The Dipole Moment of Cytochrome *c* [1]

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Vertebrate cytochromes *c* and the cytochromes *c* of insects and plants have, on average, dipole moments of 320 and 340 debye, respectively. The direction of the dipole vector with respect to the haem plane, at the solvent-accessible edge of which electron transfer presumably takes place, is conserved in these two groups—at 32° ± 7° and 22° ± 10°, respectively. The variation of dipole orientations and magnitudes observed in these species is compared with the results of a model in which charge distributions occur randomly. Since this model does not generate the observed charge asymmetries of the various cytochromes *c*, it is concluded that the dipole moment of cytochrome *c* is a feature that is evolutionarily conserved, apparently because it has an important influence on the interaction of this mobile electron carrier with its physiological electron donors and acceptors in the intermembrane space of mitochondria.

Introduction

Electrostatic interactions serve a variety of purposes in biological systems. Salt bridges and hydrogen bonds are essential in preserving the tertiary and quaternary structures of proteins (Perutz 1978; Osheroff et al. 1980; Garcia-Moreno et al. 1985). Surface charges guide charged substrates to active sites—as is the case, for example, for Cu/Zn superoxide dismutases (Koppenol 1981; Getzoff et al. 1983; Klapper et al. 1986; Desideri et al. 1988)—and also orient proteins prior to collision, to enhance the efficiency of reaction, as discussed below for cytochrome *c*. Lysines 13, 72, 86, and 27 of cytochrome *c* are conserved in evolution and probably are essential for recognition in the reactions with the physiological redox partners of the protein (Margoliash and Bosshard 1983). Finally, charges play an important role in the catalytic reaction mechanisms of, among others, lysozyme (Warshel 1981), ribonuclease, serine proteases and carbonic anhydrase (Allen 1981), actinidin, and papain (Van Duijnen et al. 1979; Kowlessur et al. 1989). Of the four aspects in which electrostatic interactions are mentioned as being involved—namely, stability, orientation, recognition and catalysis—the present study concerns itself with the second.

The charge distribution of cytochrome *c* can be described as the sum of a net charge, dipole, quadrupole, etc. Furthermore, the potential due to the net charge is proportional to $1/r$, that due to the dipole is proportional to $1/r^2$, that due to the

1. Key words: molecular evolution, dipole moment, cytochrome *c*, charge change, electrostatic interaction. Abbreviations: e = elementary charge; n and p = number of negative and positive charges, respectively, $\vec{r}_N$ and $\vec{r}_P$ = vectors from the center of mass to the centers of negative (N) and positive (P) charge, respectively; D = debye; θ = angle between dipole vectors; μ = dipole moment in debye.

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quadrupole is proportional to $1/r^3$, etc. (Böttcher 1973). Thus, at a certain distance the electrostatic interaction between the positively charged cytochrome *c* and a physiological redox partner with a negative net charge is satisfactorily described by net charges only, and this interaction leads to attraction. Closer by, the net-charge-to-net-charge-only interaction is to be replaced by that between net charge and net charge plus dipole moment. It is this interaction that orients cytochrome *c* such that the "front," which includes the haem edge, faces the redox partner (Koppenol et al. 1978; Koppenol and Margoliash 1982). It is clear that orientation requires an asymmetric charge distribution. Further orienting forces might arise from shorter-range dipole-dipole and, in an inhomogeneous field, from monopole-quadrupole forces. However, very little is known about electrostatic properties of the membrane-bound physiological redox partners, other than that these carry a negative net charge. "Docking" of cytochrome *c* onto the physiological partner cannot be described in terms of net charges and dipole moments only. Because of the short distance, higher-order multipoles are likely to be involved.

