Research article

NO-independent regulatory site of direct sGC stimulators like YC-1 and BAY 41-2272

Eva Maria Becker[1], Cristina Alonso-Alija[1], Heiner Apeler[1], Rupert Gerzer[3], Torsten Minuth[1], Ulrich Pleiβ[1], Peter Schmidt[1], Matthias Schramm[1], Henning Schröder[2], Werner Schroeder[1], Wolfram Steinke[1], Alexander Straub[1] and Johannes-Peter Stasch*[1]

Address: [1]Pharma Research Center, Bayer AG, Wuppertal, Germany, [2]Martin Luther University, School of Pharmacy, Halle, Germany and [3]DLR, Institute of Aerospace Medicine, Köln, Germany

E-mail: Eva Maria Becker - eva.becker@gmx.net; Cristina Alonso-Alija - cristina.alonso-alija@bayer-ag.de; Heiner Apeler - heiner.apeler.ha@bayer-ag.de; Rupert Gerzer - rupert.gerzer@dlr.de; Torsten Minuth - torsten.minuth.tm@bayer-ag.de; Ulrich Pleiβ - ulrich.pleiss.up@bayer-ag.de; Peter Schmidt - peter.schmidt.ps2@bayer-ag.de; Matthias Schramm - matthias.schramm.ms@bayer-ag.de; Henning Schröder - schroeder@pharmazie.uni-halle.de; Werner Schroeder - werner.schroeder.ws@bayer-ag.de; Wolfram Steinke - wolfram.steinke.ws@bayer-ag.de; Alexander Straub - alexander.straub.as@bayer-ag.de; Johannes-Peter Stasch* - johannes-peter.stasch.js@bayer-ag.de

*Corresponding author

Abstract

Background: The most important receptor for nitic oxide is the soluble guanylate cyclase (sGC), a heme containing heterodimer. Recently, a pyrazolopyridine derivative BAY 41-2272, structurally related to YC-1, was identified stimulating soluble guanylate cyclase in an NO-independent manner, which results in vasodilatation and antiplatelet activity. The study described here addresses the identification of the NO-independent site on soluble guanylate cyclase.

Results: We developed a photoaffinity label (^{3}H-meta-PAL) for the direct and NO-independent soluble guanylate cyclase (sGC) stimulator BAY 41-2272 by introducing an azido-group into the tritium labeled compound. The synthesized photoaffinitylabel directly stimulates the purified sGC and shows in combination with NO a synergistic effect on sGC activity. Irradiation with UV light of ^{3}H-meta-PAL together with the highly purified sGC leads to a covalent binding to the α_1-subunit of the enzyme. This binding is blocked by unlabeled meta-PAL, YC-1 and BAY 41-2272. For further identification of the NO-independent regulatory site the ^{3}H-meta-PAL labeled sGC was fragmented by CNBr digest. The ^{3}H-meta-PAL binds to a CNBr fragment, consisting of the amino acids 236–290 of the α_1-subunit. Determination of radioactivity of the single PTH-cycles from the sequencing of this CNBr fragment detected the cysteines 238 and 243 as binding residues of the ^{3}H-meta-PAL.

Conclusions: Our data demonstrate that the region surrounding the cysteines 238 and 243 in the α_1-subunit of the sGC could play an important role in regulation of sGC activity and could be the target of this new type of sGC stimulators.

Background

Guanylate cyclases (GTP pyrophosphate-lyase [cyclizing];

EC 4.6.1.2) catalyze the biosynthesis of guanosine 3',5'-cyclic monophosphate (cGMP) from GTP. While the

membrane bound forms are monomers and stimulated by the natriuretic peptides, the soluble guanylate cyclases (sGC) exist as heterodimers consisting of an α_1- and a β_1-subunit and containing heme as a prosthetic group [1]. Besides this major species of sGC there exist also reports of homodimeric forms of this enzyme [2,3]. By formation of cGMP as a second messenger, sGC plays an important role in smooth muscle cell relaxation [4], inhibition of platelet aggregation, retinal signal transduction [5] and synaptic transmission [6]. The enzyme is strongly activated by NO [7], by the new direct and NO-independent stimulator YC-1 [8–11], and to a lesser extend by CO [12–14,11]. Thus in several studies YC-1 was shown to be an antithrombotic agent by elevation of cGMP, VASP phosphorylation and inhibiting platelet aggregation [8,13,15–18]. Furthermore YC-1 was shown to relax precontracted aortic rings [9], even if they were made tolerant with glyceryl trinitrite [19] and to induce a dose-dependent decrease in systolic blood pressure [19]. Interestingly, in addition to the direct activation of the purified sGC by YC-1, an overadditive effect was observed by the combinations of YC-1 and NO or CO [9–11,18,20]. It was shown that YC-1 is a heme-dependent but NO-independent stimulator of sGC [11,13]. In contrast to NO and CO which directly interact with the heme group of the enzyme, YC-1 did not change the spectral characteristics of the heme moiety of sGC [11]. Therefore it is believed that YC-1 did not interact directly with the heme region of the sGC. Because YC-1 sensitises the enzyme towards their endogenous ligands NO and CO, it is proposed that YC-1 binds to an allosteric site on sGC and thereby reduces the ligand dissociation rates from the heme group [10,11,20,21].

Recently, we described the pharmacological profile of a new sGC stimulator – BAY 41-2272 with similar characteristics like YC-1, however, with a distinctly higher potency and no PDE inhibitory activity [22]. Using BAY 41-2272 as lead structure of this new pharmacological principle, we synthesized the first photoaffinity label derived from the BAY 41-2272 chemical core structure, characterized it on the purified enzyme, labeled the highly purified sGC, and identified the binding amino acids of this new type of sGC stimulators.

