Alternative Products in the Reaction of 2-Nitro-5-thiocyanatobenzoic Acid with Thiol Groups

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2-Nitro-5-thiocyanatobenzoic acid has been proposed as a reagent for converting thiol groups in proteins into their *S*-cyano derivatives. Evidence was obtained for formation of both the *S*-cyano derivative and the mixed disulphide derivative. Formation of the *S*-cyano derivative can be promoted by addition of excess of CN^- to the reaction mixture.

One of the most widely used techniques for examination of structure–function relationships in proteins is that of chemical modification of amino acid side chains (Vallee & Riordan, 1969). The role of thiol groups in proteins has been particularly well studied, mainly because of the availability of a number of specific thiol-modifying reagents such as organomercurials (Boyer, 1954) and Nbs_2^* (Ellman, 1959). It has become clear that the effect of modification of a given side chain in a protein can depend on the characteristics, such as size and charge, of the grouping introduced into the protein. Thus modification of cysteine-390 residue in pig heart aspartate aminotransferase by Nbs_2 or *N*-ethylmaleimide leads to loss of more than 95% of the enzyme activity, whereas when the side chain of the cysteine-390 residue is converted into the *S*-cyano derivative 60% of the activity is retained (Birchmeier *et al.*, 1973). Introduction of the small uncharged cyano group can thus provide a useful check on whether or not a given thiol group is essential for the catalytic activity of an enzyme. Two methods have been proposed for introduction of the cyano group at thiol groups in proteins: (i) treatment of the mixed disulphide $-\text{S}-\text{S}-\text{Ar}$ derivative (formed by reaction of the protein with Nbs_2) with excess of CN^- (Vanaman & Stark, 1970); (ii) reaction of the protein with 2-nitro-5-thiocyanatobenzoic acid (Degani & Patchornik, 1971). In the present paper, evidence is presented to show that the second method can lead to the

formation of alternative products and that careful examination of the reaction is required in each particular case.

Experimental

Tricine, 2-mercaptoethanol, GSH, Nbs_2 and bovine serum albumin (fraction V) were from Sigma (London) Chemical Co., London S.W.6, U.K. Creatine kinase (ATP–creatine phosphotransferase, EC 2.7.3.2) and glyceraldehyde 3-phosphate dehydrogenase [D -glyceraldehyde 3-phosphate–NAD oxidoreductase (phosphorylating), EC 1.2.1.12] were isolated from rabbit skeletal muscle as previously described (Price & Hunter, 1976; Amelunxen & Carr, 1967). 2-Nitro-5-thiocyanatobenzoic acid and 2-nitro-5-thio[^{14}C]cyanatobenzoic acid (specific radioactivity 1.3×10^{11} c.p.m./mol) were synthesized as described by Degani & Patchornik (1971). The u.v. spectral characteristics and m.p. of these compounds were identical with the literature values. K^{14}CN (specific radioactivity 60 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation counting was performed as described previously (Kayne & Price, 1973). Concentrations of Nbs_2^- were determined spectrophotometrically at 412 nm by using the published extinction coefficient (Ellman, 1959). All reactions were performed in 50 mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C.

Results and Discussion

The reaction of 100 μM -2-nitro-5-thiocyanatobenzoic acid with excess (1 mM) of 2-mercaptoethanol or GSH led to the production of Nbs_2^- in amounts equal to the initial concentration of 2-nitro-5-thiocyanatobenzoic acid, in agreement with previous observations (Degani & Patchornik, 1971).

However, when 2-nitro-5-thiocyanatobenzoic acid was present in excess over 2-mercaptoethanol or

* Abbreviations: Nbs_2 , 5,5'-dithiobis-(2-nitrobenzoic acid); Nbs_2^- , dianion of 2-nitro-5-thiobenzoic acid; GSH, reduced glutathione; Tricine, *N*-tris(hydroxymethyl)methylglycine. Ar represents the group

GSH, the Nbs^{2-} released was less than the initial concentration of thiol compound. Typically, with $25\text{ }\mu\text{M}$ -thiol compound and $250\text{ }\mu\text{M}$ -2-nitro-5-thiocyanatobenzoic acid, the Nbs^{2-} released was 80% (2-mercaptoethanol) or 85% (GSH) of that released by reaction of these compounds with excess of Nbs_2 . Over the 10 min period required for completion of the reactions with 2-nitro-5-thiocyanatobenzoic acid, there was no change in the concentration of these thiol compounds incubated under the reaction conditions. Thus the low extent of release of Nbs^{2-} cannot be accounted for by disappearance of available thiol groups, e.g. by oxidation. When the reaction of the thiol compounds with 2-nitro-5-thiocyanatobenzoic acid was complete (as judged by the lack of any further change in E_{412}) there was no further reaction with added Nbs_2 ($250\text{ }\mu\text{M}$).

