J. Pharm. Pharmac., 1974, **26**, 882–888 *Received March* 7, 1974

An ESR titration technique for the determination of accessible tyrosine and tryptophan residues in globular proteins*

J. B. CLOUGHLEY, M. HOOPER AND J. MAXWELL

School of Pharmacy, Sunderland Polytechnic, Sunderland SR1 3SD, U.K.

The reaction of potassium nitrosyldisulphonate (NDS) with globular proteins has been monitored by electron spin resonance. The reaction is shown to be sufficiently sensitive and reproducible to provide a rapid new method for the estimation of exposed tyrosine and tryptophan residues in these proteins at physiological pH. Under these conditions each tyrosine residue was found to consume 2 moles, and each tryptophan residue 3 moles, of NDS. The estimation of accessible groups thus obtained are in good agreement with published data obtained using other chemical probes.

Recent studies have demonstrated the involvement of exposed tyrosine and tryptophan residues in drug-protein binding (Swaney & Klotz, 1970), enzyme-substrate interactions (Di Prisco, D'Udine & others, 1970), and in immunological reactions (Little & Eisen, 1967). A useful method for investigating these reactions involves the use of chemical probes which selectively modify the exposed tyrosine or tryptophan residues coupled with a technique for measuring the proportion of such residues modified in each molecule of the protein. The classical concept of 'exposed' and 'buried' groups has now been qualified and such groups are more accurately described as being accessible or inaccessible to a particular probe reagent. The accessibility of a particular grouping to a specific chemical probe will depend upon the chemical forces influencing the amino-acid residues, the chemical nature of the probe reagent and the mechanism of the reaction between the amino-acid residue and the chemical probe. Hence a particular amino-acid residue may fail to react with a probe reagent, either because it is buried in the classical sense, or because, even if it is on the surface of the protein molecule, strong interactions with other moieties in the protein molecule or specific steric factors prevent the reaction (Kronman & Robbins, 1970).

An ideal probe reagent would be a small molecule or ion which would interact, specifically, with a particular amino-acid residue which was freely accessible, without perturbing the molecular conformation of the protein (Kronman & Robbins, 1970). Whilst the ideal may be unattainable, particularly with respect to conformational changes in the protein molecule (Lumry & Biltonen, 1969), a number of chemical probe reagents have yielded useful information about protein structure and activity.

Suitable chemical reagents include *N*-acetylimidazole (NAI) (Simpson, Riordan & Vallee, 1963), cyanuric fluoride (CYF) (Kurihara, Horinishi & Shibata, 1963) and tetranitromethane (TNM) (Sokolovsky, Riordan & Vallee, 1966). The first two reagents attack the phenolic hydroxyl group of the tyrosine residues whilst TNM is less specific and reacts not only with the phenyl ring of tyrosine, to form 3-nitrotyrosine

* A preliminary report was presented to the British Pharmaceutical Conference, 1973.

but also with tryptophan and cysteine residues (Di Prisco & others, 1970). Chemical reagents which modify tryptophan residues lack specificity and include the oxidizing agent *N*-bromosuccinimide (NBS), and the alkylating agent 2-hydroxy-5-nitrobenzyl bromide (HNBB) (Kronman & Robbins, 1970).

The present paper describes a method in which potassium nitrosyldisulphonate (NDS), a stable free radical, which is known to selectively oxidise both tyrosine and tryptophan residues (Dukler, Wilchek & Lavie, 1971; Earland & Stell, 1966; Consden & Kirrane, 1968), is caused to react with various proteins at physiological pH. Some workers (Polis, Wyeth & others, 1969; Goldstein & Polis, 1971) have reported that the reaction of NDS with proteins leads to a spin label on the macromolecule. In our hands the reaction gave only a time dependent triplet signal, typical of NDS which disappeared in the presence of excess protein, but in the presence of excess NDS reached a limiting value within 1 h for all the proteins examined. The decay of the NDS signal is readily explained by the previously mentioned reaction of NDS with accessible tyrosine and tryptophan residues present in the macromolecules. Our results show that these reactions, at physiological pH, are sufficiently reliable and reproducible to form the basis of an assay procedure for these residues.

MATERIALS AND METHODS

The following proteins were used as supplied. Protein solutions were made up in M/50 phosphate buffer at pH 7·4:

Crystallized bovine serum albumin (BSA), Pentex, lot 24, code 81–001, mol. wt 65 000.

