A C T A B I O C H I M I C A P O L O N I C A

Vol. 34 1987 No. 2

EWA WASYLEWSKA, JOANNA DULIŃSKA, VLADIMIR S. TRUBETSKOY[a], VLADIMIR P. TORCHILIN[a] and WŁODZIMIERZ S. OSTROWSKI

STABILIZATION OF HUMAN PROSTATE ACID PHOSPHATASE BY CROSS-LINKING WITH DIIMIDOESTERS*

Institute of Medical Biochemistry, N. Copernicus Academy of Medicine, Kopernika 7, 31-034 Kraków, Poland
[a]U.S.S.R. Cardiology Research Center, Moscow, 121552, U.S.S.R.

Received 10 December, 1986; Revised 11 February, 1987

1. Modification of dimeric human prostate acid phosphatase (EC 3.1.3.2) by diimidoesters leads to the formation of water-soluble preparations of high enzymatic activity, resistant to denaturing agents.

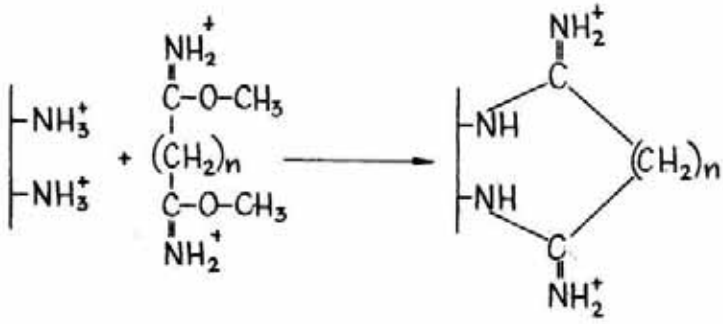
2. Monomeric, dimeric, trimeric and tetrameric species were found in SDS-polyacrylamide gel electrophoresis of the phosphatase cross-linked with dimethyl-suberimidate, and dimeric, trimeric and tetrameric enzymatically active species on thin-layer Sephadex 200 gel filtration. This molecular pattern evidenced formation of the inter-subunit covalent linkages. All molecular forms are immunoreactive against the polyclonal rabbit anti-phosphatase antibodies.

3. The catalytic properties of the modified phosphatase are almost the same as those of the native enzyme. Differences in the optical properties between the modified and the native enzymes point to slight conformational transitions in the modified enzyme.

Enzymes are commonly used in food industry, analytical chemistry, preparative organic chemistry, chemical technology, agriculture and medicine. However, their instability under technological conditions due to denaturation or inactivation by heat, proteolysis, organic solvents etc., limits their application. Therefore recently the problem of enzyme stabilization has attracted considerable attention [1]. One of the general methods of stabilizing conformation of native proteins is based on formation of the intra- and inter-molecular cross-linkages which prevent unfolding of the protein molecule and dissociation of oligomeric proteins [2]. Bifunctional reagents are often used for these purposes [3, 4].

* This work was supported in part by Grant No 09.7.1.3.2. from the Polish Academy of Sciences.

Acid phosphatase (EC 3.1.3.2) belongs to the enzymes of practical importance. It is used as a tool in sequencing of oligonucleotides and for clinical diagnosis [5]. The enzyme is a very unstable glycoprotein of M_r of about 100 000, composed of two identical subunits [6]. Higher thermostability of prostatic phosphatase and resistance to denaturating agents was achieved in our laboratory some years ago by immobilization of the enzyme on CM cellulose [7] and cross-linking with bovine serum albumin [8]. Also Torchilin *et al.* [9] succeeded in immobilization of this phosphatase on CNBr-Sepharose, Concanavalin/A-Sepharose and ethylenediamine-Sepharose. More recently, soluble, active phosphatase preparations of enhanced thermostability were obtained by modification with glutaraldehyde [10] or cross-linking with diamines of the $H_2N\text{-}(CH_2)_n\text{-}NH_2$ type [11], (Scheme 1). This paper



Scheme 1

describes the formation and some properties of soluble derivatives of human prostate phosphatase obtained by modification with diimidoesters.

Bisimidate esters are highly specific reagents for primary amino groups in proteins (Scheme 1). They are soluble in water and react under mild conditions (pH 7 - 10) yielding protein derivatives with unaltered charge and therefore of good solubility. The side reactions with thiol, phenolic, carboxyl, imidazolyl and guanoyl groups are negligible [3]. The resulting amidine is quite stable and resistant to acid hydrolysis.

The reaction conditions: protein concentration, the bifunctional agent/protein ratio, pH and ionic strength largely predetermine which kind of linkage, inter- of intra- is dominating [3, 4].

