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CHROMOGRANINS AS SUBSTRATE FOR TRANSGLUTAMINASE*†

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Abstract—The possible attachment of amines to proteins obtained from adrenal medulla was investigated using transglutaminase as the enzyme. Transglutaminase catalyzes a replacement of the amide group of certain protein-bound glutamine residues by primary amines. The chromogranins were used as the protein substrate, and proved to be equal to, or sometimes better than, β-lactoglobulin or casein as an acceptor. As replacing amines, norepinephrine, dopamine, and phenylethylamine showed the same affinity for the ox chromogranins as for β-lactoglobulin. Higher incorporation in the chromogranins than in the other two proteins was found with diamines, putrescine and cadaverine. Transglutaminase levels were estimated in total adrenal glands, the medullary and cortical tissue as well as medullary granules. The significance of the presence of transglutaminase in the adrenal medulla is discussed.

THE AMIDE groups of protein- or peptide-bound glutamine can be replaced by primary amines, under catalytic influence of transglutaminase. Native proteins show a wide variation in availability of α-glutamyl amide for this calcium-dependent replacement, and many of the known native proteins have been studied as substrates for transglutaminase. Insulin,[1] hemoglobin,[2] tropomyosin, and actin[3] were investigated. The importance of primary structural properties of glutamine peptides was stressed by Neidle and Acs[4] and Folk and Cole,[5] but even more, the conformation of protein or the polypeptide molecule was shown to influence their susceptibility to the action of transglutaminase.[6]

Among native proteins, casein and β-lactoglobulin were found to be the best substrates.[7] Both have a relatively high content of glutamic acid (near 20%); a similar composition (26–27%) was found for the chromogranins.[8] This soluble protein, stored in chromaffin granules of adrenal medulla,[9,10] is secreted, upon stimulation, in perfusates of adrenal gland, together with catecholamines and adenosine nucleotide. The presence of calcium is critical for the release of catecholamines; this metal is found in high concentrations in the granules, as was shown by Borowitz *et al.*[11] Isolated granules, when lysed,[12,13] were found to contain 80 per cent of soluble protein in the total protein fraction. Fifty per cent of this soluble protein, when purified, was obtained as a homogeneous fraction by Smith and Winkler.[14] Physical characterization by Kirshner and Kirshner[15] indicated that this chromogranin (mol. wt. —80,000)

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is composed of two nearly identical subunits with an average mol. wt. of 40,000, obtained upon reduction with mercaptoethanol.

The presence of the primary amine, the protein that could accept the amine under a catalytic action of transglutaminase, and calcium (the enzyme shows complete dependence on calcium ions) in the medullary tissue was one of the deciding factors in choosing the chromogranins as a substrate in this study.

MATERIALS AND METHODS

Transglutaminase assay. The enzyme levels were determined by the radiochemical method described by Wajda, Lee and Neidle,[16] using carbobenzoxy L-glutaminyl, L-valine methyl ester, and [^{14}C]methylamine as substrates. The activity of the enzyme was expressed in counts per minute per milligram of protein; the protein was determined in tissue homogenates by the Lowry method.[17] Homogenates were obtained using 0·25 M sucrose as the homogenizing medium; the dilutions were: brain, 1:16 (w/v); adrenal glands, medulla, and cortex, 1:20. The medullary granules, when obtained as pellet after a series of centrifugations, were diluted to 1:10 of the original wet weight of the medullary tissue.

