REVERSIBLE MODIFICATION OF TYROSYL RESIDUES IN RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE *b* BY *N*-ACETYLIMIDAZOLE

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1. Introduction

The chemical approach to the study of the structure–function relationship in rabbit muscle glycogen phosphorylase *b* (EC 2.4.1.1) has dealt mainly with the investigation on the reactivity of sulfhydryl and amino groups [1].

In the course of an investigation on the role of aromatic residues on the catalytic and allosteric properties of this enzyme, we have studied the effect of *N*-acetylimidazole on the enzymatic activity, in the absence and presence of the allosteric activator AMP.

N-acetylimidazole is a useful reagent for selective and reversible acetylation of tyrosyl residues, since, under relatively mild conditions, it reacts with amino groups to a much lesser extent than acetic anhydride [2]. In this communication, we report that this reagent, under suitable conditions, inactivates reversibly phosphorylase *b*; this inactivation is largely prevented by the presence of AMP. It appears that two tyrosyl residues are essential for catalytic activity, and that AMP selectively protects them. A partial account of these findings has been presented [3].

2. Experimental

2.1. *Materials*

Rabbit muscle phosphorylase *b* was prepared according to the method of Fischer and Krebs [4]. AMP-free phosphorylase *b* was obtained by gel filtration on a Sephadex G-25 column (1.5 × 25 cm), equilibrated in 50 mM β-glycerophosphate pH 6.8, 2 mM EDTA and 1 mM β-mercaptoethanol. The $A_{260\,nm}/A_{280\,nm}$ ratio of the eluted enzyme was always lower than 0.52 [5].

N-acetylimidazole was from Pierce; 5′-AMP, $NADP^+$, phosphoglucomutase and glucose 6-phosphate dehydrogenase were purchased from Boehringer; glycogen was from Sigma. All other chemicals were reagent-grade.

2.2. *Methods*

The enzymatic activity was measured at 20°C in the direction of glycogen breakdown; the formation of glucose 1-phosphate was measured with the coupled phosphoglucomutase and glucose 6-phosphate dehydrogenase system [6], by recording NADPH formation of 340 nm in a Gilford Model 2400-S spectrophotometer: the specific activity of the crystalline enzyme was such that 1 mg released 55 μmol of inorganic phosphate per min.

Phosphorylase *b*, at a concentration of 2 mg/ml, was reacted with *N*-acetylimidazole at 0°C and 25°C, in 50 mM β-glycerophosphate, pH 6.8, 2 mM EDTA and 1 mM β-mercaptoethanol. Aliquots were withdrawn at different times and assayed for enzymatic activity. Acetylated phosphorylase *b* was dialyzed against 50 mM β-glycerophosphate pH 6.8, 2 mM EDTA. The number of *O*-acetylated tyrosyl residues was determined spectrophotometrically in a Cary 118 spectrophotometer, using the molar $\Delta\epsilon_{278\,nm}$ = 1160 [2]. Deacetylation was carried out by incubation at 0°C with 30 mM neutralized NH_2OH; after 30 min, spectrophotometric evidence indicated no further regeneration of phenolic groups. Protein was determined according to Lowry et al. [7].

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3. Results

The extent of reaction of phosphorylase *b* with *N*-acetylimidazole was found to be strictly dependent on the reagent/Tyr molar ratio. In the presence of 100-fold excess of reagent, at 25°C, the activity decreased to 10–20% of the initial value in less than 10 min; the presence of 5 mM AMP did not prevent this inactivation. At the same temperature, in the presence of a 25-fold excess of *N*-acetylimidazole, the rate of inactivation substantially decreased; in fact, 15–20% residual activity was found after 60 min. Under these conditions, the presence of AMP afforded a slight protection (fig.1). Phosphorylase *b* was reacted with a 20-fold excess of *N*-acetylimidazole at 0°C. Fig.2 shows that at this temperature, 80% inactivation occurred in 2 hours; moreover, AMP was effective in protecting against this inactivation, maintaining the activity to 70% of the initial value.