MATERIALS AND METHODS

Reagents. Goat antirabbit IgG serum was obtained from Wytwórnia Surowic i Szczepionek (Poland). Antibody against prostatic acid phosphatase was raised in New Zealand white rabbits with purified enzyme [12]. All other chemicals were of analytical grade.

Phosphatase isolation. Prostatic acid phosphatase was isolated from seminal fluid. Whole semen was collected from volunteer normal adult men (25 to 40 years old). Cells were removed from the seminal plasma by centrifugation at 2000 *g* for 10 min. The seminal fluid was kept frozen at −20°C until use. The phosphatase was purified by affinity adsorption on tartramic acid -Sepharose 4B according to Van Etten & Saini [13].

Acid phosphatase activity was determined using 10 mM *p*-nitrophenyl phosphate as a substrate in 50 mM acetate buffer, pH 5.0, at 20°C [14].

Protein was determined by the method of Lowry *et al.* [15].

Cross-linking reaction. Dry dimethyl imidate dihydrochlorides of adipic, pimelic and suberic acids were added to the enzyme solution (0.5 mg/ml) in 50 mM borate buffer, pH 9.0, up to the concentration indicated. The reaction mixture was incubated for 1 h at 20°C and the reaction was stopped by gel filtration through the Sephadex G-50 column (8 × 1 cm) equilibrated with 10 mM Tris/HCl buffer, pH 7.0, containing 100 mM KCl [16, 17].

pH optimum. The determination was carried out at 20°C using 10 mM pNPP[1] in the following buffers (all 50 mM): glycine/HCl (pH 2.5 - 3.5), acetate (pH 4.0 - 5.5), imidazole/HCl (pH 6.0 - 7.0), Tris/HCl (pH 7.5 - 8.5) and glycine/NaOH (pH 9.0 - 10.0). Incubation time was 1 min.

Temperature optimum. The optimum for the hydrolysis reaction was determined using 10 mM pNPP in 100 mM acetate buffer, pH 5.0, in the temperature range from 20 to 80°C. Incubation time was 1 min.

Thermostability. The enzyme protein (0.5 mg/ml) in 10 mM Tris/HCl buffer, pH 7.0, containing 100 mM KCl was incubated at 60°C. At appropriate time intervals aliquots were withdrawn, transferred to ice bath, and phosphatase activity was determined at 20°C.

Denaturation. The enzyme (0.5 mg/ml in 10 mM Tris/HCl buffer containing 100 mM KCl) was denatured at 20°C for 5 min with ethanol or DMSO at concentration up to 50%. Alternatively the enzyme was denatured at pH 2.0 or at pH 10 (10 mM citrate/borate buffers) for 15 min at 20°C. The enzyme denatured with 8 M urea for 15 min at 20°C was renatured by overnight dialysis against 50 mM citrate buffer, pH 5.0, containing 1 mM EDTA and 1 mM dithiothreitol. In each case the enzyme activity was assayed by incubation for 1 min at 20°C with 10 mM acetate buffer, pH 5.0.

Gel filtration. Thin layer filtration on Sephadex G-200 Superfine gel in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl was performed

[1] Abbreviations used: pNPP, *p*-nitrophenyl phosphate; SDS, sodium dodecyl sulphate; GA, glutaraldehyde; DMSO, dimethyl sulphoxide; DMA, dimethyladipimidate; DMP, dimethyl pimelimidate; DMS, dimethyl suberimidate; TEMED, *N,N,N',N'*-tetramethylenediamine.

on 19 × 19 cm glass plates at room temperature. The phosphatase activity on Whatman 3 MM replica was localized using the mixture of sodium 2-naphtyl phosphate (1 mg/ml) and Fast Blue (1 mg/ml) in 100 mM acetate buffer, pH 5.0. For protein staining, 0.1% bromophenol blue in glacial acetic acid: methanol (1:9; v/v) was used. Molecular weight was determined from migration distance [18].

Gel electrophoresis. SDS-PAGE was performed on 10% gel slabs under conditions described by Laemmli [19] as modified by Ryrie & Gallagher [20]. The protein sample (1 mg/ml) was boiled for 2 min in 1% SDS and 2% dithiothreitol solution prior to separation. Proteins resolved during electrophoresis were either directly stained with 0.3% Coomassie Brilliant Blue or were transferred to nitrocellulose paper, BA 85 [21]. On one nitrocellulose copy proteins were stained with 0.2% Amido Black dissolved in methanol:acetic acid:water (4.5:1:4.5, by vol.). The second copy was used as an immunobinding probe. The antigens were detected on nitrocellulose paper by the double antibody method according to De Blas & Chervinski [22]. An excess of binding sites on nitrocellulose was saturated with 2% Tween 20 [23] and the reaction with the antiphosphatase rabbit serum took place under gentle rocking for 12 h in 50 mM Tris/HCl, pH 8.0, containing 0.05% Tween 20. Incubation with the peroxidase-conjugated antirabbit goat antibody [24] lasted 1 h. The peroxidase activity detected with 3,3'-diaminobenzidine as substrate according to De Blas & Chervinski [22].

