Synthesis and degradation *in vivo* of a phosphoprotein from rat dental enamel

Identification of a phosphorylated precursor protein in the extracellular organic matrix

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(Received 12 February 1985/26 April 1985; accepted 15 May 1985)

The cellular enamel organ and the cell-free organic matrix of developing enamel of female rats injected intravascularly with [^3H]serine and [^3H]proline were extracted in a number of solvents and examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and h.p.l.c. in 6M-guanidinium chloride at intervals varying from 5 min to 1 week after injection. Three major species soluble in NH_4HCO_3 with M_r values of approx. 100 000, 25 000 and 11 000 were identified in the cellular enamel organ. The M_r 100 000 and 11 000 components were not secreted but remained intracellular for periods of up to 1 week after injection of the radioactively labelled amino acids. In contrast, the M_r 25 000 species was secreted from the cells and was first detected in the extracellular organic matrix approx. 15–30 min after injection. With time, labelled components, first of M_r approx. 11 000 and subsequently approx. 6500, were detected in the organic matrix concomitant with a relative decrease in the M_r 25 000 component, demonstrating that the lower M_r species were derived from degradation of the putative extracellular precursor protein (M_r 25 000). All of the extracellular components were found to contain *O*-phosphoserine. No radioactively labelled component with an M_r greater than approx. 25 000, either an amelogenin or an enamelin, was observed in the extracellular organic matrix or in an intracellular component which subsequently was lost from the intracellular pool. The M_r of the highest M_r protein or class of proteins is calculated to be approx. 22 000–26 000 when standard proteins are used as markers, but only 15 000–18 000 when using the CNBr peptides of α_1 chains of rat tail tendon collagen as markers.

The extracellular organic matrix of developing tooth enamel from all vertebrate species studied has been shown to consist of a large number of protein components almost all of which are soluble in neutral pH, low ionic strength buffers at 4°C (NSEP) (Glimcher *et al.*, 1964; Levine & Glimcher, 1965; Levine *et al.*, 1967b; Seyer & Glimcher, 1977).

A term suggested for the proline-rich components in such extracts is 'amelogenins', referring only to those protein components rich in proline, glutamic acid, leucine and histidine (Eastoe, 1965, 1979). Since a number of the components in the neutral soluble proteins are not rich in proline, and

indeed have very different overall amino acid compositions, the two terms NSEP (Glimcher *et al.*, 1964) and amelogenins are not synonymous. The majority of these components are characterized by their high concentrations of proline, glutamic acid, leucine and histidine (Eastoe, 1960, 1963; Glimcher *et al.*, 1961, 1964; Levine & Glimcher, 1965; Levine *et al.*, 1967b; Seyer & Glimcher, 1977; Fukae & Shimizu, 1974; Elwood & Apostolopoulos, 1975; Eggert *et al.*, 1973; Fincham *et al.*, 1983; Robinson *et al.*, 1977a; Deutsch *et al.*, 1979). By using standard globular proteins as markers, the components characterized by high concentrations of these four amino acids (amelogenins) have been shown to have M_r values not exceeding approx. 25 000–40 000 (Fincham *et al.*, 1981, 1983; Belcourt *et al.*, 1983; Termine *et al.*, 1980). From a number of compositional and M_r distribution studies as a function of age and

Abbreviations used: Ser(P), *O*-phosphoserine; Thr(P), *O*-phosphothreonine; NSEP, neutral soluble enamel proteins; SDS, sodium dodecyl sulphate.

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maturity and from the apparent sequence conservation of a number of amelogenin peptides, it has been proposed that one or more independent gene product precursors of the NSEP of the extracellular organic matrix are degraded during the progressive maturation and mineralization of the enamel matrix, giving rise to the very many lower M_r components observed by a number of chemical and physicochemical techniques (Glimcher & Levine, 1966; Glimcher *et al.*, 1964, 1977; Seyer & Glimcher, 1977; Fincham *et al.*, 1982; Robinson *et al.*, 1977, 1978, 1979).

Organ cultures or embryonic rabbit whole tooth germs and of bovine embryonic enamel organ have yielded very conflicting results as far as elucidating the number, size and amino acid compositions of the putative precursor(s). Crispens *et al.* (1979), Slavkin *et al.* (1976, 1979) and Guenther *et al.* (1977) isolated four protein components from embryonic rabbit tooth germs cultured *in vitro* and pulse-labelled with several ^3H -labelled amino acids; these had M_r values ranging from 60000–65000 to 20000. However, the amino acid compositions of all four protein components were markedly different from those of the NSEP of developing rabbit enamel and of the proline-rich amelogenins: about half of the amino acid constituents of these putative precursors consisted of threonine, glycine and alanine in approximately equal amounts. On the other hand, Sasaki & Shimokawa (1979), utilizing the enamel organ of bovine embryonic teeth, isolated a single M_r approx. 25000 precursor whose amino acid composition closely paralleled that of the lower M_r species found in the organic matrix of developing bovine enamel. Labelling *in vitro* of hamster enamel with [^{35}S]methionine has also demonstrated the major intra- and extracellular amelogenin component to be M_r approx. 25000, with no evidence for higher M_r amelogenin precursors (Zeichner-David *et al.*, 1983). The situation is somewhat complicated by the fact that components of M_r greater than 26000–30000 have been observed in very small concentrations in the soluble proteins of developing enamel ('enamelin'). Their amino acid compositions, however, are very different from those of the amelogenins (Strawich & Glimcher, 1982; Termine *et al.*, 1980).

