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EFFECT OF NaF ON TYPE-1 PHOSPHATASE AGGREGATION

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In muscle cytosolic and glycogen fractions prepared in the presence of 50 mM NaF phosphorylase phosphatase was a ≈70 kDa complex instead of the 250 kDa or higher seen in the absence of NaF. A ≈70 kDa complex was also formed when purified 37 kDa phosphatase-1 catalytic subunit (but not its 33 kDa tryptic fragment) was exposed to NaF. Treating this latter complex with a cross-linker led to disappearance of the 37 kDa protein and formation of a ≈66 kDa band (detected by SDS electrophoresis), thus indicating the dimeric nature of the ≈70 kDa complex.

In skeletal muscle Pase-1 represents ca. 80% of the soluble phosphorylase phosphatase and is the only Pase in glycogen particles and microsomes [1,2,3]. In these cell fractions the same catalytic subunit (or maybe different isozymes [1]) is targeted to specific regulatory subunits: inhibitor-2 (I-2) in the cytosol, the G-subunit in the glycogen particles, a putative receptor in the microsomes [3].

NaF has been known for a long time as Pase inhibitor and is routinely included in extraction buffers to prevent dephosphorylation of proteins on serine and threonine residues by endogenous Pases. When NaF is used Pase can still be assayed following reactivation by Mn^{2+} (0.5-1 mM) added to the Pase assay buffer. Working on muscle extracts prepared with NaF we

Abbreviations. $PhMeS_2F$, phenylmethylsulfonyl fluoride; β-ME, β-mercaptoethanol; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; BSA, bovine serum albumin; Pase-1, serine protein phosphatase of type-1; I-2, phosphatase inhibitor-2.

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found a never-described effect of NaF on the aggregation of Pase-1.

MATERIALS AND METHODS

Materials. BSA, ovalbumin, carbonic anhydrase, catalase, ferritin, TPCK-treated trypsin, soybean trypsin inhibitor, ATP, $PhMeS_2F$, benzamidine, salivary α-amylase, electrophoresis protein standards and the cross-linker 3,3'-dithiobis(propionic acid N-hydroxysuccinimide ester) were purchased from Sigma. Brij-35 was from Pierce. The chemicals for electrophoresis were from Bio-Rad. The FPLC Superose 6 column was from Pharmacia.
Muscle extract and fractionation. New Zealand White rabbits (ca. 3 kg b.w.) were anesthetized with Nembutal (50 mg/kg) and exanguinated. Muscle from the back and hind limbs were quickly removed, frozen in liquid nitrogen and stores at -70^{o}. Frozen muscles were homogenized at 4^{o} in 5 mM EDTA, pH 7.5, 15 mM β-ME, 0.002% $PhMeS_2F$ and 0.1 mM benzamidine containing or not 50 mM NaF, and centrifuged at 6,000 x g for 20 min. The supernatant thus obtained was centrifuged at 100,000 x g to separate the cytosolic fraction (supernatant) from the glycogen pellet. The pellet was then digested with α-amylase in the presence or not of 50 mM NaF and centrifuged at 100,000 x g as described previously [2] thus obtaining the α-amylase supernatant.
FPLC gel filtration was performed on a Superose 6 column equilibrated at room temperature in 10 mM imidazole, 0.1 M NaCl, 0.1 mM EDTA, 5% glycerol, 0.01% Brij-35, pH 7,5, 50 mM β-ME and 0.1 mM benzamidine, containing or not 50 mM NaF.
Enzyme purifications and assays. Pase-1 catalytic subunit was purified from rabbit skeletal muscle glycogen particles [2] and phosphorylase from rabbit skeletal muscle [see 4]. Phosphorylase kinase was a gift from Prof. L. M. G. Heilmeyer Jr. (Bochum, FRG). Pase was assayed by the release of $[^{32}P]$-inorganic phosphate from phosphorylase a with or without trypsin pretreatment (20 μg/ml trypsin for 5 min at 30° followed by 120 μg/ml soybean trypsin inhibitor) in the presence or not of 0.5 mM $MnCl_2$ [4].
Cross-linking of Pase was performed with 3,3' dithiobis(propionic acid N-hydroxysuccinimide ester) [5]. After gel filtration of purified Pase-1 in the presence of NaF the fractions containing the ≈70 kDa complex were pooled, dialyzed for 4 h against 20 mM Hepes, 5 % glycerol, pH 7.4 and concentrated in Amicon Centricon 10. Then glycerol was brought to 20% and the cross-linker was added to 1 mM final concentration. The mixture was incubated for 10 min at 30^{o}, when triethanolamine pH 8.0 was added to 4 mM final concentration. The protein was then precipitated with TCA, resuspended and boiled in Laemmli buffer without β-ME. A parallel reaction was run with the addition of 300 μM β-ME.
Other methods. Electrophoresis was performed in the presence of SDS [6,7] and the gel was silver-stained [8]. Protein was determined according to Bradford [9] using BSA as standard.

