Further Studies on the Structural Differences between the Isoenzymes of Human Parotid α-Amylase*

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ABSTRACT: Crystalline preparations of human parotid amylase were separated by recycling chromatography into two families of isoenzymes: family A, comprising isoenzymes 5, 3, and 1 and family B, comprising isoenzymes 4 and 2. The isoenzymes of family A are glycoenzymes whereas those of family B are not. The carbohydrate moiety of family A contains 6 moles of glucosamine, 3 moles of fucose, 2 moles of mannose, and 2 moles of galactose per mole of glycoenzyme. No sialic acid was detected. The neutral sugars were qualitatively and quantitatively the same in amylases isolated from three individuals. The isoenzymes of both families appear to be single polypeptide chains with molecular weights of 62,000 for the glycosidated proteins of family A and 56,000 for the nonglycosidated protein of family B. No amino-terminal group was detected by the phenylthiohydantoin method nor by dinitrophenylation. No functional role for the glyco moiety has been observed. The two families of isoenzymes have the same specific enzymatic activity, exhibit the same action patterns toward the high molecular weight substrate, Superlose, and the same sensitivities to heat at 45 and 55°. The isoenzymes of each family can be transformed into more anionic forms by incubation at pH 9 in a reaction that appears to involve deamidation. Polyacrylamide disc gel patterns of peptides prepared by cyanogen bromide treatment of families A and B contain a number of common peptides but also contain peptides specific to each family. The peptides specific to the A family appear to be glycopeptides.

The existence of isoenzymes of human salivary amylase was first reported by Muus and Vnenchak (1964). Although these earlier studies were made with crystalline amylase prepared from pooled samples of whole saliva, thus allowing possible organ differences as well as individual variations, subsequent investigations have shown that amylase isoenzymes are present in saliva collected directly from the separate salivary glands of individual donors (Lamberts and Meyer, 1967; Wolf and Taylor, 1967; Steiner and Keller, 1968; Lamberts *et al.*, 1971).

Fresh samples of human parotid saliva collected from single subjects usually contain amylase in five molecular forms, designated isoenzymes 1–5 according to their electrophoretic mobilities, with the number one being assigned to the most rapidly migrating form in an anionic system (Steiner and Keller, 1968). Several additional forms, migrating more anodically than isoenzyme 1, are sometimes present in trace amounts and these have been designated Z bands (Zager, 1969; Kauffman *et al.*, 1970).

Crystalline preparations of human parotid amylase contain all of the isoenzymes found in parotid saliva. Such preparations can be separated on columns of Bio-Gel into two families: family A, containing isoenzymes 5, 3, and 1 and family B containing isoenzymes 4 and 2. We reported earlier (Kauffman *et al.*, 1970) that the isoenzymes of family A contain covalently linked carbohydrate whereas those of family B do not. In the present report we will present further structural and functional studies on the glycosidated and nonglycosidated forms of human parotid amylase.

Materials

Ovalbumin, bovine serum albumin, sodium dodecyl sulfate, $NADP^+$, DTNB,[1] and Triton X-100 were obtained from Sigma Chemical Co. Phenyl isothiocyanate was obtained from Mann Research Laboratories and was redistilled before use. Trifluoroacetic acid was purchased from J. T. Baker Laboratory Chemicals and Products and fluorodinitrobenzene from Eastman Organic Chemicals. Dithiothreitol and Hepes were obtained from Calbiochem Corp., catalase from Worthington Biochemical Corp. as well as D-galactose, D-mannose and L-fucose were purchased from Pierce Chemical Co.; [^{14}C]taurine and PPO from New England Nuclear; Noredux starch from Ganes Chemical Works, Inc., New York. Superlose was obtained from Stein-Hall and Co., New York, and further purified by the procedure of Robyt and French (1967). ATP, phosphoenolpyruvate, hexokinase, pyruvate kinase, lactic dehydrogenase, and D-galactose dehydrogenase were the products of C. F. Boehringer und Soehne GmbH, Mannheim, Germany. L-Fucose dehydrogenase was the gift of H. Schachter (University of Toronto). The ion-exchange resins AG 1-X2 and AG 50W-X8 (purified from the standard Dowex 1 and 50 resins) were obtained from Bio-Rad and equilibrated according to their specifications.

Methods

Preparation of Enzymes. Crystalline amylase was prepared from pooled or separate samples of human parotid saliva as previously described (Kauffman *et al.*, 1970). The crystalline enzyme was separated into two families, designated families

* From the Department of Oral Biology, University of Washington, Seattle, Washington 98105. *Received August 18, 1971.* This work has been supported by Public Health Grants DE-02600 to the Center for Research in Oral Biology and DE-02918 to the Regional Primate Center, both at the University of Washington, from the National Institute of Dental Research.

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[1] Abbreviations used are: PITC, phenyl isothiocyanate; FDNB, 1-fluoro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PPO, 2,5-diphenyloxazole; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDC, 1-ethyl-3(3-dimethylaminopropylcarbodiimide-HCl); CNBr, cyanogen bromide; PTH, phenylthiohydrantoin.

