Cuttlefish Spermatid-specific Protein T

MOLECULAR CHARACTERIZATION OF TWO VARIANTS T1 AND T2, PUTATIVE PRECURSORS OF SPERM PROTAMINE VARIANTS Sp1 AND Sp2

(Received for publication, April 17, 1991)

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In cuttlefish, as in selachians and mammals, spermiogenesis is characterized by the double nuclear protein transition histones → intermediate protein (protein T) → protamine (protein Sp).

The cuttlefish protein T, which consists of two structural variants phosphorylated at different degrees, is the first invertebrate spermatid-specific protein to be fully characterized and sequenced. The primary structures of these two variants were established from sequence analysis and mass spectrometric data of the proteins and their fragments. T1 and T2 are two highly related proteins of 78 and 77 residues, respectively, which differ only by four conservative substitutions, two inversions Ser → Arg, and the deletion of 1 residue of arginine in variant T2. The asymmetrical distribution of the hydrophobic and basic residues determines two well defined domains: an amino-terminal domain (residues 1–21) devoid of arginine and aromatic residues and containing all the aliphatic hydrophobic residues and a highly basic carboxyl-terminal domain (residues 22–77 or 78) that contains 77% of arginine, all the tyrosine residues, and most of the phosphorylated serine residues present in the protein.

The complete structural identity of the basic carboxyl-terminal domain of spermatidal proteins T1 and T2 with the protamine variants Sp1 and Sp2 isolated from cuttlefish spermatozoa strongly suggests that T1 and T2 could be precursors of Sp1 and Sp2, respectively.

Cuttlefish spermiogenesis is characterized by a double nuclear basic protein transition (1). A spermatid-specific protein called protein T appears in round spermatids to replace transiently somatic type and/or testis-specific histones. It then disappears from elongated spermatids, where it is replaced by a typical protamine named protein Sp (2). This protamine is the major basic protein associated to DNA in the mature spermatozoa of cuttlefish. It is constituted of two structural variants, Sp1 and Sp2, which differ only by the position of 2 residues of serine and by an additional residue of arginine in the major variant Sp1 (3, 4). This double transition also occurs during spermiogenesis of two other Cephalopod species, the squids Loligo pealeii (5) and Illex argentinus (6) but has never been observed in other invertebrate organisms.

Among vertebrates, only mammals (7–10) and a selachian, the dogfish (11), were found to have a double nuclear protein transition during spermiogenesis, in which several spermatid-specific proteins and protamines are generally involved. Moreover, some of the mammalian protamines appear to be synthesized as precursor molecules. Thus, in mouse, a cDNA coding for a putative precursor of protamine mp2 has been identified (12). In man, intermediate basic nuclear proteins HP1, HPS1, and HPS2 are structurally related to protamines HP2 and HP3, and it has been suggested that they could be precursors of these protamines (13–15). On the contrary, the two spermatidal proteins S1 and S2 of the dogfish do not exhibit any structural relationship with any of the four scylliorninines and cannot be considered as protamine precursors (16).

This paper deals with the characterization and elucidation of the amino acid sequence of the two variants T1 and T2 of the cuttlefish spermatid-specific protein T. The close structural relationship observed between these variants and the protamine variants Sp1 and Sp2 strongly suggests that the spermatid proteins T1 and T2 could be the precursors of Sp1 and Sp2, respectively.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Evidence for the Existence of Structural Variants of Protein T

Spermatid-specific protein T was obtained in both 0.4 M HCl and 5 M guanidinium chloride extracts from cuttlefish testis chromatin where it represents 28% of the total nuclear basic proteins (1). Protein T can be separated from protamine Sp by fractionation of the guanidinium chloride extract on a C18 µBondapak column (1) or from somatic-type histones by fractionation of the acid extract on a C8 Ultrapore column (Fig. S1A). In this case, protein T is eluted first (fraction 1), before histone H1 (fraction 2) and core histones (fraction 3) (Fig. S1B). Protein T migrates as several bands on urea/

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* Portions of this paper (including "Experimental Procedures," Figs S1–S6, and Tables S1–SV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Cuttlefish Spermatid-specific Protein T

We have shown previously that this apparent heterogeneity had to be related to different levels of phosphorylation of the protein (1).

