THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 24, Issue of June 13, pp. 22153–22160, 2003
Printed in U.S.A.

Biochemical Characterization of the Serum Fetuin-Mineral Complex*

Received for publication, January 22, 2003, and in revised form, April 1, 2003
Published, JBC Papers in Press, April 3, 2003, DOI 10.1074/jbc.M300739200

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The present study was carried out to characterize the fetuin-mineral complex (FMC), a high molecular mass complex of calcium phosphate mineral and the proteins fetuin and matrix Gla protein (MGP) that was initially discovered in serum of rats treated with etidronate and appears to play a critical role in inhibiting calcification *in vivo*. Fetuin purified from the FMC contains 3.3 mol of protein-bound phosphate. There is 1.3 mg of FMC/ml of serum 6 h after etidronate injection, and the FMC is 46% fetuin and 53% mineral by mass. Formation of the FMC in the first 6 h after etidronate injection does not increase serum fetuin despite the fact that 50% of serum fetuin is associated with the FMC, and clearance of the FMC in the 9–24-h interval lowers total serum fetuin by 50%. These observations suggest that the fetuin component of the FMC is derived from fetuin initially in serum and that clearance of the FMC removes the associated fetuin from circulation. One additional protein was consistently present in all preparations of the FMC, spp24 (secreted phosphoprotein 24). This 24-kDa protein is similar in domain structure to fetuin and, like fetuin and MGP, contains several residues of phosphoserine and accumulates in bone. Exogenous spp24 associated strongly with the FMC when added to serum containing it. These observations suggest that spp24 may, like fetuin and MGP, play a role in inhibiting calcification.

The present experiments are a continuation of our investigations into the mechanisms by which proteins interact specifically with mineral *in vivo* to prevent the calcification of arteries and other soft tissues. In a previous study (1), we described the discovery of a complex of calcium, phosphate, fetuin,[1] and matrix Gla protein in the serum of rats treated with the bone active bisphosphonate etidronate and showed that the appearance of this complex in serum correlates with the inhibition of bone mineralization by etidronate. The fetuin-mineral complex reaches maximal levels 6–9 h after etidronate injection and causes a 4-fold increase in total serum calcium without causing any increase in ionic calcium levels.

The proteins associated with the fetuin-mineral complex, fetuin and matrix Gla protein (MGP), have both been shown to function as potent inhibitors of calcification *in vitro*. Fetuin is a 59-kDa protein that consists of two N-terminal cystatin domains and a smaller C-terminal domain. Fetuin is synthesized by the liver and secreted into blood, where it is found at a concentration of ~1 mg/ml in the rat (2). Previous studies have demonstrated that fetuin is the major calcification inhibitor found in serum (3, 4). Fetuin is also one of the most abundant noncollagenous proteins found in mammalian bone (5–10), with a concentration of ~1 mg of fetuin/g of bone in rat (9). MGP is a small, 10-kDa vitamin K-dependent protein (11) that is secreted by a wide variety of cell types, including vascular smooth muscle cells, and is found in bone at a concentration of 0.2 mg/g in the rat (12). Genetic and biochemical studies have convincingly demonstrated that impaired MGP function causes extensive calcification of the elastic lamellae in the artery media of mice (13), rats (14), and humans (15).

Our working hypothesis is that the calcification inhibitory properties of fetuin and MGP may be related to their ability to form complexes with nascent mineral nuclei that prevent the growth of the mineral and target it for clearance from blood. This hypothesis is supported by the observation that formation of the fetuin mineral complex prevents the growth, aggregation, and precipitation of the mineral component (1) and by the observation that the fetuin-mineral complex is removed from blood in the 9–24-h interval following etidronate injection (1).

The objectives of the present investigations were to better characterize the composition of the fetuin-mineral complex, to identify additional protein components in the complex, and to determine the impact of the generation and clearance of the complex on total serum levels of fetuin. In the course of these studies we have developed the first method for isolating the native, phosphorylated form of fetuin from rat serum and the first immunoassay for the determination of rat fetuin in serum and in other biological specimens.

EXPERIMENTAL PROCEDURES

Materials—Albino male rats (Sprague-Dawley-derived) were purchased from Simonsen labs (Gilroy, CA). Etidronate was a gift from Proctor & Gamble (Cincinnati, OH). Sephacryl S300HR gel filtration media was purchased from Amersham Biosciences. Warfarin and human serum amyloid P component were purchased from Sigma, and human platelet factor 4 was purchased from Calbiochem (La Jolla, CA). NuPAGE Bis-Tris 4–12% polyacrylamide gradient gels were purchased from Invitrogen. MGP and spp24 (secreted phosphoprotein 24) were purified from bovine bone as described (16, 17). All other reagents used were reagent grade or better.

Purification of Rat Fetuin—In a typical experiment, six 40-day-old male rats were exsanguinated 6 h after receiving a subcutaneous injection of 32 mg of etidronate/100 g of body weight. The blood was allowed to clot for 30 min at room temperature, and the serum was collected by centrifugation at 1,400 × *g* for 10 min, pooled, and frozen in sixteen 1-ml aliquots at −70 °C until use. The 1-ml aliquots were centrifuged for 2 h at 16,000 × *g*. The supernatants were then removed, and the pellets were rinsed briefly with 1 ml of ice-cold 0.15 M NaCl, resuspended in 0.5 ml of HEPES buffer (20 mM HEPES buffer, pH 7.4, containing 1 mM $CaCl_2$, 2 mM Na_2HPO_4, 0.02% azide, and 0.15 M NaCl) and centrifuged for 30 min at 16,000 × *g*. After removal of the super-

* This work was supported in part by United States Public Health Service Grant AR25921. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: fetuin, α 2-HS glycoprotein; Gla, γ-carboxyglutamic acid; FMC, fetuin-mineral complex; MGP, matrix Gla protein; PVDF, polyvinylidene difluoride.

natants, the pellets were dissolved serially in a 1-ml volume of 60 mM EDTA, pH 7.5, and applied directly to a 2 × 140 cm column of Sephacryl S300HR equilibrated with 5 mM NH_4HCO_3 at room temperature. Fractions of 3 ml were collected, and the absorbance at 220 nm was determined; fetuin protein concentrations were estimated based on the assumption that a 1 mg/ml solution of fetuin has an absorbance of 10 at 220 nm. The ratio of absorbance at 220 nm to absorbance at 280 nm for purified rat fetuin is 32.4. Fetuin-containing fractions were either used directly or concentrated to dryness by speed vacuum and then resuspended in the desired volume of buffer.