A and B, respectively, by recycling chromatography on two Bio-Gel P-100 columns (2.5 × 82 cm) eluted in an ascending manner with 0.05 M potassium phosphate buffer (pH 6.8). A crystalline suspension containing 25 mg of amylase was diluted with sufficient phosphate buffer to dissolve the crystals. This solution of amylase in a final volume of 12–15 ml was applied to the Bio-Gel column and eluted at a flow rate of 12 ml/hr maintained with an LKB peristaltic pump. Fractions (3.2 ml) were collected. For some experiments the purified enzymes were dialyzed against distilled water and sterilized by passage through a Millipore filter (45 μ).

Polyacrylamide Disc Gel Electrophoresis and Densitometry. Anionic polyacrylamide disc gel electrophoresis and densitometric tracings of the gel patterns were performed as previously described (Kauffman *et al.*, 1970). Starch-slide zymograms were done as described by Allan *et al.* (1970).

Molecular Weight Determinations. Polyacrylamide disc gel electrophoresis was performed in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn (1969), using half the amount of cross-linker and 0.05 M sodium phosphate (pH 7.1)–0.1% sodium dodecyl sulfate for the electrophoresis buffer. The protein samples were incubated at 37° for 2 hr in 0.01 M sodium phosphate (pH 7), 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol. In one case, EDTA was added to the incubation mixture to 0.01 M, and the gels also contained 10 mM EDTA. After incubation approximately 0.01 mg of protein was applied to the gel. Electrophoresis was performed at a constant current of 8 mA/gel for 3.25 hr, until the marker dye had migrated to within one centimeter of the end of the gel. Bovine serum albumin, catalase, and ovalbumin with molecular weights of 68,000, 60,000, and 43,000, respectively, as cited by Weber and Osborn (1969) were used as standards for the runs. Measurements of the gel length and the distance moved by the dye were made before staining. The gels were stained for 3–5 hr with coomassie brilliant blue. After destaining, the length of the gel and positions of the protein bands were measured. Mobilities were calculated and were plotted against the known molecular weights of the standard proteins expressed on a semilogarithmic scale.

Oxidation and Reduction of Disulfide Bands. Performic acid oxidation was carried out as described by Hirs (1956). Reduction was done as described by Craven *et al.* (1965). S-Carboxymethylation was done by the procedure of Anfinsen and Haber (1961), using a 5-fold excess of iodoacetic acid.

Amino-Terminal Analyses. PHENYL ISOTHIOCYANATE REACTION. Oxidized family B (2 mg) in 4 ml of 6 M urea was allowed to react at pH 9 with 0.2 ml of 10% (v/v) PITC in redistilled dioxane in the pH-Stat at 37° for 2.5 hr (Heilmann *et al.*, 1957). The excess of reagent was extracted with benzene and cyclohexane. The aqueous layer was dialyzed against water, along with a small precipitate which appeared at the interface. After lyophilization, cyclization was carried out with 1 ml of anhydrous trifluoroacetic acid for 3 hr at room temperature. The solvent was removed *in vacuo* and the dry powder extracted with acetone. The acetone extract was taken to dryness and treated with 1 ml of 3 N HCl for 3 hr at 37°. After diluting to 1 N HCl, the phenylthiohydantoin was extracted with ethyl acetate and taken to dryness. The spectrum of an ethanol solution of the material was taken between 240 and 280 nm. The reaction was also carried out on reduced and carboxymethylated family B in the presence of 0.1 M borate (pH 9). In this experiment, the ethyl acetate extract was subjected to chromatography on starch paper with heptane–pyridine (70:30, v/v).

Fluorodinitrobenzene Reaction. Family B (5.3 mg) was reacted with 0.1 ml of FDNB in the presence of 1% sodium dodecyl sulfate and 10 mM EDTA at pH 8.5 for 2 hr at 40° in the pH-Stat. The excess reagent was extracted with ether and the protein was precipitated by acidification with HCl. The precipitate was washed with water, ether, and acetone and dried *in vacuo*. It was then hydrolyzed with 6 N HCl at 105° for 16 hr. After dilution to 1 N HCl the hydrolysate was extracted with peroxide-free ether. Thin-layer chromatography was performed on the aqueous and ether phases (Brenner *et al.*, 1965). The chromatographic systems used for the ether phase were toluene–pyridine–ethylchlorohydrin–0.8 N ammonia (10:30:60:60, v/v) in the first dimension and chloroform–*tert*-amyl alcohol–acetic acid (70:30:3, v/v) in the second dimension, which was run in a perpendicular direction to the first chromatography. The solvent used for the aqueous phase was 1-butanol–34% ammonia (80:20, v/v).

Carbohydrate Determination. Total neutral sugar was measured initially on the intact protein by the phenolsulfuric method of Dubois *et al.* (1956), as described earlier (Kauffman *et al.*, 1970). Sialic acid determinations were done as described by Svennerholm (1957); hexosamine content was quantified on the Beckman 120C amino acid analyzer after hydrolysis of the protein for 6 hr in 4 N HCl at 100° in sealed evacuated tubes.