Preparation of the chromogranins. The soluble protein from chromaffin granules was obtained using a method modified from that of Banks.[18] The medullary tissue was dissected from 60 bovine adrenal glands, care being taken to work on ice. A homogenate was prepared (1:5 w/v) in 0·3 M sucrose and centrifuged at 600 *g* for 15 min in a Sorvall centrifuge. The precipitate was discarded and the supernatant fraction was centrifuged again at 15,000 *g*. The sediment, which contains the large granules fraction, was washed by swirling with 0·3 M sucrose, and the lower (pink) layer was resuspended and centrifuged again at 15,000 *g* to remove traces of brown material which accumulated in the upper layer. The procedure was repeated once more and the remaining pink material was examined under the phase-contrast microscope; the typical chromaffin granules showed little contamination detectable by this method. When a transglutaminase assay was performed, the pellet was diluted with 0·25 M sucrose (1:10 of the original wet weight of the medullary tissue); if the material was used for further purification of the soluble protein fraction, the granules were lysed at this point by adding distilled water (60% of the original wet weight of adrenal medullary tissue). Centrifugation in the Spinco centrifuge for 20 min at 105,000 *g* followed, according to the procedure described by Helle.[19] The supernatants were dialyzed in the cold overnight against distilled water, and finally lyophilized. From 60 bovine adrenal glands, 200 g of medullary tissue was harvested, and the recovery of dry protein was 0·1 per cent. This yield is in agreement with that reported by Helle,[19] who obtained 0·1–0·3 per cent, according to the animal used. The amino acid composition of each batch of chromogranins was determined. For this purpose 1–2 mg of dry protein was hydrolyzed for 20 hr at 110° in 6 N HCl (5 ml/1 mg of protein) in vacuum-sealed tubes. The amino acid content was assayed using the Technicon amino acid analyzer. If the results of analysis indicated a preparation sufficiently similar to those of Streider *et al.*,[20] the incorporation experiments were carried out. Some preparations of the chromogranins were tested for transglutaminase activity.

Determination of ammonia released by transglutaminase. The substrate protein (10 mg/0·5 ml of distilled water) was incubated with 0·1 ml of 0·1 M $CaCl_2$, 0·1 ml of 0·5 M glutathione, 0·8 ml of 0·1 M Tris buffer (pH 7·2), and 0·5 ml of purified

enzyme preparation (2000–3000 units/mg of protein), in a total volume of 2·0 ml, for 60 min at 37°; the reaction was stopped by the addition of 0·2 ml of 50% trichloracetic acid, and the protein was removed by centrifugation at 40° for 10 min. A portion (1·3 ml) of the supernatant fluid was neutralized and made up to 5 ml with water. Two-ml portions were used for the determination by the Conway-microdiffusion technique.[21]

Determination of amide-nitrogen released by acid hydrolysis. The protein (5 mg) was hydrolyzed in 0·5 ml of 2 N HCl for 2 hr at 100°. Each sample was neutralized with 2 N NaOH and made up to 10 ml by adding distilled water. Ammonia was measured in 2-ml portions by the Conway-microdiffusion method as mentioned above. The procedure was described by Leach and Parkhill.[22]

Purification of transglutaminase. A partially purified preparation of transglutaminase was obtained according to the procedure of Waelsch and Mycek.[23] Fresh guinea pig livers were used as the source of enzyme. Ten-fold purification was usually attained, and the preparation did not change activity (2000–3000 units/mg of protein) for a week when kept at 4°. The enzyme activity was assayed using the hydroxylamine method;[24] one unit of enzyme activity corresponds to a change in absorbance of 0·0001/min of incubation time, as described by Wajda *et al.* [25]