Analytical Methods—For determination of MGP, the samples were assayed in triplicate using radioimmunoassay procedures previously described (12, 13). Calcium levels in serum and other samples were determined colorimetrically using cresolphthalein complexone (Sigma), and phosphate levels in serum and other samples were determined colorimetrically as described (18). Protein sequencing was carried out on bands transferred to PVDF membranes using a Procise 494 Sequencer (ABI division, PE Biosystems, Foster City, CA). The phosphorylation of fetuin samples was determined using a modification of the Malachite Green procedure. In a typical experiment, 60 μg of protein in 150 μl of water was combined with 20 μl of 8 M NaOH and hydrolyzed for 30 min at 100 °C. The reaction was stopped by the addition of 30 μl of 10 M HCl. 50-μl aliquots of the hydrolysate or of phosphate standards in 1.5 M HCl were placed in wells of a 96-well microtiter plate, and 100 μl of Malachite Green reagent (Biomed Research Laboratories, Plymouth Meeting, PA) was added to each well and allowed to react for 90 min at room temperature. The results were obtained by measurement of absorbance at 600 nm. The number of mol of fetuin in a sample was calculated from the absorbance at 220 nm using the assumptions that the protein portion of fetuin has a molecular mass of 36,000 and that the protein mass can be estimated from the relation that a 1 mg/ml solution of fetuin has an absorbance of 10 at 220 nm.

Radioimmunoassay for Rat Fetuin—Rabbits were immunized against purified rat fetuin by Strategic Biosolutions (Newark, DE), and the antiserum was used at a final 1:8000 dilution. The radioimmunoassay diluent, sample volumes, and procedures were identical to those used in the MGP radioimmunoassay (12), with the exception that the iodinated fetuin and the sample (serum or extract) were added to antibody at the same time. Fetuin was iodinated by incubating 10 μg of purified rat fetuin with 0.5 mCi of ^{125}I and 4 μg of iodogen (Pierce) in 150 μl of 100 mM phosphate buffer, pH 7.4, for 1h at room temperature.

Maintenance of Animals—The rats were fed rodent diet 5001 (Purina Mills Inc., St. Louis, MO), a diet that is 0.67% phosphorus and 0.95% calcium by mass. Etidronate was dissolved in water, titrated to pH 7.4 with NaOH, and administered subcutaneously to 40-day-old male rats at a dose of 8 or 32 mg/100 g of body weight. To determine the effect of warfarin on the generation of the serum protein-mineral complex, the rats received subcutaneous injections of 15.4 mg of warfarin/100 g of body weight 2 h prior to etidronate injection. For plasma analyses, blood was drawn into a heparin-containing syringe to achieve a final heparin concentration of 75 μg/ml, and plasma was collected by centrifugation at 1,400 × *g* for 10 min in a clinical centrifuge. For serum, the blood was allowed to clot for 30 min at room temperature, and the serum was collected by centrifugation at 1,400 × *g* for 10 min. The University of California, San Diego Animal Subjects Committee approved all animal experiments.

Biochemical Characterization of the Fetuin-Mineral Complex—To determine the composition of the fetuin-mineral complex at the 32 mg/100 g of body weight dose of etidronate, eight rats received etidronate at $t = 0$. Four of these rats were exsanguinated 6 h later, and four rats were exsanguinated 24 h later. Three 200-μl aliquots of pooled serum obtained at the two time points were centrifuged at 16,000 × *g* for 2 h to sediment the fetuin-mineral complex. After removal of the supernatant, the pellets were washed briefly with 200 μl of ice-cold 0.15 M NaCl and then dissolved overnight in 100 μl of 0.15 M HCl. Each solution was analyzed for calcium, phosphate, fetuin, and MGP; the numbers shown in Fig. 4 and Table II are the averages of the three individual determinations on each serum pool.

To determine the composition of the fetuin-mineral complex at the 8 mg/100 g of body weight dose of etidronate, four rats received etidronate at $t = 0$ and were exsanguinated 6 h later. To sediment the fetuin-mineral complex, three 175-μl aliquots of pooled serum were placed in polyallomer centrifuge tubes and centrifuged for 1 h in a Beckman Airfuge operated at 27 p.s.i. (~164,000 × *g*). After the removal of the supernatants, the pellets were washed briefly with 175 μl of ice-cold 0.15 M NaCl and then dissolved overnight in 70 μl of 0.15 M HCl. Each solution was analyzed for calcium, phosphate, fetuin, and MGP; the numbers shown in Table II are the averages of the three individual determinations.

Identification of Additional Constituents of the Fetuin-Mineral Complex—To identify additional proteins in the fetuin-mineral complex, the rats were exsanguinated 6 h after etidronate injection. For the samples from animals treated with 32 mg of etidronate/100 g of body weight, 1-ml aliquots of pooled serum or plasma were centrifuged at 16,000 × *g* for 2 h to sediment the fetuin-mineral complex. After removal of the supernatant, the pellets were washed briefly with 0.5 ml of ice-cold 0.15 M NaCl and resuspended in 0.5 ml of HEPES buffer. The aliquots were again centrifuged for 30 min at 16,000 × *g*, and the supernatants were removed. For samples from animals treated with 8 mg of etidronate/100 g of body weight, 175-μl aliquots of pooled serum or plasma were centrifuged at 164,000 × *g* for 1 h to sediment the fetuin-mineral complex. After removal of the supernatants, the pellets were washed briefly with 175 μl of ice-cold 0.15 M NaCl and then resuspended in 40 μl of HEPES buffer. The aliquots were again centrifuged for 1 h at 164,000 × *g*, and the supernatants were removed. The final pellets from the 8- and 32-mg doses were dissolved in SDS gel loading buffer containing 60 mM EDTA, pH 7.4, and electrophoresed on 4–12% polyacrylamide gels. The proteins were either stained with Coomassie or electrophoretically transferred to a PVDF membrane for N-terminal protein sequencing.

