Electrostatic Effects in Water-Accessible Regions of Proteins†

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ABSTRACT: A simple, point charge model has been used to calculate electrostatic effects in water-accessible regions of proteins. In order to account for the shielding effect of the dielectric medium, an empirical dielectric permittivity function of charge separation is proposed. The functional form and parametrization are based on dielectric permittivity data obtained from experimental and theoretical studies of the ratio of the first to the second dissociation constant of bifunctional organic acids and bases [Conway, B. E., Bockris, J. O., & Ammar, I. A. (1951) *Trans. Faraday Soc. 47*, 756–766]. In order to test the efficacy of this model, the effect of nearby charged groups on the dissociation constants of charged functional groups has been calculated for a number of enzymes. The reasonable agreement between calculated and observed pK shifts (i.e., δ-chymotrypsin, calculated $\Delta pK = 2.8$ and observed $\Delta pK = 2.1$; dihydrofolate reductase, calculated $\Delta pK = 1.4$ and observed $\Delta pK = 1.2$) shows that this electrostatic model with an accounting of solvent and ionic effects is able to rationalize the observed free energy changes.

There are many proteins that have an amino acid side chain with an abnormally high or low dissociation constant. This shift is attributed to the presence of nearby charged groups and/or a low-polarity microenvironment. A dramatic example is the shift of more than 4 pK units of His-159 in papain which is attributed to a salt bridge between the histidine and Cys-25 (Johnson et al., 1981a,b). As in papain, these pK shifts are often intimately associated with an enzyme's catalytic activity, and it is therefore of interest to try to understand the sources of these changes in dissociation constants. In this paper we present a simple electrostatic approach for estimating the changes in the pK values.

The relationship between the shifted and unshifted dissociation constant is given by

$$\Delta\mu_0' - \Delta\mu_0 = RT \ln (K'/K) \quad (1)$$

where $\Delta\mu_0'$ and $\Delta\mu_0$ are the standard chemical potential changes for the dissociating species in the presence and absence, respectively, of n charged groups. The situation studied here resembles the problem of calculating the ratio of the first to the second dissociation constant of a bifunctional acid or base, and therefore, we can write

$$RT \ln (K'/K) = N\Delta w = 2.303RT\Delta pK \quad (2)$$

where N is Avogadro's number and Δw is the change in electrostatic free energy caused by introduction of the charges.

A method for calculating Δw was given by Kirkwood (1934) and Kirkwood & Westheimer (1938). Shire et al. (1974a,b, 1975) have used a modified procedure for application to proteins. Kirkwood and Westheimer assumed a finite spherical shape for the ion of interest with an internal dielectric permittivity dependent on the molecular makeup of the ion and with the solvent's permittivity for the external region. However, active site clefts are far from spherical, and high-resolution X-ray studies have shown the presence of water molecules and ions in these regions (Watenpaugh et al., 1978; Finney, 1979; Baker, 1980; Dijkstra et al., 1981). In addition, charged groups such as ammonium, guanidinium, or carboxylate, considered in the present study, have ionic radii of about 1.5 Å or less, whereas the separation between these interacting groups in the active site environment will generally be greater than van der Waals distances (≥3.4 Å). Thus, according to electrostatic theory, a point charge model for which $\Delta w = q_1q_2/(\epsilon r_{12})$ (Bjerrum, 1923) should be a reasonable first approximation.

The value of the effective dielectric permittivity, ϵ, is estimated to range between 2 and 5 in nonpolar regions of protein interiors (Pethig, 1979), whereas for water it is 78 at 298 K. The presence of water at the protein surface suggests that a value of 2–5 cannot be correct there, and indeed, Kirkwood & Westheimer (1938) already suggested a much larger value. Recently, Rees (1980) has estimated an effective dielectric permittivity of about 50 when charges are separated by about 10–12 Å. The occurrence of ions and water molecules in the active site region, and the accessibility of the active site to the bulk solvent, suggests that, as for the surface, the dielectric permittivity is larger than for the protein interior.