Results

We developed the first photoaffinity label for direct and NO-independent sGC stimulators. Coming from YC-1 and BAY 41-2272 as lead structures of direct and NO-independent sGC stimulators we synthesized the ortho- (BAY 50-6038), meta- (BAY 51-9491) and para-PAL (BAY 50-8364) compounds (Fig. 1) and tested their influence on sGC activity. The meta- and para-PAL showed on the one hand a direct sGC stimulation comparable to YC-1 and on the other hand in combination with NO distinct
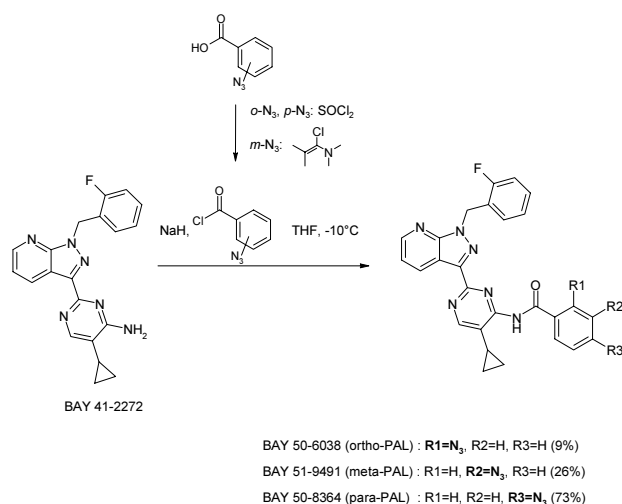

Figure 1
Synthesis of the ortho-, meta- and para-PAL compounds.

synergistic effects on sGC activity. The ortho-PAL showed the lowest sGC stimulation and in combination with NO no synergistic effect on sGC activity (Tab. 1). Both under reducing conditions and in the absence of DTT, in the sGC assay a similar sGC activating profile of the three PALs is given (Tab. 1). Because of the more dominant stimulation of sGC by the single compound both in the presence and absence of DTT the meta-PAL compound was chosen as photoaffinitylabel for the following studies (Tab. 1).

Similar to YC-1 and BAY 41-2272, meta-PAL (BAY 51-9491) concentration-dependently stimulates purified sGC and shows in combination with NO a potentiation of sGC activity, while in combination with YC-1, only additive effects could be detected. sGC stimulation by meta-PAL could be nearly completely blocked by ODQ and is dependent of the presence of the prosthetic heme-moiety of sGC (Fig. 3a). As YC-1, meta-PAL does not shift the position of the Soret band of sGC both under basal and NO stimulated conditions (Fig. 3b).

To optimize the irradiation conditions for the following photoaffinity studies, the NH- and CH-insertion qualities of meta-PAL (BAY 51-9491) were studied in the presence of different solution compounds by irradiation under 254 or 365 nm. As shown in Tab. 2, NH-insertion was more effective than CH-insertion under our conditions. UV 254 nm was chosen as irradiation source. In addition, the time dependency of the photoactivation of the meta-PAL (BAY 51-9491) under UV 254 nm (40 Watt, distance of 3 cm) was studied by thin layer chromatography (Rf of meta-PAL = 0.7 and of photolysed meta-PAL = 0.5). The meta-PAL was completely destroyed after irradiation in PAL

Table 1: Stimulation of purified sGC in the presence and absence of DTT by the NO donor SNP. YC-1 and the ortho-. meta- and para-PALs with Mg^{2+} as cofactor. Shown are the x-fold stimulations versus basal specific activity of SNP (1 μM). YC-1 (100 μM, 10 μM, 1 μM and 1 μM YC-1 in combination with 1μM SNP) and the different PALs (100 μM, 10 μM, 1 μM and 1 μM PAL in combination with 1 μM SNP). The data presented represent the mean ± SEM out of four independent experiments performed in duplicate.

Stimulation of purified sGC [fold increase vs. basal activity ± SEM] (basal specific activity [nmol/mg/min])			
Stimulator	**Concentration**	**+ DTT**	**-DTT**
Basal		1.0 ± 0.1 (64 ± 8)	1.0 ± 0.2 (150.8 ± 33.4)
SNP	1 μM	82.3 ± 10.5	17.1 ± 2.2
	100 μM	45.9 ± 7.7	44.4 ± 4.8
YC-1	10 μM	4.5 ± 0.8	7.0 ± 1.7
	1 μM	2.0 ± 0.4	1.5 ± 0.2
YC-1 + SNP	1 μM + 1 μM	98.4 ± 17.3	20.6 ± 3.6
	100 μM	14.4 ± 1.6	21.4 ± 4.2
ortho-PAL	10 μM	2.9 ± 1.5	3.1 ± 0.6
	1 μM	2.1 ± 1.0	1.0 ± 0.1
ortho-PAL + SNP	1 μM + 1 μM	85.7 ± 11.4	15.8 ± 2.0
	100 μM	54.7 ± 10.2	56.1 ± 0.1
meta-PAL	10 μM	21.3 ± 7.1	23.3 ± 4.1
	1 μM	4.5 ± 1.0	4.9 ± 0.4
meta-PAL + SNP	1 μM + 1 μM	185.6 ± 72.6	39.0 ± 5.6
	100 μM	36.6 ± 3.5	30.2 ± 4.5
para-PAL	10 μM	15.3 ± 2.7	17.3 ± 1.9
	1 μM	3.5 ± 0.4	3.8 ± 0.3
para-PAL + SNP	1 μM + 1 μM	229.1 ± 33.6	50.6 ± 5.5

buffer within 3 min. In the presence of glucose oxidase as model protein, meta-PAL was completely destroyed after an irradiation time of about 18 min (data not shown). Therefore the irradiation time for the labeling studies was chosen with 15 min under 254 nm (40 Watt) at a distance of 3 cm.

For enzyme labeling, ^{3}H-meta-PAL was synthesized as described (Fig. 2). The binding of ^{3}H-meta-PAL to highly purified sGC was studied both in the presence of different concentrations of sGC as different concentrations of ^{3}H-meta-PAL, and also in the presence of unlabeled meta-PAL (100 μM). As shown in Fig. 4, ^{3}H-meta-PAL binds concentration-dependently almost exclusively to the α_1-subunit of the sGC after irradiation. The radiolabeling of the β_1-subunit is only weakly visible in the autoradiogramm after reaction of sGC with 200 μCi ^{3}H-meta-PAL. In the presence of unlabeled meta-PAL (100 μM) the covalent binding of the ^{3}H-meta-PAL to the α_1-subunit is obviously diminished in the presence of 20 or 2 μCi ^{3}H-meta-PAL.

In addition, we examined the effects of different sGC modulators on binding of ^{3}H-meta-PAL on purified sGC (Fig. 5). Both unlabeled meta-PAL (100 μM and 10 μM), YC-1 (100 μM), BAY 41-2272 [22] but also ODQ (100 μM) diminished concentration-dependently the binding of ^{3}H-meta-PAL to the α_1-subunit. ODQ inhibit the binding of the ^{3}H-meta-PAL to the α_1-subunit, but in this case weak unspecific labeling of the β_1-subunit and the α_1-subunit was detected. ^{3}H-meta-PAL processed with sGC without irradiation showed no insertion of radioactivity (Fig. 5).

