Synthesis and application of chemically reactive proteins by the reversible modification of protein amino groups with *exo-cis*-3,6-endo-epoxy-4,5-*cis*-epoxyhexahydrophthalic anhydride

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The reversible reaction of *exo-cis*-3,6-endo-epoxy-4,5-*cis*-epoxyhexahydrophthalic anhydride (EEHPA) with free protein amino groups is described. The free protein amino groups of lysozyme can be completely blocked through the reaction of the anhydride EEHPA. The chemically less reactive epoxy groups in EEHPA-modified lysozyme remain intact during modification of the protein and can be used for many subsequent chemical reactions. Hydrolysis of the modified inactive lysozyme at pH 2.5 results in deblocking and almost complete recovery of the enzymic activity of the protein. The epoxy groups in EEHPA-modified proteins have a great many potential uses: disaggregation of supramolecular structures, conversion of hydrophobic membrane proteins or tryptic peptides into water-soluble coloured proteins or peptides, inhibition of tryptic cleavage at lysine residues, synthesis of chemically reactive proteins or enzymes for affinity chromatography or immobilized-enzyme technology, two-dimensional separation techniques for complex protein mixtures, detection of specific protein-binding sites for organic substrates or tumour diagnostics, synthesis of defined artificial glycoproteins for biophysical and cytochemical studies and chemical synthesis of radioactively labelled proteins.

Amide groups that are located close to carboxy groups are stable at neutral or alkaline pH but can be hydrolysed over the acidic pH range under mild conditions by a mechanism involving intramolecular catalysis through the carboxy group (Leach & Lindley, 1953; Butler *et al.*, 1969). The optimal situation for catalysis is an arrangement in which the carboxy and amide functions are eclipsed and unable to rotate freely. This has been shown for maleic anhydride, 2-methylmaleic anhydride, dimethylmaleic anhydride, 3,4,5,6-tetrahydrophthalic anhydride and *exo-cis*-3,6-endo-epoxytetrahydrophthalic anhydride (ETPA) (Butler *et al.*, 1969; Dixon & Perham, 1968; Riley & Perham, 1970; Gibbons & Schachman, 1976). They all react with free protein amino groups to give the corresponding dicarboxylic acid monoamides. The removal of the maleyl groups is too severe for some applications, and the 2-methyl- and dimethyl-maleyl groups may be too labile (Butler *et al.*, 1969; Dixon & Perham, 1968). In addition, the maleyl groups, 2-methylmaleyl groups and dimethylmaleyl groups

all contain activated olefinic bonds that can react with protein thiol groups by irreversible nucleophilic addition. To avoid these disadvantages it was found that ETPA seems to be the optimal molecule for the reversible modification of protein amino groups, representing a potential tool for protein chemistry (Riley & Perham, 1970).

These findings gave us the basic working hypothesis for the synthesis of an epoxy derivative of ETPA and its application in protein chemistry. From chemical and structural considerations the anhydride of such a molecule is expected to be much more reactive than its epoxy group and should react preferentially with the amino groups of the protein. The less-reactive epoxy group could be then used for many subsequent chemical reactions, which would drastically extend the range of application in protein chemistry.

Materials and methods

Reagent, virus and enzyme

exo-cis-3,6-endo-epoxy-4,5-*cis*-Epoxyhexahydrophthalic anhydride (Scheme 1a) was synthesized from *exo-cis*-3,6-endo-epoxytetrahydrophthalic anhydride and peracetic acid and re-

Abbreviations used: EEHPA, *exo-cis*-3,6-endo-epoxy-4,5-*cis*-epoxyhexahydrophthalic anhydride; ETPA, *exo-cis*-3,6-endo-epoxytetrahydrophthalic anhydride.

crystallized from chloroform [m.p. (uncorr.) 241–242°C (decomp.)]. Its structure and purity was confirmed by i.r. and n.m.r. spectroscopy [C. Becker (Institute of Organic Chemistry, University of Basel, Basel, Switzerland), unpublished work]. Egg-white lysozyme (EC 3.2.1.17), three-times-recrystallized, was obtained from Seravac Laboratories (Maidenhead, Berks., U.K.) and highly purified bacteriophage PM2 was obtained from Dr. H. Satake (Biozentrum Basel, Basel, Switzerland).

High-voltage Cellogel electrophoresis

Electrophoresis of proteins was performed on analytical cellulose acetate (Cellogel) strips, which were equilibrated against different buffer systems as described in the Results and discussion section. Electrophoresis was at 100 V/cm and 2°C for 30 min. The proteins were stained with 0.25% Amido Black 10B in methanol/water/acetic acid (9:9:2, by vol.) (Heil & Zillig, 1970).

