AN ANTIBODY PROBE FOR THE AMINO-TERMINAL REGION OF GLYCOGEN PHOSPHORYLASE

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1. Introduction

The enzymatic activity of rabbit skeletal-muscle glycogen phosphorylase is regulated by several mechanisms. Allosteric transitions and the degree of association of phosphorylase monomers are key factors in control. These regulatory characteristics are affected by whether the amino-terminal region of phosphorylase is phosphorylated or dephosphorylated. The mechanism by which the phosphorylatable amino-terminal region affects catalytic, allosteric, and association properties has not been elucidated. Some information may be obtained by studying how regulatory properties are affected when the amino-terminal region interacts with a probe that reacts specifically with the amino-terminal region. An antibody specific for the amino-terminal region could be a suitable probe for such studies. Antibodies have been utilized for exploring the conformations of many protein molecules; this topic has been reviewed recently by Anfinsen and Scheraga [1]. Preparation of antibodies against fragments of protein molecules has been performed previously [2–5]. When it is desirable to have antibodies to a conformation of the protein fragment possessed by the intact native protein, the native protein is used as the antigen in preparing the antibodies. The antibodies specific for the given protein fragment are isolated by affinity chromatography on a resin that contains covalently bound protein fragments. We have applied this general procedure for the isolation of antibodies specific for the amino-terminal region of phosphorylase. Evidence is presented for the specificity of the isolated antibody population, and the use of this antibody population in studying phosphorylase is briefly described.

2. Experimental

2.1. *Antibody preparation*

A young female goat was inoculated with 20 mg phosphorylase *a* in 2 ml 0.15 M NaCl, 0.05 M glucose, 1 mM TrisCl, pH 7.4, emulsified with 2 ml complete adjuvant. After 2 weeks, the goat was boosted with 40 mg of phosphorylase *a* in the same buffer, but without adjuvant. Two weeks later, an identical booster injection was performed. After 1 week, a 500 ml blood sample was obtained; experiments described in this paper were performed by using antibody isolated from this blood sample. Antibody against goat serum was prepared by immunizing New Zealand white rabbits with goat serum emulsified with complete adjuvant. The γ-globulin fractions of goat antisera (goat γ-globulin) and rabbit antisera (rabbit anti-goat γ-globulin) were prepared by precipitation in the presence of 35% saturated ammonium sulfate. The γ-globulin precipitate was dissolved in water, dialyzed against 0.15 M NaCl and stored at −20°C. Measurement of protein in solutions containing immunoglobulins was performed by the method of Lowry et al. [6].

2.2. *Affinity chromatography resin*

Procedures used in affinity chromatography have been modified from those described by Young et al. [2]. A peptide, (1–18)p, corresponding to the last

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18 amino-terminal residues of phosphorylase *a*, was synthesized, phosphorylated, purified, and was found to be homogeneous by methods that have been described previously [7]. The amino acid sequence of (1–18)p is NH_2-SerArgProLeuSerAspGln-GluLysArgLysGlnIleSer(PO_4)ValArgGlyLeuCOOH. Sepharose 4B (Pharmacia) that had been activated with cyanogen bromide [8] was reacted with (1–18)p in 0.1 M sodium bicarbonate, pH 7.4. The resulting affinity resin contained 0.35 μmol (1–18)p/ml.

2.3. *Glycogen phosphorylases*

Rabbit skeletal-muscle glycogen phosphorylase *b* was isolated as by Fischer and Krebs [9], and phosphorylase *a* was prepared from phosphorylase *b* by phosphorylation with rabbit-muscle phosphorylase kinase [10]. Phosphorylase *b'* was obtained from phosphorylase *a* by limited proteolysis with trypsin [11]. Phosphorylase activity was measured in the direction of glycogen synthesis either by the method of Engers et al. [12] or by incorporation of [^{14}C] glucose from [^{14}C]glucose 1-phosphate into glycogen as described by Parrish and Graves [13]. The protein concentration of phosphorylase was measured spectrophotometrically by using the extinction coefficient $E^{1\%}_{10\,mm}$ at 280 nm of 13.2 [14]. A phosphorylase monomer was assumed to be mol. wt 100 000 [15].

2.4. *^{3}H-Labelled antigens*

Phosphorylases *a*, *b*, and *b'* were tritiated by a limited reaction with [^{3}H]acetic anhydride (New England Nuclear, 400 mCi/mmol). Less than 5% of the total lysine and tryosine residues were acetylated per monomer, and greater than 70% of the enzymatic activity was recovered. An acetylated phosphopeptide, Ac(1–18)p, and an acetylated dephosphopeptide, Ac(1–18), were prepared according to procedures previously described [7]. The amino terminus of native phosphorylase is *N*-acetylated. The amino acid sequence of Ac(1–18)p is the same as (1–18)p except that the amino terminus is *N*-acetylated, and Ac(1–18) differs from Ac(1–18)p in that serine-14 is not phosphorylated. The amino termini of these peptides were acetylated with acetic anhydride; other reactive functional groups were chemically blocked. [^{3}H]Acetic anhydride was used when radioactive peptides were prepared.