The individual neutral sugars were quantitated as follows. Family A isoenzymes (0.025–0.05 μmole) from three individuals were hydrolyzed in 1 ml of 2 M trifluoroacetic acid for 2.5 hr at 100°. Hydrolysates were taken to dryness on an Evapomix and dissolved in 0.5 ml of 1 M NH_4OH. They were applied to a column (0.5 × 12 cm) of anion-exchange resin AG-1 in H_2O and eluted with 4 ml of H_2O. The eluates were concentrated to 0.5 ml and applied to a similar column containing the cation-exchange resin AG 50, eluted with H_2O, and taken to dryness before dissolving in appropriate volumes of water for assay. D-Mannose, L-fucose, and D-galactose were determined enzymatically as described by Finch *et al.* (1969) in a total volume of 1.8 ml. As a control, a glycoprotein of known composition, ovalbumin (0.05 μmole), was similarly hydrolyzed, passed through the ion-exchange columns, and the recovery of mannose determined. Unhydrolyzed samples of amylases were also assayed for fucose by the method of Dische and Shettles (1948).

Nature of the Carbohydrate Linkage. Family A (0.42 mg) was treated with 0.05 N NaOH at 40° for 6 hr. After treatment the protein was dialyzed against water overnight at 5° and tested for neutral sugars by the phenolsulfuric procedure.

Specific Enzymatic Activities. Amylase activity was routinely measured by the dinitrosalicylate method (Bernfeld, 1955) at 30° with Noredux starch (0.5%) in 0.02 M sodium phosphate buffer (pH 6.9). A standard curve relating amylase activity to micrograms of protein was constructed, using crystalline human parotid amylase prepared in this laboratory as previously described (Kauffman *et al.*, 1970). The concentration of the standard enzyme solution was based on the absorbance at 280 nm using the extinction coefficient $E_{1cm}^{1\%}$ at 280 nm of 23.3 (Hsiu *et al.*, 1964).

Heat Stabilities of Amylase Isoenzymes. For the series of experiments investigating the effect of temperature on amylase activity the buffer system was changed to 0.02 M sodium glycerophosphate–0.01 M sodium chloride (pH 6.9), since amylase appeared to be more stable in this buffer at temperatures higher than 30°. Isoenzyme families A and B were incubated for 30 min at 45 and 55° at a concentration of 0.5 μg/ml and assayed immediately at 30°. The glycero-

phosphate buffer was used in the incubation mixture and the assay mixture.

Action Patterns. The mode of action of isoenzyme families A and B on the high molecular weight substrate, Superlose, was followed by simultaneous measurement of the decrease in the blue color of the amylose–iodine complex and the increase in the reducing groups. The analytical methods employed in a similar study by Robyt and French (1967) were used except that changes in absorbance were measured with a Hitachi Perkin-Elmer spectrophotometer. The buffer was 0.02 M sodium glycerophosphate–0.01 M sodium chloride (pH 6.9) and the temperature was 40°.

Transformation of Isoenzymes. Sterile enzyme solutions (0.6 mg/ml) in 0.1 M Tris (pH 9) were incubated at 37° for intervals up to 1 week. In some studies, the enzyme was incubated at room temperature and, in one case, the incubation was carried out in the presence of 0.2 mM DTNB. As controls, solutions of the enzymes were incubated in 0.05 M potassium phosphate (pH 6.8). Disc gel electrophoresis was performed after incubation and amylase zymograms were done on the gels.

Peptide Mapping. Family B (1.5 mg) stored at 4° in water and family B (1.5 mg) which had been incubated at pH 9 at 37° for 1 week, were dialyzed and lyophilized. Both samples were digested with 0.15 mg of pepsin in 0.15 ml of 5% formic acid for 2 hr at 37°. The digests were then subjected to high-voltage paper electrophoresis at pH 6.5 for 35 min at 50 V/cm. The neutral area from this was run again at pH 1.9, 45 min at 50 V/cm. Chromatography of the basic, acidic, and neutral areas was done with a solvent containing butanol–pyridine–acetic acid–water (15:10:3:12, v/v) for 18 hr. The chromatograms were stained with cadmium–ninhydrin.

Purification of Acidic Peptide 1. Family B (10 mg) was stored at 37° pH 9 for 1 week. After dialysis against water, it was digested with pepsin as described above. The digest was applied to Whatmann No. 3MM along a 10-cm line in the center of the paper, and subjected to pH 6.5 high-voltage electrophoresis, 35 min at 3000 V. A 1-cm strip was stained with ninhydrin to locate the acidic peptide. The corresponding unstained area was cut out and stitched to another paper for chromatography as described for the peptide maps. After locating the peptide, it was eluted with 0.05 M NH_4OH, lyophilized, and subjected to hydrolysis with 6 N HCl for 16 hr. Amino acid analysis was done on a Jelco 5 AH amino acid analyzer.