Incorporation of various amines. DL-[7-^{14}C]Norepinephrine, [1-^{14}C]3,4-dihydroxyphenylethylamine hydrobromide (dopamine), [1-^{14}C]*p*-hydroxyphenylethylamine hydrobromide (tyramine), [1-^{14}C]β-phenylethylamine hydrochloride, [1,4-^{14}C]putrescine dihydrochloride, [1,5-^{14}C]cadaverine dihydrochloride, and [1,4^{14}C]spermine-tetrahydrochloride, were obtained from New England Nuclear Corp. [2-^{14}C]Histamine ring dihydrochloride and [3-^{14}C]5-hydroxytryptamine-creatine sulfate were purchased from Nuclear Chicago. Dilutions were made with the corresponding unlabelled amines to give a final concentration 0·1 M with a sp. act. of 0·05 μc/μmoles. The incubation mixture contained 10 μmoles of $CaCl_2$, 80 μmoles of Tris buffer, pH 8·3, 20 μmoles of reduced glutathione, 10 μmoles [^{14}C]amine (sp. act. 0·05 μc/μmole), 5·0 mg of protein, and 0·1 ml of purified transglutaminase (sp. act. 2000–3000 units/mg of protein). The final volume of the reaction mixture was 2·0 ml. Incubations were carried out for 1 hr at 37°; the reaction was stopped by the addition of 0·2 ml of 50% trichloroacetic acid (TCA). The protein was collected by centrifugation, washed six times with 3·0 ml of 50% TCA, heated at 90° in 5% TCA for 3 min, and washed once with 2·0 ml of absolute alcohol, 2·0 ml of ether–alcohol–chloroform mixture (2:2:1), and once with ether; it was dried under vacuum overnight. After weighing, the protein was dissolved in 2·0 ml of Soluene in a scintillation vial, and 15·0 ml of scintillation fluid was added. The radioactivity was determined using a Nuclear Chicago scintillation counter, using 0 time blanks, where the enzyme was destroyed by addition of 0·2 ml of 50% TCA. In several experiments the nonenzymatic incorporation of primary amines was also tested. In those cases, the transglutaminase preparation was replaced by the 0·1 ml of Tris buffer.

RESULTS

Tissue levels of transglutaminase. The enzyme is present in almost all tissues of the body. Liver and spleen are the richest sources of transglutaminase; 20–30 times less is found in the brain.[16] The enzyme is present in the soluble fraction of tissue homogenates, residing probably in the cytoplasm, and only traces of it can be detected in plasma.

Table 1 represents a comparative study of the levels of transglutaminase, assayed by a radio-chemical method, in brain, whole adrenal gland, adrenal cortex, adrenal medulla, chromaffin granules, and the chromogranins, obtained from different animals.

TABLE 1. TRANSGLUTAMINASE ACTIVITY IN BRAIN AND ADRENAL TISSUE

Specie	Organ	Enzyme activity in counts/min/mg of protein
Guinea-pig	Adrenal gland	2860
	Brain	405
Rat	Adrenal gland	2400
	Brain	370
Mouse	Adrenal gland	5900
	Brain	450
Sheep	Adrenal cortex	4404
	Adrenal medulla	3577
	Medullary granules	272
Ox	Adrenal cortex	6662
	Adrenal medulla	3717
	Medullary granules	250
	Chromogranins	90

* The results presented here are a composite of 3 separate experiments in the material obtained from sheep and ox. The values for guinea-pig, rat and mouse tissues are the means of 6 estimations. Radiochemical method of estimation, as described in Methods, was used.

The level of enzyme in the adrenal gland of guinea-pigs, rats, and mice was 5–10 times higher than in brain. Sheep and bovine adrenal gland showed equal activities in cortical and medullary parts of the gland; the activity in chromaffin granules obtained from the medullary tissue of sheep and ox did not differ much from the usual activity of total brain homogenates obtained from guinea pigs, rats, and mice. The level of transglutaminase in isolated sheep or bovine chromaffin granules was less than 1/10 of the average tissue level. This small quantity of transglutaminase could be a residual amount left from the supernatant enzyme. These findings suggest that the soluble protein has little or no enzymatic activity, but the transglutaminase levels of our preparation of the chromaffin granules are equal to that in brain tissue.

