Localization of Phosphorylated Highly Acidic Regions in the NH2-terminal Half of Nucleolar Protein C23*

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Protein C23 (Mr = 110,000), a putative nucleolus organizer protein, contains multiple sites of phosphorylation which are located in regions rich in aspartic and glutamic acid (Mamrack, M. D., Olson, M. O. J., and Busch, H., Biochemistry (1979) 18, 3381-3386). To localize the phosphorylated sequences (approximately 35 phosphoserines and 1 phosphothreonine) along the polypeptide chain, protein C23 was subjected to cleavage by N-bromosuccinimide at various molar ratios to tyrosine. At relatively high ratios (e.g. 25:1), a Mr, 60,000 fragment (60K) was predominant along with several lower molecular weight fragments. When 32P-labeled protein C23 was cleaved with N-bromosuccinimide under these conditions all of the 32P radioactivity in the parent molecule was contained in the 60K fragment. The 60K fragment was purified by gel filtration and reverse-phase high performance liquid chromatography. Protein C23 and the 60K fragment had approximately the same isoelectric points and similar amino acid compositions. By automated Edman degradation, protein C23 and the 60K fragment both contained amino terminal sequences, Val-Lys-Leu-Ala-Lys-Ala-Gly-Lys-Thr-, thereby placing the 60K fragment on the NH2-terminal end of the molecule. The 60K fragment was further cleaved by submaxillaris protease into a 15K NH2-terminal and 45K carboxyl-terminal fragment. The two fragments contained approximately equal amounts of 32P radioactivity. These data indicate that all of the phosphorylated groups and all of the phosphorylated acidic regions are located in the NH2-terminal half of the C23 polypeptide chain. The NH2-terminal 15K fragment contains roughly half of the sites of phosphorylation.

The nucleolus, which is the site of ribosome biogenesis (Busch and Smetana, 1970) contains proteins of a special class: the “acidic cluster” proteins. Two of these proteins, designated B23 and C23 (Orrick et al., 1973), contain regions of high net negative charge (Mamrack et al., 1977) due to the presence of nearly contiguous sequences containing only aspartic acid, glutamic acid, and phosphoserine or phosphothreonine. For example, protein C23 contains a tryptic peptide with the sequence Ala-Ala-Pro-Ala-Ala-Pro-Ala-Ser(P)-Glu-Asp-Glu-Asp-Glu-Asp-Asp-Glu-Asp-Asp-Glu-Asp-Asp-Glu-Asp-Ser(P)-Glu-Glu-Ser(P)-Glu-Glu-Glu-Val-Met-Glu-Ile-Thr-Pro-Ala-Lys (Mamrack et al., 1979).

Protein C23 is predominantly localized to the nucleolus in interphase cells (Olson et al., 1981) and is found at the nucleolus organizer regions of chromosomes (Lischwe et al., 1981). Protein C23 also preferentially stains with silver on polyacrylamide gels (Lischwe et al., 1979) under the same conditions that portions of the interphase nucleolus (Busch et al., 1979) and the nucleolus organizer regions of chromosomes (Howell et al., 1975; Goodpasture and Bloom, 1975) stain with silver. Thus, the protein may play a role in organizing the chromatin and ribonucleoprotein components of the nucleolus.

Organization of the nucleolus by this class of protein would require interaction with various macromolecules; e.g. the highly acidic regions may interact with histones or ribosomal proteins and basic or neutral regions may interact with DNA. Observations on the partitioning of protein C23 in various nucleolar fractions suggest that this may be the case. For example, a fraction of the protein is extracted with low ionic strength buffers (Rothblum et al., 1977), whereas another fraction is associated with preribosomal ribonucleoprotein particles (Prestayko et al., 1974; Olson et al., 1974b). More recent studies suggest that protein C23 preferentially binds cloned rDNA sequences upstream from the 18 S coding region. A nucleolar chromatin-associated phosphoprotein was previously designated C18 (Yeoman et al., 1973; Olson et al., 1979), but data presented in this paper suggest that proteins C18 and C23 are identical and that C18 is a tightly bound form of C23. Thus, protein C23 appears to interact with chromatin as well as with ribonucleoprotein particles, possibly serving as a bridge between the two components.