Modification of the Carboxylic Acid Groups. The modification was carried out according to the procedure of Hoare and Koshland (1967) on duplicate samples of family B before and after conversion, using either [^{14}C]taurine or norleucinamide as the nucleophile. [^{14}C]Taurine (2.6 mg) and 374 mg of unlabeled taurine were dissolved in 4.3 ml of 8 M urea. This (1 ml) was added to 1.5 mg of lyophilized protein sample. The pH was adjusted to 4.75, after which 19.2 mg of EDC was added. The pH was maintained at 4.75 for 4 hr at 25°. At the end of the reaction time, 1.0 ml of 2 M sodium acetate (pH 4.75) was added to quench the reaction with the diimide. The samples were dialyzed exhaustively at room temperature for 3 days against 8 M urea–0.5 M NaCl. They were then dialyzed against water, which resulted in precipitation of the protein. The suspensions were transferred to scintillation vials and lyophilized in the vials. The powders were dissolved in 1.0 ml of 1 N NaOH from which 0.075 ml was removed for protein determinations by the Lowry procedure (Lowry *et al.*, 1951). Scintillation fluid (16 ml) (2:1, v/v, toluene–Triton X-100 mixture containing 4 g of PPO/l.) was added and the solution was neutralized with 0.1 ml of concentrated HCl (Madsen, 1969). After several hours at 4° with occasional shaking, a monophasic solution resulted. The samples were counted in a Packard liquid scintillation spectrometer. Alternatively, protein samples (0.6 mg) were reacted with 41.5 mg of norleucinamide and 19.2 mg of EDC in 1.0 ml of 8 M urea (pH 4.75). At 45 min and again at 100 min, an additional 16.6 mg of norleucinamide and 19.2 mg of EDC were added. After 3 hr at room temperature, at pH 4.75, the samples were quenched with sodium acetate. They were then dialyzed at room temperature against water, with three changes for 24 hr. The dialyzed samples were hydrolyzed in 6 N HCl for 16 hr at 108°. Amino acid analyses were done on the Beckman 120C analyzer.

Assay for Asparaginase. Asparaginase activity was assayed with Nessler's reagent to detect the production of ammonia, using the method of Roberts (1968). Sterile family B (0.45 mg) was incubated for 4 days at 37° in 0.1 M Tris (pH 9) or in 0.05 M potassium phosphate (pH 6.8) in the presence and absence of 0.02 M asparagine. The total volume was 0.5 ml. After incubation, 0.2 ml was removed for assay. After the addition of an equal volume of H_2SO_4, the mixture was centrifuged and 0.2 ml of the supernatant fluid was brought to a volume of 4.0 ml with water. Nessler's reagent (0.5 ml) was added with mixing and the optical density of the solution was measured at 420 nm. Controls of asparagine alone were carried through the same procedure. Aliquots of the incubation mixtures were also subjected to anionic gel electrophoresis.

Cyanogen Bromide Cleavage. Isoenzyme families A and B were subjected to cyanogen bromide cleavage in 70% formic acid as described by Steers *et al.* (1965), using the conditions of Givol and Porter (1965). Protein (10 mg) was dissolved in 3.3 ml of 70% formic acid containing 20 mg of CNBr. After incubation at 25° for 24 hr the solution was diluted with water and lyophilized. The residue was suspended in water and relyophilized. The cyanogen bromide peptides were dissolved in 1 M acetic acid, a portion was removed for polyacrylamide disc gel electrophoresis and the remainder was held for chromatography.

Results

The separation of crystalline human parotid amylase into families of isoenzymes by recycling chromatography is shown in Figure 1. The resolution of the respective families achieved by coupled columns is superior to that achieved earlier by means of single gel filtration columns. Typical gel patterns of families A and B are shown in Figure 2. Approximately the same proportions of families A and B were observed in all preparations of amylase examined, whether parotid saliva was collected from single donors or pooled samples were used.

When the isoenzymes were run on polyacrylamide gels in the presence of sodium dodecyl sulfate one band was observed for each family. Figure 3 shows that when run on the same gel the band from each family was separable from the other. Measurement of experimental gels gainst the standards gave mean molecular weights of 61,856 ± 1853 for family A proteins and 56,133 ± 1433 for those of family B.[2] The mobilities of the protein bands were not affected by the presence of EDTA nor significantly altered by reduction and carboxymethylation or performic oxidation of the protein. The sodium dodecyl sulfate–polyacrylamide gels thus gave no evidence for subunits or multiple polypeptide chains. Rather, they indicate

[2] Nine experimental gels were run for each isoenzyme family.

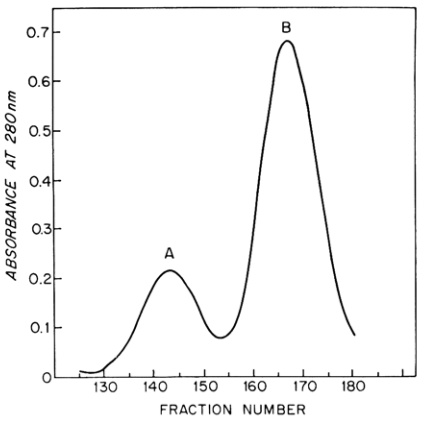

FIGURE 1: Separation of crystalline preparations of human parotid amylose into isoenzyme families A and B by recycling chromatography on Bio-Gel columns. Details of the experiment are presented in the text.

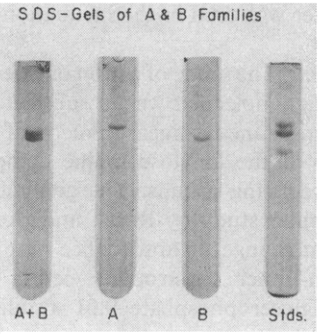

FIGURE 3: Polyacrylamide disc gels of families A and B and standard proteins run in the presence of sodium dodecyl sulfate. The standard proteins and their molecular weights are identified in the text in the section entitled Methods.

that the isoenzymes of human parotid amylase are single polypeptide chains. They also indicate that, within the standard deviations shown, the isoenzymes within each family have the same molecular weights and that the value for family A is slightly higher than for family B.