Amino acid content of the chromogranins. The granular soluble protein was obtained from 60 bovine adrenal glands freshly excised and brought from the slaughter house on ice. From about 200 g of wet weight of the medullary tissue, 200 mg of dry, lyophilized protein was usually obtained; this yield (0·1%) is in close agreement with that obtained by Helle;[19] her recoveries were from 0·1 to 0·3% according to the species used. The total number of bovine medullae used in this study was 450. When comparing the amino acid content of our preparations of the chromogranins, or ox total soluble proteins from chromaffin granules (Table 2) to those published by Streider *et al.*,[20] only small differences are noted even though we did not attempt to use

density gradient centrifugation to obtain highly purified preparations of chromaffin granules, and the final purification of the soluble protein consisted only of overnight dialysis. The values are, with minor exceptions (lower values for arginine, and histamine), much closer to those published by Streider *et al.*,[20] than to those found in β-lactoglobulin or casein. It was felt, therefore, that the general name, the *chromogranins*, can be used for this protein.

TABLE 2. AMINO ACID ANALYSIS OF PROTEINS USED AS SUBSTRATES FOR TRANSGLUTAMINASE*

Amino acid	β-Lactoglobulin	Casein	Ox chromogranins	
			Present values	Values of Streider *et al.*[20]
Glu	21·5	22·4	25·3	26·6
Lys	11·2	8·2	9·0	8·6
Asp	11·2	7·1	9·5	7·7
Pro	4·1	11·3	7·2	8·0
Leu	15·6	9·3	7·1	6·8
Arg	2·8	4·1	6·7	10·7
Ser	4·4	6·3	6·1	5·2
Ala	6·2	3·0	5·6	4·2
Gly	1·4	2·7	4·4	3·8
Val	5·8	7·2	3·6	2·7
His	1·5	3·1	2·1	3·6
Thr	5·9	4·9	3·4	2·3
Phe	3·5	5·0	3·0	2·3
Meth	3·2	3·4	1·4	1·8
Tyr	3·8	6·3	2·4	2·2
Ile	8·4	6·1	2·2	1·0
Cys	1·1	0·3	1·6	0·6
NH_3	1·3			1·8
Total	112·9	110·7	102·0	99·9

* The results are expressed as grams of amino acid per 100 g of protein, and the present values of ox chromogranins are based on 10 experiments.

The chromogranins have a rather unusual amino acid composition, being very rich in glutamic acid. Only two other animal proteins contain more glutamic acid: tropomyosin (29%),[26] and the acidic protein of brain, first isolated by Moore,[27] so-called protein S-100, which contains over 30%.

Reactivity of protein-bound amide groups. Amide groups of protein-bound glutamine are lost in the hydrolytic procedure preceding the amino acid analysis. Because they are of primary importance for transglutaminase-catalyzed reactions, a comparison of the chemical method of amide ammonia estimation with transglutaminase-catalyzed hydrolysis of glutamine residues was undertaken; a partially purified preparation of transglutaminase was used (Table 3). Three proteins used in this study as substrates were compared for availability of glutamine amide groups for transglutaminase-catalyzed exchange. Transglutaminase released equal amounts of ammonia from the chromogranins and from casein; much less was released from β-lactoglobulin. The release of ammonia by 2-hr hydrolysis in 2 N HCl is summarized in the second column, and the comparison of those two values, as present in the third column, gives the

theoretical reactivity of the 3 proteins as substrates for transglutaminase. It can be seen that the glutamine amide groups of the chromogranins, although lower in total number than in casein, show higher relative reactivity in transglutaminase-catalyzed hydrolysis.

TABLE 3. AMMONIA LIBERATION BY THE ACTION OF TRANSGLUTAMINASE OR BY ACID HYDROLYSIS*

Protein substrate	μmoles of NH_3/10 mg of protein released by:		Per cent of total amide groups released by transglutaminase
	Transglutaminase	Acid hydrolysis	
Chromogranins (bovine medulla)	0·64	4·84	13·0
β-Lactoglobulin	0·31	3·69	8·0
Casein	0·69	8·02	8·0

* Each value is the mean from 3 different experiments.

