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Electrostatic interactions of 4-carboxy-2,6-dinitrophenyllysine-modified cytochromes *c* with physiological and non-physiological redox partners

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An analysis of the effect of electrostatic properties of 4-carboxy-2,6-dinitrophenyllysine (CDNP-lysine) cytochromes *c* on their reactions with strongly and weakly binding redox partners is given. For strongly binding systems (cytochrome-*c* oxidase, cytochrome-*c* reductase, sulphite oxidase and yeast cytochrome-*c* peroxidase) the magnitude of the dipole moments of the CDNP cytochromes *c* determines their relative reactivities. For weakly binding redox agents, such as hexacyanoferrate(III), cobalt(III)tris(1,10-phenanthroline), azurin and plastocyanin, the electrostatic potential at the haem edge accounts for the greater part of the relative activities. Relative rate data were obtained from the literature. It is concluded that the dipole moment of native cytochromes *c* may account for an approx. 50-fold increase in the efficiency of its physiological activity towards membrane-bound enzymes. A correction on a formula to describe the contribution of a molecular dipole moment to the ionic strength dependence of a bimolecular rate constant (Koppenol, W.H. (1980) Biophys. J. 29, 493–508) leads to an equation nearly identical to that obtained by Van Leeuwen et al. (Van Leeuwen, J.W., Mofers, F.J.M. and Veerman, E.C.I. (1981) Biochim. Biophys. Acta 635, 434–439).

Introduction

The role of electrostatics in increasing the efficiency and selectivity of biochemical reactions is generally regarded as important [1]. Electrostatic interactions may occur at several levels, but in general coulombic attractions may first help to orient protein and substrate. Then short-range forces, if favourable, may further enhance the likelihood of the reaction. Although these concepts may be apparent in theory, it is difficult to find systems in which rate enhancement can be unambiguously observed or quantified. Changes in the electrostatic properties of proteins by chemical modification usually involve alterations of these properties at all levels: monopolar charge, dipole moment, and the micro-properties of the electric potential field simultaneously. Since we are presently investigating the electrostatic properties of proteins [2,3] in relation to the location of their reactive sites, a system in which the influence

Abbreviations: CDNP-lysine, 4-carboxy-2,6-dinitrophenyllysine; b, radius; k_B, the Boltzmann constant; k_1, bimolecular rate constant; k_{inf}, bimolecular rate constant at infinite ionic strength; P, the dipole moment; R, 'hydrated' radius of the molecules; T, absolute temperature, Z, charge (in units of e); e, the elementary unit of charge; ϵ_0, dielectric permittivity; ϵ, the static dielectric constant; θ, the angle between the dipole moment vector and the vector from the centre of mass to the site of reaction; κ, the inverse of the debye length = $0.33\sqrt{\mu}$ A^{-1}; and μ, ionic strength.

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of the above-mentioned changes can be resolved was chosen for analysis.

This report is primarily a re-evaluation of the extensive rate data compiled for the reactions of 4-carboxy-2,6-dinitrophenyllysyl derivatives of horse cytochrome *c* [4–16]. The mono-CDNP derivatives, first prepared by Margoliash and coworkers [15,16], involve a substitution of the positive charge of one of the lysines by an anionic group without otherwise affecting the tertiary structure or redox properties of the protein as a whole [17,18]. However, the dipole moments as well as details of the electrostatic field are changed [19] so that the CDNP-lysine derivatives represent a homologous series, identical in net charge, and well adapted to the identification of these influences on protein activity.

A topic central to the interpretation of these properties, the influence of a molecular dipole moment on the rate and ionic strength dependence of a reaction, will be addressed in Appendix B. At present there are conflicting theories in the literature concerning the dipolar effect [20,22]. This study may also elucidate the adaptive significance of the asymmetric charge distribution on mitochondrial cytochromes *c* in terms of the effect which their substantial dipole moments have on the efficiency of their physiological function.

Data and Treatment

The reactions of the CDNP-modified cytochromes *c* with the following haem proteins: cytochrome-*c* oxidase [4–6], cytochrome-c_1 reductase [7,8], sulphite oxidase [9], and yeast cytochrome-*c* peroxidase [10] have been measured by a variety of methods in enzymatic (steady-state) systems as well as by stopped-flow spectrophotometry. In the case of enzymatic studies the bimolecular rate constants of the CDNP-lysine cytochromes towards the physiological reaction partners are the best indicators of activity. Reactions of the CDNP-lysine derivatives with weakly binding redox agents have been carried out by stopped-flow spectrophotometry, primarily by Sykes and coworkers. These include the reactions with cobalt(III)tris(*o*-phenanthroline) and hexacyanoferrate(III) [11], cobalt(II)sepulchrate and ferrous EDTA [12], and the blue copper proteins: azurin and parsley plastocyanin [13], stellacyanin and algal plastocyanin [14]. Results obtained with yeast cytochrome b_2 [23] are also placed in this cate-