2.5. *Radioimmunoassays*

The double antibody radioimmunoassay consisted of three steps:

(i) Incubation of antibody with ^{3}H-labelled antigens.
(ii) Incubation with rabbit anti-goat γ-globulin (a wide range of rabbit anti-goat γ-globulin concentrations gave maximal precipitation of goat γ-globulin).
(iii) Incubation with normal goat γ-globulin, isolated from a goat that had not been immunized, to increase the quantity of precipitate.

Precipitates were pelleted by centrifugation and washed twice with 0.5 ml 1.6 mg/ml bovine serum albumin (Pentex crystalline) in 0.15 M NaCl, and 20 mM TrisCl, pH 7.4. The washed precipitates were dissolved in 100 μl 0.25 M NaOH and then assayed for radioactivity. All radioimmunoassay data have been corrected for controls in which antibody was not present.

3. Results

Immune goat γ-globulin was fractionated by affinity chromatography (fig.1). The affinity resin consisted of a peptide, corresponding to the last 18 amino-terminal residues of phosphorylase *a*, covalently attached to Sepharose 4B. Of the 270 mg γ-globulin applied to the column, only 0.6% was adsorbed by the column. The adsorbed protein was

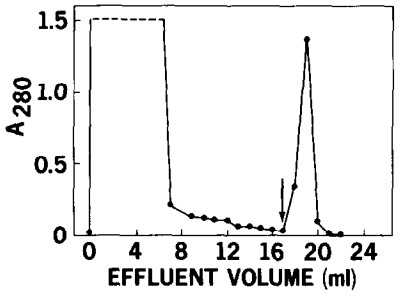

Fig.1. Elution profile of immune goat γ-globulin from Sepharose-(1–18)p. To 1.0 ml Sepharose–(1–18)p equilibrated in column buffer (0.15 M NaCl, 0.01 M KH_2PO_4, 0.01 M Na_2HPO_4, pH 7.3), 270 mg goat γ-globulin in 5.0 ml column buffer was applied. The column was washed with column buffer until the A_{280} of the effluent was no longer decreasing. The bound material was eluted by treatment with 4 M guanidine·HCl (arrow).

eluted with 4 M guanidine·HCl and immediately dialyzed against 0.15 M NaCl. Of the antibody activity against phosphorylase *a* that was applied to the column, only 2.4% was contained in the guanidine·HCl eluate. The recovery of antibody activity against Ac(1–18)p in the guanidine·HCl eluate is not known because this activity was not detectable in the γ-globulin fraction applied to the column. Measurement of antibody activity against Ac(1–18)p in the γ-globulin fraction was apparently inhibited by the high concentration of protein in this fraction. When the guanidine·HCl fraction was mixed with the γ-globulin fraction, antibody activity against Ac(1–18)p, originally present in the guanidine·HCl fraction, was not detectable. The guanidine·HCl fraction, anti-(1–18)p, was stored in 0.5 ml portions in the freezer. No loss in immunological activity towards either phosphorylase *a* or peptide antigens was observed after at least 3 months storage.

The specificity of anti-(1–18)p was analyzed by radioimmunoassay. When anti-(1–18)p was reacted with either phosphorylase or peptide antigens, soluble complexes were formed. Thus, it was necessary to introduce a second antibody, produced in rabbit against goat serum, to precipitate the complexes formed by anti-(1–18)p with these antigens. The specificity of anti-(1–18)p for [^{3}H]phosphorylase and [^{3}H]peptide antigens is presented in table 1. Phosphorylase *a* was a much better antigen than phosphorylase *b*. Phosphorylase *b'*, a derivative of phosphorylase *a* in which the last 16 amino-terminal residues have been removed, is a very poor antigen. The small amount of radioactivity found in the precipitate from the phosphorylase *b'* radioimmunoassay could be accounted for by contaminating phosphorylase *a*, which amounted to 6% for the phosphorylase *b'* preparation. This observation suggests that the amino-terminal region is necessary for interaction of phosphorylase with anti-(1–18)p. The phospho-amino-terminal peptide, Ac(1–18)p, and the dephospho-amino-terminal peptide, Ac(1–18), served equally well as antigens. Therefore, the phosphate group is not necessary for antibody interaction.