Horse and tuna cytochromes *c* have asymmetric charge distributions that result in molecular dipole moments of ~300 D (Koppenol et al. 1978; Koppenol and Margoliash 1982). This permanent dipole moment is the result mostly of the asymmetric distribution of negative charges, which are found at the "back" of the protein. The distribution of positive charges is more homogeneous. Thus, the dipole moment is created by the absence of negative charges near the solvent-accessible haem edge (Koppenol and Margoliash 1982). Since the locations and number of negative charges vary more than those of the positive charges, it is a priori not to be expected that the dipole moment is preserved. A dipole of this magnitude considerably enhances the rate of reaction that cytochrome *c* has with its major mitochondrial partners, such as cytochrome *c* oxidase and cytochrome *c* reductase (Rush and Koppenol 1988). This is not to say that all cytochromes *c* with identical net charges and identical dipole moments will have the same reactivity. Under the assumption of identical Gibbs energy changes, rates of electron transfer are dependent on the reorganization barrier, according to the Marcus theory (Marcus and Sutin 1985). On reaction with the physiological reaction partner, a conformational change (Rush et al. 1988; Hildebrandt and Stockburger 1989; Michel et al. 1989)—which involves unknown uncharged residues that are not necessarily conserved, thereby allowing for variations in the rate of electron transfer—is likely to take place.

We have calculated dipole moments of a wide variety of mitochondrial cytochromes *c* by using 59 known amino acid sequences. These dipole moments are comparable, in both direction and magnitude, to those previously computed for tuna and horse cytochromes *c* (Koppenol et al. 1978; Koppenol and Margoliash 1982). In principle, dipole moments could be preserved either by chance or, since these cytochromes *c* exhibit many similarities in their sequences, by charge conservation. Arguments are considered in favor of the hypothesis that the dipole moment is functionally important and therefore conserved by natural selection. This contention is supported by computer simulations in which charge distributions are allowed to vary randomly.

Methods

Dipole moments were calculated as described elsewhere, with the help of the following equation (Koppenol and Margoliash 1982):

$$\vec{\mu} = n(\vec{r}_P - \vec{r}_N)e + (p - n)\vec{r}_P e, \qquad (1)$$

in which $\vec{r}_P - \vec{r}_N$ is the distance between the centers of positive and negative charge.

Equation (1) applies to a macromolecule with a net positive charge. It is assumed that all lysines and arginines carry a positive charge and that all glutamic and aspartic acids are negatively charged. Furthermore, the contribution of peptide bonds (Wada 1976) in α-helices was taken into account, with the approximation of half a positive charge at the N-terminus and half a negative charge at the C-terminus (Hol et al. 1978; Sheridan and Allen 1980). All other bond dipoles are assumed to have random orientations and are ignored. Calculations that take bond dipoles into account yield results which are not significantly different from ours (Northrup et al. 1986). The amino termini of insect cytochromes *c* are not acetylated and are assumed to carry a positive unit charge at neutral pH. No charges were assigned to the pyrrole nitrogens or to the iron; therefore, our calculations apply best to the ferrous oxidation state. The difference in dipole moment between this and the ferric form is small: 3° in direction and 15 debye in magnitude (Koppenol and Margoliash 1982). The propionic acid groups of the haem were assumed to be ionized (Moore 1983; Churg and Warshel 1986). For the calculation of vertebrate cytochromes *c* we used, with the appropriate substitutions, the coordinates of the horse protein, which were generated by R. Feldmann, from the tuna structure (Takano et al. 1977). The dipole moment for horse cytochrome *c* generated in this fashion is in excellent agreement with that calculated from coordinates of the recently published structure of this protein (Bushnell et al. 1990). The insect and plant cytochromes *c* have residues before the first amino acid of vertebrate cytochromes *c*. Dipole moments for these species were generated from the structure of rice cytochrome *c*, which has a similar N-terminal extension (Ochi et al. 1983). The relative error in the magnitude of a calculated dipole moment is estimated to be 5%–10% (Barlow and Thornton 1986) and is considered to be due to the movement of charged residues. The amino acid sequences were obtained both from Dayhoff et al. (1972), Hunt et al. (1973), Dayhoff and Barker (1976), Schwartz and Dayhoff (1978), and from unpublished work of one of us (E.M.).

For simulations of charge distributions, a sphere with a radius of 18 Å was used. The surface of this sphere was divided into 411 areas of 10 $Å^2$ each. Twenty positive charges and 13 negative charges were assigned, at random, to these sites. The average dipole moment and the distribution of dipole moments were calculated from 1,000 random charge distributions. Programs were written in BASIC and were executed on an IBM-compatible personal computer.

