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Model studies of enzymatic NH_2-terminal acetylation of proteins with des-$N^{\alpha 1}$-acetyl-α-melanotropin as a substrate

(calf lens/α-amino acetylation/synthetic peptide)

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ABSTRACT **The present study describes the acetylation by an enzyme present in calf lens of a synthetic tridecapeptide [analogous to α-melanotropin (α-melanocyte stimulating hormone) but lacking the naturally occurring NH_2-terminal acetyl group: des-$N^{\alpha 1}$-Ac-α-melanotropin]. The reaction is specific for the α-amino group of the NH_2-terminal amino acid. The minimum length required for the substrate to become acetylated appears to be a sequence of five to eight amino acid residues. Modification of the internal lysine decreases the incorporation of acetate, irrespective of the size of the blocking group.**

The number of NH_2-terminally acetylated proteins described in the literature is considerable, but virtually nothing is known concerning the underlying mechanism and possible functional implications. NH_2-terminal acetylation of *Escherichia coli* ribosomal protein L_{12} (1, 2) and of ribosome-associated proteins from rat liver (3, 4) is under enzymic control. A conspicuous feature of α-NH_2-acetylated proteins is that, with rare exceptions, only NH_2-terminal alanyl, glycyl, methionyl, seryl, and threonyl residues are involved (5, 6). The lens cell-free system explored previously in our laboratory for the solution of a variety of other problems in protein biosynthesis (7, 8) appeared to be a useful system for the study of NH_2-terminal acetylation. This system must have a powerful acetylating capacity in view of the fact that approximately 75% of the water-soluble lens proteins are NH_2-terminally acetylated. The experimental approach of NH_2-terminal acetylation was until now seriously hampered by the lack of a suitable substrate. Obviously, all potential substrates isolated from natural sources, with exception of the ribosomal protein L_{12} (9), are already acetylated in NH_2-terminal position. We were able to circumvent this difficulty by using a synthetic tridecapeptide, with the same sequence as α-melanotropin (α-melanocyte stimulating hormone, α-MSH) but lacking the α-NH_2-acetyl group normally present in the native hormone. This substrate becomes readily acetylated by the lens cell-free system using acetyl-CoA as acetate donor. The acetylation is an enzymatic process and is specific for the NH_2-terminal amino group of the substrate.

MATERIALS AND METHODS

Substrates. Peptides with sequences related to the sequence of melanotropin (melanocyte stimulating hormone, α-MSH) were synthesized as described (10). The main substrate used was des-$N^{\alpha 1}$-acetyl-α-MSH:

$$R_1\text{-}\overset{1}{\text{Ser}}\text{-Tyr-Ser-Met-}\overset{5}{\text{Glu}}\text{-His-Phe-Arg-}\underset{\underset{10}{|}}{\overset{R_2}{}}\text{Trp-Gly-Lys-Pro-Val-}NH_2,$$

where R_1 = H and R_2 = H. Related peptides of different lengths and with different blocking groups were also used as substrates: α-MSH (R_1 = Ac and R_2 = H); des-$N^{\alpha 1}$-acetyl-$N^{\epsilon 11}$-acetyl-α-MSH (R_1 = H and R_2 = Ac); $N^{\epsilon 11}$-acetyl-α-MSH (R_1 = Ac and R_2 = Ac); des-$N^{\alpha 1}$-acetyl-$N^{\epsilon 11}$-Msc-α-MSH [R_1 = H and R_2 = Msc (methylsulfonylethyloxycarbonyl)]. Moreover, the following peptides have been assayed: H-Ser-Tyr-Ser-Met-OH; H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-OH, and H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH. In addition, a synthetic peptide corresponding to the first 24 residues of corticotropin (ACTH) was also used as substrate. This peptide was a gift of Organon N.V., Oss, The Netherlands.

Enzymes and Chemicals. Trypsin and chymotrypsin were obtained from Worthington; carboxypeptidase A and B from Boehringer; carboxypeptidase C from Rohm & Haas, and Pronase from Calbiochem. Dowex 50 H^+ (100–200 mesh) and ion exchanger II were products of Baker and Merck, respectively. [1-^{14}C]Acetic anhydride (109 mCi/mmol) and [1-^{14}C]acetylcoenzyme A (58 mCi/mmol) were purchased from The Radiochemical Centre, Amersham. [1-^{3}H]Acetylcoenzyme A (0.74 Ci/mmol) was obtained from New England Nuclear.