TABLE I

ELECTROSTATIC PROPERTIES OF CDNP-LYSINE-MODIFIED PROTEINS

Cytochrome	No.	Dipole moment					$\Psi H(60) - \Psi H(x)$
		Fe(III)	Fe(II)	γ_1 [a]	γ_2 [b]	γ_3 [c]	(k_BT/e)
CDNP-lysine	7	428 [e], 380 [f]	407 [e], 366 [f]	31	50	60	−0.05
	8	384 , 322	366 , 307	42	46	59	0
	13	243 , 192	222 , 179	30	48	58	−0.25
	25	286 , 383	385 , 379	30	60	81	−0.40
	27	317 , 318	314 , 316	31	59	83	−0.75
	39	462 , 467	445 , 454	19	17	48	−0.10
	60	481 , 457	458 , 439	15	22	37	0
	72	187 , 219	175 , 208	11	35	47	−0.25
	73	272 , 271	249 , 254	41	7	29	−0.05
	86	242 , 194	223 , 172	38	15	25	−0.05
	87	309 , 250	287 , 226	48	9	23	+0.05
	99	477 , 439	457 , 420	19	28	40	0

[a] Angle with respect to the dipole moment of horse ferrocytochrome *c* (deprotonated propionic acids).
[b] Angle with respect to the haem plane.
[c] Angle with respect to the sulphur atom of Cys-17.
[d] Difference in potential in front of the haem edge as calculated before [19].
[e] Propionic acids ionized.
[f] Propionic acids unionized.

gory. For the present purpose activities of the CDNP derivatives are related to the activity of the lysine-60-modified protein which is normally about as reactive as the native protein and has the largest dipole moment of the derivatized cytochromes *c*. The dipole moments of CDNP derivatives with protonated and deprotonated haem propionic acid side-chains were taken from, or calculated according to Ref. 19. This reference also lists many of the relative activities of the CDNP derivatives with haem protein reactants, although in relation to the activity of the native protein. Table I lists the dipole moments of the ferri- and ferrocytochrome *c* derivatives (for protonated and deprotonated propionic acids) and relative activities ($k_{rel} = k/k_{60}$) towards the haem protein partners.

The electrostatic potentials at the haem edge (Ψ_H) of cytochrome *c* and its derivatives were calculated as described in Ref. 19 from crystallographic coordinates of tuna cytochrome *c* [24] into which the sequence of the horse protein was substituted. A homogeneous dielectric constant of water ($\epsilon = 78$) was assumed. The haem edge was defined at the surface site through which a line passing through the plane of the haem intersects the methene carbon bridge between haem pyrroles II and III, and the back surface of the protein near lysine-99 [19]. These surface potentials are listed in Table I, and relative rate data for the weakly binding non-physiological reactants in Table III.

Theory

Two approaches to the influence of dipole moment on reaction rates have obtained different results [20,19]. For the purposes of this analysis we employ the form reached by Van Leeuwen et al. [19].

$$\ln k_I = \ln k_{inf} - \frac{e^2}{4\pi\epsilon\epsilon_0 k_B TR} \times \{Z_A Z_B \kappa f_m(\kappa) + (Z_B P_A \cos\theta_A / eR) f_d(\kappa)\} \quad (1)$$

The ionic strength screening functions for monopole and dipole interactions f_m and f_d are here left unspecified. Eqn. 1 is for reaction between a species of charge Z_B and a dipolar ion of charge Z_A and dipole moment P_A oriented at angle θ_A to a line from the site of electron transfer of ion A to its centre of mass. In the analysis of comparative activity this can be simplified, since the net charges of all CDNP derivatives are the same as well as Z_B (the charge of the common reactant) and the values of the f_m and f_d at constant ionic strength are constant. If all differences in activity are due to bulk electrostatic factors, i.e., the rates at infinite ionic strength are identical, Eqn. 1 results in the proportionality:

$$\ln k_{rel} = \ln(k_A/k_B) = \text{constant} \times (P_A \cos\theta_A - P_B \cos\theta_B) \quad (2)$$

where subscripts refer to respective properties of two CDNP derivatives. If the reaction is not over-sensitive to the precise orientation of the dipole moment, Eqn. 2 results in a single linear relation between the logarithm of relative activity and the difference in protein dipole moments.

In order to calculate Z_B, expressions for f_m and f_d are required. For reactions between macromolecules A and B, the expressions: $f_m = \{1 - \exp(-2\kappa R_B)\}/(2\kappa R_B(1 + \kappa R_A))$ and $f_d = (1 + \kappa R)^{-1}$ have been proposed [22], and are used in Eqn. 1 for the evaluation of the ionic strength dependences of cytochrome *c* and its derivatives with metalloproteins. This expression is also used to determine the magnitude of the charge Z_B of the redox partner of cytochrome *c* from relative activities of the CDNP derivatives (see text). It should be noted that Eqn. 1 does not include terms for the interaction of the net charge of cytochrome *c* with the dipole moment of the redox partner, but this will not affect the analysis unless cytochrome *c*'s of different net charge are to be compared.

Results

Fig. 1 is a schematic view of the front face of ferrocytochrome *c* showing the α-carbon positions of certain lysine residues and the sites where the dipole moments (computed for ionized haem propionates) intersect the surface. The CDNP-lysine-60 cytochrome *c* vector lies about in the centre of the cluster which can be circumscribed by a section comprising about 15% of the protein's surface

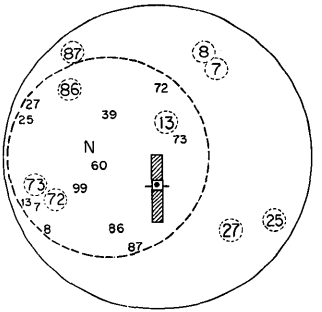

Fig. 1. A schematic representation of the front (haem edge) surface of ferrycytochrome *c* shows the intersections of the dipole moment vectors of the CDNP derivatives with the protein surface. The large patch indicates that these vectors are within a ±45° angle with respect to the centre of the patch, which is close to the position of the dipole moment vector of the CDNP-lysine-60 derivative. Front surface lysine modifications are indicated by number within the smaller dashed circles which represents their α-carbon position. The porphyrin plane is shaded and the site on the surface at which the haem crevice potential was measured is indicated.