Single charge relocations of a particular configuration were simulated by neutralizing, at random, one of the 33 charges and by then creating, at random, a charge of the same sign but at another site. During this procedure the net charge, $+7e$, and the total number of charges remained constant. Ten random charge configurations with dipole moments of 300 ± 10 D were selected for further simulations. To determine the average angular displacement of the dipole, we performed 15 sequential single relocations on each of these 10 charge configurations. These simulations were repeated 20 times. The displacements after each such change were summed and averaged, first for each of the 10 initial charge configurations and then over all 10 charge configurations.

For each of the 10 original charge distributions, we calculated the probability that a charge distribution with a vector component either <200 D or <100 D in the direction of the original 300-D dipole moment would occur after a given number of single changes. A total of 100 random sequential charge substitutions were carried out. The actual number of charge replacements is likely to be less than the number of substitutions, since a later substitution might restore a charge present in the original

charge distribution. The number of substitutions, or single relocations, and the corresponding average number of charge replacements (in parentheses) are as follows: 1 (1), 4 (3.88), 8 (7.16), 12 (10.12), 16 (12.68), 20 (15.06), and 24 (17.08).

Results

Dipole Moments of Eukaryotic Cytochromes *c*

Cytochromes *c* from vertebrates have, on average, 30 charged residues. In addition, there are two negative charges from the haem propionates. Their asymmetric distributions result in dipole moments that are ~200–400 D (table 1). Sixteen positively charged residues (lysines 5, 7, 8, 13, 25, 27, 39, 53, 55, 72, 73, 79, 86, and 87 and arginines 38 and 91) and nine negative charges (haem propionates, aspartic acids 2 and 93, glutamic acids 4, 21, 69 and 90, and the C-terminal carboxylate) are conserved in vertebrate cytochrome *c*.

In tables 1 and 2 the dipole moments are shown for 19 vertebrate and 17 invertebrate and plant cytochromes *c*. The 19 entries in the category of vertebrate cytochromes *c* apply to a total of 36 cytochromes *c*. Many sequences are identical from an electrostatic point of view but differ in a number of uncharged residues. In general, the net charges of invertebrate and plant cytochromes *c* are slightly lower—i.e., $+5e$ versus $+6e$ for the vertebrate proteins—and vary more, as is clear from the twice larger standard deviation. It appears that an angle of 32° between the haem plane and dipole vector is well conserved in vertebrate cytochromes *c*. In invertebrate and plant cytochromes *c* this angle is 22°, not significantly different from that in the vertebrate species. The dipole moment resulting from only the conserved charges in vertebrate cytochromes *c* is comparable in magnitude to that of other cytochromes *c* but, in direction, deviates from the horse protein dipole vector by 35°, and the angle with the haem plane is approximately twice the average for all cytochromes *c*, namely 56°. The results of this calculation are listed in table 3, as "Core" cytochrome *c*. Table 3 also lists the dipole moments of an annelid (brandling worm), an echinoderm (starfish) a gymnosperm (ginkgo), and a eumycophyte (baker's yeast). A diagram showing the location of the dipoles of vertebrate cytochromes *c* and of starfish cytochrome *c* is given in figure 1. For all species the positive end of the dipole vector emerges from the protein at the "left" side of the haem plane, as the protein is usually oriented with the solvent-accessible haem edge facing the viewer and with the haem propionyl side chains pointing downward.

Simulations

When 20 positive and 13 negative charges are randomly distributed on a sphere with a radius of 18 Å, the average dipole moment is 450 D. However, the probability distribution of dipole magnitudes is not symmetrical about the mean, since some charge configurations which give very high dipole moments are possible. The most probable dipole moment is ~350 D, and there is a 75% likelihood that a randomly generated dipole has a moment of 200–600 D. The average dipole moment of a charged sphere tends to increase with the number of charges on the surface and, naturally, with the radius.