Preparation of Lens Extract. The epithelial cell layer was removed from fresh calf lenses, and 12 mm of the outer cortex at the equator was punched off with the aid of a glass trephine. Homogenization was carried out in two volumes of buffer (50 mM Tris·HCl, pH 7.4, 300 mM sucrose, 50 mM KCl, 3 mM magnesium acetate) by applying 10 strokes with a Teflon homogenizer. The homogenate was centrifuged at 15,000 × *g* for 10 min at 2°. The supernatant fraction (S15) was used for subsequent experiments. In some cases an aqueous lens extract was lyophilized and used as a source of the acetylating enzyme.

Acetylation Reaction. The reaction mixture contained: 50 mM Tris·HCl, pH 7.4, 300 mM sucrose, 50 mM KCl, 0.36 mM substrate, 2.23 μM [^{3}H]acetylcoenzyme A, and S15. The final volume was 0.3 ml. Unless otherwise indicated, incubations were for 15 min at 37°. The reaction was started by the addition of labeled acetylcoenzyme A and stopped by adding $ZnSO_4$ up to a concentration of 6 mM. The precipitated proteins, mostly crystallins (11), were removed by centrifugation. The supernatant fraction, containing the substrate, was applied to a small column (1 × 9 cm) packed with ion exchanger II (Merck) in order to remove the excess [^{3}H]acetylcoenzyme A. The nonadsorbed material, including the substrate, was eluted with 0.1 M acetic acid. An aliquot of this fraction was applied to a filter paper disc (Whatman GF/2), and the radioactivity of the dried disc was measured in a Packard Tricarb 2450 Liquid Scintillation Counter.

Analysis of Labeled Chymotryptic Peptides. The incubated and labeled substrates, freed from excess labeled acetyl-CoA, were lyophilized and suspended in 0.4 ml of 0.1 M NH_4HCO_3, pH 8.9. Chymotrypsin (0.1 mg) was added and the solution was

Abbreviations: α-MSH, α-melanotropin (α-melanocyte stimulating hormone); Msc-, methylsulfonylethyloxycarbonyl; ACTH, corticotropin.

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Table 1. Acetylation of des-$N^{\alpha 1}$-Ac-α-MSH and related peptides by an S15 preparation

Exp. no.	Incubation	[^{3}H] Acetate incorporated (pmol)
1	S15	8
2	S15 + des-$N^{\alpha 1}$-Ac-α-MSH	92
3	S15 preincubated with $ZnSO_4$ + des-$N^{\alpha 1}$-Ac-α-MSH	4.5
4	S15 + $N^{\epsilon 11}$-Ac-α-MSH	15
5	S15 + α-MSH	17
6	S15 + des-$N^{\alpha 1}$-Ac-$N^{\epsilon 11}$-Ac-α-MSH	37
7	S15 + des-$N^{\alpha 1}$-Ac-$N^{\epsilon 11}$-Msc-α-MSH	36.5
8	S15 + NH_2-terminal tetrapeptide	10.5
9	S15 + NH_2-terminal octapeptide	27.4
10	S15 + NH_2-terminal decapeptide	28.10
11	S15 + NH_2-terminal peptide of ACTH (residue 1–24)	80.7

Details of the incubation and assay are described in *Materials and Methods*. The incubation mixture contained 3 mg of S15 preparation, 108 nmol of substrate, and 0.5 μCi of [^{3}H]acetylCoA at pH 7.4. After 15 min at 37° the reaction was stopped by adding $ZnSO_4$ up to a concentration of 6 mM. Excess [^{3}H]acetylCoA was removed by adsorption onto a weakly basic ion exchanger. The [^{3}H]acetate incorporated into protein was then measured. No corrections have been made for a possible loss of substrate during its purification.

incubated at 37° for 2 hr. The clear solution was lyophilized and the residue subjected to high voltage paper electrophoresis. Chymotryptic peptides of des-$N^{\alpha 1}$-Ac-α-MSH and $N^{\epsilon 11}$-Ac-α-MSH were run in parallel on the same sheet of paper. The dried electropherograms were cut into 1-cm strips and the radioactivity of each strip was measured in a Packard Tricarb 2450 Liquid Scintillation Counter. The chymotryptic peptides of the unlabeled substrates des-$N^{\alpha 1}$-Ac-α-MSH and $N^{\epsilon 11}$-Ac-α-MSH were detected by UV fluorescence, staining with ninhydrin, or specific staining for tyrosine (12).

