0273-2289/87/1503—0265$02.80

Preparation of the Stabilized Glycoenzymes by Cross-Linking Their Carbohydrate Chains

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Received April 29, 1987; Accepted August 28, 1987

ABSTRACT

Each of the three high-mannose type glycoproteins studied, acid phosphatase, invertase, and glucose oxidase, could be specifically cross-linked through its carbohydrate chains. The procedure involves periodate oxidation of carbohydrate residues followed by reaction of the generated aldehyde groups with adipic acid dihydrazide as a cross-linker. The amount and size as well as solubility of the formed polymers could be efficiently controlled by varying the reaction conditions, i.e., the oxidation degree and the concentrations of glycoproteins, cross-linker, and hydrogen ions during the cross-linking reaction. It was found that the quantity and size of polymers increased with oxidation degree and protein concentration and by lowering the pH. When the protein concentration was above and pH below certain values, depending on the glycoenzyme, insoluble polymers formed. The soluble cross-linked polymers retained a high level of original activity, and the minor decrease in specific activity noticed was shown to occur during the periodate oxidation step. The cross-linked glycoenzymes are much more resistant to denaturation by high temperature and by changes in pH, demonstrating the usefulness of this method in preparation of the stabilized glycoprotein derivatives.

Index Entries: Glycoproteins; cross-linking; stabilization; glycoenzymes; quarternary structure.

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INTRODUCTION

Intramolecular cross-linking occurs frequently in proteins, where it plays an important role in stabilization of their native conformation (*1*). There are many reagents for in vitro cross-linking of enzyme molecules (*2,3*). It has been shown that artificial intramolecular cross-linking of enzymes usually increases their stability, possibly through the enhanced rigidity of the modified protein structure (*4*). Since most enzymes are not sufficiently stable under technological conditions, the preparation of stabilized enzymes opens wider possibilities for their application.

Intra- or intermolecular cross-linking of enzyme molecules through their amino acid side chains, some of which play a role in catalysis or in maintenance of an enzymatically active conformation, quite often results in a significant loss of the enzyme activity. Glycoenzymes offer an opportunity to perform cross-linking reactions through their carbohydrate chains. Since carbohydrate chains of glycoenzymes are not directly involved in catalysis (*5–7*), it seems reasonable to expect that the original enzyme activity will be retained by cross-linking the glycoenzymes through the catalytically inactive carbohydrate chains.

In the study on the carbohydrate part of yeast acid phosphatase we have shown that the two enzyme subunits could be cross-linked through their carbohydrate chains (*8*). Here we have systematically investigated various parameters influencing the cross-linking procedure in order to find optimal conditions for the preparation of soluble cross-linked enzyme polymers. Three glycoenzymes with different degree of glycosylation were used, i.e., yeast acid phosphatase with 50% carbohydrate (*9*), yeast invertase containing 40% carbohydrate (*10*), and glucose oxidase from *Aspergilus niger* with 16% carbohydrate (*11*). The activity as well as thermal- and pH-stability of the prepared cross-linked enzyme polymers were compared to those of the native enzymes.

EXPERIMENTAL PROCEDURES

Materials

Reagents were purchased from the following suppliers: adipic acid dihydrazide, acrylamide, *N,N'*-methylene-bis-acrylamide, TEMED from Serva; sodium dodecyl sulfate from LKB. All other chemicals were from Kemika, Zagreb. Yeast invertase was a commercial preparation of Boehringer and glucose oxidase was a product of Fluka. High molecular weight protein standards were obtained from Pharmacia.

Preparation of Enzymes

Acid phosphatase was purified from yeast *Saccharomyces cerevisiae* according to the previously described procedure (*9*). The pure enzyme,

containing about 48% of neutral sugars, was used throughout this study. Yeast invertase and glucose oxidase were purified from the partially purified commercial preparations as described (*10,11*) and the purified enzymes contained 38 and 16% of neutral sugars, respectively. The neutral sugars were determined by the orcinol-sulfuric acid method (*12*), using mannose as a standard.

Cross-Linking of Glycoenzymes through Their Carbohydrate Chains

Enzyme samples in .1*M* sodium acetate buffer (pH value corresponding to optimal pH of the particular enzyme) were oxidized by sodium periodate at 4°C for 24 h in the dark. The samples were then desalted by gel filtration on a small column (1.5×8 cm) filled with Sephadex G-25 and cross-linking was performed by adipic acid dihydrazide for 24 h at room temperature. The concentrations of glycoproteins and the cross-linker as well as pH were varied as described in the legends to the figures. Where not indicated, the molar ratio of adipic acid dihydrazide to mannose was 1:1.

Electrophoresis

Three–thirty% gradient gels were prepared according to instructions of Pharmacia Fine Chemicals. Electrophoresis was run for 10–15 h at constant voltage (125 V) in .1*M* Tris-borate buffer pH 8.3 with 1 m*M* EDTA. For SDS electrophoresis .1% SDS was added to the same buffer and electrophoretically introduced into 3–30% gradient gels before application of the samples.

