A Kinetic Method for the Study of Solvent Environments of Thiol Groups in Proteins Involving the Use of a Pair of Isomeric Reactivity Probes and a Differential Solvent Effect

INVESTIGATION OF THE ACTIVE CENTRE OF FICIN BY USING 2,2'- AND 4,4'-DIPYRIDYL DISULPHIDES AS REACTIVITY PROBES

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1. Whereas the second-order rate constants for the reaction of the thiolate ion of 2-mercaptoethanol with 4,4'-dipyridyl disulphide (k_{4PDS}) and with 5,5'-dithiobis-2-nitrobenzoate dianion increase with decreasing dielectric constant of the solvent, or remain unchanged, the rate constant for the analogous reaction with 2,2'-dipyridyl disulphide (k_{2PDS}) decreases. This anomalous solvent effect and other unusual physico-chemical properties of 2,2'-dipyridyl disulphide are discussed. 2. The differential effect of solvent on the reactions of thiolate ion with the 2,2'- and 4,4'-dipyridyl disulphides is shown to provide a method of characterizing solvent environments of thiol groups in proteins by a reactivity-probe method that should not suffer from the usual drawback associated with the existence of steric or binding effects of unknown magnitude. Application of the method to ficin (EC 3.4.22.3) suggests that its active-centre thiol group resides in a relatively hydrophobic environment. 3. The pH- k profile for the reaction of ficin with 4,4'-dipyridyl disulphide is reported.

Kinetic study of the reactions of protein nucleophiles with site-specific reagents can provide information about local microenvironment and its effect on chemical reactivity, knowledge of which is essential to our understanding of enzyme catalysis (see Brocklehurst, 1979, and references cited therein). One of the problems inherent in the use of reactivity probes is that when it is not possible to isolate binding effects, perhaps for reasons of limited solubility, it is usually not possible to distinguish steric effects in enzyme-reagent association from active-centre-solvent effects. The present paper reports an approach to the solution of this problem involving the use of a pair of thiol-specific isomeric reactivity probes (2,2'- and 4,4'-dipyridyl disulphides) whose reactions with thiolate ions respond very differently to changes in solvent environment. In many cases the steric requirements of the two probe molecules for association with a given site on a given protein should be very similar, and the ratio of the reactivities of a protein thiolate ion towards the two reagents provides a basis for the assessment of the nature of its solvent microenvironment. The

hydrophobic nature of the catalytic site of the thiol proteinase ficin (EC 3.4.22.3) is demonstrated by using this technique.

Materials and Methods

Ficin

The enzyme was prepared from the dried latex of *Ficus glabrata* as described by Malthouse & Brocklehurst (1976).

1,4-Dioxan (dioxan)

The 'special for spectroscopy' product of BDH Chemicals (Poole, Dorset, U.K.) was passed down a column (2.8 cm $\times$ 8 cm) of alumina immediately before use to remove peroxides. The alumina had been activated by heating for 1 h at 175°C.

Deuterated materials

$^2\text{H}_2\text{O}$, (minimum isotopic purity 99.7%), NaO^2H (40% in $^2\text{H}_2\text{O}$, minimum isotopic purity 99%) and

^2HCl (38% in $^2\text{H}_2\text{O}$, minimum isotopic purity 99.5%) were products of BDH Chemicals. Materials used for the preparation of buffers in $^2\text{H}_2\text{O}$ (glycine and Tris) were dried at 110°C for 2.5 h and then stored over finely ground CaCl_2 *in vacuo*.

Other materials and methods

These have been described previously: spectral characteristics of the disulphides and their thiolysis products (Brocklehurst & Little, 1973), buffers, determination of second-order rate constants (k) by stopped-flow spectral analysis and determination of the characterizing parameters of pH- k profiles by an optimization procedure (Malthouse & Brocklehurst, 1976; Shipton & Brocklehurst, 1978). All solutions were degassed as a routine before stopped-flow experiments were performed. In kinetic runs the concentration of 2-mercaptoethanol or ficin was usually $10\text{--}20\ \mu\text{M}$ in the mixing chamber and the concentration of the disulphide was usually $200\text{--}500\ \mu\text{M}$.