Studies were also carried out to see whether the addition of purified MGP, platelet factor 4, secreted phosphoprotein 24, or serum amyloid P component to serum from etidronate-treated rats would increase the level of each in the fetuin-mineral complex. In a typical experiment, serum was obtained from rats 6 h after injection of 32 mg of etidronate/100 g, and 200 μl of this serum was placed into each of three tubes. One tube received 5 μg of purified platelet factor 4, the second received no addition, and the third received 5 μg of platelet factor 4 and 50 mM EDTA. Each tube was centrifuged for 2 h at 16,000 × *g*. The supernatants were then removed, and the tubes were rinsed briefly with 200 μl of ice-cold 0.15 M NaCl. The three pellets were then dissolved in SDS gel loading buffer containing 60 mM EDTA, and 50% of the dissolved pellet was electrophoresed on a 4–12% polyacrylamide gel; the control lane contained 2.5 μg of platelet factor 4 alone. This procedure was repeated to test the possible incorporation of 20 μg of secreted phosphoprotein 24 and 6 μg of purified human serum amyloid P component into the fetuin-mineral complex. For MGP, 100 μg of the protein was added to 200 μl of serum, and only 10% of the dissolved pellet was loaded onto the gel.

RESULTS

Purification of Fetuin from the Serum of Etidronate-treated Rats—The native form of rat fetuin was purified from the serum fetuin-mineral complex for use in immunizations and immunoassays. The first step in purification was sedimentation of the fetuin-mineral complex by centrifugation of serum from etidronate-treated rats. In agreement with previous studies (1), the resulting pellets were primarily fetuin with significant contamination by serum albumin (Fig. 1). To remove albumin and other nonspecifically associated contaminants, the pellets were resuspended in HEPES buffer, and the fetuin-mineral complex was again sedimented by centrifugation. As seen in *lane 3* of Fig. 1, this stratagem removed most of the albumin contaminant with good recovery of fetuin. The final pellets were dissolved in 60 mM EDTA, pH 7.5, and fractionated by gel filtration over Sephacryl S300HR. As shown in Fig. 2, the major protein component recovered from this purification step has an apparent molecular mass of 59 kDa, which is the same molecular mass previously found for rat fetuin. N-terminal sequence analysis of the pooled peak fractions 87–90 revealed the sequence APQGAGLGFR, which matches the N-terminal sequence of rat fetuin (19). These four peak fractions contain ~6 mg of rat fetuin, which is 65% of the fetuin estimated to be present in the fetuin-mineral complex in the initial 16-ml serum sample. As can be seen in Table I, rat fetuin purified by these procedures has an average phosphorylation of 3.3 mol P/mol, which is close to the number of potential sites of serine phosphorylation by the secretory pathway protein kinase (20). In contrast, commercial samples of fetuin purified from fetal bovine serum and human serum had only 0.7 and 0.9 mol P/mol, respectively.

Biochemical Characterization of the Fetuin-Mineral Complex—A specific radioimmunoassay was developed for the

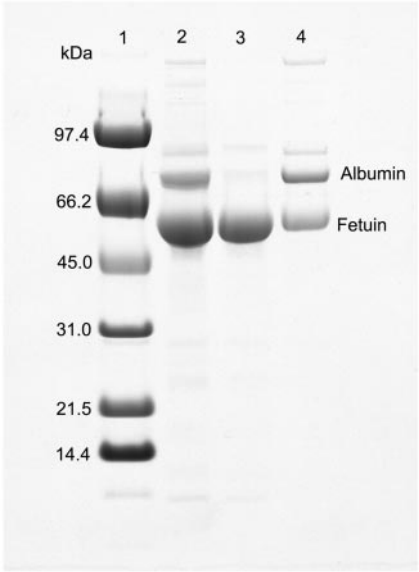


FIG. 1. **Isolation of the fetuin-mineral complex by the centrifugation of serum from etidronate-treated rats.** Serum was obtained from 40-day-old male rats 6 h after subcutaneous injection with 32 mg of etidronate/100 g of body weight, and the fetuin-mineral complex was sedimented by centrifugation of this serum for 2 h at 16,000 × *g*. After removal of the supernatants, some pellets were resuspended in HEPES buffer and centrifuged an additional 30 min. Pellets from the first and second centrifugation steps were dissolved in SDS gel loading buffer containing 60 mM EDTA and electrophoresed using 4–12% polyacrylamide gels. *Lane 1*, Bio-Rad low molecular mass markers. *Lane 2*, pellet from the first centrifugation step. *Lane 3*, pellet from the second centrifugation step. *Lane 4*, supernatant from the second centrifugation step (see "Experimental Procedures").

quantitative detection of rat fetuin using protein purified by the above procedures for immunoassay standards and for raising antibodies in rabbits. As shown in Fig. 3, the fetuin radioimmunoassay detects fetuin amounts ranging from 5 to 100 ng, and the dose-dilution curve for rat serum parallels the curve for the purified rat fetuin standard.

The fetuin radioimmunoassay was used to determine the composition of the fetuin-mineral complex found in rat serum at two different etidronate doses. The complex found in serum at 6 h after treatment with the 32 mg of etidronate/100 g dose can be sedimented by centrifugation for 2 h at 16,000 × g, and Table II gives the amounts of calcium, phosphate, fetuin, and MGP found in the resulting pellet fraction. Assuming that the mass of the complex can be approximated by the sum of the calcium, phosphate, and fetuin masss, each 1 ml of serum from rats treated with the 32-mg dose of etidronate contains 1.3 mg of the complex, and the complex is 46% fetuin and 53% mineral by mass.

The fetuin-mineral complex found in serum at 6 h after treatment with the lower, 8 mg of etidronate/100 g dose cannot be sedimented by centrifugation for 2 h at 16,000 × *g* (1), and experiments were accordingly carried out to determine whether higher speed centrifugation would sediment the complex found in serum at the 8-mg dose. These experiments showed that centrifugation for 1 h at 164,000 × *g* sedimented the complex found in serum at the 8-mg dose and that the resulting pellet had the same translucent appearance as the pellet formed at the 32-mg dose. Table II gives the amounts of calcium, phosphate, fetuin, and MGP found in this pellet fraction. Assuming that the mass of the complex can be approximated by the sum of the calcium, phosphate, and fetuin masss, each 1 ml of serum from rats treated with the 8-mg dose of etidronate contains 0.46 mg of the complex, and the complex is 62% fetuin and 37% mineral by mass.