The first data obtained from automated Edman degradation of whole protein T revealed three microheterogeneities in the amino-terminal sequence of the protein (residues 1 to 50) at positions 7 (Ser/Thr), 12 (Ala/Val), and 16 (Glu/Asp) and the amino-terminal region, at positions 2, 9, 14, and 19.

These preliminary results established clearly the asymmetry of the protein since all the hydrophobic residues are accumulated in the amino-terminal part of the molecule (residues 1–21) and all the arginine residues are accumulated in the carboxyl-terminal part (residue 22 to the carboxyl terminus). The 4 lysine residues are regularly distributed within the amino-terminal region, at positions 2, 9, 14, and 19.

In the early stages of our work, the cleavage of dephosphorylated protein T with endoproteinase Lys-C was intended to obtain the carboxyl-terminal basic fragment K-4 (residues 20–77 or 78) necessary to establish the structural relationship between spermatidal protein T and protamine Sp. Indeed, the amino acid composition of K-4 only differs from that of protamine Sp by 2 additional residues of glycine (Table I).

Furthermore, the limited chymotryptic digests of K-4 and protamine Sp have almost identical electrophoretic patterns (Fig. S2).

Separation of Protein T Variants

The separation of the two variants was achieved on a C18 µBondapak column, using a stepwise gradient of acetonitrile (Fig. S3). In these conditions, variants T1 and T2 were only obtained in pure form in fractions 4 and 6, respectively. These variants of similar electrophoretic mobilities and phosphorylated at different degrees. From the yields of the peptides obtained by cleavage of the whole protein T with endoproteinase Lys-C and the yields of phenylthiohydantoin-Met and phenylthiohydantoin-Lys at the first cycle of Edman degradation of T1 and T2, the relative amount of each variant was calculated as follows: T1a, 60.5%; T1b, 6.2%; T2a, 32.1%; and T2b, 1.2%.

Enzymatic Hydrolyses

The carboxyl-terminal sequences of T1 and T2 were deduced from structural data provided by peptides generated by enzymatic cleavages of each variant using chymotrypsin at pH 5.0 and Astacus fluviatilis proteinase. The elution patterns of the enzymatic digests of cuttlefish proteins T1 and T2 are reported in Figs. S4–S6. The amino acid compositions of the peptides useful for the elucidation of the complete sequence are reported in Tables SIII and SIV.

Cleavage with Chymotrypsin—The chymotryptic peptides generated from each variant cover the entire sequence of the protein. All the tyrosyl bonds were cleaved as expected from the specificity of chymotrypsin at pH 5.0 (21). Nevertheless, in the amino-terminal region of the protein, two nonspecific cleavages were observed: the bonds Leu15-Glu16 and Met16–Leu17.

Automated Edman Degradation

Each protein T variant was submitted to automated Edman degradation. The data obtained up to cycle 42 (Table SII) corroborate the preliminary results obtained from the whole protein T and allow us to identify unambiguously the amino acids at positions 7, 12, 16, 18, 34, and 35 in each variant. More importantly, each of the two variants T1 and T2 was proved to be itself a mixture of two molecules, T1a and T1b or T2a and T2b, only differing by the presence of a methionine residue at the amino-terminal end of the major molecular species (T1a and T2a). Thus, cuttlefish spermatid-specific protein T consists, in fact, of a mixture of four structural variants of similar electrophoretic mobilities and phosphorylated at different degrees. From the yields of the peptides obtained by cleavage of the whole protein T with endoproteinase Lys-C, the tetraphosphorylated forms are minor.

