ACTIVE AND INACTIVE MODIFICATIONS OF TRYPSIN INDUCED BY ACETYLATION*

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SUMMARY

The acetylation of trypsin with acetic anhydride results in a heterogeneous mixture of acetylated derivatives. That fraction which is most fully acetylated (85–90 %) is enzymically inactive, whereas the partially acetylated (about 50 %) fraction is fully as active as unmodified trypsin. These two forms of trypsin may be further differentiated on the basis of their solubility properties, chromatographic behavior on DEAE-cellulose, and optical rotation. The implication of these results with respect to the structural requirements for enzymic activity is discussed.

INTRODUCTION

Chemical modification of proteins[1,2] has, in many instances, proved to be a useful tool in delineating the specific groups responsible for their biological activity. In the case of trypsin, FRAENKEL-CONRAT *et al.*[3] and NORD and his associates[4,5] have reported that the acetylation of amino groups does not interfere with the catalytic function of this enzyme. In this laboratory, however, it has been consistently observed that the acetylation of trypsin, employing acetic anhydride under the same conditions prescribed by these authors[3–5], does result in a noticeable loss of activity. In an attempt to resolve these discrepancies, the acetylation of trypsin has now been subjected to closer scrutiny. The evidence to be presented here shows that the reaction of trypsin with acetic anhydride leads to a heterogeneous mixture containing active and inactive species of modified protein which can be further differentiated on the basis of certain physical and chemical properties.

EXPERIMENTAL

Trypsin was acetylated with acetic anhydride in the manner originally described by FRAENKEL-CONRAT *et al.*[3] and likewise adopted by NORD's group[4,5**]. Since this paper deals with conflicting results obtained in this laboratory and elsewhere, the experimental conditions assume considerable importance and will be described in detail.

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** The specificity of this procedure for the acetylation of the amino groups of trypsin has been established by URAKI *et al.*[6] using labelled acetic anhydride. Although O-acetyl groups are also introduced, these are unstable under the alkaline conditions employed in the enzymic assay.

An aqueous solution of 200 mg of twice crystallized salt-free trypsin (Worthington, lot No. 641) dissolved in 40 ml of ice-cold, half-saturated sodium acetate was treated with 0.5 ml acetic anhydride. The latter was added intermittently over a period of 1 h with the enzyme solution immersed in an ice bath and agitated with a magnetic stirrer. The solution was then exhaustively dialyzed at 4° against 0.001 *N* HCl (pH 3) and lyophilized. This preparation will be referred to as "1x-acetyltrypsin".

Where a second acetylation was desired, lyophilization was omitted, and sufficient solid sodium acetate was added to the dialyzed solution (volume about 100 ml) to bring it to half-saturation followed by the intermittent addition of 0.8 ml acetic anhydride over a period of 90 min in the same manner as above. Dialysis against 0.001 *N* HCl and lyophilization gave a preparation designated as "2x-acetyltrypsin".

During dialysis at pH 3, a considerable amount of protein precipitated. Since this insoluble fraction was subsequently found to have very little activity, preparations of acetyltrypsin were also made in which this insoluble protein was removed by centrifugation after each dialysis stage of the preparation. This insoluble fraction could be redissolved at pH 10 for purposes of analysis.

The treatment of trypsin or acetyltrypsin preparations with calcium ions involved the dispersion of the sample in 0.005 *M* borate buffer, pH 8.0, containing 0.05 *M* $CaCl_2$, to give a concentration of 5 mg of protein/ml of buffer. Under these conditions, an inactive fraction of the protein remained insoluble and could be separated by centrifugation. This fraction could be subsequently solubilized by adjusting an aqueous suspension of the precipitate to pH 8.0.

The enzymic activity of the various fractions was determined on benzoyl L-arginine ethyl ester (BAEE) using the spectrophotometric procedure of SCHWERT AND TAKENAKA[7]. 0.05 *M* $CaCl_2$ was incorporated into the substrate solution in order to provide the activating influence of calcium ions[8,9] to systems which had not been previously fractionated with $CaCl_2$. One activity unit is arbitrarily defined as an absorbance change of 1.0 per min at 37°, and the specific activity as the number of activity units per mg protein. The protein concentration (mg/ml) was obtained by multiplying the absorbance of the protein solution at 280 mμ by a factor of 0.6[10], or by multiplying the N content, determined according to LANNI *et al.*[11], by 6.25. A mol. wt. of 24,000 was assumed for converting protein into moles of trypsin or acetyltrypsin[12]. The extent of acetylation was estimated by comparing the number of free amino groups present in the initial and final materials according to the ninhydrin method of TROLL AND CANNAN[13]. The applicability of the ninhydrin reaction for the evaluation of the amino groups of trypsin and its acetylated derivatives has been pointed out by NORD *et al.*[14] and PECHÉRE AND NEURATH[15].