The binding of [^{3}H]phosphorylase *a* to anti-(1–18)p in the presence of different amounts of non-radioactive Ac(1–18)p is plotted in fig.2. The interaction of phosphorylase *a* with anti-(1–18)p would be inhibited by 50% if 1200 pmol Ac(1–18)p were

Table 1
Antibody specificity for phosphorylase antigens and peptide antigens

Antigen	Antigen in precipitate (%)
Phosphorylase *a*[a]	61
Phosphorylase *b*[a]	27
Phosphorylase *b'*[a]	4.5
Ac(1–18)p[b]	40
Ac(1–18)[b]	42

[a] Radioimmunoassay: The incubation mixture (455 μl) contained 73 pmol (27 000–37 000 cpm) [^{3}H]phosphorylase, 27 μg anti-(1–18)p, 0.8 mg bovine serum albumin, 75 μmol NaCl, and 10 μmol TrisCl, pH 7.4. After incubation for 30 min at 37°C, 4.0 mg (50 μl) rabbit anti-goat γ-globulin was added. After 10 min at 37°C, 250 μg (5 μl) normal goat γ-globulin was added, and the mixture was incubated at 37°C for 30 min. Precipitates were washed and analyzed for radioactivity as described in the text

[b] Radioimmun assay: The incubation mixture (455 μl) contained 700 pmol (3000 cpm) [^{3}H]peptide, 85 μg anti-(1–18)p, 75 μmol NaCl and 10 μmol TrisCl, pH 7.4. After incubation for 60 min at 37°C, 4.0 mg (50 μl) rabbit anti-goat γ-globulin was added. After 30 min at 37°C and 1 day at 4°C, 250 μg (5 μl) normal goat γ-globulin was added and the mixture was incubated at 37°C for 30 min and at 4°C for 1 day. Precipitates were washed and analyzed for radioactivity as described in the text

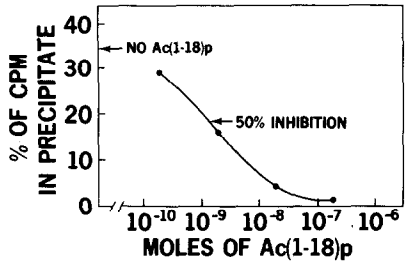

Fig.2. Inhibition of phosphorylase *a* binding to anti-(1–18)p by non-radioactive Ac(1–18)p. [^{3}H]Phosphorylase *a* (74 pmol, 38 000 cpm) was incubated with different amounts of Ac(1–18)p, 11 μg anti-(1–18)p, 0.8 mg bovine serum albumin, 75 μmol NaCl and 10 μmol TrisCl, pH 7.4, in total vol. 455 μl. After 30 min at 37°C, 4.0 mg (50 μl) rabbit anti-goat γ-globulin was added. After 10 min at 37°C, 250 μg (5 μl) normal goat γ-globulin was added and the mixture was incubated at 37°C for 30 min. Precipitates were washed and analyzed for radioactivity as described in the text.

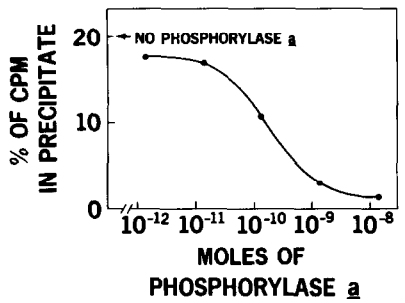

Fig.3. Inhibition of Ac(1–18)p binding to anti-(1–18)p by phosphorylase *a*. [^{3}H]-Ac(1–18)p (700 pmoles, 3000 cpm) was incubated with different amounts of phosphorylase *a*, 33 μg anti-(1–18)p, 75 μmol NaCl and 10 μmol TrisCl, pH 7.4, in total vol. 455 μl. After 60 min at 30°C, 1.6 mg (20 μl) rabbit anti-goat γ-globulin was added. After 30 min at 30°C and 1 day at 4°C, 250 μg (5 μl) normal goat γ-globulin was added and the mixture was incubated at 30°C for 30 min. Precipitates were washed and analyzed for radioactivity as described in the text.

Table 2
Antibody inhibition of phosphorylase enzymatic activity[a]

Phosphorylase	Anti-(1–18)p (μg)	Inhibition (%)	
		5′-AMP absent	5′-AMP present
a	16	40	6.1
a	33	62	23
b′	33	–	5.7

[a] Phosphorylase (0.35 μg) was pre-incubated with anti-(1–18)p in 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.06 M NaCl, pH 6.8, in total vol. 50 μl. After 30 min at 30°C, an aliquot was removed and assayed for enzymatic activity in the presence and absence of 1 mM 5′-AMP by using the [^{14}C]glucose 1-phosphate assay described by Parrish and Graves [13]. Inhibition was measured relative to experiments in which normal goat γ-globulin was present in place of anti-(1–18)p

present; 74 pmol [^{3}H]phosphorylase *a* were present in the assays. Therefore, under the conditions of the radioimmunoassay, phosphorylase *a* can compete more effectively than Ac(1–18)p for anti-(1–18)p. At the highest concentration of Ac(1–18)p tested, phosphorylase *a* binding to anti-(1–18)p was inhibited by 97%.

