DINITROPHENYLATION OF A SINGLE CYSTEINE SIDE CHAIN IN PHOSPHORYLASE *b* FROM RABBIT MUSCLE WITH CONCOMITANT BLOCKING OF AMP BINDING

H.G. BÄUMERT, H. FASOLD, F. KELLER, M. HALBACH and F. ORTANDERL

Institut für Physiologische Chemie der Universität Würzburg and *Institut für Biochemie der Universität Frankfurt/Main, Germany*

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1. Introduction

The inactivation of rabbit muscle phosphorylase *b* by dinitrofluorobenzene (DNFB) at pH 7.5–8.0 was investigated by several groups [1–4]. One sulfhydryl, which seems to be covered up by pyridoxal phosphate in the native enzyme, could be identified by this method in apo-phosphorylase, and the sequence of a peptic peptide around this cysteine was determined [1]. At least one SH group was found to be implicated in AMP binding of the *b* form of the enzyme, however, a clear decision between at least two corresponding peptic sequences could not be made [2, 3, 5].

As reported in a preliminary communication [4], we have found that the inactivation of phosphorylase *b* by DNFB proceeds more slowly and more specifically at pH 6.0–6.5. A subsequent thiolysis by mercaptoethanol [6] removes all dinitrophenyl groups except one from the modified enzyme.

2. Materials and methods

Phosphorylase *b* and phosphorylase *b* kinase were prepared from rabbit muscle as described by Fischer and Krebs [7, 8]. All other auxiliary enzymes, as well as ATP, AMP, NADH, and glucose-phosphates were obtained from Boehringer, Mannheim. Glycogen was purchased from Merck & Co., Darmstadt, and pretreated to remove AMP [9].. [^{14}C]DNFB (10–20 mCi/mmole), and [^{14}C]glucose-phosphate (2.7 mCi/mmole) were obtained from the Radiochemical Centre, Amersham. ϵ-DNP-lysine, *O*-DNP-tyrosine, and im-DNP-histidine were prepared as described in the literature [10]. S-DNP-cysteine was purchased from Mann Biochemicals, N.Y. Phosphorylase activity was measured either in the direction of glycogen degradation [9], or of glycogen synthesis in the modifications previously described [11]. Peptic digestion, fingerprints, autoradiography, scintillation counting, amino acid analyses, and sedimentation runs in a Spinco model E analytical ultracentrifuge, equipped with schlieren optics were carried out as described in a previous communication [11]. For glycogen binding investigation runs, the cell of the analytical ultracentrifuge contained, in final concentrations, 2–11 mg/ml of phosphorylase, freed from AMP by gel filtration [11], 2.5 mg/ml of glycogen, and 0.05 M sodium glycerophosphate-HCl buffer, pH 6.8. The total volume always was 0.8 ml. Rotor speed was 44 770 rpm, and pictures were taken at 2 min intervals. For evaluation of glycogen binding characteristics, the areas of the phosphorylase and glycogen peaks were measured when the distance of the glycogen peak from the center of the rotor was 6.4 mm. Binding constants for AMP were determined as described by Kastenschmidt et al [12]. For the determination of the binding constants for glucose-1-phosphate, the same method was used. The columns of Sephadex G-25 were equilibrated with 0.005–0.01 M glucose-1-phosphate in this case, and enough of the ^{14}C-labelled substrate was added to bring the radioactivity to 3000 dpm/ml of the elution and equilibration buffer. Dansylation of isolated peptides and the Edman degradation procedure followed the usual methods [13].

The procedure cited in [10] was used for hydrazinolysis of peptides, the carboxyl terminal amino acid was identified by automated amino acid analyses. Protein concentrations were determined by the method of Lowry et al. [14]. The pyridoxal phosphate content of phosphorylase samples was measured after precipitation of 4–20 mg of the protein, in 4 ml of solution, by addition of 0.5 ml of 60% perchloric acid. After centrifugation for 5 min at 3000 rpm, spectra of the supernatant were taken between 260 and 400 nm, and a molar extinction coefficient at 295 nm of 6 2501 $mole^{-1}\,cm^{-1}$ was used for calculations [15, 16], against control values of pyridoxal phosphate standards and phosphorylase *b*. Labellings of the enzyme with [^{14}C]DNFB were carried out with 40 mg portions of the protein, dissolved in 10 ml of 0.005 M sodium glycerophosphate-HCl buffer, pH 6.2. 18.6 mg of DNFB, containing 2 μCi of the labelled compound, and dissolved in 0.4 ml of ethanol, were added, and the mixture was stirred for the desired time, while the pH was controlled, and readjusted if necessary. The solution was then passed quickly through short columns of Sephadex G-50 (4.0 x 20 cm), equilibrated with the same buffer. 1 μl of mercaptoethanol was added per ml of the eluted protein solution, the pH was raised to 7.5 by addition of 2 N NaOH, and the solution was incubated at 30° for 1 hr. In preliminary experiments, the pH was varied between 7.0 and 8.2, and the temperature between 25 and 40°. Only small changes in the amount of radioactivity removed from the protein were observed. Aliquots of the solution for determinations of the residual enzymatic activity were removed at this point. The mixture was then brought to pH 2 by addition of 6 N HCl and dialyzed thoroughly against 0.001 N HCl. After lyophilization, samples of the material were weighed for scintillation counting, after digestion by digestin (Merck & Co., Darmstadt), and peptic digestion for fingerprinting [11]. For conversion of labelled phosphorylase *b* to the *a* form, the procedure of Krebs and Fischer [8] was followed. After incubation with ATP and phosphorylase *b* kinase, aliquots of the solution containing 50 mg of protein were passed over 1 X 20 cm columns of Sephadex G-50 equilibrated with 0.01 M sodium glycerophosphate-HCl buffer, pH 6.8, containing 0.001 M sodium fluoride. The enzymatic activity of the eluted protein was determined in the absence and presence of AMP [11], and the sedimentation constant of the protein was measured in the analytical ultracentrifuge to establish the full conversion to phosphorylase *a*.