Various preparations or fractions of acetyl-trypsin were examined chromatographically on diethyl-aminoethyl (DEAE)-cellulose in essentially the same manner as described elsewhere[16], namely stepwise elution with solutions of increasing ionic strength in the following sequence: 0.005 *M* borate buffer, pH 8.0; 0.01 *M* borate–0.02 *M* NaCl, pH 8.0; 0.02 *M* phosphate–0.10 *M* NaCl, pH 6.8; and 1% NaOH. Eluates obtained with each of these solutions will be referred to as fractions I, II, III, and IV respectively. Fractions from the column were examined for protein by measuring absorbance at 280 mμ and for enzymic activity on BAEE. Column fractions could not be reliably analyzed with ninhydrin by the method of TROLL AND CANNAN[13] since the level of free amino groups in the protein solutions obtained

directly from the column were far below the sensitivity of this method. All samples were adjusted to pH 8.0 or dialyzed against 0.005 *M* borate buffer, pH 8.0, prior to their application to the column. Suspensions or precipitates which were not initially completely soluble at pH 8.0 were first dissolved in alkaline solution (pH not exceeding 10) and then readjusted to pH 8.0. The same column, after re-equilibration with the starting buffer, was used for all the analyses reported here. All chromatographic runs were made in a cold room maintained at a temperature of 4–7°.

RESULTS

In Table I are recorded the activity and amino groups of various preparations or fractions obtained by the acetylation of commercial trypsin (preparation 1). If the insoluble precipitate which formed during the dialysis of the acetylated protein was not removed after the first acetylation, preparation 3 was obtained which had 81 % of its amino groups blocked and had lost about 37 % of its original activity. A second acetylation, without removal of insoluble protein, blocked additional amino groups, but at the expense of a 50 % loss in activity (preparation 4).

If, on the other hand, the insoluble precipitate was removed after dialysis, preparations 5-S and 6-S were obtained after the first and second acetylations

TABLE I

AMINO GROUPS AND ACTIVITY OF PREPARATIONS OF TRYPSIN AND ACETYLATED TRYPSIN

Preparation	*Sub-fractions*	*Designation*	*Protein distribution %*	*Amino groups*		*Specific activity*
				Moles/mole protein	*% blocked*	
Trypsin	None	1	—	12.5	0	21.7
Ca-treated trypsin	Supernate	2-S	65	12.3	0	35.0
	Precipitate	2-P	35	12.6	0	0
1x acetyltrypsin	None*	3	—	2.4	81	13.7
2x acetyltrypsin	None*	4	—	1.5	88	10.5
1x acetyltrypsin	Suspension after dialysis	5	100	2.6	79	14.0
	Supernate	5-S	56	4.9	61	23.8
	Precipitate	5-P	44	1.9	85	1.5
2x acetyltrypsin**	Suspension after dialysis	6	100	3.4	73	19.2
	Supernate	6-S	86	4.2	66	20.8
	Precipitate	6-P	14	1.1	91	1.2
Ca-treated 2x acetyltrypsin***	Supernate	7-S	55	6.0	52	33.4
	Precipitate	7-P	45	1.9	85	1.3
1x acetyltrypsin§	None	8		3.4	73	28.3
2x acetyltrypsin§	Suspension after dialysis	9	100	2.4	81	21.7
	Supernate	9-S	75	3.6	71	28.0
	Precipitate	9-P	25	1.1	91	1.3

* Precipitate formed during dialysis was not removed.

** Only the supernate from the 1x-acetyltrypsin (preparation 5-S) was acetylated.

*** The lyophilized supernate from 2x-acetyltrypsin (preparation 6-S) was treated with calcium as described in the text.

§ The lyophilized supernate from Ca-treated trypsin (preparation 2-S) was acetylated. No precipitation was observed during dialysis after the first acetylation.

respectively. The activities of both of these preparations were quite comparable to the activity of the original trypsin*. This enrichment of activity was evidently due to the removal of a considerable amount of the inert protein precipitating out during dialysis (preparations 4-P and 6-P). This inert protein was further characterized by its high degree of acetylation, 85–90 %, compared to the soluble, active fractions which were acetylated to the extent of only 60–65 %.