The phosphorylation of protein C23 in regions which already carry a high negative charge is a puzzling phenomenon. The added negative charge may be expected to facilitate interactions of these regions with basic regions of other proteins. Since the level of phosphorylation of protein C23 correlates with the synthetic activity of the nucleolus (Kang et al., 1975; Ballal et al., 1975; Olson et al., 1978), the role of phosphorylation may be a regulatory one. These studies are an initial attempt to cleave protein C23 into structural domains and to locate the phosphorylated acidic regions along the polypeptide chain.

MATERIALS AND METHODS

1. M. O. J. Olson, Z. Rivers, and S. T. Case, manuscript in preparation.

2. Portions of this paper (including "Materials and Methods," part of "Results," Figs. 3-5, and Table III) 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 available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1849, cite the authors, and include a check or money order for $3.90 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
RESULTS AND DISCUSSION

Cleavage of Protein C23 with NBS—In order to optimize conditions of cleavage, protein C23 was treated with various concentrations of NBS, calculated as ratios to tyrosine. By amino acid composition each molecule of protein C23 was calculated to contain 8 residues of tyrosine and only traces of tryptophan (Tables I and II) based on \( M_r = 110,000 \) (Tsutsui et al., 1980). All reactions were carried out at room temperature in 1968. Upon radiometric analysis for 1 h, although preliminary kinetic studies indicated that the reaction was complete in a few minutes. At relatively low ratios of NBS:tyrosine (9:1 or lower) protein C23 was cleaved into four major fragments estimated to have \( M_r = 88,000, 78,000, 70,000, \) and 60,000 (Fig. 1). In addition, four smaller fragments, 27K, 21K, 19K, and 12K, appeared. At the 9:1 ratio the 70K fragment was predominant. At higher NBS:Ty ratios, starting at approximately 12:1, the 60K fragment became the predominant fragment (Fig. 1). This fragment was stable up to a ratio of approximately 25:1, above which it also began to disappear. Also appearing at higher NBS concentrations were two other lower molecular weight bands (23K and 13K), while the 27K band disappeared. No bands of intermediate molecular weight (between 27,000 and 60,000) were seen at any of the NBS concentrations. At very high ratios (30:1 or above) the 60K as well as lower molecular weight fragments began to disappear. At least two observations regarding the chemistry of the reaction require comment. First, certain potential cleavage sites appeared more susceptible to cleavage than others. By amino acid analysis, protein C23 was shown to contain 8 tyrosine residues and only traces of tryptophan after hydrolysis with mercaptoethanesulfonic acid. However, sequence studies in progress have revealed the presence of a tryptophan residue on the NH₂-terminal side of the 12K fragment.4 If cleavage occurs at all of these 9 sites, a minimum of 10 fragments should be seen. However, under conditions which are optimal for the production of the 60K fragment, only 6 or 7 major fragments were seen. The sum of the molecular weights of these fragments is more than 110,000, implying partial cleavage. It can be seen from Figs. 1 and 2 that higher concentrations of NBS were required to produce the 60K fragment than to generate the extensions of the 60K fragment (70K, 78K, and 88K). Thus, the bond that connects the 60K fragment to the carboxyl-terminal half of the molecule must be more stable (or less susceptible) than the bonds that are cleaved to produce the larger fragments.

The second observation on the NBS cleavage reaction is that much higher than the theoretical values of NBS:tyrosine were required for extensive cleavage: i.e. more than 20 mol of NBS/mol of tyrosine as opposed to the theoretical value of 3:1 (Ramachandran and Witkop, 1967). The high concentrations of NBS required may be due to the NBS reacting with methionine and histidine, the latter of which is not cleaved unless the reaction mixture is heated at pH 3–4 at 100°C.

Location of Phosphorylated Residues—When protein C23 was analyzed for phosphoseryl and phosphothreonyl by the \( \beta \) elimination procedure (Richardson et al., 1978), it was found that the protein contained 3.5 mol % phosphoseryl and 0.1 mol % phosphothreonine (Table I). On a residue/110,000 dalton basis, this is approximately 35 residues of phosphoserine and 1 residue of phosphothreonine. Analyses of the partial acid hydrolysate of \( ^{32}P \)-labeled protein C23 by thin layer chromatography confirmed that the bulk of the radioactivity was in phosphoserine. Radioactive phosphothreonine was not found.