The formulation of the dielectric permittivity in terms of distance from a charge is expressed by the Laplace equation for regions where there is no excess charge, i.e., $\nabla^2\psi + \text{grad}\epsilon\cdot\text{grad}\psi/\epsilon = 0$ (Owen, 1963), where ψ is the potential. By itself, however, this equation cannot give us the functional form of $\epsilon(r)$. Indeed, the determination of the dielectric permittivity at microscopic distances is beset with considerable difficulties (Pollock et al., 1980). In part, this is so because two mutually inconsistent quantities are juxtaposed: On the one hand, we need to evaluate the interaction energy between atoms or molecular fragments embedded in a medium at microscopic distances where the correct description is given by quantum mechanics, but on the other hand, Coulomb's law contains a macroscopic parameter, the bulk dielectric permittivity.

However, functional forms for $\epsilon(r)$ have been suggested previously on empirical grounds or from more fundamental considerations: Gelin & Karplus (1979) have used $\epsilon(r) = r$ in a study of side-chain torsional potentials, Warshel & Levitt (1976) used $\epsilon(r) = 1 + r$, Warshel (1979) suggested $\epsilon(r) = 2 + (r - 1)^2$ in a study of the proton pump in *Halobacterium halobium*, and van Duijnen et al. (1979) have taken $\epsilon(r) = 1 + (r - r_0)/r$ where r_0 is a cutoff separation below which $\epsilon = 1$. In view of Rees' (1980) results, however, van Duijnen's and the linear forms are too low for solvent-exposed regions. In addition, the lack of asymptotic behavior of the linear and quadratic forms also makes them unacceptable for the present application. Additional discussion on the theoretical basis and

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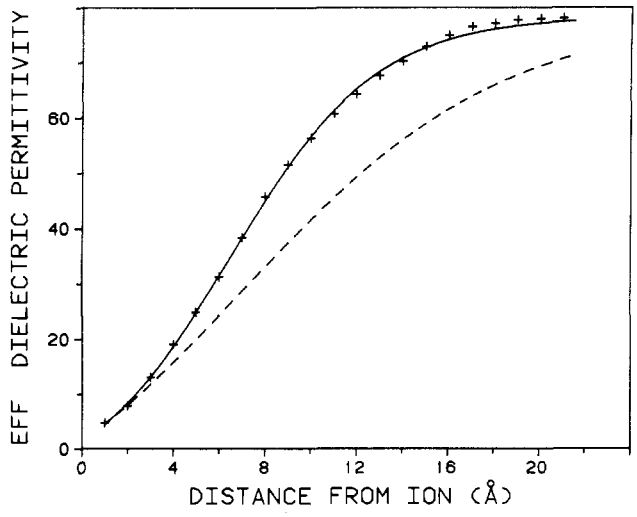


FIGURE 1: Effective dielectric permittivity as a function of distance from a monovalent ion. Data points (+) from Conway et al. (1951); (—) ϵ_{WDS}: $\epsilon_{H_2O} = 78.4$, $A = -8.5525$, $k = 7.7839$, $\lambda = 0.003627$. (---) ϵ_s: $\epsilon_{H_2O} = 78.4$, $A = -20.929$, $k = 3.4781$, $\lambda = 0.001787$ (parameters defined in eq 3 and 4).

functional form of $\epsilon(r)$ has been given by Pollock et al. (1980) and Ehrenson (1982).

Formulation and Parametrization of an Effective Dielectric Permittivity Function

In eq 1 and 2 we noted the similarity between evaluating changes in pK in enzymes and calculating the relation of the first to second dissociation constants in bifunctional organic acids and bases. For the latter, considerable experimental and theoretical data are available from which an effective dielectric permittivity as a function of distance can be deduced. Conway et al. (1951) reported the mean dependence of ϵ upon distance from a univalent ion in water. Their tabulation is based on the mean of data published by Webb (1926), Debye (1925), and Schwarzenbach (1936) and is reproduced in Figure 1. These results assume a point charge for the ion. As pointed out by Conway et al. (1951) the ϵ vs. r relationships derived by the three authors are somewhat different, so that implicit in using the mean values is a proportional uncertainty which of necessity is also inherent in the results reported in this paper. Conway's tabulation gives $\epsilon(r)$ from 1 to 21 Å in 1-Å steps, and we shall use these data to carry out the electrostatic calculations reported below.