Because of these contradictory findings, we were concerned that organ cultures of embryonic tooth germ and dissected enamel organ may not have faithfully reproduced the conditions *in vivo* of a complex tissue such as enamel, where the secretory function of the ameloblasts, which synthesize the enamel proteins, is highly dependent on the stage of extracellular and cellular development and maturation (Reith, 1961, 1970; Warshawsky & Smith, 1974). We therefore decided to investigate

the nature of the protein precursor(s) of enamel matrix *in vivo*, taking advantage of the fact that the erupted incisor teeth of rats grown continuously and have at their anatomical base functional secretory ameloblasts which are continuously and actively synthesizing new developing enamel matrix proteins (Glimcher *et al.*, 1977; Robinson *et al.*, 1979). Preliminary results have been reported (Strawich & Glimcher, 1982).

Experimental procedures

Animals, radioactive labelling of the enamel proteins, and tissue preparation

Groups of 12 Sprague–Dawley rats weighing approx. 150g each were injected intra-arterially via the common carotid arteries or intravenously into the jugular veins with both 0.8mCi of L-[^3H (G)]serine and 0.6mCi of L-[2,3- ^3H]proline. In each of several experiments, 12 animals were killed at each of the following time periods after injection: 5, 15, 30, 60 and 120 min and 8, 24, 48, 72 and 168 h. Immediately after killing, both the upper and lower incisor teeth were removed and rinsed with normal saline solution containing the following proteinase inhibitors: 1 mM-phenylmethanesulphonyl fluoride, 10 mM-N-ethylmaleimide, 1 mM-benzamidinium hydrochloride, 0.1 mM-DL-dithiothreitol and 1 mM-levamisole (Sigma).

Extraction of the cellular proteins of the enamel organ

The enamel organ was carefully separated from the buccal surfaces of the lower, developing portion of the incisor teeth. In the initial experiments, the tissue was placed immediately into a solution of 0.05M- NH_4HCO_3 , pH 7.4, at 2°C containing the proteinase inhibitors and 0.2% Triton X-100 and homogenized. The homogenized suspension was then sonicated at 30 pulses for intervals of 20 s to prevent foaming. The suspension was then extracted at 2°C for 48–72 h in 0.05M- NH_4HCO_3 , pH 7.5, and the supernatant separated by centrifugation at 30000g for 60 min. Triton was removed on a column (1 cm $\times$ 8 cm) of SM-2 Bio Beads (Bio-Rad) equilibrated with 0.05M- NH_4HCO_4 at 4°C and the eluate was freeze-dried. The column was regenerated with methanol. In later experiments, the tissue was either first extracted in 0.3M-EDTA, pH 7.5 at 2°C, or sequentially extracted in EDTA, NH_4HCO_3 , occasionally 0.3M-acetic acid and finally in 6M-guanidinium chloride, pH 7.5 (see below).

Extraction of the extracellular proteins of the enamel matrix

After removal of the enamel organ, the calcified, developing, cheesy enamel was carefully scraped off the distal portion of the buccal surface of the

incisor teeth. Light-microscopic examination of this region after removal of the enamel organ showed no evidence of a cellular layer remaining on the surface of the tooth. Similarly, examination of the tooth after the enamel had been scraped off showed a small quantity of residual enamel matrix remaining, establishing that significant amounts of enamel organ and dentin had not been included in the extracellular enamel matrix sample.

In the initial experiments, the undecalcified, developing enamel was immediately placed in a solution of 0.3M-EDTA, pH 7.5 at 2°C, containing the proteinase inhibitors and extracted for 48–120h. After dialysis against water containing the proteinase inhibitors, the EDTA-soluble, non-diffusible (approx. 1200 M_r cut-off) proteins were freeze-dried.

The EDTA-insoluble residue obtained by centrifugation of the EDTA-extracted enamel was further extracted for 2–3 days in a solution of 0.05M-NH₄HCO₃, pH 7.4 at 4°C, containing the proteinase inhibitors, and the supernatants and residues obtained after centrifugation were freeze-dried. The proteins which are soluble in 0.05M-NH₄HCO₃ (NSEP) accounted for more than 95% of the total protein content of the developing enamel.