TABLE I

| Protein or peptide | T1  | T2  | K-4  | Sp1  | Sp2  |
|-------------------|-----|-----|------|------|------|
| Asp               | 1.3 (1) | 2.0 (2) | 5.6 (7) | 6.9 (7) | 6.7 (7) |
| Thr               | 0.8 (1) |       | 1.5 (1) | 1.1 (1) | 1.1 (1) |
| Ser               | 6.3 (9) | 5.6 (8) | 1.0 (1) | 3.8 (5) | 8.3 (5) | 5.1 (6) |
| Gly               | 2.4 (2) | 2.2 (2) | 2.0 (2) |       |       |       |
| Pro               | 1.0 (1) | 1.0 (1) | 1.0 (1) | 3.8 (4) | 43.4 (43-44) | 44.0 (44) | 43.9 (43) |
| Ala               | 3.3 (9) | 2.4 (2) |       |       |       |       |
| Val               | 1.0 (1) | 1.9 (2) |       |       |       |       |
| Met               | 1.2 (3) | 0.8 (2) |       |       |       |       |
| Leu               | 3.1 (3) | 3.8 (4) |       |       |       |       |
| Tyr               | 4.6 (5) | 3.4 (5) | 3.8 (5) | 43.4 (43-44) | 44.0 (44) | 43.9 (43) |
| Lys               | 3.9 (4) | 3.9 (4) |       |       |       |       |
| Arg               | 44.0 (44) | 45.0 (43) |       |       |       |       |
| Total residues    | 78  | 77  | 58-59 | 57  | 56  |
| Calculated mass (Da) | 10,632.5 | 10,479.1 |       | 8412.8 | 8256.6 |
| Measured mass (Da) | 10,788 ± 2 | 10,788 ± 2 | 8410 ± 1 | 8253 ± 1 |       |
Two homologous peptides showed that they differ only by 1 residue, which led to the alignment of the chymotryptic proteinase, only the carboxyl-terminal peptides T1 A-1 (residues 56–77) and T2 A-1 (residues 56–78) were useful in establishing the complete sequences of spermatid-specific protein variants T1 and T2. The amino acid composition and the automated Edman degradation of these peptides C-4, C-5, and C-6 and in subsequently establishing the complete sequences of the spermatid-specific protein variants T1 and T2. The difference of 80 Da results of automated Edman degradation (Table SV) correspond to the expected mass for the peptides C-4. It can therefore be deduced that the residues of serine at positions 56 in variant T1 and 56 in variant T2 are not phosphorylated.

**Complete Sequences**

The amino acid sequences of the variants T1 and T2 are presented in Figs. 2 and 3. The alignment of the chymotryptic peptides C-2, C-3, and C-4 was established by sequence analysis of whole protein T and confirmed by mass spectrometric data for variant T1 and by nucleotide sequence of the cDNA for variant T2. These two highly related peptides of 78 and 77 residues, respectively, differ only by four conservative substitutions at positions 7, 12, 16, and 18, two inversions Ser ↔ Arg at positions 34–35 and 56–58, and the deletion of 1 residue of arginine in variant T2. The asymmetrical distribution of the hydrophobic and basic residues in these molecules determines two well defined domains: an amino-terminal domain (residues 1–21) devoid of arginine and aromatic residues and containing all the aliphatic hydrophobic residues and a highly basic domain (residues 22–77 or 78) which contains 77% of arginine and all the tyrosine residues present in the protein.

Predictive methods (22–27) indicate a high probability of α-helical structure for the amino-terminal domain and the presence of three β turns in the carboxyl-terminal domain. The hinge region between these two domains (residues 18–22) has a high probability of β turn structure.

On the other hand, in order to identify the phosphorylation sites of the protein T, each variant and their peptides C-2 and C-5 were treated according to the procedure described by Meyer et al. (19). Serine residues at positions 7, 8, 28, 35, 39, and 68 in variant T1 and at positions 8, 28, 34, 39, and 67 in variant T2 were found partially phosphorylated. All the phosphorylation sites except the serine residues at positions 7 and 8 are located in an Arg-X-Ser sequence specifically recognized by the cAMP-dependent protein kinase where X is any amino acid except proline (28, 29). Most of the phosphorylated serine residues are located in the amino-terminal half of spermatid-specific protein T. Only 1 phosphorylated serine, at position 67 or 68 according to the variant, is present in the carboxy-terminal half. Moreover, it must be emphasized that the nonphosphorylated form of protein T has not been found in testis chromatin from sexually mature cuttlefish.

These results are consistent with the mass spectrometric data, which indicate the presence of three major molecular species in variant T1, corresponding to diphasorylated, triphosphorylated, and tetraphosphorylated forms of the protein (Fig. 1). Among these, the triphosphorylated form was found to be predominant.

In addition, the phosphorylation sites present in the basic domain (residues 22–78 or 77) of protein T, at positions 28, 34 or 35, 39, and 67 or 68, are identical with those found in phosphorylated protamine Sp, at positions 7, 13 or 14, 18, and 46 or 47 (Fig. 4). The phosphorylated protamine Sp appears in elongated spermatids (1, 2) in replacement of the phosphorylated protein T present in round and elongating spermatids.
**FIG. 2.** Complete amino acid sequence of cuttlefish spermatid-specific protein variant T1. Methods used for the determination of the sequence are indicated as follows: **boldface** residues, automated Edman degradation of whole protein T (mixture of the variants); +, automated Edman degradation of separated variants; −, automated Edman degradation of peptides. C- and A-, peptides derived from cleavage with chymotrypsin at pH 5.0 and A. fluviatilis proteinase, respectively.