Changes in Total Serum Fetuin Levels Associated with the Formation and Clearance of the Fetuin-Mineral Complex—The radioimmunoassay for rat fetuin was also used to determine the time course of formation and clearance of the fetuin-mineral complex from the serum of rats treated with 32 mg of etidronate/100 g of body weight. In agreement with previous studies (1), total serum calcium increased 4-fold over the first 9 h following etidronate injection and then fell to control levels between 9 and 24h (Fig. 4*A*); changes in serum phosphate paralleled the changes in serum calcium (data not shown). As seen in Fig. 4B, centrifugation of these serum samples showed that the changes in total serum calcium are due to the appearance and subsequent clearance of the fetuin-mineral complex from serum. Total serum fetuin levels were not significantly changed over the first 6 h following etidronate injection (Fig. 4*A*), despite the fact that almost half of the circulating fetuin becomes associated with the complex by the 6-h time point (Fig. 4*B*); this result shows that the fetuin associated with the serum mineral complex arises from the fetuin in circulation prior to etidronate injection. Fig. 4*A* also shows that total serum fetuin levels fall by ~40% in the 6–24-h interval after etidronate injection, and Fig. 4*B* shows that this reduction in total serum fetuin is associated with the disappearance of the fetuin-mineral complex from serum in the 6–24-h interval.

Identification of Additional Constituents of the Fetuin-Mineral Complex—Experiments were carried out to assess the possible presence of additional protein components in the fetuin-mineral complex. To ensure that only those proteins that are actually associated with the fetuin-mineral complex were identified, the initial pellets formed upon centrifugation of serum from etidronate-treated rats were resuspended in buffer and repelleted prior to analysis (*cf.* Fig. 1). Fig. 5*A* shows the Coommassie staining pattern obtained when SDS gels were overloaded with the fetuin-mineral complex found in rats treated with the 32-mg dose of etidronate. To identify protein components, the gel electrophoresis step was repeated, and the proteins were transferred to a PVDF membrane. The proteins identified by N-terminal sequencing of bands cut from this membrane are shown in Fig. 5*A*. In addition to the dominant fetuin component, four additional components were consistently seen in the SDS gels carried out on pellet fractions from rats treated with the 32-mg etidronate dose: matrix Gla protein, secreted phosphoprotein 24, serum amyloid P component, and prothrombin. Platelet factor 4 was also consistently present in SDS gels carried out on pellets formed by centrifugation of serum from different rats but was not seen in pellets formed by centrifugation of heparin plasma from the same animals, which is consistent with the fact that plasma levels of the protein are ~1000 times lower than serum levels (21). Hemoglobin was detected in some pellets but not others; the samples that contained hemoglobin consistently had a small red component at the bottom of the pellet, which suggests that these samples may have had some erythrocyte contamination. The addition of EDTA to serum prior to centrifugation eliminated all bands associated with the fetuin-mineral complex (data not shown).

Fig. 5*B* shows the results obtained when SDS gels were overloaded with the fetuin-mineral complex found in the serum of rats treated with the 8-mg dose of etidronate. As seen, the pattern is markedly similar to the pattern with the 32-mg etidronate dose, with comparable bands of matrix Gla protein, platelet factor 4, and secreted phosphoprotein 24. Hemoglobin was present in some but not all of the serum samples, and its presence again correlated with the presence of a small red component at the bottom of the pellet. The major difference between the composition of the pellets formed by centrifugation

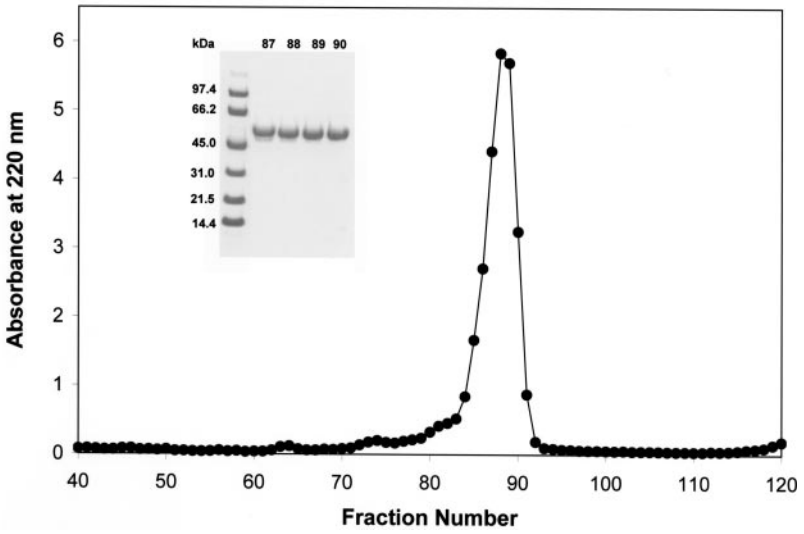


FIG. 2. **Purification of rat fetuin by Sephacryl S300HR filtration of the EDTA-treated fetuin-mineral complex.** 16 ml of serum was obtained from rats 6 h after injection with 32 mg of etidronate/100 g of body weight. The fetuin-mineral complex was sedimented by centrifuging 1-ml aliquots of this serum for 2 h at 16,000 × *g*, and the resulting pellets were resuspended in HEPES buffer and recentrifuged. The final pellets were dissolved in 60 mM EDTA, pH 7.5, and purified over a 2 × 140-cm column of Sephacryl S300HR equilibrated with 5 mM NH_4HC0_3 at room temperature. Fraction size was 3 ml. *Inset*, SDS gel electrophoresis of the fractions 87–90 on a 4–12% polyacrylamide gel. Load was 10 μg/lane.

TABLE I

Comparison of the amount of protein-bound phosphate found in fetuin purified from the serum of etidronate-treated rats with that found in bovine and human fetuin.