Analysis of the Total Enzymatic Digest of Labeled Substrates. The incubated and labeled substrates were lyophilized and suspended in 0.4 ml of 0.1 M NH_4HCO_3, pH 8.9, and digested by successive addition of chymotrypsin, Pronase, and carboxypeptidase A, B, and C (100 μg of each enzyme) during a 12-hr period at room temperature. The enzymatic digest was fractionated on a small column of Dowex 50 H^+ (0.5 × 7 cm). The nonadsorbed material was eluted with water to obtain the "acidic" fraction, which contained NH_2-terminally blocked amino acids and peptides. All the adsorbed material was eluted with 0.5 M NH_4OH to obtain the "basic" fraction containing the free amino acids and peptides. The eluates were lyophilized and analyzed by high voltage electrophoresis and paper chromatography. Reference [^{14}C]acetyl-amino acids were prepared by treatment of 1 mmol of the amino acid with 1 mmol of [^{14}C]acetic anhydride in boiling acetic acid (13).

Protein Determination. Proteins were estimated according to Lowry *et al.* (14), with bovine serum albumin as a standard.

RESULTS

Acetylation of des-$N^{\alpha 1}$-Ac-α-MSH by a lens extract: Specificity of the acetylation reaction

The different substrates were incubated with an S15 preparation and labeled acetylcoenzyme A at 37° as described in *Ma-*

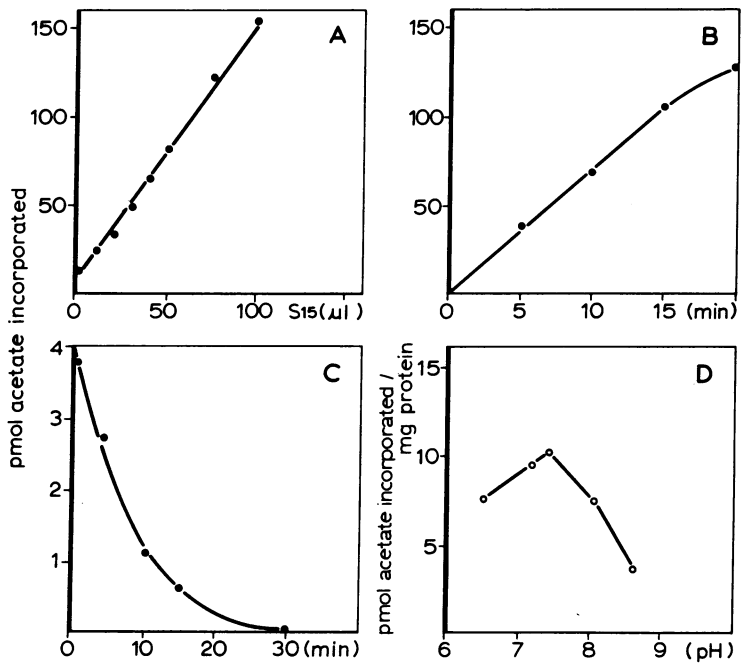

FIG. 1. Characteristics of the acetylation reaction. Details of incubation are described in *Materials and Methods*. The difference in [^{3}H]acetate incorporation obtained with an S15 preparation incubated in the presence and absence of substrate is considered to be due to enzymatic acetylation. (A) Effect of supernatant concentration. Different amounts of the S15 preparation (89 μg of protein per μl) were incubated with [^{3}H]acetylCoA and des-$N^{\alpha 1}$-Ac-α-MSH at 37° for 15 min, and the amount of incorporated acetate was measured. (B) Kinetics of the reaction. 160 μl of S15 preparation (89 μg of protein per μl) were incubated. At different time intervals an aliquot fraction of 40 μl was removed and the reaction was stopped by adding $ZnSO_4$ up to a concentration of 6 mM. (C) Heat inactivation. The S15 preparation (4 μg/μl) was incubated at 55°. At different time intervals aliquots were removed and chilled in melting ice. The extent of acetylation of des-$N^{\alpha 1}$-Ac-α-MSH by these heat-treated S15 preparations was measured after an additional incubation for 15 min at 37°. (D) Effect of pH. A lyophilized S15 preparation was dissolved in 50 mM Tris·HCl, 30 mM sucrose, 50 mM KCl, 30 mM MgOAc, at different pH values. The acetylation reaction was performed for 15 min at 37°.

terials and Methods. The presence of 50 mM KCl should minimize the nonenzymatic acetylation of ϵ-NH_2 groups of lysine (15).