Treatment of 2x-acetyltrypsin (preparation 6-S) with borate buffer containing calcium ions resulted in the precipitation of an additional amount of inactive, highly acetylated protein (preparation 7-P). The soluble supernatant solution (preparation 7-S) now had a specific activity of 33.4, far in excess of that of the original trypsin (21.7), and only about 50 % of its amino groups were acetylated.

Since the activity of the original trypsin preparation was less than that of its acetylated derivative which had been treated with calcium ions, it was thought that either calcium was acting as an activator or that the trypsin preparation employed in these studies was relatively impure. The first possibility was considered unlikely for the following reasons: (a) the magnitude of the increased activity was much greater than the 25 % usually attributed to calcium[17], (b) the removal of calcium ions from preparation 7-S by prolonged dialysis did not reduce its specific activity and (c) all assays were conducted in the presence of 0.05 *M* $CaCl_2$ (see experimental). On the other hand, treatment of trypsin with calcium under the same conditions resulted in the formation of an insoluble, inactive fraction (preparation 2-P), which constituted about one-third of the total protein, and a soluble fraction (preparation 2-S) which was about 1.5 times as active as the commercial enzyme preparation. The activity of this soluble fraction was now comparable to the activity of the calcium-treated acetyltrypsin referred to earlier.

The acetylation of preparation 2-S, the active fraction of calcium-treated trypsin, produced little or no precipitable protein during dialysis after the first acetylation (preparation 8). After the second acetylation, however, 25 % of the protein became insoluble (preparation 9-P). As before, this insoluble fraction was highly acetylated, about 90 %, and essentially devoid of activity. The supernatant (fraction 9-S) was only 71 % acetylated and had an activity which was somewhat less than that of the starting enzyme preparation 2-S.

In contrast to solubility behavior, which served a useful but obviously limited means of distinguishing the various fractions of acetyltrypsin, chromatography on DEAE-cellulose permitted a more detailed examination of the composition of these protein fractions. The various chromatographic patterns are shown in Fig. 1–3, and Table II provides additional data regarding the distribution and recovery of the protein and activity in these chromatographic experiments.

Fig. 1 serves to compare two preparations of 2x-acetyl-trypsin, one (preparation 4) in which the insoluble protein which precipitates out during dialysis was not removed, and the other (preparation 6-S) in which this fraction was eliminated. The principal difference between these two patterns is the reduction in the size of fraction IV as a result of removing this insoluble, highly acetylated protein. As a consequence of this redistribution of protein, the active component in fraction III is proportionately greater in preparation 6-S. Of the three components in fraction III, the first

* It is this observation, perhaps, which may have led other investigators[2–5] to conclude that acetylation does not seriously impair the activity of trypsin.

TABLE II

PROTEIN DISTRIBUTION AND ACTIVITY DATA OF CHROMATOGRAPHIC EXPERIMENTS

Preparation*	Original sample		Column fractions						
			% protein of the original					Activity in fraction III	
	Protein mg	Specific activity	I	II	III	IV	Total recovery	Spec.**	Total % recovered
4 (Fig. 1A)	8.9	11	4	22	35	50	111	31	83
6-S (Fig. 1B)	7.9	21	0	2	56	32	90	34	84
7-S (Fig. 2A)	7.7	33	0	4	83	16	103	36	86
7-P (Fig. 2B)	7.0	1	1	9	21	63	94	6	94
8 (Fig. 3A)	5.8	27	0	7	67	22	96	33	84
9 (Fig. 3B)	7.2	21	0	11	58	40	109	32	77

* See Table I for description of the various preparations.
** Based on activity and protein content of tube corresponding to the active peak.

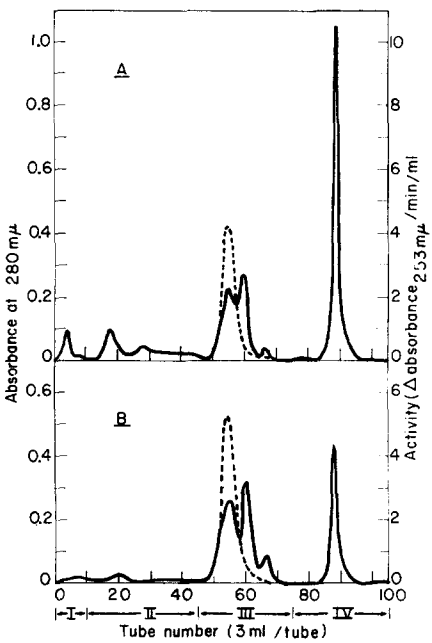

Fig. 1. Chromatographic behavior of 2x-acetyltrypsin showing the effect of removing protein which precipitates out during dialysis. Patterns A and B were obtained with preparations 4 and 6-S respectively described in Table I. Distribution of protein (based on absorbance at 280 mμ) is shown in solid curve, and activity is shown in dashed curve. Changes in elution schedule are indicated by roman numerals below the abcissae. See experimental section for further explanation.

