THE REACTIVITY OF SH GROUPS WITH A FLUOROGENIC REAGENT

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Received 20 January 1970

1. Introduction

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) reacts with amino groups to yield a highly fluorescent derivative [1]. Reaction with the SH groups of cysteine and glutathione yields a less fluorescent product.

This communication describes the use of this reagent as a specific probe for the chemical reactivity of SH groups in some model compounds and proteins.

2. Materials and methods

NBD-Chloride was synthesised by the method of Boulton et al. [2]. The recrystallised product had a m.p. and IR spectrum identical to that reported [2]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [3] phosphorylase *b* [4], and F-actin [5] were prepared from rabbit muscle. Apo-GAPDH was prepared by charcoal treatment of the holoenzyme [6]. Glutamate dehydrogenase was obtained from Boehringer. All other reagents were obtained from commercial sources and were of the highest available purity.

Fluorescence measurements were made on a Zeiss spectrofluorimeter or on an instrument designed in this laboratory [7]. Reaction rates were measured with a Hilger-Gilford kinetic spectrophotometer.

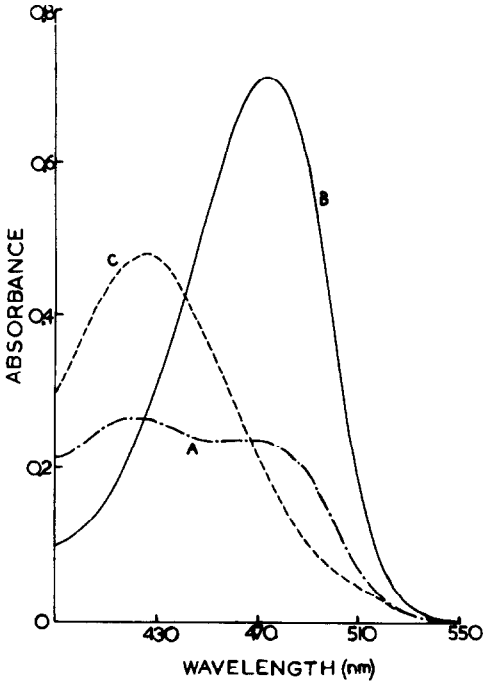

Fig. 1. Absorption spectra of reaction products.
(A) -.-.- 143 μM cysteine + 93 μM NBD-chloride reacted at pH 4.2 for 36 hr.
(B) —— (A) after adjustment of pH to 7.0.
(C) ---- Spectrum of *N*-acetyl-*S*-NBD cysteine at pH 7.0.

3. Results

NBD-Chloride reacts rapidly with the SH groups of *N*-acetyl cysteine and glutathione at pH 7.0 in 50 mM sodium citrate buffer containing 1 mM EDTA. Under these conditions no reaction was observed with *S*-methyl cysteine, methionine, cystine, α-*N*-acetyl lysine amide, alanine, serine, histidine, tyrosine, tryp-

Table 1
Rate constants and stoichiometry of reaction of NBD-chloride with model compounds and proteins.

Compound	k(l mole^{-1} min^{-1})	Groups reacted per mole protein
N-Acetyl cysteine	110	–
Glutathione	325	–
Apo GAPDH (3.54 μM)	1250 (Fast set) 270 (Slow set)	1.3 6.3
Apo GAPDH (3.54 μM) + NAD^+ (7.0 μM)	580 (Fast set) 185 (Slow set)	1.2 6.6
Phosphorylase *b* (5.4 μM)	250	4.0
Phosphorylase *b* (5.4 μM) + AMP (25 mM)	460	2.1
Phosphorylase *b* + glucose-1-phosphate (10 mM)	350	4.0
*L-Glutamate dehydrogenase	no reaction	

The solutions contained 50 mM sodium citrate buffer pH 7.0, 1 mM EDTA.
* in 0.1 M sodium phosphate buffer pH 7.6.

Table 2
Spectral characteristics of NBD derivatives.