The binding of [^{3}H]-Ac(1–18)p to anti-(1–18)p in the presence of different amounts of non-radioactive phosphorylase *a* is represented by fig.3. Binding of [^{3}H]Ac(1–18)p to anti-(1–18)p was inhibited by 93% at the highest concentration of phosphorylase *a* tested. In other experiments, not described in this paper, phosphorylase *b* was able to cause nearly complete inhibition of the interaction of Ac(1–18)p with anti-(1–18)p, whereas phosphorylase *b*′ was not an inhibitor. These results provide strong evidence that the antibodies against phosphorylase *a* in the anti-(1–18)p fraction are specific for determinants located within the last 18 amino-terminal residues of the enzyme.

Because of the importance of the phosphorylated amino-terminal region in affecting catalytic properties of phosphorylase *a*, the effect of anti-(1–18)p on enzymatic activity of phosphorylase *a* and *b*′ was measured (table 2). Phosphorylase *a* normally is active with or without 5′-AMP present. Interaction of anti-(1–18)p with phosphorylase *a* inhibits catalytic activity measured in the absence of 5′-AMP much more than catalytic activity measured in the presence of 5′-AMP. The presence of 1–10 mM 5′-AMP during pre-incubation of anti-(1–18)p with phosphorylase *a* had no effect on the interaction of anti-(1–18)p with phosphorylase *a*. Therefore, it is unlikely that the greater activity when assayed in the presence of 5′-AMP can be explained by disruption by 5′-AMP of the interaction of anti-(1–18)p with phosphorylase *a*. The catalytic activity of phosphorylase *b*′, which is dependent on the presence of 5′-AMP for activity, was only slightly inhibited. Contaminating phosphorylase *a* in the phosphorylase *b*′ preparation could account for this inhibition.

4. Discussion

A peptide, (1–18)p, corresponding to the last 18 amino-terminal residues of glycogen phosphorylase *a*, was covalently linked to Sepharose 4B. Antibodies, anti-(1–18)p, that are bound to and eluted from a column of this affinity resin react specifically with determinants contained within the last 18 amino-terminal residues of phosphorylase. The evidence presented supporting this conclusion is:

(1) The amino-terminal phosphopeptide, Ac(1–18)p,

could essentially completely inhibit the interaction of anti-(1–18)p with phosphorylase *a*.

(2) Phosphorylase *a* could cause nearly complete inhibition of the interaction of Ac(1–18)p with anti-(1–18)p.

(3) Phosphorylase *b'*, which is missing the last 16 amino-terminal residues of native phosphorylase, did not have significant interaction with anti-(1–18)p and could not inhibit the interaction of Ac(1–18)p with anti-(1–18)p.

The isolated anti-(1–18)p fraction had more immunoreactivity with phosphorylase *a* than with phosphorylase *b;* however, phosphopeptide, Ac(1–18)p, and dephosphopeptide, Ac(1–18)p, were about equal as antigens. Therefore, the greater immunoreactivity of phosphorylase *a* relative to phosphorylase *b* is not because the phosphate group is directly involved in the antibody interaction. The difference probably is due to conformational changes in the protein induced by phosphorylation of the amino-terminal region. Phosphorylase *a* competes more effectively for anti-(1–18)p than does Ac(1–18)p. Thus, Ac(1–18)p may lack the conformational integrity or part of the antigenic determinant(s) possessed by phosphorylase *a*. Further speculation regarding the meaning of these observations cannot be made until the specificity of the anti-(1–18)p fraction has been characterized in greater detail. Studies are being conducted in this laboratory to determine how much of the amino-terminal region and which amino-acid residues are necessary for complexation with anti-(1–18)p.

When phosphorylase *a* was pre-incubated with anti-(1–18), catalytic activity of the enzyme in the absence of 5'-AMP was inhibited much more than catalytic activity measured in the presence of 5'-AMP. Activity in the presence of 5'-AMP is not dependent on whether the amino-terminal region is phosphorylated. Therefore, the antibody is able specifically to interfere with the functioning of the phosphorylated amino-terminal region in activating phosphorylase, without greatly interfering with the catalytic process. A more detailed analysis of the effect of anti-(1–18)p on the conformation and the regulatory properties of phosphorylase *a* may provide information that will help explain how the phosphorylated amino-terminal region is involved in activating phosphorylase in the absence of 5'-AMP.

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