activation process which accompany variations in reaction partners. This approach has previously been used to analyze the reactivities of inorganic complexes with electron-transfer proteins, and a detailed discussion of its applicability has already been published (Wherland & Gray, 1976, 1977; Goldberg & Pecht, 1976); thus, only a summary is presented here.

The Marcus theory correlates the rate constant of an outer-sphere electron-transfer reaction, k_{12}, with the electron self-exchange rate constants of the two reactants, k_{11} and k_{22},

and the equilibrium constant for the electron-transfer reaction, K, through the expression:

$$k_{12} = (k_{11}k_{22}K)^{1/2} \quad (10)$$

This is the simplest form of the equation, but it is adequate for this analysis. The two electron self-exchange rate constants, k_{11} and k_{22}, are characteristic of the activation processes which the individual metalloproteins must undergo in order to transfer an electron. If the electron-transfer process is independent of the reaction partners, then these parameters will be true constants; if, on the other hand, there is kinetic selectivity then these parameters will vary with the reaction studied and a greater selectivity will be expressed in a higher k_{11}.

For the present application, eq 10 can be restated:

$$\log (k_{11}) + \log (k_{22}) = \log (k_{12}^2/K) \quad (11)$$

with the known parameters on the right. For any given reaction, k_{11} and k_{22} cannot be separated and only their product is predicted; however, for a series of reactions involving fewer proteins than there are cross-reactions there is enough information to predict optimum individual parameters. (Note that the logarithmic formulation is equivalent to the usage of free energies of activation and reaction instead of rate and equilibrium constants.)

The data analyzed are presented in Table II and also include several values from the literature. For the series of 13 electron-transfer reactions listed, log (k_{12}^2/K) was calculated and the optimum log k_{11} values were found by minimizing the sum of the squares of the differences between log $(k_{11}k_{22})$ and log (k_{12}^2/K). The number of parameters was limited to six and the resulting k_{11} (M^{-1} s^{-1}) values are: cytochrome c_{553}, 2.8×10^8; cytochrome c_{551}, 4.6×10^7; *Ps.* azurin, 9.9×10^5; *Alc.* azurins, 2.6×10^5; plastocyanins, 6.6×10^2; and cytochrome *c*, 1.5×10^2.

The fit to the data using the six k_{11} values is good. This is especially striking since fewer parameters were used than there were proteins. Within the Marcus formalism there was no evidence for kinetic selectivity between any of the 13 reaction partners analyzed. This absence of selectivity indicates that there is no sequential electron transport between a blue copper protein and a cytochrome in the two cases where it has been proposed, cytochrome c_{553} and plastocyanin (Bishop, 1971) and cytochrome c_{551} and azurin (Horio et al., 1961). The former conclusion has also been indicated for the *Bumilleriopsis* system by establishing that plastocyanin is absent from this organism (Kunert & Böger, 1975) and by the reciprocity of formation of cytochrome c_{553} and plastocyanin in *Scenedesmus* chloroplasts (Bohner & Böger, 1978). Looking next at the numerical values of the self-exchange constants, the most striking pattern is the separation into four categories. The two small cytochromes, c_{551} and c_{553}, give k_{11} values two orders of magnitude higher than the azurins, which in turn are three orders of magnitude higher than the plastocyanins, with cytochrome *c* giving the slowest value. This pattern indicates that despite general similarities, the plastocyanins and azurins have developed a somewhat different electron-transfer pathway. On the one hand, the copper site itself is probably closely similar in the plastocyanins and azurins (Colman et al., 1978; Adman et al., 1978). On the other hand, the reactivity of a low molecular weight copper complex with the same ligands and coordination geometry is expected to be considerably higher than observed for the proteins (Wherland & Gray, 1977). Thus, the variability between the plastocyanins and the azurins must arise from differences in their protein envelope surface properties and probably also in the "kinetic accessibility" of the copper site. Kinetic accessibility has been defined (Wherland & Gray, 1976, 1977) by the reactivity of proteins with $Fe(EDTA)^{2-}$ and is taken to reflect the proximity to which the metal site can be approached by a reagent which does not interact favorably with the protein surface.

