Revised Amino Acid Sequence for a Heat-Stable Enterotoxin Produced by an Escherichia coli Strain (18D) that is Pathogenic for Humans

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The amino acid sequence of heat-stable enterotoxin produced by enterotoxigenic Escherichia coli 18D has been revised. Amino acids originally assigned to positions 11 and 18, i.e., Tyr and Asn, respectively, were found to occupy positions 18 and 11, respectively. Thus all heat-stable enterotoxins composed of 18 amino acids sequenced to date from human, porcine, and bovine isolates of E. coli have identical primary structures, i.e., Asn-Thr-Phe-Tyr-Cys-Glu-Leu-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr. Furthermore, all 18- and 19-amino-acid heat-stable enterotoxins from E. coli share an almost identical core sequence, i.e., 14 of the 15 carboxy-terminal amino acid residues are identical.

Enterotoxigenic Escherichia coli can elaborate several toxins which cause intestinal secretion and diarrhea (2, 9). In addition to a high-molecular-weight, cholera toxin-like, heat-labile enterotoxin, these include two distinct families of low-molecular-weight, heat-stable enterotoxins designated STa (or STI) and STb (STII) (2, 13). These two families are genetically distinct and can be differentiated by host susceptibilities, by differing solubilities in methanol, and by mechanism of action (2, 9, 10). The suckling mouse-active, methanol-soluble STas seem to fall into two classes, one composed of enterotoxins containing 18 amino acid residues and the other containing 19 residues (1, 3, 8, 11, 12, 16). These two STa classes differ in the amino-terminal end of the molecule and share antigenic determinants and biologically active core sequences which reside in the carboxy-terminal amino acid residues (3, 17).

The first STa to be purified to homogeneity (15) and sequenced (3) was obtained from a human isolate of enterotoxigenic E. coli, strain 18D. It was reported by one of us (3) to consist of 18 amino acid residues and to contain Tyr in position 11 and Asn in position 18, i.e., 11-Tyr, 18-Asn. The assignment of Asn to the carboxy terminus was based upon the presence of 2 mol of Asn per mol of toxin; the unassigned Asn was placed at residue 18 although at that time it was not possible to measure a released phenylthiohydantoin-aminoc acid at the 18th cycle of the Edman degradation. Carboxypeptidase data demonstrated the release of Asn.

Subsequently, stable toxins have been purified and sequenced by other investigators from other human, bovine, and porcine isolates (1, 8, 11, 12, 16). Currently, gene analysis (14) and protein sequence data on the various 18-amino-acid STas produced by other strains consistently differ from our original sequence in the 11th and 18th (carboxy-terminal) residues, i.e., 11-Asn, 18-Tyr (Fig. 1).

Since all but one (16) of these sequences originated from toxins produced by other enterotoxigenic E. coli strains, we felt the difference could be explained by the existence of at least two distinct genomes. Furthermore, Kliepststein et al. (6) synthesized STa according to our published sequence, 11-Tyr, 18-Asn, and obtained a toxin as active in the suckling mouse bioassay as the native toxin. Immunization with this synthetic peptide provided protection against challenge with purified STa, synthetic STa, and enterotoxigenic E. coli strains of human origin known to produce STa (6). Thus, we had at that time no reason to doubt the original sequence.

In an effort to determine the minimum toxin fragment required for full biological activity, we attempted to remove the carboxy-terminal residue from native toxin with carboxypeptidase Y but were unsuccessful. Upon carboxymethylation of the reduced toxin, however, we clearly demonstrated release of Tyr by carboxypeptidase. This observation was in agreement with data presented by Dreyfus et al. (4) which showed that five STas purified from different strains contained carboxy-terminal Tyr. On the basis of this observation and the growing number of sequenced STas uniformly containing 11-Asn, 18-Tyr, we decided to resequence STas from strain 18D.

Automated Edman degradation was performed on a lot of toxin purified (15) after the original sequenced lot, using a Beckman spinning cup sequencer. We found Asn at position 11 and (with very low yield) Tyr at position 18, the carboxy terminus. In conjunction with the carboxypeptidase data we concluded that the amino acid sequence from the preparation indeed differed from that of the original toxin. We therefore decided to resequence the lot of toxin which was used in the original published work (3). Because of the small amount of toxin remaining and because of the solvent losses associated with the spinning cup sequenator, an Applied Biosystems automated gas-phase protein sequencer was used.

An initial sequence determination on the original lot of purified STa identified Asn at position 11 and Tyr at position 18, although the overall release of phenylthiohydantoin-amino acids was low (only 10% of the amino-terminal residue was released as phenylthiohydantoin-Asn). Thus, a modification was incorporated in the procedure. The immobilized toxin was exposed to gaseous trifluoroacetic acid before sequence determination to increase accessibility of the N-terminal amino acid. Hence, a second sequence determination was made on the original toxin lot. Upon reanalysis, the identical sequence was obtained, with an

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approximately threefold increase in the yield of derivatized amino acids. On the basis of these recent carboxypeptidase and sequence data, we therefore conclude that the original sequence is in error. The correct sequence is: Asn-Thr-Phe-Tyr-Cys-Glu-Leu-Cys-Glu-Cys-Tyr-Pro-Ala-Cys- Ala-Gly-Cys-Asn.

This revision greatly simplifies the relationship between the STAs sequenced to date, since complete homology is now found with all 18-amino-acid toxins examined, and one need not postulate two classes of 18-amino-acid-containing STAs. With the exception of the fourth amino acid residue from the carboxy terminus, the carboxy-terminal 15 amino acids are identical in all 18- and 19-amino-acid STAs thus far sequenced (Fig. 1b and c).

Recently, Takao et al. (16) have purified and sequenced STAs from the same strain, enteroenterotoxigenic E. coli 18D, that we have described. Their sequence demonstrates Asn at position 11 and Tyr at position 18, differing from our original sequence but in total agreement with results presented here. In further studies, this group synthesized STAs with both sequences (5, 17, 18). Both preparations demonstrated biological activity, but in their hands the synthesized STAs with 11-Tyr, 18-Asn was 30 to 40 times less active in the suckling mouse bioassay than the synthesized peptide with the 11-Asn, 18-Tyr sequence (17). This latter peptide was as active as native purified toxin.

As mentioned above, Klipstein et al. (6) have synthesized STAs according to our original published sequence, i.e., 11-Tyr, 18-Asn, and found their synthetic toxin to have the same antigenic properties and biological activity as native toxin, which we now believe differs at residues 11 and 18. In further studies, Klipstein et al. (7) showed that the biological and antigenic properties of synthetic STAs can be easily altered by rearrangement of the disulfide bridges. Thus, the apparent differences in data of the Osaka group (5, 17, 18) and Klipstein et al. (6) may reflect the differences in procedures used by the two groups to synthesize these toxins. It is apparent that the presence of six Cys residues may require very specific synthesis conditions for the generation of synthetic peptides with disulfide bridges in the appropriate positions to yield biological activity comparable to that of native toxin. Thus, it is possible that the methods used by the Osaka group to successfully synthesize fully active 11-Asn, 18-Tyr STAs may not be optimal to yield the appropriate tertiary structure for fully active 11-Tyr, 18-Asn STAs. As was done by Ronneberg et al. (11), it is interesting to speculate whether the amino acids at positions 11 and 18 play a significant role in the biological activity of the native toxin produced in vivo. Although we cannot answer this question definitively, the synthetic toxin data of Klipstein et al. (6) would lead us to believe that positions 11 and 18 can be occupied by Asn or Tyr interchangeably with little change in the biological activity of the toxin.

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**LITERATURE CITED**


