Time dependent formation of acetylcholinesterase isozymes

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Summary. 4 electrophoretically and chromatographically distinguishable forms of 11S acetylcholinesterase were generated during storage of an 11S preparation of the enzyme.

It has been observed[2-4] that electrophoretically homogeneous acetylcholinesterase (AChE) spontaneously converts in a matter of weeks to months into 3 or more electrophoretically distinguishable forms. The reaction occurs either in the frozen or solution state. With increasing storage time, the bands constitute a greater fraction of the total activity. Little is known about the process which generates the bands nor the properties of the products. This communication describes the first partial characterization of 4 such AChE polymorphs.

Materials and methods. The AChE preparation employed here has been isolated from frozen electric eel tissue (*E. electricus*), partially purified and stored under toluene as described earlier[4] for 5 years. At the time of examination, this preparation had retained 70% of its initial activity. Fresh, control AChE preparations were extracted as outlined by Kothari et al.[5], the 11S form isolated by sucrose gradient centrifugation and stored at 4 °C (no toluene).

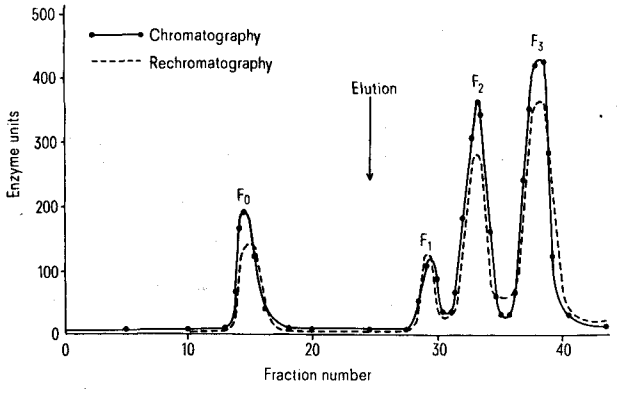
Protein was determined by the method of Lowry et al[6]. Enzyme activity was measured by the spectrophotometric method of Ellman et al.[7]. 1 unit of activity is defined as one μmole of acetylthiocholine iodide hydrolyzed per minute. The sedimentation coefficient of AChE was estimated by sucrose density gradient sedimentation as described before[4]. Polyacrylamide gel electrophoresis was carried out at 0–2 °C using 7% gels at pH 9 in the Buchler apparatus following the instructions in the Buchler manual (Buchler Instruments, Inc., Fort Lee, N.J.). Gels were electrophoresed for 1 h at 1.25 mA/tube. 10–15 units of AChE (100 μl) were applied, and after entering the running gel (80 mm long), were electrophoresed at 2.5 mA/h for 16 h. Enzyme activity bands were visualized by the method of Uriel[8]. Column chromatography was carried out at room temperature (~ 22 °C). Generally, 1 ml of AChE (2500 units) was loaded onto a 2 × 16 cm hydroxylapatite column (Bio-Gel HT, Bio-Rad Laboratories, Richmond, Ca.) previously equilibrated with 0.01 M Na-phosphate buffer (pH 7.6) in 0.1 M NaCl. The column was washed at a flow rate of 10–15 ml/h with at least 3 bed volumes of the equilibrating buffer to remove any loosely retained AChE. The adsorbed AChE was then eluted with a linear gradient consisting of 100 ml each of 0.01 M–0.5 M Na-phosphate buffers (pH 7.6) in 0.1 M NaCl. Fractions of 5.0 ml were collected and the protein content and AChE activity were estimated. Peaks of AChE activity were pooled individually, concentrated against Carbowax PEG-4000 and dialyzed overnight at 4 °C against 10 volumes of the equilibrating buffer. All chemicals were reagent grade or better. Eels were obtained from Paramount Research Supply Co., Ardsley, N.Y. Acetylthiocholine iodide was a Sigma product (Sigma Chemical Co., St. Louis, Mo.).

Results and discussion. The figure shows the hydroxylapatite chromatographic profile of the 5-year-old AChE preparation. About 10–15% of the applied AChE is not adsorbed to the column. The amount of this nonadsorbed fraction was not altered by lowering the phosphate concentration to 0.001 M, increasing the amount of

Properties of the various AChE isozymes

Isozymes	Sedimentation coefficient	K_m (10^{-4}) Mean ± SD	Electrophoretic mobility*
F_0	11.1S	1.7 ± 0.2	1.0
F_1	11.1S	0.97 ± 0.09	1.3
F_2	11.1S	1.3 ± 0.01	1.5
F_3	11.1S	1.9 ± 0.05	1.6
	Average:	1.5 ± 0.4	
	11.0S	1.4 ± 0.2	1.5

Fresh 11S AChE *normalized to F_0.



Chromatography of 11S isozymes on hydroxylapatite.