In a separate group of experiments, both the enamel organ and the calcified enamel matrix were each independently and sequentially extracted at 2°C, first in 0.3M-EDTA, pH 7.5, for 3–5 days (EDTA-soluble proteins), then in 0.05M-NH₄HCO₃, pH 7.5, for 2–4 days (NSEP), occasionally in 0.3M-acetic acid for 2 days [acid-soluble proteins (Glimcher *et al.*, 1964)], and finally in 6M-guanidinium chloride, pH 7.5, for 3–5 days. All solutions contained the proteinase inhibitors.

The cellular proteins of the enamel organ and the extracellular proteins of the enamel matrix from the incisors of over 300 rats which had not received radioactively labelled amino acids were prepared by the techniques described above for the animals labelled with [³H]serine and [³H]proline.

Analytical techniques

SDS/polyacrylamide-gel electrophoresis. The radioactively labelled proteins from both the cellular enamel organ and the extracellular enamel matrix were subjected to SDS gel electrophoresis in 4M-urea, acrylamide concentration 7.5%, and stained with Coomassie Blue. Other gels containing the radioactively labelled fractions were sliced into 1 mm sections and digested with 0.2 ml of 60% HClO₄ and 0.1 ml of 30% H₂O₂ in tightly capped vials at 60°C with agitation. The incubation was carried out for 5 h. The vials were cooled and 10 ml of Aquasol-2 was added to each sample. The samples were dispersed in 10 ml of Aquasol-2 and

then counted in a Beckman model LS8000 scintillation counter. ¹⁴C-labelled standards [cytochrome *c* (M_r 12300), carbonic anhydrase (M_r 30000), ovalbumin (M_r 46000), albumin (M_r 69000) and phosphorylase *b* (M_r 92500)] were used to calculate the approximate M_r values of the proteins extracted from the enamel organ and from the enamel matrix, respectively. In addition to the standard M_r markers, CNBr digests of the α_1 chain of rat tail tendon and skin collagens were prepared and the individual CNBr peptides isolated by ion-exchange chromatography (Piez *et al.*, 1963; Bornstein & Piez, 1965; Bornstein, 1969). Specifically, the CNBr peptides used as standards to calibrate the M_r of the extracellular putative precursor enamel-matrix protein were α_1 CB₈ (M_r 24000), α_1 CB₇ (M_r 23080), α_1 CB₆ (M_r 18100), and α_1 CB₃ (M_r 13980).

Gel filtration. Molecular sieve chromatography was carried out on a column (1.6 cm × 100 cm) of Sephadex G-100 equilibrated with 0.05M-NH₄HCO₃/6M-urea, pH 8.2. The column was operated at 4°C at a flow rate of 14 ml/h.

Ion-exchange chromatography. The major fraction (fraction II) of the NSEP from unlabelled animals eluted by G-100 molecular sieving was chromatographed on a column (1.5 cm × 10 cm) of Cellex T equilibrated with 0.01M-glycine/NaOH buffer, pH 9.5, in 6M-urea. The column was eluted with a 0–0.5M-NaCl gradient in a total volume of 500 ml at a flow rate of 60 ml/h at 4°C. The major fraction was rechromatographed twice and, in some instances, three times.

H.p.l.c. Samples obtained by Cellex T ion exchange chromatography were further purified by h.p.l.c. molecular sieving carried out using two TSK 3000 columns (0.75 cm × 30 cm) (Beckman HPLC model 334) equilibrated with 20 mM-Mes/6M-guanidinium chloride buffer, pH 6.5, containing 5 mM-dithiothreitol. The column was operated at a flow rate of 1 ml/min.

Amino acid analyses. Samples of the proteins were hydrolysed in 6M-HCl in sealed tubes evacuated under N₂ for 24 h at 110°C and analysed for complete amino acid composition on a Beckman 121-M automatic amino acid analyser. Other samples were hydrolysed in 4M-HCl at 105°C for 6 h and the contents of Ser(P) and Thr(P) determined on a Beckman 121-M analyser as previously described (Cohen-Solal *et al.*, 1978, 1979).

Identification of [³H]Ser(P). Samples of NSEP from radioactively labelled animals killed 30–60 min after injection and shown by SDS/polyacrylamide-gel electrophoresis to consist of a single component of M_r approx. 25000, and samples obtained from animals killed up to 1 week and longer after radioactive labelling and found to contain principally the M_r 11000 and 6500

components, were hydrolysed in 4M-HCl at 110°C for 6h, and *O*-phospho[³H]serine was isolated by preparative amino acid analysis (Cohen-Solal *et al.*, 1978, 1979).