After every simulated single charge relocation, the dipole vector differed by angle θ from the original. The average displacement from the original vector after M substitutions in a configuration of T charges is approximated by the empirical function

$$\theta = \cos^{-1}(1-M/T), \qquad (2)$$

in which (1−M/T) is the fraction of charged residues conserved from the original configuration. After seven replacements the dipole will, on average, differ in orientation, by $\theta = 45°$ for an original dipole of 300 D. After the degree of charge replacement which corresponds to the variations between plants and insects (M/T = ½), the average deviation is 60°. The mean deviations of $\theta = 11°$ (within vertebrates) and $\theta = 22°$ (among plants and insects) are much lower than either of these averages.

Large deviations from the average occur if charged residues which make large vector contributions to the dipole are not conserved. In this respect not all randomly generated distributions with dipole moments of 300 D are equivalent, since some contain a higher proportion of such charges than do others and thus are more susceptible to change after a few charge relocations. In this respect it is important to realize that the lysines at the front—i.e., residues 13, 27, 72, 79, and 86—are conserved and make relatively large contributions to the dipole moment of the molecule. As equation (2) indicates, the natural conservation of a protein dipole moment increases with the total number of charges. The high proportion (22%–28%) of charged amino acids can make it difficult to distinguish between conservation of dipole moment as a consequence of natural selection and conservation of dipole moment as a result of chance.

If one starts with a random 300-D charge configuration, there is a 70% chance that, after seven substitutions (a range roughly spanning the charge variation among vertebrates), a variant with a dipole-moment component of <200 D in the original direction should have occurred. A dipole moment of this magnitude is close to the lower limit observed in all dipole-moment calculations. There is a 30% chance that a variant with a dipole moment <100 D would be formed. After half of all initial charges have undergone redistribution, these percentages increase to 87% and 68% for the 200-D and 100-D limits, respectively. In general, the actual range of dipole moments and their close clustering within 5% of the protein surface—a percentage which represents a cone of ~30° originating at the center of the mass—is duplicated in ~20% of simulations after seven or eight substitutions. Larger divergences are more common, but the observed range is not unusual. However, after random replacement of half of the charges, such as between insects and plants (table 2), our simulations indicate that the convergence—of both magnitude and orientations—observed in tables 1 and 2 is extremely unlikely.

Discussion

As shown by Barlow and Thornton (1986), the magnitude of the average dipole moments—317 ± 41 D for vertebrate cytochromes *c* and 343 ± 41 D for insect and plant cytochromes *c*—is not exceptional for a protein with the size and the number of charges of cytochrome *c*. Our simulations, which are similar to theirs, confirm this. Also in qualitative agreement with our findings is the observation that the polarity of cytochromes *c* has been kept at a particular level and that substitutions which would lead to too high a polarity change do not occur (Vogel 1971). In this context, polarity is defined as the average polarity of all amino acids of cytochrome *c*.

If the dipole moment were not a selectively conserved feature, then, by chance, variants would tend to arise with a survival likelihood independent of the overall charge configuration. Peetz et al. (1986) showed that in the case of cytochrome *c* the proportion of amino acid substitutions resulting in charge changes (37.0%) is similar to that predicted by a model of random substitution (40.3%). This would suggest that the direction of the dipole moment would not be conserved. However, many amino acids are invariant, and those that are not are found mostly at the "back" surface of cytochrome *c*, 180° away

Table 1
Dipole Moments of Cytochromes *c* from Vertebrates, Relative to That of Horse Cytochrome *c*

Species[a]	Differences			Net Charge (*e*)	Dipole Moment (D)	Angle with	
	Sequence[b]	Charge[c]	Conservative Changes[d]			Dipole of Horse Cytochrome *c*	Haem
Zebra-donkey	1	0	0	6 (21+, 15−)	299	0°	34°
Horse	0	0	0				
Human-chimpanzee	12	5	1	6 (19+, 13−)	349	22°	31°
Baboon	12	5	1				
Rhesus monkey	11	5	1				
Capuchin monkey-spider monkey	12	6	3	5 (19+, 14−)	328	9°	28°
Rabbit	6	4	1				
Gray kangaroo	7	3	1	6 (19+, 13−)	349	6°	32°
Mouse-rat	5	3	1				
Camel-whale-guanaco	5	2	0				
Mouse (testes)	11	5	0	5 (19+, 14−)	267	19°	26°
Bat	7	5	0	7 (20+, 13−)	232	4°	31°
Dog	6	4	0	6 (20+, 14−)	252	20°	16°
Elephant seal	6	5	0	5 (19+, 14−)	250	33°	4°
Pig-cow-sheep	3	1	0	5 (19+, 14−)	357	5°	30°