Results and Discussion

Comparison of the properties of 2,2'- and 4,4'-dipyridyl disulphides

Some applications of pyridyl disulphides such as 2,2'-dipyridyl disulphide (compound I) and 4,4'-dipyridyl disulphide (compound II) in the study of thiol enzymes have been reviewed (Brocklehurst, 1979).

One of the most striking characteristics of compound (I) is its ability to increase its electrophilicity by more than 10^3 -fold consequent on its mono-protonation. The fact that this characteristic is displayed to a very much smaller degree by compound (II) (Brocklehurst & Little, 1972; Brocklehurst *et al.*, 1979) led us to investigate the effect of organic solvent on the reactions of the two disulphides with 2-mercaptoethanol in anticipation of a differential effect.

The dependence on pH of the second-order rate constant (k) for the reaction of 2-mercaptoethanol with 2,2'-dipyridyl disulphide in aqueous solution, in aq. 25% (v/v) dimethylformamide and in aq. 25% (v/v) dioxan is shown in Fig. 1(a). The most striking feature of Fig. 1(a) is the decrease in k [the plateau value of k at high pH which is the rate constant characteristic of the reaction of the thiolate ion of 2-mercaptoethanol with compound (I)] with decrease in the dielectric constant of the solvent (values of which are: for water, 78.5; for dimethylformamide, 36.7; and for dioxan, 2.21; see Reichardt, 1965). This decrease in k would not be predicted for a reaction involving charge dispersal in the transition state (eqn. 1) (see, e.g., Reichardt, 1965):

The effects of organic solvent on the reactions of 2-mercaptoethanol with 4,4'-dipyridyl disulphide (Fig. 1b) and with 5,5'-dithiobis-2-nitrobenzoate dianion (compound III) (Fig. 1c) contrast markedly with the results shown in Fig. 1(a). The small increase in k with decrease in dielectric constant predicted for a reaction of the type shown in eqn. (1) is clearly shown in Fig. 1(c). Although the data in Fig. 1(b) do not provide convincing evidence for an increase in k , a decrease like that shown in Fig. 1(a) is clearly absent.

The $\text{p}K_a$ values provided by the pH- k profiles of Fig. 1 [in aqueous solution (a) 9.65 ± 0.02 , (b) 9.80 ± 0.1 and (c) 9.51 ± 0.05 ; in 25% (v/v) dioxan (a) 10.04 ± 0.05 , (b) 9.80 ± 0.1 and (c) 10.13 ± 0.04] correspond to the $\text{p}K_a$ value of 2-mercaptoethanol determined by study of the pH-dependence of thiolate ion absorbance at 237 nm [in aqueous solution 9.64 ± 0.03 and in 25% (v/v) dioxan 10.01 ± 0.07 ; Jencks & Salvensen (1971) reported $\text{p}K_a$ values of 9.61 for 2-mercaptoethanol in H_2O and 9.96 in $^2\text{H}_2\text{O}$].

In view of the anomalous solvent effect exhibited by the reaction of thiolate ion with 2,2'-dipyridyl disulphide, it is noteworthy that a marked difference in character between this disulphide and its 4,4'-dipyridyl isomer is suggested also by some of their physicochemical properties. The long-wavelength u.v.-absorption band of 4,4'-dipyridyl disulphide is centred at 247 nm in a region near to that of pyridine (253 nm in water; see Jaffé & Orchin, 1962, p. 563). The u.v.-absorption spectrum of 2,2'-dipyridyl disulphide contains an absorption band with λ_{max} 233 nm and an additional band with λ_{max} 281 nm. The position of the absorption band of pyridine is relatively insensitive to solvent (e.g. λ_{max} is slightly blue-shifted to 252 nm in hexane; Jaffé & Orchin, 1962, p. 363). Changing the solvent from water to dioxan results in a blue-shift of the u.v.-absorption band of 4,4'-disulphide from 247 to 242 nm. The same solvent change leaves the 281 nm band of the 2,2'-isomer unchanged and provides a red-shift of the lower-wavelength band from 233 to 238 nm. The dipole moment of 4,4'-dipyridyl disulphide (in benzene solution at 25°C) is 1.90 D (Pappalardo & Ronsisvalle, 1973), which is similar to that of pyridine (2.2 D; see Albert, 1959). The dipole moment of 2,2'-dipyridyl disulphide, however, is considerably larger (3.99 D; Pappalardo & Ronsisvalle, 1973) and similar to that of nitrobenzene (3.97 D; see Fieser & Fieser, 1961). The intrinsic $\text{p}K_a$ for the first protonation of 4,4'-dipyridyl disul-