Purified rat, bovine, and human fetuin were subjected to alkaline hydrolysis, and the amount of phosphate released was determined using the Malachite Green method (see "Experimental Procedures").

Source of fetuin	Phosphate/mol fetuin	Number of potential serine phosphorylation sites[a]
	mol	
Etidronate-treated rat serum	3, 3.3, 3.6[b]	4
Fetal calf serum	0.7[c]	6
Human plasma	0.9[d]	4

[a] Number of serine residues having the SX (Glu/Ser(P)) phosphorylation recognition motif.
[b] Values obtained for three different preparations of fetuin purified from the serum of etidronate-treated rats.
[c] The same value was obtained for bovine fetuin purchased from Sigma and Calbiochem.
[d] Value obtained for human fetuin from Calbiochem.

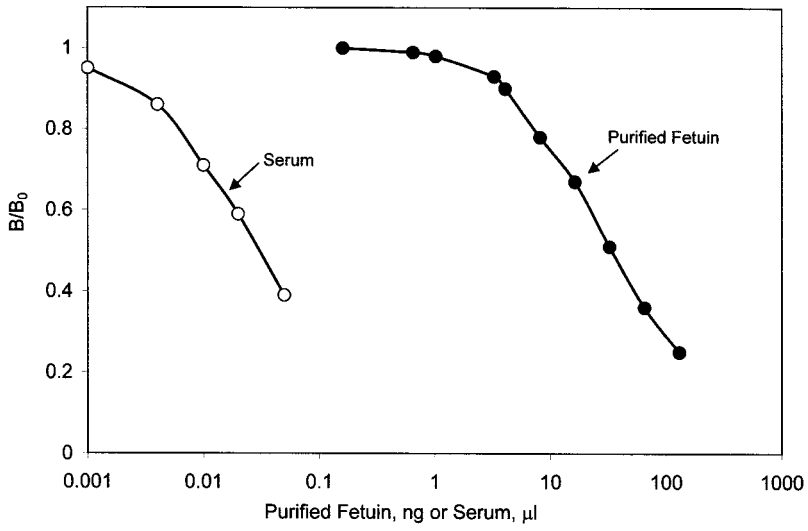


FIG. 3. **Radioimmunoassay of rat fetuin and its detection of fetuin antigen in normal rat serum.** Relative fraction of ^{125}I-labeled rat fetuin bound to antibody (B/B_o) at increasing amounts of purified rat fetuin (●) and at increasing volumes of rat serum (○).

of serum from rats treated with the 8- and 32-mg etidronate doses was the presence of serum amyloid P component at the 32-mg dose but not at the 8-mg dose.

Additional studies were carried out to see whether the addition of purified MGP, platelet factor 4, secreted phosphoprotein 24, or serum amyloid P component to serum from etidronate-treated rats prior to centrifugation would increase the level of each in the fetuin-mineral complex pellet formed after centrifugation. As seen in Fig. 6, the addition of purified MGP, secreted phosphoprotein 24, and platelet factor 4 to serum prior to sedimentation of the fetuin-mineral complex increased the intensity of staining in the respective protein bands (*lane 2*). In contrast, the addition of serum amyloid P component to serum did not increase the intensity of staining in this protein band (Fig. 6). It should be noted that the addition of exogenous human platelet factor 4 increases the intensity of only one of the two platelet factor 4 bands present in the original pellet because rat platelet factor 4 is a mixture of glycosylated (9 kDa) and nonglycosylated (7 kDa) components, whereas the human protein consists only of a 9-kDa form (22).

The importance of the vitamin K-dependent γ-carboxylation of matrix Gla protein was assessed by subcutaneous injection of 15.4 mg of warfarin/100 g of body weight 2 h prior to injection of the 32-mg etidronate dose. The serum was obtained 6 h after

TABLE II

Composition of the fetuin-mineral complex found in the serum of etidronate-treated rats

Serum was obtained from rats 6 h after injection with etidronate doses of 8 mg or 32 mg/100 g body weight. The fetuin-mineral complex was obtained by centrifugation for 2 h at 16,000 × *g* (32 mg dose) or by centrifugation for 1 h at 164,000 × *g* (8-mg dose). The resulting pellets were assayed for calcium, phosphate, fetuin, and MGP. The results are expressed as the amount of complex found in 1 ml of serum and are the average ± S.D. of three individual determinations on each serum pool of (see "Experimental Procedures").

Etidronate dose	Composition of fetuin-mineral complex			
	Calcium	Phosphate	Fetuin	MGP
mg	*μg*	*μg*	*μg*	*μg*
32	314 ± 8	357 ± 41	578 ± 20	6.3 ± 1.1
8	75 ± 6	97 ± 10	283 ± 30	7.2 ± 0.6