It is convenient to fit Conway's data to a functional form, and insight into an appropriate relationship can be obtained by noting that $\epsilon(r)$ has sigmoidal character: For small r values $\epsilon(r)$ increases rapidly and $d\epsilon/dr$ can be described by a linear function of ϵ. For $r > 10$ Å, ϵ approaches the bulk value of the dielectric permittivity of water asymptotically with $d\epsilon/dr$ proportional to $\epsilon_{H_2O} - \epsilon(r)$, where ϵ_{H_2O} is the dielectric permittivity of water. The functional form for the entire range of r can then be rationalized by a differential equation of the form

$$d\epsilon/dr = \lambda(\epsilon - A)(\epsilon_{H_2O} - \epsilon) \quad (3)$$

Solution of eq 3 yields (Batschelet, 1971)

$$\epsilon(r) = A + B/[1 + k \exp(-\lambda B r)] \qquad B = \epsilon_{H_2O} - A \quad (4)$$

where k is a constant of integration and ϵ_{H_2O} has the value of 78.4 at 25 °C, leaving three parameters to be determined by least-squares fitting. The function fitted to the Webb, Debye, and Schwarzenbach data, ϵ_{WDS}, is shown in Figure 1.

The conditions in proteins are somewhat different than for the ionic situation considered by Conway et al. (1951). In the latter the ions are completely accessible to water whereas this is not the case in proteins where the bulk of the macromolecule and the shape of the active site region limit the accessibility of water and ions. This limited accessibility should result in a slower rate of increase of ϵ with distance, which is also suggested from the fact that Rees (1980) reports an effective mean permittivity of 51.5 at 12.6 Å although ϵ_{WDS} yields 64 at this distance. In order to account for these effects, we define a scaled permittivity, $\epsilon_s(r)$, by reevaluating the three parameters in eq 4 from the following conditions:

$$\epsilon_s(1 \text{ Å}) = \epsilon_{WDS}(1 \text{ Å}) \quad (5a)$$

$$\epsilon_s(3.4 \text{ Å}) = \epsilon_{eff}(3.4 \text{ Å}) \quad (5b)$$

$$\epsilon_s(12.6 \text{ Å}) = 51.5 \quad (5c)$$

where $\epsilon_{eff}(3.4$ Å) is calculated from the pK change in papain, mentioned in the introduction, by using eq 1 and 2 and the Bjerrum (1923) formula $\epsilon = q_1 q_2/(\Delta w r)$. At 12.6 Å (eq 5c), the value of ϵ_s is taken to be the mean value obtained from Rees' determination in cytochrome c. The scaled dielectric permittivity function is also shown in Figure 1.

Applications

The validity of our empirical permittivity function was tested with a number of proteins that exhibit an acidic or basic group with a shifted pK. Both the X-ray structure and the altered pK values of the test cases are known. The atomic coordinates were obtained from the protein data bank (Bernstein et al., 1977), or from the Atlas of Macromolecular Structure (Feldman, 1976), except the coordinates for chicken heart mitochondrial aspartate aminotransferase (AAT), which were kindly provided by Prof. Jansonius. The following conventions are used for calculating distances between charged groups (unless noted otherwise): In histidine the midpoint of the imidazole ring or the midpoint between $N_{\epsilon 1}$ and $N_{\delta 1}$ is taken as the origin; for carboxyl groups and guanidium groups the central carbon atom is used.