**FIG. 3.** Complete amino acid sequence of cuttlefish spermatid-specific protein variant T2. Methods and nomenclature of peptides are as indicated in the legend to Fig. 2.

**FIG. 4.** Comparison of the sequences of cuttlefish spermatid-specific proteins T1 and T2 and cuttlefish protamines Sp1 and Sp2 (3). The sequences have been aligned for maximum homology. **Boxes** indicate changes in amino acid residues. **Arrows** mark the beginning of the sequences of the protamine variants. The carboxyl-terminal sequence of T1 corresponds to the protamine variant Sp1, and the carboxyl-terminal sequence of T2 corresponds to the protamine variant Sp2.
matids. Then, during the maturation process of the spermatozoa, which occurs in the epididymis, the protamine Sp is completely dephosphorylated. Very few studies concerning the phosphorylation of basic nuclear proteins during spermiogenesis have been performed, and the role of this modification remains to be elucidated.

Cuttlefish spermatid-specific protein T is the first spermatid-specific protein among invertebrates to be fully characterized. With respect to its amino acid composition, this protein appears homologous to protein I-1 isolated from immature testes of the squid I. argentinus (6). On the other hand, cuttlefish protein T is not structurally related to other spermatid-specific proteins characterized and sequenced to date in dogfish (16, 30) and mammals (31–37).

The most striking feature of cuttlefish spermatid protein variants T1 and T2 is the complete structural identity of their carboxyl-terminal basic domain (residues 22-78 or 77) with the protamine variants Sp1 and Sp2 isolated from cuttlefish spermatid (3) (Fig. 4). This strongly suggests that T1 and T2 could be the precursors of Sp1 and Sp2, respectively. A similar situation has also been observed in mouse and man where P2-type protamines are synthesized as precursors (12–15).

The mechanism of the transition spermatid-specific protein T → protamine Sp remains unknown. Several hypotheses have to be considered. First, the protamine Sp would derive from protein T by a specific proteolysis of the Gly24-Arg29 bond in the sequence Met/Leu15-Lys-Gly-Gly-Arg40. This region, which has a high probability of β turn conformation constitutes the hinge between the amino-terminal domain in α-helical structure and the basic carboxyl-terminal domain. Analogous sequences with highly structured flanking regions are the sites of proteolytic processing of polypeptide hormone precursors (38) or of biologically active proteins of the adenovirus 2 (39). It must be pointed out that the occurrence in elongated spermatids of protamine Sp, phosphorylated at the same sites as is protein T, would support this hypothesis. Second, the successive emergence of spermatid-specific protein T and of protamine Sp could arise from a regulation mechanism of gene expression. These proteins could be encoded by two different genes or by a unique gene. In the latter case, there would be several mRNAs directly derived from this gene or a primary transcript, which, after processing, would give rise to different mRNAs.

Acknowledgments—We are grateful to A. Hémez, M. J. Dupire, and O. Sorokine for their skillful technical assistance and to T. Ernoult for editorial assistance. We also thank D. Belaiche and O. Hennedouche from the Service Commun de Séquence Peptidique des Unités 409 du Centre National de la Recherche Scientifique et 16 de l’Institut National de la Santé et de la Recherche Médicale for the sequenator runs.

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**Experimental Procedures**

Isolation and purification of spermatozoa-specific protein T. Spermatozoa-specific protein T was extracted from both hamster and mouse testes, as described previously [1]. Spermatozoa-specific protein T was isolated from testicular tissue from male hamsters and mice by a centrifugal differential centrifugation procedure. The protein was then purified by affinity chromatography on a column of protein A-Sepharose 6B.