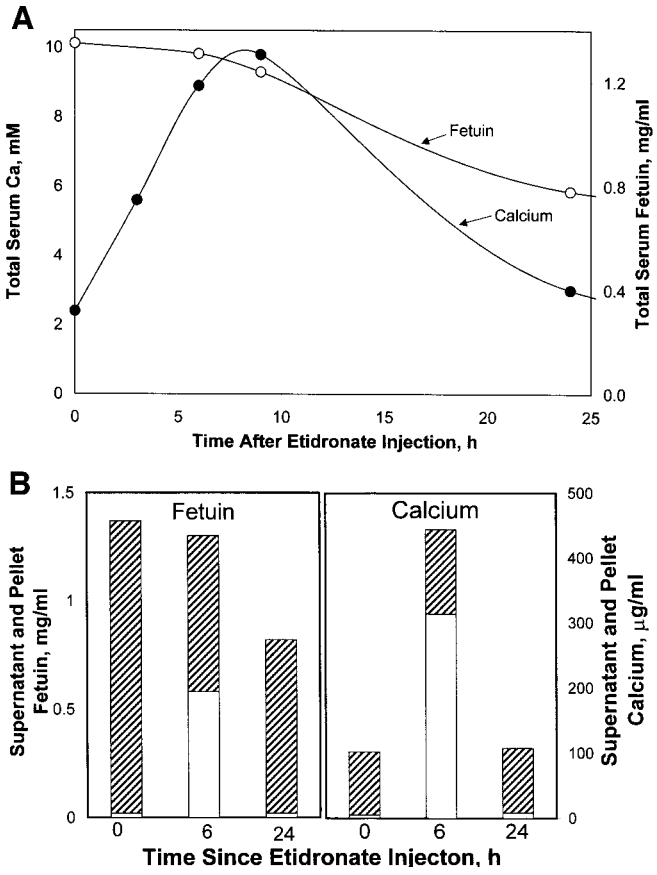


FIG. 4. **Effect of a dose of 32 mg of etidronate/100 g on serum levels of fetuin and calcium in the rat.** 40-day-old male Sprague-Dawley rats were given subcutaneous injections of etidronate at a dose of 32 mg/100 g of body weight at $t = 0$. *A*, blood was removed from four animals at the indicated times and analyzed to determine the levels of calcium and fetuin (see "Experimental Procedures"). Each data point is the average of the individually determined levels in the four experimental animals. ●, total serum calcium (mM); ○, total serum fetuin (mg/ml). *B*, blood was removed by exsanguination from four untreated control rats and from four rats at 6 and 24 h after etidronate injection. The fetuin-mineral complex was sedimented by centrifuging serum at 16,000 × *g* for 2 h, and the resulting pellets were analyzed for fetuin and calcium as described under "Experimental Procedures." The *cross-hatched region* of each bar denotes the average supernatant level of calcium and fetuin in the four rats, and the *open region* of each bar denotes the average pellet level.

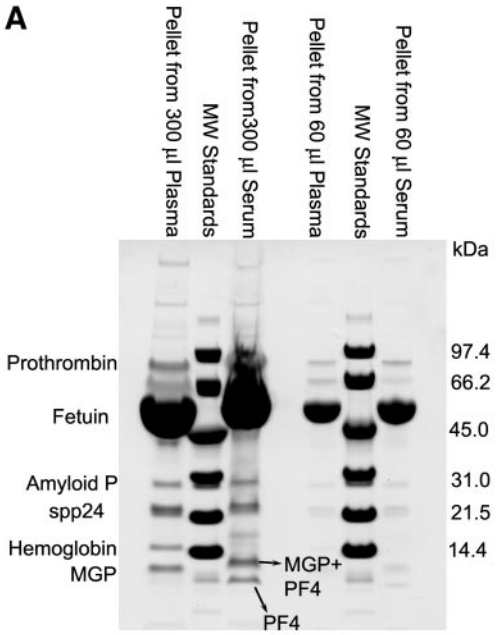
etidronate injection, and the samples were analyzed as described for the 32-mg etidronate dose in Fig. 5. The resulting Coomassie-stained gel showed that warfarin treatment caused the complete loss of the MGP band but had no effect on the intensity of the fetuin, secreted phosphoprotein 24, platelet factor 4, or serum amyloid P component bands (data not shown). These results show that the vitamin K-dependent γ-carboxylation of MGP is required for binding to the fetuin-mineral complex and also show that the accumulation of other minor protein components in the complex is not dependent on the presence of MGP.

DISCUSSION

Composition of the Fetuin-Mineral Complex—To our knowledge, the present studies are the first to describe the isolation of fetuin from rat serum. The procedure is rapid and simple, yielding 6 mg of the purified protein from the serum of 6 etidronate-treated rats in 3 days. Because the biologically relevant serum fetuin-mineral complex is the starting point for this purification procedure and no denaturants are employed during purification, it is likely that the resulting purified rat fetuin is the biologically active, native structure of the protein. In contrast, previous studies have purified fetuin from the extracellular matrix of rat bone and have used denaturants in the purification procedures (5, 9). In the present study we have used the purified, native structure of rat fetuin to make the first quantitative immunoassay for the determination of rat fetuin levels in biological fluids and extracts.

Fetuin purified from the fetuin mineral complex in rat serum by these procedures has 3–3.6 molecules of protein-bound phosphate. Because fetuin has not been purified from rat serum in previous studies, there is no published data on the basal phosphorylation of the total pool of serum fetuin in the rat. Serum fetuin arises from the hepatocyte, however, and previous studies have used metabolic labeling with ^{32}P to demonstrate that the fetuin secreted from rat hepatocytes is indeed phosphorylated (19, 23). Although the extent of phosphorylation could not be determined by the procedures used in these earlier studies, it was shown that the phosphorylation was restricted to phosphoserine residues in the protein (23). A phosphorylated fetuin has recently been isolated from human serum and shown to contain ~1 mol P/mol (24). The protein-bound phosphate in this human fetuin preparation was localized to two partially phosphorylated serine residues, serines 120 and 312. Both serine residues lie in the sequence Ser-Xaa-(Glu/Ser(P)), which is the recognition motif for phosphorylation by the secretory pathway protein kinase (20) and is the location of phosphoserine in virtually all other presently known secreted phosphoproteins (25). These two serines are conserved in the rat fetuin, and probably account for 2 of the 3–3.6 molecules of protein-bound phosphate found in the protein. There are two additional sites of potential serine phosphorylation by the secretory pathway protein kinase in rat fetuin, serines 310 and 307, and it is likely that the remaining 1–1.6 mol of protein-bound phosphate found in rat fetuin can be accounted for by phosphorylation of these residues.

Previous studies showed that the fetuin-mineral complex formed at the 32-mg/100 g of mass dose of etidronate can be sedimented by centrifugation at 16,000 × *g*, whereas the complex formed at the 8-mg dose cannot. We have here found that centrifugation at the higher, 164,000 × *g* conditions of an Airfuge will sediment the fetuin-mineral complex from serum obtained from rats treated with the 8-mg etidronate dose and have used the quantitative immunoassay for rat fetuin to com-



