CHIMIA 45 (1991) Nr. 1/2 (Januar/Februar)

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Eingegangen am 11. Dezember 1990

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Chimia 45 (1991) 43–45

Gas-phase Sequencing of Photoimmobilized Peptides

Vibhuti Klingler-Dabral[2]), **André Collioud**[1]), **Johann Schaller**[1]), **Franz Herbst**[2]), **Wolf-Georg Forssmann**[2]), and **Hans Sigrist**[1])*

Abstract. Aminopropylated glass-fiber discs were derivatized with the photosensitive reagents 3-(trifluoromethyl)-3-[*m*-(isothiocyano)phenyl]diazirine and *p*-azidophenyl isothiocyanate, respectively. Photolabel-derivatized solid supports were photoactivated in the presence of a hexapeptide. Gas-phase sequence analysis of washed fiber discs yielded end-to-end sequences with photoactivated disks only, indicating efficient photo-immobilization of the applied peptide. The quantity of amino acid released with each *Edman*-degradation cycle conferred with the established philicity of amino-acid side chains for photogenerated carbenes. The procedure provides a hitherto unexplored way to immobilize biomolecules (polypeptides) through statistically occurring carbene or nitrene insertions.

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This study reports on the preparation and characterization of photoactivable supports, which carry monomolecular layers of carbene- or nitrene-generating photoreagents. Photo-activation of photolabel-derived supports in the presence of a hexapeptide leads to covalent polypeptide immobilization by carbene/nitrene insertion. Evidence is presented that photocoupled peptides can be sequenced by *Edman*-degradation-based gas-phase microsequencing.

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Photoactivable Glass Fibers: Preparation and Loading

The bifunctional photoreagents 3-(trifluoromethyl)-3-[*m*-(isothiocyano)phenyl]diazirine (TRIMID) and *p*-azidophenyl isothiocyanate (API) were synthesized as described in [7][8]. GF/C glass fiber sheets (*Whatman*) were aminopropylated according to *Aebersold et al.* [2] yielding 14 nmol of NH_2 groups per mg glass fiber. Dried aminopropylated glass-fiber sheets were cut into discs of 1.2 cm in diameter.

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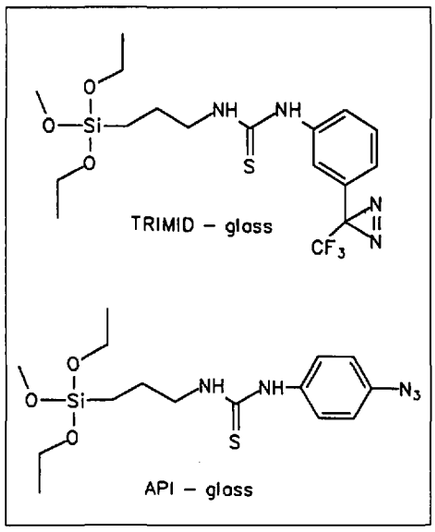

Fig. 1. *Structure of aminopropylated glass fibers thiocarbamoylated with 3-(trifluoromethyl)-3-[*m*-(isothiocyano)-phenyl]-diazirine* (TRIMID-glass) or p-*azidophenylisothiocyanate* (API-glass)

photoactivated. Released amino acids were identified and spectrophotometrically quantified as phenylthiohydantoin derivatives.

Results

Philicity of Amino-Acid Side Chains for Photogenerated Nitrenes and Carbenes

Amino-acid binding capacities of carbene- and nitrene-generating supports (*Fig. 1*) were investigated by analyzing net amino-acid retention upon photocoupling of radioactive tyrosine or alanine. ^{14}C-tyrosine (166 pmol) or ^{14}C-alanine (110 pmol) were added per mg photoactivable support of either system. Analysis of net radiolabel binding revealed that photogenerated nitrene supports bind 8.2% of applied tyrosine and 1.2% of applied alanine. Amino-acid binding to photogenerated carbene supports was in line with previously reported results [10] (3.6% of applied tyrosine and 2.2% of applied alanine). Since the conditions for photoactivation of TRIMID-glass and API-glass differ significantly in light intensity, duration, and the spectrum of activating light, it is at present not possible to comparatively qualify the two supports. However, the results clearly document that both types of photoactivable glass fiber are capable of establishing covalent links with amino acids. The newly described photo-activable supports are thus applicable for polypeptide coupling.