To identify the binding residues of the ^{3}H-meta-PAL at the α_1-subunit, sGC was labeled with ^{3}H-meta-PAL and the labeled protein was fragmented by CNBr cleavage. The resulting protein fragments were separated by electrophoresis (10–20% SDS-PAGE), transferred to a PVDF membrane, stained with Coomassie-blue and exposed to Imaging-Plates (Fig. 6). The resulted autoradiogramm shows five highly labeled protein fragments (CNBr I-V) with molecular weights of 51.8 / 35.9 / 30.2 / 11.7 and 6.2 kDa (Fig. 6) which were identified and excised from the Coomassie-blue stained Western blots. Sequencing of this bands showed, that they contain different fragments of both the α_1- as the β_1-subunit (Tab. 4). For better separation of peptides in the lower molecular weight range, the same fragments were separated on a TRIS-Tricine-gel. In this autoradiogramm we detected three highly labeled protein fragments (CNBr VI-VIII) with molecular weights of 14.7 / 11.7 and 6.2 kDa (Fig. 7). Before sequencing of the different labeled sGC fragments, both intact subunits
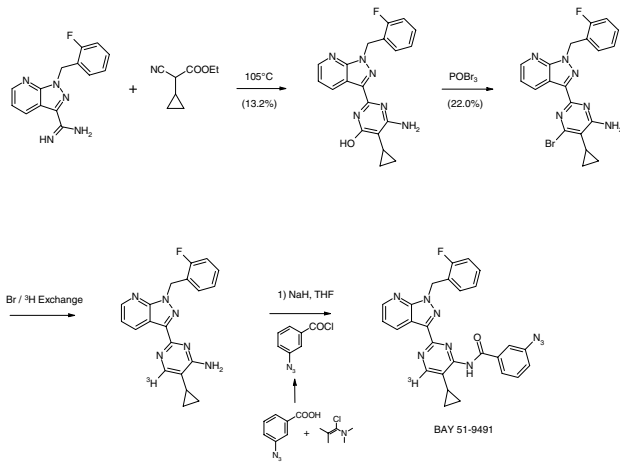
Figure 2
Synthesis of the ³H-meta-PAL.

Figure 3
a) Stimulation of purified sGC by meta-PAL (0.3–100 μM) in the absence and presence of DEA/NO (0.01, 0.1, and 1 μM), YC-1 (30 μM) and ODQ (10 μM) and stimulation of heme-depleted sGC by meta-PAL in the presence of DTT and Mg^{2+} as cofactor. The specific activity of sGC is expressed as x-fold stimulation vs. basal activity (basal activity in the presence of Mg^{2+}: 152 nmol/mg/min). The data presented represent means ± SEM, from 4 independent experiments performed in duplicate. b) Heme spectra (OD: optic density) of sGC under basal conditions and in the presence of the NO donor DEA/NO and the meta-PAL. These data are representative for three independent determinations.

of sGC were sequenced. We observed, that the first 20 coded amino acids of the α_1-subunit probably belong to the presequence. The β_1-subunit corresponded to the published sequence. Because of the selective labeling of the α_1-subunit by the ³H-meta-PAL (Fig. 4 and 5), in Tab. 3 only the theoretically CNBr fragments of the α_1-subunit (AS 20-690) are aligned and signed after their position. It is known that CNBr fragments show different migration properties in SDS-PAGE related to the used molecular weightmarkers. Therefore in the figures we used the calculated molecular weights from sequencing to mark the labeled CNBr fragments. The sequenced bands are marked in Fig. 6 and 7. The results of the sequencing of the different fragments are shown in Tab. 4. The overview of the determined α_1-sequences shows, that the ³H-meta-PAL specifically binds to the CNBr VIII = CNBr-2 fragment of the α_1-subunit (Tab. 3 and 4) with the amino acids 236–290.

To more closely specify the binding site of the ³H-meta-PAL, the single PTH-cycles from the sequencing of the CNBr VIII fragment (Fig. 7 and Tab. 4) were collected, lyophilised and the radioactivity of each fraction was counted. Beside an unspecific "wash out", we determined a distinct increase of radioactivity in the cycles 3 and 8 (> 10-fold over basal measured activity), which correspond to the cysteines 238 and 243 (Fig. 8). Thereby ³H-meta-PAL is bound to sGC after irradiation by the cysteines 238 and 243 of the α_1-subunit of the sGC (Fig. 9).

The putative target sequence of bovine sGC differs from rat and human by the substitution of an arginine at position 238 instead of cysteine. Therefore we investigated if BAY 41-2272 is active against the bovine enzyme or in iso-
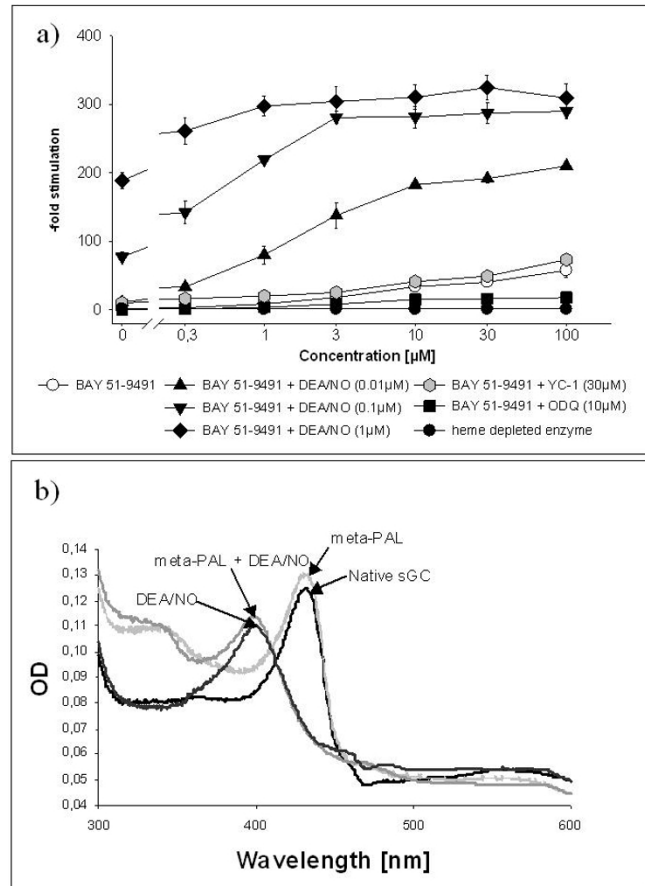
lated bovine vascular preparations. We prepared crude sGC containing fraction from bovine lung by performing the homogenization and ion-exchange steps of the method described earlier [11] with a specific basal activity of 0.15 nmol/mg/min. Using this preparation we examined the characteristic of sGC stimulation by BAY 41-2272 alone and in the presence of high concentrations of DEA/NO (Fig. 10). This study shows that BAY 41-2272 stimulates directly the crude sGC-containing fraction from bovine lung in a concentration dependent manner. In combination, BAY 41-2272 and the NO donor DEA/NO potentiates over a wide range of concentrations.