Enzyme Activities

Acid phosphatase was measured with 10 m*M* p-nitrophenyl phosphate as substrate and glucose oxidase with 180 m*M* glucose, according to the published procedures (*13,14*). Invertase activity was assayed as described by Bernfeld (*15*).

RESULTS AND DISCUSSION

Cross-linking of glycoenzymes through their carbohydrate chains, according to the procedure described by us (*8*), involves periodate oxidation of carbohydrate residues followed by reaction of the generated aldehyde groups with adipic acid dihydrazide. Depending on conditions employed, this can lead to various derivatives of an oligomeric glycoenzyme, such as: (a) uncross-linked enzyme molecules that have reacted with only one hydrazide group of adipic acid dihydrazide; (b) intramolecularly cross-linked enzymes with or without intersubunit cross-linking; and (c) inter-

molecularly cross-linked soluble oligomers or insoluble polymers. It is clear that in most cases a mixture of several forms will be present after the cross-linking reaction.

We have examined various reaction conditions, i.e., the degree of oxidation, protein, and cross-linker concentrations and pH during the cross-linking reaction, in order to optimize the procedure for preparation of soluble intermolecularly cross-linked oligomers. Three dimeric glycoenzymes, containing high-mannose type sugar chains, were selected for this study, and cross-linked polymers thus formed were monitored by gradient gel electrophoresis.

The course of the cross-linking reaction was first tested as a function of the degree of sugar chain oxidation. It was reported that in glucose oxidase at most 50–60% of mannose residues could be oxidized by periodate (*11*), and on the basis of our results (*8*) it was calculated that about 60% of acid phosphatase mannose could be oxidized by periodate. Mannose residues in invertase are probably oxidized to a similar extent, since the structures of acid phosphatase and invertase carbohydrate chains are rather similar (*8*). As can be seen from Fig. 1A, the amount and size of intermolecularly cross-linked invertase oligomers increased uniformly (except lane 6) with the degree of oxidation, and at the maximal oxidation the enzyme was converted completely to higher oligomers. Similarly, we have previously shown that acid phosphatase was converted completely to oligomers, even at a lower degree of oxidation (*8*). A significant amount of glucose oxidase was intermolecularly cross-linked (Fig 1B). However, even at the maximal oxidation it was not possible to convert this enzyme, containing much less carbohydrate than acid phosphatase and invertase, completely to higher oligomers. We have obtained similar results with peroxidase and glucosyltransferase, which are glycosylated to a comparable extent as glucose oxidase (unpublished work).

The effect of protein concentrations on products of the cross-linking reaction was further examined and results for acid phosphatase, invertase, and glucose oxidase are shown in Fig. 2. As expected, the amount and molecular weight of intermolecularly cross-linked oligomers increased with the protein concentration. This is best seen with invertase (Fig. 2B). The transition is not so gradual with glucose oxidase and acid phosphatase, but the increase in protein concentration resulted in higher intensity of the oligomer bands (Fig. 2C, lanes 7–4) or in a decreased amount of the penetrating material (Fig. 2A). At the concentration of 5.2 mg/mL, acid phosphatase was converted almost completely into partially insoluble, high molecular weight polymers that could not enter the gel (Fig. 2A, lane 5). When cross-linking was performed at higher protein concentrations, all three enzymes formed insoluble derivatives. In contrast to acid phosphatase and invertase, it was not possible to transform glucose oxidase completely to higher oligomers, although the enzyme was maximally oxidized and cross-linked at considerably higher protein concentration

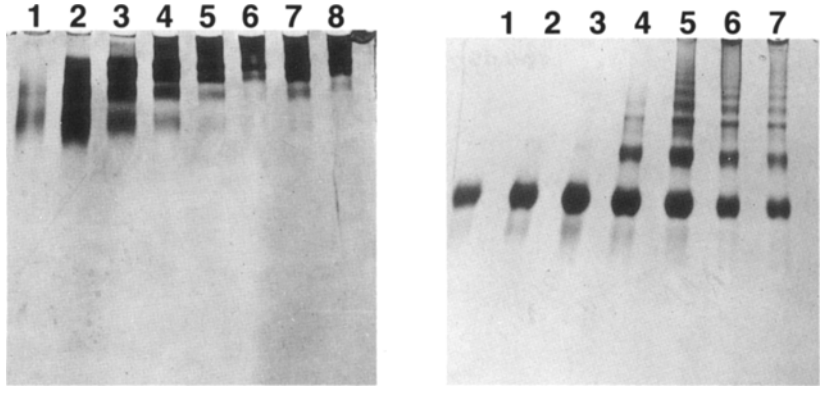

Fig. 1. Electrophoresis of cross-linked invertase (A) and glucose oxidase (B) oxidized by various amounts of periodate. Invertase (3 mg) and glucose oxidase (10 mg) were oxidized by various amounts of sodium periodate and cross-linked by adipic acid dihydrazide in 2 mL of .1*M* sodium acetate buffer, pH 4.6, as described in Experimental procedures. The molar ratio of periodate to mannose was: (1) native enzyme; (2) .1:1; (3) .2:1; (4) .3:1; (5) .5:1; (6) .75:1; (7) 1:1; and (8) 2:1.