Optical methods. Fluorescence emission spectra recorded with Aminco SPF 500 spectrofluorimeter, at the excitation wavelength of 290 nm: the native and modified phosphatase (0.09 mg/ml) were dissolved in 10 mM citrate/borate buffer of pH 2.0, 5.0 and 7.0. The differential spectrum was measured using the Yanaco spectrophotometer over the wavelength range from 210 to 400 nm: both forms of phosphatase (0.09 mg/ml) were dissolved in 10 mM citrate/borate buffer, pH 5.0.

RESULTS

A homologous series of dimethyl imidoesters of adipic, pimelic and suberic acids at 5 mM concentration was used to obtain cross-linked preparations of prostatic acid phosphatase. The modification process caused an about 10% decrease in enzymatic activity. The modified preparations were tested for thermostability at 60°C (Fig. 1). The stabilization effect depended on the carbon chain length of imidates, and was the highest with the suberic acid diimidoester (DMS). This compound was chosen for further cross-linking experiments.

The DMS modified enzyme preparation was subjected to SDS-polyacrylamide gel electrophoresis following reduction of -SS-bonds with dithiothrei-

tol. Densitometric scans of the electrophoretograms of the native (A) and cross-linked (B) enzyme (Plate 1) prove the appearance of protein fractions of molecular weight of 48 000, 95 000 and 180 000. Similar patterns were obtained for the phosphatase cross-linked with the shorter imidates over the concentration range from 5 to 30 mM. This means that the subunits are covalently bound forming dimers, trimers and tetramers, although monomers are also found in the cross-linked preparations.

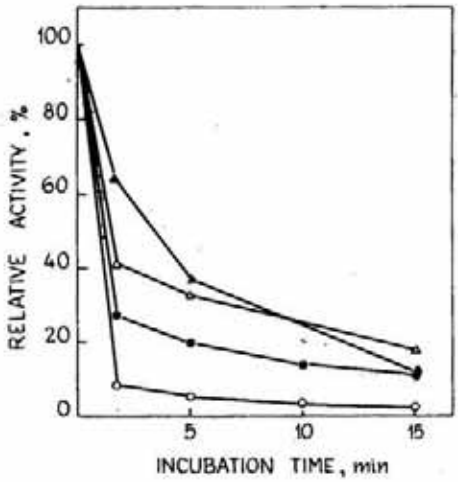


Fig. 1. Thermoinactivation at 60°C of native phosphatase and phosphatase preparations cross-linked with different diimidoesters, at concentration of 5 mM. The native enzyme (○) and the enzyme (0.5 mg/ml, i.e. 5×10^{-6}M) treated with dimethyl adipimidate (●), dimethyl pimelimidate (△) and dimethyl suberimidate (▲)

Thin layer gel filtration on Sephadex G-200 was used to analyse the DMS-modified phosphatase under non-denaturing conditions. The Whatman 3 MM replica stained for protein and phosphatase activity (Fig. 2) demonstrates three enzymatically active fractions of the following molecular weight: 100 000, 135 000 and 200 000. This indicates that cross-linking of the phosphatase by DMS produced several oligomers of subunits (dimers, trimers and tetramers) all showing enzymatic activity.

To investigate conformational changes due to modification, optical properties of native and cross-linked phosphatase were studied. The fluorescence emission spectra of both forms of the enzyme at pH 2.0, 5.0 and 7.0 (Fig. 3) show that the increase of the emission maximum of the native enzyme with increasing pH was similar to that observed for other proteins [25] whereas the cross-linked product showed the highest fluorescence maximum at pH 2.0. At the same time this maximum was shifted towards shorter wavelength by about 3 nm. These evident changes in spectral properties of the cross-linked enzyme were confirmed by the difference spectra (Fig. 4).

