Steroidogenic activity of peptide I was determined by the method of Vernikos-Danellis *et al.* (1966). The *in vitro* melanotropic activity was estimated by the procedure of Shizume *et al.* (1954). The isolated fat cells from rabbit and rat adipose tissues were used for lipolytic assay by the method of Ramachrandran and Lee (1970). Results are summarized in Table I. Highly purified sheep ACTH, obtained by the method previously described (Birk and Li, 1964), was used as the reference standard.

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Molecular Weight and Subunit Structure of Hagfish Transferrin†

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ABSTRACT: The molecular weight and subunit structure of hagfish transferrin was studied by sedimentation equilibrium, sedimentation velocity, determination of iron-binding capacity, sodium dodecyl sulfate–gel electrophoresis, and gel chromatography in a denaturing medium. Contrary to a previous report, the protein was found to consist of a single polypeptide chain of molecular weight in the range 75,000–80,000. Two similar or identical iron-binding sites, with optical and electron paramagnetic resonance spectra closely resembling those of human transferrin, are present in the native protein.

The transferrins comprise a class of proteins characterized by the ability to bind specifically, tightly, and reversibly a variety of transition metal ions of which the most important is Fe(III). Most studies of transferrins isolated from a variety of physiologic fluids and species have shown that they consist of single polypeptide chains of mol wt 75,000–80,000 (Green and Feeney, 1968; Querinjean *et al.*, 1971; Mann *et al.*, 1970), on which two identical binding sites are disposed (Aasa *et al.*, 1963; Aisen *et al.*, 1966). This has generated speculation about the possibility of gene duplication during the evolution of the proteins.

Recently Palmour and Sutton (1971) have reported that transferrin isolated from the serum of the California hagfish, a primitive vertebrate, has a molecular weight of only 45,000 and a single binding site for Fe(III). Because of the potential importance of this finding in the evolutionary biochemistry of proteins we have undertaken a further investigation of the molecular weight and subunit structure of hagfish transferrin.

Experimental Procedures

Freshly frozen hagfish serum was obtained in two separate batches from Pacific Biomarine Supply Co., the source used by Palmour and Sutton (1971). Transferrin was separately isolated from each batch by the method described by Palmour and Sutton (1971), using ^{59}Fe as a radioactive tracer during the isolation procedure. Over 98% of the counts recovered after the initial gel filtration step were present in the fraction used for the preparation of transferrin by ion-exchange chromatography. The final yield of purified protein in each preparation was about 1 mg/ml of serum. The optical

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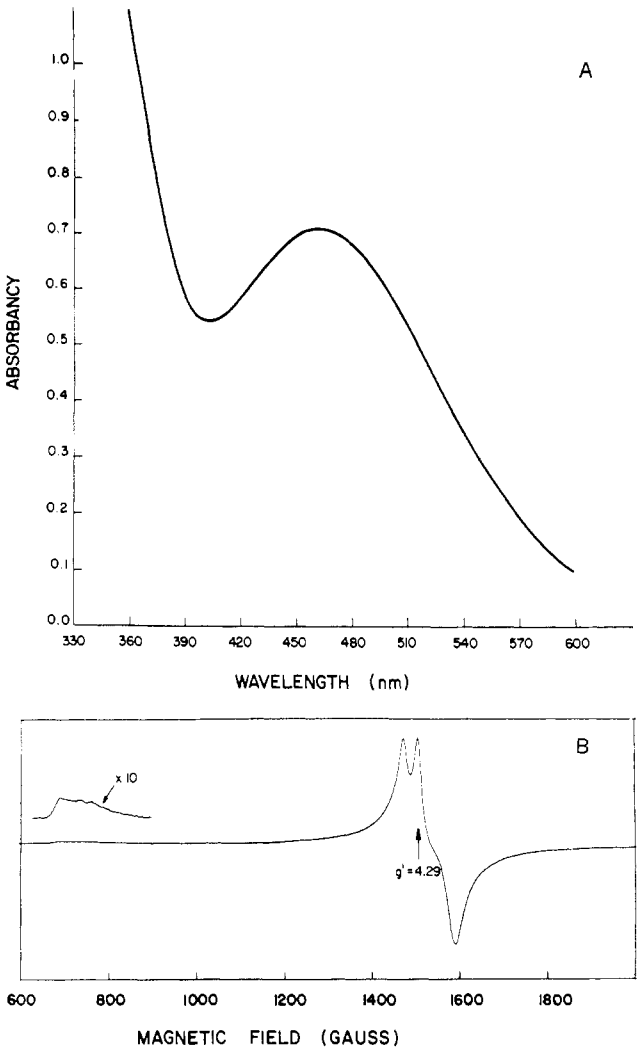