Table 1 shows that a low amount of radioactivity is incorporated into proteins when the S15 preparation is incubated with [^{3}H]acetylCoA in the absence of substrate (Exp. 1). This incorporation may be due to enzymatic and/or nonenzymatic acetylation of endogenous lens proteins. When des-$N^{\alpha 1}$-Ac-α-MSH is added to the incubation mixture (Exp. 2), the incorporation of [^{3}H]acetate is 10 times higher. Preincubation of the S15 preparation with 6 mM $ZnSO_4$ (Exp. 3) greatly decreases the incorporation of labeled acetate. The data strongly suggest that des-$N^{\alpha 1}$-Ac-α-MSH is enzymatically acetylated by the lens extract.

On des-$N^{\alpha 1}$-Ac-α-MSH two potential sites for NH_2-acetylation are available: the free amino group of the NH_2-terminal serine and the ϵ-NH_2 group of lysine-11. In order to determine whether one or both of these residues become acetylated by the S15 preparation, we incubated des-$N^{\alpha 1}$-Ac-α-MSH, α-MSH, and $N^{\epsilon 11}$-Ac-α-MSH with an S15 preparation and [^{3}H]acetyl-CoA. The amount of acetate incorporated when $N^{\epsilon 11}$-Ac-α-MSH is used as a substrate (Exp. 4) is of the same order as the incorporation of acetate in lens protein (Exp. 1) plus the nonenzymatic acetylation of des-$N^{\alpha 1}$-Ac-α-MSH (Exp. 3).

Exp. 5 shows that α-MSH also is not a substrate for the acetylating enzyme, indicating that the lens system does not promote acetylation of the ϵ-NH_2 group of lysine-11. However, des-$N^{\alpha 1}$-Ac-$N^{\epsilon 11}$-Ac-α-MSH (Exp. 6) and des-$N^{\alpha 1}$-Ac-$N^{\epsilon 11}$-Msc-α-MSH (Exp. 7), which both have free NH_2-termini but carry a blocking group on lysine-11, are acetylated by the S15 preparation, although to a lesser degree than the completely unblocked des-$N^{\alpha 1}$-Ac-α-MSH. Although the presence of the blocking group on residue 11 affects the process of NH_2-terminal acetylation (irrespective of the size of this blocking group), it appears that the acetylation reaction is specific for the α-NH_2 terminus of peptides.

In order to get some information about the minimum length required for the substrate to become acetylated, we tested the NH_2-terminal tetra-, octa-, and decapeptides of des-$N^{\alpha 1}$-Ac-α-MSH. As shown in Table 1 (Exps. 8, 9, and 10), the tetrapeptide cannot be acetylated by the S15 preparation, whereas the octa- and decapeptides become acetylated to some degree.

Furthermore, a longer peptide (consisting of the residues 1–24 of ACTH), of which the 13 NH_2-terminal residues are identical to des-$N^{\alpha 1}$-Ac-α-MSH, is as good a substrate as des-$N^{\alpha 1}$-Ac-α-MSH itself.

Attempts to acetylate histones in the S15 preparation gave negative results.

Some characteristics of the acetylation process are given by the experiments depicted in Fig. 1. Acetylation of des-$N^{\alpha 1}$-Ac-α-MSH is proportional to the amount of S15 added, at least up to 100 μl (corresponding to about 9 mg of protein) (Fig. 1A). The reaction shows a linear increase for about 15 min (Fig. 1B); it is a thermolabile process (Fig. 1C), with an optimum pH near 7.4 (Fig. 1D).

Localization of the acetyl group incorporated in des-$N^{\alpha 1}$-Ac-α-MSH

The localization of the acetyl group(s) in incubated des-$N^{\alpha 1}$-Ac-α-MSH and related peptides was established by analysis of either labeled chymotryptic peptides or labeled acetyl amino acids obtained after extensive digestion with chymotrypsin, Pronase, and carboxypeptidases.