Results

Isolation and purification of unlabelled developing rat enamel proteins

Four major fractions were obtained from the unlabelled NSEP by molecular sieve chromatography on G-100 Sephadex in 6M-urea or 6M-guanidinium chloride. The first, a very minor fraction, consisted principally of the low-proline-containing enamelines (Termine *et al.*, 1980; Strawich & Glimcher, 1982). Fraction II, the major component, had the composition of a typical proline-rich amelogenin. When fraction II was chromatographed on a column of Cellex T resin, three (rarely four) major peaks were eluted (Strawich & Glimcher, 1982). SDS gel electrophoresis of the Cellex T peaks revealed that each of the three or four peaks contained one major band (M_r approx. 25000). The Cellex T fraction (the second, or the third when there were four) was also the richest in the highest M_r (approx. 25000). The major Cellex T fraction was rechromatographed on Cellex T at least twice more. In most instances, the rechromatographed fraction peak contained only the M_r 25000 major peak by SDS gel electrophoresis. However, when a large amount of protein was applied to the gel, a faint additional higher M_r band (M_r 70000–75000) was also observed. The M_r 70000–75000 component was essentially eliminated by repeated h.p.l.c. sieving on three TSK 3000 columns (each 0.75 cm × 30 cm) run in series in 6M-guanidinium chloride. Similar behaviour has also been found in the case of embryonic bovine enamel, in which instance preparative gel electrophoresis has shown the M_r 70000–75000 component to be principally an aggregate of the M_r 25000 component and other nonamelogenin proteins (E. Strawich & M. J. Glimcher, unpublished work). Strong association of the protein components of developing enamel in dissociative salts has been reported previously (Mechanic, 1971).

The apparent M_r of the major component calculated from SDS gel electrophoresis and from h.p.l.c. molecular sieving in 6M-guanidinium chloride is approx. 22000–26000. However, when the M_r is calculated using the CNBr peptides of the α_1 chain rat tail tendon or skin collagen as standard markers, its apparent M_r is calculated to be approx. 15000–18000. Comparable results have been obtained on SDS gel electrophoresis using the CNBr peptides of collagen for similar enamel fractions from developing bovine enamel prepared

Table 1. *Major amino acid constituents of the M_r approx. 25000 component of developing rat incisor enamel*
Values represent a typical example of a single experiment; those for Ser(P) show recovery and are not adjusted for losses due to acid hydrolysis.

Amino acid	Residues/1000 total residues
Glu	178
Pro	277
Leu	95
His	70
Ser(P)	4–5

by Cellex T ion exchange chromatography and by h.p.l.c. molecular sieving (Strawich & Glimcher, 1985) and for the single protein component synthesized by calf enamel ameloblasts *in vitro* (Sasaki & Shimokawa, 1979). In the case of bovine enamel (Strawich & Glimcher, 1985), the M_r calculated from sedimentation-equilibrium ultracentrifugation is also approx. 17000–18000. However, in order to relate the data obtained here with the data previously reported in the literature (Termine *et al.*, 1980; Fincham *et al.*, 1983), the major amelogenin component will be referred to as the M_r 25000 component.

The major amino acid composition of a twice-rechromatographed Cellex T component further purified by h.p.l.c. filtration, and consisting essentially wholly of the M_r 25000 component, is shown in Table 1. Ser(P) (approx. 4–5 residues/1000 total residues) was identified in this and other samples containing only the M_r 25000 component. Reduction and alkylation of the extracts obtained from the enamel organ did not alter their SDS gel electrophoresis and h.p.l.c. molecular sieve properties.

Identification of ³H-labelled proteins of rat enamel

In the first group of experiments both the homogenized and sonicated cellular enamel organ containing the ameloblast layer, and the decalcified organic matrix of enamel, were separately extracted in 0.05M-NH₄HCO₃, pH 7.5, and the extracts subjected to SDS/polyacrylamide-gel electrophoresis containing 4M-urea. Fig. 1 depicts typical examples of the distributions of the ³H-labelled components. The results quite dramatically show that, of the three major ³H-labelled protein components in the cellular fraction (Figs. 1a₁, 1b₁), only the M_r 25000 component is secreted into the extracellular organic matrix (Figs. 1b₂, 1c₂, 1d₁, 1d₂). In the experiment shown, an M_r 25000 component is detected in the extracellular matrix by 30min (Fig. 1b₂) and by 2h the M_r 25000 component originally present in the enamel organ is absent from this cellular layer (Fig. 1d₁). The progressive and eventually complete disappear-

Fig. 1. Incorporation of [³H]serine and [³H]proline into NH₄HCO₃-soluble proteins of cellular enamel organ and organic matrix of rat incisor teeth