RESULTS AND DISCUSSION

In muscle cytosolic and glycogen fractions (α-amylase supernatant) Pase was a high molecular mass complex (250 kDa or

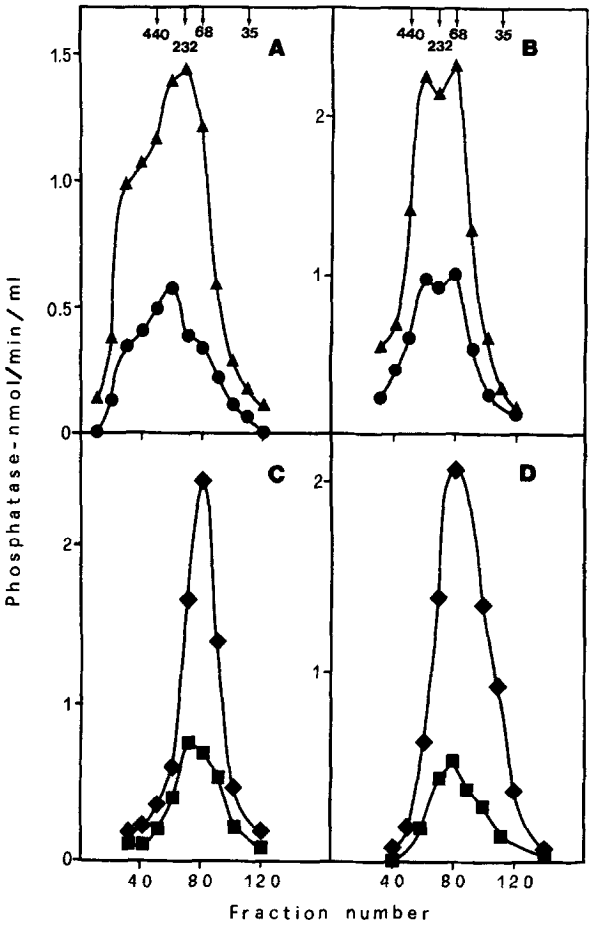

Fig. 1. Pase complexes from crude muscle fractions. FPLC gel filtration of: A, α-amylase supernatant and B, cytosolic fraction in the absence of NaF; C as in A and D as in B, but with 50 mM NaF in extraction and column buffers. 200 µl samples were applied on a Superose 6 column equilibrated as described in Methods at a flow rate of 0.4 ml/min. 64 µl fractions were collected after discarding the first 12 ml. Pase was assayed as spontaneous activity (●) or after activation with Mn^{2+} (■), trypsin (▲) or trypsin and Mn^{2+} (◆). Standards: ferritin (440kDa), catalase (232 kDa), BSA (68 kDa) and Pase-1 catalytic subunit (35 kDa).

higher) which required trypsin for full activation (Fig. 1, A and B and [2]). However, using buffers containing 50 mM NaF Pase decreased to ≈70 kDa and required Mn^{2+} in addition to trypsin for full activation (Fig. 1, C and D). Sodium pyrophosphate, another Pase inhibitor, had the same effect (not shown). In the cytosol the ≈70 kDa complex may represent catalytic subunit-I-2 complex [10]. In α-amylase supernatant the catalytic subunit is