Characterization of Labeled Chymotryptic Peptides. Four

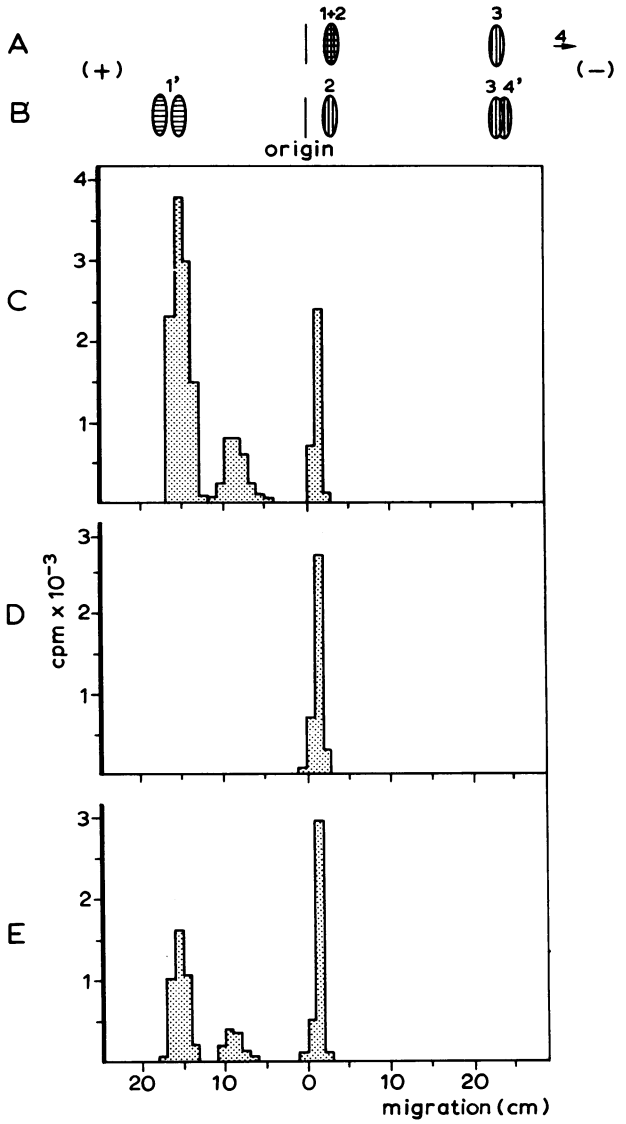

FIG. 2. High voltage paper electrophoresis of chymotryptic peptides of [^{14}C]acetylated substrates. Electrophoresis was carried out for 105 min at 50 V/cm in pyridine–acetic acid–water (25:1:225 by volume), pH 6.5. A and B represent chymotryptic digests of synthetic des-$N^{\alpha 1}$-Ac-α-MSH and $N^{\epsilon 11}$-Ac-α-MSH, respectively. Vertical hatching indicates spots stained with ninhydrin; horizontal hatching indicates spots giving a positive tyrosine staining. C, D, and E represent the radioactivity profiles of the electrophoresed chymotryptic digests of des-$N^{\alpha 1}$-Ac-α-MSH, α-MSH, and des-$N^{\alpha 1}$-Ac-$N^{\epsilon 11}$-Ac-α-MSH, respectively, incubated with an S15 preparation and acetylCoA as described in *Materials and Methods* except that [^{14}C] instead of [^{3}H]acetylCoA was used as acetate donor.

chymotryptic peptides with different electrophoretic mobilities can be obtained from des-$N^{\alpha 1}$-Ac-α-MSH: (*1*) H-Ser-Tyr-OH; (*2*) H-Ser-Met-Glu-His-Phe-OH; (*3*) H-Arg-Trp-OH; and (*4*) H-Gly-Lys-Pro-Val-NH_2 (Fig. 2A). Peptide *4* migrated off the paper, but could be detected when the time of electrophoresis was shortened. Cleavage may occur between methionine and glutamic acid in peptide *2*, but this will not change the electrophoretic pattern. When the α-NH_2 of serine-1 and the ϵ-NH_2 of lysine-11 are acetylated ($N^{\epsilon 11}$-Ac-α-MSH), the electrophoretic mobilities of peptides *1* and *4* become completely different (*1'* and *4'* in Fig. 2B) and thus provide an appropriate tool to discriminate between α- and ϵ-NH_2-acetylation. In fact, chymotryptic digestio of $N^{\epsilon 11}$-Ac-α-MSH yields two negatively