FIGURE 1: (A) Optical spectrum of 1.5×10^{-4} M hagfish transferrin saturated with Fe(III). The light path was 1 cm, and the reference cuvet contained buffer. (B) Electron paramagnetic resonance spectrum of 2.2×10^{-4} M hagfish transferrin saturated with Fe(III), obtained at 77°K. The spectrometer settings were: microwave frequency, 9.059 GHz; microwave power 15 mW; modulation amplitude 10 g; time constant, 3 sec; sweep time 16 min.

and electron paramagnetic resonance (epr) spectra of the iron-saturated hagfish protein, shown in Figure 1, were quite similar to those of the human protein (Aasa *et al.*, 1963).

To obtain a direct comparison of the subunit structure and size of human and hagfish transferrins, gel chromatography of the reduced alkylated proteins was carried out in a denaturing medium following procedures given by Mann and Fish (1972). The supporting gel was Bio-Gel A-5, 100–200 mesh (Bio-Rad Laboratories), in a 90 × 1.5 cm column, and the eluting solvent was 6 M guanidine hydrochloride dissolved in 0.05 M acetate buffer at pH 4.75. Reduction and alkylation of the protein samples prior to chromatography was accomplished by the methods of Mann and Fish (1972). The human serum transferrin was labeled with ^{125}I by the technique of Katz (1961), before the reduction and alkylation step. This permitted the human protein to be used in amounts sufficiently small so that its contribution to the absorbancy at 280 nm was negligible. The iodination procedure has been shown not to affect the chromatographic behavior of denatured transferrin (Aisen and Leibman, 1972).

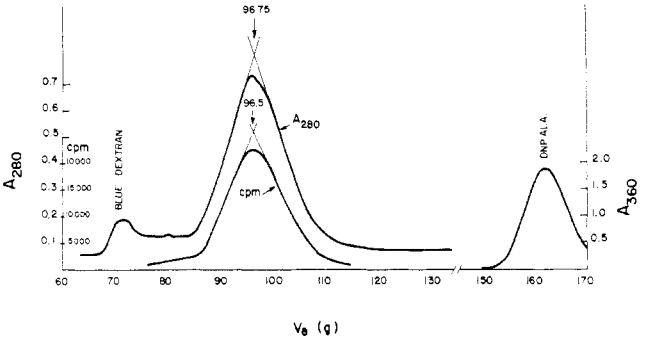

FIGURE 2: Simultaneous gel chromatography in 6 M guanidine hydrochloride of hagfish transferrin and ^{125}I-labeled human transferrin. Both proteins were reduced and alkylated. The supporting medium was a 6% agarose gel (Bio-Gel A-5m, 100–200 mesh). Dnp-alanine (DNP-ALA) was used to determine the total volume of the column accessible to solvent.

Disc electrophoresis in sodium dodecyl sulfate gels followed the methods given by Maizel (1971). A 10% polyacrylamide gel prepared in 0.18 M Tris-HCl buffer (pH 8.8), containing 0.1% sodium dodecyl sulfate was used as the separating gel, while the spacer gel consisted of 3.3% polyacrylamide in 0.06 M Tris-HCl buffer (pH 6.8). The electrode buffer was 0.05 M Tris–0.37 M glycine (pH 8.5) containing 0.1% sodium dodecyl sulfate. Samples were heated at 100° for 2 min in the presence of 2% sodium dodecyl sulfate and 5% β-mercaptoethanol prior to electrophoresis. For the determination of molecular weight reference proteins were obtained from Worthington Biochemical Corp. (phosphorylase *a*) and from Pharmacia Fine Chemicals Inc.

Dry weight determinations for the measurement of the absorptivity of hagfish apotransferrin at 280 nm were carried out in duplicate on preparations dialyzed against six changes of distilled water, or on preparations dialyzed against four changes of 0.005 M Tris-HCl buffer (pH 7.0), with appropriate corrections for the dry weight of the buffer salt. Samples were dried to constant weight in a convection oven set at 106°. Results by the two procedures differed by less than 0.1%. The average value of $A_{280\ nm}^{1\%}$ for hagfish apotransferrin was found to be 10.2. This value was used for determination of protein concentration in the experiments reported in this paper, and is in reasonable agreement with the value of 10.78 given by Palmour and Sutton (1971). Titrations of the iron-binding capacity of hagfish transferrin were carried out using Fe(III)–nitrilotriacetate (1:2 complex, pH 5.0) as the titrant.