Table 2: CH- and NH-insertion of meta-PAL dissolved in the different solvents after quantitative LC/MS (results are given as % peak area of insertion product vs. total peak area).

solvent	NH-insertion		CH-insertion	
	irradiation wavelength		irradiation wavelength	
	254 nm	365 nm	254 nm	365 nm
diethylamine	87%, 91%	32%, 23%	-	-
dipropylamine	81%, 91%	21%, 18%	-	-
piperidine	61%	26%	-	-
toluene	-	-	49%	-
ethylbenzole	-	-	8%	2%

Table 3: Sequence of the marked CNBr fragments in figures 5 and 6. MW: molecular weight; AA: amino acid; the several amino acids are shown in the one-letter-code. Sequenced fragments of the α_1-subunit are signed as in Tab. 4, while CNBr fragments of the β_1-subunit are defined by the position of the determined AA in the subunit. The CNBr-2 fragment of the α_1-subunit, represented in all labeled bands,

CNBr-fragment	α_1-subunit	β_1-subunit	Sequence from the N-terminus	MW [kDa]
CNBr I	**CNBr -1 to 8**		APGQVPTEPIEE...	51.8
CNBr II	**CNBr -1 to 4**		APGQVPTEPIEE...	35.9
CNBr III	**CNBr -1 and 2**		APGQVPTEPIEE...	30.2
CNBr IV	CNBr 16		PRYCLFGNNVT...	11.2
	CNBr 8		IYIVESSAILFLG...	12.5
	CNBr -2 and 3		PPCFRSECTEF...	11.7
		AA 505-619	EIAGQVQVDGE...	12.8
CNBr V	CNBr -10 to 13		LNALYTRFDQQ...	6.5
	CNBr 3		LDRDLAILQLG...	5.5
	CNBr 2		PPCFRSECTEF...	6.2
		AA 2-69	YGFVNHALELL...	7.7
		AA 116-164	RAPSFRCTDAE...	5.5
		AA 445-503	KIVNLLNDLYTR...	6.7
		AA 538-592	PRYCLFGNTVN...	6.2
CNBr VI	**CNBr -2 to 6**		PPCFRSECTEF...	14.7
	CNBr 16		PRYCLFGNNVT...	11.2
		AA 505-599	EIAGQVQVDGE...	10.4
CNBr VII	**CNBr -2 and 3**		PPCFRSECTEF...	11.7
	CNBr 8		IYIVESSAILFLG...	12.5
CNBr VIII	**CNBr 2**		PPCFRSECTEF...	6.2
		AA 445-480	KIVNLLNDLYTR...	4.3
		AA 2-69	YGFVNHALELL...	7.7
		AA 1-69	MYGFVNHALEL...	7.7

Table 4: Theoretical CNBr fragments of the α_1-subunit of the sGC. MW: molecular weight; AA: amino acid; the several amino acids are shown in the one-letter-code.

CNBr fragment	MW [kDa]	AA number	AA position in the α_1 subunit	Sequence from the N-terminus
CNBr-1	24	215	21–235	APGQVPTEPIEE...
CNBr -2	6.2	54	236–290	PPCFRSECTEFV...
CNBr -3	5.5	47	291–338	LDRDLAILQLG...
CNBr -4	0.2	2	339–340	TM
CNBr -5	0.3	3	341–343	LNM
CNBr -6	2.5	19	344–363	QFVIRVRRWDNL...
CNBr -7	0.6	6	364–369	DLKGQM
CNBr -8	12.5	111	370–481	IYIVESSAILFLG...
CNBr -9	2.4	22	482–504	LFSDIVGFTAICS...
CNBr -10	5.2	46	505–551	LNALYTRFDQQ...
CNBr -11	0.4	4	552–555	ALKM
CNBr -12	0.1	1	556	M
CNBr -13	0.8	7	557–563	ELSNEVM
CNBr -14	0.9	9	564–572	SPHGEPIKM
CNBr -15	1.8	17	573–590	RIGLHSGSVFAG...
CNBr -16	11.2	99	591–690	PRYCLFGNNVT...

Discussion

To identify the binding site of the new class of direct and NO-independent sGC stimulators like YC-1 and the recently described more potent compound BAY 41-2272 [22] we focus in this paper in addition to results, already shown in Stasch et al. [22], on the detailed description of the development of the first photoaffinity label by introducing a azidobenzoic acid at different positions (ortho-, meta-, and para-PAL) into the BAY 41-2272 core structure. As shown, all tested photoaffinitylabels stimulate the purified enzyme with similar characteristics as YC-1. sGC activity increases from the ortho- to the para- to the meta-PAL compound. In contrast to the ortho-PAL compound, the meta- and para-PAL compounds show synergistic effects on sGC activity in combination with NO as described for YC-1 and BAY 41-2272 [11,22] (Tab. 1). Therefore we conclude from these structure-activity-relationship, that the meta- and para-PALs in contrast to the ortho-PAL match structurally with the new binding site of the sGC, essentially regarding to the synergistic activation of sGC in combination with its physiological stimulator NO and thereby are useful tools in the identification of the new binding site in sGC. In addition to Stasch et al.[22], we demonstrate here different potential PALs, which differ in their chemical structure and sGC stimulation characteristics.

In forward to avoid an unproductive destruction of the PAL during the labeling of the enzyme [34], stimulation of sGC was not only tested under standardized conditions [11] but also verified under modified conditions in the absence of DTT. Even under this adapted conditions used for the labeling of the enzyme, the PAL compounds stimulate the purified enzyme with similar properties as YC-1 and BAY 41-2272. Under these conditions meta-PAL reveals to be the most potent compound with the same characteristics on stimulation of sGC as YC-1 and BAY 41-2272. As expected, meta-PAL shows no influence on the position of the Soret band of sGC both under basal and NO-stimulated conditions. Thereby meta-PAL belongs to the new class of direct, NO-independent sGC stimulators, which stimulate sGC by a heme-dependent mechanism without a direct interaction with the heme-moiety [11,22].