Previous studies (Mamrack et al., 1977) showed that in tryptic digests of protein C23, more than 85% of the \( ^{32}P \) radioactivity was in highly acidic peptides. To locate these along the polypeptide chain, \( ^{32}P \)-labeled protein C23 was digested with various ratios of NBS:Ty. Fig. 2a indicates that as the NBS ratio was increased, virtually all the \( ^{32}P \) radioactivity moved into the 60K fragment. At lower NBS concentrations some \( ^{32}P \) radioactivity was found in the 70K, 78K, and 88K fragments. By densitometric scans of the autoradiograph it was shown that all of the radioactivity in the original C23 molecule was transferred to the 60K fragment at NBS:Tyr ratios of 24:1 (Fig. 2b). Thus, all of the \( ^{32}P \)-labeled groups and the acidic regions where they reside are located in approximately one-half of the molecule, i.e. the 60K fragment. Furthermore, since the radioactivity resides only in the large fragments (70K, 78K, and 88K) at low concentrations of NBS, these fragments are probably extensions of the 60K fragment.

Characteristics of the 60K Fragment—The similarity of the 60K fragment to the parent molecule is illustrated by a comparison of amino acid compositions. Table I indicates that the mole percentages of acidic and basic amino acids are similar in both polypeptides. The number of potentially acidic amino acids greatly exceeds the number of basic amino acids. Therefore, since the isoelectric points are only slightly acidic (approximately 6.4), a large number of the former must be present as glutamine and asparagine.

To demonstrate that the highly acidic phosphorylated regions can be accommodated in the 60K fragment the residues of the major tryptic phosphopeptides are tabulated in Table II along with residue numbers of C23 and 60K. The tryptic

| Amino acid | C23 | C18 | 60K | 15K | 45K |
|------------|-----|-----|-----|-----|-----|
| Asx        | 11.7| 11.3| 13.2| 9.9 | 14.6|
| Thr        | 5.5 (5.4)| 5.1 | 5.9 | 6.7 | 4.8 |
| Ser        | 5.8 (2.3)| 6.1 | 6.4 | 9.0 | 7.1 |
| Gix        | 18.1| 18.0| 19.2| 17.2| 18.4|
| Pro        | 5.2 | 5.6 | 8.5 | 8.7 | 7.0 |
| Gly        | 9.8 | 10.4| 7.5 | 7.0 | 7.0 |
| Ala        | 10.0| 9.4 | 11.4| 11.0| 10.9|
| Val        | 5.8 | 5.5 | 6.4 | 7.2 | 5.8 |
| Met        | 1.2 | 1.0 | Trace| Trace| Trace|
| Ile        | 2.4 | 2.7 | 1.8 | 0.6 | 1.1 |
| Leu        | 5.5 | 5.7 | 3.6 | 1.5 | 3.3 |
| Tyr        | 0.8 | 0.5 | Trace| Trace| Trace|
| Phe        | 3.4 | 3.6 | 1.7 | 0.4 | 1.5 |
| Lys        | 11.5| 10.6| 10.6| 17.6| 13.1|
| His        | 0.5 | 0.6 | 0.8 | 0.7 | 1.5 |
| Arg        | 2.8 | 3.8 | 3.7 | 2.5 | 3.8 |
| P-Thrd     | Trace| Trace| Trace| Trace| Trace|
| P-Ser      | 3.5 | —   | —   | —   | —   |
| P-Thre     | 0.1 | —   | —   | —   | —   |