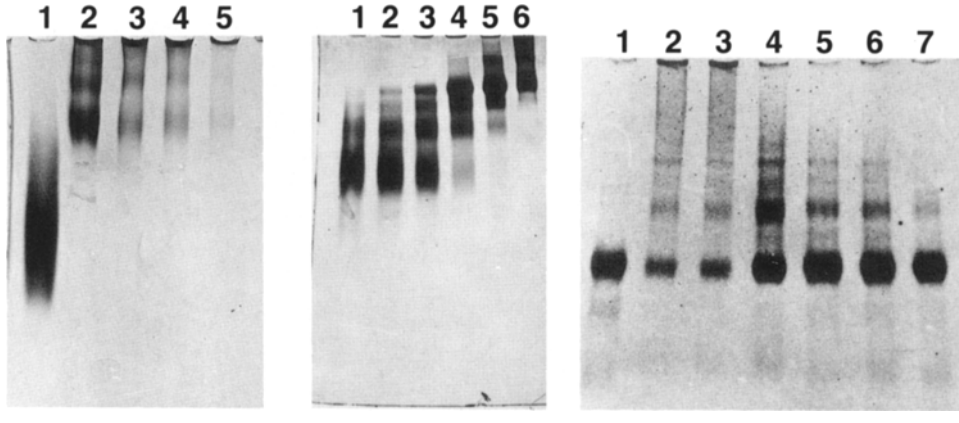

Fig. 2. Electrophoresis of acid phosphatase (A) invertase (B) and glucose oxidase (C) cross-linked at different protein concentrations. The enzymes were oxidized by periodate added in the following molar ratios to mannose: .3:1 (acid phosphatase and invertase) and 1:1 (glucose oxidase). Cross-linking was performed at various protein concentrations in .1*M* acetate buffer, pH 4.6, as described in the Experimental procedures. The protein concentrations were: (A): (1) native enzyme; (2) .65 mg/mL; (3) 1.3 mg/mL; (4) 2.6 mg/mL; and (5) 5.2 mg/mL. (B): (1) native enzyme; (2) .0015 mg/mL; (3) .015; (4) .15 mg/mL; (5) 1.5 mg/mL; and (6) 3.0 mg/mL. (C): (1) native enzyme; (2) 15 mg/mL; (3) 7.5 mg/mL; (4) 5.0 mg/mL; (5) 2.5 mg/mL; (6) .6 mg/mL; and (7) .15 mg/mL.

than the first two enzymes. At protein concentration of .15 mg/mL, very little intermolecularly cross-linked glucose oxidase was obtained (Fig. 2C, lane 7). Intermolecular cross-linking of invertase as well as acid phosphatase (*8*) could be avoided only when cross-linking was performed at about 100-fold lower concentration than in the case of glucose oxidase (Fig. 2B, lane 2). It should be noted that native invertase as well as acid phosphatase show rather diffuse bands after electrophoresis (Figs. 1A, 2A, and 2B). As demonstrated previously (*7,16*), this is caused by the heterogeneity in the carbohydrate part of these glycoenzymes. Moreover, besides the dimer, higher oligomers are also present in the invertase preparation (Fig. 2B, lane 1). This has already been reported by other authors (*17*).

The influence of pH on the cross-linking reaction was tested as well. Invertase was cross-linked at all pH values tested, but glucose oxidase was only weakly cross-linked at pH 8 (Fig. 3B, lane 6). The cross-linking was more efficient at pH below 6.2, as the amount of the nonpenetrating polymers increased by lowering the pH (Fig. 3A and B). At pH 4.6 and 3.8 glucose oxidase and invertase, respectively, were converted to partially insoluble derivatives. At even lower pH values completely insoluble derivatives of both enzymes were formed. Acid phosphatase behaved in the same manner (not shown).

Thus, the optimal pH for the cross-linking reaction lies in the acid region, several pH units lower than the pH optimum of the reaction between aldehyde and amino groups, which is around neutral pH. The rate-limiting step of this reaction (Schiff base formation) at high pH is acid catalyzed removal of proton from the tetrahedral intermediate, whereas at low pH the rate limiting step becomes the nucleophilic attack of the unprotonated amino group (*18*). Since the pK of the hydrazide group is around 2.6 (*19*), the reaction of aldehyde groups with adipic acid dihydrazide is fast at acidic pH because of the high concentration of unprotonated hydrazide.