Attempts to determine the amino-terminal residue in family B by the phenylthiohydantoin method and by dinitrophenylation met with negative results. When either oxidized or reduced and carboxymethylated protein was reacted, no PTH-amino acid was formed, as evidenced by the lack of a peak at 270 nm in the absorption spectrum of the phenylthiohydantoin extract, and by the absence of PTH-amino acids on paper chromatography. Dinitrophenylation of the protein and chromatography of the ether and aqueous extracts revealed only ϵ-DNP-lysine and *O*-DNP-tyrosine. No α-amino-substituted amino acid could be found.

We reported earlier (Kauffman *et al.*, 1970) that the two families of amylase isoenzymes had identical amino acid compositions but that family B contained less than 1 mole of neutral sugar/mole of enzyme and no hexosamine, whereas family A contained several covalently linked neutral sugars, namely, galactose, mannose, and fucose as well as glucosamine. Neither family of isoenzymes contained sialic acid. We have since improved our quantification procedures and have investigated the neutral sugar content in family A isoenzymes isolated from three separate individuals.

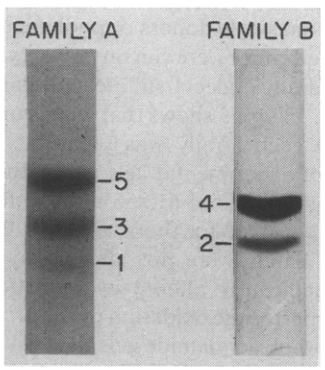

FIGURE 2: Anionic polyacrylamide disc gel patterns of isoenzyme families A and B. The direction of migration is from the top to the bottom of the figure.

Hexosamine determinations showed 0.018 μmole of glucosamine in 0.003 μmole of family A proteins analyzed, or 6 moles of glucosamine/mole of protein. No other hexosamine was detected. Table I shows that the neutral sugar content was the same for the three individuals examined. Thus, the composition of the carbohydrate moiety of amylase isoenzyme family A, as presently perceived, is 6 moles of glucosamine, 3 moles of fucose, 2 moles of mannose, and 2 moles of galactose per mole of glycoenzyme. If the glucosamines are present as N-acetylated derivatives, as is the usual case, the molecular weight of the carbohydrate moiety is approximately 2300

TABLE I: Neutral Sugar Determinations of Amylase from Three Subjects.[a]

Subject	μM Family A Hydrolyzed	μM Mannose Recovd	Moles of Mannose/ Mole of Family A
	D-Mannose Assays		
K	0.050	0.114	2.28
C	0.025	0.048	1.92
	L-Fucose Assays		
		μM Fucose Recovd	Moles of Fucose/ Mole of Family A
K	0.025	0.069	2.76
C	0.025	0.075	3.00
T	0.025	0.079	3.16
	D-Galactose Assays		
		μM Galactose Recovd	Moles of Galactose/ Mole of Family A
K	0.025	0.050	2.00
C	0.025	0.054	2.16
T	0.025	0.054	2.16

[a] A single determination was made on each sample by the enzymatic procedures for mannose, galactose, and fucose. In the case of fucose the samples were analyzed also by the Dische–Shettles procedure, with the same results.

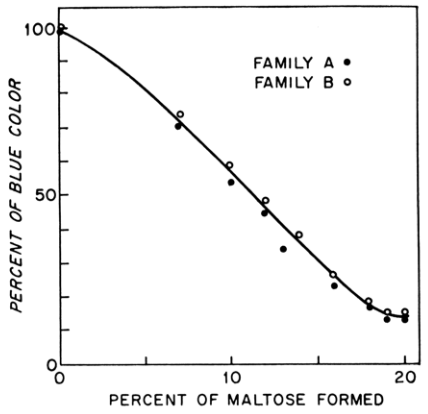

FIGURE 4: Action patterns of isoenzyme families A (●) and B (○) toward the high molecular weight substrate, Superlose. Experimental details are presented in the text.

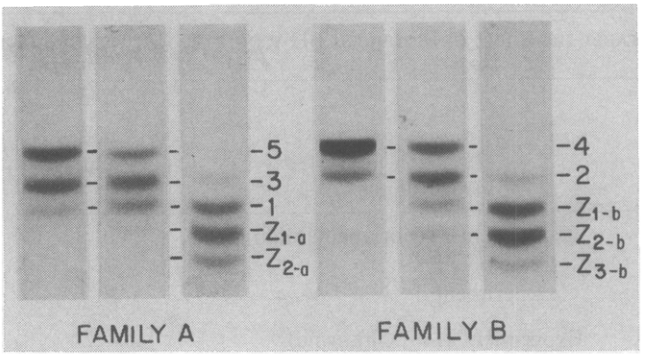

FIGURE 6: Anionic gel patterns of isoenzyme families A and B incubated 1 week at pH 6.8, 5° (left); pH 6.8, 37° (middle); and pH 9.0, 37° (right). The direction of migration is from the top to the bottom of the figure.

which would account, at least in part, for the observed difference in molecular weights of isoenzyme families A and B. The fact that a single band was observed when family A was examined on sodium dodecyl sulfate–polyacrylamide gels suggests that all of the isoenzymes of family A are glycoenzymes and that the carbohydrate is uniformly distributed. The carbohydrate moiety was not cleaved from the protein by alkaline treatment, suggesting an N-glycosidic linkage to the protein.