Gas-Phase Sequence Analysis of a Photocoupled Hexapeptide

The hexapeptide H–Leu–Trp–Met–Arg–Phe–Ala–OH (10 nmol per disc) was spotted to each of the supports and photo-activated as described in *Experimental* . Subsequent sequencing of washed discs indicated that the polypeptide was retained on photoactivated discs only, initial yields being 10.9% for the carbene-coupled peptide and 5.7% for the hexapeptide immobilized *via* photogenerated nitrenes (*Fig. 2*). The results further showed that end-to-end sequencing of photo-immobilized peptides is feasible and suggest that carbene- and nitrene-insertion re-

Preparation of TRIMID Glass

Aminopropylated glass-fiber discs were incubated with TRIMID dissolved in $CHCl_3/Et_3N$ 200:0.1 (*v/v*) with a 10-fold molar excess of photolabel over available NH_2 groups. Thiocarbamoylation of the supports was carried out for 20 min at 50° on a labshaker. Photolabel-derivatized glass fibers were washed with $CHCl_3$ (16 ml) followed by 4 mM cysteine, pH 7.4 (4 ml) and $CHCl_3$ (6 ml).

Preparation of API-Glass

Aminopropylated glass-fiber discs were incubated in 17 mM API in cyclohexane for 90 min at 40°. Modified discs were washed with 90 ml of $CHCl_3$, dried with N_2, and stored at –20° [9].

The protein-(amino-acid)binding capacity of photolabel-derivatized discs was determined by analyzing light-dependent immobilization of L-(U-^{14}C)-tyrosine (*Amersham*, 8.33 GBq mmol^{-1}) and L-(U-^{14}C)-alanine (*Amersham*, 2.78 GBq mmol^{-1}), resp. The hexapeptide H–Leu–Trp–Met–Arg–Phe–Ala–OH (*Serva*) dissolved in 15 µl of H_2O was spotted to wet (5 µl EtOH) photoactivable glass-fiber discs. Before photoactivation, applied substances were dried under N_2.

Photoactivation

TRIMID-derivatized glass-fiber discs were placed between two quartz slides in a custom-made holder. The holder was positioned 45 cm from the light source (*HBO 350* Hg lamp, *Osram*). Samples were photoactivated (controlled lamp power 200 W) under Ar with filtered light (*Schott WG 320* and a 1 cm layer of sat. $CuSO_4$ in H_2O; transmission band width 320–550 nm) for 16 min (8 min each filter face). API-derivatized glass-fiber discs were photoactivated with consecutive flashes from an electronic flash unit of a camera (*Optatron 350, Loewe Opta*) with the protecting screen removed. Samples were placed 4 cm apart from the light source and exposed with 40 flashes (20 for each disc face). Photoactivated glass-fiber discs were thoroughly washed with solvents, applied in the following sequence and quantities: 1 M NaCl (20 ml), H_2O (10 ml), EtOH (10 ml), $CHCl_3$ (10 ml), toluene (10 ml).