Using eq 1 and 2 the free energy for n point charges is given by

$$\Delta w = \sum_{j>i}^{n} \frac{q_i q_j}{\epsilon(r_{ij}) r_{ij}} \quad (6)$$

where $\epsilon(r_{ij})$ is the effective dielectric permittivity computed from eq 4 for the charged pair separated by r_{ij}. Equation 6 describes the situation where the charged active group is surrounded by $n - 1$ charges which shift the dissociation constant from its normal value. The standard pK value can be the solution value or its estimated value in the protein environment in the absence of the charged groups. pK shifts can also occur when a conformational change takes place. In this case Δw is computed from eq 6 for each conformation, and the effective change in electrostatic free energy, $\Delta\Delta w$, is the difference in free energy between the two conformations. Another source of pK change is a substrate or inhibitor which alters the number of charged groups in the active site cleft. Here also, $\Delta\Delta w$ is obtained from the difference in Δw between the two conformational states with differing number of charges. Finally, in order to compare calculated with measured pK shifts, it is necessary to correct for ionic strength effects (Rees, 1980). In the present work these effects were estimated from a simple Debye–Hückel screening potential (Laidler, 1978).

Table I tabulates the data for five proteins where no conformational changes occur. The calculations are carried out by using ϵ_{WDS} and ϵ_s, and for comparison the ΔpK obtained by using Warshel's (1979) form of $\epsilon(r)$ has also been included.

Table I: Shift in Dissociation Constant of Active Groups in Five Proteins[a]

	R (Å)	I	ΔpK (exptl)	ϵ_{WDS}	Δw_{net} (kcal/mol)	Δw_0 (kcal/mol)	ΔpK_{WDS}	ϵ_s	ΔpK_s	ϵ_W	ΔpK_W
papain[b] Sγ(Cys-25)–Nδ(His-159)	3.40	0.01	4.8	15.18	6.43	5.75	4.2	13.30	4.8	7.76	8.2
δ-chymotrypsin[c] Cγ(Asp-194)–Nα(Ile-16)	3.64	0.1	2.1	16.51	5.52	3.78	2.8	14.26	3.2	8.97	5.1
dihydrofolate reductase (*E. coli*)[d] His 141–Cγ(Glu-139)	4.50	0.15	1.2	21.61	3.41	1.92	1.4	17.80	1.7	14.25	2.9
lysozyme[e] Cγ(Asp-52)–Cδ(Glu-35)	6.48	0.02	1.1	34.74	1.47	1.09	0.8	26.35	1.0	32.03	0.9
myoglobin[f] His 36–Cδ(Glu-38)	5.28	0.01	1.4	26.62	2.36	1.98	1.5	21.12	1.8	20.32	1.7

[a] Δw_{net} and Δw_0 are unscreened and screened electrostatic energies, respectively, and I is ionic strength; $\epsilon_W = 2 + (r - 1)^2$ from Warshel (1979). [b] The pK of His-159 was determined by using Cys-25-SCH_3 papain and succinylpapain by NMR and fluorescence spectroscopy as ~3.5 and ~8.5; T = 25 °C (Lewis et al., 1976; Johnson et al., 1981a,b; Lewis et al., 1981). [c] An apparent pK of 10 has been assigned to the amino group of Ile-16 in the active form of the enzyme whereas the inactive form has a pK of 7.9 determined from pH/activity profile; T = 25 °C (Hess, 1971; Blow, 1971; Fersht, 1972). [d] His-141 shows a moderately high pK (7.2–7.4). Normal His pK values in this enzyme are about 6 (His-45, -114, and -149) determined by NMR; T = 25 °C (Poe et al., 1979). [e] pH difference titration of native and the β-ethyl ester derivative of Asp-52 results in a change of the apparent pK of Glu-35 by 1.1 units. Determined by acid/base titration; T = 25 °C (Parsons & Raftery, 1972; Imoto et al., 1972). [f] His-36 is assigned a standard pK of 6.6. Observed pK values in protein determined by NMR; T = 25 °C (Botelho et al., 1978).

For each case Δw_{net} is calculated from eq 6, and then the Debye–Hückel screening is applied to obtain Δw_0 from which ΔpK is calculated. The values of Δw are also given in Table I for the case where ϵ_{WDS} was used.