Time refers to period after intravascular injection. Animals weighing approx. 150g were injected intra-arterially via the common carotid arteries or jugular veins with both 0.8mCi of L-[³H]serine and 0.6mCi of L-[2,3-³H]proline. Immediately after sacrifice and after a rinse with saline and proteinase and phosphatase inhibitors, the dissected enamel organ and immature developing enamel were extracted at 2°C in 0.05M-NH₄HCO₃ at pH 7.5 (enamel organ) and in 0.3M-EDTA, pH 7.5, first and then subsequently in 0.05M-NH₄HCO₃ (enamel matrix). The data shown are the proteins soluble in NH₄HCO₃. Similar results were obtained when the enamel organ was first extracted in 0.3M-EDTA, pH 7.5, at 2°C, before extraction in 0.5M-NH₄HCO₃, pH 7.5, at 2°C.

ance of the M_r 25000 component from the cell layer and the corresponding appearance and progressive increase in concentration of an M_r 25000 component in the extracellular matrix, are consistent with the conclusion that they represent the same component.

The ^3H -labelled component of M_r 25000 migrates identically on SDS gel electrophoresis with unlabelled rat and bovine amelogenin precursor, components of M_r approx. 25000, and is co-eluted with these latter components on h.p.l.c. sieving. No radioactivity was ever detected in an NH_4HCO_3 -soluble component of the organic matrix with M_r higher than 25000 using SDS gel electrophoresis on h.p.l.c. sieving at any time period after injection of the radioactive-labelled amino acids.

Within approx. 8 h in the experiment shown (Fig. 1e₂) (sometimes as short as 2 h in other experiments), there is evidence that degradation of the M_r 25000 component begins and that the degradation increases with time, as shown by the increase in the proportions of an M_r approx. 11000 and later an M_r approx. 6500 fraction relative to the M_r 25000 component (Fig. 1f₂, 1g₂, 1h₂).

[^3H]Ser(P) was identified in 0.05M- NH_4HCO_3 extracts containing only the single M_r 25000 component, and after much longer periods of time (> 1 week) in samples which contained only the M_r 6500 component, and the M_r 11000 and 6500 components.

To examine further the possibility that there exists a higher M_r extracellular precursor component of the amelogenins (high proline-containing components), and/or a second independent gene product for the enamelines (high M_r , low-proline containing components), both the enamel organ and the enamel matrix were first separately and

exhaustively extracted in 0.5M-EDTA, pH 8.0, a procedure which we have found selectively solubilizes the higher M_r protein components from enamel (E. Strawich & M. J. Glimcher, unpublished work). After this extensive extraction in EDTA, both the enamel organ and the enamel matrix were then sequentially extracted in 0.05M- NH_4HCO_3 , 0.3% acetic acid, and finally in 6M-guanidinium chloride. The four methods of extraction remove about 98–99% of the total protein present in the enamel. It was later found that omitting the acetic acid step and extracting the NH_4HCO_3 -insoluble proteins directly in guanidinium chloride produced the same results.

The results of these experiments, as analysed by SDS gel electrophoresis and, in some instances, by h.p.l.c. molecular sieving, were similar to those already described. No evidence was obtained from analyses of the extracts obtained by any of the solvents for the presence of an extracellular protein which was secreted or an extracellular protein which had an M_r greater than 25000. SDS/polyacrylamide-gel electrophoresis of the EDTA extracts of the enamel organ revealed that at 5 min in almost all instances, only two components (M_r approx. 100000 and 11000) were present (Fig. 2a). Much less frequently, a small peak was also detected in an M_r 25000 fraction (Fig. 2b). The EDTA extractions of the enamel organ for longer periods of time, up to 8–24 h after injection of the radiolabelled amino acids, also consisted of two major components of M_r approx. 100000 and 11000 similar to those depicted in Fig. 2(a). A typical example after 8 h is depicted in Fig. 2(c). In those few instances in which a small radioactive peak corresponding to an M_r 25000 component was observed in the 5–30 min extracts with EDTA, this component was found to be absent from later

Fig. 2. Incorporation of [^3H]serine and [^3H]proline into EDTA-soluble proteins of enamel organ of rat incisor teeth (a) and (b) are separate experiments. (c) is typical of all experiments. Time refers to period after intravascular injection. Experimental procedures are identical with those described in Fig. 1. Enamel organ was extracted with 0.3M-EDTA, pH 7.4, at 2°C. Proteins are those soluble in EDTA.

extracts. Extraction of the enamel organ with 0.05M-NH₄HCO₃ after solubilization of the EDTA-soluble proteins revealed an M_r distribution pattern similar to that seen after NH₄HCO₃ alone: the consistent emergence as early as 5 min after injection of the radiolabelled amino acids of an M_r 25000 component in addition to the M_r approx. 100000 and 11000 components (Fig. 3a). Within 2 h, the M_r 25000 component could no longer be detected in the enamel organ (Fig. 3b). Further extraction of the enamel organ with 6M-guanidinium chloride at all time intervals (5 min–24 h), showed either a single M_r approx. 100000 component (Fig. 4) or a principal component of M_r approx. 100000 and a very minor component of M_r 95000 with no evidence of the M_r 25000 component. This indicates that essentially all of this latter species is solubilized by sequential extraction in EDTA and NH₄HCO₃ (a small variable amount in EDTA and the vast majority in NH₄HCO₃).