| Asx + Glx/Lys + His + Arg | 2.0 | 2.0 | 2.1 | 1.3 | 1.8 |

* Threonine was corrected for 7% destruction during hydrolysis.
* Serine was corrected for 10% destruction. The number in parentheses indicates value after subtracting the phosphothreonine content.
* Phosphoserine + phosphothreonine were determined by the change in serine and threonine after \( \beta \) elimination.
* —, not determined.
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| Amino acid | C23 | 60K | Acidic tryptic phosphopeptides | Total |
|------------|-----|-----|-------------------------------|-------|
|            | residues/mol | A | B | Ca | Cb | D |     |
| Asx        | 115.9 | 73.4 | 4.2 | 11.9 | 11.8 | 11.6 | 11.8 | 52 |
| Thr        | 33.5  | 32.8 | 0.9 |          |   | 1   |     |     |
| Ser        | 58.7  | 35.6 | 1.1 | 1.7 | 1.3 | 1.3 | 1.1 | 13 |
| Glx        | 179.3 | 106.8 | 6.2 | 13.1 | 12.8 | 13.8 | 21.1 | 67 |
| Pro        | 51.5  | 47.3 | 1.2 | 2.1 | 3.0 | 2.1 | 1.0 | 9  |
| Gly        | 97.1  | 41.7 |          |   |     |     |     |     |
| Ala        | 99.1  | 63.4 | 0.8 | 1.1 | 8  |     |     |     |
| Val        | 57.5  | 35.6 | 1.0 | 1.9 | 1.0 | 1.8 | 1.9 | 8  |
| Met        | 11.9  | Trace | 0.9 |          |   | 1   |     |     |
| Ile        | 23.8  | 10.0 | 1.0 |          |   |     |     |     |
| Leu        | 54.5  | 20.0 | 1.0 |          |   |     |     |     |
| Tyr        | 7.9   | Trace |          |   |     |     |     |     |
| Phe        | 33.7  | 9.4   | 1.0 |          |   | 2   |     |     |
| Lys        | 113.9 | 58.9 | 1.1 | 2.1 | 1.0 | 2.0 | 1.0 | 7  |
| His        | 4.9   | 4.4   |          |   |     |     |     |     |
| Arg        | 27.7  | 20.6  |          |   |     |     |     |     |
| Trp        | (1)*  | ND    |          |   |     |     |     |     |
| P-Ser      | 34.6  | ND    | 1-2*   | 1-3 | 1-3 | 1-3 | 1-2 | 5-13 |
| F-Thr      | 1.9   | ND    |          |   |     |     |     |     |
| Total      | 993   | 545  | 15 | 35 | 37 | 42 | 40 | 169 |

*The phosphorylated tryptic peptides were isolated by DEAE-cellulose and pH 1.8 paper electrophoresis as described by Mamrack et al. (1979). The compositions of peptides Ca and Cb were taken from Mamrack et al. (1979). Peptides A, B, and D are the remaining major phosphorylated acidic components described by Mamrack et al. (1979).

†Phosphorylated residues were not subtracted from serine and threonine residues.

The total value for serine is based on the maximal phosphoserine ratios in each of the peptides. See footnote g.

Methionine and tyrosine are destroyed by NBS treatment.

Based on the finding of trace amounts after mercaptoethanesulfonic acid hydrolysis and the presence of a tryptophan residue encountered in sequence studies.

ND, not determined.

The phosphoserine content is based on the number of electrophoretic forms of the same peptide as calculated according to the method of Mamrack et al. (1979).

Phosphopeptides were isolated by DEAE-Sephadex chromatography followed by pH 1.8 paper electrophoresis (Mamrack et al., 1979). In these peptides, which range in length from 15 to 42 residues, 60–80% of the amino acids are potentially acidic. In addition, each peptide contains from 1 to 3 phosphoserine residues. The phosphoserine content was based on the number of electrophoretic forms of each peptide, i.e., peptides with identical compositions had different electrophoretic mobilities. Table II shows that all 5 acidic phosphopeptides can fit into the 60K fragment, assuming that multiple copies of each are not present. The 5 peptides also have a maximum of 13 phosphoryl groups, or less than half of the phosphoserines in the molecule. Therefore, additional phosphoryl groups must reside in regions other than the highly acidic tryptic peptides.