To examine whether intermolecularly cross-linked oligomers were in part the result of Schiff base formation between sugar aldehyde groups, generated by periodate oxidation and protein amino groups, the samples of oxidized enzymes were incubated without adipic acid dihydrazide under otherwise identical conditions as samples with the cross-linking reagent. As shown in Fig. 4, oxidized invertase without the cross-linker formed a very low amount of higher oligomers at low pH, increasing slightly at pH values above 7. Very similar results were obtained with acid phosphatase (not shown). In the glucose oxidase samples, however, Schiff base oligomers were not formed in the whole pH range tested (not shown). These results clearly show that intermolecularly cross-linked oligomers formed at acidic pH are the result of adipic acid dihydrazide reaction. Furthermore, Schiff base oligomers, although stable during electrophoresis in the buffer containing a primary amine (.1*M* Tris), are

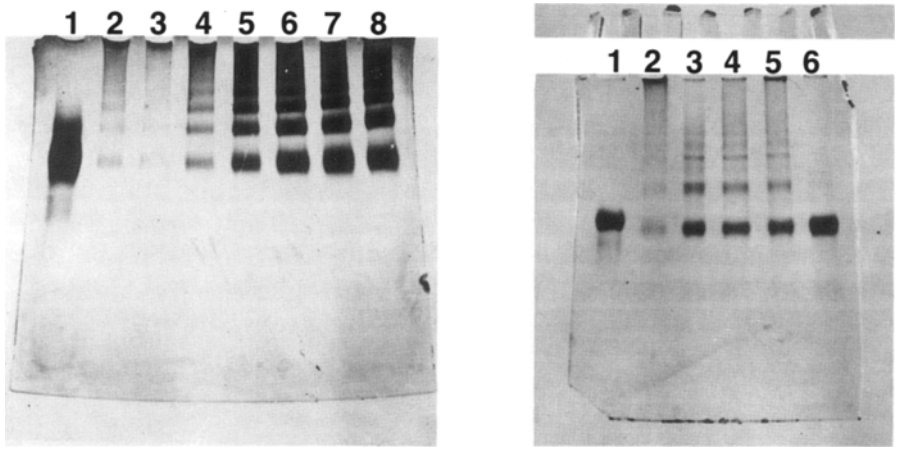

Fig. 3. Electrophoresis of invertase (A) and glucose oxidase (B) cross-linked at different pH. Invertase (6 mg) was oxidized by periodate added in .3:1 molar ratio to mannose and cross-linked by adipic acid dihydrazide in 2 mL of .1*M* buffer of various pH. (1) native enzyme; (2) pH 3.6; (3) pH 4.6; (4) pH 5.4; (5) pH 6.2; (6) pH 7.0; (7) pH 8.0; and (8) pH 9.5. Glucose oxidase (28 mg) was oxidized by periodate added in 1:1 molar ratio to mannose and cross-linked by adipic acid dihydrazide in 2 mL of .1*M* buffer of various pH values. (1) native enzyme; (2) pH 4.6; (3) pH 5.4; (4) pH 6.0; (5) pH 7.0; and (6) pH 8.0.

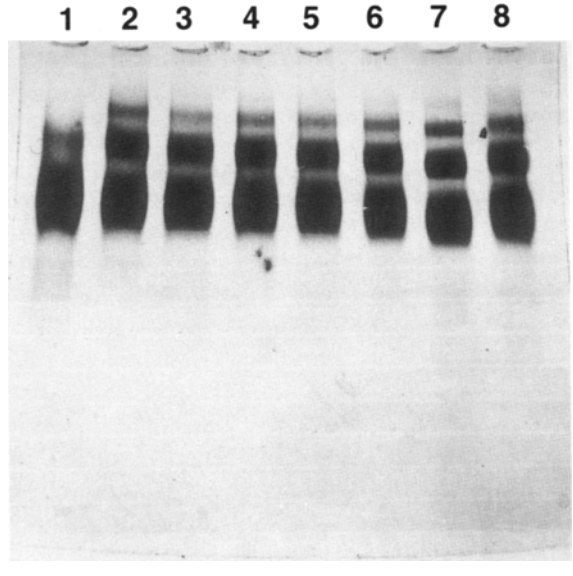

Fig. 4. Electrophoresis of oxidized invertase incubated at various pH values without addition of the cross-linking reagent. The other reaction conditions were the same as those described in Fig. 3A.

probably not stable in the presence of adipic acid dihydrazide because the latter forms more stable derivatives with oxidized sugar aldehyde groups, either hydrazones or more likely carbinol hydrazides (*19,20*).

The amount of the intermolecularly cross-linked oligomers can also be controlled by concentration of the cross-linking reagent. By increasing adipic acid dihydrazide concentration the amount of oligomers increases and the optimal concentration, expressed as molar ratio to mannose, was found to be 1:1 to 2:1 (data not shown). At higher concentrations of the cross-linking reagent, the amount of oligomers decreases and, as shown in Fig. 5, intermolecular cross-linking of glucose oxidase could be completely prevented by high concentrations of adipic acid dihydrazide. This is probably because of the fact that all aldehyde groups of the glycoenzyme molecule reacted with only one hydrazide group of the cross-linker.