Moreover we characterize the insertion characteristics of the meta-PAL. Concomitant with the literature, our meta-PAL showed more potent NH- than CH-insertion qualities and thereby is qualified for protein conjugation reactions [35]. During irradiation with UV light (254 nm), under our adapted conditions the meta-PAL completely disintegrated within a time of maximal 18 min under release of nitrogen, detected by thin-layer-chromatography. Beyond the fast reaction of azido compounds after irradiation with UV, the quality of a photoaffinitylabel depends on the stability of the compound and the radioactive label and of the covalent binding properties of the compound at or at least in the environment of the binding site [35]. Our compound is stable for several weeks stored at 70°C (data not shown). Moreover, under reducing conditions, where the sGC is distinctly stimulated by the meta-PAL, and thereby the active conformation of the sGC should be
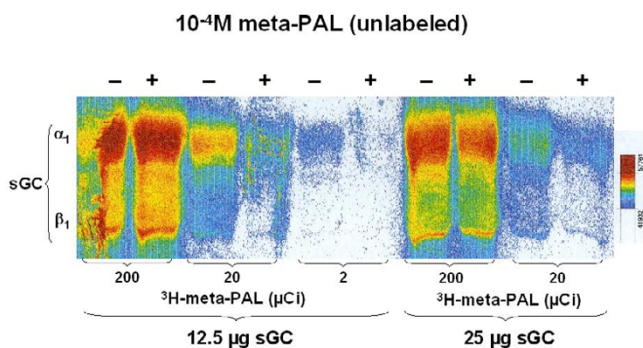
Figure 4
Autoradiogram of ^{3}H-meta-PAL (2, 20 and 200 μCi) labeled sGC (12.5 and 25 μg) in the presence and absence of meta-PAL (100 μM) after separation in both subunits by SDS-PAGE.

provided, the ^{3}H-meta-PAL binds not only concentration-dependently but also specifically to the α_1-subunit of the sGC. Ideally, a classical receptor binding study would be the best aproach to characterize the specific as well as unspecific binding to sGC. However, due to the physikochemical properties of the radioligand (e.g. low solubility, lipophilic character, low specific activity) and the difficulty that the receptor sGC is a soluble cytosolic enzyme, it was not possible to separate free and bound radioligand. For this reasons we were not able to establish this study. Binding to the α_1-subunit of the enzyme was inert versus all used analytical steps (e.g. TCA precipitation, acid CNBr proteolyse, SDS-PAGE) and thereby underlines the quality of this new compound.

In a further study, the inhibition of this specifically binding of the ^{3}H-meta-PAL to the α_1-subunit of the sGC by several sGC modulators was examined. Thereby we could show that the ^{3}H-meta-PAL binds only after irradiation to the α_1-subunit of the sGC, implicating that the ^{3}H-meta-PAL binds as a consequence of irradiation and not by an unspecific interaction to the enzyme. After preincubation with the purified sGC, the unlabeled meta-PAL(10 and 100 μM) as well as YC-1 (100 μM) or BAY 41-2272 [22] inhibited the insertion of the ^{3}H-meta-PAL in the α_1-subunit, underlining the specificity of the binding. As shown in Fig. 4, incubation with 200 μCi (125 μM) ^{3}H-meta-PAL in the presence of 100 μM unlabeled PAL showed only slight competition due to the small access of unlabeled compound. For this reason further competition studies were performed with 12.5 μM ^{3}H-meta-PAL (Fig. 5). In addition, the specific inhibitor of sGC, ODQ [36], strongly diminished the binding of the ^{3}H-meta-PAL to the α_1-subunit. In this case there could be detected an equivalent binding of the ^{3}H-meta-PAL both to the α_1- and the β_1-subunit. It is described that ODQ inhibits sGC activity by

oxidation of the heme moiety from Fe^{2+} to Fe^{3+} or by competition with the NO binding site [37,38]. Therefore we propose that after interaction with ODQ the native heme environment of the sGC is changed (e.g. by oxidation), destroying the binding site for direct and NO-independent sGC stimulators and therefore ^{3}H-meta-PAL unspecifically binds to both subunits of the sGC. The slightly stronger labeling of the beta subunit would result in this context from the possible higher available concentration of free meta-PAL. This explanation is underlined by the observation, that the heme-depleted enzyme is insensitive towards stimulation by meta-PAL and other direct and NO-independent sGC stimulators like BAY 41-2272 or YC-1 [13,22]. Recently, Martin et al. [46] showed that the stimulation of sGC by the new type of sGC stimulators like YC-1 has both heme-dependent and heme-independent components. The stimulation of sGC by YC-1 is not completely blocked by ODQ, in particular in higher concentrations of sGC stimulators. They conclude, that the binding of ODQ and YC-1 are not mutually exclusive processes and that their binding sites do not overlap. These data corroborate our findings.

In addition, the ^{3}H-meta-PAL labeled sGC was fragmented by CNBr digest to identify the binding site of the ^{3}H-PAL at the α_1-subunit more closely. In this study, the chemical digest of the labeled sGC shows five highly labeled protein fragments on the Photo-Imaging-Plate of the gradient gel in the molecular range between 6.2 and
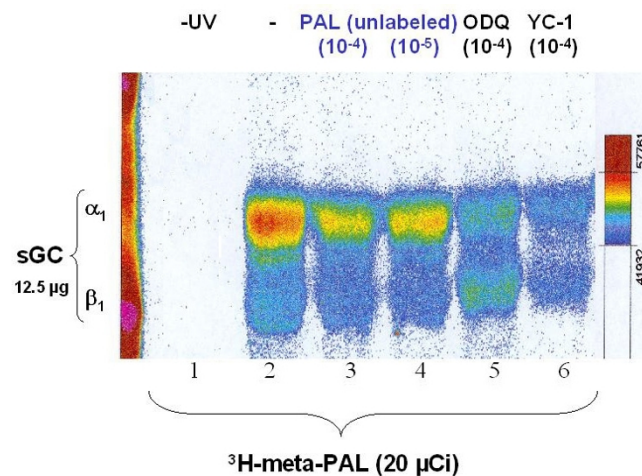

Figure 5
Autoradiogram of ^{3}H-meta-PAL labeled sGC after separation by SDS-PAGE. Lane1: 12.5 μg sGC with ^{3}H-meta-PAL without irradiation; Lane 2: 12.5 μg sGC with ^{3}H-meta-PAL after irradiation; Lane 3: as 2 but in the presence of 100 μM unlabeled meta-PAL; Lane 4: as 2 but in the presence of 10 μM unlabeled meta-PAL; Lane 5: as 2 but in the presence of 100 μM ODQ; Lane 6: as 2 but in the presence of 100 μM YC-1.