NH2-terminal Sequences of Protein C23 and the 60K Fragment—Protein C23 was subjected to NH2-terminal sequence analysis on the Beckman sequencer. Valine was the only major NH2-terminal amino acid found in the first cycle. Lysine was found at positions 2, 5, and 8 to make a relatively basic NH2-terminal sequence: NH2-Val-Lys-Leu-Ala-Lys-Ala-Gly-Lys-Thr-. No conclusive identifications were made beyond residue 9, despite several attempts at automated Edman degradation. Although there was some evidence for Asn and Gly at positions 10 and 11, respectively, this has not yet been proven. Asparaginyl-glycyl peptide bonds are converted under acidic conditions to cyclic imides or β-aspartyl peptide bonds which are resistant to Edman degradation (Bornstein and Balian, 1977). Although this bond should be cleavable by hydroxylamine, using this approach has not yet been successful on protein C23. Protein C18 was also subjected to sequencing under the same conditions. The sequence was identical to that of protein C23 for nine cycles, suggesting that they are identical polypeptides. This is further supported by the similarity of amino acid compositions presented in Table I. The isolated 60K fragment also had the same NH2-terminal sequence as protein C23. Thus, the 60K fragment must be placed at the NH2-terminal end of the protein.

Further Cleavage of the 60K Fragment—To further localize the phosphorylated acidic regions the 60K fragment was subjected to limited cleavage with submaxillaris protease which cleaves only at arginine (Schenken et al., 1977). Although the 60K fragment contains numerous arginine residues (Table I), the submaxillaris protease produced two major fragments, 15K and 45K (Fig. 6). NH2-terminal sequence analysis indicated that the 15K fragment had the same NH2-terminal sequence as the parent molecule, thereby placing it at the NH2-terminal end of the molecule.

To determine the distribution of sites of phosphorylation between the two subfragments of the 60K fragment, 32P-labeled material was subjected to submaxillaris protease digestion. After separation by electrophoresis and autoradiography, radioactivity was found in both fragments (Fig. 6). By densitometric scans of the autoradiograph, approximately equal amounts of radioactivity were present in both the 15K and 45K fragments. This indicates that half of the phosphoryl groups in the molecule are located within 15,000 daltons from the NH2-terminus of the molecule. The remainder are distributed in an unknown manner throughout the 45K fragment.

Amino acid compositions of the 15K and 45K fragments were also generally similar (Table I). Although the 15K frag-
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FIG. 2. Distribution of radioactivity in NBS fragments from 32P-labeled protein C23. Digestion and electrophoresis conditions were essentially the same as those in the legend to Fig. 1. a, autoradiograph of sodium dodecyl sulfate Laemmli-type gel containing 12% polyacrylamide. Numbers at bottom indicate ratios of NBS:tyrosine. b, plot of data obtained from densitometric scans of autoradiograph in a. The areas of all peaks of radioactivity are presented as percentage of total area. The areas of the intermediate bands (88K, 78K, and 70K) were combined.

ment contained somewhat higher percentages of basic amino acids, both fragments were rich in charged amino acids which lends support to the idea that charged residues are rather generally distributed throughout the NH2-terminal half of the C23 polypeptide chain.

Protein C23 has also been found associated with nucleolar preribosomal ribonucleoprotein particles (Prestayko et al., 1974; Olson et al., 1974b). In addition, it has been proposed that the highly acidic regions may interact with histones on nucleosomes to possibly organize nucleolar chromatin (Mammack et al., 1979). Since this is a large polypeptide chain, it is possible that separate domains of the molecule participate in interactions with several other classes of nucleolar macromolecules. The finding that the phosphorylated acidic regions reside in one end of the molecule lends support to this idea. Another nuclear protein, high mobility group protein 1 has a continuous run of 41 acidic residues in the COOH-terminal half of the molecule (Walker et al., 1978).

Labhart and Koller (1982) have proposed that in the amplified rDNA of Xenopus oocytes some factor prevents histones from binding to DNA, thereby promoting transcription. Labhart and Koller further suggest that nucleoplasmin (Krohne and Franke, 1980a, b) which is capable of neutralizing the charge on histones (Laskey et al., 1977) is a candidate for that factor. It seems feasible that protein C23 with its highly negatively charged regions could play such a role in mammalian nucleoli.

These studies show that it is possible to cleave protein C23 into large fragments and purify a number of them, which opens up the possibility of performing functional studies on portions of this molecule. Preliminary studies indicate that some of the NBS-derived fragments retain DNA binding activity. Thus, it should be possible to use this or similar methods of cleavage and fractionation to determine which regions of the protein interact with various other macromolecules in the nucleolus.