Chicken-turkey	11	6	0	5 (20+, 15−)	291	0°	34°
King penguin	12	6	1				
Pekin duck	10	6	1				
Emu	12	6	1				
Snapping turtle	11	6	1				
Pigeon	11	6	1	6 (20+, 14−)	277	14°	28°
Ostrich	11	6	1	4 (20+, 16−)	323	8°	42°
Bullfrog	13	5	1	7 (20+, 13−)	225	10°	25°
Tuna	18	9	1	6 (18+, 12−)	353	32°	38°
Bonito	17	8	0	6 (18+, 12−)	396	22°	40°
Dog fish	16	6	0	9 (20+, 11−)	360	34°	50°
Lamprey	15	5	0	7 (20+, 13−)	299	12°	44°
Average	9.1	4.4	0.6	6 (19+, 13−)	317	11°	32°
Standard deviation	4.5	2.2	0.8	1 (1+, 1−)	41	10°	7°

[a] Species names, for animals listed in alphabetical order, are as follows: baboon, *Papio papio;* bat, *Miniopteris schreibersi;* bonito, *Katsuwonis pelamis;* bullfrog, *Rana catesbeiana;* camel, *Camelus dromedarius;* capuchin monkey, *Cebus apella;* chicken, *Gallus gallus;* chimpanzee, *Pan troglodytes;* cow, *Bos taurus;* dog, *Canis familiaris;* dogfish, *Squalus suckleyi;* donkey, *Equus asinus;* elephant seal, *Mirounga leonina;* emu, *Dromaius novae-hollandiae;* gray kangaroo, *Macropus cangaru;* guanaco, *Lama guanicoe;* horse, *Equus caballus;* human, *Homo sapiens;* king penguin, *Aptenodytes patagonia;* lamprey, *Entosphenus tridentatus;* mouse, *Mus musculus* (strain BALB/C); ostrich, *Struthio camelus;* pekin duck, *Ana platyrhyncos;* pig, *Sus scrofa;* pigeon, *Columba livia;* rabbit, *Oryctolagus cuniculus;* rat, *Rattus norvegicus;* rhesus monkey, *Macaca mulata;* sheep, *Ovis aries;* snapping turtle, *Chelydra serpentina;* spider monkey, *Ateles fusiceps;* tuna, *Thunnus germo;* turkey, *Meleagris gallopavo;* whale (California gray), *Rhachianectes glaucus;* and zebra, *Equus quagga.*

[b] Number of residues different from those of the sequence of horse cytochrome *c*.

[c] Number of amino acids involving charges that differ from those in the sequence of horse cytochrome *c*.

[d] Number of conservative charge changes, such as glutamate for aspartate, etc.

Table 2
Dipole Moments of Cytochromes *c* from Invertebrates and Plants, Relative to That of Rice Cytochrome *c*

Species[a]	Differences			Net Charge (*e*)	Dipole Moment (D)	Angle with	
	Sequence[b]	Charge[c]	Conservative Changes[d]			Dipole of Rice Cytochrome *c*	Haem
Rice	0	0	0	2 (15+, 13−)	393	0°	20°
Tobacco hornworm moth	46	19	4	7 (18+, 11−)	330	43°	16°
White cabbage butterfly:							
Wild type	44	18	3	6 (18+, 12−)	272	30°	12°
Mutant	44	18	3	4 (17+, 13−)	334	9°	12°
Mediterranean Fruit fly	51	22	5	6 (19+, 13−)	286	27°	11°
Fruit fly	51	21	4				
Locust	43	19	3	5 (19+, 14−)	334	41°	37°
Blowfly	46	17	4	7 (19+, 12−)	335	25°	36°
Screw worm fly	47	17	4				
Mung bean	12	4	1	5 (16+, 11−)	367	23°	29°
Pumpkin	10	4	1	5 (17+, 12−)	360	19°	33°
Cauliflower	12	5	1	4 (17+, 13−)	409	13°	29°
Elder	14	5	1	1 (15+, 14−)	406	25°	33°
Cotton	12	3	1	4 (15+, 11−)	336	7°	20°
Castor	14	5	2	6 (15+, 9−)	296	19°	15°
Tomato	11	4	1	4 (15+, 11−)	333	13°	19°
Sunflower	15	6	1	3 (14+, 11−)	389	20°	11°
Maize	9	3	1	4 (16+, 12−)	392	16°	25°
Buckwheat	16	7	2	5 (16+, 11−)	330	9°	13°
Average	28	11	2.4	5 (17+, 12−)	243	22°	22°
Standard deviation	18	7	1.4	2 (2+, 1−)	41	10°	10°