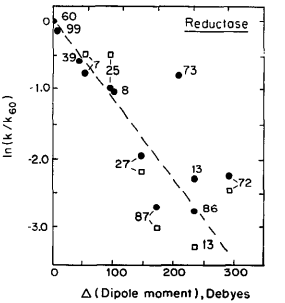

Fig. 2. Two sets of relative activity data for the reaction of CDNP ferrocytochromes *c* with (●) ubiquinol cytochrome-*c* reductase and (□) purified cytochrome c_1 are plotted against the difference in dipole moments relative to that of the lysine-60 modified protein. The respective ionic strengths were 0.01 [7] and 0.03 M [8]. The data were plotted from values in Table I with the assumption that haem propionic acids are protonated.

area, or a cone with a vertex angle of 90°, as indicated on the figure. The haem edge is within the dipole cluster, but its angles with respect to the dipole vectors vary from about 20° to 60°. Modifications of back surface lysines displace the dipole vector only slightly, while frontal modifications have a much greater effect. The degree of propionate ionization and the protein oxidation state have only a small effect on the locus of the dipole moment vectors.

In Figs. 2–4 the relative activities of the CDNP derivatives (Tables II and III) are plotted versus the difference in magnitude of the dipole moment with respect to CDNP-lysine-60 cytochrome *c*. Fig. 2 combines two data sets for the relative activities of CDNP ferricytochromes *c* towards ubiquinol cytochrome-*c* reductase (enzymatic) and purified cytochrome c_1 (stopped flow). Fig. 3A represents enzymatic measurements of the cytochrome-*c* oxidase reaction at 25 mM ionic strength and Fig. 3B, stopped-flow measurements of the reaction of ferrocytochrome *c* with oxidase at 133 mM ionic strength. In Fig. 3 the dipole moment differences for cytochromes with both protonated and deprotonated propionates have been used to

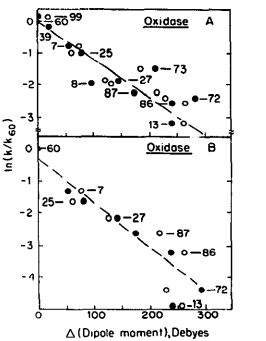

Fig. 3. Relative activity vs. dipole moment difference plots for the reactions of CDNP ferrocytochrome *c* derivatives with cytochrome *c* oxidase. Data in A represent enzymatically measured activities at 0.025 M ionic strength, and in B direct stopped-flow measurements at 0.133 M ionic strength. ○, dipoles computed assuming both ferrocytochrome *c* haem propionates are ionized; ●, unionized groups.

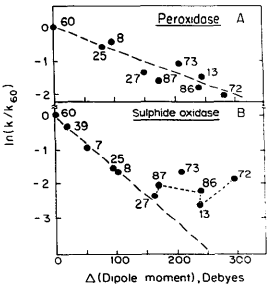

Fig. 4. Relative activity data for the reactions of (A) CDNP ferrocytochromes *c* with yeast cytochrome-*c* peroxidase and (B) CDNP ferrocytochrome *c* with cytochrome *c* sulphite oxidase. The dipole moment differences assume unionized haem propionic acid groups. Data were plotted from Tables I and II. In B a pattern of reactivity for front face CDNP modifications is high-lighted.

show that the variation of this parameter appears not to be significant. Figs. 4A and B represent the order of activity with yeast peroxidase and with sulphite oxidase, respectively. The indicated slopes in these figures require net charges on the physiological redox partners of $-70e$ (cytochrome-*c* reductase), $-50e$ and $-72e$ (cytochrome-*c* oxidase, Fig. 4A and B, respectively), $-14e$ (cytochrome-*c* peroxidase) and $-50e$ (sulphite oxidase), assuming a charge of $+6e$ on CDNP ferrocytochromes. Ionization of the propionic acids lowers the net charge by $2e$, such that the above charges appear to be lower limits. With the exception of peroxidase, a radius of 40 Å was assumed for all redox partners in the calculation of their charge. Published data [26] for the charge ($-14e$), radius (25 Å, and dipole moment (425 debyes) of yeast cytochrome-*c* peroxidase were used to calculate the slope indicated in Fig. 3B, using the full equation derived by Van Leeuwen [22].