As already demonstrated, intermolecular vs intramolecular cross-linking could be controlled by protein concentration during cross-linking; at low protein concentration intermolecular cross-linking was prevented

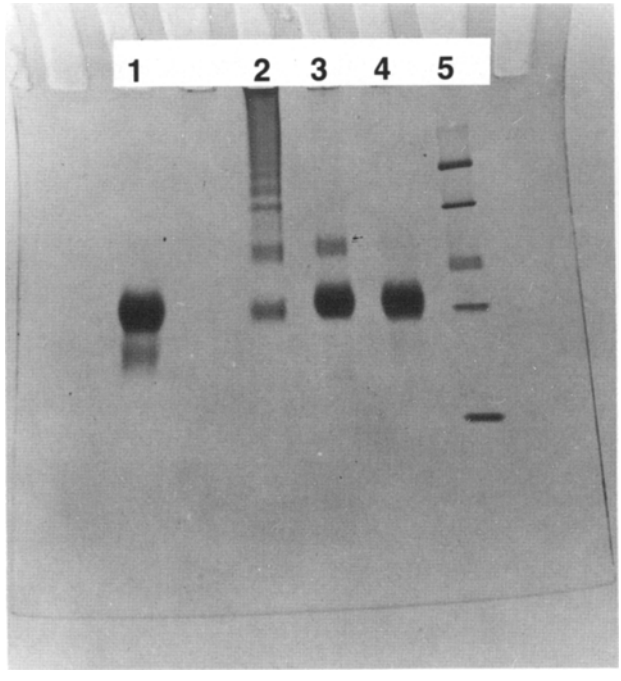

Fig. 5. Electrophoresis of glucose oxidase cross-linked by addition of an excess of the cross-linking reagent. Glucose oxidase was oxidized by periodate added in 1:1 molar ratio to mannose and cross-linked in .1*M* acetate buffer, pH 4.6, by various amounts of adipic acid dihydrazide. The molar ratios of adipic acid dihydrazide to mannose were: (1) native enzyme; (2) 1:1; (3) 10:1; and (4) 25:1. Lane (5) are High molecular weight protein standards: Thyroglobulin (669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000), and bovine serum albumin (67000).

(Figs. 2B and 2C). In naturally occurring oligomeric enzymes intramolecular cross-linking can involve intra- and intersubunit cross-linking. We have previously shown that efficient intersubunit cross-linking of acid phosphatase could be achieved. This was proved by SDS electrophoresis, where only the dimer was detected in the sample cross-linked at low protein concentration (*8*). A similar result was obtained with invertase (not shown). However, only a low amount of intersubunit cross-linked glucose oxidase was formed in dilute solution, under optimal conditions for cross-linking. As shown in Fig. 6, on SDS electrophoresis, the enzyme migrated mainly as a monomer. Moreover, even the small quantity of the dimer observed could be a result of dissociation of the two subunits from the intermolecularly cross-linked tetramer, which was present in low amounts in the preparation. The fact that glucose oxidase subunits, in contrast to acid phosphatase and invertase, were not cross-linked through their carbohydrate chains, indicates that these chains were spatially separated

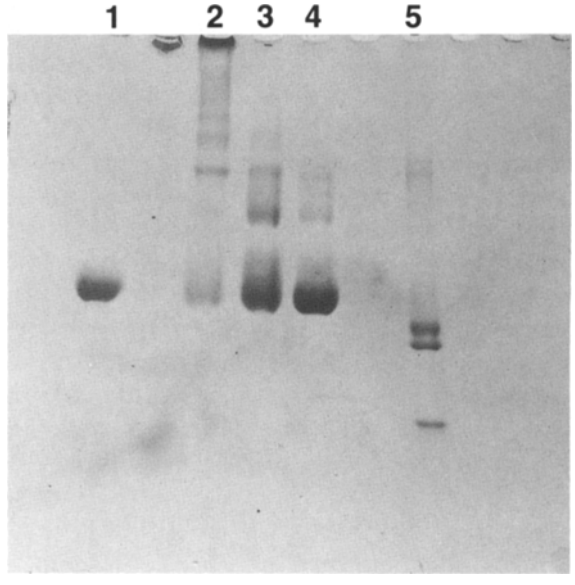

Fig. 6. SDS electrophoresis of glucose oxidase cross-linked at various protein concentrations. Glucose oxidase was oxidized by periodate, added in 1:1 molar ratio to mannose, and cross-linked at various protein concentrations by adipic acid dihydrazide at pH 4.6. The protein concentrations were: (1) native enzyme; (2) 14 mg/mL; (3) .5 mg/mL; and (4) .1 mg/mL. Lane (5) are High molecular weight standard proteins (Pharmacia) treated by SDS under mild conditions, as described by the supplier, in order to get the following protein markers: Thyroglobulin (330000), ferritin (220000), bovine serum albumin (67000), catalase (60000), and lactate dehydrogenase (36000).

and therefore could not be cross-linked by a relatively short reagent like adipic acid dihydrazide. Thus, it seems that this procedure might be useful in the study on proximity of sugar chains in other oligomeric glycoproteins, i.e., in dimeric glycoprotein hormones, whose carbohydrate chains apparently influence their biological activity (*21*). From the result shown in Fig. 6, it appears that the carbohydrate chains in glucose oxidase are not directly involved in stabilization of the quarternary structure, as was suggested for acid phosphatase (*8*) and invertase (*8,17*).