[a] Species names, for animals listed in alphabetical order, are as follows: blowfly, *Lucila cuprina;* buckwheat, *Fagopyrus esculentus;* castor, *Ricinus communis L.;* cauliflower, *Brassica oleracea;* cotton, *Gossypium barbadense L.;* elder, *Sambucus nigra, L.;* fruit fly, *Drosophila melanogaster;* locust, *Schistocerca gregaria;* maize, *Zea mays;* mediterranean fruit fly, *Ceratitis capitata;* mung bean, *Phaseolus aureus L.;* pumpkin, *Cucurbita maxima;* rice, *Oryza sativa;* screw worm fly, *Haematobia irritans;* sunflower, *Helianthus annuus L.;* tobacco hornworm moth, *Manduca sexta;* tomato, *Lycopersicon esculentum;* and white cabbage fly, *Pieris brassica.*

[b] Number of residues different from those of the sequence of horse cytochrome *c*.

[c] Number of amino acids involving charges that differ from those in the sequence of horse cytochrome *c*.

[d] Number of conservative charge changes, such as glutamate for aspartate, etc.

Table 3
Dipole Moments of Miscellaneous Cytochromes *c*

Source (species name)	Net Charge (*e*)	Dipole Moment (D)	Angle with Rice (r) or Horse (h) Dipole Moment	Angle with Haem
"Core"	7 (16+, 9−)	365[a]	35° (h)	56°
Starfish (*Asteria rubens*)	9 (20+, 11−)	183	19° (h)	33°
Brandling worm (*Eisenia foetida*)	6 (18+, 12−)	250	18° (r)	18°
Ginkgo (*Ginkgo biloba*)	7 (16+, 9−)	335	12° (r)	27°
Baker's yeast (iso-1) (*Saccharomyces cerevisiae*)	6 (20+, 14−)	522	50° (r)	29°

[a] This result is from all charges that are invariant in vertebrate cytochromes *c*—namely, lysines 5, 7, 8, 13, 25, 27, 39, 53, 55, 72, 73, 79, 86, and 87, arginines 38 and 91, the two haem propionates, aspartic acids 2 and 93, glutamic acids 4, 21, 69, and 90, and the C-terminus.

from the solvent-accessible haem edge. These restrictions limit the extent of possible variations in both the direction and the magnitude of the dipole moment. Furthermore, certain changes which would seem to be important have little, if any, influence. For instance, the brandling worm protein has a "reversed" salt bridge: instead of the salt bridge between glutamate 61 (or aspartate) and lysine 99, one finds the salt bridge between lysine 61 and glutamate 99. This might also apply to charge reversals—such as (1) lysine 4 and glutamate 7 (in plants) versus glutamate 4 and lysine 7 (in vertebrates and insects) and (2) lysine 100 and glutamate 104 (in mammals) versus aspartate 100 and lysine 104 (in the birds)—even though these charge pairs do not appear to form salt bridges.