51.8 kDa and three highly labeled bands in the Photo-Imaging-Plate of the TRIS-Tricine-gel in the molecular range between 6.2 and 14.7 kDa. These bands were identified and excised from the Coomassie-blue stained blot and sequencing of these bands showed, that they contain different fragments of both the α_1- as the β_1-subunit. Because the ^{3}H-meta-PAL binds specifically to the α_1-subunit of the sGC and thereby all identified β_1-sequences could be discarded, the overview of the determined α_1-sequences shows, that the ^{3}H-meta-PAL binds specifically to the CNBr VIII fragment, consisting of the amino acids 236–290 of the α_1-subunit. To more closely identify the binding site of the ^{3}H-meta-PAL, the single PTH-cycles from the sequencing of the CNBr VIII fragment were collected and the cysteines 238 and 243 of the α_1-subunit were detected as binding sites of ^{3}H-meta-PAL.

As shown in Fig. 9, these cysteines are conserved between the amino acid sequences of rat and human sGC [39,40] and this region could play an important role in regulation of sGC activity by this type of sGC. However, the putative target of bovine sGC differs from rat and human by the substitution of an arginine at position 238 instead of cysteine. As shown in previous studies with YC-1 [11] and verified in this study using crude bovine lung derived sGC extracts, we know that cysteine 238 is not essential for the stimulation of sGC by neither YC-1, nor BAY 41-2272, nor
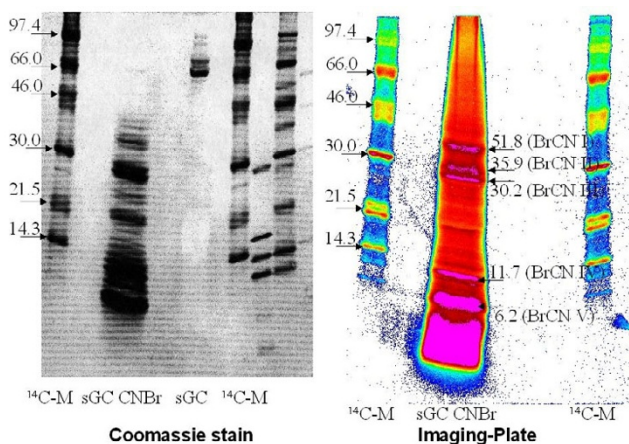

Figure 6
Coomassie-blue stained Western Blot and autoradiogram of photoaffinity labeled sGC fragments after CNBr digest and SDS-PAGE on a 10–20% gradient gel (6 h exposition on Imaging-Plate). Shown are a ^{14}C-labeled rainbow molecular weightmarker (^{14}C-M), further molecular weightmarker, photoaffinity labeled sGC (200 µg) after CNBr digest (sGC CNBr) and for control undigested sGC (sGC; 5 µg). The arrows show the molecular weightmarkers with their molecular weights (kDa) or the ^{3}H-meta-PAL labeled sGC fragments (BrCN I-V) with their molecular weights, as calculated after sequencing.
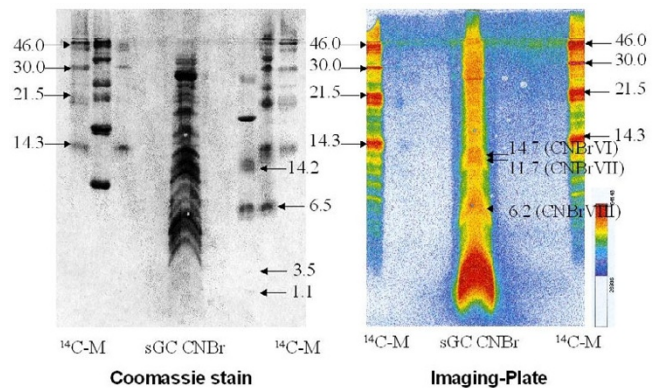

Figure 7
Coomassie-blue stained Western Blot and autoradiogram of photoaffinity labeled sGC fragments after CNBr digest, SDS-PAGE on a TRIS-Tricin-gel (16.5%) (48 h exposition on Imaging-Plate screened by a folio of contamination monitors). Shown are a ^{14}C-labeled rainbow molecular weightmarker (^{14}C-M), further molecular weightmarkers, and photoaffinity labeled sGC (200 µg) after CNBr digest (sGC CNBr). The arrows show the molecular weightmarkers with their molecular weights (kDa) or the ^{3}H-meta-PAL labeled sGC fragments (CNBr VI-VIII) with their molecular weights, as calculated after sequencing.

the PAL compound. However, is must be considered that the potencies of BAY 41-2272 on human, rat and bovine sGC could be different. For such comparison, the highly purified guanylate cyclases from these three species are needed. The mechanism of sGC stimulation is rather more complicated than simple interaction with one or two amino acids since the heme moiety bound to the His-105 of the β_1-subunit is essential for activating the enzyme by YC-1, BAY 41-2272 and the PAL compound. There is a distance of 9 Å between the photolabile azido group and the pharmacophore of the compound. For this reason a covalent binding of the ^{3}H-PAL to reactive cysteines slightly outside the binding pocket would not be surprising. Nevertheless, we assumed that Cys-238 and Cys-243 are in the direct region of access of ^{3}H-PAL because no other labeled amino acids have been detected and a diffusion over a wide distance can be excluded.

Recent studies with YC-1 imply that YC-1 binds to an allosteric site on sGC and thereby increases the maximal catalytic rate and sensitises the enzyme towards its gaseous activators NO and CO [11,13]. Studies about the kinetics and equilibra of sGC in the presence of YC-1 led to a mechanistic model, which attributes a crucial role to the proximal bond that connects the heme iron to the histidine 105 of the β_1-subunit of sGC [41,42], but also requires protein control of the distal environment [43]. Firstly a small shift in the soret absorption feature of the sGC by YC-1 is observed. It is discussed, that this small
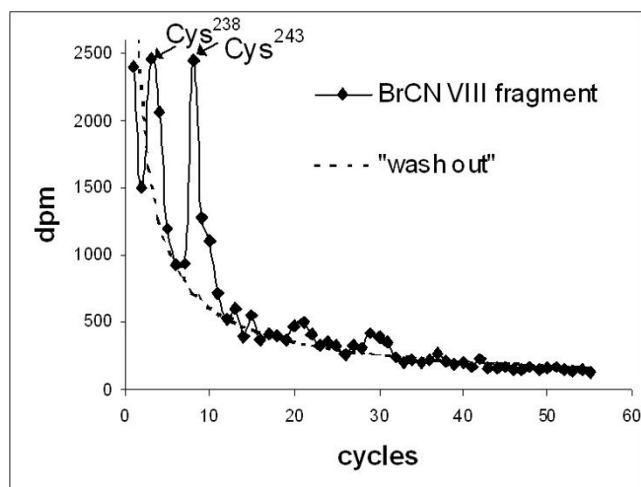
Figure 8
Course of the counted radioactivity of the single PTH-cycles of the sequencing of the CNBr VIII band. Presented are the measured dpm (decompositions per min, counting time 2 h). The pointed line represents the potential regression of the unspecific bound radioactivity, which is washed of the membrane during the first cycles ("wash out").

shift could be evoked by the replacement of the His-105 as the proximal heme ligand by another base, either another amino acid side chain or even YC-1 itself [43].