We have measured activities of the enzymes that were subjected to periodate oxidation only, as well as activities of the soluble cross-linked polymers, in order to distinguish between effects of each of the two reactions on enzymes' activities (Table 1). It can be seen that the remaining activity depends mainly upon the degree of oxidation. The decrease of activity after periodate oxidation is most probably caused by oxidation of certain sensitive amino acid residue(s). This is in agreement with the re-

Table 1
Activities of the Enzymes after Periodate Oxidation and after Cross-Linking

Enzyme	Molar ratio of periodate to mannose	Remaining activity after oxidation,[a] %	Remaining activity after cross-linking,[a] %
Glucose oxidase	Native	100	100
	.1 : 1	100	99
	.2 : 1	99	100
	.3 : 1	93	97
	.5 : 1	95	94
	.7 : 1	92	89
	1.0 : 1	90	88
Invertase	Native	100	100
	.1 : 1	93	91
	.2 : 1	93	86
	.3 : 1	90	85
	.4 : 1	91	80
	.5 : 1	88	78
	.7 : 1	82	74
Acid phosphatase	Native	100	100
	.1 : 1	92	89
	.2 : 1	89	85
	.3 : 1	82	78
	.4 : 1	78	71
	.5 : 1	69	60
	.7 : 1	59	55

[a] Aliquots used for activity measurements were taken from desalted samples before and after the cross-linking reaction which was performed as described in the Experimental procedures. Standard deviation was 3–5%.

cently reported oxidation of methionine residues in proteins by periodate (*22*). On the other hand, the loss of activity because of the cross-linking reaction itself is negligible (Table 1).

Accordingly, the remaining activity of glycoenzymes cross-linked through their carbohydrate chains is generally high—usually not the case when the cross-linking is performed through the protein part. We have examined the cross-linked glycoenzymes for their thermal stability and, as shown in Fig. 7, the cross-linked polymers of invertase and glucose oxidase are much more stable than the native enzymes. The stabilization is a result of the cross-linking reaction and not of periodate oxidation, since the oxidized enzymes are essentially as stable as the native ones. In contrast to glucose oxidase and invertase, oxidized acid phosphatase was less thermally stable than the native enzyme, but the subsequent cross-linking increased the stability to the level of the native enzyme (not shown). All three cross-linked glycoenzymes were more resistant to denaturation by high (9.5–11) pH, and acid phosphatase displayed the most pronounced stabilization effect (Fig. 8). At acid pH, the stabilization effect was less pronounced (not shown). Accordingly, the cross-linking of glycoenzymes through the carbohydrate chains generally increases their stability except in those cases, illustrated by the lower thermal stability of oxidized acid phosphatase, when the enzyme is destabilized during the periodate oxidation step.

The results obtained demonstrate the potential applicability of the described method for preparation of the stabilized water-soluble glycoenzyme derivatives. The high level of the original activity retained (Table 1) supports the assumption that this approach is superior to the cross-linking through the protein part. Our studies were done on high mannose type glycoproteins, but the procedure can be applied to the complex type glycoproteins as well, because at least terminal monosaccharides will be oxidized by periodate. This approach may be beneficial also when working with only partially purified glycoproteins, since the protein impurities are not expected to interfere with the cross-linking reaction.

We are currently investigating some of several possible applications of the stabilized glycoenzymes. Thus, since they contain a large number of free hydrazide groups on their sugar chains, these cross-linked glycoenzymes might be suitable for detection of aldehydes in biological systems, as described with enzymes containing hydrazide groups linked to the protein part (*23,24*).

ACKNOWLEDGMENT

We are grateful to Peter Brodelius and Stephen Cliffe for their critical reading of the manuscript. This work was supported by a grant from Self-Managed Community of Interest for Scientific Research of SR Croatia.