The linear dependence in all the plots seems sufficient to justify most of the assumptions used in Eqn. 2. The increasing scatter from linear dependence shown by front-face modifications (lysines 25, 86, 87, 13, 72) in Fig. 1 might reflect greater difficulty in resolving the enzyme kinetics of the more weakly binding CDNP derivatives. The generally high activity of CDNP-lysine-73 cytochrome *c* and the inversion of the expected relative activities of CDNP-lysine-72 and CDNP-lysine-13 cytochrome *c* which is observed are persistent discrepancies in most data sets. In Fig. 3B the front face modifications exhibit a particular

TABLE II

RELATIVE ACTIVITIES OF CDNP-LYSINE-MODIFIED CYTOCHROMES *c* TOWARDS PHYSIOLOGICAL REDOX PARTNERS

Redox partner	Cytochrome, CDNP-lysine											
	7	8	13	25	27	39	60	72	73	86	87	99
Cytochrome *c* reductase												
enzymic [a]	0.51	0.36	0.10	0.36	0.14	0.60	1	0.11	0.44	0.07	0.07	0.87
stopped-flow [b]	0.63	–	0.04	0.65	0.11	–	1	0.09	–	0.06	0.05	–
Cytochrome *c* oxidase												
enzymic [c]	0.47	0.13	0.04	0.40	0.16	0.88	1	0.05	0.22	0.08	0.10	1.2
stopped flow [d]	0.26	–	0.01	0.19	0.11	–	1	0.01	–	0.04	0.08	–
Yeast cytochrome *c* peroxidase [e]	–	0.63	0.24	0.57	0.24	–	1	0.13	0.33	0.16	0.19	–
Sulphite oxidase [f]	0.41	0.20	0.06	0.21	0.11	0.74	1	0.16	0.17	0.11	0.13	–

[a] Ref. 7 (measured enzymatically at $\mu = 100$ mM).
[b] Ref. 8 (measured by stopped-flow at $\mu = 300$ mM).
[c] Refs. 5 and 6 (measured enzymatically at $\mu = 25$ mM).
[d] Ref. 4 (measured by stopped-flow at $\mu = 133$ mM).
[e] Ref. 10 (enzymic measurements at $\mu = 50$ mM).
[f] Ref. 9 (enzymatic measurements at $\mu = 50$ mM).

TABLE III

RELATIVE RATES OF CDNP-LYSINE-MODIFIED CYTOCHROMES *c* WITH SMALL IONS, COPPER PROTEINS AND CYTOCHROME b_2

Reactant	Cytochrome, CDNP-lysine								
	7	13	25	27	39	60	72	86	87
$Fe(CN)_6^{3-}$ [a]	0.77	0.30	0.60	0.39	–	1	0.25	0.59	0.70
$FeEDTA^{2-}$ [b]	0.86	0.68	0.75	0.39	–	1	0.31	0.83	0.84
$Ascorbate^{2-}$ [c]	0.67	0.51	0.51	–	0.63	1	0.33	0.74	0.67
$Fe(CN)_5NH_2pyr^{3-}$ [a]	–	0.81	–	–	–	1	0.65	–	1.1
$Fe(CN)_5imid^{3-}$ [a]	–	0.44	–	–	–	1	0.41	–	1.1
$Co(phen)_3^{3+}$ [a]	1.2	2.1	1.7	6.5	–	1	2.6	1.0	1.1
$Co(sep)^{2+}$ [b]	1.3	1.4	1.6	2.7	–	1	1.3	1.2	1.1
O_2^- [d]	–	0.88	–	0.63	–	1	0.23	0.45	0.71
Azurin [e]	1.2	4.0	2.0	4.7	–	1	6.0	1.2	1.0
$Plastocyanin^{7-}$ [e] (parsley)	0.80	0.50	0.57	0.54	–	1	0.87	0.57	0.60
$Plastocyanin^{2+}$ [f] (*Anabena Vulgaris*)	1.4	6.3	2.5	3.5	–	1	6.8	1.2	1.2
Stellacyanin [f]	1.0	1.7	–	2.7	–	1	7.1	1.1	1.1
Cytochrome b_2 [g]	–	0.41	–	0.40	–	1	0.52	0.76	0.60

[a] Ref. 10.
[b] Ref. 11.
[c] E. Margoliash, unpublished data ($\mu = 2$ mM).
[d] Ref. 25.
[e] Ref. 12.
[f] Ref. 13.
[g] Ref. 23.

pattern of activity towards sulphite oxidase. The enzyme kinetics in this system are relatively straightforward [9] and the deviation from the dipolar slope cannot be attributed to experimental limitations.

A consequence of assigning a dominant influence to dipolar effects is the very high negative charge assigned to some of the substrates. The temperature and ionic strength dependences of the reactions of the CDNP ferrocytochromes *c* with

TABLE IV

COMPARISON OF EXPERIMENTAL AND CALCULATED IONIC STRENGTH DEPENDENCES FOR REACTIONS OF CDNP FERROCYTOCHROME *c* WITH CYTOCHROME-*c* OXIDASE

Experimental data were taken from Fig. 4 of Ref. 4. Calculated values are best fits for five values in the ionic strength range from 0.08 M $< \mu <$ 0.22 M obtained with Eqn. 1, the tabulated values of k_{inf}, and $z(\text{oxidase}) = -72e$.

Cytochrome, CDNP-lysine	k(0.08 M) ($M^{-1}\cdot s^{-1}$)		k(0.22 M) ($M^{-1}\cdot s^{-1}$)		k_{inf} [a] ($M^{-1}\cdot s^{-1}$)
	experimental	calculated	experimental	calculated	
7	$1.4\cdot10^7$	$1.7\cdot10^7$	$1.0\cdot10^6$	$1.2\cdot10^6$	$3.0\cdot10^4$
13	$1.8\cdot10^5$	$1.7\cdot10^5$	$1.5\cdot10^4$	$2.0\cdot10^4$	$2.0\cdot10^3$
25	$1.1\cdot10^7$	$1.3\cdot10^7$	$7.2\cdot10^5$	$8.0\cdot10^5$	$2.6\cdot10^4$
27	$4.3\cdot10^6$	$4.8\cdot10^6$	$3.7\cdot10^5$	$4.2\cdot10^5$	$2.2\cdot10^4$
60	$5.0\cdot10^7$	$5.8\cdot10^7$	$2.5\cdot10^6$	$2.8\cdot10^6$	$6.0\cdot10^4$
72	$5.3\cdot10^5$	$5.3\cdot10^5$	$5.6\cdot10^4$	$6.7\cdot10^4$	$1.0\cdot10^4$
87	$3.3\cdot10^6$	$3.6\cdot10^6$	$3.1\cdot10^5$	$3.6\cdot10^5$	$2.3\cdot10^4$