Fig. 1. Effect of solvent on the second-order rate constant (k) for the reaction of 2-mercaptoethanol with (a) 2,2'-dipyridyl disulphide, (b) 4,4'-dipyridyl disulphide and (c) 5,5'-dithiobis-2-nitrobenzoate dianion at 25°C at $I 0.1$

The continuous and broken lines are theoretical for $k = \bar{k} / \{1 + ([H^+]/K_a)\}$. (a) O, Water, $\bar{k} = 7.42 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 9.65$; ●, 25% (v/v) dimethylformamide, $\bar{k} = 5.72 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 10.00$; ■, 25% (v/v) dioxan, $\bar{k} = 4.13 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 10.04$. (b) O, Water; ●, 25% (v/v) dioxan, $\bar{k} = 13.90 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 9.80$. (c) O, Water, $\bar{k} = 11.96 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 9.51$; ●, 25% (v/v) dioxan, $\bar{k} = 17.33 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_a = 10.13$.

phide is 4.8 (molecular pK_a 5.1; see Brocklehurst & Little, 1973), which is similar to the pK_a of pyridine (approx. 5.2; see Perrin, 1965). The pK_a for the first protonation of 2,2'-dipyridyl disulphide, however, is much lower (2.15; molecular pK_a 2.45; see Brocklehurst & Little, 1973). The fact that the molecular pK_a for the first protonation of 2-(2-pyridyl)pyridine (compound IV) (approx. 4.4; Krumholz, 1951) is not much lower than the pK_a of pyridine (compound V) suggests that the presence of sulphur in 2,2'-dipyridyl disulphide is an important factor in providing the low pK_a of 2,2'-dipyridyl disulphide. These differences in physicochemical properties of the two isomeric pyridyl disulphides, together with the substantially different (approx. 100-fold) increases in electrophilicity that result from their protonation (Brocklehurst & Little, 1972; Brocklehurst *et al.*, 1979) and the different solvent effects here reported, point to an important sulphur–nitrogen interaction in 2,2'-dipyridyl disulphide that is lack-

ing in 4,4'-dipyridyl disulphide. Comparison of the pK_a values of the 2,2'- and 4,4'-dipyridyl disulphides with those of the reference compounds (V)–(XI) (see Jones & Katritzky, 1958; Albert & Barlin, 1959; Shipton & Brocklehurst, 1978) suggests that both sulphur atoms in 2,2'-dipyridyl disulphide might contribute to the interaction responsible for the unique properties of this compound. The location of the nitrogen atom relative to one or both of the sulphur atoms is clearly of crucial importance and points to some type of sulphur–nitrogen interaction, presumably through space. Study of the structure of 2,2'-dipyridyl disulphide in the crystal by X-ray diffraction (Raghavan & Seff, 1977) shows that both sulphur atoms lie approximately in the plane of each pyridyl ring, the angle between the two ring planes being 80.3°. Raghavan & Seff (1977) discussed the possibility of a π -interaction between the pyridyl ring and the disulphide group, but concluded that no evidence for this type of interaction