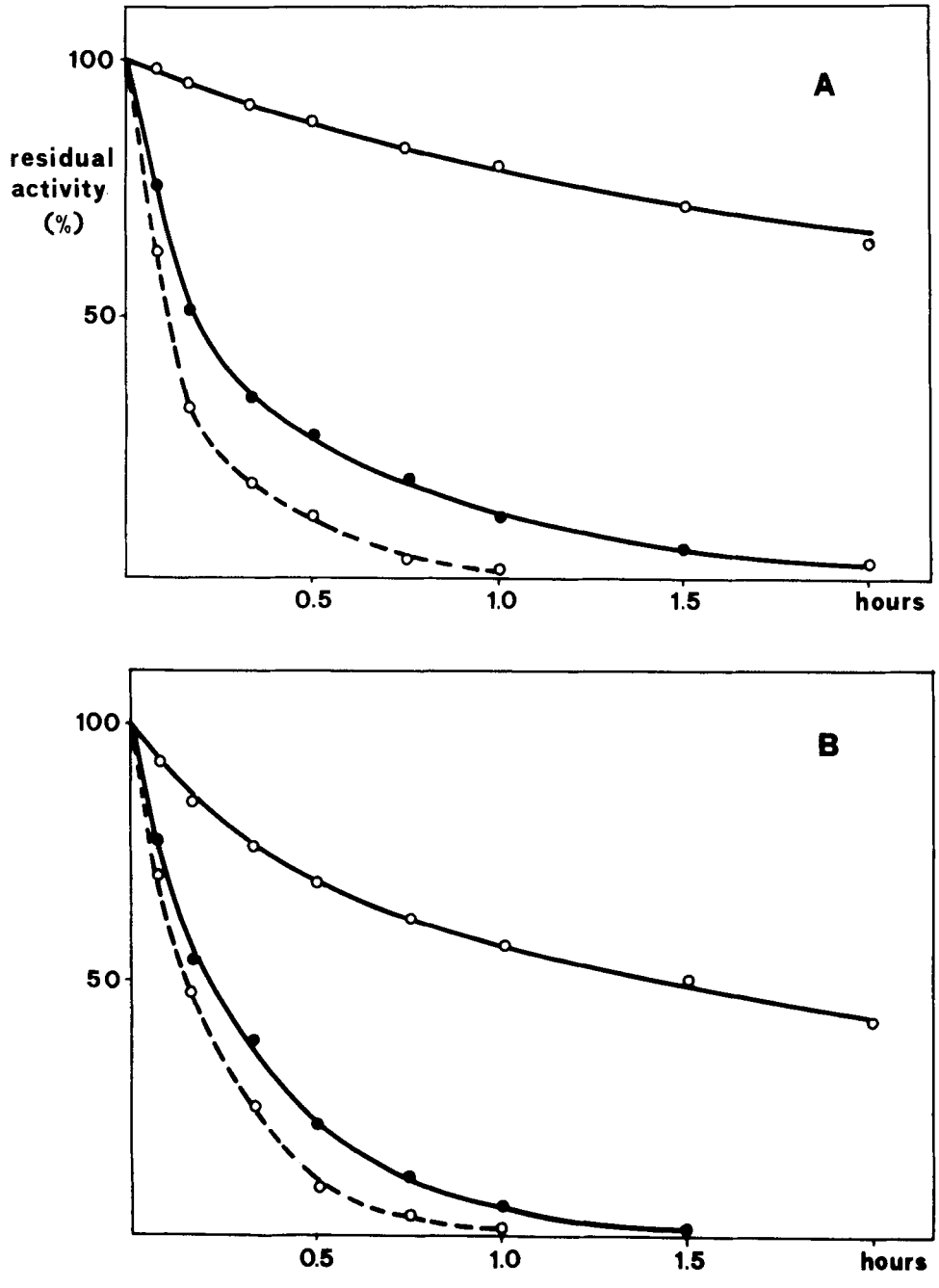

Fig. 7. Thermal stability of cross-linked invertase (A) and glucose oxidase (B). Invertase (3 mg/mL) was oxidized by periodate in .3:1 molar ratio to mannose. The oxidation of glucose oxidase (14 mg/mL) was done with the equimolar amount (to mannose) of periodate. Both enzymes were cross-linked in .1*M* sodium acetate buffer pH 4.6. The stability of the cross-linked enzymes was determined by measuring the remaining activity, at several intervals, after incubation at 60°C (invertase) or 65°C (glucose oxidase). Native (○——○), oxidized (●——●) and cross-linked (○——○) enzymes.