Ultracentrifugal studies were carried out with the Spinco Model E analytical ultracentrifuge. Sedimentation was measured at 20° and a rotor speed of 59,780 rpm, at protein concentrations of 2, 3.5, 4, and 6.5 mg per ml. Sedimentation equilibrium studies were carried out at 20°, with a rotor speed of 23,150 rpm for 18 hr by the meniscus-depletion method of Yphantis (1964). Sedimentation equilibrium analyses were also made by a conventional low-speed method (Richard *et al.*, 1968), using a rotor speed of 6166 rpm. The diffusion constant was measured by the area–height method of Wagner and Scheraga (1956) from the sedimentation velocity patterns. For all ultracentrifugation studies a 0.1 M Tris-HCl buffer at pH 7.5 was used. The value of $\bar{v}_2$ was taken as 0.721 (Palmour and Sutton, 1971).

The optical spectrum of hagfish transferrin was recorded with a Cary Model 14 spectrophotometer. A Varian Model

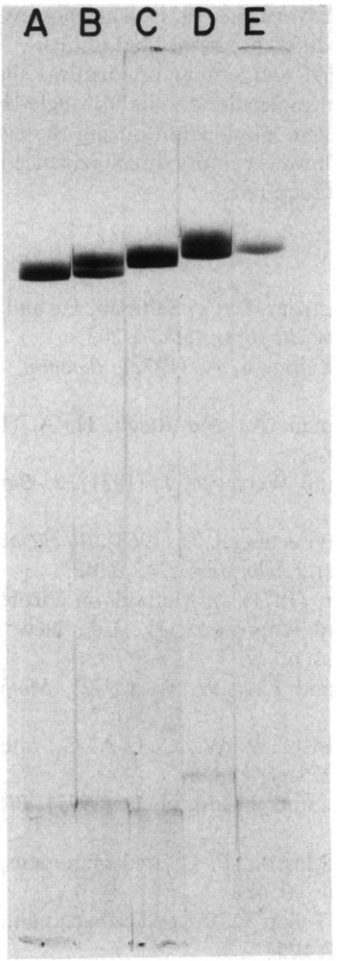

FIGURE 3: Sodium dodecyl sulfate–gel electrophoresis of hagfish and human transferrins. (A) Preparation I of hagfish transferrin; (B) preparation I + human transferrin; (C) human transferrin; (D) preparation II of hagfish transferrin + human transferrin; (E) preparation II.

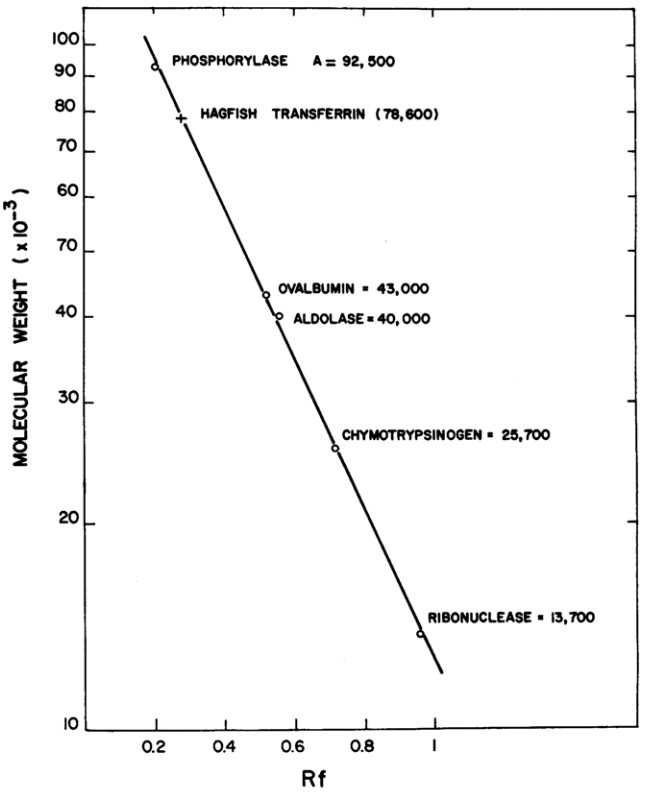

FIGURE 4: Molecular weight determination of hagfish transferrin by electrophoresis in sodium dodecyl sulfate–polyacrylamide gel.