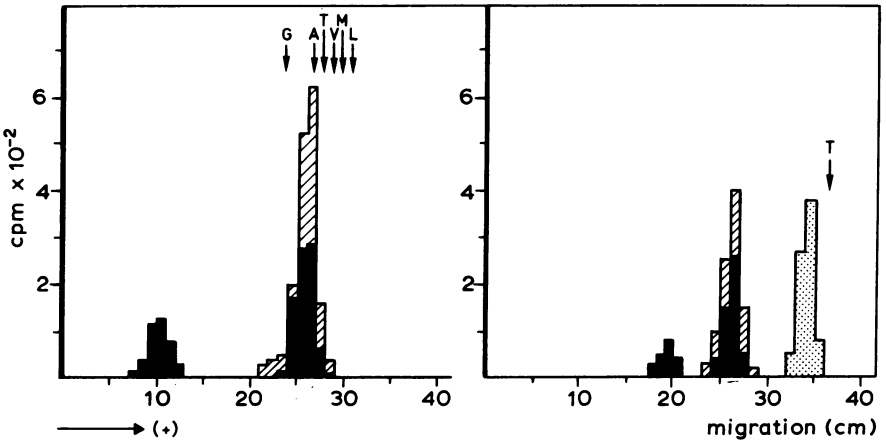

FIG. 3. Identification of acetyl amino acids after extensive digestion of [^{3}H]acetylated substrates. Des-$N^{\alpha 1}$-Ac-α-MSH was incubated with an S15 preparation and [^{3}H]acetylCoA as described in *Materials and Methods*. The ^{3}H-labeled substrates were digested to their constituent amino acids. The enzymatic digest was then fractionated by chromatography on a Dowex 50 H^+ column. Thereafter, the "acidic" fraction was subjected to electrophoresis and chromatography. (*Left*) High voltage electrophoresis in pyridine–acetic acid–water (25:1:225 by volume), pH 6.5, at 50 V/cm for 90 min. The patterns of reference [^{14}C]acetylserine (hatched area) and of the total enzymatic digest of incubated des-$N^{\alpha 1}$-Ac-α-MSH (black area) are shown. The positions of reference [^{14}C]acetyl derivates of glycine (G), alanine (A), threonine (T), valine (V), methionine (M), and leucine (L) are indicated by arrows. (*Right*) Descending chromatography in butanol–acetic acid–water (12:3:5 by volume). The patterns of reference [^{14}C]acetylserine (hatched), of [^{14}C]acetylalanine (dotted), and of the total enzymatic digest of incubated des-$N^{\alpha 1}$-Ac-α-MSH (black) are shown together with the position of [^{14}C]acetylthreonine (T).

charged peptides of equal intensity, which are both ninhydrin-negative and tyrosine-positive. The reason for this phenomenon and the question of which of the two peptides corresponds to genuine Ac-Ser-Tyr is still unsolved. However, the results shown in Fig. 2C and D confirm that in any case no acetylation of the ϵ-NH_2 group of lysine occurs. Both des-$N^{\alpha 1}$-Ac-α-MSH (Fig. 2C) and α-MSH (Fig. 2D) have a free ϵ-NH_2 group available, but do not show any trace of labeling in the position of the chymotryptic peptide *4*. When the incubation is performed in the presence of a substrate with a free NH_2-terminus (Fig. 2C and E), two labeled peptides migrate towards the anode. Both must have a blocked NH_2-terminus. The most slowly moving peptide might result from incomplete cleavage. The fastest moving peptide comigrates with the NH_2-terminal chymotryptic peptide of synthetic $N^{\epsilon 11}$-Ac-α-MSH. Because of the remaining ambiguity due to the two anodal chymotryptic peptides *1'* of $N^{\epsilon 11}$-Ac-α-MSH, it was necessary to unequivocally identify the acetylated amino acid. Comparison of Fig. 2C and E with Fig. 2D allows the conclusion that under the conditions of our experiments no constituent of the lens becomes acetylated that would coincide with acetyl-Ser-Tyr.

Characterization of Labeled Acetyl Amino Acids in the Incubated Substrate. The total enzymatic digests of incubated substrates were applied onto a small Dowex 50 H^+ column. About 95% of the radioactivity was recovered in the "acidic" fraction, which contains NH_2-terminally blocked amino acids and peptides. After high voltage electrophoresis, the radioactivity was found in two positions (Fig. 3, left): the fast moving spot comigrates with standard [^{14}C]acetylalanine and [^{14}C]acetylserine; the slowly moving one has not been identified. [^{14}C]acetylalanine and [^{14}C]acetylserine can be resolved by chromatography. Fig. 3, right, clearly shows the presence of *N*-[^{3}H]acetylserine in the extensive enzymatic digest of incubated des-$N^{\alpha 1}$-Ac-α-MSH. Again, a second peak of unknown identity is present. These results demonstrate that incubation of des-$N^{\alpha 1}$-Ac-α-MSH with an S15 preparation results in NH_2-acetylation of the substrate and that the reaction is specific for the NH_2-terminal amino acid.