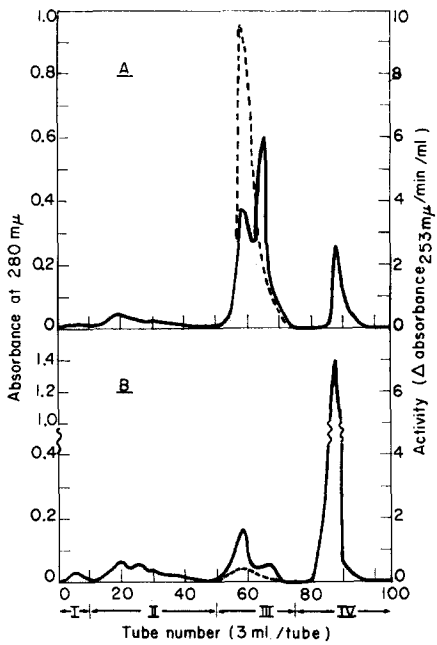

Fig. 2. Chromatographic behavior of fractions of 2x-acetyltrypsin treated with calcium. Patterns A and B were obtained with preparations 7-S and 7-P respectively described in Table I. See caption to Fig. 1 for further explanation of figure.

peak to emerge appears to contain most of the activity which, in terms of specific activity, is comparable to that of the calcium treated trypsin described earlier*.

The effect of treating preparation 6-S with calcium is shown in Fig. 2A where

* Unmodified trypsin is not absorbed by DEAE-cellulose under the conditions employed here.

a further diminution in fraction IV and a proportionate increase in fraction III may be noted. That the protein precipitated by calcium is in fact fraction IV is evident from Fig. 2B. The small residual activity associated with the calcium-precipitated protein is evidently due to contamination with fraction III.

The chromatographic experiments depicted in Fig. 3 were designed to answer the question as to whether the inert protein, as represented by fraction IV, was derived from contaminants pre-existent in the trypsin or arose as a consequence of the acetylation of the active component itself. The starting material in this case was trypsin which had been pre-treated with calcium to remove inactive impurities (preparation 2-S). This preparation was judged as being essentially homogeneous since a single peak was obtained when it was chromatographed on carboxymethylcellulose*. Fig. 3A depicts the pattern obtained after the first acetylation. About 20 % of the protein was converted to inactive protein as represented by fraction IV. After the second acetylation, Fig. 3B, this fraction had increased to 40 %. This progressive increase in fraction IV occurred largely at the expense of fraction III which contains the active component.

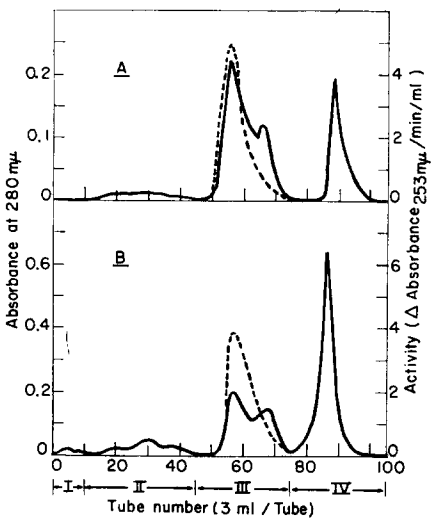

TABLE III

OPTICAL ROTATION OF ACETYLTRYPSIN AND TRYPSIN PREPARATIONS

*Preparation**	$[\alpha]_{25}^{D}$
6	— 64.9
7-S	— 40.0
7-P	— 80.0
Trypsin	— 45.4
Trypsin in 8 *M* urea	—124.8

* See Table I for description of preparations. All preparations were dialyzed, lyophilized, and redissolved in 0.1 *M* borate buffer, pH 8.0, prior to measurement. Final concentration of protein, 0.5 %. Length of polarimeter cell, 10 cm.