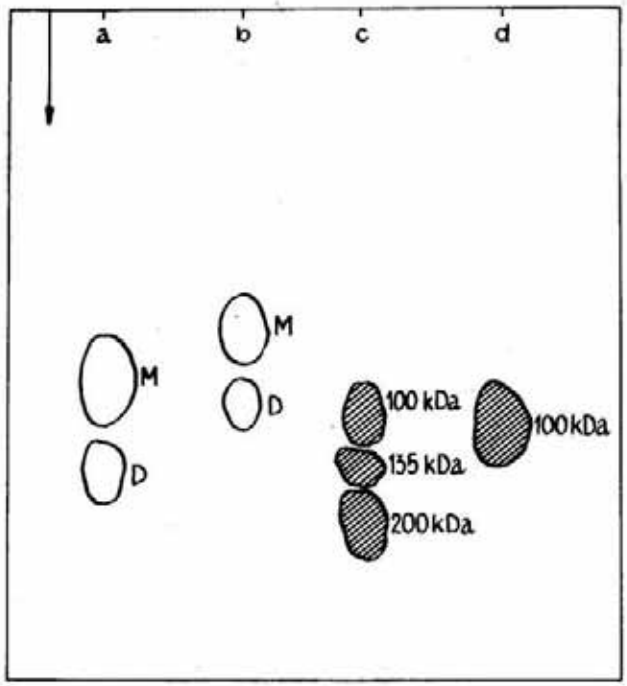


Fig. 2. Thin-layer chromatography on Sephadex G-200 Superfine gel of the native and dimethyl suberimidate modified phosphatase. The separation was performed in 10 mM Tris/HCl buffer, pH 7.0, containing 100 mM KCl. The Whatman 3 MM replica were stained for protein (outlined) and for phosphatase activity (hatched). The separated proteins: (a) bovine serum albumin, (b) ovalbumin, (c) modified phosphatase, and (d) native phosphatase (M, monomer; D, dimer)

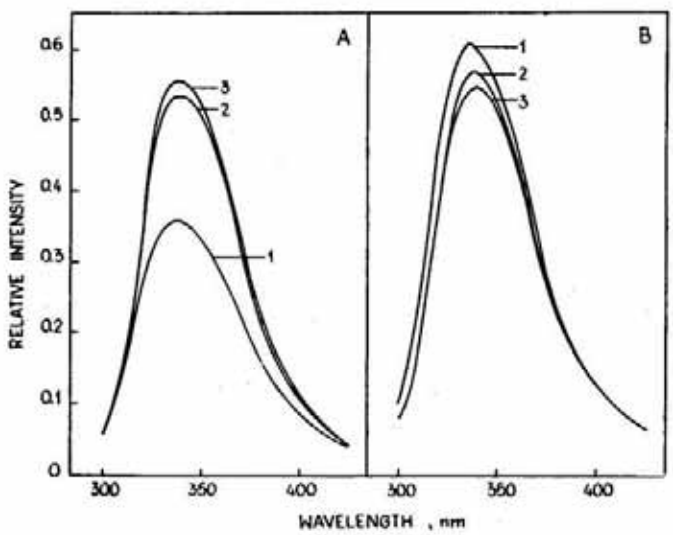


Fig. 3. Tryptophanyl fluorescence emission spectra of the native phosphatase (A) and phosphatase preparation cross-linked with dimethyl suberimidate (B). Excitation wavelength 290 nm. The spectra were taken at pH 2.0 (1), 5.0 (2) and 7.0 (3)

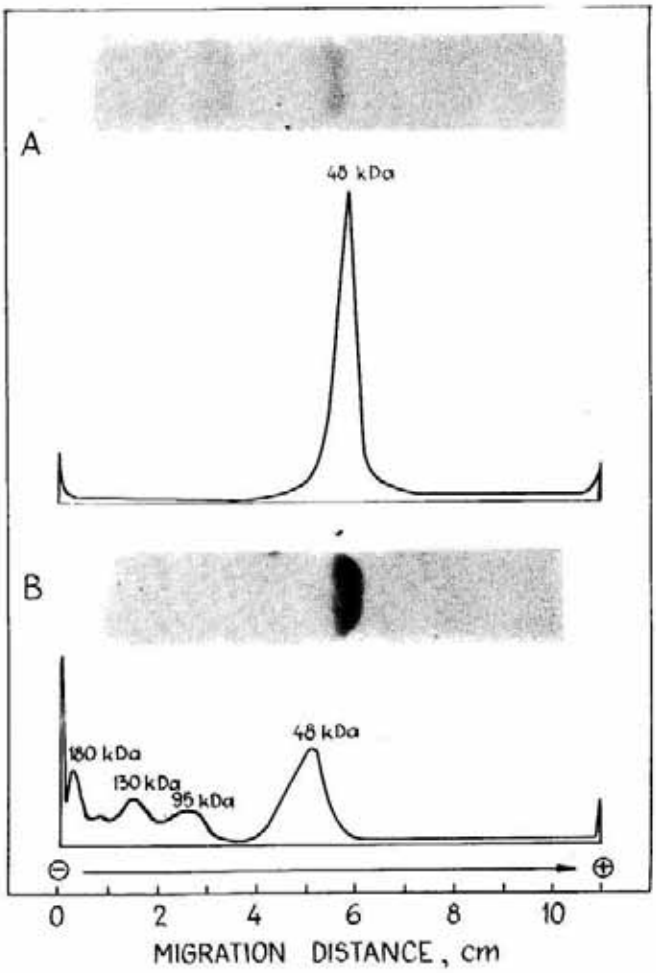


Plate I. SDS-Polyacrylamide gel electrophoretic patterns of native phosphatase (A) and phosphatase cross-linked with dimethyl subermidate (B). For details see Methods