Radioactivity was first detected in the organic matrix of the enamel in all three solvents at approx. 12–30 min. In the EDTA experiments, in almost all instances, the only labelled band in the EDTA extract corresponded to a component of M_r approx. 11000 (Fig. 5). This was true in specimens examined from 12–15 min after injection to 24 h and longer after injection of the radiolabel. In a very few instances, a little radioactivity was detected in a position corresponding to a component of M_r approx. 25000. No radioactivity was

ever detected in a component of M_r greater than 25000, despite the fact that identical EDTA extracts (and NH₄HCO₃ extracts as well) both showed Coomassie-Blue stained protein bands with M_r values much greater than 25000, when similarly run on SDS gel electrophoresis. As with similar experiments on developing bovine enamel, amino acid analyses revealed that these high- M_r components were not amelogenins but enamelinins. NH₄HCO₃ extracts of the organic matrix after first extracting them in EDTA showed a distribution of radioactive bands similar to that shown in Figs. 1(b₂)–1(h₂): a single M_r 25000 band starting at 12–30 min after injection of the radiolabel which remained as a single peak for 2 h and often for 8 h or longer. After this, one notes the appearance of an M_r 11000 component and later still an M_r 6500 component similar to that depicted in Figs. 1(e₂)–1(h₂). Further extensive extraction of the organic matrix with guanidinium chloride 12–30 min after the injection of the radiolabel solubilized a small amount of the M_r 25000 component (Fig. 6a). At 2–8 h after the injection of the radiolabel, an additional M_r 11000 component was also observed (Fig. 6b). As was true of the EDTA and NH₄HCO₃ extracts, no component of M_r greater than 25000 was ever observed in the guanidinium chloride extracts at any time period after injection of the radiolabelled amino acids.

At 5 min and less after the injection of the radioactively labelled amino acids, essentially all

Fig. 3. Incorporation of [³H]serine and [³H]proline into NH₄HCO₃-soluble proteins of enamel organ of rat incisor teeth after first extracting the EDTA-soluble proteins

(a) Experiment depicted is 5 min after intravascular injection of radiolabelled amino acids; (b) 2 h after intravascular injection. Experimental procedures identical with those described in Fig. 1. Enamel organ was first extracted in 0.3M-EDTA, pH 7.5, 2°C and then in 0.05M-NH₄HCO₃, pH 7.5, 2°C. The proteins are those solubilized in the NH₄HCO₃ extractant.

Fig. 4. Incorporation of [^3H]serine and [^3H]proline into guanidinium chloride-soluble proteins of enamel organ of rat incisor teeth after first extracting tissue with EDTA and NH_4HCO_3 .

Experiment shown is 5 min after intravascular injection of radiolabelled amino acids. Experimental procedures are identical with those described in Fig. 1. The enamel organ was first extracted in 0.3M-EDTA, pH 7.5, 2°C , then 0.05M- NH_4HCO_3 , pH 7.5, 2°C , and finally with 6M-guanidinium chloride, pH 7.5, 2°C . The proteins shown are those solubilized in the 6M-guanidinium chloride.

of the radioactivity was recovered in the enamel organ. After 8h, approx. 35% of the radioactivity was recovered in the enamel organ and 65% in the enamel matrix. Approx. 98% or greater of the total radioactivity in both the enamel organ and in the enamel matrix was solubilized by the EDTA, NH_4HCO_3 , and guanidinium chloride solutions.

Discussion

Several previously unanswered questions about the proteins of developing enamel have been settled by the present experiments *in vivo* and additional light was shed on the biochemistry of this interesting tissue and the unique group of proteins which make up its extracellular structural framework.

The data *in vivo* obtained in the present study clearly demonstrate that no protein component (amelogenin or enamelin) of $M_r > 25000$ is detected in extracellular rat enamel matrix newly synthesized *in vivo*, or in an intracellular compo-

Fig. 5. Incorporation of [^3H]serine and [^3H]proline into EDTA-soluble proteins of organic matrix of rat incisor teeth 15 min after intravascular injection of radiolabelled amino acids.