Incorporation of amines into the chromogranins, β-lactoglobulin, and casein. Figure 1 presents the transglutaminase-catalyzed incorporation of norepinephrine into β-lactoglobulin and the chromogranins with respect to time. The reaction was complete after 1 hr of incubation.

A number of amines were incorporated (Table 4). Norepinephrine and dopamine were incorporated to the same extent as tyramine into all 3 proteins. Higher values of incorporation were noticed when β-phenylethylamine and histamine were used as

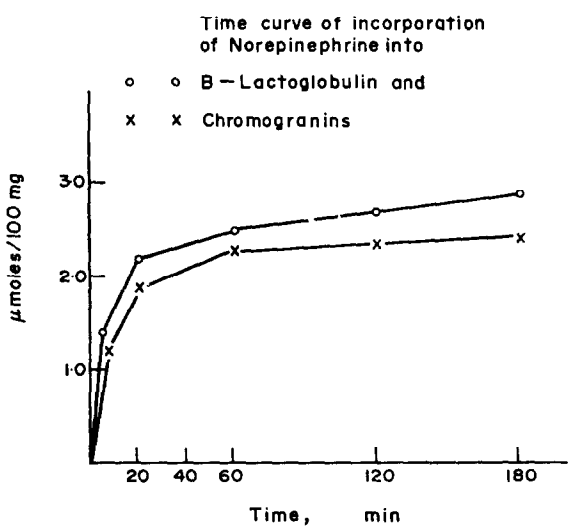

FIG. 1. Each point on the graph was obtained from 3 separate experiments. The conditions of incorporation are the same as described in Methods.

TABLE 4. TRANSGLUTAMINASE CATALYZED INCORPORATION OF PRIMARY AMINE (μmoles/100 mg OF PROTEIN)*

Radioactive amine	Protein substrate		
	β-Lactoglobulin	Casein	Ox chromogranins
Norepinephrine	2·66	1·40	2·33
Tyramine	3·34	4·12	2·45
Dopamine	3·46	3·33	3·29
β-Phenylethylamine	5·57	4·62	4·80
5-Hydroxytryptamine	1·33	0·78	0·47
Histamine	4·32	9·14	6·88
Putrescine	4·99	3·57	8·88
Cadaverine	4·01	5·00	5·66
Spermine	5·95	7·11	7·79

* Each value is a mean of 3–6 separate experiments. Incubation conditions are described in Methods.

replacing amines. Three diamines (putrescine, cadaverine, and spermine) were incorporated better than most other amines; this observation was made before by others.[7] High values of histamine incorporation were different in the 3 substrates, very high in casein and lower in β-lactoglobulin. Serotonin was the least active of all 3 substrates as the replacing amine.

Displacement studies. Taking into account the fact that many amines have the ability to release norepinephrine, and that tyramine was particularly active in this respect,[28–30] an attempt was made to establish the ability of the radioactive amine to displace the cold amine. This was done by preincubating in the presence of cold amine (at 10 times the concentration of radioactive amine) followed by an incubation of additional usual quantities of the radioactive amine. Only putrescine and norepinephrine completely occupied all the sites normally accepting the radioactive amine. The inhibitory effect of β-phenylethylamine was also high, and a lower percentage of inhibition was noticed when tyramine, histamine, or dopamine was used for preincubation (Table 5).

Incorporation of amine without addition of transglutaminase. Some of the primary amines used were also tested for such incorporation into β-lactoglobulin and the chromogranins. Very small incorporation was noticed, but values obtained were scarcely above those recorded at 0 time blanks when putrescine and tyramine were used. Only in the case of norepinephrine was such acceptance significant, reaching 20 per cent of incorporation in the chromogranins when transglutaminase was added to the incubation mixture.

DISCUSSION

The chromogranins obtained from bovine adrenal medulla were used as the substrate in calcium-dependent exchange reactions, involving the glutamine amide nitrogen and a suitable replacing primary amine, under catalytic action of transglutaminase. The detailed mechanism of this reaction was investigated with various protein substrates,[1,2] and we assumed it to be identical with the chromogranins.