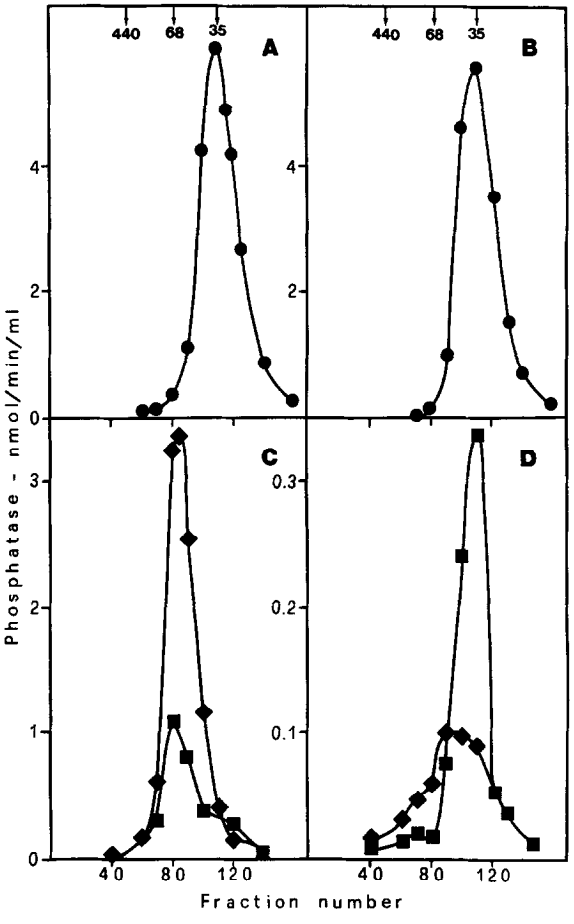

Fig. 2. FPLC gel filtration of purified Pase-1 catalytic subunit. A and C, native catalytic subunit and B and D, catalytic subunit treated with trypsin before gel filtration. A and B, gel filtration in the absence or, C and D, in the presence of 50 mM NaF as in Fig.1. Pase was assayed as spontaneous activity (●) or after activation with Mn^{2+} (■) or trypsin and Mn^{2+} (◆).

associated to the 165 kDa G-subunit and dissociation should yield isolated catalytic subunit, unless some other component is present or a dimer is formed .

To test the hypothesis of formation of a dimer we used Pase-1 catalytic subunit purified from glycogen particles [2], which is 35 kDa by gel filtration (Fig. 2, A and [2]) and 37 kDa by SDS gel electrophoresis (Fig. 3, lane c and [2]). Following incubation in the presence of 33 mM NaF at 30 ^{o} for 20 min, gel filtration in the presence of NaF showed that a ~70 kDa complex

had been formed which required trypsin and Mn^{2+} for reactivation (Fig. 2 C). Addition of ATP (0.7 mM) and $MgCl_2$ (1.3 mM) to the incubation mixture increased the yield of the following gel filtration, but it was not required for complex formation. On the other hand no such complex was formed when the catalytic subunit had been treated with trypsin (20 µg/ml for 5-10 min) before exposure to NaF (Fig. 3, D) and the enzyme recovered was reactivated by Mn^{2+} alone, though with a low yield. Trypsin-treatment (which decreases the SDS electrophoresis Mr from 37 k to 33 k [2]) did not influence the gel filtration Mr in the