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Phosphorylated Acidic Regions of Protein C23

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Materials and Methods

Phosphorylated acidic regions of protein C23 were identified using an antibody directed against the N-terminus of the protein. The antibody was raised in rabbits against a synthetic peptide corresponding to the N-terminal sequence of the protein. The antibody was coupled to Sepharose beads and used to purify the phosphorylated regions from a bovine brain extract.

Phosphorylated acidic regions were identified by mass spectrometry. The purified protein was digested with trypsin and analyzed by MALDI-TOF mass spectrometry. The masses of the phosphorylated peptides were compared to the theoretical masses of the peptides calculated from the sequence of the protein.

Results

The antibody was used to purify the phosphorylated acidic regions from a bovine brain extract. The purified protein was digested with trypsin and analyzed by MALDI-TOF mass spectrometry. The masses of the phosphorylated peptides were compared to the theoretical masses of the peptides calculated from the sequence of the protein. The results indicated that the phosphorylated acidic regions were located in the regions of the protein corresponding to the N-terminal sequences of the peptides identified by the antibody.

Discussion

The results suggest that the phosphorylated acidic regions of protein C23 are involved in the regulation of protein function. Further studies are needed to determine the specific function of these regions in the regulation of protein activity.

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Figure 1. Identification of phosphorylated acidic regions of protein C23. The purified protein was digested with trypsin and analyzed by MALDI-TOF mass spectrometry. The masses of the phosphorylated peptides were compared to the theoretical masses of the peptides calculated from the sequence of the protein. The results indicated that the phosphorylated acidic regions were located in the regions of the protein corresponding to the N-terminal sequences of the peptides identified by the antibody.
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Figure 1. Isoelectric focusing of proteins C23 and the 60 K fragment. Myelinated proteins C23 or the 60 K fragment were applied to the tube type isoelectric focusing gel containing 18 polyacrylamide and pH 4.5 ampholytes. Proteins samples were injected to the cathode end and focusing was carried out for 12 h. The pH gradient was determined by one of a microelectrode applied directly to the gel at 0.5 cm intervals. The gel was sliced into 0.5 cm sections and the slices were counted in a scintillation counter. (a) protein C23; (b) 60 K fragment.

Figure 2. HPLC of the bombesin-like protease digest of the 60 K fragment. The 60 K fragment was digested with enzyme as described in the Methods and Materials section, freeze dried and applied to the HPLC column as in Figure 3a. The peaks containing essentially pure 65 K and 60 K fragments are indicated. FF indicates flow through.

Table III: HPLC TERMINAL SEQUENCE ANALYSIS OF PROTEINS C23 AND C6 AND THE 60 K AND 15 K FRAGMENTS

| Step | C23 | C6 | 60 K | 15 K |
|------|-----|----|------|------|
| 1    | Val | Val | Val  | Val  |
| 2    | Lys | Lys | Lys  | Lys  |
| 3    | Leu | Leu | Leu  | Leu  |
| 4    | Ala | Ala | Ala  | Ala  |
| 5    | Lys | Lys | Lys  | Lys  |
| 6    | Ala | Ala | Ala  | Ala  |
| 7    | Gln | Gln | Gln  | Gln  |
| 8    | Leu | Leu | Leu  | Leu  |
| 9    | Thr | Thr | Thr  | Thr  |

1The HPLC-terminal sequences of C23, 60 K and the 15 K fragment were analyzed only by HPLC of free amino acids released by the sequencer. Values in parentheses indicate total yield of free amino acids recovered. Theoretical values are: C23, 12.5 K mole; C6, 1.5 K mole; 60 K, 1.2 K mole; 15 K, 1.2 K mole.

2Qualitative analysis of sequencer steps by gas chromatography are listed, lysine is not detected by this technique.

3Trypsin digestion was conducted in 95% ethanol and analyzed on the amino acid analyzer. Under these conditions, tyrosine is converted to alanine, methionine is dimethylated and threonine is converted to x-methylthreonine (DeLini et al., 1975).