The Nbs^{2-} released (0.27 mol/subunit) by reaction of excess of 2-nitro-5-thiocyanatobenzoic acid ($250\text{ }\mu\text{M}$) with creatine kinase (at subunit concentrations of $25\text{--}75\text{ }\mu\text{M}$) was only 28% of that released by reaction of the enzyme with excess of Nbs_2 (0.95 mol/subunit). After completion (10 min) of the reaction with 2-nitro-5-thiocyanatobenzoic acid there was no subsequent reaction with added Nbs_2 ($250\text{ }\mu\text{M}$).

On the basis of these results, it is proposed that the reaction of 2-nitro-5-thiocyanatobenzoic acid with thiol groups can proceed via two possible pathways, as shown in eqn. (1). The reacting thiol group is shown in its ionized form, by analogy with the reaction of thiol groups with Nbs_2 (Brocklehurst *et al.*, 1972):

In the cases of 2-mercaptoethanol and GSH, reaction (a) predominates, whereas with creatine kinase (b) is the major reaction.

If the thiol compound is present in excess over 2-nitro-5-thiocyanatobenzoic acid, further reaction of the disulphide could occur (eqn. 2):

Under these conditions the release of Nbs^{2-} would be stoichiometric with respect to 2-nitro-5-thiocyanatobenzoic acid, as observed in the case of 2-mercaptoethanol and GSH.

Further evidence for the reaction scheme shown in eqn. (1) in the case of creatine kinase was obtained as follows.

(i) *Disulphide formation.* The disulphide R-S-S-Ar has an absorption maximum near 330 nm ($\epsilon_{330} = 7.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). This value was determined by reaction of creatine kinase with Nbs_2 and is in close agreement with the previously published value

Fig. 1. Absorption spectra of creatine kinase derivatives

Creatine kinase ($50\text{ }\mu\text{M}$ subunit concentration) was reacted with 2-nitro-5-thiocyanatobenzoic acid ($250\text{ }\mu\text{M}$) and the mixture dialysed. —, Absorption spectrum of the product (dialysis residue); ----, absorption spectrum of the product when 25 mM-KCN was included in the reaction mixture (the final subunit concentrations of the enzyme were 43 and $40\text{ }\mu\text{M}$ respectively).

(Birchmeier *et al.*, 1973). Creatine kinase was reacted with excess of 2-nitro-5-thiocyanatobenzoic acid and then dialysed or gel-filtered (Sephadex G-25). The absorption spectrum of the protein-containing fraction clearly showed the presence of an absorption peak at 330 nm , characteristic of the disulphide (Fig. 1). The disulphide formed was calculated as 0.67 mol/subunit by using the above value of the extinction coefficient.

(ii) *Cyanide incorporation.* 2-Nitro-5-thio[^{14}C]-cyanatobenzoic acid ($100\text{ }\mu\text{M}$) was reacted with creatine kinase ($75\text{ }\mu\text{M}$ -subunits). The release of Nbs^{2-} corresponded to 0.27 mol/subunit . After gel filtration of the reaction mixture, the incorporation of [^{14}C]-cyano groups into the enzyme was determined as 0.24 mol/subunit , in close agreement with the release of Nbs^{2-} .

(iii) *Stoichiometry of modification.* The sum of the concentrations of products, R-S-S-Ar (0.67 mol/subunit) and R-S-CN (0.24 mol/subunit), is very nearly equal to the concentration of thiol groups that react rapidly with Nbs_2 (0.95 mol/subunit). It is unlikely that other thiol groups on the enzyme are involved in the reaction with 2-nitro-5-thiocyanatobenzoic acid,

since previous reaction of the the enzyme with iodoacetamide leads to quantitative protection against subsequent reaction with 2-nitro-5-thiocyanatobenzoic acid. Iodoacetamide is known to react specifically with the same thiol group on each subunit of the enzyme as does Nbs₂ (Watts, 1973; Price & Hunter, 1976).