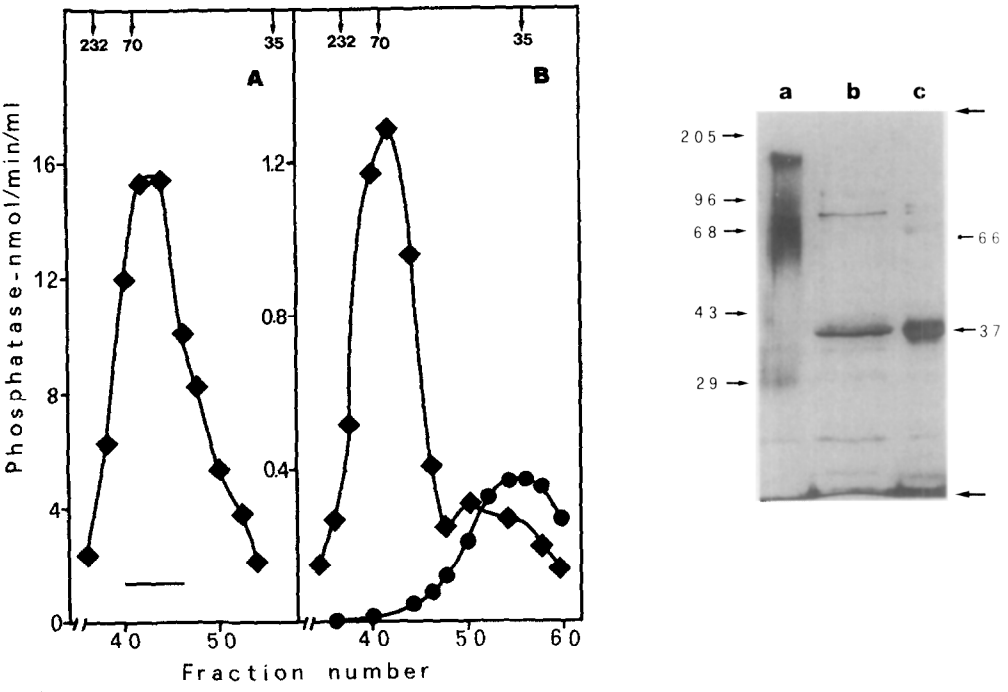

Fig. 3. A, gel filtration on FPLC of purified Pase-1 catalytic subunit in the presence of 50 mM NaF as described in Fig. 2, C; the active fractions were pooled as shown, dialyzed extensively against gel filtration buffer not containing NaF and subjected to a second gel filtration, shown in B, in the absence of NaF. Gel filtration was as in Fig. 1 but 128 µl fractions were collected. Pase was assayed as spontaneous activity (●) or after activation with trypsin and Mn^{2+} (◆). Gel filtration standards: catalase (232 kDa), Pase-1 catalytic subunit-I-2 complex (70 kDa), Pase-1 catalytic subunit (35 kDa). SDS gel electrophoresis of the ≈70 kDa complex obtained in A: lane a, after cross-linking; lane b, after cross-linking in the presence of 300 mM β-ME; lane c, without cross-linking. Electrophoresis standards: myosin (205 kDa), phosphorylase (96 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa).

absence of NaF (compare Fig.2 B and D). In the presence of NaF Pase catalytic subunit (or its tryptic fragment) was still able to bind to I-2 (not shown), indicating that NaF did not seem affect the interaction between catalytic subunit and I-2.

To test the reversibility of the ≈70 kDa complex induced by NaF we dialyzed and then gel-filtered in the absence of NaF the ≈70 kDa complex obtained in Fig. 3 A. The results (Fig. 3, B) showed that even after removal of NaF most of the activity was still in the ≈70 kDa aggregate, except for the small, newly formed 35 kDa peak; this latter peak was fully active likewise the catalytic subunit we first exposed to NaF. Complete dissociation of the ≈70 kDa complex was achieved only by boiling in electrophoresis sample buffer (Fig. 3, lane c), thus indicating the non-covalent nature of the association between catalytic subunits. The dimeric nature of the ≈70 kDa complex was further proven by cross-linking experiment. After extensive dialysis of the active pool of Fig. 3 A in the absence of β-ME and concentration on Amicon Centricon 10, the reversible cross-linker 3,3′ dithiobis(propionic acid N-hydroxysuccinimide ester) was added as described in Methods. Fig. 3, lane a, shows that cross-linking took to complete loss of the 37 kDa protein and appearance of a new widespread band of average Mr ≈66 k. Cross-linking in the presence of 300 mM β-ME prevented the formation of such band (Fig. 3, lane b).

Altogether these data indicate that: 1) exposure of Pase-1 catalytic subunit to NaF induces dimerization; however, removal of NaF is accompanied by little dissociation; 2) the dimer is not formed when the 33 kDa tryptic fragment of catalytic subunit is used, indicating the involvement of the ≈5 kDa portion removed by trypsin in dimer formation; 3) in crude fractions NaF induces dissociation of the high Mr Pase complex with formation

of a ≈70 kDa complex which, at least in the case of the glycogen-bound Pase, might also represent a dimer of catalytic subunit.

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