Compound	Absorption maximum (nm)	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence polarisation	Relative fluorescence intensity
N-Acetyl-*S*-NBD-cysteine	425	430	545	0.31	1.00 (0% EtOH) 1.70 (90% EtOH)
S-NBD-glutathione	420	420	540	0.31	1.55
N-NBD cyclohexylamine	475	470	545	0.05	40.0
Apo GAPDH	425	425	525	0.38	2.92
Phosphorylase *b*	420	430	525	0.41	–

Relative fluorescence intensities were measured by excitation at the absorption maximum and observation of emission at 350 μM. The buffer was described in table 1.

tophan or glutamic acid over a period of 2 hr at 25°C. The absorption spectrum of the product of reaction with *N*-acetyl cysteine is shown in the fig. and has a maximum at 425 nm. The reaction product with cysteine (in the presence of excess NBD-chloride) has an absorption maximum at 475 nm and a shoulder at 425 nm. With excess cysteine the product has a single maximum at 475 nm. When the latter reaction is carried out at pH 4.2 in the citrate buffer, two distinct maxima at 425, 475 nm are observed. On raising the pH to 7.0 the 425 nm peak disappears and the 475 nm peak is rapidly enchanced (fig.).

The reaction of NBD-chloride with *N*-acetyl cysteine (followed by absorption at 420 nm) is first order in each reactant and is 30 times faster at pH 8.0 than at pH 5.5. Several proteins react rapidly with NBD-chloride and under pseudo first-order conditions the course of reaction can be analysed by the method of Freeman

and Radda [8]. The rate constants and numbers of groups reacting are summarised in table 1.

The spectral and fluorescence properties of the products of reaction with the proteins are similar to those of the products of reaction with *N*-acetyl cysteine and glutathione (table 2). The numbers of groups reacting in each kinetic set (table 1) are derived on the assumption that the absorbance change at 420 nm on reaction (13×10^6 cm^2/mole) is the same in the proteins as in the model compounds. The SH contents of native and modified GAPDH and phosphorylase *b*, as determined after denaturation by sodium dodecyl sulphate by the method of Ellman [9], support this assumption. Table 1 also shows that the reactivities of the SH groups of GAPDH and phosporylase *b* are markedly affected by specific ligands.

4. Discussion

It is evident that this reagent is specific for SH groups under our conditions. Under other conditions NH_2 groups will react [1]. These two reaction products can be clearly distinguished on the basis of their spectral and fluorescence properties (table 2). Cysteine, even at neutral pH, will rapidly form the *N,S*-bis-NBD derivative. The mechanism of this reaction is likely to involve an intramolecular $S \rightarrow N$ transfer. This is supported by the following observations:

a) *S*-Methyl cysteine does not react with NBD-chloride.

b) At pH 4.2, in the presence of excess cysteine, two absorption maxima are observed in the spectrum (fig.) which correspond to the maxima of SH and NH_2 derivatives (table 2). On raising the pH to 7.0 a single peak corresponding to the NH_2 derivative is observed. The spectra of the SH and NH_2 derivatives are pH independent in this range.

c) The NBD group cannot be transferred from *N*-acetyl-*S*-NBD-cysteine to *S*-methyl cysteine.

d) Glutathione forms only the *S*-derivative. In this case intramolecular migration would involve an unfavourable ten-membered ring whereas in cysteine migration occurs in a five-membered ring. A similar $S \rightarrow N$ transfer has been observed in *S*-DNP-cysteine [10].

The specificity of this reagent for SH groups is supported by its lack of reaction with glutamate dehydrogenase which has no reactive SH groups but one very reactive NH_2 group per subunit [11]. Many SH groups of proteins exhibit a higher reactivity than the model compounds studied. Within one protein it is possible to distinguished SH groups with different reactivities and specific ligands often alter the course of the reaction. For example, addition of the effector AMP to phosphorylase *b* brings about protection of two SH groups per enzyme molecule and enhances the reactivity of the other two, whereas glucose-1-phosphate, a substrate, enhances the reactivity of all four. This provides a sensitive method for detecting ligand-induced structural changes.

In addition, the fluorescence of the product is environmentally sensitive (table 2). This sensitivity to environment has been used to follow the transition of G to F-actin which is accompanied by an 1.8 fold enhancement of the NBD-actin fluorescence [12].

Acknowledgements

We thank the Science Research Council for financial support.

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