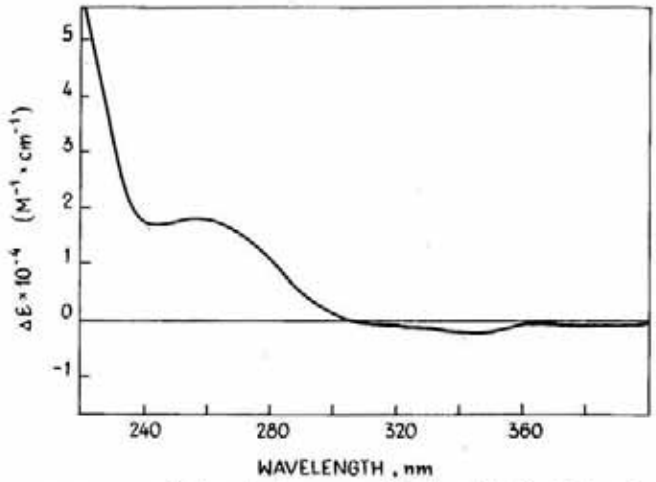


Fig. 4. Difference spectrum of phosphatase cross-linked with dimethyl suberimidate against the native enzyme at pH 5.0.

The differences observed could be interpreted as evidence for formation of the intra-molecular covalent bonds.

The immunological properties of the modified phosphatase were practically unchanged. All fractions of the cross-linked phosphatase separated on SDS-PAGE were bound to the polyclonal antiphosphatase antibodies (Plate 2).

The enzymatic properties of the modified prostate acid phosphatase were slightly changed as compared to those of the native protein; K_m values were the same. The modification of phosphatase with DMS raised its pH optimum from 5.0 to 5.5 (Fig. 5A). Although the temperature

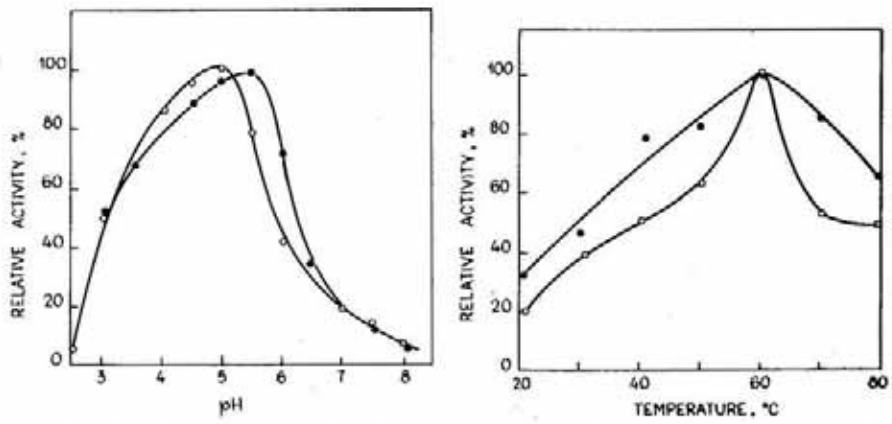


Fig. 5. pH (A) and temperature (B) optima for the native (○) and modified (●) phosphatase

optimum remained unchanged after cross-linking (Fig. 5B) the activity of the modified enzyme both below and above optimum temperature was higher as compared with the native enzyme.

As shown in Fig. 6 the cross-linked prostatic acid phosphatase was much more resistant to ethanol and DMSO than the native enzyme.

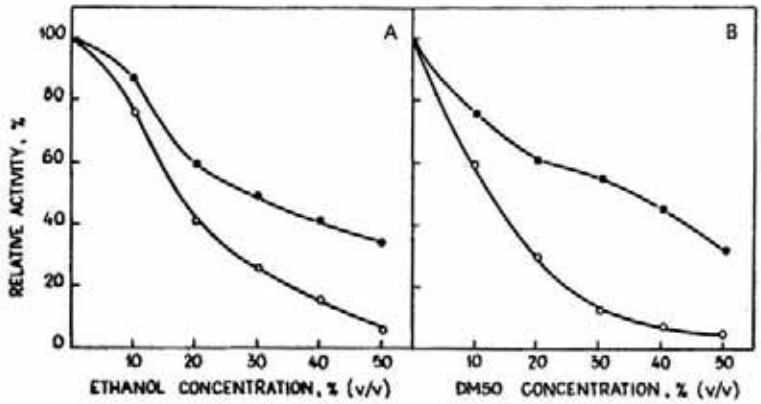


Fig. 6. The effect of ethanol (A) and dimethyl sulphoxide (B) on the activity of native (○) and dimethyl suberimidate cross-linked phosphatase (●)