DISCUSSION

NH_2-terminal acetylation of proteins has been reported to be a postinitiational event (5) and appears to be a rather common phenomenon in prokaryotic and eukaryotic cells (1, 2, 16, 17). It has been shown in this laboratory that, at least for lens proteins, acetylation takes place while the nascent chain is still on the ribosome (18). From these studies it appeared that the approximate length of the growing polypeptide chain is between 20 and 25 amino acid residues when NH_2-terminal acetylation starts. This length would be requied for a polypeptide to protrude from the ribosome. Furthermore, it could unequivocally be demonstrated that the translation product of the 14S α-crystallin messenger becomes acetylated in NH_2-terminal position independent of the heterologous system that is used for translation (19–21). The latter observation strongly supports the idea that NH_2-terminal acetylation of a protein is not limited to the cell species in which it normally occurs.

The results described in this paper demonstrate the enzymatic acetylation of a synthetic tridecapeptide, des-$N^{\alpha 1}$-Ac-α-MSH, by a calf lens extract using [^{3}H]acetylcoenzyme A as acetate donor. Two potential sites for NH_2-acetylation are available on the substrate: the α-NH_2 group of the NH_2-terminal serine and the ϵ-NH_2 group of lysine-11. Our studies of the incorporation of labeled acetate into this substrate, which was modified by blocking one or both of the amino groups, indicated that acetylation took place preferentially at the α-NH_2 group of the terminal amino acid residue. Identification of the acetyl group after enzymatic digestion of the labeled substrate confirms the assumption that acetylation occurred at the NH_2-terminal seryl residue and that no acetate is incorporated into the ϵ-NH_2-lysyl group of residues.

The acetylation reaction shows an optimum pH around 7.4 and is a thermolabile process, which suggests enzymatic control of the reaction. This is sustained by the findings that nonenzymatic acetylation of proteins requires a more alkaline pH and is a thermostable process (22).

The minimum length required for the substrate to be acetylated seems to be between five and eight amino acid residues.

A maximum incorporation of acetate is obtained with des-$N^{\alpha 1}$-Ac-α-MSH. Blocking the ϵ-NH_2 group of lysine-11 results in a decrease of NH_2-terminal acetylation, irrespective of the size of the blocking group (e.g., acetyl or methylsulfonylethyloxycarbonyl). Furthermore, the NH_2-terminal tetracosapeptide of corticotropin (ACTH), of which the sequence of the first 13 residues is identical to that of des-$N^{\alpha 1}$-Ac-α-MSH, seems to be as good a substrate as des-$N^{\alpha 1}$-Ac-α-MSH itself. Native ACTH can also be acetylated to some extent in the bovine lens systems (unpublished results).

Earlier observations revealed that the translation product of lens α-crystallin messenger becomes NH_2-terminally acetylated in a variety of heterologous cell-free systems. Hence it is very likely that the acetylation of native α-MSH in the pituitary gland is brought about by an enzyme similar to the one present in the lens system. It therefore seems that the specificity of the enzyme is rather broad in view of the dissimilar NH_2-terminal sequences of α-MSH and α-crystallin. Amino acids that are involved in NH_2-terminal acetylation are, almost exclusively, alanine, glycine, serine, threonine, and methionine, although, on the other hand, proteins having such an NH_2-terminal amino acid are not necessarily acetylated. Comparison of the NH_2-terminal sequences of a series of α-NH_2-acetylated proteins does not provide obvious similarities which might account for the acetylation.

Enzymatic ϵ-NH_2 acetylation of lysyl residues has been proven to occur in a variety of proteins (3, 23, 24), but it has most extensively been studied in histones. Proposals suggesting the initiation of protein synthesis by acetylated amino acids as a general mechanism in eukaryotes (25) or as a special case for histones (26) have been refuted (27). Nevertheless, very recently this abandoned idea has been put forward again (28).

The present results provide an experimental approach to the problem of NH_2-terminal acetylation of eukaryotic proteins.

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