Assay of protein amino groups

Free protein amino groups were determined with 2,4,6-trinitrobenzenesulphonic acid (Habeeb, 1966).

Assay of protein thiol groups

Free protein thiol groups were determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1958).

Renaturation of unblocked lysozyme

Renaturation of unblocked and reduced egg-white lysozyme was performed as described by Epstein & Goldberger (1963).

Results and discussion

Modification of lysozyme with EEHPA and subsequent reactions

Lysozyme (10 mg/ml) was dissolved in 0.1 M-sodium phosphate buffer, pH 8.8 (approx. 0.15 M- NaH_2PO_4 was adjusted to pH 8.8 with NaOH solution and diluted to 0.1 M), and allowed to react with a 10-fold molar excess of EEHPA over free protein amino groups. The reagent was added in six identical lots to the enzyme solution with continuous stirring at 2°C while the pH was kept constant at pH 8.6–8.8 with 1 M-NaOH. When the reaction was completed, the modified protein (Scheme 1b) was dialysed overnight against 10 mM-sodium phosphate buffer, pH 7.5, at 4°C to remove excess of reagent. It was found that the 10-fold excess of the reagent over free protein amino groups was sufficient for the complete blocking of the amino groups, which was measured by assay with 2,4,6-trinitrobenzenesulphonic acid sodium salt (Habeeb, 1966). A 5-fold excess of EEHPA over protein amino groups was sufficient to block 90% of the amino groups, a finding that was in close agreement with the

modification of lysozyme in the presence of ETPA as described by Riley & Perham (1970). Thus in all subsequent modifications a 10-fold excess of the reagent was used for complete blocking. In order to prove the integrity of the epoxy group in the EEHPA-modified lysozyme and to examine the potential use of this reagent for further chemical reactions, the protein was dialysed overnight against 6 M-urea/10 mM-sodium phosphate buffer, pH 9.5, at 4°C and then allowed to react with 0.1 M-sodium [^{35}S]sulphide for different times at 20 or 37°C (Fig. 1). Under these conditions the epoxide is converted into the corresponding α -hydroxysulphonic acid sodium salt derivative, which was demonstrated independently by direct sulphitolysis of EEHPA and n.m.r. measurements (results not shown). The sulphitolysis of modified lysozyme is shown in Scheme 1(c). During sulphitolysis the disulphide bridges in lysozyme are converted into S-sulphonates, as described by Cecil & McPhee (1959). As the reduced lysozyme is poorly soluble in aqueous solutions, urea or guanidinium chloride was used in these model assays in order to keep the protein in solution (Epstein & Goldberger, 1963). From the maximal values obtained from the time curves of the incorporation of sodium [^{35}S]sulphite into the modified lysozyme (46 220 c.p.m. and 48 890 c.p.m. per 100 μl portion at 20 or 37°C respectively), from the amount of protein (140 μg /portion) and from the specific radioactivity of sodium sulphite in the assay mixture (4.72×10^5 c.p.m./ μmol), we calculated the number of sulphonic acid groups per molecule of lysozyme, which as a result reflected complete availability or modification respectively of all EEHPA epoxy groups after the subtraction of the S-sulphonates that were formed by sulphitolysis of the disulphide bridges in the protein. This number was also consistent with the number of free protein amino groups in the lysozyme molecule. The sulphonic acid derivative of EEHPA-modified lysozyme was purified from the incubation mixture by overnight dialysis against 10 mM-sodium phosphate buffer, pH 7.5, at 4°C. The rates of the conversion of the epoxy group of EEHPA-modified lysozyme into the sulphonic acid derivative in the presence of sodium sulphite at 20 or 37°C were both relatively low. This can be explained by stereochemical hindrance of the electrophilic reaction at the epoxy group, which is located very close to the *exo-cis*-3,6-*endo*-epoxy group of the molecule. It was found in a different set of experiments (results not shown) that this conversion could be achieved much more quickly by using *cis*-4,5-epoxyhexahydrophthalic anhydride. However, the use of such a reagent turned out to be disadvantageous because reaction of the epoxy residues occurred with internal groups (thiol groups) of the protein, causing irreversible modifications.

Scheme 1. Chemical reactions of *exo-cis*-3,6-*endo*-epoxy-4,5-*cis*-epoxyhexahydrophthalic anhydride (EEHPA) with free protein amino groups and subsequent chemical modifications

Fig. 1. Kinetics of sulphitolysis of the epoxy group of EEHPA-modified lysozyme at 20°C (●) or 37°C (○). Portions of the incubation mixtures were collected at different times. The protein (140 µg/portion) was precipitated in the presence of 85% (v/v) ethanol, collected by centrifugation and its radioactivity counted in a liquid-scintillation spectrometer. The s.d. from three independent experiments was calculated to be maximally $\pm 8\%$.