3. Results

When phosphorylase *b*, freed from AMP, was labelled by [^{14}C]DNFB with subsequent thiolysis as described in Methods, the plot of residual activity against the amount of dinitrophenyl residues incorporated furnished the curve shown in fig.1. The labelled protein was digested with pepsin at several stages of inactivation, and fingerprints of these peptide mixtures were autoradiographed (fig. 2). The spot denoted as peptide 1 was first to become labelled in these experiments, and only after more than 30% inactivation, peptide 2 appeared in the autoradiographs, while after more than 70% inhibition several additional labelled peptides were observed. When the mercaptoethanol thiolysis step was omitted, three equally labelled peptides were observed even during the initial stages of inactivation, a fourth peptide spot appeared on the autoradiographs after about 20% inactivation.

Peptide 1 was isolated from peptic digests by preparative high voltage electrophoreses at pH 1.9, and a series of fingerprints after the usual method [11]. Amino acid analysis, however, gave evidence for a

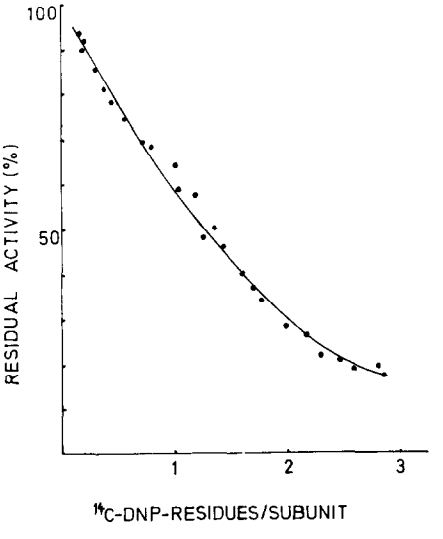

Fig.1. Inactivation of phosphorylase *b* by dinitrofluorobenzene. Enzymatic activity was determined in the presence of saturation concentrations of 5'-AMP, as described in Methods.

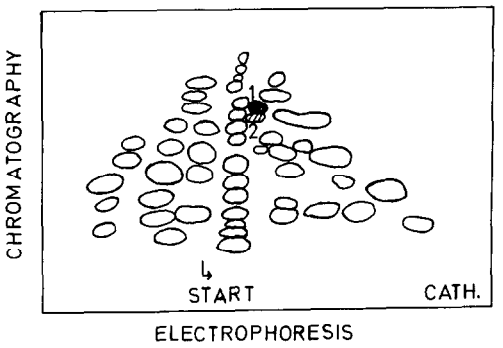

Fig.2. Fingerprint of peptic digest of [^{14}C]DNP-phosphorylase *b*. 5–8 mg of the peptide mixture were applied, and high-voltage electrophoresis was carried out in 0.1 M pyridine–acetate buffer, pH 6.5, at 100 V/cm in the first dimension. For chromatography in the second dimension, n-butanol–pyridine–acetic acid–water (30:20:6:4 by vol) was used.