Renaturation of both enzyme forms totally inactivated by 8 M urea was achieved by 16 h dialysis against 50 mM citrate buffer, pH 5.0, containing 1 mM dithiothreitol and 1 mM EDTA. The recovery of catalytic activity was 85% for the native and 57% for the modified phosphatase. After 15 min treatment at pH 2.0 or pH 10 the modified enzyme retained

Table 1

Effect of denaturing agents on the activity of prostatic phosphatase

Treatment	Activity of the enzyme	
	native (%)	modified (%)
None (control)	100	100
Urea, 8 M	0	0
Urea + 16 h dialysis	85	57
pH 2.0	6.7	64.7
pH 10	9.2	23.6

much higher activity than the native enzyme (Table 1). It is not clear why the activity of renatured modified enzyme is lower than the renatured native enzyme. The above results demonstrate that the cross-linked product of prostate phosphatase was more resistant to denaturation under various conditions.

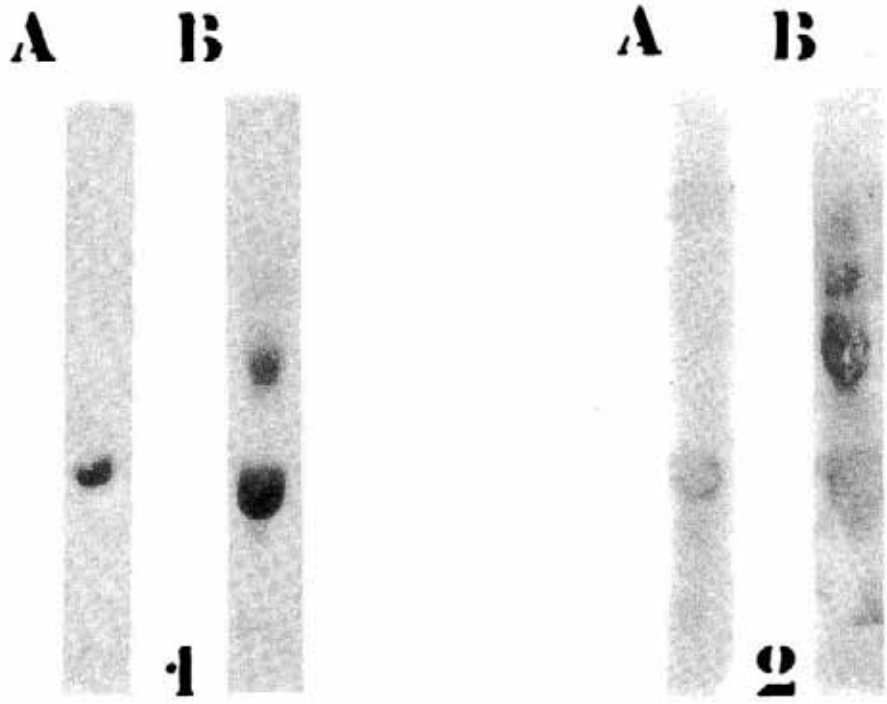


Plate 2. Nitrocellulose replicas of a SDS-polyacrylamide gel stained for protein (1) and immunoreactivity (2) after electrophoretic separation of the native (A) and the cross-linked preparation (B). Peroxidase activity was measured of the antigen-antibody-anti-IgG serum complex bound to peroxidase. For details see Methods

DISCUSSION

Practical application of enzymes requires their stabilization by the methods not involving the use of carriers. The best of such methods is based on formation of covalent bonds which make the globular protein molecules more rigid and resistant to denaturing agents [1, 2, 26].

Imidoesters of dicarboxylic acid are often used for immobilization and stabilization of protein molecules because stable and acid-resistant amidine derivatives formed in the reaction of diimidoesters with the primary amino group of lysine residues (Scheme 1) possess the same charge as the unmodified lysine [3, 4]. Diimidoesters can stabilize the tertiary structure of protein due to intra-subunit linkages, as well as quarternary structure formed by the inter-subunit covalent bonds. Moreover, diimidates were used for studying the subunit composition of oligomeric proteins [16, 27, 28], for determination of the distance between lysine residues [29], for investigating the protein-protein interaction [30] and stabilization of monomeric and oligomeric proteins [17, 31].