[a] A best fit value. Higher values probably reflect, in part, dipole-dipole interactions with oxidase.

cytochrome-*c* oxidase (Fig. 2 B) are consistent with this. Eyring plots for native and derivatized cytochromes were parallel [4], showing that the differences in activity of the CDNP derivatives depend almost entirely upon the entropy of activation ($\Delta S^{\dagger}$) which varies from 30 e.u. (CDNP-lysine-60 cytochrome *c*) to 21 and 24 e.u. (CDNP-lysine-13 and -72 cytochrome *c*). Over this range of dipole moments ($P_{60} - P_{72} = 260$ debyes), the interaction with a charge $-72e$ accounts for a difference in $\Delta S^{\dagger}$ of 7 e.u. and in $\Delta H^{\dagger}$ of -0.5 Kcal/mol, the latter quantity being within the reported experimental uncertainty (16.5 $\pm$ 0.5 kcal/mol). These calculations are described in Appendix A. The charge of approx. $-72e$ on the oxidase preparation can also be supported by application of Eqn. 1 to the ionic strength dependence data reported in Fig. 4 of Ref. 4. Over a short range, $0.08\ M < \mu < 0.22\ M$, the CDNP-lysine-60 cytochrome *c* rate constant increases by a factor of twenty and that of CDNP-lysine-72 cytochrome *c* by a factor of about 10. This decrease in slope is explained by the difference in protein dipole moment. A comparison of experimental and recalculated values for the extreme points, with best fit values of k_{inf}, are given in Table IV. Other ionic strength data reported in these studies are consistent with high charges on the physiological redox partners, some of which likely comes from surfactant molecules associated with the protein preparation.

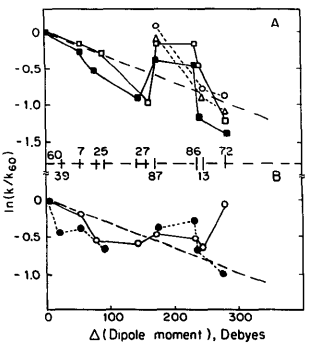

Fig. 5. In the upper figure, relative rate data from Table III for anionic redox agents: (■) $Fe(CN)_6^{3-}$, (□) $FeEDTA^{2-}$, (○)$Fe(CN_5NH_2pyr^{3-}$, and (△) $Fe(CN)_5imid^{3-}$ are plotted as in the previous figures. In the lower figure are plotted data for parsley plastocyanin^{7-} (○) and ascorbate ion^{1-} (●). The slopes indicated by dashed lines are for the dipolar trends expected of $Fe(CN)_6^{3-}$ (upper) and plastocyanin (lower). The CDNP modifications are identified on the centre scale. Some experimental points here and in Fig. 6 are slightly displaced for purposes of clarity.

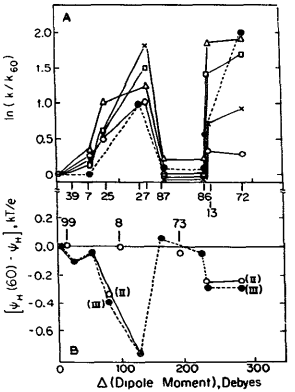

Fig. 6. In A, data from Table III for cations are plotted as before. The points are for (○) Co(III)sepulchrate^{2+}, (×) Co(III)phenanthroline^{3+}, (□) Azurin$^{0/1-}$, (△) *Anabena variabilis* plastocyanin^{3+}, and (●) Stellacyanin^{7+}. In the lower figure, B, the left hand scale indicates the difference in haem crevice potentials plotted against the same dipole difference scale as the upper, with the position of the various CDNP modifications indicated above. The connected points are for residues for which corresponding relative rate data are plotted above and in Fig. 5. Values for other CDNP derivatives are plotted with open circles. Differences in haem-crevice potential values between oxidized and reduced cytochrome *c* are indicated in the lower figure.

In Figs. 5 and 6A the relative rate data for CDNP derivatives with anionic and cationic nonbinding reactants are plotted against the same scale as the previous figures. The distinct pattern of activity exhibited in Fig. 5 is reversed in Fig. 6A such that each CDNP modification influences the reaction in an opposite and roughly equal