Crystallized human serum albumin (HSA), Cohn fraction V, Pentex, lot 31, code 82–301, mol. wt 69 000.

Crystallized bovine pancreas ribonuclease, BDH, mol. wt 13 700.

Crystallized bovine pancreas trypsin, BDH, mol. wt 24 000.

Hen egg white lysozyme, BDH, mol. wt 14 600.

Crystallized porcine insulin, Allen & Hanbury's Ltd., batch No. 747007, 23·6 units mg^{-1}. mol. wt 5730.

Potassium nitrosyldisulphonate was supplied by K & K Laboratories and found to be 98·4% pure by iodimetric titration. 1-Nitroso-2-naphthol was obtained from BDH.

Electron spin resonance (esr) measurements were made using a Hilger and Watts Microspin Spectrometer with 1,1-diphenylpicrylhydrazyl (DPPH) as an external reference. The same crystal of DPPH was used as the external reference in every esr measurement. Fluorescence measurements were made using an Aminco Bowman spectrofluorimeter and absorption measurements were made with a Unicam SP 1800 spectrometer.

Titration procedure

As an illustration one experiment using BSA will be described. 10μl aliquots of BSA solution (0·65% w/v, 1×10^{-4} mol $litre^{-1}$) were transferred by micropipette into six capillary tubes (i.d. 1 mm) and varying quantities of freshly prepared NDS solution (0·05%, $1{\cdot}866 \times 10^{-3}$ mol $litre^{-1}$) added to each tube, beginning with 7·0μl and proceeding by 0·2μl increments to 8·0μl, to provide a range of protein/NDS molar ratios. (This narrow range of molar ratios was selected after preliminary

experiments covering much wider molar ratios had been made in an analogous manner.) The solutions were then made up to 25μl with buffer, thoroughly mixed, and the tubes sealed. After 1 h at room temperature (20°) the esr spectrum of each tube was recorded, Fig. 1A. The molar ratios of the system (in this case tube 4 containing 7·6μl of NDS solution) just generating a detectable triplet signal was used to calculate the number of molecules of NDS reacting with one molecule of protein. All the determinations were carried out in a similar manner using a range of concentrations of both protein solutions (0·08–0·73% w/v, $1{\cdot}0 \times 10^{-5}$–5×10^{-4} mol litre^{-1}) and NDS solutions (0·025–0·10% w/v, $9{\cdot}33 \times 10^{-4}$–$3{\cdot}73 \times 10^{-3}$ mol litre^{-1}) and inter-tube increases of 0·2–0·5 μl of NDS solution. The external reference facilitated the locating of the low intensity triplet peaks, in the background noise, at the end point. Under these conditions the concentration of NDS radicals was linearly related to the differential peak height of the signal (Fig. 1B) and the detection limit for the NDS radical was $1{\cdot}8 \times 10^{-11}$ mol litre^{-1}.

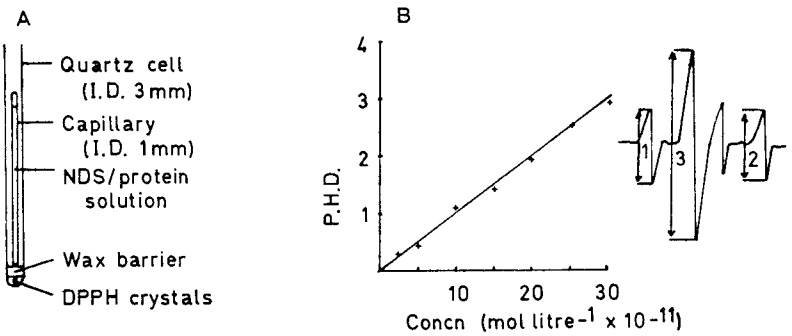

FIG. 1A. Arrangement of external reference with respect to protein—NDS sample in esr spectrometer.