There is little, if any, difference between the self-exchange values for cytochromes c_{551} and c_{553}, but a huge difference between these two and horse heart cytochrome *c*. The two procaryotic cytochromes are much smaller than the mammalian, with cytochrome c_{553} being one of the smallest of the *c* type cytochromes known. It has been shown, at least for cytochrome c_{551}, that the difference in molecular weight is primarily accounted for by a large deletion rather than by changes throughout the molecule. Cytochrome c_{553} has not been sequenced, but extrapolating from the strong conservation of certain residues and regions in the cytochrome *c* family, it is reasonable to expect that it is similar to cytochrome c_{551}, possibly with a further deletion near the N terminus of the sequence. For cytochrome c_{551}, the x-ray structure has shown (Dickerson et al., 1976) that the large deletion has caused further opening of the heme crevice, which makes it more accessible. A second feature which may make horse heart cytochrome *c* generally less reactive is its high positive charge relative to the other cytochromes. Multiple electrostatic interactions are expected to be important in protein-protein interactions (Kirkwood & Shoemaker, 1952a,b; Salemme, 1977); thus, despite the high ionic strength and the finding that the single electrostatic interactions in small molecule-protein electron-transfer reactions are relatively unimportant (Wherland & Gray, 1976, 1977) this possibility should not be discounted and should be further pursued, especially in the context of the interaction of these small electron carriers with their respective membrane-bound complex partners. The presence of a second area of relatively exposed heme with a much different electrostatic and neutral amino acid side-chain environment appears to open a high reactivity pathway to general protein-protein electron transfer in the two procaryotic cytochromes.

An extensive set of data on electron-transfer reactions of low molecular weight coordination complexes with proteins has been collected and analyzed by the Marcus theory formalism (Cummins & Gray, 1977; Goldberg & Pecht, 1976; Wherland & Gray, 1976, 1977). It is of interest to compare the results and conclusions of these previous studies with those from the present work. The small molecule data are summarized by Cummins & Gray (1977) and include data on horse heart cytochrome *c*, cytochrome c_{551}, bean plastocyanin, and *Ps.* azurin. The k_{11} values calculated from the protein-protein reaction data are larger than those calculated from the small molecules for azurin and cytochrome c_{551}, but among the lowest values calculated for cytochrome *c* and bean plastocyanin. Thus, relative to the lower molecular weight reagents which lead to the highest k_{11} values ($Co(phen)_3^{3+}$, $Fe(CN)_6^{3-}$, and $Ru(NH_3)_5(py)^{3+}$), the protein-protein derived k_{11} values are higher for the bacterial proteins and lower for the chloroplast and mitochondrial proteins. This may indicate that the soluble proteins studied in this work are better models for the native partners in the bacterial system than in the mitochondrial or chloroplast systems. Azurin and cytochrome c_{551} may well react at near their optimum rates while plastocyanin and cytochrome *c* react via a consistent but inefficient mechanism with the proteins used in this study. It is not clear what feature of the proteins causes this dichotomy but also yields such consistent k_{11} values, since with the low molecular weight reagents, no such consistent k_{11} values were found. A further point in the comparison is that there is no clear correlation with

the "kinetic accessibility" scale as defined by the cross-reactions with $Fe(EDTA)^{2-}$.

The last three entries in Table II are data from the literature which do not fit the correlation of the previously analyzed data. The parsley plastocyanin–cytochrome *c* value is in disagreement by four orders of magnitude, while the parsley plastocyanin–cytochrome c_{551} result is in good agreement. An attempt was made to measure the bean plastocyanin–cytochrome *c* cross-reaction rate constant, but because the observed rates were rather slow, only two sets of concentrations could be analyzed. Assuming an equilibrium constant of 34 as predicted from the potentials, a rate constant for reduction of bean plastocyanin by cytochrome *c* of about $3.5 \times 10^4\ M^{-1}\ s^{-1}$ was calculated. This value is much closer to that predicted, but also rather high. These results may indicate that the k_{11} value for cytochrome *c* will prove to be more erratic, like the results with small molecules. Alternatively they may reflect a more complex mechanism, since cytochrome *c* has a high, net positive charge and plastocyanin a large, net negative charge. Better data over a wider concentration range are needed to properly test for complex formation. The last and most significant system involves cytochrome *f* from parsley. In this case no comparison may be made from our data. However, applying the Marcus theory analysis and calculating the apparent k_{11} value for cytochrome *f* (using the cross-reaction rate constants and equilibrium constants of Table II and the k_{22} values derived for azurin and plastocyanin from the rest of the reactions) gives a disagreement of five orders of magnitude between the two calculated k_{11} (cytochrome *f*) values. Thus, the effective k_{11} value must vary for at least one of the proteins. Since the higher reactivity accompanies the reaction with plastocyanin, this is the only available example which might indicate true selectivity. Since this reaction involves the blue copper protein which showed low reactivity with the cytochromes in this study, compared to the reactivity with small molecules, it is one of the best candidates to have a latent, efficient mechanism.