Sequence Analysis

N-Terminal sequence analysis of the photocoupled hexapeptide was carried out using an *Applied Biosystems 477A Protein Sequencer*. Extensively washed fiber discs were sequenced without addition of polybrene. Control samples were identically treated but not

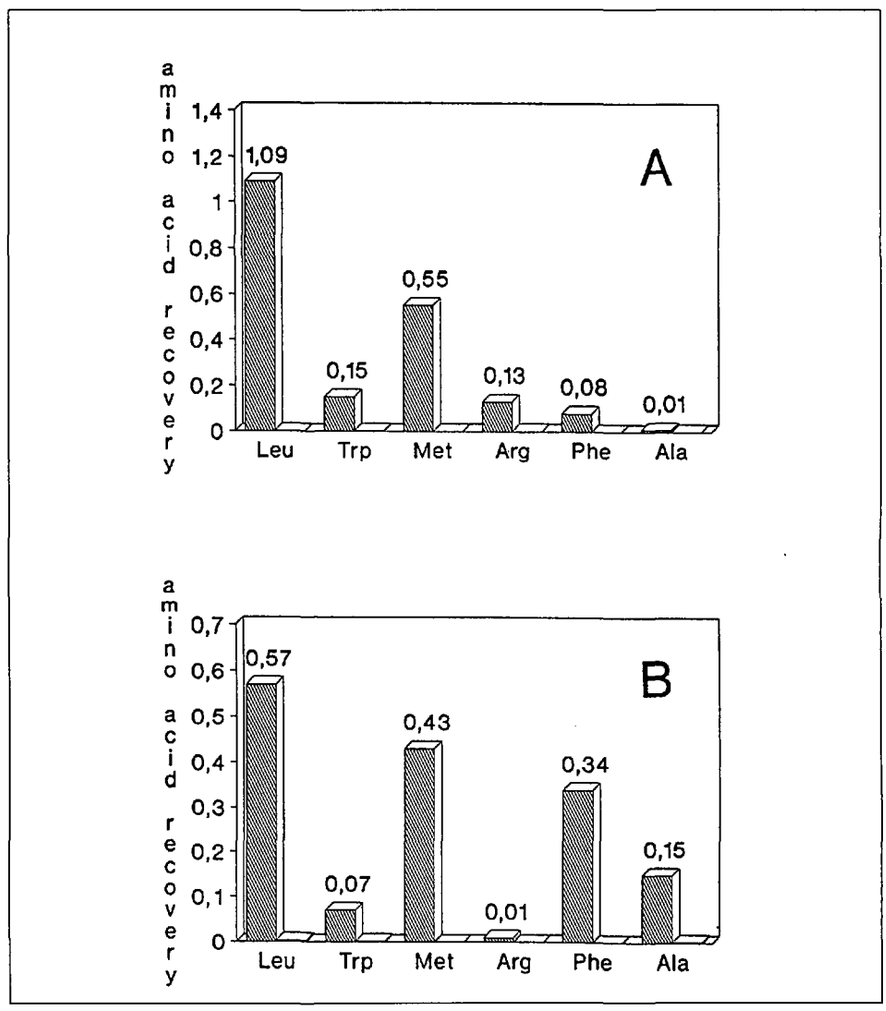

Fig. 2. *The hexapeptide H–Leu–Trp–Met–Arg–Phe–Ala–OH* (10 nmol, 15 µl) *was spotted to TRIMID-glass* (*A*) or API-glass (*B*). Impregnated supports were photoactivated, thoroughly washed and submitted to automated sequence analysis. Amino-acid recoveries (nmol) are depicted for photoactivated (▒) and control samples without photoactivation (■).

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actions occur with all constituting amino-acid side chains (*Fig. 2*). That the recovery of tryptophan in the second *Edman*-degradation step is lower than the following methionine (*Fig. 2 A*) is in accordance with the observed high carbene philicity of tryptophan [10]. An apparent limitation of the newly described immobilization system is the low recovery of applied peptide due to the limiting number of available photoactive functions. Experiments designed to augment photolabel density are in progress.