A limitation of the present study is that many evolutionary branches not less significant than those represented by vertebrates, insects, and the angiosperm plants have not been considered. Our results indicate that, among any one of the three groups mentioned, it is possible that the dipole moment is conserved by chance but that, over the wide range of charge variations which distinguish the insects (or vertebrates) from plants, that this is unlikely. However, the particular phyla from which most of the sequences have been taken may be atypical. In this respect four examples from table 3 are useful. The cytochromes *c* of the brandling worm (an annelid), starfish (an echinoderm), *Ginkgo biloba* (a gymnosperm plant), and yeast (a eumycophyte) have dipole moments comparable to those of the others. This makes it more likely that a dipole moment within a certain range of magnitude and orientation is indeed a common characteristic of all eukaryotic cytochromes *c*.

If a dipole moment is advantageous in the reactions between cytochrome *c* and its mitochondrial redox partners, it might be supposed that larger dipole moments are better. However, kinetic studies of the electron-transfer function of cytochrome *c* suggest that optimal steady-state reactivity requires optimal "off" rates as well as optimal "on" rates. There are, in fact, clear-cut examples of diminished steady-state turnover rates resulting from slow "off" rates (Garber and Margoliash 1990). Furthermore, within the narrow confines of the intermembrane space of mitochondria, the function of cytochrome *c* in transporting electrons from reductase to oxidase will also depend on its mobility. Indeed, cytochrome *c* is likely to remain in proximity to, if not actually bound to, the anionic

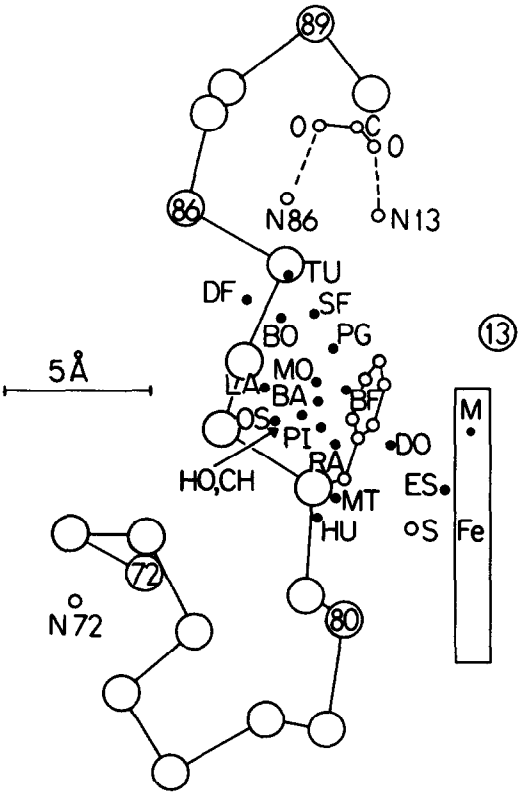

FIG. 1.—Diagram of left front face of cytochrome *c*, showing α-carbon atoms of residues 13 and 72–90 (large circles); ε-nitrogens of lysines 72, 86, and 13; sulfur of cyteine 17 (small circles); and haem and center of mass (M). Two salt bridges between glutamate 90 and lysines 13 and 86 are indicated near the top of the figure. Dipole vectors originate in M. The locations where these vectors intersect a plane perpendicular to the haem at a distance of 10 Å from M are indicated by small blackened circles. Abbreviations used for the various species are: BA, bat; BF, bullfrog; BO, bonito; CH, chicken; DF, dogfish; DO, dog; ES, elephant seal; HO, horse; HU, human; LA, lamprey; MO, mouse, somatic; MT, mouse, testicular; OS, ostrich; PG, pigeon; PI, pig; RA, rabbit; SF, starfish, and TU, tuna. Except for starfish, the species name of which is in table 3, complete species names are given in table 1. As shown in table 1, other species have cytochromes *c* with dipole moments that are identical. For instance, the dipole vector for chicken cytochrome *c* is identical to that of turkey, king penguin, pekin duck, emu, and snapping turtle.

surface of the phospholipid membrane (Swanson et al. 1983). The mobility of cytochrome *c* over a charged interface decreases with increasing dipole magnitude, as shown by the observation that a set of singly modified 4-carboxy-2,6-dinitrophenyllysine cytochromes *c* elute from a carboxymethyl cellulose column in reverse order of their dipole-moment magnitude (Brautigan et al. 1978). Their reactivities with cytochrome *c* oxidase and cytochrome *c* reductase preparations are, however, in direct order of their dipole magnitudes (Rush and Koppenol 1988), because all these singly modified cytochromes *c* bind to the enzymes less strongly than does the unmodified protein. The latter may be presumed to operate in an optimal mode.