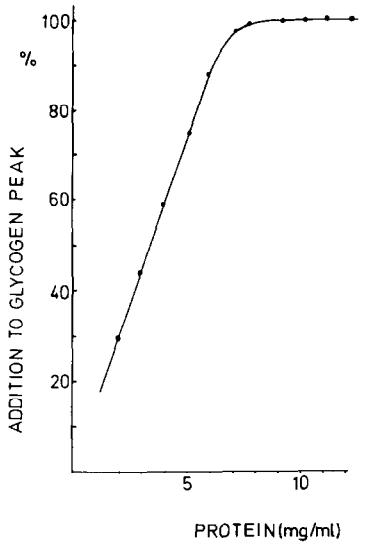

Fig.3. Evaluation of glycogen binding of phosphorylase *b* and DNP-phosphorylase *b* in the analytical ultracentrifuge. Experimental details are given in Methods.

mixture of peptides. Therefore, the material was re-digested with trypsin. The fingerprint then revealed a single new [^{14}C]DNP-peptide besides a small amount of material running at the position of the original peptide 1, and possessing the same amino acid composition as peptide 1, as well as two new ninhydrin-positive, unlabelled peptides. The tryptic DNP-peptide consisted of only three amino acids: glycine, identified as the amino end group by the dansyl method, and by its removal by an Edman degradation step, ϵ-DNP-cysteine, calculated in amount by the radioactivity of the peptide, and identified in high voltage electrophoresis at pH 1.9, or paper chromatography in butanol–acetic acid–water (70:15:15) in comparison with ϵ-DNP-lysine, im-DNP-histidine, O-DNP-tyrosine, and synthetic S-DNP-cysteine, after hydrolysis of the peptide, finally arginine, identified as the carboxyl terminal amino acid by hydrazinolysis and amino acid analysis.

To characterize the mode of inactivation by modification of a single cysteine side chain, attempts were made to measure the binding constants for the more tightly bound ligands of phosphorylase *b*. Precise K_D values could be determined for glucose-1-phosphate and AMP from the results of gel filtration experiments, as described in Methods. In the case of AMP, the value of 5×10^{-5} M for the native enzyme was changed to 2×10^{-2} for a DNP-phosphorylase *b* with 20% residual activity, while the value for glucose-1-phosphate, 8×10^{-4}, was the same for both proteins. To compare the binding characteristics for glycogen, constant amounts of glycogen were sedimented in ultracentrifugational runs, and increasing amounts of the native or modified enzyme were added to the solution. Partial binding of the enzyme was observed from the increase of the area of the glycogen peak. From the relative areas of this peak and the phosphorylase *b* peaks, a characteristic and reproducible curve (fig.3) of glycogen binding was obtained, identical for both proteins. As the inhibition of enzymatic activity might also have resulted from an increased tendency of the modified phosphorylase to dissociate into the subunits, the sedimentation behaviour of the DNP enzyme was carefully studied at varying concentrations. No monomers could be detected when the protein concentration was lowered to 0.05 mg/ml. At higher concentrations, the modified enzyme aggregated to tetramers to the same amount as native phosphorylase *b*.

From this evidence we concluded that only the AMP binding site had been affected by the modification of a single cysteine side chain. The same modification should then have but small effect on phosphorylase *a*. To test this, DNP-phosphorylase *b*, inhibited to 15–20% residual activity, was incubated with phosphorylase *b* kinase and ATP as described in Methods. The product possessed the expected s_{20w} value in the analytical ultracentrifuge, and sedimented as a homogeneous peak. This protein showed the specific activity of a sample of phosphorylase *a* prepared

in the same manner from native *b* enzyme in the absence of AMP, but could not be activated further by saturation concentrations of AMP. The experiment at the same time excluded the possibility of nonspecific denaturation of the enzyme by the dinitrophenylation procedure. Furthermore, the pyridoxal phosphate content of the DNP-enzyme, determined as described in Methods, was not lowered beneath the content of the native enzyme.

4. Discussion

The application of side-chain specific reagents, as compared to the use of quasisubstrates, usually entails careful control experiments to exclude inhibition of the enzyme by unspecific denaturation. In this case, we presume the inhibited DNP-phosphorylase *b* to remain in the native conformation of the enzyme, as it will return to full activity upon conversion to the *a* form, and the binding constants for the substrates glucose-1-phosphate and glycogen were found not to be changed in direct binding studies.

Our experiments endeavoured to establish a selective labelling of the AMP binding site in phosphorylase *b* with the aid of fluorodinitrobenzene and subsequent thiolysis. Since this reaction usually removes all DNP residues attached to cysteine, histidine, and tyrosine side chains, and only a single side chain remained modified, as was demonstrated by the peptic fingerprints, the label was at first presumed to be bound to a lysine side chain. Analysis of the labelled peptide, however, clearly demonstrated the modification of a sulfhydryl group. The dinitrophenylation of this cysteine very probably within, or close to the AMP binding site, results in a thioether bond well protected against thiolysis, much better than all other modified cysteines. A plausible explanation would postulate a niche of the enzyme at the AMP binding site, suitable for the accommodation of the nucleotide. The dinitrophenyl residue, becoming attached to a sulfhydryl within or at the brink of this crevice, might then protect the bond against the subsequent attack of mercaptoethanol. Currently, an X-ray crystallographic analysis of phosphorylase *a* and *b* is under way [17], and its results may be expected to furnish final evidence for or against this geometry of the AMP binding site.

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