Isoenzyme families A and B were examined for functional differences by several criteria. No differences were observed in specific enzymatic activities (2800 maltose units/mg) nor in the mode of action of the respective enzymes toward the high molecular weight amylose, Superlose. Figure 4 shows curves relating loss in the blue color of the amylose–iodine complex (dextrinizing activity) to the increase in reducing power expressed as maltose groups (saccharifying activity). The curves reflect the average number of catalytic events, following the first, during the lifetime of the enzyme–substrate complex and can be seen to be the same for the two families. The respective families also exhibit similar responses to incubation at 45 and 55°, as depicted in Figure 5.

Amylase isoenzymes 1–5 and traces of the fast anionic Z bands are present in fresh parotid saliva and, in the one individual systematically studied, the proportions of the isoenzymes in fresh saliva remained constant over a 2-year period (Kauffman *et al.*, 1970). The proportions can be altered however by prolonged storage in the cold or incubation at 37°. As shown in Figure 6 the original isoenzymes of both families are transformed during incubation into more rapidly migrating anionic proteins. The proteins formed on incubation correspond in migration rate to those present originally in minor or trace amounts. Thus, in family B, isoenzyme 4 disappears and seems to give rise initially to a protein migrating like isoenzyme 2 and eventually to forms corresponding to the original Z bands. Similarly, in family A, the conversion appears to proceed from isoenzymes 5 to 3 to 1 and finally to the fast anionic Z bands, respectively. Zymograms have shown that all of the new proteins have amylolytic activity and gels run in the presence of sodium dodecyl sulfate revealed that the molecular weights had not been significantly altered during incubation.

The relative proportions of isoenzymes before and after incubation, as determined by densitometric tracings of stained gels, are shown in Table II. The reaction is dependent on pH as well as temperature. Whereas the proportions remain relatively constant during storage at neutral pH in the cold (4°), incubation for 7 days at pH 6.8, 37° caused some conversion. Incubation for 7 days at pH 9.0, 37° effected an

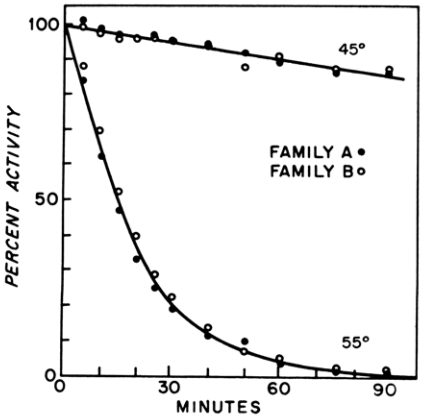

FIGURE 5: Stability of isoenzyme families A (●) and B (○) toward incubation at 45 and 55° in 0.01 M NaCl–0.02 M glycerophosphate buffer at pH 6.9. Conditions of assay are described in the section entitled Methods.

TABLE II: Proportions of Isoenzymes before and after Incubation Isoenzymes.[a]

	5	3	1	Z_{1-a}[b]	Z_{2-a}	Z_{3-a}
Family A, 6.8, 5°	46	43	11			
Family A, 6.8, 37°	26	42	25	7		
Family A, 9.0, 37°		6	27	44	20	3
		4	2	Z_{1-b}[b]	Z_{2-b}	Z_{3-b}
Family B, 6.8, 5°		79	21			
Family B, 6.8, 37°		41	42	17		
Family B, 9.0, 37°			8	36	46	10

[a] Values are expressed as percentages of total based on planimetric area measurements on densitometric tracings of stained gels. Incubations were carried out for 1 week in 0.05 M potassium phosphate buffers at 6.8 or 0.1 M Tris at pH 9.0. [b] The Z bands of the A family do not migrate identically with those of the B family.

TABLE III: Effect of Storage at pH 9 on Free Carboxyl Content of Family B.

Substance	Isoenzyme Proportions (% of Total)					Nucleophile Incorpd (Moles/Mole of Enzyme)
	4	2	Z_{1-b}	Z_{2-b}	Z_{3-b}	
Experiment A (norleucinamide)						
Control	88	12	0	0	0	20.1
Experimental	30	45	21	4	0	21.7
Experiment B ([^{14}C]taurine)						
Control	87	13	0	0	0	46
Experimental	2	23	45	27	3	51

almost complete transformation from the original isoenzymes to the more anodic forms. Qualitatively similar changes occur at pH 9, at 25 and 5°, respectively, but much more slowly. When the storage was carried out at pH 9 and 5°, no loss in activity occurred over 124 days despite significant changes in the gel patterns. It should be noted that these solutions had been passed through Millipore filters prior to incubation and that all subsequent operations were conducted under sterile conditions.

Two lines of evidence indicate that the transformation of isoenzymes involves partial deamidation of the amylase molecules. (1) Peptide maps of family B prepared before and after incubation at pH 9, 37° revealed two new acidic peptides in the incubated samples, and the partial loss of one neutral and one slightly basic peptide. The acidic peptides are shown in Figure 7. The peptide designated "1" was purified by paper chromatography, eluted, and hydrolyzed for amino acid analyses as described under Methods. It was found to contain the following residues: Asx 3.8, Glx 1.1, Gly 1.1, Cys/2 0.6, and Lys 0.9. (2) The number of reactive carboxyl groups available for modification by Hoare and Koshland's procedure (1967) is increased upon incubation. Table III shows the number of nucleophilic groups incorporated into protein using norleucinamide and [^{14}C]taurine, respectively. With both reagents the number of nucleophilic groups incorporated, which is a measure of the free carboxyl groups, is higher in incubated samples. That higher values were obtained with taurine than with norleucinamide is consistent with the observations of Hoare and Koshland (1967) who suggest that the difference may result from steric hindrance occasioned by the larger norleucine derivative.