In further experiments the epoxy groups of EEHPA-modified lysozyme and untreated lysozyme (control) were allowed to react with 0.1 M-dithiothreitol/4 M-guanidinium chloride/10 mM-sodium phosphate buffer, pH 9.5, for 16 h at 37°C (Scheme 1d). The thiol derivative of EEHPA-blocked lysozyme and unblocked lysozyme were purified from the incubation mixture by extensive dialysis under N_2 against 4 M-guanidinium chloride/10 mM-sodium phosphate buffer, pH 7.5, for 72 h at 4°C, and the thiol groups in the assays were determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1958). As a result the epoxy groups in the modified enzyme were converted quantitatively into the corresponding α -hydroxy thiol derivatives, which was consistent with the reaction of the epoxy groups in the presence of the sodium sulphite. After high-voltage Cellolog electrophoresis in the presence of 6 M-urea/0.1% (v/v) 2-mercaptoethanol/50 mM-sodium phosphate buffer, pH 7.0, the mobilities (m) were -2.5 cm (migration to the cathode) for unmodified lysozyme, $+2.2$ cm (migration to the anode) for EEHPA-modified lysozyme (Scheme 1b), $+2.6$ cm for the reaction product of EEHPA-modified lysozyme and dithiothreitol (Scheme 1d), and $+3.9$ cm for the reaction product of EEHPA-modified lysozyme and sodium sulphite (Scheme 1c).

Unblocking of EEHPA-modified lysozyme

For unblocking the EEHPA-modified lysozyme (Scheme 1b) or its reaction product with sodium sulphite (Scheme 1c) or dithiothreitol (Scheme 1d) were dialysed against two changes of 0.1% (v/v) 2-mercaptoethanol/6 M urea/0.2 M-sodium citrate buffer, pH 2.5 (approx. 0.25 M-citric acid was ad-

justed to pH 2.5 with NaOH solution and diluted to 0.2 M), for 30 h at 25°C, as described by Riley & Perham (1970). Some protein was precipitated during the first 2 h of dialysis at pH 2.5, but was all back in solution after about 5 h.

After high-voltage Cellolog electrophoresis in the presence of 6 M-urea/0.1% (v/v) 2-mercaptoethanol/50 mM-sodium phosphate buffer, pH 7.0, the mobilities (m) were -2.6 cm for unmodified lysozyme (control), -2.6 cm for unblocked lysozymes in Scheme 1(b) and 1(d), and $+2.1$ cm for the unblocked lysozyme in Scheme 1(c). From these results it became obvious that the modified lysozymes in Scheme 1(b) and 1(d) could be unblocked completely by mild hydrolysis. This was not the case for the modified lysozyme in Scheme 1(c), where the epoxy groups were converted into sulphonic acid residues after incubation with sodium sulphite.

It was found in a different set of experiments (results not shown) that the hydrolysis (unblocking) of modified lysozyme was always incomplete under mild conditions when strong anionic residues were introduced into the modifier through the reaction with the epoxide. Under these conditions, complete unblocking occurred only in the presence of strong acid (1 M-HCl), increased temperature (50–70°C) and increased times of hydrolysis (2–5 days), whereby hydrolysis of protein was observed.

Dialysis at pH 2.5 for 30 h was sufficient to regenerate more than 90% of the protein amino groups of the modified lysozymes in Scheme 1(b) and 1(d) as measured with 2,4,6-trinitrobenzenesulphonic acid, which is consistent with the results of hydrolysis experiments described by Riley & Perham (1970).

Finally, unmodified but reduced lysozyme (control) and the unblocked lysozymes in Scheme 1(b) and 1(d) were dialysed against 0.1 M-acetic acid as described by Epstein & Goldberger (1963). The relative specific enzymic activities were 0.0 for the modified lysozymes in Scheme 1(b) and 1(d), 0.92 for the unblocked lysozyme in Scheme 1(b), 0.94 for the unblocked lysozyme in Scheme 1(d) and 1.0 for unmodified reduced lysozyme (control) after reactivation.

Some applications of chemically reactive proteins

(i) *Dissociation of supramolecular structures with highly hydrophobic membrane proteins into water-soluble coloured proteins and separation of the subunits.* The lipid-containing bacteriophage PM2 contains a superhelical DNA, four water-insoluble structural proteins, and phosphatidylethanolamine and phosphatidylglycerol as the major lipids (Hinnen *et al.*, 1974).