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enzyme loaded 4fold, using columns of increasing capacity (45 × 2.4 cm, 45 × 4 cm), or adding 0.5% TX-100 and 1 M NaCl to the wash (to disassociate aggregates). These results indicate that the appearance of this fraction in the wash is not due to overloading or aggregation. We designated this fraction: F_0. The remaining activity was quantitatively eluted in 3 fractions: F_1, F_2 and F_3 (figure). This chromatogram was quantitatively and qualitatively reproducible, e.g., the average coefficient of peak height variation was 5%. Isolation and rechromatography of an individual peak fraction resulted in elution at the same point it eluted from originally. This result shows that any possible interconversion between the isolated forms does not take place detectably within a 36 h time frame. Freshly isolated 11S enzyme quantitatively eluted from the column as 1 peak at a point identical with F_2.

The table shows some of the properties of these multiple forms. The forms all have a sedimentation coefficient of 11.1 showing that they are not sizeozymes. The K_m values for acetylthiocholine iodide are slightly different. Fresh 11S has a K_m value of 1.5×10^{-4} M which is similar to the average K_m value of the forms. The electrophoretic mobility of the chromatographically isolated forms (normalized with respect to F_0) does not show a simple linearity of migration versus fraction number. Freshly prepared 11S AChE migrates identically to F_2. Examination of a gel electrophoretogram of the 5-year-old preparation done when the preparation was fresh showed only 1 enzyme activity band. Since electrophoresis conditions were slightly different, we do not know whether it migrated identically to F_2. The original material was shown to be a true acetylcholinesterase since it hydrolyzed butyryl choline at a rate orders of magnitude below the rate it hydrolyzed acetylcholine. Similar tests on F_0, F_1, F_2 and F_3 confirmed that they, too, were true acetylcholinesterases. The apparent identity of mol. wt, utilization of the same substrate and difference in electrophoretic mobility clearly allow F_0, F_1, F_2 and F_3 to be termed isozymes. The origin of this multiplicity and charge difference is not clear. In this connection, the following treatments were tried in an attempt to produce isozymes from fresh 11S enzyme: 30 min incubation at 45 °C; repeated (5–10 times) freeze thawing; 60 min incubation at 37 °C with either 50 μg papain, 25 μg trypsin or 25 μg pepsin (at a protease to AChE ratio of 1/50, 40–60% loss of activity was observed); addition of soybean trypsin inhibitor or phenylmethylfulfonyl fluoride (1.5 mg/100 ml of extract); extraction with 1% TX-100; chromatography over a 10fold range of protein concentration; and incubation with 1 M Guanidine-HCl at 4 °C followed by dialysis against 5000 volumes of 0.01 M Na-phosphate (pH 7.6) in 1 M NaCl (96% loss of activity resulted). Some of these treatments are well known to produce isozymic forms from tetrameric enzymes with different subunits. The others were designed to investigate the effects of exogenous proteases, detergent removal of lipids, and protein concentration on the formation of the isozymes. In no case was more than 1 electrophoretic or chromatographic band seen. These experiments rule out such simple origins for the isozyme bands and suggest that further research is necessary to elucidate the generative process. Though we do not know the actual time dependence for the appearance of each of the forms, it is clear that in studies of genetic and/or tissue specific AChE isozymes, a possible storage problem should be considered and tissues examined at as early a date as is possible.

Modification of theophylline-induced lipolysis in human fat cells after trypsination

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Summary. Trypsin-treatment of human fat cells results in the potentiation of the lipolytic response and the cAMP accumulation induced by theophylline ($5 \cdot 10^{-4}$ M) but not of those induced by theophylline ($5 \cdot 10^{-3}$ M). The amount of cAMP formed after exposure to theophylline ($5 \cdot 10^{-3}$ M) plus norepinephrine ($5 \cdot 10^{-6}$ M) remains, however, 2.6fold higher in trypsin-treated human fat cells than in the control ones.

Recently, we have shown that the exposure of human fat cells to trypsin, although not affecting the lipolytic response of these cells to dibutyryl cyclic AMP, resulted in a marked increase in both lipolysis and 3′–5′ cyclic AMP (cAMP) synthesis induced by catecholamines[2]. Contrasting with these findings, we found that trypsin-treatment of rat fat cells maintained a normal lipolytic response to catecholamines[2].

In the present studies, we have investigated the influence of trypsin-digestion on the lipolytic response of human adipocytes exposed to theophylline, a lipolytic agent inhibiting phosphodiesterases (PDE)[3] and acting therefore on the lipolytic process at a step localized between the sites of action of catecholamines and cAMP.

Materials and methods. Human omental adipose tissue was obtained from overnight fasted, non-obese patients of both sexes (30–50 years old), having no clinical and biochemical evidence of endocrine disease and undergoing abdominal surgery. Procedure for the premedication, anesthesia and adipose tissue collection were as described previously[4].

Human fat cells were isolated following a modification[2] of the method of Rodbell[5]. Procedures used for the preparation of trypsin-treated fat cells, their incubation, the determination of lipolysis, the extraction and the determination of cAMP, as well as the origin of the material used, have been described previously in detail[2]. As the

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