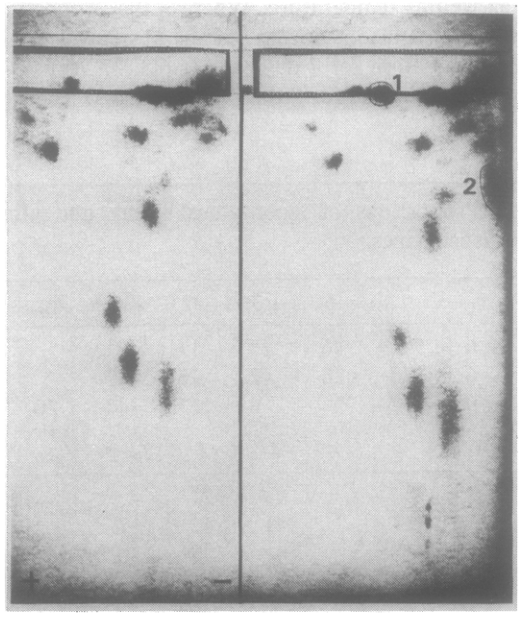

FIGURE 7: Peptide maps of acidic peptides obtained by peptic digestion of family B, before and after transformation of the isoenzymes. The map on the left is of the protein before incubation; on the right, after incubation of the isoenzymes. Electrophoresis at pH 6.5 was in the horizontal direction, chromatography was in the vertical direction. Further details are in the text.

Freshly collected samples of human parotid saliva contain immeasurable amounts of asparaginase and none appears upon incubation of sterilized saliva for 5 days at 37°. Incubation of nonsterilized saliva for 4 days at 37° resulted in very low levels of activity when measured by the asparaginase procedure of Roberts (1968). The amounts measured were 0.05 unit/ml at pH 6.8, 0.075 unit/ml at pH 8.0, and 0.02 unit/ml at pH 9.0. However, to further explore the role of amidases in the transformation of amylase isoenzymes, asparagine was included in the sterile incubation mixtures of family B isoenzymes and Nessler's reagent used to measure ammonia production at the end of the incubation period. Four-days room-temperature incubation at pH 6.8 effected only slight changes in isoenzyme gel pattern but yielded an increase in optical density at 420 nm of 1.172 over the sample without asparagine. Incubation at pH 9 resulted in extensive conversion of isoenzymes but an increase of only 0.028 in optical density at 420 nm. In separate experiments the conversion of asparagine to aspartic acid at pH 6.8 but not at pH 9.0 was demonstrated by high-voltage electrophoresis of the incubation mixtures. Thus deamidation of added asparagine occurs during incubation but exhibits a different pH dependency from the isoenzyme transformations.

Isoenzyme families A and B were reacted with cyanogen bromide and the peptides examined on polyacrylamide disc gels in the presence of 6 M urea. The gel patterns were very reproducible and are shown in Figure 8. Cationic gels contained nine peptides whereas eleven peptides were resolved in the anionic system, consistent with the presence of 10 methionine residues/mole. Most of the peptides were common to both families but each family of isoenzymes contains one or two peptides specific to itself, as indicated in Figure 8. In preliminary experiments the cyanogen bromide peptides have been partially purified on columns of Bio-Gel. Each

fraction has been examined for absorbancy at 280 nm, electrophoretic gel pattern, and sugar content. The fractions containing the peptides specific to family A also contain the carbohydrate.

Discussion

Most of the α-amylases of vertebrate origin studied to date appear to consist of single polypeptide chains with molecular weights of approximately 55,000. In addition to the present report on human parotid amylases, investigation of possible subunit structure have been made with amylases purified from human pancreas (D. Stiefel and P. J. Keller, manuscript in preparation), rabbit parotid and pancreas (Malacinski and Rutter, 1969), rat parotid and pancreas (Sanders and Rutter, 1971), and pig pancreas (Cozzone *et al.*, 1970; Robyt *et al.*, 1971). Of these the only report of subunits has come from Robyt *et al.* (1971) who concluded that porcine pancreatic α-amylase consists of subunits with molecular weights of approximately 25,000 held together by intermolecular disulfide bonds. The latter authors attribute the differences between their results and those of Cozzone *et al.* (1970), who also worked with porcine pancreatic amylase, to the use of a more efficient reducing agent, namely, dithiothreitol in place of β-mercaptoethanol, and to conducting the reaction at pH 8.5 instead of 7.1.

In the case of human parotid amylase neither reduction, which was carried out at pH 8.5 in 8 M urea with a molar ratio of mercaptoethanol to protein of 1000, nor oxidation with performic acid effected a decrease in molecular weight. The absence of subunits such as those reported for pig pancreas is thus unlikely to result from incomplete scission of intermolecular disulfide bonds. Any differences in subunit structure would thus appear to be due to organ or species variations rather than procedural differences. It has not been ruled out however, that limited proteolysis of porcine pancreatic amylase might have contributed to the subunits observed by Robyt *et al.* (1971) following reduction of the disulfide bonds.