In conclusion, the available data for protein–protein electron-transfer reactions prove to be remarkably self-consistent in that they can be formally well represented by the simplest outer-sphere electron-transfer model. Each of the proteins considered in this study (with the exception of cytochrome *f*) shows a single mechanism of electron transfer in their reactions with each other. The same proteins, when they react with a variety of inorganic transition-metal complexes, show a variety of mechanisms, based on comparison of the calculated reactivity index k_{11} of the Marcus theory treatment. For the azurins and for cytochrome c_{551}, the reactivity (k_{11}) with other proteins is higher than that with the small molecules, while for cytochrome *c* and plastocyanin the reactivity is lower than with most of the small molecules. Assuming that the reactivity of the plastocyanins and mammalian cytochrome *c* is high and comparable to that of the azurins and cytochromes c_{553} or c_{551} in vivo, the reactions between these latter proteins and their physiological partners must involve types of interaction not available in this in vitro comparison. The natural choice for an additional type of interaction is the involvement of the membrane and the spatial organization provided by the mitochondrion or chloroplast. For the bacterial proteins, the reactivity is quite high. The high reactivity alone cannot be taken as evidence that two proteins are physiological partners, as proteins which cannot be specific partners show high reactivity, but neither can the lack of kinetic selectivity eliminate, for example, the *Ps.* azurin and cytochrome c_{551} from being partners. If these two proteins are specific partners, however, specific recognition is probably not involved. The constancy of the calculated k_{11} values for these reactions is clear evidence that kinetic selectivity does not operate for any of the combinations tested.

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Supplementary Material Available

Tables of data and fitting results (3 pages). Ordering information is given on any current masthead page.

References

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol.* (in press).

Ambler, R. P., & Brown, L. H. (1967) *Biochem. J. 104,* 784.

Ambler, R. P. (1971) in *Recent Developments in the Chemical Study of Protein Structures* (Previero, A., Pechere, J.-F., & Coletti-Previero, M. A., Eds.) p 289, INSERM, Paris, 1971.

Ambler, R. P., & Wynn, M. (1973) *Biochem. J. 131,* 485.

Bishop, N. I. (1971) *Annu. Rev. Biochem. 40,* 197.

Bohner, H., & Böger, P. (1978) *FEBS Lett. 85,* 337.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272,* 313.

Cummins, D., & Gray, H. B. (1977) *J. Am. Chem. Soc. 99,* 5158.

Dickerson, R. E., & Timkovich, R. (1976) *Enzymes, 3rd Ed., 11,* Chapter 7.

Dickerson, R. E., Timkovich, R., & Almassy, R. J. (1976) *J. Mol. Biol. 100,* 473.

Goldberg, M., & Pecht, I. (1976) *Biochemistry 15,* 4197.

Greenwood, C., Finazzi-Agro, A., Guerrieri, P., Avigliano, L., Mondovi, B., & Antonini, E. (1971) *Eur. J. Biochem. 23,* 321.

Gupta, R. K. (1973) *Biochim. Biophys. Acta 292,* 291.

Horio, T., Sekuzu, I., Higashi, T., & Okunuki, K. (1961), *Haematin Enzymes* (Falk, J. E., Lemberg, R., & Morton, R. K., Eds.) p 302, Pergamon Press, Oxford.

Jovin, T. M. (1975) in *Trends in Biochemical Fluorescence Spectroscopy* (Chen, R., & Edelhoch, H., Ed.) p 305, Marcel Dekker, New York, N.Y.

Kirkwood, J. G., & Shoemaker, J. B. (1952a) *Proc. Natl. Acad. Sci. U.S.A. 38,* 855.

Kirkwood, J. G., & Shoemaker, J. B. (1952b) *Proc. Natl. Acad. Sci. U.S.A. 38,* 863.

Kunert, K.-J., & Böger, P. (1975) *Z. Naturforsch. C 30,* 190.

Lach, H.-J., Rüppel, H. G., & Böger, P. (1973) *Z. Pflanzenphysiol. 70,* 432.

Marcus, R. A., & Sutin, N. (1974) *Inorg. Chem. 14,* 213.

Margalit, R., & Shejter, A. (1973) *Eur. J. Biochem. 32,* 492.

Milne, P. R., Wells, J. R. E., & Ambler, R. P. (1974) *Biochem. J. 143,* 691.

Morton, R. A., Overnell, J., & Harbury, H. A. (1970) *J. Biol. Chem. 245,* 4653.

Ramshaw, J. A. M., Brown, R. H., Scawen, M. D., & Boulter, D. (1973) *Biochim. Biophys. Acta 303,* 269.

Rosen, P., & Pecht, I. (1976) *Biochemistry 15,* 775.

Salemme, F. R. (1977) *Annu. Rev. Biochem. 46,* 299.