could be deduced from the length of the S-S bond in 2,2'-dipyridyl disulphide, which is similar to that of S-S bonds in other disulphides where no significant π -interaction can exist. Sheppard & Taft (1972) used fluorine n.m.r. shifts for the series $p\text{-FC}_6\text{H}_4\text{S}$ (which included compounds such as XII-XIV) to assess relative contributions to π -interaction of π acceptor orbitals (d -orbitals) on sulphur as against p - π donation of an unshared electron pair to the aromatic ring. They concluded that π -interaction does occur in this series, which includes an aromatic disulphide (compound XII), and that, although, in general, electron withdrawal by the substituent X will increase π (p - d) acceptor interaction, i.e. $\text{Ar} \rightarrow \ddot{\text{S}}$ and may decrease π (p - p) donor interaction, i.e. $\text{Ar} \leftarrow \ddot{\text{S}}$, considerations other than the inductive effect of X are involved. For example, X substituents in which sp -carbon is bonded to sulphur as in compounds (XIII) and (XIV) appear to increase the importance of $\text{Ar} \leftarrow \ddot{\text{S}}$ π (p - p) donation relative to $\text{Ar} \rightarrow \ddot{\text{S}}$ π (p - d) acceptor interaction even though these substituents are relatively electron-attracting. It seems possible that one or both of these types

of interaction may be responsible for the unique properties of 2,2'-dipyridyl disulphide discussed above. The propensity that neutral sulphur donor ligands (as against thiolate anions) have for back-bonding interactions with metal ions has been discussed by Brown & Kallen (1972) and by Kuehn & Taube (1976), and Sheppard & Taft (1972) have pointed out that two of the d -orbitals in aryl sulphur fluorides such as ArSF_3 are in the best possible orientation for overlap with the aromatic π -orbitals. The change in the balance of π (p - p) and π (p - d) interactions consequent on protonation of 2,2'-dipyridyl disulphide may contribute to the large increase in electrophilicity that monoprotection of the neutral reagent provides.

Reaction of ficin with 2,2'- and 4,4'-dipyridyl disulphides and a basis for probing solvent environments

The data in Table 1 suggest that the ratio of the second-order rate constants for the reactions of a thiolate ion with 4,4'- and 2,2'-dipyridyl disulphides ($R = \bar{k}_{4\text{PDS}}/\bar{k}_{2\text{PDS}}$) may provide an indication of solvent polarity. Thus for 2-mercaptoethanol R is 1.9

Table 1. Kinetic parameters for the reactions of 2-mercaptoethanol and of ficin with some disulphide reactivity probes at 25°C at I 0.1
The pK_a values are the kinetically determined parameters characteristic of the thiol; the values of the second-order rate constant, $\tilde{k}$, characterize the reaction of thiolate ion with unprotonated disulphide.

Solvent	Thiol	Parameters				$R = \frac{\tilde{k}_{4PDS}}{\tilde{k}_{2PDS}}$
		2,2'-Dipyridyl disulphide (2PDS) pK_a $10^{-4} \tilde{k} (M^{-1} \cdot s^{-1})$	4,4'-Dipyridyl disulphide (4PDS) pK_a $10^{-4} \tilde{k} (M^{-1} \cdot s^{-1})$	5,5'-Dithiobis-2- nitrobenzoate dianion pK_a $10^{-4} \tilde{k} (M^{-1} \cdot s^{-1})$		
H ₂ O	2-Mercaptoethanol	9.65 ± 0.02	9.80 ± 0.09	9.51 ± 0.05	11.96 ± 0.27	1.9
² H ₂ O	2-Mercaptoethanol	10.07 ± 0.16	10.13 ± 0.01	—	—	2.0
25% (v/v) dimethyl- formamide	2-Mercaptoethanol	10.00 ± 0.02	—	—	—	2.4*
25% (v/v) dioxan	2-Mercaptoethanol	10.04 ± 0.05	9.80 ± 0.09	10.13 ± 0.04	17.33 ± 0.43	3.4
H ₂ O	Ficin	8.61 ± 0.09	8.15 ± 0.05	—	—	4.3

* Assuming $\tilde{k}_{4PDS}$ to be unchanged from its value in aqueous media as it is in 25% (v/v) dioxan.