E-9 epr spectrometer was used to obtain the epr spectrum. For these studies the buffer employed was 0.1 M KCl–0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid at pH 7.4–7.5.

Results

Human serum transferrin labeled with ^{125}I, and hagfish serum transferrin, each reduced and alkylated, were virtually indistinguishable in their behavior during gel filtration chromatography in a denaturing solvent (Figure 2). The hagfish protein was detected by its absorbancy at 280 nm. The human protein was detected by its radioactivity; in the quantity used for this experiment its contribution to absorbancy at 280 nm was negligible. Each protein produced a single peak, one superimposable on the other. It may be concluded, therefore, that the molecular weights of the subunits of each protein are identical.

This result is confirmed by electrophoretic studies in the dodecyl sulfate gel, where the human and hagfish proteins behaved similarly (Figure 3). When hagfish transferrin was run with a mixture of protein standards, the subunit molecular weight was calculated to be 78,600 (Figure 4). A second preparation, run separately with standards, yielded a molecular weight of 80,000 by the same method. The results of the ultracentrifugation studies also indicate that the molecular weight is in the range 75,000–80,000 (Table I).

From the iron-binding studies shown in Figure 5, 17.8 mg of protein bound 0.42 μmole of Fe(III). The equivalent weight of protein for the binding of one Fe(III) is then about 42,000. Like the human protein, therefore, hagfish transferrin contains two binding sites per molecule, disposed on a single polypeptide chain.

Discussion

The yields of transferrin we obtained from hagfish serum are comparable to those we obtain from mammalian sera, from which 30–40% of the total transferrin can be recovered in pure form. The optical and epr spectra of our preparations of

TABLE I: Ultracentrifugal Studies of Hagfish Transferrin.

Method	Mol Wt
Sedimentation–diffusion	75,600
($s^0_{20,w}$ = 5.80 × 10^{-13} sec^{-1})	
($D^0_{20,w}$ = 6.70 × 10^{-7} cm^2 sec^{-1})	
Meniscus depletion	
C_0 = 0.5 mg/ml	75,000
C_0 = 0.7 mg/ml	76,400
C_0 = 0.4 mg/ml (second preparation)	76,500
Low-speed equilibrium	
C_0 = 1.84 mg/ml	83,200
C_0 = 3.47 mg/ml	86,300
Extrapolated to infinite dilution	79,700

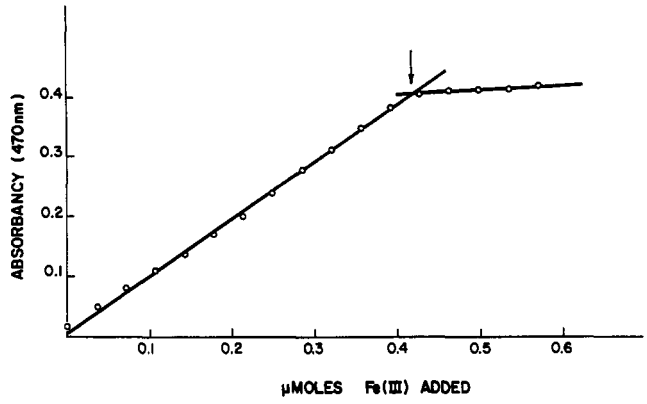

FIGURE 5: The iron-binding capacity of hagfish transferrin. The sample cuvette contained 17.8 mg of protein, dissolved in 2.0 ml of buffer (0.1 M KCl–0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5).

hagfish transferrin are similar to, although not quite identical with, corresponding spectra of human serum transferrin. We did not detect evidence of another iron-binding protein in hagfish serum during the course of our purification procedures. We feel confident, therefore, that we have isolated the major Fe(III)-binding protein of hagfish serum. It is possible, however, that a second iron-binding protein, distinct from transferrin, is present in hagfish serum and was a constituent of the preparation studied by Palmour and Sutton (1971). Otherwise, we are unable to account for the discrepancies between our results and those reported by Palmour and Sutton (1971). Substantially the same procedures have been used for the determination of molecular weight and subunit structure. We chose to use the nitrilotriacetate complex of Fe(III) as the titrant in measuring iron-binding capacity because of the rapidity with which Fe(III) in this form is bound to transferrin (Bates and Wernicke, 1971). This, however, should lead to no significant difference in the final results.

The finding that two similar or identical sites are present in the transferrin molecule, despite its single-chain structure, is suggestive of gene duplication during the evolution of the protein. As yet, however, more direct evidence to corroborate this hypothesis is required.

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