Siegelman, M. H., Rasched, I. R., Kunert, K.-J., Kroneck, P.,

& Böger, P. (1976) *Eur. J. Biochem. 64,* 131.
Strehlow, H., & Jen, J. (1971) *Chem. Instrum. 3,* 47.
Sutin, N. (1977) *Adv. Chem. Ser., No. 162,* 156.
Wherland, S., & Gray, H. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2950.
Wherland, S., & Gray, H. B. (1977) in Biological Aspects of Inorganic Chemistry (Cullen, W. R., Dolphin, D., & James, B. R., Eds.) p 289, Wiley, New York, N.Y.
Wilson, D. F., Erecinska, M., Lindsay, J. G., Leigh, J. S., Jr., & Owen, C. S. (1975a) *Proc. FEBS 10th Meet. 40,* 195.
Wilson, M. T., Greenwood, C., Brunori, M., & Antonini, E. (1975b) *Biochem. J. 145,* 449.
Wood, P. M. (1974) *Biochim. Biophys. Acta 357,* 370.
Yamanaka, T. (1966) *Biochemistry of Copper* (Peisach, J., Aisen, P., & Blumberg, W. E., Eds.) p 275, Academic Press, New York, N.Y.

Size of Histone Gene Transcripts in Different Embryonic Stages of the Sea Urchin, *Strongylocentrotus purpuratus*†

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ABSTRACT: Histone genes in sea urchins are organized into a repeat unit of DNA with a modal length of 6–7 kilobases. Since all five histones are encoded in the unit, it is possible that a large initial transcript containing the sequences of the complete repeat is cleaved into 9S histone mRNAs. We prepared total RNA from embryos labeled for 90 min or 3 h with ^{14}C-labeled nucleosides and for the last 10 min of this period with [^{3}H]uridine. The RNA was sedimented in 85% formamide gradients and the position of the histone transcripts was then determined by hybridization with the histone DNA recombinant, pCO1. Unless care was taken to denature the RNA, considerable aggregation of the histone transcripts was observed. Using denaturing conditions, no high molecular weight histone transcripts were identified in RNA from the late cleavage embryo; all hybridizable RNA was in the 7–12S region of the gradient. Treatment of the embryos with toyocamycin or proflavin, or growth at 9 °C did not alter the results. Transcripts from four-cell embryos were also devoid of high molecular weight histone RNA. However, RNA prepared from mesenchyme blastula embryos did contain high molecular weight transcripts which hybridized to the histone DNA. These results were obtained when the hybrids were not treated with RNase. The high molecular weight RNA was markedly enriched in newly synthesized transcripts as determined from the high $^{3}H/^{14}C$ ratio of hybridized counts. These transcripts are further differentiated from the 7–12S RNA in that they are twice as sensitive to RNase when in hybrid form and can be recycled through another round of 85% formamide gradients retaining, for the most part, their large size.

The histone genes of the sea urchin are organized into a repeating structure which contains coding sequences for all five major histones (Weinberg et al., 1975; Birnstiel et al., 1975; Kedes et al., 1975b; Cohn et al., 1976; Wu et al., 1976; Schaffner et al., 1976; Portmann et al., 1976; Holmes et al., 1977). Superficially, at least, this structure resembles a prokaryotic operon in that related proteins are encoded on adjacent stretches of DNA. The structure might serve as a template for a multigene transcript which would then be processed into the mRNAs. The availability of cloned histone gene fragments (Kedes, 1975; Clarkson et al., 1976; Overton & Weinberg, 1978) provides a pure hybridization probe to identify newly synthesized histone transcripts. Hybridization of labeled RNA to the probe, present in DNA excess, should indicate the amount of newly synthesized RNA in the fraction tested. This procedure allows a direct measurement of the labeled RNA and not merely a determination of the total complementary RNA concentration. It is particularly important to design a method to distinguish old and new transcripts (Curtis & Weissmann, 1976; Ross, 1976), especially since aggregation is of great concern in these experiments (Nemer et al., 1974). In our experiments, we have distinguished the newly made RNA from older transcripts by labeling embryos for a long period with one isotope and for a short period with a second isotope.

A multi-gene histone transcript would be a convenient means of coordinate regulation of protein synthesis. However, as we report here, we have not been able to identify a large transcript in the late cleavage stage where there is a high level of histone mRNA synthesis. We have, on the other hand, demonstrated that part of the newly synthesized histone transcript in the mesenchyme blastula is greater than 18S in size. This developmental stage is characterized by a shift in the synthesis of particular H2A, H2B, and H1 subtypes (Cohen et al., 1975) made on different mRNAs which are encoded by different genes (Weinberg et al., 1978; Newrock et al., 1978; Kunkel & Weinberg, 1978). A fundamental change in the expression of histone genes appears to occur at this embryonic stage.

Materials and Methods

Embryo Development and Preparation of Labeled RNA. Eggs of *S. purpuratus* (Pacific Biomarine Supply Co.) were fertilized and cultured at 17 °C. Embryos which were cultured for 9 h at 17 °C developed to the 128 cell stage, just starting to form a blastocoel. These embryos are designated to be in the "late cleavage" stage. Embryos cultured for 22.5 h at 17 °C

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