B. Determination of peak height differential (PHD) and the region of linearity with NDS concentration. PHD is the sum of the vertical heights (in mm) of the low field and high field components of the NDS triplet divided by the amplitude (in mm) of the DPPH reference singlet.

$$\text{PHD} = \frac{1+2}{3}.$$

Tyrosine estimations

Two separate assay techniques utilizing the Gerngross reaction of tyrosine with 1-nitroso-2-naphthol were used. The fluorimetric method of Waalkes & Udenfriend (1957) (see also Das Gupta & Boroff, 1966) was applied to both NDS treated (i.e. with the quantity corresponding to the stoichiometric ratios given in Table 1) and untreated protein after acid hydrolysis (Pederson & Foster, 1969). Each result in Table 2 is an average of several determinations each made in triplicate. The second was the more recent absorptiometric assay of Uehara, Mannen & Kishida (1970), each determination in Table 2 being the average of experiments made in triplicate.

RESULTS AND DISCUSSION

The results of the NDS titrations are given in Table 1. For each protein a single result is given from which the stoichiometric ratio can be determined by inspection For example the concentrations of BSA and NDS, in mol litre^{-1}, are those derived from the example given in the methods section, after allowing for the purity of the

Table 1. *The determination of the NDS/Protein stoichiometric ratios by titration with NDS solution.*

	Insulin	HSA	BSA	Trypsin	Ribonuclease	Lysozyme
Protein (mol litre^{-1})	$1{\cdot}10 \times 10^{-10}$	$4{\cdot}0 \times 10^{-11}$	$4{\cdot}0 \times 10^{-11}$	$1{\cdot}60 \times 10^{-10}$	$2{\cdot}00 \times 10^{-10}$	$2{\cdot}00 \times 10^{-10}$
NDS (mol litre^{-1})	$4{\cdot}51 \times 10^{-10}$	$6{\cdot}09 \times 10^{-10}$	$5{\cdot}67 \times 10^{-10}$	$2{\cdot}848 \times 10^{-9}$	$1{\cdot}224 \times 10^{-9}$	$2{\cdot}408 \times 10^{-9}$
Stoichiometric ratio	4·1	15·2	14·2	17·8	6·1	12·0
No. of determinations	5	12	9	18	7	10
Mean stoichiometric ratio	4·1 ± 0·1	15·3 ± 0·2	13·8 ± 0·2	17·7 ± 0·2	5·8 ± 0·3	13·2 ± 0·2

NDS used. The total numbers of determinations made for each protein are reported and the mean stoichiometric ratio together with the standard deviation derived from these measurements. Variations in the concentrations of the protein and NDS solutions did not affect the stoichiometric ratio, indicating that the reaction between NDS and the macromolecules was not concentration dependent within the limits given in the methods.

Table 2 gives the results of the tyrosine assays, for the various proteins, after treatment with NDS under the same conditions as those for the esr experiments. The assay procedures determined the percentage of total tyrosine residues remaining after treatment with NDS; by subtracting the average of the results obtained from the two methods from 100, the mean percentage of total tyrosine residues oxidized by NDS was obtained. From the known composition of the proteins, this percentage figure was equated with the number of tyrosine residues affected by treatment with NDS. For example BSA showed 80·3% [(81·2 + 79·3) ÷ 2] tyrosine residues remaining after treatment with NDS; therefore 19·7% (100–80·3%) of the total residues had been oxidized, this means that four (rounding to the nearest whole number) out of the twenty tyrosine residues in BSA had been oxidized by NDS.

The calculation of the number of exposed tyrosine residues in the various proteins, Table 3, was based on the assumption that each exposed tyrosine residue reacted with two moles of NDS. This figure has been derived from experiments with isolated

Table 2. *Estimation of the number of tyrosine residues oxidized by reaction with the stoichiometric amount of NDS.*

	Insulin	HSA	BSA	Trypsin	Ribo-nuclease	Lysozyme
Absorptiometry: mean % tyrosine assayed	51·3 ±0·9	65·8 ±0·8	81·2 ±0·9	39·5 ±1·6	48·8 ±1·1	31·3 ±1·6
No. of determinations	6	6	6	6	6	6
Fluorimetry: mean % tyrosine assayed	50·5 ±1·8	66·5 ±2·9	79·3 ±1·3	41·8 ±1·1	52·1 ±1·3	27·9 ±2·5
No. of determinations	6	9	9	6	6	6
Mean % oxidized by NDS (100-mean)	49·1	33·8	19·7	59·3	49·5	70·4
No. of accessible residues	1·96	6·10	3·94	5·93	2·97	2·11