manner depending on the charge type of the reactant (azurin is an exception, it being neutral or slightly negatively charged yet behaving as a cation). The different types of activity indicate that each modification influences an electrostatic property of the protein at the reactive site, making it more or less favourable to the approach of a charged reactant. That this site must be the haem edge is apparent from the correspondence between the difference in surface electrostatic potentials at this site, plotted in Fig. 6B, and the respective variations in activity shown in Figs. 5 and 6A. A further observation is that the magnitude of the effect is largely independent of the net charge on the reactant, which might suggest that only a limited portion of the charge of the reaction partner interacts with the haem-crevice surface potential. Also the influence of the dipole moment, while hardly discernible in the cation pattern, appears to give a distinct bias to the pattern of the small anions, $FeEDTA^{2-}$ and $Fe(CN)_6^{3-}$, and is clearly important in the activity of the CDNP-lysine derivatives towards parsley plastocyanin ($-7e$) shown in Fig. 4B. Parsley plastocyanin shows anomalously high activity towards CDNP-lysine-72-modified ferrocytochrome *c*. It is perhaps significant that the pattern exhibited in its reactions with CDNP-lysine-27, CDNP-lysine-87, CDNP-lysine-86, CDNP-lysine-13, and CDNP-lysine-72 cytochrome *c* is very similar to that of sulphite oxidase which, though highly charged, binds weakly with ferrocytochrome *c*.

Discussion

We have presented an interpretation of the CDNP derivative data which assigns no particular effect to any of the specific modifications but only to their influence on the protein dipole moment of the potential near the haem edge. A crucial assumption in this theoretical treatment of physiological reactions is that orientation according to the dipole vector takes place. Recently, a circular dichroism study confirmed that in the reaction with photosynthetic reaction centres horse cytochrome *c* is oriented in exactly such a way [29]. The relative importance of haem edge potential or dipole moment in influencing activity seems to depend on whether the redox partner is strongly or weakly binding though, as in the case of sulphite oxidase, both influences seem to be important. The dipolar trend in the reactions with strongly binding proteins is clearly the dominant influence. From Figs. 2 and 3, an approx. 50-fold increase in rate of reaction towards its physiological partners is expected from the presence of a dipole moment of approx. 300 debyes, to the extent that the reactions in homogeneous solution are fair models for reactions at the membrane surface.

The treatment given the experimental data in Figs. 2–6 presents two theoretical problems. Firstly there is the dependence on dipole magnitude alone exhibited by some of the proteins. The dipole orientations vary considerably in angle from any fixed point on the surface of cytochrome *c* as is evident from Fig. 1. Secondly, there is the dichotomy between dipole moment dependence for the large, highly charged proteins and the almost exclusive dependence on surface electrostatic field strength exhibited by small ions or weakly charged proteins. Some qualitative arguments which might explain these phenomena are presented below.

Mathematically tractable treatments of the dipolar effect on reaction rates assume hard-sphere reactants with a point reactive site and an electrostatic field resulting from a net charge and a point dipole. It is generally assumed that the reaction rate depends on the concentration of reactant species in the reactive orientation and this may be calculated by equilibrium methods. That is, the reaction probability is relative to the Boltzmann probability of attaining the reactive orientation, which is a valid assumption when the reactions are much slower than diffusion controlled. Let us consider the reactions to be composed of four stepwise processes: (1) association of cytochrome *c* and the reaction partner, S; (2) orientation of the complex to the reactive site of S (cyt. $c \cdot S^*$); reorientation of cytochrome *c* to its reactive site (cyt. $c^* \cdot S^*$); and (4) electron transfer, resulting to products, as shown below.

(1) cyt. $c + S \rightleftharpoons$ cyt. $c \cdot S$

(2) cyt. $c \cdot S \rightleftharpoons$ cyt. $c \cdot S^*$

(3) cyt. $c \cdot S^* \rightleftharpoons$ cyt. $c^* \cdot S^*$

(4) cyt. $c^* \cdot S^* \rightarrow$ products

The first two processes might be considered as isoenergetic for the CDNP derivatives and do not contribute to the relative activity. In step 3, cytochrome c must reorient in a strong field where its dipole moment determines the probability of any orientation. Therefore the configuration cyt. $c^* \cdot S^*$ is reached N times through rotational diffusion during the lifetime of the state cyt. $c \cdot S^*$, and N is proportional to $\exp(-W_p \cos\theta / k_B T)$ where $W_p \cos\theta$ is the dipolar energy. If the probability of reaction every time cyt. $c^* \cdot S^*$ is attained is p, then the overall probability of reaction during the lifetime of cyt. $c \cdot S^*$ is $1-(1-p)^N$. When p is large ($p \to 1$) this overall probability is relatively independent of N and when it is small, as in the fully reversible case, the likelihood of reaction should be proportional to N. In the case of cytochrome c's highly efficient reactions with its oxidase and reductase, the former is probably true. The dipole moment exerts a positive effect on the rate because it restrains cytochrome c to generally frontal orientations where random motions usually result in reaction. As the dipole magnitude decreases, these same motions are more likely to extend to a region of highly unreactive orientations, such as the negatively charged back surface, which could cause separation of cytochrome c from its redox partner. This may account for the absence of a strict interaction angle dependence in the experimental data. Computer simulations of diffusion-controlled reactions of dipolar molecules indicate that broadening in the effective 'reactive patch' decreases the dependence on dipole angle [27]. The experimental dipole dependence does indicate that orientations are controlled by bulk electrostatic properties of the cytochrome c and that, for the most part, there is little evidence that any one of the front-face lysines are crucial to alignment or binding of the redox partners [28].