Our results on modification of the human prostate acid phosphatase with diimidoesters of adipic, pimelic and suberic acids show that the modified phosphatase was soluble and enzymatically and immunologically active. Irrespective of DMS (5 - 30 mM) and protein (0.1 - 0.5 mg/ml) concentration, the molecular composition of the cross-linked phosphatase was, as shown by SDS-PAGE, the same as that of the native enzyme. The presence of monomers, dimers, trimers and tetramers points to the formation of inter-subunit covalent linkages and proves that, in our preparation, not all subunits were covalently linked (Plate 1). Under non-denaturing conditions active dimers, timers and tetramers were revealed and dimeric molecule was not dissociated (Fig. 2). Dissociation of subunits was previously found in the phosphatase modified with glutaraldehyde [10]. The composition of this preparation depended on concentration of the cross-linking agent (0.25 - 3%) and included monomers and dimers, or all molecular forms at the two extreme concentrations, respectively [10]. However, the phosphatase modified with 1,12-dodecamethylene diamine showed on SDS-polyacrylamide gel not only the monomer or dimer but also the multipolymer forms [11].

The fluorescence emission spectra proved conformational transitions of the phosphatase due to the diimidoester cross-linking. The opposite effect of pH on the absorbance at the maximum of tryptophanyl fluorescence emission spectrum and a shift at pH 2.0 to a shorter wavelength of the modified phosphatase evidenced alterations in tryptophan environment after cross-linking [32].

The u.v. difference spectrum (Fig. 4) has a maximum at about 257 nm characteristic for phenylalanine, but the shape of the curve and the absorbance

at 270 - 300 nm must be related also to tyrosine and tryptophan residues [33, 34].

The shift of the pH optimum from 5.0 to 5.5 denotes a slight alteration in the catalytic centrum of the modified phosphatase. Modification of this enzyme by diamine resulted in the shift of pH optimum to lower values [11]. One can assume that higher resistance to organic solvents (Fig. 6) and heat (Fig. 1) of the DMS-cross-linked phosphatase is due to stabilization of the native conformation by covalent linkages [35, 36]. Also the phosphatase modified with amines or glutaraldehyde were more resistant to thermoinactivation than the native enzyme [10, 11].

The binding of polyclonal rabbit antiphosphatase antibodies to all molecular species of the diimidoester cross-linked prostate phosphatase (Plate 2) proved that the structure of the antibody binding site in the phosphatase molecule is not disturbed by covalent linkages.

The cross-linking of human prostate acid phosphatase by diimidoesters is a convenient method for stabilization of that labile enzyme protein; it avoids denaturation of the enzyme and provides opportunities for its use in biotechnology and biochemical diagnostics.

REFERENCES

1. Torchilin, V. P. & Martinek, K. (1979) Enzyme stabilization without carriers. *Enzyme Microb. Technol.*, **1**, 74 - 82.
2. Klibanov, A. M. (1979) Enzyme stabilization by immobilization. *Anal. Biochem.*, **93**, 1 - 25.
3. Han, K. K., Richard, C. & Delacourte, A. (1984) Chemical cross-links of proteins by using bifunctional reagents. *Int. J. Biochem.*, **16**, 129 - 145.
4. Peters, K. & Richards, F. M. (1977) Chemical cross-linking: reagents and problems in studies of membrane structure. *Ann. Rev. Biochem.*, **46**, 523 - 551.
5. Ostrowski, W. (1980) Human prostatic acid phosphatase: physicochemical and catalytical properties; in *Male Accessory Sex Glands* (Spring-Mills, E. & Hafez, E. S. E., eds.) pp. 197 - 213, Elsevier North Holland Biomedical Press.
6. Luchter-Wasyl, E. & Ostrowski, W. (1974) Subunit structure of human prostatic acid phosphatase. *Biochim. Biophys. Acta*, **365**, 349 - 359.
7. Gryszkiewicz, J. Dziembor, E. & Ostrowski, W. (1970) An active insoluble complex of acid phosphomonoesterase from human prostate gland with carboxymethylcellulose. *Bull. Pol. Ac.: Biol.*, Cl II, **18**, 439 - 444.
8. Gryszkiewicz, J. & Dziembor-Gryszkiewicz, E. (1974) Active insoluble prostatic phosphatase cocrosslinked with bovine serum albumin by glutaraldehyde. *Bull. Pol. Ac.: Biol.*, Cl VI, **22**, 657 - 662.
9. Torchilin, V. P., Galka, M. & Ostrowski, W. (1977) Comparative studies on immobilization of human prostatic acid phosphatase. *Biochim. Biophys. Acta*, **483**, 331 - 336.
10. Gryszkiewicz, J., Dziembor, E. & Ostrowski, W. (1978) Modification of human prostatic acid phosphatase by glutaraldehyde. *Bull. Pol. Ac.: Biol.*, **26**, Cl II, 215 - 220.