Table 3. *Number of exposed tyrosine residues.*

Protein	Total	Number of accessible tyrosine residues				
		NDS	NAI	C	TNM	Solvent perturbation
Insulin	4	2	—	2 [e]	—	2[k]
HSA	18	6	—		—	
BSA	20	4	4[a]	-	—	6[l]
Trypsin	10	6	7[b]	[f]	—	6–8[m]
Ribonuclease	6	3	3[c]	1+1+1[g]	3[i]	3[n]
Lysozyme	3	2	2[d]	2[h]	2·6[j]	

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molecules (Zimmer, Lankin & Horgan, 1971; Ishii, 1972). We were able to confirm this figure using tryptophan-free proteins such as insulin and ribonuclease. For example one molecule of insulin reacted with four molecules of NDS (Table 1) which indicates that two out of the four tyrosine residues in insulin are exposed to this reagent. This figure is in good agreement with that in Table 2. Furthermore, the absorption at 390 nm, associated with the oxidation of tyrosine to an *o*-quinone derivative (Dukler & others, 1971), was found to reach a maximum value after 1 h when the equivalent of two molecules of NDS had reacted with each accessible tyrosine residue in these proteins. This observation confirms that within 1 h all the exposed tyrosine residues had reacted with NDS. For the remaining proteins it was found that, after allowing 2 moles of NDS for each tyrosine residue that had been oxidized, 3 moles of NDS were consumed by each exposed tryptophan residue. This figure, which is the basis of the results reported in Table 4, is also in close agreement with that obtained from studies on the isolated molecule (Zimmer & others, 1971). For example, 1 mole of BSA consumed 14·0 moles NDS (figure in Table 1 rounded to nearest whole number) and 4 tyrosine residues (figure in Table 2 rounded to nearest whole number), requiring 8 moles NDS, were oxidized. The remaining 6 moles NDS are accounted for by reaction of NDS with the two tryptophan residues in the macromolecule. Under our experimental conditions the thiol and disulphide groups in the various proteins would not be expected to be oxidized (Polis & others, 1969; Stell, personal communication).

Table 3 also gives the number of accessible tyrosine residues, in the various proteins, determined using other chemical and physico-chemical methods. Whilst the close agreement between the results with NDS and NAI and CYF might be expected, since the last two reagents, like NDS, initially attack the phenolic hydroxyl group of

Table 4. *Number of accessible tryptophan residues.*

Protein	Total	Number of accessible tryptophan residues NDS	NBS	HNBB	Solvent perturbation
Insulin	0	—	—	—	—
HSA	1	1	—	—	
BSA	2	2	—	—	1–2[e]
Trypsin	4	2	3·7[a]	4·2[c]	2–3[f]
Ribonuclease	0	—	—	—	—
Lysozyme	6	3	6[b]	1·5[d]	3–4[g]

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tyrosine, the close agreement with the results from solvent pertubation studies seems to indicate that the unexposed tyrosine residues are deeply buried within the molecule and consequently cannot interact with a solvent or an external reagent. A three-dimensional model of ribonuclease derived from X-ray studies shows the presence of three exposed tyrosine residues (Kartha, Bello & Harker, 1967) and a similar model of lysozyme (Blake, Johnson & others, 1967) shows three tyrosine groups on the surface of this molecule one of which is not available for oxidation by NDS since its phenolic group is involved in hydrogen bond formation with a neighbouring serine hydroxyl or aspartine group (Kronman & Robbins, 1970; Di Prisco & others, 1970).

Table 4 gives the number of accessible tryptophan residues in the different proteins as determined by various methods. These results are not in such close agreement as those for the tyrosine residues. This probably reflects the sensitivity of the tryptophan residues to the different reagents and reaction conditions used. The model of lysozyme already referred to shows three exposed tryptophan residues in agreement with our determination, and it is generally agreed, in binding studies, that the tryptophan residues in HSA and BSA are exposed (Swaney & Klotz, 1970; Takenaka, Aizawa & others, 1972), as our results indicate.

The good agreement between the studies reported here and those of other workers suggests that these results apply to the substantially unperturbed protein molecules. Preliminary electrophoretic studies do not indicate any gross conformational changes in the proteins under our experimental conditions.

Acknowledgements

The authors wish to express their thanks to Mr. J. G. P. Stell of the Department of Pharmaceutical Chemistry, Bradford University, for helpful discussions and the supply of crystalline insulin and to Dr. C. Jackson for his help and advice particularly with the esr measurements.

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