The results reported in Table I show that the values of ΔpK calculated by using ϵ_{WDS} and ϵ_s are both in reasonable agreement with the measured values. The slightly higher ΔpK values obtained by using ϵ_s are due to the decreased accessibility of solvent as expressed by eq 5. At the same time it is clear that ϵ_W leads to ΔpK values which are too large for charge separations below 5 Å, overestimating the change in dissociation constant by up to 3.5 orders of magnitude. In the range 5–8 Å, ϵ_W gives approximately correct estimates of ΔpK, but for $r > 8$ Å it predicts values of ϵ which are too large. Thus, at r = 12.6 Å, ϵ_W = 136.6 which is to be compared with Rees' (1980) value of 51.5. The other forms of $\epsilon(r)$ mentioned above also lead to serious overestimation of ΔpK because the values of $\epsilon(r)$ for $r > 3$ Å are too small.

The His-36–Glu-38 interaction in myoglobin has been studied previously by Botelho et al. (1978) by using Shire's (1974) approach for calculating pK values. Botelho assumed a conformational change in solution, bringing the carboxyl group of Glu-38 to within H-bonding distance of $N_{\epsilon 1}$ of His-36. Using this hypothetical structure (charge separation of 2.85 Å) we calculated a ΔpK of 5.6 which is much too large. However, assuming no conformational changes from the X-ray structure, we obtained a ΔpK_{WDS} value of 1.5. Thus, in contradiction to Botelho's assumption, the present results suggest no important structural changes between the crystal and solution structure in the environment of His-36 and Glu-38 in myoglobin.

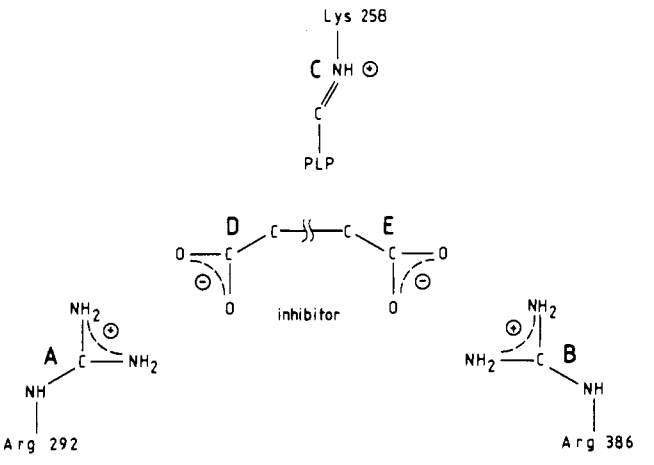
Ohe & Kojita (1980) have measured dissociation constants of various histidine side chains in deoxy hemoglobin and (carbon monoxy)hemoglobin. In some cases the pK values are shifted due to structural changes in the two forms. Electrostatic free energy changes for this type of interaction were calculated, and two interactions are reported in Table II. The values to be compared with experiment are the $\Delta\Delta pK$ values, and it is seen that here too the present electrostatic model is able to rationalize the observed changes in dissociation constants.

The most complicated case we have examined is the active site of mitochondrial aspartate aminotransferase. The active site structure is presented schematically in Figure 2. In the native enzyme there is a weak interaction between the guanidinium groups of Arg-292 and Arg-386 with the protonated form of the Schiff's base, shifting the equilibrium to the unprotonated form. But the effect is small due to the large separation between the charged groups. When the active site

Table II: Difference in Dissociation Constant of Histidine Groups in Deoxyhemoglobin and (Carbon monoxy)hemoglobin[a]

	interaction	R (Å)	ϵ_s	ΔpK_s	ϵ_{WDS}	ΔpK_{WDS}
deoxy-HB	His-89α–Lys-139α	7.61	29.70	−0.49	40.21	−0.36
HB(CO)		4.69	17.64	−1.80	21.61	−1.47
$\Delta\Delta pK$		1.6[b]		1.3		1.1
deoxy-HB	His-146β–Asp-94β	4.90	18.48	1.61	22.89	1.30
HB(CO)		11.51	45.07	0.14	60.23	0.10
$\Delta\Delta pK$		1.1[b]		1.5		1.2

[a] Calculations at T = 37 °C, I = 0.1. [b] Experimental values from Ohe & Kojita (1980). Measurements at T = 37 °C, I ~0.1.