In contrast to [21] who postulated the YC-1 binding site in analogy to the forskolin binding site in the adenylate cyclase within the catalytic region of the α_1- subunit of the sGC in the region of cysteine 596, we showed that the new generated photoaffinitylabel for direct and NO-independent sGC stimulators binds to the cysteines 238 and 243 in the N-terminus of the α_1-subunit of the sGC and thereby in the near of the heme binding region. Hobbs [44] postulated an intramolecular sixth ligand of the heme, which oscillates on and off the sixth coordinate, thereby conferring some sort of ligand specificity (i.e. NO and CO). The binding of direct sGC stimulators to this site could block this intramolecular binding site and thereby simplify the binding of NO and CO to the enzyme. This model is concomitant with the sensitising of the enzyme towards NO by this class of stimulators. Very recently our findings have been supported [45] showing the important role of an additional heme binding domain for sGC activation by the NO-independent sGC stimulator YC-1. This domain is in the α-subunit in vicinity of the PAL labeled region.

Conclusion
In summary, using photoaffinity labelling, we identified the region of the cysteines 238 and 243 in the α_1 subunit of sGC as the target for NO-dependent sGC stimulators. However, the relevance of the identified region as a regu-

latory unit remains to be confirmed by mutational analysis and co-crystallization studies.

Materials and Methods
Synthesis of YC-1 and the photoaffinity labels (PALs)
YC-1 ((3-(5-hydroxymethyl-2 furyl)-1-benzylindazole) was synthesized as described [23] and was used as a 10 mM stock solution in DMSO. Three PAL candidates (BAY 50-6038, BAY 50-8364 and BAY 51-9491) were synthetized from the common intermediate BAY 41-2272 [24,25] (Fig. 1). The azide derivatives were obtained by deprotonation of the BAY 41-2272 primary amine with sodium hydride followed by reaction with the corresponding azide benzoyl chloride. The ortho- and para-azidobenzoyl chlorides were synthesized by reaction of the corresponding ortho- and para-azidobenzoic acid with thionyl chloride. In order to obtain the meta-azido derivative, the benzoyl chloride was synthesized in situ by reaction of the corresponding azidobenzoic acid [26] with 1-chloro-N,N-2-trimethylpropenylamine [27] (Fig. 1). The synthesis of the corresponding [3H]-BAY 51-9491 was accomplished as depicted in Fig. 2. Cyclization of the amidine with ethyl cyano(cyclopropyl)acetate [28] afforded the 6-amino-4-hydroxypyrimidine derivative, which was converted to the bromo precursor by reaction with phosphoroxide tribromide. Br/^{3}H exchange followed by formation of the amide bond as detailed above yielded the [3H]- BAY 51-9491.

Purification of soluble guanylate cyclase (sGC) and determination of sGC activity
sGC was highly purified from a baculovirus / Sf9 expression system and enzyme activity was measured by formation of [^{32}P]-cGMP from [α-^{32}P]-GTP modified according to Gerzer [29] in the presence of Mg^{2+} as the divalent metal cation as described [11]. Incubations were performed in the presence and absence of 1 mM DTT. All measurements were performed in duplicate and were repeated three times unless otherwise indicated. The specific activity of sGC was calculated as nmol cGMP formed per mg protein
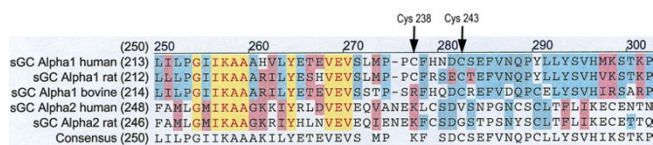

Figure 9
Sequence alignment of 3 α_1 subunits (human, rat, bovine) and 2 α_2 subunits (human, rat). Invariant amino acids are shown in red on yelow background. Amino acids building the consensus are shown in black on blue background and amino acids similar to the consensus are shown in black on red background. The two labeled cysteines are marked with arrows.
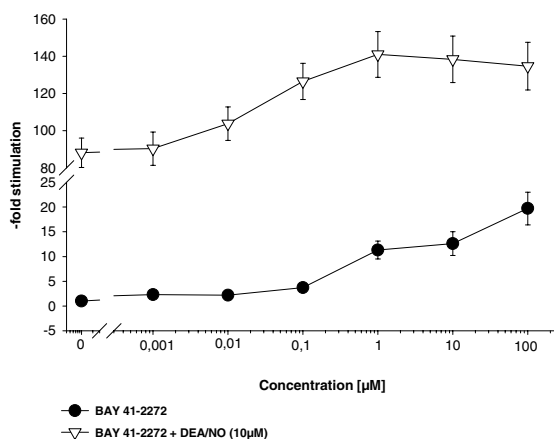
Figure 10
Stimulation of the crude sGC preparation from bovine lung by BAY 41-2272 in the absence and presence of DEA/NO (10 μM). The specific activity of sGC preparation is expressed as x-fold stimulation vs. basal activity (basal activity in the presence of Mg^{2+}: 152 nmol/mg/min). The data presented represent means ± SEM, from 4 independent experiments performed in duplicate.

per min incubation time. For characterisation of the different sGC stimulators the specific activity of sGC was expressed as x-fold stimulation vs. specific basal activity. The highest DMSO concentration in the test was 1% (v/v) and did not elicit any effect per se on cGMP production.

Spectroscopic studies
UV/Vis spectra were recorded from 300 to 600 nm on a DU 640 spectrophotometer (Beckman, Munich, Germany). NO was introduced via an aqueous solution of DEA/NO. A 100 mM stock solution of meta-PAL in DMSO was prepared and added in a final concentration of 10 μM, resulting in a final DMSO concentration of 0.1% (v/v), which does not alter the properties of the enzyme.