Since the type of reaction shown in eqn. (1b) is known to be reversible by addition of excess of CN⁻ (Degani & Patchornik, 1971), it is possible that the reaction of thiol groups with 2-nitro-5-thiocyanatobenzoic acid might be displaced in favour of reaction (1a) by performing the reaction in the presence of excess of CN⁻. The net result would be displacement of the equilibrium shown in eqn. (3) to the right:

This expectation was confirmed for the reaction of GSH (25 μM) with 2-nitro-5-thiocyanatobenzoic acid (250 μM). In the presence of 50 or 25 mM-KCN, the yield of Nbs²⁻ was almost 100% of that released by reaction of GSH with excess of Nbs₂. With smaller concentrations of added KCN, the yield of Nbs²⁻ was lower (e.g. with 10 mM-KCN the yield was 94%). There was some evidence for a very slow reaction between CN⁻ and 2-nitro-5-thiocyanatobenzoic acid under these conditions; appropriate control experiments were thus performed in all cases.

In the reaction of creatine kinase (50 μM-subunits) with 2-nitro-5-thiocyanatobenzoic acid (250 μM), the presence of 25 mM-KCN increased the yield of Nbs²⁻ to approx. 1 mol/subunit and decreased the disulphide formed to less than 0.05 mol/subunit (Fig. 1). By using added K¹⁴CN of the same specific radioactivity as the 2-nitro-5-thio[¹⁴C]cyanatobenzoic acid, the incorporation of cyano groups into the enzyme (either from 2-nitro-5-thiocyanatobenzoic acid or from added CN⁻) could be determined and was found to be in reasonable agreement (±15%) with the measured release of Nbs²⁻ during the reaction. However, the reaction in the presence of excess of CN⁻ is almost certainly more complex than is indicated in eqns. (1) and (3), for the following reasons: (i) in the presence of excess of CN⁻, the production of Nbs²⁻ reached a value of 1 mol/subunit after about 40 min, but the reaction continued at a slow rate for at least 1 h longer; (ii) after release of 1 mol of Nbs²⁻/subunit, the reaction mixture was gel-filtered and the enzyme found to contain a significant concentration (0.3–0.4 mol/subunit) of thiol groups which would react rapidly with added Nbs₂. These findings suggest that interchange reactions may be occurring between neighbouring thiol groups on the enzyme, or that other slowly reacting thiol groups on the enzyme are being modified. It should be noted that there are similar difficulties in interpreting the results of experiments in which the disulphide (formed by reaction of creatine kinase with Nbs₂) is treated with excess of

CN⁻; this reaction also regenerates a similar concentration of thiol groups which react rapidly with Nbs₂. Further investigation of these reactions is required before the specificity of cyanide incorporation into the enzyme can be definitely established.

Creatine kinase which has been reacted with 2-nitro-5-thiocyanatobenzoic acid (in the absence of added CN⁻) retains 1% or less of the activity of unmodified enzyme. Since the disulphide derivative (the major product) has been shown to be totally inactive (Smith *et al.*, 1975), this implies that the *S*-cyano derivative (the minor product) cannot possess more than 4% of the activity of unmodified enzyme. This is somewhat surprising, in view of the report that introduction of the more bulky –S–CH₃ moiety at this thiol group gives a derivative with 18% of the activity of unmodified enzyme (Smith *et al.*, 1975).

The balance of the alternative pathways (eqns. 1a and 1b) in the reaction of 2-nitro-5-thiocyanatobenzoic acid with thiol groups may well depend on the environment of the thiol group concerned. In the case of the model compounds, 2-mercaptoethanol and GSH, the *S*-cyano derivative is the major product. The disulphide derivative is formed predominantly, however, in the case of creatine kinase. When creatine kinase is denatured (by treatment with 6M-guanidinium hydrochloride) and reacted with excess of 2-nitro-5-thiocyanatobenzoic acid, the yield of Nbs²⁻ is approx. 80% of that given by reaction of the denatured enzyme with excess of Nbs₂. This yield is comparable with that given by the model compound GSH on reaction with excess of 2-nitro-5-thiocyanatobenzoic acid under these conditions. Two other proteins studied, glyceraldehyde 3-phosphate dehydrogenase and bovine serum albumin, give very different yields of Nbs²⁻ on reaction with excess of 2-nitro-5-thiocyanatobenzoic acid (60 and 12% respectively of that produced by reaction of these proteins with Nbs₂).

From these results it is clear that the reagent 2-nitro-5-thiocyanatobenzoic acid cannot be relied on to convert thiol groups into the *S*-cyano derivatives quantitatively. In each case the balance of the alternative reactions (eqn. 1) should be checked by determination of the incorporation of [¹⁴C]cyanide by using radioactively labelled reagent and by spectrophotometric determination of the disulphide derivative. It is possible to favour formation of the *S*-cyano derivative by addition of excess of CN⁻ to the reaction mixture, but it is important to check the specificity of the modification reaction carefully, before firm conclusions regarding the role of thiol groups in proteins can be drawn.

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