Experimental procedures are identical with those described in Fig. 1. The immature enamel was first successively extracted in 0.3M-EDTA. The proteins soluble in EDTA are depicted in the Figure.

nent which subsequently is secreted and/or disappears from its intracellular site, a finding consistent with organ culture studies *in vitro* using bovine embryonic tooth germs (Sasaki & Shimokawa, 1979). The synthesis *in vivo* data reported here are also consistent with the results reported earlier that the highest M_r species of amelogenin present in developing enamel is a component of M_r approx. 25000–30000 as determined by gel filtration and SDS gel electrophoresis (Fincham *et al.*, 1983; Termine *et al.*, 1980). No evidence for an M_r 40000 component (Fincham *et al.*, 1981) was obtained. The conclusions of Guenther *et al.* (1977), Crispens *et al.* (1979), and Slavkin *et al.* (1976, 1979) that the extracellular enamel protein precursor of the major group of proteins (amelogenins) in the enamel matrix is an M_r 65000 protein which is successively degraded to 58000, 22000 and 20000 components as mineralization and maturation progresses are not supported by the present data. This may be due to the fact that their studies were done on rabbit enamel, which recent studies have suggested is composed principally of enamelines rather than amelogenins (Zeichner-David *et al.*, 1984). This is also consistent with the finding that the amino acid compositions of all of the fractions isolated by these latter authors are entirely different from those of the amelogenin components of bovine, rat and other species (Levine & Glimcher, 1965; Fincham *et al.*, 1983) and similar in some respects to the enamelines (Termine *et al.*, 1980). Although we conclude that it is unlikely that any of

Fig. 6. Incorporation of [^3H]serine and [^3H]proline into guanidinium chloride-soluble proteins of organic matrix of rat incisor teeth (a) 15 min and (b) 8 h after intravascular injection of radiolabelled amino acids

Experimental procedures are identical with those described in Fig. 1. The immature enamel was first successively extracted in 0.3M-EDTA, pH 7.5, 2°C, 0.05M- NH_4HCO_3 , pH 7.5, 2°C, before extraction in 6M-guanidinium chloride, pH 7.5, 2°C. The proteins depicted are those solubilized in the 6M-guanidinium chloride. Similar results were obtained when the immature enamel was extracted in 0.05M-acetic acid after the NH_4HCO_3 extraction and before the guanidinium chloride extraction.

the components isolated by Crispens *et al.* (1979), Guenther *et al.* (1977), and Slavkin *et al.* (1976, 1979) represent precursors of the major proteins or peptides (amelogenins) found in developing enamel of rats or any of the other dozen or more species studied (Levine & Glimcher, 1967b), it is possible that the components isolated by these investigators represent precursors of the higher M_r , low proline-containing enamelines.

There are several possible explanations for our inability to detect radiolabelled high- M_r enamelines in the present experiments. Clearly the first must be that the enamelines were present in such small amounts that they were not adequately labelled by the two radioactive amino acids used. Although this explanation is plausible, the fact that both serine and glycine would be labelled by the injection of [^3H]serine, both of which are very prominent amino acid constituents of the enamelines, and the fact that labelling was also unsuccessful when five times the dose of radioactive amino acids were injected, also casts some doubt on the likelihood of this explanation. On the other hand, studies *in vitro* of the synthesis of enamel proteins in hamsters labelled with [^{35}S]methionine have shown radioactive protein bands corresponding principally to the amelogenins but also to a much lesser extent to the enamelines (Zeichner-David *et al.*, 1983).

Another possibility is that the enamelines are derived from the plasma membrane, Tomes processes and possibly intracellular organelles of

the ameloblasts and other enamel organ cells, since it has been well documented that the ameloblasts undergo very marked structural changes as a function of enamel development (Reith, 1961, 1970; Warshawsky & Smith, 1974). Under these circumstances the enamelines, although present in the enamel matrix, would not technically be enamel proteins in the sense that enamel proteins are considered to be synthesized and normally transported from the ameloblasts to the extracellular matrix. The increase in the relative proportions and concentrations of the enamelines with respect to the amelogenins, at the earliest stage of amelogenesis (Robinson *et al.*, 1979) and again after maturation of the enamel, and the degradation and probable release of plasma membrane and intracellular proteins with ameloblast maturation and tissue mineralization, would also support this view.

Similarly, since proline comprises approx. one-third of the amino acids present in the amelogenin precursor and since [^3H]proline was used as the radioactive marker, it seems unlikely that failure to label amelogenins of $M_r > 25000$ can be attributed to the fact that there was insufficient radioactivity available to incorporate into precursor(s) of $M_r > 25000$. On the other hand, since 98% or more of the total tissue radioactivity was recovered in the sequential extractions with EDTA, NH_4HCO_3 and guanidinium chloride, any high- M_r amelogenin or enamelin extracellular precursor would necessarily have to be present in only very small

amounts and might therefore have not incorporated sufficient radioactivity for such a species to be detected. In addition, the chances of observing such a precursor would be made even less likely if its rate of synthesis and degradation were rapid. Clearly, further studies of intracellular synthesis and processing and extracellular secretion (Zimmerman *et al.*, 1980) of the enamel proteins, by isolated ameloblasts in different stages of development, will be necessary to elucidate conclusively the synthetic and degradative pathways of the enamel proteins.