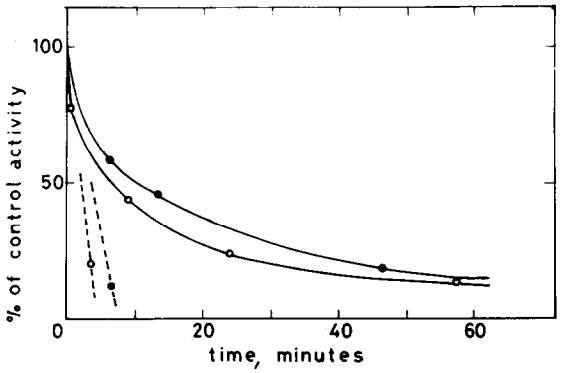

Fig.1. Reaction of phosphorylase *b* with *N*-acetylimidazole at 25°C. 1 mg enzyme was incubated as described under Methods, in the absence (○) and presence (●) of 5 mM MAP. The *N*-acetylimidazole/Tyr molar ratio was 25 (continuous curves) and 100 (dotted curves).

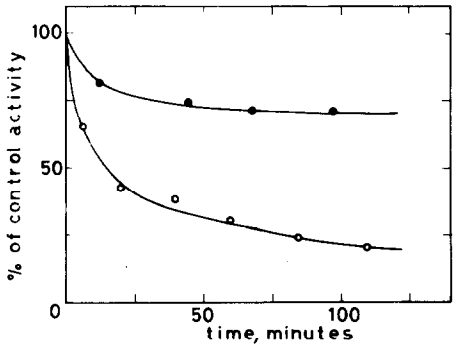

Fig.2. Reaction of phosphorylase *b* with *N*-acetylimidazole at 0°C, in the absence (○) and presence (●) of 5 mM AMP. The *N*-acetylimidazole/Tyr molar ratio was 20.

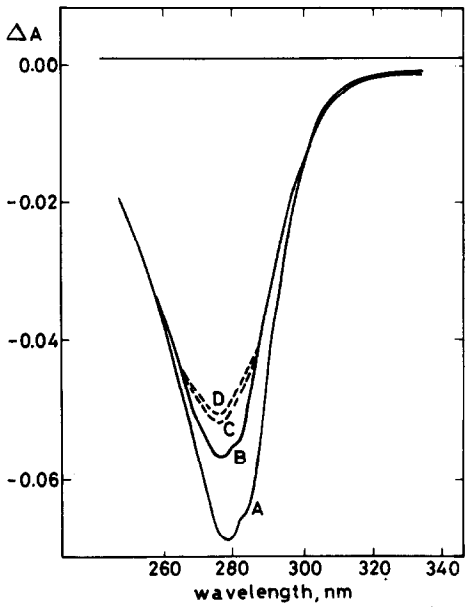

Fig.3. Difference spectra of phosphorylase *b* acetylated in the absence (A) and presence (B) of 5 mM AMP. (C) and (D), same as (A) and (B), after deacetylation with 30 mM NH_2OH at 0°C.

Following extensive dialysis, the residual activity was found to be 22% in the sample reacted in the absence, and 83% in that reacted in the presence of AMP.

The number of tyrosyl residues modified was determined spectrophotometrically. The modified samples and dialyzed control samples of phosphorylase *b* were adjusted to a final protein concentration of 1 mg/ml. Fig.3 reports the difference spectra of the enzyme modified in the absence (curve A) and presence (curve B) of AMP; the presence of the ligand caused a lesser extent of acetylation. The results indicate that 8.8 tyrosyl residues/95 000 were modified in the absence of AMP, whereas in its presence 7.0 residues/95 000 were acetylated. Curves C and D refer to the difference spectra of the two samples, following partial deacetylation with 30 mM NH_2OH at 0°C. This mild treatment regenerated 3.0 and 1.2 residues/95 000, respectively, with a complete recovery of enzymatic activity in both cases (table 1).