When the reaction partner is much larger than cytochrome c or its electrostatic field much stronger, it is convenient to regard the process as in steps 2 and 3 above in which the smaller, more mobile, ion reorients itself in the field of the larger. Smaller ions that react at rates much less than diffusion-controlled, distribute themselves in the ionic cloud around cytochrome c and their distribution nearby in bulk solution is approximated by the dipolar field. Very close to the surface, however, the dipole approximation breaks down and local electrostatic potentials determine the tendency of an ion to move towards any particular point. The rate will depend on this local electrostatic effect and the net dipole moment, since the latter property of the field determines the concentration of ions in nearby solution available for diffusion to the reactive site and its therefore most sensitive to the ionic strength. It might be inferred from the data in Figs. 5 and 6 that when dipole interactions are relatively weak, the surface potential 'maps' the distribution of counter-ion charges on the protein's surface. The dipole moment appears to influence the relative effect exerted by the CDNP-lysine-25 and CDNP-lysine-27 modifications on anion and cation reactivity (Figs. 5 and 6), since their dipoles would tend to attract anions strongly to the left side of the haem edge in Fig. 1 where repulsion by these modified groups is diminished. Presumably, there is an opposite effect for cations that increases the rate of their reactions with cytochromes c modified at lysines-25 and -27. Similar effects may explain the deviations exhibited by the relative activity of CDNP-lysine-72 cytochrome c towards parsley plastocyanin and sulphite oxidase.

A previous description of electrostatic influences on the rates of CDNP-modified cytochrome c with strongly binding redox partners has several limitations. Koppenol and Margoliash [19] relate the electrostatic work of reorienting the dipole of the modified cytochrome c to the original orientation of the native cytochrome c dipole in the surface electrostatic field of the physiological reaction partner to a rate process by assuming this work to be a part of the temperature dependent (enthalpic) term in the Arrhenius expression. Electrostatic factors are reflected most strongly in the entropy of activation as described in Appendix A. More importantly, the reorientation work refers to differences in states in which the dipole moments of varying strengths are reoriented within the electrostatic field of the protein redox partner. Since both initial and final (reoriented) states have different energies for different CDNP derivatives depending on the dipole magnitude, this is not a valid basis of comparison. This topic is dealt with in Appendix B concerning the relative activities of

dipoles at a charged membrane interface.

It has been found that the ionic strength dependences of small ion reactions with native cytochrome *c* tend to reflect their interaction with the protein dipole moment [2]. However, as outlined in Appendix A, the electrostatic potential at the surface is an ionic-strength-dependent property which should contribute to the variation of rates with ionic strength, though less strongly than the dipole and monopole moments which define the electrostatic field in bulk solution. Further experiments and more detailed theoretical treatments are obviously required to quantify the importance of surface potentials or 'site charges' to the reaction rates and binding properties of proteins.

Appendix A

The dipolar contribution to the electrostatic Gibbs energy of activation, $\Delta G_{el}^{\dagger}$, for reaction between an ion of charge Ze and a species with dipole moment P can be expressed by Eqn. 1-A:

$$\Delta G_{el}^{\dagger}(\text{dipolar}) = \frac{\Pi ZeP \cos\theta f_d(\kappa)}{4\pi\epsilon\epsilon_0 R^{\dagger 2}} \qquad (1\text{-A})$$

where Π is Avogadro's number and $R^{\dagger}$ is the radius of the reactive complex. The electrostatic enthalpy and entropy of activation [30] are related by Eqns. 2-A and 3-A, where $[\mathrm{d}(\ln\epsilon)/\mathrm{d}T]_P = 0.0046\ \mathrm{K}^{-1}$ at 25 °C in water.

$$\Delta H_{el}^{\dagger} = \Delta G_{el}^{\dagger}[1 + T(\mathrm{d}(\ln\epsilon)/\mathrm{d}T)_P] = -0.37\,\Delta G_{el}^{\dagger} \qquad (2\text{-A})$$

$$T\Delta S_{el}^{\dagger} = \Delta H_{el}^{\dagger} - \Delta G_{el}^{\dagger} = -1.37\,T\Delta G_{el}^{\dagger} \qquad (3\text{-A})$$

In general, electrostatic entropy makes by far the largest contribution to the rate of a reaction. For a series of reactants in water at ~ 25 °C which vary in activity only due to the differences in their dipole moments, the difference in the activation parameters can be determined by Eqns. 1A–3A. The difference in the activation parameters between CDNP-lysine-60 ($P = 458$ debye) and CDNP-lysine-72 ($P = 200$ debye) cytochrome *c* reacting with a protein of charge $-72e$ and radius 40 Å at an ionic strength 0.133 M ($f_d(\kappa) = 0.31$) gives $\Delta S_{el}^{\dagger}(60) - \Delta S_{el}^{\dagger}(72) = 7.02$ e.u. and $\Delta H_{el}^{\dagger}(60) - \Delta H_{el}^{\dagger}(72) = 0.5$ kcal/mol.

Appendix B

Eqn. 2 asserts that the dipolar term is proportional to the electrostatic energy of a monopole in the potential field of the dipole at reaction radius, R, in analogous fashion to the monopolar term. An earlier derivation by Koppenol [20] based on the monopolar (Debye and Hückel, [31]) and dipolar (Kirkwood [32]) activities of ions and employing the Brønsted-Bjerrum relation [33] leads to a fundamentally different result. The Kirkwood expression for the activity of a dipole, in a slightly simplified form valid for a ion of large radius, R, and a dipole moment, P, is given in Eqn. 1-B.

$$\ln\gamma = \frac{\kappa^2}{4\pi\epsilon\epsilon_0 R}(3/8)P^2(1+\kappa R+(\kappa R)^2/2)^{-1} \qquad (1\text{-B})$$

The following, brief, derivation of the monopole–dipole dependence of a bimolecular reaction using 1-B is given to support Eqn. 2.