TABLE 5. PERCENTAGE INHIBITION OF AMINE INCORPORATION BY OTHER PRIMARY AMINES*

Protein substrate	Radioactive amine (final concn 5×10^{-3} M; incub. time 30 min)	Cold amine (final concn 5×10^{-2} M; incub. time 60 min)	Percentage inhibition
Ox chromogranins	Norepinephrine	β-Phenylethylamine	94·0
	Norepinephrine	Tyramine	84·5
	Norepinephrine	Histamine	86·6
	5-Hydroxytryptamine	Tyramine	78·7
	5-Hydroxytryptamine	Dopamine	74·3
	β-Phenylethylamine	Norepinephrine	99·0
	β-Phenylethylamine	Putrescine	99·0
β-Lactoglobulin	β-Phenylethylamine	Putrescine	99·0
	5-Hydroxytryptamine	Tyramine	89·5
	5-Hydroxytryptamine	Dopamine	83·5
Casein	β-Phenylethylamine	Putrescine	99·0
	5-Hydroxytryptamine	Tyramine	82·1
	5-Hydroxytryptamine	Dopamine	81·7

* Cold amine was preincubated with the protein for 30 min. After adding the radioactive amine further incubation of 30 min at 37° followed. The results are composite of 2 estimations.

A high content of glutamic acid (over 26%) in the chromogranins (Blaschko *et al.*;[31] Helle[10]) suggested that the protein might have more reactive glutamine residues than β-lactoglobulin or casein, 2 proteins considered to be very good substrates for transglutaminase.

The results of this study confirm this working hypothesis, and it is of interest to note that, as judged from the amount of ammonia liberation, the amide groups of the chromogranins show higher reactivity for transglutaminase than the other 2 substrates. In the chromogranins 13 per cent of the amide groups was reactive in transglutaminase-catalyzed hydrolysis whereas only 8 per cent of the total amide groups of the other 2 proteins was released as ammonia in enzymatic hydrolysis.

The role of the chromogranins in the adrenal vesicles which store the catecholamines is not clear. They are present in large quantity and are released with relative ease; half of the total content of the soluble protein in the granules consists of the chromogranins,[15] and 80 per cent of the total protein content is found in soluble fraction.[12] The chromogranins represent a good natural substrate for the transglutaminase, and for the purpose of this study they were obtained quickly and in a relatively pure state. The incorporation of amines by transglutaminase is not easily reversible *in vitro*, although the possibility exists that factors unknown at present may increase the reverse reactions of amine incorporation. Irreversible binding of biogenic amines to proteolipids was studied by Mokrasch *et al.*,[32] and a suggestion was presented at that time that an endogenous enzymatic reaction could be responsible for the release of covalently bound amine, by an enzyme similar in action to that discovered in snake venom.[33] Undoubtedly, more research is needed before we can answer this question.

Transglutaminase is present in adrenal tissue and requires Ca^{2+} ions for its activity. The possible role of transglutaminase in the secretory process, however, is difficult

to accept, as the binding of primary amine to the protein was proved to be of covalent nature. A certain amount of binding of the amine cannot be ruled out completely. Borowitz,[34] in a recent study of possible involvement of calcium in binding of catecholamines in bovine adrenal medulla, gives evidences that calcium is part of the binding complex. At the same time, kinetic studies of Folk *et al.*[35] on transglutaminase support the proposition that the enzyme functions in combination with the divalent cation as a metal–enzyme complex.

The covalent binding of amines to the substrate, the fact that only the primary amines are involved in the reaction catalyzed by transglutaminase, and also the small quantity of norepinephrine (2·3 μmoles/100 mg of protein) that can be bound to the suitable protein all argue against transglutaminase playing a role in catecholamine storage. Nevertheless, the presence of transglutaminase in adrenal medulla might have a different, important role in the metabolism of the gland.

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