Discussion

Gas-phase sequencing procedures currently applied in protein chemical analysis are based mainly on noncovalent interactions between the polypeptide (to be sequenced) and an adsorbing solid support (impregnated glass, PVDF membranes). Alternatively, classical solid-phase sequencing techniques utilize covalent group-specific reactions for chemical polypeptide immobilization [3]. In either case, proteins are spotted or blotted onto supporting discs or membrane material. Due to side reactions with buffer components, efficient blotting of polypeptides to functionalized membranes is difficult. Furthermore, removal of blotting buffer or detergents before sequence analysis may lead to uncontrolled polypeptide loss. The described photo-activable supports provide a new and attractive way to immobilize polypeptides (and biomolecules in general) by nonspecific random covalent interactions [11]. Immobilized photoreagents serve as molecular glue. Photolabel-derivatized supports are 'chemically inert' until light exposure. With reference to the diazirine-derivatized (TRIMID) glass, the activating light (350 nm) is not destructive for protein structure and function [12].

This research was supported by *EMBO* (fellowship to *V. K.-D.*), the *Sandoz Foundation*, and the *Swiss National Science Foundation* (grants No. 3.391-0.86 and 31-26234.89).

Received: December 11, 1990

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Chimia 45 (1991) 45–49

Inhibition of the Nicotinic Ion Channel by Arachidonic Acid and Other Unsaturated Fatty Acids in Chromaffin Cells from Bovine Adrenal Medulla**

Markus U. Ehrengruber and **Peter Zahler***

Abstract. The effects of arachidonic and other *cis*-unsaturated fatty acids were investigated in cultured chromaffin cells from bovine adrenal medulla with respect to Ca^{2+} influx and secretion of catecholamines. As previously shown, arachidonic acid generated during the signal transduction is essential for the fusion of the chromaffin granules with the plasma membrane and by this enables secretion [10–12]. However, if one adds arachidonic acid externally before the agonist, this leads to a partial inhibition of the cholinergic induced secretion. Preincubation with *cis*-unsaturated fatty acids totally blocks the cholinergic induced Ca^{2+} influx but not the Ca^{2+} influx evoked by the voltage gated Ca^{2+}channel. This inhibition is not due to activation of protein kinase C by arachidonic acid, since preincubation with PMA does not block the receptor-dependent Ca^{2+} influx. Preincubation with 5-, 12, 15-HETE and 12-HPETE does not inhibit the receptor-dependent Ca^{2+} influx, indicating that eicosanoids are not responsible for the inhibition. This statement is supported by the fact that BW755C does not reverse the arachidonic acid-evoked blockade of the receptor-dependent Ca^{2+} influx. These results demonstrate that *cis*-unsaturated fatty acids and especially arachidonic acid inhibit the receptor-dependent Ca^{2+} influx by blocking the nicotinic cation channel. The generation of arachidonic acid in the physiological process of the stimulus-response coupling may, thus, contribute to the relaxation, of the cell by preventing further Ca^{2+} influx.

Introduction

Chromaffin cells (CC) from adrenal medulla secrete catecholamines upon stimulation by cholinergic agonists, *e.g.* acetylcholine (ACh) or by depolarization of the plasma mebrane with high K^+ [1]. Stimulation of the cells leads to membrane depolarization and causes a transient increase of membrane permeability to Ca^{2+} (Ca^{2+} influx) which is necessary for the initiation of the secretion [2]. The cells contain nicotinic and muscarinic receptors, both of them beeing activated by ACh [3]. Muscarinic receptors exhibit a higher affinity for ACh [4] and activate the phosphatidylinositol cycle in bovine adrenal medulla without inducing catecholamine secretion [5]. The nicotinic receptor is a heteropentameric complex containing the ligand-binding structure and a cation-selective channel [6]. The supposed physiological sequence of events leading to the large transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) is initiated, when ACh binds to the nicotinic receptor and

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**Abbrevations used:* AA: arachidonic acid; ACh: acetylcholine; BW755C: 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline; $[Ca^{2+}]_i$: cytosolic free calcium concentration; CC: chromaffin cells; HETE: hydroxyicosatetraenoic acid; HPETE: hydroperoxyicosatetraenoic acid; PKC: protein kinase C; PMA: phorbol-12-myristate-13-acetate; UFA: unsaturated fatty acid.