Separation between point charges in maleate

	A	B	C	D
B	8.99			
C	9.22	8.31		
D	4.34	6.63	5.25	
E	6.78	4.31	4.66	2.93

Separation between point charges in α-ketoglutarate

	A	B	C	D
B	11.09			
C	11.08	10.72		
D	4.52	9.17	6.62	
E	8.09	5.37	5.89	4.52

FIGURE 2: Schematic diagram and geometries of the active site and inhibitor model of AAT for calculating electrostatic interactions. Distance in angstroms. PLP = pyridoxal 5′-phosphate; inhibitors = α-ketoglutarate and maleate.

Table III: AAT: Change in Schiff's Base Dissociation Constant with Different Inhibitors[a]

inhibitor	form of protein	Δw_{WDS} (kcal/mol)	ΔpK_{WDS}	Δw_s (kcal/mol)	ΔpK_s	ΔpK (exptl)
α-ketoglutarate	native	−0.58	−0.42	−0.78	−0.57	
	native + inhibitor	1.80	1.32	2.32	1.70	
	effective change	2.38	1.74	3.10	2.27	1.8–2.6[b]
maleate	native	−0.58	−0.42	−0.78	−0.57	
	native + inhibitor	3.34	2.45	4.01	2.94	
	effective change	3.92	2.88	4.79	3.51	
			2.2[c]		2.7[c]	>2.0[d]

[a] Δw_{WDS} and Δw_s are calculated with ϵ_{WDS} and ϵ_s, respectively; T = 25 °C, I = 0.023, except as noted. [b] Pig heart AAT: Braunstein (1973), Jenkins & D'Ari (1966), and Haddad et al. (1977). Chicken heart AAT: Eichele et al. (1978). [c] I = 0.1. [d] Preliminary measurements in crystals at $I \sim 0.1$; Picot et al., private communication.

is occupied by a substrate or inhibitor, the negative charges on the carboxyl groups provide a strong electrostatic stabilization of the guanidinium groups favoring the protonated form of the Schiff's base. The dissociation constant increases by 2 orders of magnitude (Braunstein, 1973; Eichele et al., 1978; Jenkins & D'Ari, 1966; Haddad et al., 1977). Distances were evaluated according to the model presented in Figure 2 for two inhibitors for which coordinates are available (J. N. Jansonius et al., unpublished results), and electrostatic free energies were calculated for free and liganded enzyme. The calculated changes in pK are shown in Table III. For the α-ketoglutarate inhibitor no changes in conformation are observed, whereas for the maleate (and presumably for the substrates) Arg-386 moves in such a way as to decrease the volume of the active site and bring the charged groups closer together (Kirsch et al., 1984). For the α-ketoglutarate derivative it is seen that the calculated pK shifts are well within the range of the experimentally observed shift. Preliminary experimental results indicate that for the maleate derivative the pK shift is greater than two (D. Picot and J. N. Jansonius, unpublished results). This is in agreement with expectation since the closer approach of the charged guanidinium groups to the maleate molecule increases the electrostatic interaction energy between the involved ionized groups.

Conclusions

The most important conclusion to be drawn from the results presented in this paper is that in solvent-accessible regions attenuation of electrostatic effects by the environment is often greater than commonly assumed. Thus, these regions are not similar to nonpolar regions of the protein interior, nor is it appropriate to assume an environment similar to a dense gas or any other environment which would lead to a low, solvent-independent, dielectric permittivity. Moreover, apart from catalysis, this attenuation is relevant for several other aspects of macromolecular behavior. For example, protein chains in the process of folding also must interact with solvent, and the attenuation of Coulombic interactions needs to be considered. Finally, the formation of supramolecular structures (protein–protein or protein–nucleic acid complexes) is influenced by this attenuation as well.

In this model the effects of all noncharged species present in solution, and also all parts of the protein not explicitly entering into the electrostatic interaction, are lumped together into a single effect described by $\epsilon(r)$. Because of this the interpretation of $\epsilon(r)$ as used here is limited, and it is somewhat surprising that this crude approach appears to give roughly correct qualitative results, although the actual values may be in error by as much as 1 pK unit.