Fig. 3. Chromatographic behavior of acetyltrypsin prepared from calcium-treated trypsin. Pattern A was obtained with 1x-acetyltrypsin and B with 2x-acetyltrypsin (preparations 8 and 9 respectively as described in Table I). See caption to Fig. 1 for further explanation of figure.

Optical rotations were measured on buffered solutions (pH 8.0) of 2x-acetyltrypsin (preparation 6) and the soluble and insoluble fractions derived therefrom by treatment with calcium ions (preparations 7-S and 7-P respectively). These data are recorded in Table III. The preparation of 2x-acetyltrypsin was more levorotatory than trypsin itself, and no doubt reflects the contribution of the Ca-soluble and Ca-insoluble fractions. The rotation of the Ca-insoluble fraction was twice that of the Ca-soluble fraction, the latter agreeing within experimental error with the value for trypsin. The rotation of urea-denatured trypsin is shown for comparison.

* Unpublished experiments.

DISCUSSION

The most obvious conclusion to be drawn from this study is that the acetylation of trypsin, performed under the conditions described by FRAENKEL-CONRAT *et al.*[3], results in a heterogeneous mixture of proteins. A partial resolution of this mixture may be achieved by virtue of the fact that the most highly acetylated species are insoluble at pH 3 in the absence of salt, conditions which obtain during the dialysis of the acetylated protein solution. This insoluble fraction, which had 85–90 % of its amino groups blocked, was virtually devoid of activity. On the other hand, the protein which remained soluble under these conditions had only 60–65 % of its amino groups blocked by acetylation. This fraction was fully as active as the starting trypsin preparation but only two-thirds as active as a preparation of trypsin which had been purified by treatment with calcium.

Chromatographic experiments revealed that the almost fully acetylated protein corresponded to a fraction which required 1 % NaOH for its elution from DEAE-cellulose. This fraction was still present to considerable extent in the soluble supernatant, but could be further reduced by subsequent treatment with 0.05 *M* $CaCl_2$. By this means, it was possible to obtain a preparation of acetyltrypsin which was virtually as active as the calcium purified trypsin but which now had only half of its amino groups blocked.

The chromatographic patterns provide evidence for the existence of at least three to four major components whose order of elution from an anionic exchange adsorbent is most likely determined by their anionic properties. Since the acetylation of amino groups would be expected to enhance the anionic character of a protein, the emergence of each component would occur in order of increasing degree of acetylation. The active component was in all cases associated with the first peak to be eluted as fraction III, and the peaks which followed would be the more fully acetylated proteins which were inactive.

It becomes pertinent to inquire as to the reasons why acetylation beyond a critical limit, which seems to be about half of the amino groups of trypsin, should lead to a loss in enzymic activity. Two possible explanations may be considered: (a) Changes in the secondary or tertiary structure of the molecule due to charge or steric effects induced by replacing amino groups with uncharged acetyl groups, and (b) the blocking of less reactable amino groups which are involved in the catalytic function of the enzyme. Evidence in support of (a) is derived from the observation that the specific rotation of the highly acetylated, inactive derivative of trypsin is twice that of the partially acetylated but active derivative which is similar to native trypsin. It has become increasingly apparent from recent studies on the chemical modification of proteins that pronounced changes in physical properties, indicative of denaturation, may be the principal cause for losses in biological activity[18–22]. Although sufficient evidence to exclude (b) is not available from these studies, it is pertinent to recall an analogous situation with such –SH enzymes as urease[23] and aldolase[24] where a decrease in catalytic activity does not occur until the less reactive –SH groups have become blocked. Here again the evidence points to secondary changes in the structure caused by the blocking agent, as being primarily responsible for this loss in activity[24].

The observations recorded here may have a direct bearing on the report by

NORD *et al.*[14] that acetyltrypsin retains its activity while undergoing extensive self-digestion. If the inert protein resulting from "over-acetylation" does in fact represent a form of denatured protein, it is possible that the latter is more susceptible to attack by the active, partially acetylated species. This view is supported by the observation of SRI RAM AND MAURER[20] that acetylated bovine serum albumin was more rapidly digested by trypsin and chymotrypsin than the unmodified protein, although, in the case of trypsin, a consideration of its known specificity would suggest contrawise. This explanation gains further support from the experiments of WOOTON AND HESS[25] and LIENER[16] which also demonstrated the existence of inactive and active components in autolysates of acetyltrypsin, and which prompted these authors to conclude that it is the hydrolysis of this inactive protein which probably accounts for the liberation of free amino groups without any substantial loss in enzymic activity.

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