Evaluation of CH- and NH-insertion qualities of the meta-PAL
Meta-PAL (BAY 51-9491) was dissolved in diethylamine, dipropylamine, piperidine, toluene, or ethylbenzole in a concentration of 1 mg/ml and degassed by three thaw-freeze-cycles. 1 ml of each solution was given in a well of a 24 well PET plate (Wallac, Turku, Finland) and irradiated with a UV hand lamp at 254 or 365 nm in a distance of 3 cm for 2 h (254 nm: N8K UV 254 nm hand lamp, Benda, Wiesloch, Germany; 365 nm: Universal UV lamp, CAMAG, Berlin, Germany). Solutions were evaporated in a Speed Vac (Bachofer, Reutlingen, Germany) and qualita-

tively and quantitatively analysed by LC / MS (column: symmetry C18, 2.1 × 150 mm; eluent: A: acetonitrile, B: 0.6 g HCl (30%) in 1 l MilliQ water; gradient: time 0: 10% A and 90% B flow: 0.6 ml/min, time 4 min: 90% A and 10% B flow: 0.6 ml/min, time 9.5 min: 10% A and 90% B flow: 0.8 ml/min; at 50°C). Resulting products were considered as NH- or CH-insertion products if their molecular weights correspond to the calculated weight of the insertion product and were given as % peak area of total peak area. To evaluate the irradiation time required for N2 degradation, meta-PAL (1 mg/ml) was dissolved in PAL buffer (final 50 mM TEA/HCl, 0.1 mM EGTA, 1 mM cGMP, 3 mM MgCl2, 200 μM GTP, pH 7.4) in the absence and presence of 0.3 mg/ml glucose oxidase as model protein and irradiated as described at 254 nm. Every minute an aliquot was removed and fragmented compounds were separated from meta-PAL by thin layer chromatography (stationary phase: silicagel 60 layered glass plate 5 × 10 cm (Merck, Darmstadt, Germany); mobile phase: acetoacetate).

Labeling of purified sGC
12.5 or 25 μg sGC (purity about 91%) were dissolved in PAL buffer (final 50 mM TEA/HCl, 0.1 mM EGTA, 1 mM cGMP, 3 mM $MgCl_2$, 200 μM GTP, pH 7.4) and incubated with 200, 20 or 2 μCi ^{3}H-meta-PAL (specific activity: 7.9 Ci/mmol ≅ 0.29 MBq/nmol) in the presence or absence of 100 μM meta-PAL in a volume of 80 or 100 μl in a 24 well PET plate (5 min, 37°C). Samples were irradiated at 254 nm (distance 3 cm, 20°C, 15 min) and the reaction was stopped by adding 20 or 25 μl SDS-containing stop solution (312.5 mM TRIS/HCl / 10% (w/v) SDS / 50% (v/v) glycerine / 250 mM DDT / 0.025% (w/v) bromphenolblue, pH 6.8) and heating (5 min at 80°C).

In a further study 12.5 μg sGC were incubated with 20 μCi ^{3}H-meta-PAL in the presence and absence of the different sGC modulators as indicated, irradiated and stopped as described. For controls 12.5 μg sGC were incubated with 20 μCi ^{3}H-meta-PAL without irradiation and 12.5 μg glucose oxidase were irradiated as described in the presence of 20 μCi ^{3}H-meta-PAL. Separation was performed on a 7,5% SDS-PAGE (PROTEAN II cell, Bio-Rad, München, Germany) using a modified Laemmli method [30]. After electrophoresis, proteins were fixed for 20 min in methanol/acetic acid/ MilliQ water (30/10/60), dried and exposed to BAS-TR 20/25 Imaging-Plates (Ray Test, Straubenhardt, Germany) for 15 days as described [31]. After exposure, the imaging plates were scanned (BAS 5000 Scanner, Ray Test, Straubenhardt, Germany). Evaluation was performed by visual classification of radiographic intensities, displayed by use of pseudo-colours, starting with blue for lowest detectable concentrations up to red for highest concentrations as shown in each figure by a gradation bar.

CNBr fragmentation of labeled sGC

200 µg sGC were labeled as described with 1600 µCi ^{3}H-meta-PAL, and the reaction was stopped by the addition of final 10% TCA. The labeled protein was precipitated (4°C, 30 min), centrifuged (14,000 rpm, 30 min; Centrifuge 5415C, Eppendorf, Hamburg, Germany) and washed two times with ice cold ethanol:ether (1:1). The protein pellet was dissolved in formic acid (70%) and reacted with a few crystals of CNBr in the dark (24 h, 20°C) under oxygen-free nitrogen. After evaporation in a Speed Vac (Bachofer, Reutlingen, Germany) for three times after the addition of 1 ml MilliQ water, the pellet was dissolved in 100 µl sample buffer (62.5 mM TRIS/HCl / 2% (w/v) SDS / 10% (v/v) glycerine / 50 mM DDT / 0.0025% (w/v) bromphenolblue, pH 6.8), heated (5 min, 80°C) and the protein fragments were separated on a 10–20% gradient SDS-PAGE or a 16.5% TRIS-Tricine-gel [32], respectively. The digested protein fragments were transferred to a PVDF membrane (Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad, München, Germany) [33], stained with 0.025% (w/v) Coomassie-blue-R / 40% (v/v) MeOH and destained in 50% (v/v) MeOH. Dried membranes were exposed to BAS-TR 20/25 Imaging-Plates (Ray Test, Straubenhardt, Germany) for 6 h (10–20% gradient gel blot) without or for 48 h with a screen of metal folio for contamination monitors (0.9 mg/cm^{2}Steiner, Erndtebrück-Schameder, Germany) (16.5% TRIS-Tricine-gel blot) [31]. After exposure, the Imaging Plates were scanned and evaluation was performed as described above.

N-terminal sequence analysis

N-terminal sequence analyses were performed using the gas-liquid-solid-phase protein sequencer Procise™ from Applied Biosystems (Forster City, CA., U.S.A.). The sequencer program for blot sequencing was used according to the manufacturer s standard protocol (User's manual set Procise™, 1994; Applied Biosystems, Forster City, USA). The detection of PTH-amino acids was performed on-line using an RP-18-PTH-column (220 mm × 2 mm, 5 µm-material) from Applied Biosytems. The PTH-amino acids were identified and quantified by a 50 pmol PTH-standard. The data were collected and integrated using the sequencer data system (Protein sequencing protocols; ed. Bryan John Smith; Humana Press INC.; Totowa, New Jersey).

Sequence analysis

PVDF-membrane pieces were washed twice with 100 µl 50% (v/v) MeOH before sequencing. The cyanogen bromide fragment CNBrVIII was sequenced over 60 cycles using the sequencer program for blot sequencing. The single PTH-amino acids were collected (fraction collector Superrac 2211, Pharmacia) lyophilised, dissolved in acetonitril and MeOH and transferred to Combust-Cones, filled with two Combust-Pads (Packard, Illinois, USA). The radioactivity of each fraction was determined after combustion (Oxidizer model 307, Oximate 80, Packard, Illinois, USA) and dissolution of resulting gases in 15 ml scintillator (Monophase Scintillator, Packard) with a counting time of 2 h in a liquid scintillation counter (LS-6500, Beckmann, München, Germany).

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