The mean deviations (and standard deviation of the mean) for the eight cases where the experimental values are known are −0.09 (±0.45) and 0.26 (±0.44) for ΔpK_{WDS} and ΔpK_s, respectively. Thus, the ϵ_s values calculated from eq 5 underestimate the total dielectric effect. The fact that ϵ_{WDS} gives a mean deviation close to zero suggests that additional effects are in operation which tend to compensate for the reduced accessibility of water to the charged groups in active site clefts. For the examples presented here all these effects seem to cancel so that the net result is a screening effect similar to that of water. It must be kept in mind, however, that this cancellation may be fortuitous and that for other systems the dielectric permittivity may behave quite differently. Finally it is noted that an electrostatic point charge model has clear limitations. Especially for interactions involving separations considerably less than van der Waals distances, this type of model probably breaks down. In such cases contributions from polarization, dispersion, charge transfer, and overlap repulsion effects (Mehler, 1980) need to be included explicitly in the calculation of Δw.

Acknowledgments

We thank Professor J. F. Kirsch for interesting discussions and suggesting the original problem, namely, the prediction of the observed change in pK in AAT. We also thank Professor J. N. Jansonius for his encouragement and helpful comments. Finally, we thank Dr. W. W. Parson for noting some errors and discrepancies in the original formulation of eq 3 and 5.

Registry No. AAT, 9000-97-9; δ-chymotrypsin, 9004-07-3; dihydrofolate reductase, 9002-03-3; papain, 9001-73-4; lysozyme, 9001-63-2; α-ketoglutaric acid, 328-50-7; maleic acid, 110-16-7.

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Indole Fluorescence Quenching Studies on Proteins and Model Systems: Use of the Inefficient Quencher Succinimide†

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ABSTRACT: We have compared the quenching of the fluorescence of proteins by acrylamide and succinimide, two chemically similar quenchers. We find that the ratio of the apparent rate constants for succinimide and acrylamide quenching, $\gamma_{S/A}$, ranges from ~0.1 to ~0.7. Proteins having relatively buried tryptophan residues, such as ribonuclease T_1, cod parvalbumin, and aldolase, are found to have small values of $\gamma_{S/A}$ (i.e., succinimide quenches with a much smaller rate constant than acrylamide); proteins with relatively solvent-exposed tryptophan residues, such as glucagon and adrenocorticotropin, are found to have larger values of $\gamma_{S/A}$. We interpret this range of $\gamma_{S/A}$ values as being due to either (a) a critical size dependence of the dynamic penetration of quencher through a protein matrix (succinimide being larger than acrylamide) and/or (b) an inherent dependence of the succinimide quenching reaction on the microenvironment of the indole ring. The latter interpretation is supported by studies of the solvent dependence of the quenching of the fluorescence of indole and 5-methoxyindole by succinimide and acrylamide. These studies show that whereas acrylamide is an efficient quencher in all solvents investigated, succinimide is a relatively inefficient quencher in aprotic solvents. Thus, both of the above molecular bases for poor quenching of interior tryptophan residues in proteins by succinimide (i.e., a critical size dependence of a microenvironment dependence) are consistent with the fluorescence quenching process occurring within the globular structure of proteins by a dynamic penetration mechanism, as opposed to an unfolding mechanism by which interior residues would be periodically exposed to the solvent.

Studies of indole fluorescence quenching by added solutes have, in recent years, provided valuable information regarding the structure and dynamics of proteins in solution (Lehrer, 1971; Lehrer & Leavis, 1978; Lakowicz & Weber, 1973; Lakowicz et al., 1983; Eftink & Ghiron, 1976a, 1977, 1981). The most significant finding with this technique is that even tryptophan (Trp) residues that are presumably deeply buried within globular proteins are able to be quenched by the uncharged quenchers oxygen and acrylamide with quenching rate constants on the order of 10^9 M^{-1} s^{-1}. These results have been interpreted as indicating that the quenchers are able to penetrate into the matrix of globular proteins, with this penetration

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Received December 6, 1983. This work was supported by National Science Foundation Grants PCM-8206073 and PCM-7726614. A preliminary report of this work has appeared (Eftink & Ghiron, 1983).

0006-2960/84/0423-3891$01.50/0