Loss of protein from enamel and enamel protein degradation

Early analyses of developing and adult mature enamel demonstrated a considerable loss (>95%) of protein as a function of age and maturation (Deakins, 1942; Stack, 1960). This has been confirmed in a number of more recent studies in a variety of species by different workers (Glimcher & Levine, 1966; Glimcher *et al.*, 1964, 1977; Weidman & Eyre, 1971). In the case of bovine and human enamel, not only was it shown that >99% of the initial protein content by weight of developing enamel is lost during maturation to adult enamel, but also that the vast majority of the remaining protein components consist of very low M_r (500 or less) peptides (Glimcher & Levine, 1966; Glimcher *et al.*, 1964, 1977; Weidman & Eyre, 1971), indicating marked degradation of enamel proteins with maturation. Further examination of the protein components of embryonic enamel obtained either from different aged animals or from different portions of the same tooth representing various stages of aging and maturation (Robinson *et al.*, 1977, 1978; Glimcher *et al.*, 1977; Fincham *et al.*, 1982) all showed a consistent pattern: a decrease in proline concentration, a decrease in the relative proportion of the larger (M_r 25000) components and an increase in the proportion of the smaller (M_r 6000–10000) components.

It has been assumed from these results, especially the increasing proportion of lower M_r components relative to the M_r 25000 components (Fincham *et al.*, 1982), as well as more recent amino acid sequence studies (Fincham *et al.*, 1983), that the lower M_r fractions are derived from the M_r 25000 species. However, it is not possible to reach such a conclusion from these types of data. To do so requires that only one M_r 25000 precursor component be present at some stage of enamel development, and that subsequently lower M_r species be observed. To date, at no stage of enamel development has only a single precursor species ever been observed; even in the youngest enamel studied, multiple components have been present. In the present study, radiolabelling of the proteins

synthesized by the ameloblasts *in vivo* has allowed us to observe a stage of enamel matrix development where the structural enamel proteins consist of only a single M_r 25000 component; moreover, we have been able to follow the status of this component with time. The steady progression of this single precursor component to two lower- M_r fractions and the progressive increase in the proportion of the lower M_r components relative to the higher M_r putative precursor with time, establishes for the first time that the amelogenins with M_r lower than 25000 are indeed degradative products of the M_r 25000 precursor. In addition, we can conclude that the loss of protein from enamel during maturation and mineralization is indeed preceded and accompanied by a progressive degradation of an original protein precursor. This would explain the very large number of components with very similar amino acid compositions (high proline, glutamic acid, histidine, leucine, i.e., amelogenins) observed by molecular sieving, ion-exchange chromatography and amino acid sequencing (Fincham *et al.*, 1981; Mechanic *et al.*, 1967; Papas *et al.*, 1977; Seyer & Glimcher, 1977).

The identification of Ser(P) and [3 H]Ser(P) in the M_r 25000 component and in the M_r 11000 and 6500 fractions establishes that the M_r 25000 precursor component is a phosphoprotein and is consistent with the conclusion that the lower M_r phosphoprotein components identified earlier (Levine *et al.*, 1967a; Seyer & Glimcher, 1971) are also derived from the degradation of the M_r 25000 species.

The question of the true M_r of the precursor amelogenin protein which we have referred to as the M_r 25000 [or 26000–40000 (Termine *et al.*, 1980; Fincham *et al.*, 1983)] component still needs to be resolved. We have used the term ' M_r 25000' since this is the term currently employed in the literature (Fincham *et al.*, 1981, 1983; Termine *et al.*, 1980). However, equilibrium sedimentation experiments in 6M-guanidinium chloride (Strawich & Glimcher, 1985) of the single precursor amelogenin protein precursor as well as recent amino acid sequence studies (Takagi *et al.*, 1984) agree with the results reported here using the collagen CNBr peptides of the α_1 chains of rat tail tendon collagen as markers, namely, that the true M_r of the amelogenin precursor is approx. 16000–18000. This is consistent with the earlier studies by Eggert *et al.* (1973) and Seyer & Glimcher (1977).

We thank Dr. Barry Reit for his considerable help with the animal experiments, Dr. Diane Brickley-Parsons for carrying out the [3 H]Ser(P) determinations, and Ms. Thea Pickering and Ms. Linda Radonsky for their technical assistance. This work was supported in

part by grants from the National Institutes of Health (AM34078-03) and from the New England Peabody Home for Crippled Children, Inc.

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