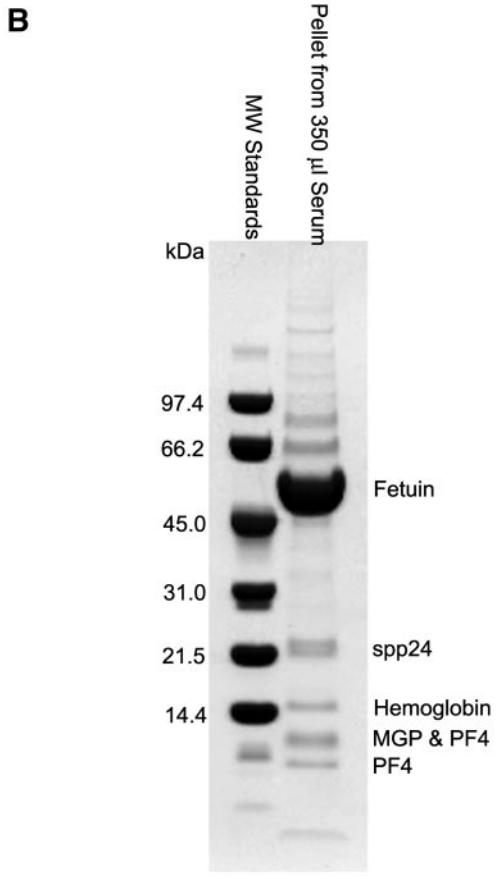


FIG. 5. **Identification of additional proteins in the fetuin-mineral complex.** To identify additional proteins in the fetuin-mineral complex, serum and heparin plasma were obtained from rats 6 h after injection with 8 or 32 mg of etidronate/100 g of body weight. The fetuin-mineral complex was sedimented by centrifuging serum or plasma at 16,000 × *g* for 2 h (32-mg dose) or at 164,000 × *g* for 1 h (8-mg dose). After removal of the supernatant, the pellets were washed briefly with ice-cold 0.15 M NaCl and resuspended in HEPES buffer and again centrifuged at the respective centrifugal forces. The final pellets were dissolved in SDS gel loading buffer containing 60 mM EDTA, pH 7.5, electrophoresed on 4–12% polyacrylamide gels, and either stained with Coomassie or transferred to a PVDF membrane for N-terminal sequence analysis. *A*, electrophoresis of pellets from the indicated volume of serum or plasma from rats treated with 32 mg of etidronate/100 g. *B*, electrophoresis of the pellet from 350 µl of serum from rats treated with 8 mg of etidronate/100 g. The proteins identified by N-terminal sequence analysis are shown. *MW*, molecular mass.

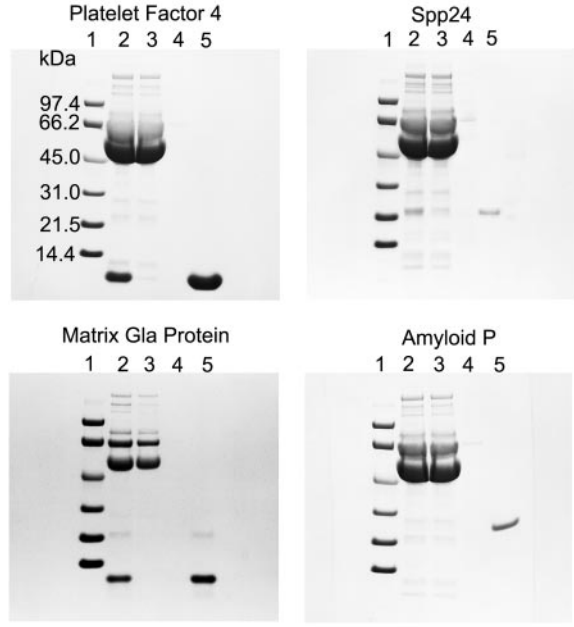


FIG. 6. **Incorporation of purified matrix Gla protein, platelet factor 4, and secreted phosphoprotein 24 into the fetuin-mineral complex.** To confirm the association of proteins with the fetuin-mineral complex, purified MGP, platelet factor 4, spp24, and serum amyloid P component were each added to serum obtained from rats 6 h after injection with 32 mg of etidronate/100 g of body weight. As a negative control, another aliquot of the test protein was placed in an aliquot of the same serum that had been adjusted to 50 mM EDTA to dissolve the fetuin-mineral complex. The serum was then centrifuged for 2 h at 16,000 × *g* to sediment the fetuin-mineral complex, and the resulting pellets were dissolved in SDS gel loading buffer, electrophoresed on 4–12% polyacrylamide gels, and stained with Coomassie. *Lanes 1*, Bio-Rad low molecular mass markers; *lanes 2*, pellet from serum containing the test protein; *lanes 3*, pellet from serum alone; *lanes 4*, pellet from serum containing the test protein and EDTA; *lanes 5*, the purified test protein alone.

pare the composition of this complex to that formed at the 32-mg dose. These measurements (Table II) showed that the mass ratio of fetuin to mineral is approximately twice as great for complex formed at the 8-mg dose than for the complex formed at the 32-mg dose. The higher mass fraction of fetuin in the complex formed at the 8-mg dose could account for the fact that a higher centrifugational force is needed for its sedimentation, because the density of protein is far lower than that of mineral. Although the reason for the difference in fetuin to mineral ratio of the complexes formed at the 8- and 32-mg etidronate doses is not presently known, one possibility is that the complex formed at the 8-mg dose is smaller and so has a higher surface to volume ratio and a greater number of surface sites for fetuin binding for a given mass of mineral.

Impact of the Generation and Clearance of the Fetuin-Mineral Complex on Total Serum Fetuin Levels—The present studies show that total serum fetuin levels are not significantly changed over the first 6 h following etidronate injection despite the fact that almost half of the circulating fetuin becomes associated with the complex by the 6-h time point (Fig. 4). The simplest explanation for this observation is that the fetuin component of the complex arises from serum fetuin. Our working hypothesis (26) is that the fetuin-mineral complex arises in the bone remodeling compartment when the acute inhibition of

bone mineralization by etidronate coupled with the ongoing bone resorption by osteoclasts causes a sharp rise in the concentrations of calcium and phosphate in the aqueous phase of the compartment. This rise leads to the spontaneous formation of calcium phosphate crystal nuclei whose growth is then arrested by formation of a complex with fetuin. This hypothesis is supported by the observations that fetuin inhibits the precipitation of calcium phosphate mineral phases from supersaturated solutions (3) and that the fetuin-mineral complex is formed in this process (29). Fetuin is, however, a serum protein made in the liver and not in bone, and the question might therefore arise as to how serum fetuin can be engaged in the formation of a complex with crystal nuclei in a bone compartment. The answer to this paradox may lie in the fact that the bone remodeling compartment is itself thought to be a vascular compartment (27, 28), with direct connections to the vascular system and with evidence of blood cells within the compartment. The continued flow of blood through the bone remodeling compartment could therefore supply the reservoir of serum fetuin for the continued formation of the fetuin-mineral complex within the compartment.