Consider the reaction of two ions, A and B, with respective charges Z_A and Z_B, in which A has a dipole moment P_A oriented at angle θ to its reactive site. In step 1 of Scheme I, B is brought into contact with A at a site at right angles to the dipole vector of A to form an intermediate state AB′. In step 2, A is rotated through an angle $90 - \theta$ to bring B into contact with the reactive site on its surface and form the transition state $AB_{\phi}^{\dagger}$.

Scheme I

With AB′ as an intermediate we can separate monopolar and dipolar contributions to the activity of each state. From the Brønsted-Bjerrum relation:

$$\ln k = \ln k_0 + \ln\frac{\gamma_A\gamma_B}{\gamma_{AB'}} + \ln\frac{\gamma_{AB'}}{\gamma_{AB}^{\dagger}} \qquad (2\text{-B})$$

AB′, of total radius $R = R_A + R_B$, is formed in a manner consistent with the assumptions of the

Brønsted-Debye-Hückel equation [20], since only monopolar forces are involved in its formation and therefore the second term in Eqn. 2-B is equivalent to the monopolar term in Eqn. 1. In the artifice of the Brønsted-Debey-Hückel relation AB′ is considered as a single species of radius R and charge $Z_A + Z_B$. Therefore the total dipole moment of AB′ is considered the same as that of A and dipolar activities cancel. In evaluating the last term in Eqn. 2B this construction of AB′ is changed and the charge Z_B is regarded as located at radius $R = R_A + R_B$ from the centre of A. The monopole activity remains constant in step 2. The total dipole moments of AB′ and AB† are, respectively; $P_{AB'} = [P_A^2 + (Z_B eR)^2]^{1/2}$, and $P_{AB\dagger}) = [P_A^2 + (Z_B eR)^2 + 2\,P_A Z_B eR\cos\theta]^{1/2}$. Use of Eqn. 1-B to evaluate $\ln(\gamma_{AB'}/\gamma_{AB\dagger})$ gives, after simplification, Eqn. 3-B.

$$\ln\frac{\gamma_{AB'}}{\gamma_{AB\dagger}} = \frac{Z_B e P_A \cos\theta}{4\pi\epsilon\epsilon_0}\left[\frac{3\kappa^2}{4(1+\kappa R+(\kappa R)^2/2)}\right] \qquad \text{(3-B)}$$

This result leads to:

$$\ln k_1 = \ln k_0 + \frac{Z_A Z_B e^2}{4\pi\epsilon\epsilon_0 k_B}\left[\frac{\kappa}{1+\kappa R_A}\right] \times\frac{Z_B e P_A\cos\theta}{4\kappa\epsilon\epsilon_0 k_B}\left[\frac{3\kappa^2}{4(1+\kappa R+(\kappa R)^2/2)}\right] \qquad \text{(4-B)}$$

which is similar to Eqn. 5-B proposed by Van Leeuwen et al. [21]:

$$\ln k_1 = \ln k_0 + \frac{Z_A Z_B e^2}{4\pi\epsilon\epsilon_0 k_B}\left[\frac{\kappa}{1+\kappa R_A}\right] + \frac{Z_B e P_A\cos\theta}{4\kappa\epsilon\epsilon_0 k_B}\left[\frac{R_B\kappa^2}{R(1+\kappa R)}\right] \qquad \text{(5-B)}$$

except for the dipolar screening factor, which in Kirkwood's expression 1-B is probably valid only at low ionic strength.

An earlier derivation by Koppenol [20] does not employ the intermediate AB′ but implicitly assumes that the Brønsted-Debey-Hückel-Kirkwood expression can be derived from Debye-Hückel activity coefficients when the charges of A and B are not superimposed in AB′. Eqn. 14 of Ref. 20 can be arrived at when the dipolar activity coefficient of reactant A replaces that of AB′ in the last term of Eqn. 2-B. This leads, among other anomalies, to an asymmetry in the predicted ionic strength dependences of oppositely charged and like-charged monopolar ions.

Under physiological conditions, cytochrome *c* reacts with its oxidase and reductase embedded in the inner mitochondrial membrane. The anionic phospholipid groups in the membrane together with the charges on the redox partner impose an electric field, that will tend to orient the reactive site of cytochrome *c* towards its redox partners. The relation between protein dipole moment and rate derived for homogeneous reaction also applies to reaction at a charged surface. If a dipolar cytochrome is brought into contact with the charged surface at a 90° orientation of the dipole vector with respect to the field, the electrostatic work required to attain this initial state (analogous to AB′ in Scheme I) will be the same for any magnitude of the dipole. The electrostatic work, W_{el}, in reorienting any such dipole from this initial orientation to its reactive site is equivalent to $PE\cos\theta$. The change in activity of the protein in going from its initial to its final and reactive orientation is related to W_{el} by

$$W_{el} = -\Pi k_B T\ln(\gamma_f/\gamma_i) = PE\cos\theta \qquad \text{(6-B)}$$

where γ_i and γ_f are the initial and final activities, respectively. Since values of γ_i are independent of the dipole moment magnitude, it can be readily shown from the Brønsted-Bjerrum relation [33] that the relative activities of dipolar proteins towards a surface site will exhibit the dependence as given by Eqn. 2.

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