11. Dziembor-Gryszkiewicz, E., Maksimenko, A. V., Torchilin, V. P. & Ostrowski, W. S. (1983) Stabilization of human prostatic acid phosphatase by cross-linking with diamines. *Biochem. Int.*, **6**, 627 - 633.
12. Paoli, C., Rinaldi, F., Tarli, P. & Nevi, P. (1981) Purification of human prostatic acid phosphatase and preparation of a specific antiserum in the rabbit. *Ital. J. Biochem.*, **30**, 242 - 250.
13. Van Etten, R. L. & Saini, M. S. (1978) Selective purification of tartrate-inhibitable acid phosphatases: rapid and efficient purification of human and canine prostatic acid phosphatase. *Clin. Chem.*, **24**, 1525 - 1529.
14. Ostrowski, W. (1968) Further characterization of acid phosphomonoesterase of human prostate. *Acta Biochim. Polon.*, **15**, 213 - 225.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265 - 275.
16. Davies, G. E. & Stark, G. R. (1970) Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **66**, 651 - 656.
17. Trubetskoy, V. S. & Torchilin, V. P. (1985) Artificial and natural thermostabilization of subunit enzymes. Do they have similar mechanism? *Int. J. Biochem.*, **17**, 661 - 663.
18. Wasyl, Z., Luchter, E. & Bielański, W., Jr. (1971) Determination of the effective radius of protein molecules by thin-layer gel filtration. *Biochim. Biophys. Acta*, **243**, 11 - 18.
19. Laemmli, U. K. (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, (*London*), **227**, 680 - 685.
20. Ryrie, I. J. & Gallagher, A. (1979) The yeast mitochondrial ATP-ase complex. Subunit composition and evidence for a latent protease contaminant. *Biochim. Biophys. Acta*, **545**, 1 - 14.
21. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350 - 4354.
22. De Blas, A. L. & Cherwinski, H. M. (1983) Detection of antigens on nitrocellulose paper. Immunoblots with monoclonal antibodies. *Anal. Chem.*, **133**, 214 - 219.
23. Bjerrum, O. J. & Larsen, K. P. (1983) Some recent developments of the electroimmunochemical analysis of membrane proteins. Application of zwittergent, Triton X-114 and Western blotting technique; in *Modern Methods in Protein Chemistry* (Tschesche, H., ed.), pp. 79 - 124, Walter de Gruyter, Berlin, New York.
24. Nakane, P. K. & Kawaci, A. (1974) Peroxidase-labelled antibody. A new method of conjugation. *J. Histochem. Cytochem.*, **22**, 1084 - 1091.
25. Udenfriend, S. (1962) Fluorescence of simple proteins; in *Fluorescence assay in biology and medicine*. (Kaplan, N. O. & Scheragan, H. A., eds.) pp. 201 - 207, Academic Press, New York, London.
26. Torchilin, V. P. (1983) Immobilized enzymes and the use of immobilization principles for drug targeting; in *Targeted Drugs* (Goldberg, E., ed.) pp. 127 - 151, John Wiley and Sons Inc.
27. Hajdu, J., Bartha, F. & Friedrich, P. (1976) Crosslinking with bifunctional reagents as a means for studying the symmetry of oligomeric proteins. *Eur. J. Biochem.*, **68**, 373 - 383.
28. Anderson, W. M. & Fischer, R. R. (1981) The subunit structure of bovine heart mitochondrial transhydrogenase. *Biochim. Biophs. Acta*, **635**, 194 - 199.
29. Hajdu, J., Wyss, S. & Aebi, H. (1977) Properties of human erythrocyte catalases after crosslinking with bifunctional reagents. *Eur. J. Biochem.*, **80**, 199 - 207.
30. Brett, M. & Findlay, J. B. C. (1979) Investigation of the organization of rhodopsin

in the sheep photoreceptor membrane by using cross-linking reagents. *Biochem. J.*, **177**, 215 - 223.

31. Lambooy, P. K. & Steiner, R. F. (1982) The crosslinking of phosphorylase kinase. *Arch. Biochem. Biophys.*, **213**, 551 - 556.
32. Gabel, D., Steinberg, I. Z. & Katchalski, E. (1971) Changes in conformation of insolubilized trypsin and chymotrypsin, followed by fluorescence. *Biochemistry*, **10**, 4661 - 4669.
33. Yanari, S. & Bovey, F. A. (1960) Interpretation of the ultraviolet spectral changes of proteins. *J. Biol. Chem.*, **10**, 2818 - 2826.
34. Wetlaufer, D. B. (1962) Ultraviolet spectra of proteins and amino acids. *Adv. Prot. Chem.*, **17**, 303 - 390.
35. Torchilin, V. P., Maksimenko, A. V., Smirnov, V. N., Berezin, I. V., Klibanov, A. M. & Martinek, K. (1978) The principles of enzyme stabilization. III. The effect of the length of intra-molecular cross-linkages on the thermostability of enzymes. *Biochim. Biophys. Acta*, **522**, 277 - 283.
36. Singer, S. J. (1962) The properties of proteins in nonaqueous solvents. *Adv. Prot. Chem.*, **17**, 1 - 68.