Because the protein dipole moment, like the net charge, is a summed effect of all charges which, in the course of evolution, change in type and position, it is necessary to consider how cytochromes *c* may conserve advantageous electrostatic properties. In figure 2 the average dipole moments are plotted against the net charges of cytochromes *c*. As

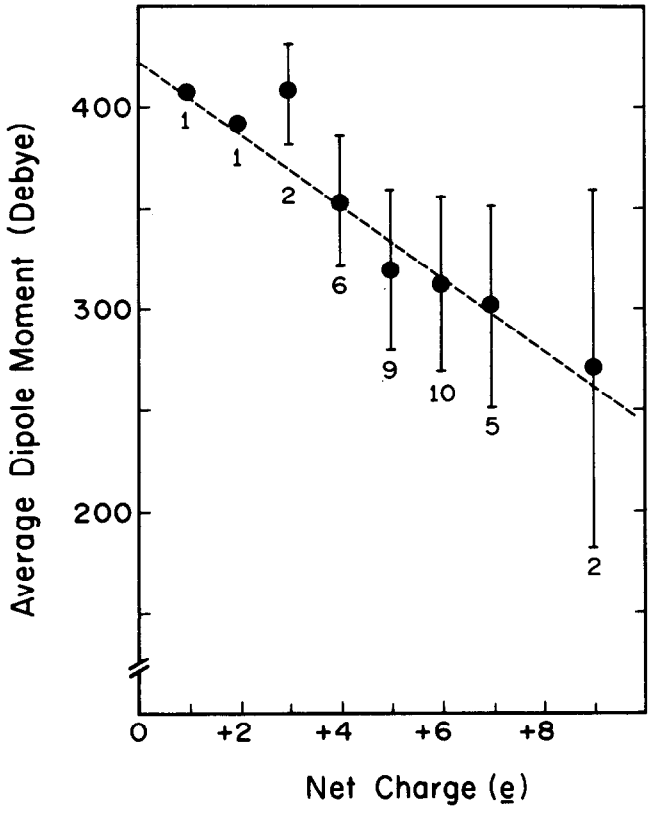

FIG. 2.—Average dipole moments plotted as function of net charge. Electrostatically identical cytochromes *c* from related species were counted as 1. Error bars indicate standard deviations, and the number beneath is the number of cytochromes *c* examined with that net charge.

the net charges increase, the dipole moment tends to decrease. The overall variation of charge types (from +1e to +9e) is much greater than the range of observed dipole moments, which would lead to the expectation that the evolutionary variation of charges should have a much greater effect on the protein's dipole moment than is, in fact, observed. Since charged residues at the back show greater variation than those at the front, charge changes fixed in evolution tend to require a compensating effect—that is, the elimination of a positive charge from the back decreases the net charge but increases the dipole moment. For example, the replacement of the positive charge of lysine 60 of horse cytochrome *c* by a negative charge, by modification with 4-chloro-2,6-dinitrobenzoate, causes little change in reactivity, because the 120-D increase in dipole moment compensates for the decrease in net charge (Rush and Koppenol 1988). Such an electrostatic balance is consistent with the protein's functional requirement for both mobility and reactivity.

Some of the conservation of charges on the front surface of the molecule might be related to other functions of electrostatic interactions mentioned in the Introduction, functions such as stability and recognition. In addition, if individual charge changes on the front produce very large decreases in either reaction rate or mobility, which would correspond to a binding affinity with regard to physiological reaction partners lying below or above an optimal range, then such variations are not likely to be fixed in evolution, even if they do not affect the tertiary structure, reduction potential, or

other important properties of the molecule. In conclusion, it would seem that, in the cytochrome *c* molecule, the dipole moment, like the reduction potential (Gao et al. 1989), is a collective property which has been conserved in the course of biological evolution, most probably because of its functional significance.

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