Treatment of intact bacteriophage PM2 with EEHPA, subsequent reaction with dithiothreitol as

described above, removal of excess of dithiothreitol by extensive dialysis under N_2 and incubation with an aromatic disulphide (with $A_{\max}$ at 505 nm and a molar absorption coefficient of $32\,000\text{ M}\cdot\text{cm}^{-1}$) at a concentration of 0.005 M, completely dissociated the virus.

The structural proteins were converted into water-soluble red proteins (with $A_{\max}$ at 590 nm and a molar absorption coefficient of $15\,000\text{ M}\cdot\text{cm}^{-1}$; Scheme 1e).

Under these conditions the positive charge of one lysine residue is converted into four negative charges and the molecular weights of the individual proteins were increased drastically (by $C_{28}H_{21}N_2O_{14}S_5$, which is equivalent to 769/free protein amino group) after the modification with such a complex organic residue (Scheme 1e). The protein subunits and the water-soluble modified phosphatidylethanolamine could then be separated either by polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate or by Sephadex column chromatography in aqueous solution. The stained protein subunits with a deep-red colour could be monitored visually and they migrated or were eluted according to their different molecular weights.

Unblocking of the separated proteins occurred as described under 'Unblocking of EEHPA-modified lysozyme' above, but in two steps; because we found that unblocking of EEHPA-modified proteins was difficult when strong anionic groups were present in the modifier, the disulphide bridges between the dithiothreitol residue and the dye were first cleaved in the presence of high concentrations of reducing agents [2% (v/v) 2-mercaptoethanol or 0.05 M-sodium dithionite] at 20°C. The dye was removed by extensive dialysis in the presence of the reducing agents at 4°C for 24 h, and final unblocking was performed with 8 M-urea/2-mercaptoethanol/sodium citrate buffer, pH 2.5, as described above.

(ii) *Detection of sites that bind reagents containing thiol groups.* We have found that the fluorescent dye Cyanin RS (Scheme 1g) and derivatives specifically bind to surface proteins of transformed human cells and as a consequence specifically stain some malignant transformed human cells isolated from glioma, meningioma, adenoma, sarcoma and leukaemic cells (D. Schäfer & R. Schäfer, unpublished work). In order to analyse specific binding sites on surface proteins of transformed cells, human leukaemic cells were incubated first with the fluorescent dye described above, followed by removal of excess of the dye by dialysis of the incubation mixture. The cells were then modified in the presence of 5 mM-EEHPA at pH 7.8 in balanced salt solution (0.8% NaCl) and subsequently incubated at 37°C for 16 h. Under this condition the thiol residue of the fluorescent dye that was membrane-associated had a chance to react covalently with the epoxy groups of

the EEHPA-modified surface proteins of the malign cell. After this step the transformed human leucocytes were collected by centrifugation, dissociated in the presence of sodium dodecyl sulphate and separated by polyacrylamide-slab-gel electrophoresis. In the case of the transformed leucocytes the fluorescent dye was bound predominantly to a glycoprotein that migrated as a 96 kDa polypeptide (as revealed by fluorescent detection and calibration with marker proteins).

(iii) *Further applications.* Proteins or enzymes that are modified with sub-stoichiometric amounts of EEHPA over protein amino groups can be linked covalently to cellulose at pH 9–10 by using the reactive epoxy group of the modifier in the protein. Such applications can be used for affinity chromatography or immobilized-enzyme technology (Scheme 1f).

Proteins with defined sequence or cell-surface components can be modified with EEHPA and converted into defined glycoproteins by using the reactive epoxy group in the EEHPA-modified proteins for the covalent linkage to selected polysaccharides at pH 9–10 (Scheme 1f). Under these conditions the effect of glycosylation can be studied biophysically or cytochemically under defined conditions.

As described above under 'Modification of lysozyme with EEHPA and subsequent reactions', proteins that are modified with EEHPA can be chemically labelled by incubation with radioactive compounds that are able to react with epoxides. Furthermore, the reversible modification of proteins with EEHPA can be also used for separation of complex protein mixtures by two-dimensional polyacrylamide-gel electrophoresis.

EEHPA has a great potential in sequence work, as a method of inhibiting tryptic cleavage at lysine residues, as a means of converting water-insoluble lysine-containing peptides into water-soluble and coloured peptides after reaction of the tryptic-digest EEHPA-modified lysyl peptides with dithiothreitol and disulphide dimers of aromatic dyes.

A summary of the various chemical reactions described above is given in Scheme 1.

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