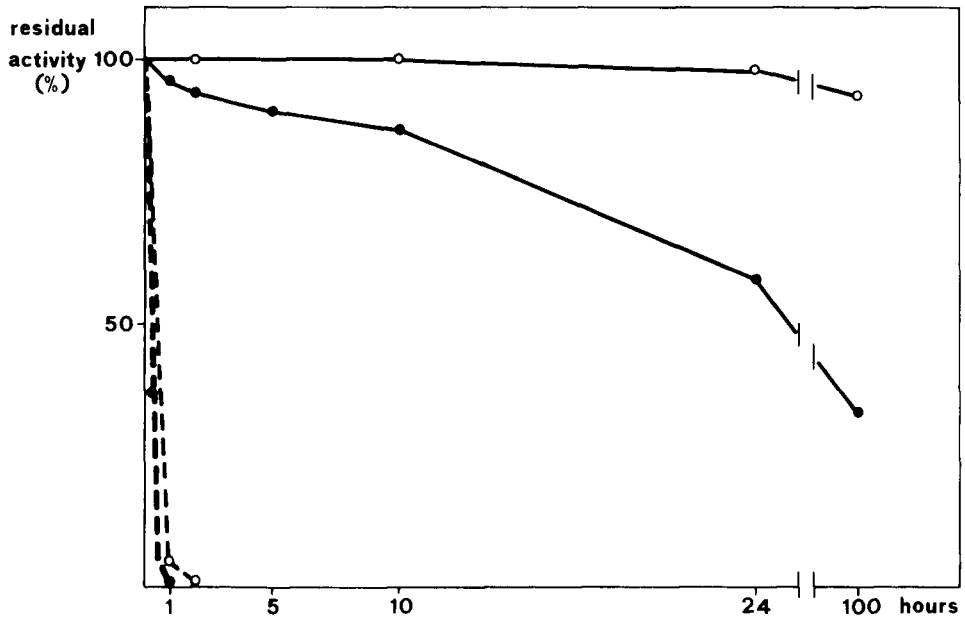

Fig. 8. pH stability of the cross-linked acid phosphatase. The enzyme (1.3 mg/mL) was oxidized with periodate in .3:1 molar ratio to mannose and cross-linked in .1*M* sodium acetate buffer pH 4.6. The enzyme was then incubated at 30°C and pH 9.5 or 11.0. At times indicated, aliquots were withdrawn, diluted with acetate buffer pH 3.8 and analyzed for the remaining activity. Native acid phosphatase at pH 9.5 (○———○) and 11.0 (●———●); the cross-linked enzyme at pH 9.5 (○———○) and 11.0 (●———●).

REFERENCES

1. Kauzmann, W. (1959), *Adv. Prot. Chem.* **14,** 1-63.
2. Wold, F. (1972), *Meth. Enzymol.* **25,** 623-651.
3. Peters, K., and Richards, F. M. (1977), *Ann. Rev. Biochem.* **46,** 523-551.
4. Zaborsky, O. R. (1974), in *Enzyme Engineering* (Pye, E. K., and Wingard, L. B., Jr., eds.), vol. 2, pp. 115-122, Plenum, New York.
5. Tarentino, A. L., Plumer, T. H., Jr., and Maley, F. (1974), *J. Biol. Chem.* **249,** 818-824.
6. Chu, F. K., Trimble, R. B., and Maley, F. (1978), *J. Biol. Chem.* **253,** 8691-8693.
7. Barbaric, S., Mrsa, V., Ries, B., and Mildner, P. (1984), *Arch. Biochem. Biophys.* **234,** 567-575.
8. Kozulic, B., Barbaric, S., Ries, B., and Mildner, P. (1984), *Biochem. Biophys. Res. Commun.* **122,** 1083-1090.
9. Barbaric, S., Kozulic, B., Ries, B., and Mildner, P. (1984), *J. Biol. Chem.* **259,** 878-883.
10. Brown, J. A., Segal, H. L., Maley, F., Trimble, R. B., and Chu, F. (1979), *J. Biol. Chem.* **254,** 3689-3691.
11. Pazur, J. H., Kleppe, K., and Cerpure, A. (1965), *Arch. Biochem. Biophys.* **111,** 351-357.

12. Francois, C., Marshall, R. D., and Neuberger, A. (1962), *Biochem. J.* **83,** 335–341.
13. Mildner, P., Ries, B., and Barbaric, S. (1975), *Biochem. Biophys. Acta* **391,** 67–74.
14. Weethal, M. M., and Hersh, L. S. (1970), *Biochem. Biophys. Acta* **206,** 54–60.
15. Bernfeld, P. (1951), *Adv. Enzymol.* **12,** 379–428.
16. Trimble, R. B., and Maley, F. (1984), *Anal. Biochem.* **141,** 515–522.
17. Chu, F. K., Watorek, W., and Maley, F. (1983), *Arch. Biochem. Biophys.* **223,** 543–555.
18. Feeney, R. E., Blankenhorn, G., and Dixon, H. B. F. (1975), *Adv. Prot. Chem.* **29,** 135–203.
19. Inman, J. K. (1974), *Meth. Enzymol.* **34,** 30–58.
20. Hansske, F., Sprinzl, M., and Cramer, F. (1974), *Bioorg. Chem.* **3,** 367–376.
21. Pierce, J. G., and Parsons, T. F. (1981), *Ann. Rev. Biochem.* **50,** 465–495.
22. Yamasaki, R. B., Osuga, D. T., and Feeney, R. E. (1982), *Anal. Biochem* **126,** 183–189.
23. Gershoni, J. M., Bayer, E. A., and Wilchek, M. (1985), *Anal. Biochem.* **146,** 59–63.
24. Keren, Z., Berke, G., and Gershoni, J. M. (1986), *Anal. Biochem.* **155,** 182–187.