in water, 2.4 in 25% (v/v) dimethylformamide and 3.4 in 25% (v/v) dioxan. The lack of a deuterium isotope effect in this reaction (Table 1) suggests that the transfer of a proton from solvent to the 2-pyridyl nitrogen atom is a post-transition state process. The dependence on pH of the apparent second-order rate constant for the reaction of ficin with 4,4'-dipyridyl disulphide is shown in Fig. 2. This profile differs markedly from the analogous

Fig. 2. Dependence on pH of the second-order rate constant (k) for the reaction of ficin with 4,4'-dipyridyl disulphide at 25°C at I 0.1

The continuous line is theoretical for $k = \tilde{k} / \{1 + ([H^+]/K_a)\}$ in which $\tilde{k} = 9.69 \times 10^3 M^{-1} \cdot s^{-1}$ and $pK_a = 8.15$. The small positive deviation from this form of pH-dependence contrasts markedly with the much larger positive deviation displayed by the analogous profile for the reaction of ficin with 2,2'-dipyridyl disulphide (see Malthouse & Brocklehurst, 1976). For this reaction k at the optimum in acidic media (pH approx. 3) is approx. $8 \times 10^4 M^{-1} \cdot s^{-1}$ and its pH-dependence is given by:

$$k = \frac{\tilde{k}_1}{\left(1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}\right)} + \frac{\tilde{k}_2}{\left(1 + \frac{[H^+]}{K_{II}} + \frac{K_{III}}{[H^+]}\right)} + \frac{\tilde{k}_3}{\left(1 + \frac{[H^+]}{K_{III}}\right)}$$

in which $\tilde{k}_1 = 1.11 \times 10^5 M^{-1} \cdot s^{-1}$, $\tilde{k}_2 = 400 M^{-1} \cdot s^{-1}$, $\tilde{k}_3 = 2.23 \times 10^3 M^{-1} \cdot s^{-1}$, $pK_I = 2.42$, $pK_{II} = 3.82$ and $pK_{III} = 8.61$.

profile for the reaction of ficin with 2,2'-dipyridyl disulphide reported previously (Malthouse & Brocklehurst, 1976) in two important respects. Firstly, the value of k at the optimum in acidic media is only approx. $400\text{ M}^{-1}\cdot\text{s}^{-1}$ for the 4,4'-dipyridyl disulphide reaction, whereas it is approx. $80000\text{ M}^{-1}\cdot\text{s}^{-1}$ for the 2,2'-dipyridyl disulphide reaction. This large difference in rate in acidic media reflects mainly the much larger increase in electrophilicity provided by protonation of the 2,2'-dipyridyl isomer (see above).

The second striking difference in the two pH- k profiles is the larger value of k in the plateau region at high pH in the case of the 4,4'-dipyridyl disulphide reaction. Although both dipyridyl disulphide reagents should be closely similar in size and shape, $\tilde{k}_{4\text{PDS}}$ is $9690\text{ M}^{-1}\cdot\text{s}^{-1}$, whereas $\tilde{k}_{2\text{PDS}}$ is only $2230\text{ M}^{-1}\cdot\text{s}^{-1}$. The value of $R = \tilde{k}_{4\text{PDS}}/\tilde{k}_{2\text{PDS}} = 4.3$ for the reactions of the ficin thiolate ion with the unprotonated disulphide reagents points to a relatively hydrophobic environment for this active-centre residue. This result is in accord with most views of the active centres of the thiol proteinases (see Lowe, 1976).

Applications of disulphides containing the 2-pyridyl moiety in the isolation and characterization of thiol enzymes have been reviewed (Brocklehurst, 1979). Some of these, i.e. those that make use of the large increase in electrophilicity of the reagent that results from protonation, are applicable only to thiols with environments that endow them with low $\text{p}K_{\text{a}}$ values. The application reported in the present paper has no such restriction, since it depends on reaction of the unprotonated disulphides and should be useful for the study of any thiol-containing macromolecule.

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