Amylase isoenzymes 1–5 are present in all samples of parotid saliva examined immediately after collection and can be demonstrated in cationic electrophoretic systems run at pH 3.8 as well as anionic systems at pH 8.9. They thus appear to have existence *in vivo*. However, forms electrophoretically identical with the more anodic members of both families can be produced *in vitro* by reactions that appear to entail deamidation. If, in fact, the anionic forms produced *in vitro* are identical with those made *in vivo*, a provisional view of the molecular relationships between the amylase isoenzymes might be that the A and B families comprise glycosidated and nonglycosidated forms of amylase, respectively, and that the isoenzymes within each family differ mainly in their amide content. This could markedly reduce the number of genetic variations involved in the formation of the amylase isoenzymes. No conversion has ever been observed between the isoenzymes of different families.

The very low levels of asparaginase and the discrepancy in pH dependencies suggest that the deamidation of the amylases is nonenzymatic. Robinson *et al.* (1970) have studied the rates of nonenzymatic deamidation of model peptides and shown that the reactions depend strongly on the nature of the neighboring residues. Further work will be needed to relate these observations to the sequence of amino acid residues in the acidic peptides formed during transformation of the isoenzymes. It is of interest that the original isoenzymes

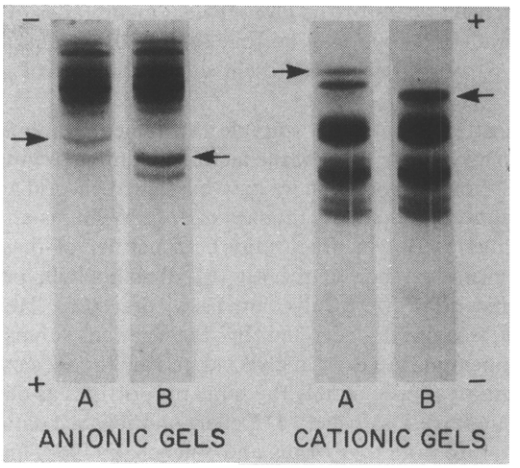

FIGURE 8: The electrophoretic gel patterns of peptides obtained by cyanogen bromide cleavage of families A and B. Gels contained 6 M urea. Electrophoresis was performed as described in the text. Arrows indicate the regions of difference between the two families.

can be altered to the degree shown in Figure 6 and Tables II and III of this paper without losses in specific enzymatic activity.

Flatmark (1967, 1968) has shown that different amide groups in cytochrome *c* are deamidated at different rates and that the deamidated forms of the enzyme have more open structures and are more susceptible to degradation than the original molecule. He, and subsequently Robinson *et al.* (1970), propose controlled deamidation as a means of promoting degradation of a protein afters its useful lifetime in a physiological environment is over. The pH dependency of the deamidation that appears to accompany the transformation of amylase isoenzymes is not typical of most intracellular processes but it is compatible with the pH of the duodenum where amylases are presumably degraded. Perhaps the phenomenon plays some role in degradation of the 1–2 g of salivary amylase ingested daily.

No biological role has been found to date for the carbohydrate moiety on the isoenzymes of amylase family A. The glycoenzymes have been found in all samples of human parotid saliva examined and in approximately the same proportion. The composition of the carbohydrate moiety appears to be constant within and between individuals. The question arises as to what determines that a certain constant proportion of the amylase molecules will be glycosidated and the remainder will not.

One possible explanation is that due to some genetically determined alteration at the polypeptide level, only the proteins of family A possess the acceptor site for the carbohydrate. If this were true, glycosidation would be complete in terms of acceptor sites.

As an experimental approach to this hypothesis we have prepared cyanogen bromide peptides from families A and B, respectively, and are currently engaged in purification of the peptides and glycopeptides unique to each family of isoenzymes. Determination of their amino acid compositions and sequences should help to elucidate the molecular basis for the existence of glycosidated and nonglycosidated forms of amylase.

A number of investigators, including Eylar (1965), have suggested that the role of the carbohydrate moiety in many glycoproteins is to serve as "passports" facilitating exit from the cell and that this mechanism of secretion has been super-

seded in acinar cells by the glycoprotein membranes of zymogen granules. It thus may be that the glycosidated forms of human parotid amylase represent vestigial forms of the enzyme.

In an attempt to find a suitable experimental animal with which to investigate some of the factors that might regulate the glycosidation phenomenon we have examined parotid amylase from baboons and rhesus monkeys (B. L. Williams and P. J. Keller, manuscript in preparation). In neither of these subhuman primates, nor in the rat (M. Robinovitch, personal communication) were glycoamylases detected. However, amylase isoenzymes secreted by the human submaxillary gland appear also to exist in glycosidated and nonglycosidated forms and in approximately the same proportions as observed in human parotid saliva (P. J. Keller, unpublished data). It is interesting to note that Muus and Vnenchak (1964) analyzed crystalline preparations of amylase from whole human saliva for neutral sugars, hexosamines, and sialic acid. All three tests gave results that corresponded to less than one-third of a group per 50,000 g of amylase. Muus and Vnenchak (1964) suggested at that time however that a fraction of the human salivary amylase might contain carbohydrate.

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