Table 1
Reactivity of tyrosyl residues of phosphorylase *b* (34 Tyr/95 000) with *N*-acetylimidazole

	N-acetylimidazole/Tyr = 100, t = 25°C				*N*-acetylimidazole/Tyr = 20, t = 0°C			
	No. of modified Tyr	% of control activity	No. of Tyr regenerated by NH_2OH	% of control activity	No. of modified Tyr	% of control activity	No. of Tyr regenerated by NH_2OH	% of control activity
Unprotected enzyme	16.3	0	10.1	0	8.8	17	3.0	100
AMP-protected enzyme	8.9	0	3.2	0	7.0	78	1.2	100

A greater extent of deacetylation could be achieved at room temperature, using higher concentrations of NH_2OH; however, these conditions did not restore the activity and furthermore inactivated the control. The disappearance of the characteristic 330 nm band indicated that the pyrydoxal-5′-phosphate moiety was affected by this treatment.

Table 1 also shows that extensive acetylation caused the modification of 16.3 out of the 34 tyrosyl residues/95 000; half of these residues was protected by the presence of AMP. The resulting loss of activity, regardless of the presence of the activator (see also fig.1) was, however, irreversible, despite the regeneration by NH_2OH of 10.1 and 3.2 residues/95 000, respectively. Also in this case, spectra of acetylated phosphorylase *b* showed that pyrydoxal-5′-phosphate was affected by the excess reagent.

4. Discussion

Reaction of phosphorylase *b* with *N*-acetylimidazole has been reported by Vulfson and Skolysheva [8]. These authors used a molar excess of reagent ranging from 100 to 1000. Under these conditions, extensive modification of lysyl as well as tyrosyl residues occurred, alongside with enzyme inactivation. They concluded that amino and phenolic groups are essential for the activity.

Our results show that, upon acetylation of phosphorylase *b* under mild conditions, a direct correlation between activity and extent of tyrosyl acetylation could be found in the presence and absence of the allosteric activator AMP. In the absence of this ligand, approximately 9 out of 34 tyrosyl residues per monomer (mol.wt. = 95 000) were acetylated with a parallel loss of activity of 78%; in the presence of AMP only 7 residues were acetylated, and the loss of activity was reduced to 17% of the control. Deacetylation regenerated 3 of the 9 tyrosyl residues modified in the absence of AMP, and 1 of the 7 residues acetylated in its presence; in both cases, a full recovery of activity was achieved. It appears, therefore, that 2 out of the 9 acetylated residues are essential for maintaining the enzymatic activity, and that the presence of AMP can selectively protect them.

N-acetylimidazole can react also with amino groups: acetylation of some reactive amino groups of phosphorylase *b* cannot be excluded. However, *N*-acetylated residues cannot be deacetylated by NH_2OH. Therefore, the full reactivation obtained upon deacetylation clearly demonstrates that such groups would not have an essential role in maintaining the activity.

References

[1] Graves, D. J. and Wang, J. H. (1972) in: The Enzymes, 3rd Ed. (Boyer, P. D., ed.), Vol. 7, pp. 435–482, Academic Press, New York, London.
[2] Riordan, J. F., Wacker, W. E. C. and Vallee, B. L. (1965) Biochemistry 4, 1758–1765.
[3] di Prisco, G., Cacace, M. G. and Zito, R. (1975) 10th FEBS Meeting, Paris, Abstr. no. 649.
[4] Fischer, E. H. and Krebs, E. G. (1962) Meth. Enzymol. 5, 369–373.
[5] Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry 7, 3590–3608.
[6] Helmreich, E. and Cori, C. F. (1964) Proc. Natl. Acad. Sci. US. 51, 131–138.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[8] Vulfson, P. L. and Skolysheva, L. K. (1967) Biokhimiya 32, 582–589.