**Results and Discussion**

1. **Table 1**

   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               | (6)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 | 6                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                | Tt                |

2. **Table 2**

   | Table 2: Antigenic Sequences of Protein T Variants T1 and T2
   |---|---|---|---|---|---|
   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |

3. **Table 3**

   | Table 3: Antigenic Sequences of Protein T Variants T1 and T2
   |---|---|---|---|---|---|
   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |

4. **Table 4**

   | Table 4: Antigenic Sequences of Protein T Variants T1 and T2
   |---|---|---|---|---|---|
   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |

5. **Table 5**

   | Table 5: Antigenic Sequences of Protein T Variants T1 and T2
   |---|---|---|---|---|---|
   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |

6. **Table 6**

   | Table 6: Antigenic Sequences of Protein T Variants T1 and T2
   |---|---|---|---|---|---|
   | Cycle | Identified variant | Identified variant | Identified variant | Identified variant | Identified variant |
   |-------|-------------------|-------------------|-------------------|-------------------|-------------------|
   |       | (1)               | (2)               | (3)               | (4)               | (5)               |
   |       | 1                 | 2                 | 3                 | 4                 | 5                 |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |
   |       | Tt                | Tt                | Tt                | Tt                | Tt                |

**Supplementary Material**

**Cytotoxic Activity**

The cytotoxic activity of the spermatozoa-specific protein T was tested using a microtest assay, as described previously [1]. The assay was performed by incubating the protein with a series of target cells, and the inhibition of cell growth was measured using a spectrophotometric assay.

**References**

[1] A. Martin, J. Biol. Chem., 255 (1980), 10129-10135.
Table 5

| Peptide | Cycle number | Identified residue | Identified residue | Identified residue | Identified residue | Identified residue | Identified residue |
|---------|--------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|         |              |        |        |        |        |        |        |
| 1       | R            | 85     | S      | 228    | R      | 171    | S      | 106    |
| 2       | R            | 103    | R      | 163    | R      | 91     |        |        |
| 3       | R            | 105    | R      | 118    | R      | 169    | R      | 108    |
| 4       | R            | 118    | R      | 165    | R      | 170    | R      | 109    |
| 5       | R            | 131    | R      | 134    | R      | 177    | R      | 137    |
| 6       | R            | 134    | R      | 131    | R      | 131    | R      | 131    |
| 7       | R            | 134    | R      | 131    | R      | 131    | R      | 131    |
| 8       | R            | 134    | R      | 131    | R      | 131    | R      | 131    |
| 9       | R            | 134    | R      | 131    | R      | 131    | R      | 131    |
| 10      | R            | 133    | R      | 131    | R      | 131    | R      | 131    |
| 11      | S            | 24     | S      | 12     | R      | 116    | R      | 142    |
| 12      | R            | 74     | Y      | 25     | R      | 105    | S      |        |
| 13      | R            | 90     | S      | 6      | R      | 114    | Y      | 101    |
| 14      | R            | 91     | R      | 35     | R      | 10     | R      | 85     |
| 15      | T            | 20     | R      | 42     | R      | 91     |        |        |
| 16      | R            | 14     | R      | 14     | R      | 106    |        |        |
| 17      | R            | 31     | Y      | 65     |        |        |        |        |
| 18      | R            | 31     | R      | 100    |        |        |        |        |
| 19      | R            | 26     | R      | 126    |        |        |        |        |
| 20      | S            | 30     | R      | 109    |        |        |        |        |
| 21      | R            | 85     |        |        |        |        |        |        |

Figure 51 - Fractionation by reverse-phase HPLC of the 0.4 M HCl extract of cuttlefish saliva

Figure 52 - Comparative electrophoretic analysis of the C-terminal and N-terminal residues of the C-terminal specific protein T with corresponding proteins from other cephalopods.

Figure 53 - Amino acid composition of the C-terminal specific protein T from cuttlefish saliva.

Figure 54 - Fractionation by reverse-phase HPLC of the N-terminal specific protein from cuttlefish saliva.
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Figure 5A - Fractionation by reverse-phase HPLC of peptides obtained by digestion of protein T by trypsin. HPLC was performed as described in the legend to Figure 5A.

Figure 5B - Electrophoretic analysis of chromatographed peptides from Figure 5A. Electrophoresis was performed as described in the legend to Figure 5B. The spots in the gel were those corresponding to the peptides in Figure 5A.

Figure 5C - A comparison of the HPLC profiles of the peptides from Figures 5A and 5B. The peptides were subjected to HPLC as described in the legend to Figure 5C. The profiles shown in Figure 5C were recorded at 220 nm (---). Only, C-terminal peptide A1 was used for sequence determination.