Previous studies showed that the fetuin-mineral complex is largely removed from blood during the 6–24-h interval following etidronate injection (1). The present studies confirm these observations and show for the first time that the removal of the complex from blood is associated with a reduction in total fetuin levels. Because the magnitude of this reduction is approximately the same as the amount of fetuin found in the complex at 6 h (Fig. 4), it seems likely that the reduction in serum fetuin during this interval is due to the removal of the fetuin-mineral complex in the 6–24-h period. These observations indicate the existence of a specific mechanism for the removal of the entire fetuin-mineral complex from serum. Although it seems likely that removal of a complex the size of the fetuin-mineral complex from blood involves receptor-mediated endocytosis, it is not clear what targets the complex for removal from blood, what receptor is involved, or which cells are engaged in removal.

Identification of Other Proteins Associated with the Fetuin-Mineral Complex—SDS gel electrophoresis experiments showed that the fetuin-mineral complex has a highly reproducible, simple pattern of Coomassie-stained protein bands, with bands at 10, 13, 24, 30, 59, 70, and 84 kDa. N-terminal protein sequencing revealed that the 10-kDa band is the nonglycosylated form of platelet factor 4, the 13-kDa band is a mixture of matrix Gla protein and the glycosylated form of platelet factor 4, the 24-kDa doublet band is secreted phosphoprotein 24, the 30-kDa band is serum amyloid P component, the 59-kDa band is fetuin, and the 84-kDa band is prothrombin; the 70-kDa band could not be identified. Three of these proteins, MGP, spp24, and platelet factor 4, were found in the fetuin-mineral complex at both etidronate doses. Each of these proteins clearly has a high affinity for the complex, because the addition of each protein to serum caused the protein to become associated with the fetuin-mineral complex (Fig. 6). One protein, serum amyloid P component, may not be a true component of the complex, because it was present in the complex formed at the 32-mg etidronate dose but not at the 8-mg dose and because exogenous serum amyloid P component failed to accumulate on the fetuin-mineral complex.

MGP, spp24, and platelet factor 4 are minor components of serum, and the amount of each in the fetuin-mineral complex is probably evidence of their affinity for it. Previous studies have shown that serum MGP levels in untreated control rats are ~0.5 μg/ml and rise to 20 μg/ml within 9 h of etidronate injection (1). Essentially all of the serum MGP in etidronate-treated rats is associated with the fetuin-mineral complex. SDS gel electrophoresis indicates that the levels of platelet factor 4 and spp24 in the fetuin-mineral complex are comparable with the level of MGP, which shows that the amount of platelet factor 4, MGP, and spp24 associated with the complex in the serum of etidronate-treated rats are all probably ~20 μg/ml. Because the level of platelet factor 4 in serum is also ~20 μg/ml, most of the platelet factor 4 in serum is probably associated with the fetuin-mineral complex. Although the concentration of spp24 in rat serum is not known, the level in calf serum is ~1 μg/ml. The presence of far higher levels of spp24 associated with the fetuin-mineral complex than found in calf serum indicates either that rat serum contains much higher levels of the protein than calf or that serum levels of spp24 are, like serum levels of MGP, also elevated by etidronate injection. The presence of prothrombin in the fetuin-mineral complex may be less significant, because prothrombin is a major constituent of plasma and the amount of prothrombin found in the fetuin-mineral complex consequently accounts for less than 1% of total serum levels of the protein.

Platelet factor 4 is a heparin-binding protein that is expressed by megakaryocytes and platelets and is released into serum during platelet activation. Although platelet factor 4 has been reported to have a variety of activities *in vitro*, including activity as a chemokine and as a neutrophil adhesion molecule (21), its physiological function remains obscure. It is therefore possible that platelet factor 4 could play an active role in suppressing calcification at sites of platelet aggregation where platelet factor 4 is released. However, because we could detect no platelet factor 4 associated with the fetuin-mineral complex in plasma, platelet factor 4 is unlikely to be a significant factor in the metabolism of the fetuin-mineral complex in the general circulation.

Secreted phosphoprotein-24 has several intriguing similarities to fetuin that suggest that the proteins could have similar functions. Both proteins are synthesized in the liver and accumulate in the extracellular matrix of bone (17). Both proteins have cystatin domains, one for spp24 and two for fetuin. Both proteins have a peptide segment containing several phosphoserine residues that follows the last cystatin domain, and in both proteins the serine residues that are phosphorylated match the recognition motif for the secretory pathway protein kinase (20). Finally, both proteins have an extended C-terminal sequence following the last cystatin domain and the region of serine phosphorylation, a 62-residue segment for spp24 and a 27-residue segment for fetuin. These structural similarities between fetuin and spp24 suggest that the two proteins may have complementary functions in arresting calcification in serum.

It is intriguing to note that all three proteins that are consistent constituents of the fetuin-mineral complex, fetuin, matrix Gla protein, and secreted phosphoprotein 24 contain phosphoserine residues that match the recognition motif for the secretory pathway protein kinase (17, 24, 25). As isolated from bone, MGP and spp24 are partially phosphorylated at each target serine residue, however, and fetuin is not phosphorylated. We have previously speculated that incomplete serine phosphorylation in these proteins could be evidence that changes in serine phosphorylation regulate their activity in the extracellular milieu of bone (25). Because the fetuin associated with the fetuin-mineral complex is essentially fully phosphorylated, it is tempting to speculate that the fully phosphorylated form of the protein may be the active form and that regulated dephosphorylation may occur in bone to inactivate its calcification inhibitory activity of the protein. This hypothesis suggests that matrix Gla protein and secreted phosphoprotein

24 associated with the fetuin-mineral complex should also be fully phosphorylated at each target serine residue. Studies are in progress to isolate sufficient amounts of matrix Gla protein and spp24 from the fetuin-mineral complex to determine the extent of serine phosphorylation in each protein.

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